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AcrS Is a Potential Repressor of *acrA* Expression in *Escherichia coli* and Its Deletion Confers Increased Kanamycin Resistance in *E. coli* BW25113

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AcrAB-TolC is a major efflux pump complex in the resistance nodulation-division family that is responsible for antibiotic resistance in *Escherichia coli*. Previous research has shown that deletion of the pump component protein, AcrA, leads to decreased drug resistance in *E. coli*. It is proposed that AcrS regulates the expression of AcrA, however, how *acrA* is regulated transcriptionally remains unknown. In this paper, the effect of *acrS* deletion on kanamycin resistance in *E. coli* was studied. Resistance to kanamycin in *E. coli* JW0452-3 (Δ*acrA*), *E. coli* JW3232-1 (Δ*acrS*) and *E. coli* BW25113 (wild-type) was compared using minimum inhibitory concentration (MIC) assays. MIC assay results showed that the Δ*acrS* strain had a higher resistance to kanamycin compared to both Δ*acrA* and the wild-type strain. These results suggest that AcrS might be a potential repressor of *acrA* in *E. coli* and the deletion of *acrS* leads to higher antibiotic resistance, possibly due to increased expression of AcrA, a critical component of AcrAB-TolC.

One of the main mechanisms underlying antibiotic resistance in *E. coli* involves the activity of multi-drug efflux pumps. Multi-drug efflux pumps are membrane transport proteins that can expel a range of antibiotics from the cytoplasm due to their broad substrate specificity, and thus mediate the acquisition of multi-drug resistance in *E. coli* (1). AcrAB-TolC is a major multi-drug efflux pump in *E. coli* that is responsible for intrinsic drug resistance (2). The pump belongs to the resistance nodulation-division (RND) family, which is especially effective in generating antibiotic resistance through the use of proton-motive force (3). AcrAB-TolC is a three component system, which includes periplasmic AcrA, inner membrane transporter AcrB, and outer-membrane channel TolC (4). The efflux pump genes are constitutively expressed at low levels in antibiotic-free environments and are intrinsically upregulated when antibiotics are introduced (5). The efflux pump genes are encoded on the *acrAB* and *tolC-ygiAB* operons, which are upregulated by the transcriptional regulators MarA, SoxS and Rob (6).

Several research studies investigated the physiological impacts of AcrAB-TolC gene mutations, as well as the negative regulators of gene expression. It was found that deletion of any of the pump component genes reduced antibiotic resistance and increased antibiotic sensitivity of *E. coli* (7). In particular, *acrA* mutants were sensitive to a broad range of antibiotics, including kanamycin and ampicillin (7). One proposed explanation is that *acrA* mutants have increased outer membrane permeability, which enables molecules to flow through more readily (5). This finding suggests that *acrA* expression determines the intrinsic antibiotic resistance of *E. coli* (5). To investigate the regulation of *acrA* expression, several possible repressors of *acrA* are proposed, such as AcrS (2). The *acrS* gene is approximately 663 bp in size and is located in the *acrEF* operon (2). It was suggested that AcrS may bind to the *acrA* promoter and act as a transcriptional repressor of the efflux pump, but the relationship remains poorly understood.

Hirakawa et al. found that AcrS specifically binds to the same 24 bp palindromic DNA sequence of *acrA* promoter recognized by AcrR, a transcriptional repressor of the *acrAB* operon (2). They also investigated the relationship between AcrS and drug susceptibility by exposing *E. coli* strains that either over-expressed *acrS* or carried *acrS* mutations to antibiotics, such as tetracycline and novobiocin (2). Hirakawa et al. found that overexpression of *acrS* decreased the minimum inhibitory concentration (MIC), which suggested that AcrS may have a repressive role on *acrA* expression (2). On the contrary, mutation of *acrS* increased MIC for novobiocin due to possible *acrA* upregulation (2).

Similarly, Emami et al. investigated drug susceptibility of Δ*acrS* to kanamycin and found increased MIC following pre-treatment (8). To explain this observation, they proposed that the loss of *acrS* may lead to the upregulation of *acrA* expression, but they could not establish a direct relationship (8). Thus, the exact role of AcrS on *acrA* expression under the presence of kanamycin is unclear.

In this paper, we investigate the relationship between AcrS and *acrA* expression by comparing kanamycin MICs between Δ*acrS*, Δ*acrA*, and wild-type *E. coli* strains without kanamycin pre-treatment. We hypothesized that AcrS represses the expression of *acrA* in *E. coli* and the deletion of *acrS* results in the upregulation of *acrA* expression, resulting in increased MICs for kanamycin.

MATERIALS AND METHODS

Bacterial strains and culturing methods. *E. coli* JW0452-3 (Δ*acrA::kan*) was ordered from the Coli Genetic Stock Centre (CGSC) at Yale University. *E. coli* JW3232-1 (Δ*acrS*::kan) and *E. coli* BW25113 (wild-type) were obtained from the MICB 421 culture collection of the Microbiology and Immunology Department at the University of British Columbia. All strains were grown on Luria Bertani (LB) medium containing antibiotics when appropriate and were incubated at 37°C. One L of LB medium was

Gene		Primer Sequence (5' to 3')	Gene Size (bp)	Product Size (bp)
***acrS* -F**	Forward	GCTGGCAACAGAAACAGACA	663	1338
	Reverse	GCTTCACTTTTCGCCAGTTC		
acrS -N	Forward	CCCGCCATATTCATTAACCA	663	321
	Reverse	CTTGACGGCTGGATTAGAGC		
***acrA* -F**	Forward	AAAACTGACTCAGGCGCGTA	1194	N/A
	Reverse	CAGCGAGGTGGATGATACCC		
acrA -N	Forward	GAGTACGATCAGGCTCTGGC	1194	233
	Reverse	GACCGCTAATCGGAGAGGTG		
rpoS	Forward	TCGCTTGAGACTGGCCTTTCTG	993	800
	Reverse	CGGAACCAGGCTTTTGCTTGAATG		

TABLE 1 Primer sequences, gene size, and product size of primers used for colony PCR confirmation of *acrS* deletion in *E. coli* JW3232-1 and *acrA* deletion in JW0452-3 cells. Flanking (F) and nested (N) primers were used.

made by dissolving 10 g of tryptone, 5 g yeast extract and 10 g of sodium chloride in distilled water.

Preparation of antibiotic stock solutions. 100 mg/ml ampicillin sodium salt (Sigma-Aldrich®) and 50 mg/ml kanamycin monosulfate (Sigma-Aldrich®) stock solutions were prepared by dissolving each antibiotic in distilled water. The antibiotic stock solutions were then sterilized by filtering the solutions through 0.22 µm nitrocellulose filters (EMD Millipore) and stored at -20°C.

Confirmation of *acrS* and *acrA* deletion. The *acrS* gene present in JW3232-1 and the *acrA* gene present in JW0452-3 were knocked out by Ara et al. and replaced with a 1.3 kb kanamycin resistant cassette (9). Removal of *acrA* in JW0452-3 and *acrS* in JW3232-1 was confirmed using colony PCR and gel electrophoresis. The primers used in the PCR reaction included flanking primer pairs that hybridize upstream and downstream of *acrS* and nested primer pairs that hybridized within *acrS*. Flanking and nested primer pairs were also designed for the PCR reaction of *acrA*. The primer sequences and expected fragment sizes of nested and flanking primers are shown in Table 1. PrimerQuest Tool by Integrated DNA Technologies was used to design primers and all designed primers were ordered from Integrated DNA Technologies.

PCR amplification and gel electrophoresis. The primer sequences, gene size, and product size of *acrA* and *acrS* are shown in Table 1. The PCR reactions were performed as outlined by the iGEM Colony PCR protocol (10). The thermal cycle was programmed for 1 cycle of initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1.5 mins, extension at 72°C for 5 mins; and 1 cycle of final extension at 72°C for 5 mins. All PCR products were electrophoresed in 1.0% (w/v) agarose gel at 85 V. The agarose gel was made with SYBR® Safe DNA Gel Stain in 0.5x TBE buffer (Thermo Fisher Scientific) to allow visualization of the gel using the Alpha Innotech® MultiImage light cabinet.

Removal of kanamycin resistance cassette from *E. coli* JW3232-1 and JW0452-3 cells. pCP20 plasmid was isolated from pCP20-containing DH5α *E. coli* cells provided by Dr. David Oliver using the PureLink® Quick Plasmid Miniprep Kit (Thermo Fisher Scientific), following the protocol provided with the kit. One hundred ml of TSS broth was prepared as shown in S. Table 1, according to instructions from Chung et al. (11). The pCP20 transformation protocol was adapted from Deatherage (12). *E. coli* JW3232-1 and JW0452-3 cells were grown overnight in 5 ml LB broth containing 50 µg/ml kanamycin. Half of a ml of the overnight cultures was added to 100 ml LB containing 50 µg/ml kanamycin and were allowed to grow to OD_{600} 0.3-0.5. Cells were then placed on ice before being spun down in Oak Ridge tubes. The pellet was resuspended in 1 ml of TSS per 10 ml of original broth. For transformation, 150 µl aliquots of the two strains were prepared in 1.5 ml Eppendorf tubes, then 200 ng, 100 ng, and 50 ng of pCP20 plasmid were added. Cells were incubated on ice for 30 minutes and were heat shocked at 42°C for 2 minutes. Afterwards, 0.8 ml of ice-cold TSS was added to each tube. Transformed cells were then aliquoted into 5 ml cuvettes and left for one, two, and three hour periods at 30°C for outgrowth, before being plated on LB agar plates containing 100 µg/ml ampicillin. Plates were left at 30°C overnight. Ampicillin resistant colonies were streaked on LB agar plates and incubated at 42°C to activate FLP recombinase and inhibit plasmid replication. Isolated colonies were selected from the LB agar plates and streaked on LB agar, LB agar containing ampicillin (100 µg/ml), and LB agar containing kanamycin (50 µg/ml) plates to select for successful transformants that only grew on LB agar, but not kanamycin and ampicillin. LB agar plates and LB agar plates containing kanamycin were grown at 37°C whereas LB agar plates with ampicillin were grown at 30°C overnight.

Determination of minimal inhibition concentrations of kanamycin for *E. coli* BW25113, Δ*acrA* and Δ*acrS* strains. The MIC assay method was adapted from the Hancock laboratory protocol (12). *ΔacrS*, Δ*acrA*, and BW25113 were grown overnight in 5 ml LB broth, then 0.5 ml of overnight culture was placed in 100 ml LB broth and grown to OD_{600} 0.2-0.3. Three MIC assays were performed; the first MIC assay used 2-fold serial dilutions of kanamycin starting at 30, 45, 50 or 56 µg/ml. Since the serial dilution method did not give sufficient resolution between samples, the protocol was altered so that concentrations decreased in 2.5 µg/ml increments. The second MIC assay had kanamycin concentrations ranging from 12.5 to 30 µg/ml, plated in triplicate. The third MIC assay used kanamycin concentrations ranging from 20 to 37.5 µg/ml, also plated in triplicate; two biological replicates for each strain were prepared for the third MIC assay. The pass conditions for the second and third MIC assays were as follows: to be counted as a "pass," growth had to be observed in two out of three replicates for a given strain and kanamycin condition, and an OD_{600} of over 0.1 (after blank subtraction) had to be measured for those same wells. One column of LB broth without added culture or antibiotics was plated as a sterility control and blank. Five µl of bacterial culture was added to each well containing antibiotics and the plates were grown at 37°C overnight. In all assays, growth was confirmed by both visual inspection and using on the plate reader (BioTek Epoch) at 600 nm.

Determination of growth kinetics of *E. coli* BW25113, *ΔacrS* and Δ*acrA* strains. *ΔacrS*, *ΔacrA* and BW25113 cells were grown overnight in 5 ml LB broth. Half a ml of overnight culture was then placed in 100 ml LB broth and incubated at 37°C on a shaking

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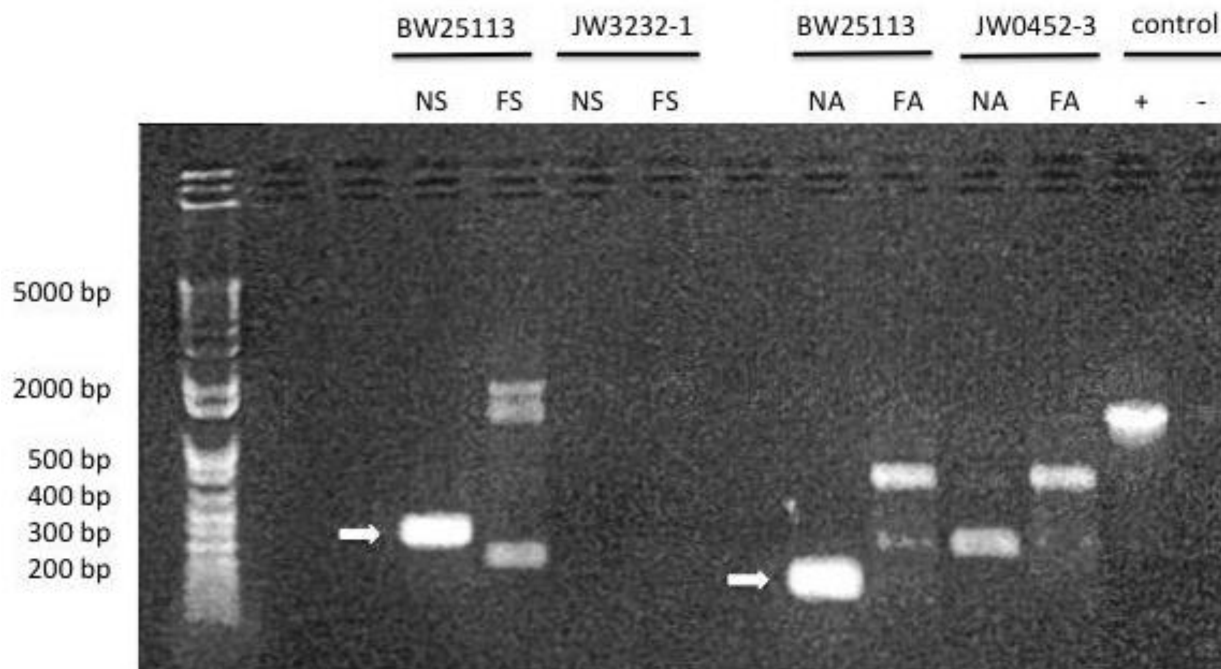

FIG 1 Agarose gel electrophoresis of colony PCR products of BW25113, JW3232-1, and JW0452-3 using *acrS* nested and flanking primers to confirm *acrS* deletion in JW3232-1 or *acrA* nested and flanking primers to confirm *acrA* deletion in JW0452-3. "NS" refers to *acrS* nested primers, "FS" refers to *acrS* flanking primers, "NA" refers to *acrA* nested primers and "FA" refers to *acrA* flanking primers. Water was used as the negative control and *rpoS* was used as the positive control.

platform set at 100 rpm. We measured OD_{600} at 20-30 minute intervals with a spectrophotometer (Ultrospec 3000 UV/Visible Spectrophotometer) using 1 ml quartz cuvettes to measure turbidity.

RESULTS

Confirmation of *acrS* deletion in *E. coli* JW3232-1 and *acrA* deletion in JW0452-3. Colony PCR and gel electrophoresis was performed to confirm *acrS* deletion in *E. coli* JW3232-1 and *acrA* deletion in JW0452-3 cells. As shown in Fig. 1, the BW25113 colony PCR using *acrS* nested primers produced a 300 bp fragment and *acrS* flanking primers produced 1500 bp and 200 bp fragments. The JW3232-1 colony PCR using *acrS* nested primers and flanking primers showed no bands. The BW25113 colony PCR using *acrA* nested primers produced a 200 bp fragment and *acrA* flanking primers produced a 400 bp fragment. The 200 bp fragment is the expected product size of *acrA*. The JW0452-3 colony PCR using *acrA* nested primers and flanking primers resulted in 300 bp and 400 bp bands respectively, but showed no bands in the 200 bp region. It was predicted that bands at 300 bp and 400 bp were due to unspecific primer binding since the bands were not in the expected *acrA* fragments. The negative control showed no bands, suggesting no contamination or self-annealing of DNA. Taken together, the results confirm the deletion of *acrS* in JW3232-1 and the deletion of *acrA* in JW0452-3.

***ΔacrS* shows increased kanamycin resistance compared to *E. coli* BW25113.** In order to test if *acrS* is a repressor of *acrA*, the kanamycin resistance between *ΔacrA, ΔacrS* and BW25113 was compared. Results from the first MIC assay, as shown in S. Table 2, were largely inconclusive; *ΔacrS* had a higher MIC in only one condition compared to BW25113 and there was no clear overall trend. The only notable trend was the decreased MIC seen in *ΔacrA.* These inconclusive results were likely due to the large increments between kanamycin concentrations from the serial dilution method.

The second MIC assay had the highest kanamycin concentration at 30 µg/ml and the lowest at 12.5 µg/ml. The second MIC assay showed *ΔacrS* with the highest MIC of over 30 µg/ml, BW25113 strain with a MIC of 27.5 µg/ml and *ΔacrA* with the lowest MIC of 20 µg/ml (Table 2). However, the MIC of *ΔacrS* could not be concluded from the second MIC assay because the value was beyond the range of the assay. As a result, a third MIC assay containing higher kanamycin concentrations was done.

TABLE 2 Second and third MIC assay results using *ΔacrA, ΔacrS*, and BW25113.

Strain	Second MIC Assay Kanamycin MIC (µg/ml)	Third MIC Assay Kanamycin MIC (µg/ml)
BW25113 (Wild-type)	27.5	27.5
JW3232-1 (*ΔacrS*)	>30	32.5
JW0452-3 (*ΔacrA*)	20	≤20

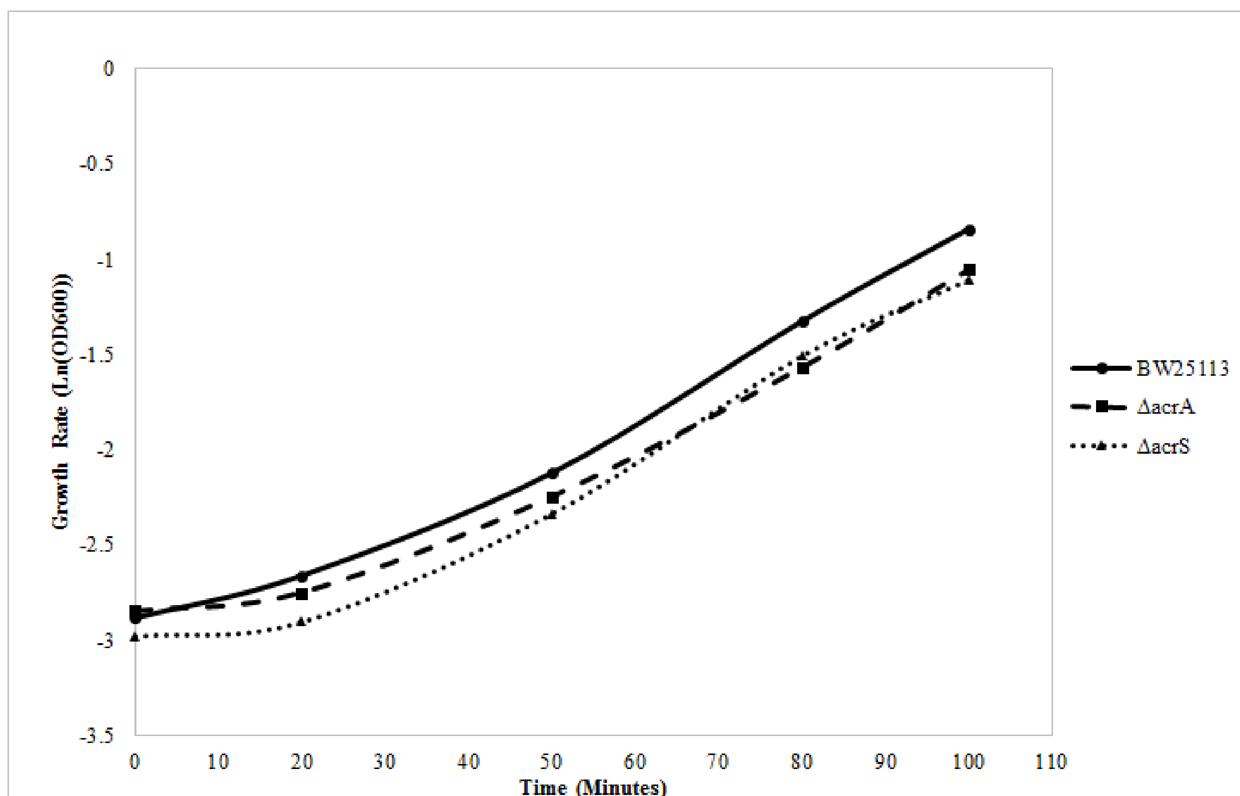

FIG 2 Growth kinetics of *E. coli* BW25113, *ΔacrS*, and *ΔacrA* strains grown at 37°C with OD_{600} measurements taken at 20 to 30 minute intervals. Growth rate is defined as the ln of the OD_{600} at a particular time point. Curves are present to help visualize data but do not represent a mathematical relationship.

For the third MIC assay, 2.5 µg/ml increments between kanamycin concentrations were used once again with 37.5 µg/ml as the highest concentration of kanamycin and 20 µg/ml as the lowest. The results for this experiment can be found in Table 2. Although one experimental 96-well plate showed a kanamycin MIC of 35 µg/ml for *ΔacrS*, the MIC was determined to be 32.5 µg/ml because both biological replicates grew at 30 µg/ml. *ΔacrA* failed to meet the modified pass conditions for both biological replicates in the third MIC assay. As such, it was determined that the MIC is less than or equal to 20 µg/ml. This supports the MIC of 20 µg/ml that was observed in the second MIC assay. MIC of 27.5 µg/ml was obtained for BW25113 and was also reported in the second MIC assay. The results show that the *ΔacrS* had an MIC that was consistently higher than that of BW25113. Additionally, the MIC of the *ΔacrA* strain is lower than the BW25113 strain. To ensure that none of the tested strains had growth advantages, growth kinetics were monitored as outlined in the methods. As shown in Fig. 2, the growth kinetics of *ΔacrS*, Δ*acrA* and *E. coli* BW25113 cells were similar, indicating that no strain had a growth advantage. Taken together, the MIC assay results suggest that the kanamycin resistance for *ΔacrS* is greater than *E. coli* BW25113 and that *ΔacrA* has a lower resistance than both *ΔacrS* and BW25113.

DISCUSSION

The objective of this paper was to determine whether AcrS is a potential repressor of *acrA* in *E. coli*. Based on the observation that AcrS binds to the same sequence on the *acrA* promoter recognized by the AcrR repressor (2), we hypothesized that AcrS is a repressor of *acrA* and that the deletion of *acrS* results in increased kanamycin resistance due to upregulation of *acrA* expression.

The MIC assay was used to compare kanamycin resistance between Δ*acrS,* Δ*acrA*, and BW25113 strains. If AcrS is a repressor of *acrA,* we expect higher MICs for Δ*acrS* compared to Δ*acrA* and BW25113 strains due to possible upregulation of *acrA* in Δ*acrS*, which leads to increased production of AcrA, a critical component of AcrAB-TolC. Table 2 shows that Δ*acrS* indeed had the highest MIC out of all the strains, which suggests that loss of *acrS* may lead to upregulation of *acrA* expression and results in higher expression of the AcrAB-TolC pump. However, a direct relationship between *acrS* and upregulation of *acrA* expression cannot be established since the MIC assays only provide qualitative measurements. Specifically, they only measure the phenotypic consequences of *acrS* deletion, and as such do not reveal what is occurring at transcriptional, translational, or posttranslational levels. We attempted to perform RT-qPCR in order to quantitatively measure the

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increase in *acrA* expression in Δ*acrS;* however, the results were inconclusive.

In addition, AcrS is suggested to be a repressor of *acrEF* operon (2). AcrEF-TolC is another drug-efflux pump that also has a broad substrate range, which includes kanamycin (2). Thus, the higher MIC observed for Δ*acrS* could be attributed to the upregulation of *acrEF*, as well as the upregulation of *acrA*.

The growth curve presented in Fig. 2 indicates that all three strains grew at similar rates. Interestingly, both Δ*acrS* and Δ*acrA* showed a lag period before exponential growth begins, which was not present for BW25113. We believe this further supports our MIC assay data and conclusions. Despite the slight growth disadvantage of Δ*acrS* and Δ*acrA*, Δ*acrS* continued to grow in higher kanamycin concentrations than BW25113.

In this paper, we report the MIC of kanamycin for BW25113 is 27.5 µg/ml, which is significantly higher than what was reported by Alian et al., who reported a MIC of 16 µg/ml for BW25113 (14). However, there are notable differences between our method and theirs. Alian et al. cultured their assay plates at 30°C and diluted their overnight cultures to an OD_{595} of 0.005 before plating, which has a two order magnitude difference from our method and may have led to the differences in the MIC observed for BW25113. Despite this difference, our method gives consistent and precise results, as evident from our second and third MIC assays. The MIC assays consisted of three independent experiments and were comparable across all experiments (Table 2). Alian et al. also reported a lower kanamycin MIC for Δ*acrA* compared to BW25113 and is confirmed in this study (14).

RT-qPCR was performed to measure the expression levels of *acrA* in *ΔacrS, ΔacrA*, and BW25113 strains, but the results were inconclusive as *GAPDH*, the positive control, was amplified in some samples and *acrA* did not amplify in any of the samples. Since *acrA* was not amplified in any samples, the hybridization temperature may be too high, preventing the *acrA* primers from annealing and amplifying *acrA*. The failure to amplify *acrA* is less likely due to incorrect primer sequence since Viveiros *et al.* performed RT-qPCR using the same primers and was able to detect *acrA* expression (17). The failure to amplify *GAPDH* may be due to using cDNA made from impure RNA, and less likely due to unoptimized cycling conditions and *GAPDH* primers since *GAPDH* was amplified in some samples. Future studies should be done to optimize the RT-qPCR cycling conditions and RNA extraction to study how *acrS* affects *acrA* expression.

In conclusion, our data indicate that the kanamycin resistance for Δ*acrS* is greater than BW25113 and Δ*acrA,* suggesting that AcrS may be a potential repressor of *acrA*. However, we could not conclude on whether increased kanamycin resistance of Δ*acrS* is due to the upregulation of *acrA* as no transcriptional analysis was conducted. Regardless, our study indicates that the loss of *acrS* is important for the intrinsic antibiotic resistance of *E. coli* and supports the results from previous investigations.

FUTURE DIRECTIONS

The minimum inhibitory concentration assays performed could only confirm that *ΔacrS* had a higher resistance to kanamycin than *ΔacrA* and BW25113, but did not fully explain how *acrS* may affect expression of *acrA*. Future studies should perform RT-qPCR again with the *E. coli* strains and qPCR primers (S. Table 3) used in this study. If AcrS is indeed a repressor of *acrA,* then Δ*acrS* strain would be expected to have the highest *acrA* expression as AcrS is not present to repress the constitutively expressed *acrA*. Δ*acrA* strain would have no *acrA* expression as the *acrA* gene is deleted and there should be no amplification of *acrA*. *E. coli* BW25113 should show the baseline level of *acrA* expression under normal conditions since AcrS and *acrA* are both present in this wild-type strain.

In addition, kanamycin was the only antibiotic used in this study to evaluate differences in antibiotic resistance between strains. Other classes of antibiotics, such as penicillins, ansamycins, or tetracyclines, should be tested to fully elucidate the role of *acrS* in regulating *acrA* and to confirm the trend of higher MICs of Δ*acrS* mutants. The AcrAB efflux pump system has been shown to be responsible for the resistance to multiple classes of drugs including ampicillin, rifampicin, tetracyclines, chloroamphenol, and nalidixic acid (18). If AcrS represses *acrA* expression and affects the function of AcrAB-TolC pump, the Δ*acrS* strain should also show a higher resistance to these classes of antibiotics compared to Δ*acrA* and wild-type *E. coli.*

As well, this study focused on the AcrAB-TolC multi-drug efflux pump. AcrEF-TolC complex is a paralog of AcrAB-TolC in *E. coli* that is expressed at low levels (15). Considering the homology between AcrAB and AcrEF, *acrS* might also be involved in the regulation of AcrEF-TolC complex. A similar study should be done to investigate the role of *acrS* in the AcrEF-TolC efflux pump complex using *ΔacrS* and *ΔacrEF.*

Lastly, our paper described higher minimum inhibitory kanamycin concentrations for both *ΔacrA* and BW25113 than reported by both Alian et al. (14) and Sidhu et al. (16). It could be of interest to use BW25113 to compare our MIC assay method to theirs to see if the discrepancy can be replicated. If it can be replicated, it is also worth investigating why such differences occur, so that the most accurate MIC assay method can be used for future studies.

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