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Plasmid-Mediated Overexpression of AcrS May Decrease Kanamycin Resistance in *Escherichia coli*

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SUMMARY *Escherichia coli* encodes efflux pumps that confer resistance to antimicrobial compounds. Previous studies have shown that efflux pumps AcrAB and AcrEF have similar structures and functions, and may be repressed by homologous transcriptional repressors. The transcriptional repressor AcrR, represses the *acrAB* operon. A gene encoding a less-studied putative transcriptional repressor AcrS, and *acrR* are both located upstream of *acrEF* and *acrAB*, respectively. Deleting *acrS* in *Escherichia coli* has also shown to result in increased levels of *acrE* mRNA when cultured in the presence of sub-inhibitory concentrations of kanamycin. Given this evidence that AcrS may repress *acrE*, we hypothesize that increasing AcrS expression will result in increased repression of *acrE* and confer a decrease in kanamycin resistance. An inducible expression vector containing *acrS* was constructed and transformed into wild-type *E. coli* strain BW25113, as well as strains bearing deletions of *acrS* or *acrE*. Resistance to kanamycin was tested using a minimum inhibitory concentration assay. Results from this study suggest that overexpressing AcrS decreased kanamycin resistance in *E. coli* BW25113. This supports the hypothesis that AcrS may be a transcriptional repressor of *acrE* in *E. coli* BW25113.

INTRODUCTION

Multidrug efflux pumps are one of the primary mechanisms underlying antibiotic resistance in *Escherichia coli* (1). Efflux pumps are constitutively expressed in stress-free conditions, but can be upregulated in the presence of antimicrobial compounds, such as antibiotics, dyes, and detergents (2, 3). In Gram-negative bacteria, these multidrug efflux pumps are part of the resistance-nodulation-division (RND) superfamily which include AcrAB and AcrEF (4). Both AcrAB and AcrEF are encoded on their respective *acrAB* and *acrEF* operon (5). These pumps function as a tripartite system and are composed of an inner membrane component, a periplasmic component, and an outer membrane channel; TolC is the common outer membrane channel for various RND pumps in *E. coli* and functions with both AcrAB and AcrEF (4). AcrA and AcrE are the periplasmic proteins, while AcrB and AcrF are the inner membrane channels (4, 5). The well-characterized transcriptional repressor AcrR, and putative repressor AcrS, are transcribed divergently upstream of *acrAB* and *acrEF*, respectively (5).

AcrB and AcrF have a 77% amino acid sequence similarity and both function as multidrug efflux pumps (6). Given this, AcrR and AcrS are proposed to have analogous functions (6). In previous studies, AcrS was shown to repress transcription of both *acrAB* and *acrEF* (5, 6). Belmans *et al.* tested these results by deleting *acrS* on kanamycin resistance in *E. coli* BW25113 (7). In their study, deleting *acrS* notably resulted in increased kanamycin resistance. However, expression levels of *acrA* and *acrE* were not measured and therefore the increase of kanamycin resistance could not be attributed to either or both AcrAB and AcrEF. Hay *et al.* measured the expression levels of *acrA* and *acrE* in *E. coli* Δ*acrS*Δ*kan* in the presence of sub-inhibitory concentrations of kanamycin to determine which efflux pump is

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responsible for resistance (2). They determined that *acrE* expression increased whereas *acrA* expression levels remain unchanged. These results show that in *E. coli*, AcrEF is possibly induced by kanamycin whereas AcrAB is not. Based on this data, we hypothesized that overexpressing the putative transcriptional repressor AcrS will result in increased repression of *acrE,* conferring a decrease in kanamycin resistance.

METHODS AND MATERIALS

Bacterial strains. *E. coli* BW25113 and mutants JW3232-1 (Δ*acrS*Δ*kan*) and JW3233-2 (Δ*acrE*Δ*kan*) (hereinafter referred to as *E. coli* Δ*acrS*Δ*kan* and Δ*acrE*Δ*kan*, respectively) were obtained from the Microbiology and Immunology Department at the University of British Columbia. *E. coli* BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan* were streaked on LB agar with and without 50 µg/ml kanamycin to screen for kanamycin sensitivity.

Genotypic confirmation. Primers used to confirm whether *acrS* was present or absent in all three strains were provided by Hay *et al.* (Table S1) (2). Genomic DNA was isolated from 1 mL overnight cultures of *E. coli* BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan* with PureLink® Genomic DNA Mini Kit (Invitrogen) as per the manufacturer's instructions. DNA concentrations were quantified with a Nanodrop3000 spectrophotometer. Genomic DNA from each strain was used as a template to amplify *acrS.* 50 µl PCR reactions were performed containing 5 µl 10X PCR Buffer - Mg, 1.5 µl 50 mM $MgCl_2$, 1 µl of 10 mM dNTP mix, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 0.2 µl of Platinum® Taq DNA Polymerase (Invitrogen), 1 µl of DNA template and 39.3 µl of sterile water. Positive controls consisted of pUC19 template DNA and its respective primers, while template DNA was replaced with sterile water for the negative controls. The T100 thermocycler (Bio-Rad Laboratories) conditions were programmed for an initial denaturation at 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 20 seconds, with a final extension at 72°C for 3 minutes. The PCR products were loaded on a 1% agarose gel stained with SYBR® Safe DNA gel stain in 0.5X TBE (Invitrogen) and subjected to electrophoresis in 1X TBE buffer at 100 V for 40 minutes. Amplicons were visualized and imaged with UV light using the Alpha Imager (ProteinSimple).

Induction of pBAD24sfGFPx2 with L-arabinose. To confirm that pBAD24 was inducible with L-arabinose, GFP fluorescence in *E. coli* BW25113 containing pBAD24sfGFPx2 was measured after L-arabinose induction. 3 mL overnight cultures were diluted 1:100 and regrown until OD_{600} reached 0.1-0.2. The cultures were then induced by adding 10% w/w L-arabinose stock solution to achieve final concentrations of 10%, 1%, 0.1%, 0.01%, 0.001%, and 0% L-arabinose followed by growth at 37°C for three hours. The control consisted of cells with 0% L-arabinose to visualize baseline expression of GFP. The presence of fluorescence was tested with an Axiostar Plus microscope. 200 µl of each culture was placed into a 96-well plate, and GFP fluorescence was quantified with the Infinite® 200 Pro Series Tecan microplate reader. A control for the plate reader was not included. Excitation and emission wavelengths were set at 488 nm and 510 nm, respectively.

Construction of pBAD24::*acrS*. Primers used to amplify *acrS* were designed using the parent strain BW25113 (NCBI accession number CP009273) and NEBuilder Assembly Tool. Two sets of primers were ordered from Integrated DNA Technologies, one set to amplify *acrS* from *E. coli* BW25113 for Gibson Assembly® (New England Biolabs) and the other to verify proper insertion of *acrS* into pBAD24 (Table S1) (8).

PCR amplification of *acrS* was performed in 50 µl reactions containing 10 µl of 5X Phusion HF buffer, 1 µl of 10 mM dNTPs, 2.5 µl of 10 uM forward primer, 2.5 µl of 10 uM reverse primer, 1 µl of template DNA, 1.5 µl of DMSO, 0.5 µl of Phusion® High-Fidelity DNA Polymerase (New England Biolabs), and 31 µl of sterile water. Positive controls consisted of pUC19 template DNA and its respective primers, while template DNA was replaced with sterile water for the negative controls. Thermocycler conditions were programmed for an initial denaturation at 98°C for 30 seconds, followed by 30 cycles of

denaturation at 98°C for 10 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. The PCR products were loaded on a 1% agarose gel stained with SYBR® Safe DNA gel stain in 0.5X TBE (Invitrogen) and subjected to electrophoresis in 1X TBE buffer at 60 V for 1.5 hours. Amplicons were visualized and imaged with UV light using the Alpha Imager (ProteinSimple). The PCR product was purified using the Purelink® PCR Purification Kit and DNA concentration was quantified using a Nanodrop3000 spectrophotometer.

E. coli DH5α containing pBAD24 was inoculated into 3 mL LB-ampicillin and grown overnight at 37°C. As per the manufacturer's instructions, pBAD24 was isolated using the PureLink® Quick Plasmid Miniprep Kit (Invitrogen). Plasmid concentration and purity were quantified using a Nanodrop3000 spectrophotometer. Restriction enzyme double digestion was performed to linearize pBAD24 for plasmid construction. The 50 µl reaction contained 1 µl of Xba1, 1 µl of Nco1, 27 µl of isolated pBAD24 DNA, 5 µl of 10X NEBuffer, 16 µl sterile water, and was incubated at 37°C for 1 hour.

Insertion of purified *acrS* DNA into the digested pBAD24 plasmid was performed using the Gibson Assembly® Cloning Kit. The 20 µl reaction volume included 1.35µl of pBAD24, 2.83 µl of *acrS* DNA, 10 µl of 2X Gibson Assembly Master Mix, and 5.82 µl of sterile water. A positive control was prepared in a 20 µl reaction containing 10 µl of Positive Control solution provided with the kit and 10 µl of 2X Gibson Assembly Master Mix. The samples were incubated in the thermocycler at 50°C for 60 minutes. The samples were transformed as per the manufacturer's instructions into chemically competent DH5α cells, plated on LB-ampicillin plates supplemented with 1% glucose, and incubated overnight at 37°C.

pBAD24::*acrS* (hereinafter referred to as pMAPS) constructed from Gibson Assembly was isolated from all isolated colonies using PureLink® Quick Plasmid Miniprep Kit and DNA concentration and purity were quantified using a Nanodrop3000 spectrophotometer. 10 µl of plasmid DNA from each clone was transferred into separate PCR tubes and sent for Sanger sequencing at GENEWIZ. The nucleotide sequence of pMAPS was analyzed using the bioinformatics tool Geneious, for detection of mutations. The universal pBAD24 forward and reverse primers option was selected for all reactions.

Growth curve. Overnight cultures of *E. coli* BW25113 containing pBAD24 (empty vector), pMAPS, or pBAD24sfGFPx2 were diluted 1:100 in 6 mL LB-ampicillin media. The pBAD24 (empty vector) served as a control to test whether regular expression levels resulted in any toxicity. L-arabinose was added to one set of cultures to a final w/w concentration of 1% while another set of cultures were not induced with L-arabinose. All samples were incubated on a shaking platform at 37˚C until cells reached stationary phase, indicated by plateaued OD_{600} readings. OD_{600} values were measured at 30 minute intervals in a 96-well plate using the Microplate Reader Model 3550 (Bio-Rad Laboratories).

Minimum inhibitory concentration of kanamycin. The method for the MIC assay was adapted from Hay *et al.*'s protocol (2). *E. coli* BW25113 containing pMAPS, Δ*acrS*Δ*kan* containing pMAPS, Δ*acrS*Δ*kan* containing pBAD24, and Δ*acrE*Δ*kan* containing pMAPS were grown overnight in 3 mL LB-ampicillin media, followed by a 1:100 dilution. The strains were grown until OD_{600} values were between 0.1-0.2 at which point L-arabinose was added to a final w/w concentration of 1%, followed by incubation at 37°C for an additional hour. 2-fold serial dilutions of kanamycin were used to achieve the following concentrations: 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, and 0.4 µg/ml. Each strain was inoculated in duplicate in a 96-well plate with LB-ampicillin supplemented with or without 1% L-arabinose. 5 µl of the appropriate cultures were added to the corresponding wells. One column containing only LB-ampicillin media was included as a negative control. The plates were grown overnight for 24 hours at 37˚C. Growth was detected visually and confirmed with Microplate Reader Model 3550 (Bio-Rad Laboratories).

RESULTS

***E. coli* BW25113, Δ*acrS*Δ*kan,* and Δ*acrE*Δ*kan* matched the expected genotypes.** In order to compare kanamycin resistance when AcrS is overexpressed, kanamycin sensitivity was

determined. BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan* were streaked on LB-agar plates containing 50 µg/mL kanamycin. No growth was observed, confirming that all three strains were kanamycin sensitive (data not shown). To determine the presence or absence of *acrS* in the strains, PCR was performed to amplify *acrS* and the resulting amplicons were visualized via agarose gel electrophoresis (Figure 1). PCR amplification of *acrS* in the three strains produced the expected results. BW25113 and Δ*acrE*Δ*kan* were expected to have the same amplicon size of approximately 321 bp since both strains contain *acrS*. No band was expected for Δ*acrS*Δ*kan* because *acrS* is absent in the genome. Our results indicated that BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan* matched their expected genotypes.

Induction of pBAD24sfGFPx2 with L-arabinose. Green fluorescent protein (GFP) fluorescence was observed in BW25113 cells containing pBAD24sfGFPx2 to confirm that the *araBAD* promoter is inducible with L-arabinose. After inducing the cells with varying concentrations of L-arabinose, the cells were observed under a fluorescence microscope. Cells were visibly green for all concentrations of L-arabinose except the control (data not shown). Fluorescence was quantified, and the results show that 1% L-arabinose is sufficient for induction of gene expression from the pBAD promoter.

Construction of pMAPS expression vector. To insert *acrS* into pBAD24, *acrS* was amplified from genomic DNA of strain BW25113. PCR amplification of *acrS* using overhang *acrS* primers produced the expected band size of approximately 663 bp (Figure 2). The purified PCR amplicons were then inserted into pBAD24 using the Gibson Assembly method (8). Transformation of the assembled plasmids into chemically competent cells with subsequent selection on LB-ampicillin plates resulted in six colonies. Sanger sequencing of the isolated plasmids from the six colonies indicated that only one colony (colony E) contained wild-type *acrS* (Table S2). The other five colonies contained an identical missense mutation leading to a codon change of alanine to threonine at position 2. This mutation was

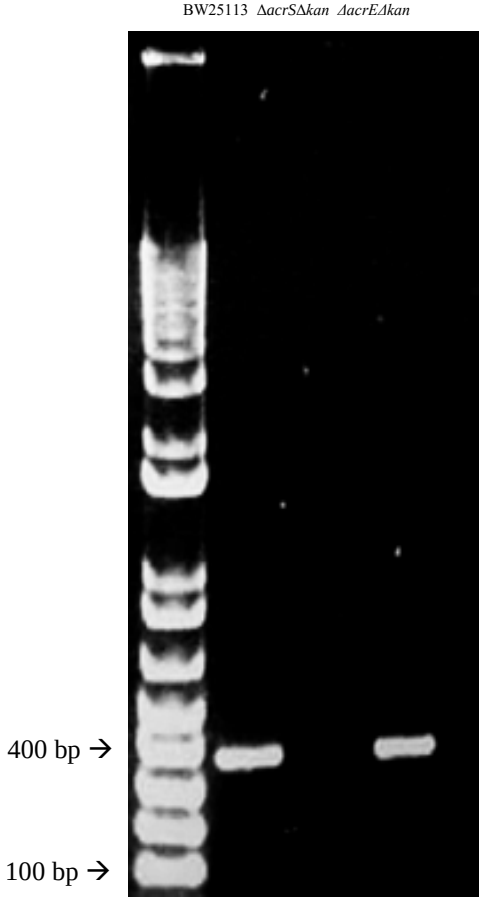

FIG. 1 PCR products of various strains run on 1% agarose gel stained with SYBR® Safe DNA Gel Stain run in 1X TBE for 90 minutes at 60V. Primers targeted a sequence within the *acrS* gene.

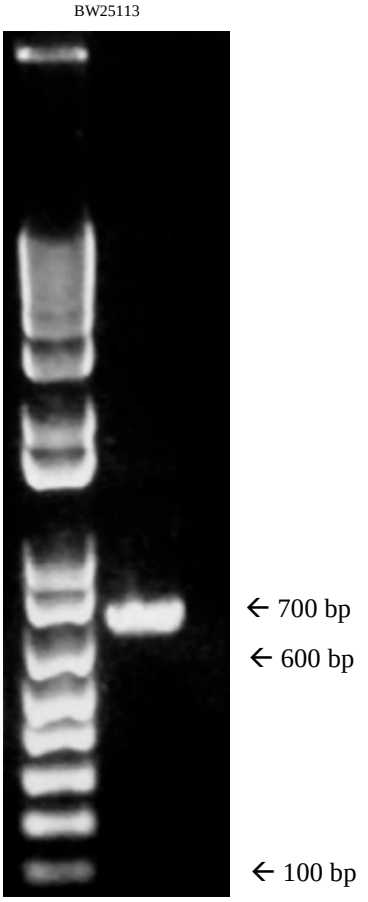

FIG. 2 PCR product of BW25113 run on 1% agarose gel stained with SYBR® Safe DNA Gel Stain run in 1X TBE for 90 minutes at 60V. Primers contained overhang sequences complementary to pBAD24 for downstream Gibson assembly reaction.

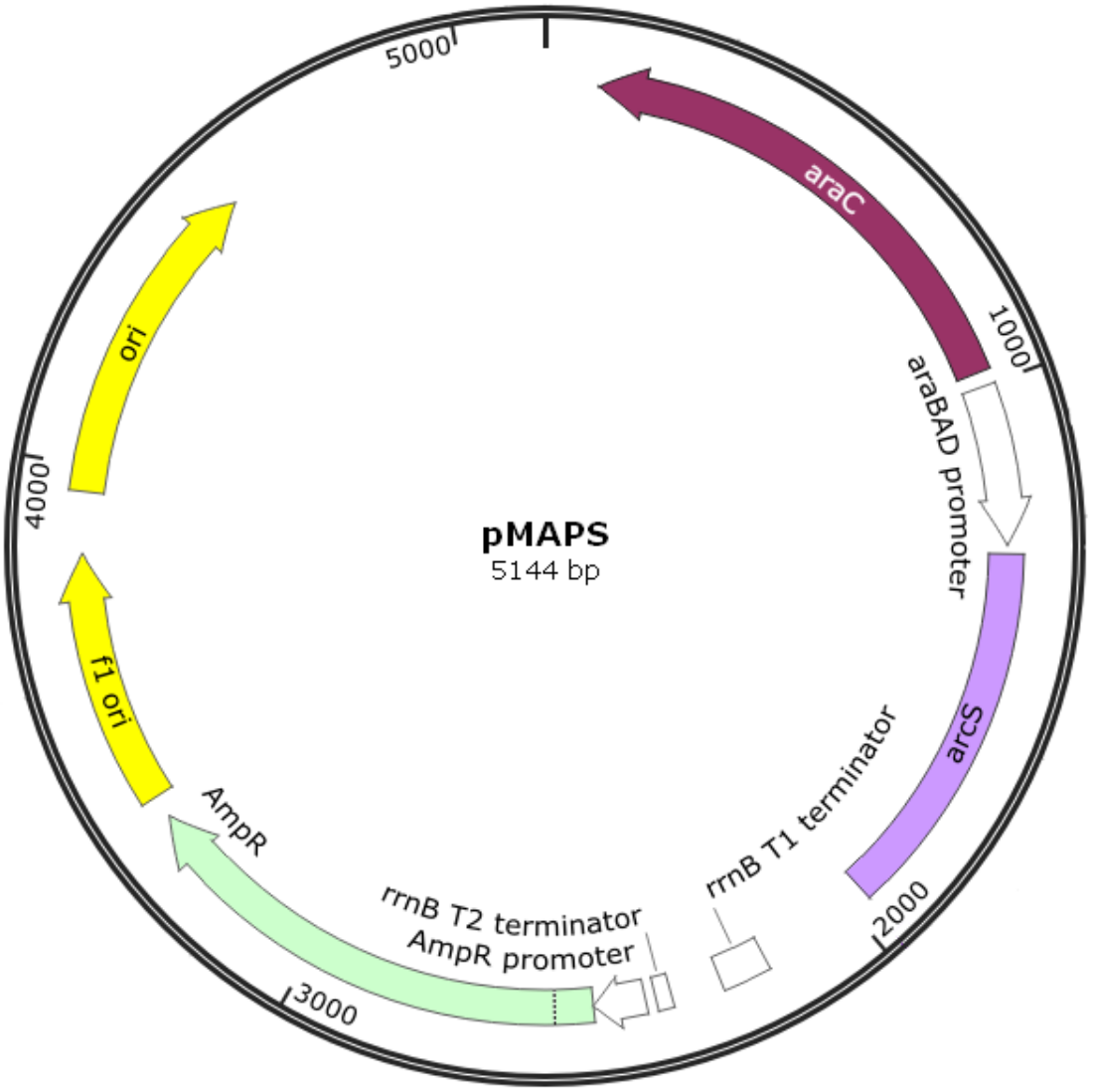

FIG. 3 Plasmid map for pMAPS. Final product after performing Gibson assembly with pBAD24 and amplified *acrS*, contains arabinose inducible promoter, and ampicillin selection marker.

located at the beginning of the *acrS* gene that overlapped with the respective primer sequence. The insertion of *acrS* into pBAD24 resulted in the construction of the intended expression vector, pMAPS (Figure 3). PCR was also performed using flanking *acrS* primers on isolated pMAPS to reconfirm the presence of *acrS* in the plasmid (Table S1). Gel electrophoresis of the amplicons on 1% agarose gel produced a band of approximately 663 bp confirming that *acrS* was inserted into pMAPS (data not shown).

Growth rates of *E. coli* BW25113 with pMAPS, pBAD24 and pBAD24sfGFPx2 in the presence and absence of L-arabinose are comparable. To test whether the presence of 1% L-arabinose and overexpression of AcrS were toxic to *E. coli* BW25113 cells, a growth curve was generated for BW25113 containing pMAPS, pBAD24 and pBAD24sfGFPx2. The cells were cultured in LB with 0% and 1% L-arabinose concentrations (Figure 4). The empty vector (pBAD24) served as a negative control. Growth curves of strains transformed with pMAPS and pBAD24 were similar in the 0% L-arabinose condition. The growth rate of strains transformed with pMAPS was slightly higher than pBAD24 induced with 1% L-arabinose (Figure 4). Cells containing pMAPS had similar growth rates in the presence and absence of L-arabinose, indicating that L-arabinose was not toxic. The similar growth rates of cells

transformed with pMAPS in the presence and absence of inducer showed that the overexpression of AcrS with L-arabinose did not inhibit cell growth. Lastly, the similarity between the growth curves of cells containing pBAD24sfGFPx2 or pMAPS suggested that overexpression of an alternative protein, GFP, resulted in a similar effect as overexpression of AcrS. These data further support the conclusion that overexpression of AcrS is not toxic to *E. coli* BW25113.

Increased expression of AcrS in *E. coli* BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan* containing pMAPS decreases the MIC of kanamycin. To measure the effect of overexpression of AcrS on kanamycin resistance, MIC assays were performed on strains BW25113 + pMAPS, Δ*acrS*Δ*kan* + pMAPS**,** Δ*acrS*Δ*kan* + pBAD24, and Δ*acrE*Δ*kan* + pMAPS (Table 1). Growth inhibition was scored with an overnight OD_{600} value of 0.1 or less. Our results suggest that strains BW25113 + pMAPS, Δ*acrS*Δ*kan* + pMAPS and Δ*acrE*Δ*kan* + pMAPS show decreased resistance to kanamycin when AcrS expression was induced with L-arabinose. Notably, Δ*acrE*Δ*kan* + pMAPS had the greatest decrease in MIC. Δ*acrS*Δ*kan* with pBAD24 showed the greatest resistance to kanamycin, although there was an unexpected increase of the MIC of kanamycin in the presence of L-arabinose (Table 1). Lastly, OD_{600} values of Δ*acrE*Δ*kan* + pMAPS did not consistently increase when subjected to decreasing kanamycin concentrations (Figure S2). Overall, these data suggest that expression of AcrS results in decreased kanamycin resistance. However, induction of AcrS expression in Δ*acrE*Δ*kan* resulted in decreased kanamycin resistance. This suggests that AcrS could potentially also repress other genes involved in kanamycin resistance, although future studies will need to be done to investigate this unexpected result.

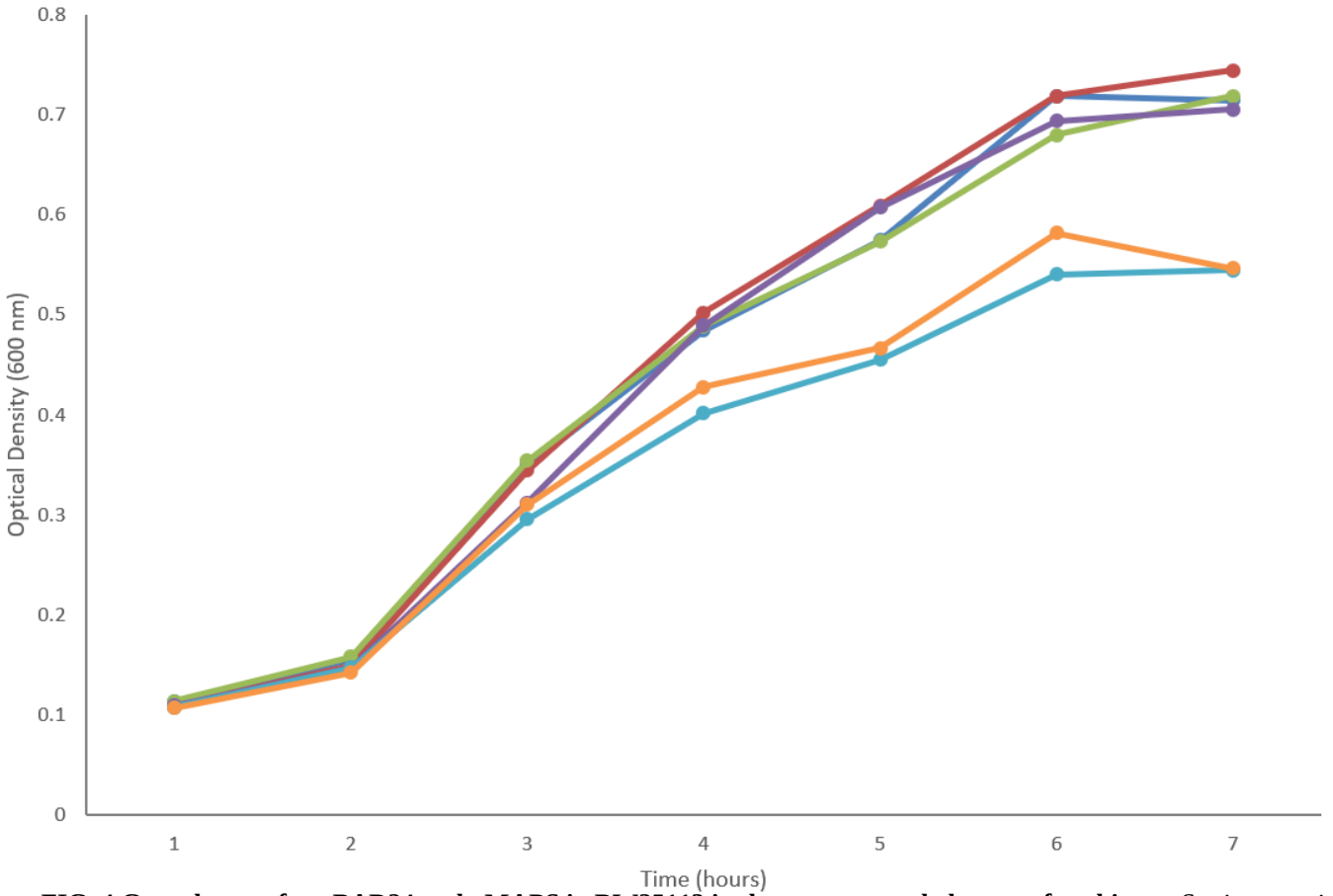

FIG. 4 Growth rates for pBAD24 and pMAPS in BW25113 in the presence and absence of arabinose. Strains contained pMAPS in 0% arabinose (blue), pBAD24 in 0% arabinose (red), pBAD24sfGFPx2 in 0% arabinose (green), pMAPS in 1% arabinose (purple), pBAD24 in 1% arabinose (teal), and pBAD24sfGFPx2 in 1 % arabinose (orange). Optical density values measured by using the Ultrospec 3000 UV/Visible Spectrophotometer.

DISCUSSION

The objective of our study was to determine whether overexpression of AcrS, a putative transcriptional repressor of AcrE, affects kanamycin resistance in *E. coli*. Two models have been previously proposed to describe the relationship between AcrS and kanamycin resistance (2, 7). Figure 5 depicts the various models of efflux pump regulation systems in relation to kanamycin resistance in *E. coli*. Belmans *et al*. proposed a model where AcrS is a repressor of *AcrAB* (Figure 5A) (7). Their results supported this model by showing decreased kanamycin resistance when *acrA* was deleted and increased kanamycin resistance when *acrS* was deleted. However, mRNA levels of *acrE* were not measured when *acrS* was deleted. Therefore, it could not be determined whether AcrS represses *AcrAB*, *AcrEF*, or both operons. To determine which operon is repressed by AcrS, Hay *et al.* measured mRNA levels of *acrA* and *acrE* in *acrS* deletion mutants (2). In their mutant, no change of *acrA* and increased *acrE* mRNA expression was observed. Their data suggested a new model (Figure 5B) where AcrS solely represses *acrEF* and not *acrAB*.

To test this new model, we sought the effect of kanamycin resistance from AcrS overexpression. This was performed by constructing an arabinose-inducible plasmid carrying the amino acid coding sequence of *acrS*. This plasmid was transformed into wild-type *E. coli* strain BW25113 as well as two strains bearing either a deletion of *acrS* or *acrE*. The expression vector was constructed using the medium copy number pBAD24 plasmid (9). This plasmid contains the *araBAD* promoter which is inducible by L-arabinose; in its absence, transcription occurs at very low levels (10). Conversely, glucose decreases the concentration of the catabolite 3',5'-cyclic AMP which further decreases transcription of the gene insert, resulting in a tightly regulated expression system (10). This expression vector also encodes an ampicillin resistance gene which serves as a selection marker (11).

Overexpression of proteins can lead to cell death (12). In addition, plasmid copy numbers can also decrease growth rate of cells due to a metabolic burden (13). To test whether pMAPS and overexpression of AcrS affects the growth rate of *E. coli* and thereby influence MIC assays, a growth curve was performed. Our data suggests that the presence of either or both pMAPS and AcrS expression does not impair growth in *E. coli* (Figure 4). Given this, our MIC assay results were likely not confounded by cell growth rates.

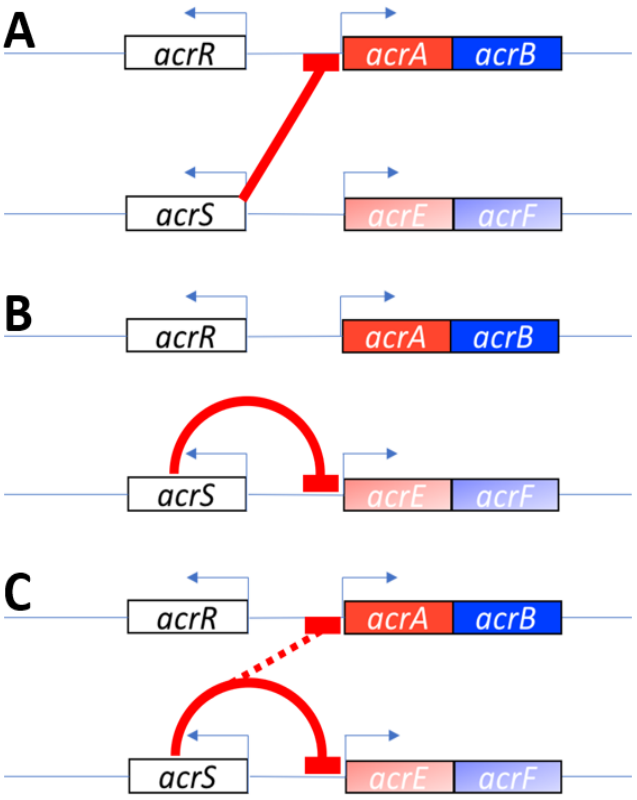

FIG. 5 Various models for the regulation by AcrS. (A) AcrS repressing only the *acrAB* operon. (B) AcrS repressing only the *acrEF* operon. (C) Overexpressed AcrS repressing both *acrEF* and *acrAB*. Dashed line indicates low-binding affinity, only present under high concentrations of AcrS. Solid line indicates binding at regular biological conditions.

Given that *acrS* deletion in *E. coli* BW25113 resulted in increased kanamycin resistance, we hypothesized that overexpression of AcrS would result in decreased kanamycin resistance (2). Since RND efflux pumps require a periplasmic component to function in antibiotic exudation, the deletion of the periplasmic component AcrE, was expected to result in no change of kanamycin resistance with AcrS overexpression (4). MIC assays were performed on strains BW25113 + pMAPS, Δ*acrS*Δ*kan* + pMAPS, Δ*acrS*Δ*kan* + pBAD24 (empty vector), and Δ*acrE*Δ*kan* + pMAPS to determine the effect of AcrS overexpression on kanamycin resistance (Table 1). This experiment was only performed once, therefore the obtained data is preliminary and should be analyzed cautiously.

Based on our working model shown in Figure 5B, we expected Δ*acrS*Δ*kan* + pBAD24 to show the highest level of kanamycin resistance which would not change with the introduction of inducer (L-arabinose) because the putative transcriptional repressor AcrS, is absent in this strain. In the Δ*acrS*Δ*kan* + pMAPS, we predicted that AcrS expression would result in a decrease of kanamycin resistance since *acrS* was complemented with pMAPS. Lastly, we expected BW25113 + pMAPS to show a lower kanamycin resistance than Δ*acrS*Δ*kan* + pMAPS due to basal level expression of *acrS* from the native chromosomal copy of the gene.

Consistent with this model, in the absence of L-arabinose inducer, the kanamycin MIC of strains BW25113 + pMAPS, Δ*acrS*Δ*kan* + pMAPS, Δ*acrE*Δ*kan* + pMAPS and Δ*acrS*Δ*kan* + pBAD24, were 18.8, 25.0, 12.5, and 18.8, respectively (Table 1). BW25113 was more sensitive than the Δ*acrS*Δ*kan* strain, which is consistent with expected basal level expression of AcrS from the genomic copy in the BW25113 strain. The MIC of the *ΔacrE*Δ*kan* strain was approximately 2-fold lower than the BW25113 and *ΔacrS*Δ*kan* strains, which was expected due to the absence of the necessary AcrE pump component. The observation that the *ΔacrS*Δ*kan* mutant containing the empty vector showed a similar MIC to the BW25113 strain suggests that under the conditions tested, the plasmid copy of *acrS* did not notably decrease kanamycin resistance.

With the addition of inducer (1% L-arabinose), the MIC of BW25113 + pMAPS, Δ*acrS*Δ*kan* + pMAPS, Δ*acrE*Δ*kan* + pMAPS and Δ*acrS*Δ*kan* + pBAD24, were 7.8, 18.8, 0.6, and 25.0 µg/mL, respectively (Table 1). As expected, induction of *acrS* resulted in a decrease of MIC for the BW25113 and *ΔacrS*Δ*kan* strains which is consistent with the proposed role of AcrS as a repressor of the gene encoding AcrE. Consistent with this observation, induction of *ΔacrS*Δ*kan* + pBAD24 did not show a decrease in the MIC. Interestingly, while we expected kanamycin resistance of the *ΔacrE*Δ*kan* mutant to remain constant in the presence of inducer, we observed a large decrease in the MIC from 12.5 (uninduced) to 0.6 (induced) µg/mL. This is inconsistent with the model (Figure 5B) in which AcrS specifically represses AcrE. Therefore, we propose a novel model that could possibly explain this observation (Figure 5C). In our model, biological concentrations of AcrS in the

TABLE 1 Minimum inhibitory concentrations of kanamycin for strains grown in the presence (1%) or absence (0%) of L-arabinose inducer. Growth inhibition was assessed as wells that did not reach optical density values greater than 0.1. Concentrations were tested in duplicate for each strain. Data are from one experiment only.

	MIC Kanamycin (µg/mL)	
***E. coli* strain**	**Uninduced**	**Induced**
BW25113 + pMAPS	18.8	7.8
Δ*acrS*Δ*kan* + pMAPS	25.0	18.8
Δ*acrE*Δ*kan* + pMAPS	12.5	0.6
Δ*acrS*Δ*kan* + pBAD24	18.8	25.0

presence of kanamycin preferentially represses *acrEF*, but overexpression of AcrS may result in binding to low-affinity operator regions, thereby repressing expression of alternate efflux pump operons. It is conceivable that AcrS negatively regulates other genes responsible for resistance to kanamycin. Overexpressing well-known transcriptional repressors of other genes which result in unexpected results are not uncommon (14). Authors from a previous study suggest that other factors are likely involved with the transcriptional control of their gene of interest to explain their results (14). However, it is also reasonable that our results may simply be experimental artifact due to AcrS overexpression. This experiment should be repeated to test our new proposed model.

It is worth noting that Hay *et al.* also obtained unexpected MIC values for Δ*acrE*Δ*kan* (2). In their study, *acrS* was deleted and not overexpressed. Our study followed their MIC assay protocol, however they determined MIC values by visual confirmation whereas we quantified OD values. Their Δ*acrE*Δ*kan* strain resulted in inconsistent kanamycin MIC values of 3.1 µg/mL and 6.3 µg/mL.

While our proposed model may be compelling, it should be viewed cautiously until the kanamycin MIC experiment has been repeated. Due to time constraints, the kanamycin MIC assay could only be performed once with duplicate samples for each concentration. Moreover, MIC assays were not performed with an Δ*acrA*Δ*kan* strain. Therefore, at this moment, whether AcrS represses *acrAB* under high concentrations cannot be proposed with confidence. Lastly, kanamycin MIC values from our study may not be very precise given that few antibiotic concentrations were used in our assay.

In conclusion, we have created (1) a novel vector (pMAPS), and (2) transformed *E. coli* strains with this plasmid enabling regulator induction of AcrS expression. Using these strains, we have tested whether induction of AcrS is sufficient to decrease kanamycin resistance. Our data shows that induction of AcrS is sufficient to decrease kanamycin resistance, which is consistent with our hypothesis that AcrS represses *acrE*. However, a decrease of the MIC in the *acrE* mutant following induction of AcrS was not expected. The results suggest that high concentrations of AcrS may repress other genes involved in kanamycin resistance, such as *acrAB*. In this case, overexpressing AcrS may cause it to function similarly to other repressor proteins and bind to their respective efflux pump operons. Whether this is a biological function of AcrS in *E. coli* or an experimental artifact due to overexpression is unknown.

Future Directions In this study, we have presented preliminary data suggesting that expression of AcrS in *E. coli* strain BW25113 and Δ*acrS*Δ*kan* may result in decreased minimum inhibitory concentrations of kanamycin. Future studies should repeat the MIC assays with more replicates, specifically focusing on the effects of AcrS overexpression in Δ*acrE*Δ*kan* mutants. Additional concentrations of kanamycin at equal intervals (50 ug/mL, 40 ug/mL, 30 ug/mL etc.) may also be used to determine a more precise MIC of kanamycin. Furthermore, studies could determine the relative abundance of AcrE and AcrA in *E. coli* BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan* when pMAPS is expressed in the presence and absence of L-arabinose. This could be performed by a Western Blot. If the intensity of the AcrE protein band decreases when AcrS is overexpressed compared to no overexpression, the results in our current study that suggest AcrS represses *acrE* would be supported.

To examine our proposed model, *acrE* and *acrA* mRNA expression levels could also be measured by RT-qPCR when AcrS is overexpressed. If our proposed model that AcrS preferentially binds to the *acrEF* operon is correct, we should see a decrease in both *acrA* and *acrE* mRNA levels at high expression of AcrS. Conversely, a low expression of AcrS should only decrease *acrE* while *acrA* mRNA levels should remain constant. An alternative project could also investigate at a double mutant of *acrA* and *acrE* in *E. coli* BW25113 overexpressing AcrS to test whether alternate mechanisms of kanamycin resistance exist. A confirmation that AcrS indeed binds to either *acrEF* and *acrAB* operons can be done using a chromatin immunoprecipitation (ChIP) assay.

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CONTRIBUTIONS

All four authors claim equal contribution to this manuscript and are considered co-first authors.

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