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Generation of an *acrAacrE* double-knockout in *Escherichia coli* and its role in kanamycin resistance

Ada Ang, Cathy Park, Joshua De Guzman, Shaneel S. Kumar

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY AcrAB and AcrEF are multidrug efflux pumps found in *Escherichia coli* that are thought to mediate the export of kanamycin, and therefore contribute to kanamycin resistance. Previous studies found that expression of *acrE* increased in response to increasing concentrations of kanamycin. Another study found that the loss of *acrA* or *acrE* was shown to have no effect on kanamycin resistance, indicating that AcrAB and AcrEF may have compensatory effects on each other. The goal of this study was to generate an *acrAacrE* double-knockout in *E. coli* and determine if this double-knockout conferred any differences in kanamycin resistance compared to the single-knockouts and wild-type. We hypothesized that by knocking out both the *acrA* and *acrE* genes, no compensatory effects would occur to support kanamycin resistance. To test this, we first generated an *acrAacrE* double-knockout by using lambda Red (λ-Red) recombination to remove the *acrA* gene from an *acrE* single-knockout strain. To determine differences in kanamycin resistance, MIC assays were performed on all the strains. The *acrAacrE* double-knockout was successfully generated and Sanger sequencing confirmed the deletion of *acrA*. However, MIC results were not conclusive as they did not show any difference in kanamycin resistance between strains. Based on these results, it is still unclear whether AcrAB and AcrEF have compensatory effects on each other, since there was no significant difference in MIC between the strains.

INTRODUCTION

Gram-negative bacteria such as *Escherichia coli* deploy multi-component bacterial drug efflux pumps belonging to the resistance-nodulation-division (RND) superfamily that play a key role in antibiotic resistance (1). Two tripartite systems belonging to this family include the AcrAB-TolC and AcrEF-TolC systems. AcrA and AcrE are the periplasmic components of these efflux pumps (2). AcrA interacts with TolC and AcrB, while AcrE interacts with TolC and AcrF. TolC is found on the outer membrane, while AcrB and AcrF are found in the inner membrane, as depicted in Fig 1a (2, 3). Together, these efflux pumps use the proton-motive force to translocate substrates from the cell interior to the extracellular environment (4). AcrAB and AcrEF are thought to mediate the export of kanamycin and therefore contribute to kanamycin resistance (5). As a result, without *acrA* or *acrE* expression, neither AcrAB nor AcrEF, respectively, should be able to function as efflux pumps and the organism should theoretically be more susceptible to kanamycin, although this is not empirically observed due to potential compensatory effects discussed later (5). *E. coli* has its own mechanism to regulate *acrAB* and *acrEF* expression, which depends on the regulatory proteins, AcrR and AcrS (6). AcrR represses the *acrAB* operon, while AcrS represses the *acrEF* operon (6). Both repressor genes are located upstream of their respective operons, as shown in Fig 1b (7). A study by Hay *et al.* found that the deletion of *acrS* leads to increased kanamycin resistance (6). This is expected because removal of the *acrEF* repressor would result in increased expression and synthesis of the AcrEF efflux pump, allowing for greater removal of kanamycin from the cell and thus increased resistance. Additionally, a study by Baeva *et al*. found that overexpressing AcrS leads to decreased kanamycin resistance due to increased repression of *acrE,* which results in fewer AcrEF efflux pumps being expressed (7). While previous research has already established that AcrS plays a role in mediating kanamycin resistance, the roles of AcrE and AcrA in mediating kanamycin resistance are slightly counterintuitive and thus still being

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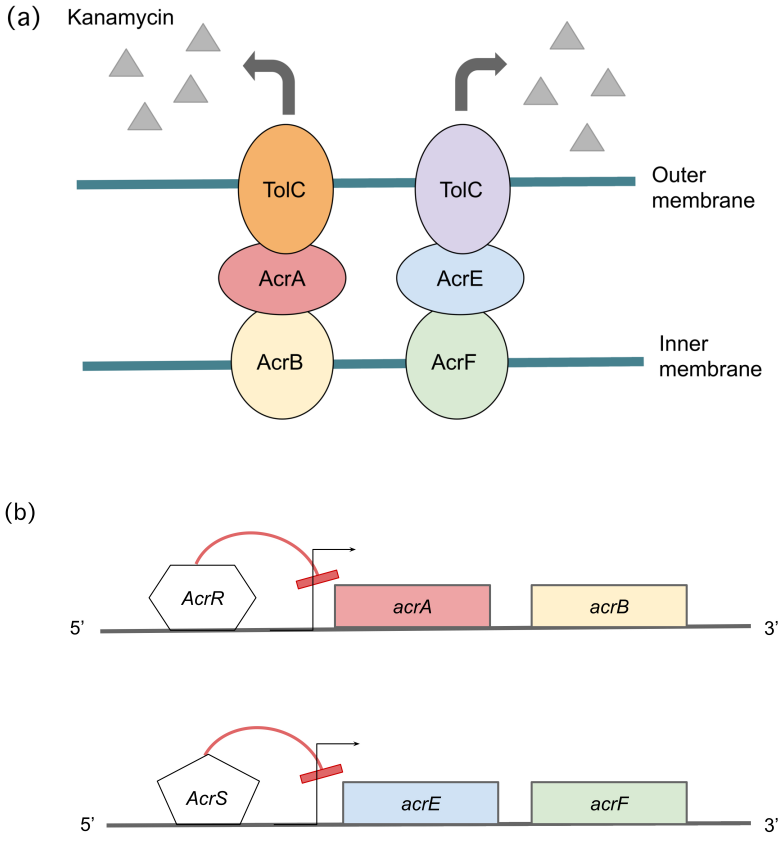

FIG. 1 Structure of AcrAB and AcrEF efflux pumps and regulation of their operons. (a) AcrA and AcrE are the periplasmic components of the efflux pump which connect TolC (outer membrane channel) to the inner membrane proteins, AcrB and AcrF respectively. The multidrug efflux pumps encoded by two operons, *acrAB* and *acrEF*, form tripartite systems, which are thought to contribute to the *E. coli* cell's kanamycin resistance. (b) The *acrAB* and *acrEF* operons are repressed by the upstream transcriptional regulators, AcrR and AcrS respectively.

elucidated (6, 7). For example, *acrE* expression has been shown to increase with increasing kanamycin concentrations, while the expression of *acrA* remains unchanged (6). The unchanged expression levels of *acrA* indicate that the AcrAB efflux pump may not be as involved in mediating kanamycin resistance as the AcrEF efflux pump (6). However, another study determined that single deletions of *acrA* and *acrE* confer similar effects on kanamycin resistance (5). To elaborate, Gabeff *et al*. found that deletion of *acrA* or *acrE* had no effect on kanamycin resistance. This may be a result of compensatory effects of AcrAB and AcrEF on kanamycin resistance when either is lost (5). Indeed, in the event of a single deletion, it may be possible for the other efflux pump to be upregulated and to continue exporting kanamycin out of the cell, resulting in an unchanged resistance to kanamycin. Thus, the generation of a *ΔacrAΔacrE* mutant is crucial in assessing the effects of *acrA* and *acrE* on kanamycin resistance, without the ambiguities caused by potential compensatory effects between AcrAB and AcrEF.

The primary aims of this project are as follows: first, to generate a double-knockout mutant wherein *acrA* and *acrE* were both deleted (*ΔacrAΔacrE*), and second, to perform a kanamycin MIC assay on this mutant. The double-knockout was generated using the lambda Red (λ-Red) recombination technique involving the deletion of the *acrA* gene from a *ΔacrE* mutants. A kanamycin MIC assay was also conducted on an *acrS* knockout mutant (*ΔacrS*), an *acrE* knockout mutant (*ΔacrE*), and an *acrA* knockout mutant (*ΔacrA*) compared to the wild-type strain. The purpose of this assay was to compare the relative MICs of the aforementioned strains (wild-type, *ΔacrS, ΔacrA, ΔacrE, ΔacrAΔacrE*) and to determine whether or not deleting *acrA* and *acrE* together resulted in a phenotype with lower kanamycin resistance. Based on a study by Hay *et al*., we expected the *ΔacrS* mutant to have increased kanamycin resistance, and therefore, have a higher MIC than the wild-type (6). We also expected their respective deletions (*ΔacrA* and *ΔacrE*) to result in a kanamycin resistance equivalent to that of the wild-type due to the compensatory effects of either protein when the other is nonfunctional, as described by Gabeff *et al*. (5). For example, if AcrAB and AcrEF are upregulated in *ΔacrA* and *ΔacrE* mutants respectively

and can compensate for the absence of the deleted efflux pump by overexpressing the retained efflux pump then the kanamycin resistance of these single-deletion mutants should be similar to that of the wild-type. On the other hand, a *ΔacrAΔacrE* mutant is expected to have a lower kanamycin resistance because upregulation of *acrA* and *acrE* would no longer be possible, preventing such compensatory effects between AcrAB and AcrEF.

As mentioned earlier, the generation of the *acrAacrE* double-knockout mutant involved the use of λ-Red recombination technology. λ-Red recombination is a process that replaces a specific chromosomal sequence with a selectable antibiotic resistance gene flanked by homologous arms of 40 or more base pairs (bp) (8). In this study, we replaced the *acrA* gene with a chloramphenicol resistance cassette (CmR) in a *ΔacrE* mutant to generate an *ΔacrAΔacrE* mutant. The homologous recombination process that replaced *acrA* with the CmR cassette was mediated by λ-Red proteins, whose genes are found on the plasmid pKD46. Plasmid pKD46 encodes λ-Red proteins, three of which are essential for mediating the process of λ-Red recombination, namely the 5′→3′ exonuclease (Exo), the single-stranded DNA (ssDNA)-binding protein (Beta), and the degradation-inhibiting protein (Gam) (8). In terms of experimental procedure, *ΔacrE* cells were first transformed with pKD46 to generate *ΔacrE* pKD46^{+} cells, which were then transformed with PCR products containing the CmR cassette flanked by 40 bp *acrA* regions. The three λ-Red proteins work in tandem to facilitate the homologous recombination process. Gam acts as an inhibitor of host nucleases (RecBCD and SbcCD), and thus protects the dsDNA substrates that are to be recombined (9). While Exo degrades dsDNA from each end in a 5'→3' direction and creates 3’ ssDNA tails, Beta stabilizes ssDNA strands longer than 35 nucleotides and pairs them with complementary sequences (9). In the case of our experiment, Exo created 3’ ssDNA tails on both the *acrA* gene in the chromosome and the regions of *acrA* homology on the CmR cassette PCR products, and Beta annealed these homologous ssDNA segments to facilitate a double crossover event, as illustrated in Fig 2. In this double crossover event, the 40 bp regions of *acrA* homology flanking the CmR cassette in the PCR product replaced their respective regions of homology in the *acrA* gene of the chromosome, which led to the insertion of the CmR cassette into the chromosome. At the end of this process, the CmR cassette insertion was still flanked by 40 bp regions of *acrA* in the chromosome; however, this region is outside the coding region of the *acrA* gene and therefore the *acrA* gene was rendered non-functional, resulting in an *acrA* deletion. Since this *acrA* deletion occurred in a *ΔacrE* mutant, the result was the generation of an *acrAacrE* double-knockout mutant. Fig

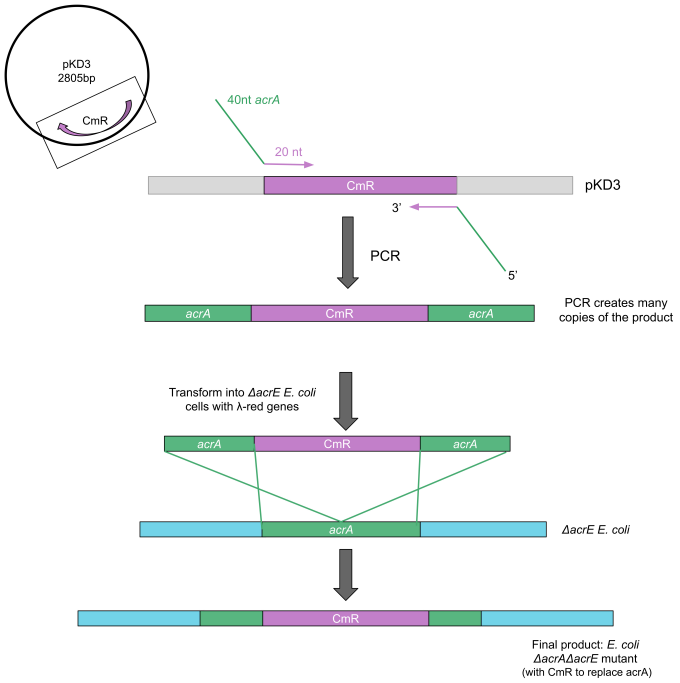

FIG. 2 λ-Red recombineering process used to create the *acrAacrE* double-knockout. First, the CmR cassette of the pKD3 plasmid was PCR-amplified using primers which could anneal to both the CmR and *acrA* genes. The PCR product consisted of the CmR cassette with *acrA* regions flanking either ends. The PCR products were transformed into competent *ΔacrE* cells expressing the λ-Red genes, and homologous recombination replaced the *acrA* gene with the CmR cassette, removing the entire *acrA* gene to generate a deletion mutant.

2 provides a mechanistic illustration of the basic processes underlying the λ-Red recombination technique described above.

We hypothesized that the lack of *acrA* and *acrE* in the *ΔacrAΔacrE* mutant would result in the lowest kanamycin resistance among all the strains in this study. This is because the absence of key components in the AcrAB and AcrEF efflux pumps will prevent any compensatory effects on kanamycin resistance since both *acrA* and *acrE* genes would not be present in the genome to be upregulated. Lastly, due to the aforementioned previous research and underlying biological models of *acrS*, *acrA*, and *acrE*, we expected to see a decreasing trend in kanamycin resistance in the following order: *ΔacrS* > wild-type = *ΔacrA* = *ΔacrE* > *ΔacrAΔacrE*. However, results revealed that all strains exhibited a wild-type-like kanamycin MIC, suggesting that these two systems do not play a role in kanamycin resistance or susceptibility.

METHODS AND MATERIALS

Bacterial strains. *E. coli* BW25113 and mutants JW3232-1 (*ΔacrSΔkan*), JW3233-2 (*ΔacrEΔkan*), and ASQ12W-2 (*ΔacrAΔkan*) (referred to as wild-type, *ΔacrS*, *ΔacrE*, and *ΔacrA*, respectively, throughout this paper) were obtained from the Microbiology and Immunology Department at the University of British Columbia. The *ΔacrS*, *ΔacrE*, and *ΔacrA* mutants are Keio strains in which the kanamycin resistance cassette has been removed to avoid interfering with the downstream MIC assay involving kanamycin (10).

Genotypic confirmation of strains. The above strains were verified for the absence of *acrS*, *acrE*, and *acrA* in their respective mutants. First, genomic DNA was isolated from each strain using PureLink® Genomic DNA Mini Kit (Invitrogen) as per the manufacturer's instructions. Each strain's DNA was quantified using the Nanodrop3000 spectrophotometer. Polymerase chain reaction (PCR) was performed using each strain's genomic DNA as the template and primer sets that amplified the deleted gene of each respective mutant (performed using the T100 thermocycler by Bio-Rad). The resulting PCR products were run on a gel electrophoresis apparatus and the gel was imaged under UV light (ChemiDoc™ Touch Imaging System by Bio-Rad) to visualize the bands (or lack thereof) to confirm the genotype of each strain. The gel results confirmed the genotypes of all the strains except the *ΔacrA* mutant, whose band results were slightly ambiguous.

Primers. Two sets of primers were designed using the genomic sequence of parent strain BW25113 (NCBI accession number CP009273) and ordered from Integrated DNA Technologies. The sequences of these primers are shown in Table 1. Forward and reverse *acrA*-CmR primers were designed to have a 20 bp region complementary to the pKD3 CmR

TABLE. 1 Primer sequences used for PCR and Sanger sequencing. The following legend shows the important regions in the primer sequences: **BOLD** = homologous to region flanking *acrA*; UNDERLINED= homologous to region flanking CmR.

Primer Name	Primer Seq
Forward *acrA*-CmR primer	5'-**ACCATTGACCAATTTGAAATCGGACAC TCGAGGTTTACAT**CCTGTTGATACCGGGAAGCC-*3'*
Reverse *acrA*-CmR primer	5'-**GATAAAGAAATTAGGCATGTCTTAAC GGCTCCTGTTTAAG**TTTAAATGGCGCGCCTTACG-*3'*
Forward *acrA* primer for sequencing	5'-**ACCATTGACCAATTTGAAATCGGACACTCGAGGTTTACAT**-3'
Reverse *acrA* primer for sequencing	5'-**GATAAAGAAATTAGGCATGTCTTAACGGCTCCTGTTTAAG**-3'

cassette as well as a 40 bp *acrA* homologous sequence at the 5' end. These primers were used to amplify the CmR cassette from pKD3 and generate PCR fragments with the pKD3-derived CmR cassette flanked by *acrA* regions. Forward and reverse *acrA* primers for sequencing only consist of the *acrA* homologous sequences and were used to amplify the *acrA* region from gDNA of double-knockouts. These same primers were used for sequencing the *acrA*-CmR PCR fragments, as well as the amplified *acrA* region to confirm the absence of *acrA* from the double-knockouts.

Generation of an *acrAacrE* double-knockout mutant. The plasmids used in this study are shown in Table 2. Plasmids pKD46 and pKD3 were grown overnight in LB with 100 µg/mL ampicillin in a 30°C shaker, and in LB with 25 µg/mL chloramphenicol in a 37℃ shaker, respectively. Plasmid pUC19 was also grown overnight in LB with 100 µg/mL ampicillin in a 37℃ shaker, and was used as a positive control group in downstream transformations and plating. All three plasmids were extracted using the Invitrogen™ PureLink® Quick Plasmid Miniprep Kit as per the manufacturer's instructions. The concentration of each plasmid was quantified using the Nanodrop3000 spectrophotometer. Plasmids pKD46 and pUC19 were transformed into competent *ΔacrE* cells, which were prepared using the Hancock Lab's $CaCl_2$ Transformation of *E. coli* protocol (13). The transformation was carried out in accordance with this protocol, except for step 8, wherein pKD46 was incubated with competent *ΔacrE* cells in a 30℃ shaker rather than a 37℃ shaker, since pKD46 is temperature-sensitive (11). Following the incubation, pKD46-transformed *ΔacrE* cells were plated on LB agar with 100 µg/mL ampicillin as a neat sample, 1/10 dilution, and 1/100 dilution. As positive controls, pKD46 transformants were plated on LB agar, pUC19 transformants were plated on LB agar with and without 100 µg/mL ampicillin, and *E. coli* with no plasmid (water and *ΔacrE* cells) were plated on LB agar. As a negative control, *E. coli* with no plasmid were also plated on LB agar with 100 µg/mL ampicillin. All plates were incubated at 30℃ overnight and observed for isolated colonies the following day.

The *ΔacrE* pKD46$^+$ transformants grown on LB agar with ampicillin were prepared for electroporation with pKD3's PCR product. PCR was performed on pKD3 using primers to amplify the CmR cassette which would be taken up by the *ΔacrE* pKD46$^+$ cells during electrocompetent transformation. After purification of the resulting PCR product, it was then transformed by electroporation into *ΔacrE* pKD46$^+$ cells by following OpenWetWare's protocol to create the *acrAacrE* double-knockout mutant (14). Successful transformants were screened on chloramphenicol plates (10 and 25 ug/mL). Genomic DNA was isolated from an overnight cultures of the *ΔacrAΔacrE* mutants using the PureLink® Genomic DNA Mini Kit (Invitrogen) as per the manufacturer's instructions. Isolated genomic DNA was

TABLE. 2 List of plasmids. This table provides information about each plasmid that was used in the study.

Plasmid	Description	Size (bp)	Carrier Strain	Growth Condition	Reference
pKD3	Contains chloramphenicol resistance cassette and ampicillin resistance gene	2805	*E. coli* BW25141	Streaked on Luria-Bertani (LB) agar with 25 µg/mL chloramphenicol and grown at 37°C	(11)
pKD46	Contains lambda-Red recombination genes and ampicillin resistance gene	6329	*E. coli* BW25141	Streaked on LB agar with 100 µg/mL ampicillin and grown at 30°C	(11)
pUC19	Contains ampicillin resistance gene	2686	*E. coli* DH5α	Streaked on LB agar with 100 µg/mL ampicillin and grown at 37°C	(12)

sent for sequencing to confirm the *acrAacrE* double-knockout.

PCR of pKD3's CmR cassette. Primers were designed to amplify the CmR cassette and its promoter within the pKD3 plasmid, and generate PCR fragments with the pKD3-derived CmR cassette flanked by *acrA* regions at both ends. Table 1 shows the sequences of both of these primers, where they are listed as "Forward *acrA*-CmR primer" and "Reverse *acrA*-CmR primer". Both of these primers have a 20 bp region at the 3' end to amplify the CmR cassette from pKD3. In addition, they have a 40 bp region at the 5' end homologous to the flanking regions of *acrA*, which is necessary for homologous recombination.

For the PCR reaction, a PCR Master Mix was prepared using SuperFi Buffer, 10 mM dNTP mix, and Platinum SuperFi DNA Polymerase. PCR was performed in reactions which included the Master Mix, forward primer, reverse primer, template DNA (pKD3), and nuclease-free water. The negative control reaction was prepared similarly, but with replacement of template DNA with nuclease-free water. The PCR was run under the following settings using the T100 thermocycler by Bio-Rad: one cycle of initial denaturation at 98℃ for 30 seconds; 35 cycles of denaturation at 98℃ for 5 seconds, annealing at 64.7℃ for 10 seconds, and extension at 72℃ for 15 seconds; then one cycle of final extension at 72℃ for 5 minutes. The PCR products were purified using ThermoFisher's GeneJET PCR Purification Kit as per the manufacturer's instructions. To verify that the correct gene had been amplified, the PCR products were loaded on a 1% agarose gel stained with SYBR® Safe DNA Gel Stain in 0.5X TBE (Invitrogen) and run in 0.5X TBE buffer at 150 V for 1 hour. Bands of amplicons were visualized and imaged by UV light (ChemiDoc™ Touch Imaging System by Bio-Rad). Lastly, the PCR products were sent for Sanger sequencing to determine that the correct gene (CmR cassette) had been amplified.

Genomic DNA isolation and confirmation of double-knockout generation. Isolated colonies from different transformants of the *ΔacrAΔacrE* mutant were picked and each colony was inoculated in LB with 10 or 25 µg/mL chloramphenicol and grown in a 37˚C shaker for over 16 hours to generate overnight cultures. Two chloramphenicol concentrations were used in case one would yield better isolated colonies than the other. A chloramphenicol concentration too low might allow the growth of colonies without the CmR cassette, while a concentration too high might yield no colonies at all. Genomic DNA was extracted from the double-knockout using PureLink Genomic DNA Mini Kit, as per the manufacturer's instructions. To confirm the generation of the double-knockout, PCR was performed on the genomic DNA to amplify the *acrA* region containing the CmR cassette; amplification of this PCR product indicated that the CmR cassette had been inserted into the genome and the *acrA* gene had been removed. Primers were designed to flank the *acrA*

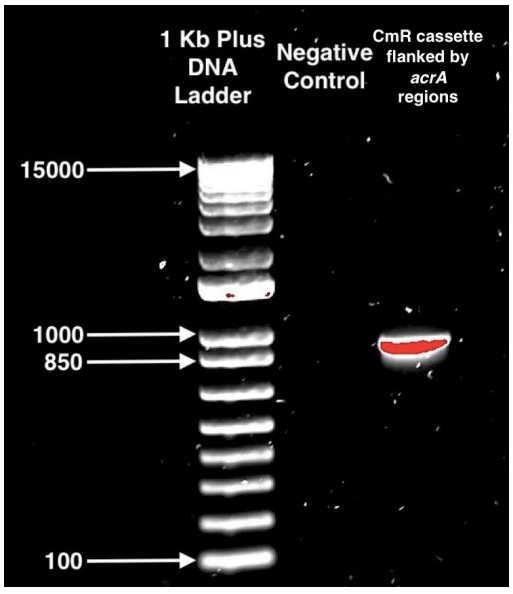

FIG. 3 Generating the pKD3-derived CmR cassette fragment flanked by *acrA* regions. The PCR product of the CmR cassette amplified from pKD3 was loaded on a 1% agarose gel stained with SYBR® Safe DNA Gel Stain and ran in 0.5X TBE for 60 minutes at 150 V. The negative PCR control, which included sterile water instead of the amplified template DNA, did not show any bands. The sample, which included the PCR-amplified template DNA, contained a band of ~900 bp, which matches the expected size of the CmR cassette flanked by *acrA* regions.

gene, which contained the CmR cassette insertion. Both the forward and reverse primers consisted of a 40 bp sequence homologous to the region directly flanking *acrA*. Table 1 shows the sequences of these primers, which are listed as "Forward *acrA* primer for sequencing" and "Reverse *acrA* primer for sequencing". A negative control was prepared in the same way as the samples, except nuclease-free water was used instead of the template DNA.

An annealing temperature of 70.8°C was used for the PCR reactions. The PCR products were purified using ThermoFisher's GeneJET PCR Purification Kit as per the manufacturer's instructions. To verify that the correct gene had been amplified, the PCR products were loaded on a 1% agarose gel stained with SYBR Green Gel Stain in 1X TAE. Bands of amplicons were visualized and imaged by UV light (ChemiDoc™ Touch Imaging System by Bio-Rad). Lastly, the PCR products were sent for sequencing to confirm the replacement of the *acrA* gene with the CmR cassette.

MIC assay with kanamycin. The MIC assay was performed based on methods found in protocols from Hay *et al*. and Baeva *et al.* (6, 7). Overnight cultures of the wild-type strain and mutants *ΔacrE*, *ΔacrS*, *ΔacrA*, and three biological replicates of the *ΔacrAΔacrE* mutant were prepared. Each overnight culture was diluted one hundred-fold and continued to grow until an OD_{600} of 0.1-0.2 was reached. Two-fold serial dilutions of kanamycin were prepared at the following concentrations: 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, and 0.4 µg/mL. 5 µL of each culture was added to each concentration of kanamycin in technical triplicates in a 96-well plate. As a negative control, one well of LB broth without culture was prepared. As a positive control, wells of LB broth with culture were prepared for each strain. The plates were incubated overnight for 24 hours at 37˚C. The wells were detected for growth by measuring the OD_{600} using the Microplate Reader.

RESULTS

Generation of the *acrAacrE* double-knockout. After PCR of pKD3 with the forward and reverse *acrA*-CmR primers listed in Table 1, the resulting PCR product was run on a 1% agarose gel to confirm that its size matched that of the CmR cassette with *acrA* flanking regions. A negative control was also included in the PCR reaction, with the same components as the sample but using sterile water in place of the template DNA. Comparison of the sample's band against the DNA Ladder indicated that the PCR product was between 850 and 1000 bp in size, as shown in Fig 3. As such, the sample's band could be approximated to a size of ~900 bp, which matches the expected size of the PCR product (908 bp). Additionally, the PCR product was sent for Sanger sequencing using the forward and reverse *acrA* primers for sequencing (Table 1). Results confirmed the expected PCR product sequence (Fig S1). Both the gel and sequencing results provided a confirmation of the successful amplification of the CmR cassette from pKD3, which allowed the subsequent transformation of the PCR product into *ΔacrE* mutants to generate the *acrAacrE* double-knockout.

After genomic DNA extraction of six biological replicates of the *acrAacrE* double-knockout mutant, PCR of all genomic DNA samples was performed to verify the insertion of CmR into the chromosome. Successful amplification of the *acrA* region and visualization of the band on an agarose gel would suggest that *acrA* had been replaced by CmR. A negative control was also included in the PCR reaction, with the same components as the sample but using sterile water in place of the template DNA. All six samples showed bands of similar size, approximately between 1000 and 1500 bp, as indicated by the DNA ladder in Fig 4. These bands were slightly larger than the expected size of the CmR cassette with *acrA* regions flanking both ends (908 bp). However, the DNA ladder had smeared and therefore was not a good indicator of band size. This may have distorted the band sizes of the samples in comparison to the ladder, making them appear larger than they actually were. To provide a solid confirmation of the replacement of *acrA* with the CmR cassette, all six PCR products were also sent for Sanger sequencing with the same primers used for PCR. Results matched the expected PCR product sequence (Fig S1), and therefore confirmed the successful generation of *ΔacrAΔacrE* mutants.

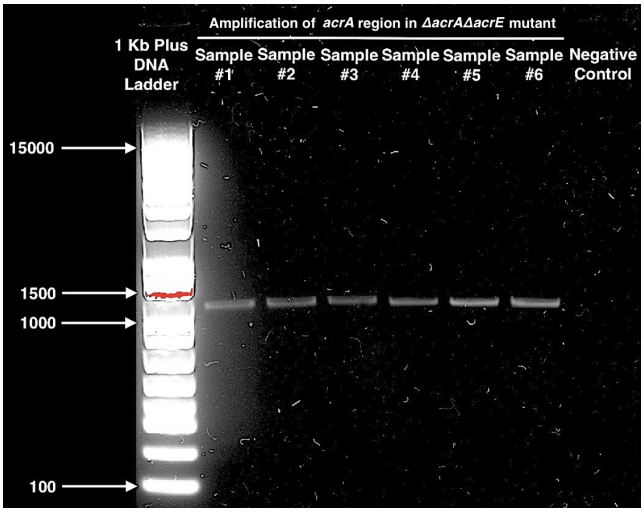

FIG. 4 Gel confirmation of *acrA* deletion in *ΔacrAΔacrE* strain. PCR products of the inserted CmR cassette with flanking *acrA* regions were obtained from six genomic samples of the newly-generated *ΔacrAΔacrE* mutants by amplifying the *acrA* region within the genomic DNA. To confirm the correct size, the PCR fragments were loaded on a 1% agarose gel stained with SYBR® Green DNA Gel Stain and ran in 1X TAE for 60 minutes at 150 V. As expected, there were no bands in the negative control, which included sterile water instead of template DNA. The samples contained bands of similar sizes, between 1000 and 1500 bp, which were slightly larger than the expected size of 908 bp. This discrepancy could be attributed to the smearing of the DNA ladder. Purified products were subsequently sent for Sanger sequencing to confirm insertion of the CmR cassette and deletion of the *acrA* gene.

Single and double-knockouts of major *acr* genes exhibited similar MIC levels. At each kanamycin dilution, MIC assays were conducted using three technical replicates but only one biological replicate for the wild-type, *ΔacrS*, *ΔacrA*, and *ΔacrE* strains. However, three biological replicates of the *ΔacrAΔacrE* mutant were tested. The results, summarized in Table 3, showed that each strain's MIC was 12.5 µg/mL, indicating that all of the strains had the same MIC level. Moreover, there was no marked decrease in the kanamycin resistance of the *ΔacrAΔacrE* mutant in comparison to the others. However, due to the lack of sufficient biological replicates, the MICs generated for the wild-type, *ΔacrS*, *ΔacrA*, and *ΔacrE* were not confirmative.

DISCUSSION

In this study, we aimed to create a *ΔacrAΔacrE* mutant and to determine whether kanamycin resistance was significantly altered compared to the wild-type strain and other mutants, such as single knock-out mutants, *ΔacrA*, *ΔacrE*, and *ΔacrS.* The generation of the double-knockout was accomplished by employing λ-Red recombineering technology, while the differences in kanamycin susceptibility and resistance were assessed by performing MIC assays. Based on the observation that *ΔacrA* and *ΔacrE* mutants have similar MIC values due to potential overcompensation of efflux activity in AcrAB and AcrEF when either is lost, we hypothesized that deleting both *acrA* and *acrE* would increase kanamycin susceptibility compared to the other strains due to the lack of compensatory effects (5). Ultimately, the results we obtained did not support our original hypothesis because they indicated that there were no significant differences in kanamycin susceptibility among the strains.

The kanamycin MIC assay results revealed that the MIC of all the strains under investigation was 12.5 µg/mL. This indicated that there were no significant differences in kanamycin resistance among the strains. Most importantly, this showed that the kanamycin resistance of the *ΔacrAΔacrE* mutant was the same as that of the wild-type strain, as well as those of the *ΔacrA*, *ΔacrE*, and *ΔacrS* mutants. These results did not support our hypothesis that the *ΔacrAΔacrE* mutant would have the lowest MIC and thus the lowest kanamycin resistance of all. Although we followed a similar MIC assay protocol as Hay *et al*. (with the exception of completing only one biological replicate for most strains and three of the double-knockout, rather than four for each), we did not yield comparable results (6). Thus, we ascribe the differences in our MIC assay results to the insufficient number of biological replicates performed, rather than deviations from the MIC assay protocol outlined by Hay *et al*. (6). As shown in Table 3, the wild-type, *ΔacrS*, *ΔacrA*, and *ΔacrE* strains were each assayed using one biological replicate only whereas the *ΔacrAΔacrE* mutant was assayed using three biological replicates. The main issue with performing only one biological replicate is the inconsistency and lack of confidence in the results obtained. Indeed, since only one biological replicate of the single mutants and wild-type strain was performed, any

differences in results due to small sampling size would not have been buffered, and thus the confidence level of each strain's MIC is low. As a result, the MICs of all five strains cannot be adequately compared at a level of high significance, nor can they be used to confirm any compensatory effects in the biological model of the AcrAB and AcrEF efflux pumps.

Although some aspects of the MIC assay results align with conclusions from various studies, other aspects deviate from the findings of certain studies. For example, while the MICs we obtained for the wild-type strain, *ΔacrA*, and *ΔacrE* (12.5 µg/mL) do not match the MICs obtained by Gabeff *et al*. for the same strains (8 µg/mL), the fact that the MICs for all three strains in our study are consistent is meaningful because Gabeff *et al*. also reported consistent MICs across these three strains (albeit slightly different values) (5).

Furthermore, certain aspects of the MIC results for *ΔacrS* either corroborate or deviate from results reported by previous studies. Specifically, while the MIC we obtained for *ΔacrS* (12.5 µg/mL) aligns with that reported by Hay *et al*. (12.5 µg/mL), the MIC value for *ΔacrS* should not be the same as the other strains (6). Indeed, Hay *et al*. reported that the MIC for *ΔacrS* is much higher than that of the wild-type strain (3.1 µg/mL) and *ΔacrE* (3.1-6.3 µg/mL) (6). This is where our results deviate from those shown by Hay *et al*., not only in the MIC values of the wild-type strain and *ΔacrE* but also in the fact that our MIC value for *ΔacrS* is not higher than those of the other strains when it theoretically should be. Since *acrS* codes for the repressor of the *acrEF* operon, deletion of *acrS* should lead to increased expression of *acrE* and thus confer increased kanamycin resistance, as shown by Hay *et al*. (6). However, our results do not reflect this. Lastly, the MIC value that we obtained for the *ΔacrAΔacrE* mutant (12.5 µg/mL) refutes our original hypothesis that it would have the lowest MIC of all the strains.

We had hypothesized that the deletion of both *acrA* and *acrE* would have mitigated any compensatory effects that AcrAB and AcrEF may have had on each other's loss, because there would have been no expression of *acrA* and *acrE* to begin with. Thus, we hypothesized that the *ΔacrAΔacrE* mutant would be the least resistant to kanamycin and thus have the lowest MIC. Ultimately, this outcome was not obtained due to any of the speculated reasons above, although the primary reason may have been the lack of sufficient biological replicates performed. Overall, the results of the MIC assay are largely inconclusive and do not provide support to the idea that *acrA* and *acrE* are upregulated in each other's absence because the MIC of the *ΔacrAΔacrE* mutant was found to be the same as those of the *ΔacrA* and *ΔacrE* mutants.

Lastly, one of the limitations associated with this study was the ambiguous genotypic confirmation of the *ΔacrA* mutant. The band results obtained from the gel electrophoresis of the *ΔacrA* mutant were not conclusive enough to confirm its identity. However, we continued using this strain throughout the course of the experiment. Therefore, if the *ΔacrA* mutant was not actually the strain that we assumed it to be, then this could have distorted the results of our MIC assay by giving us inaccurate values for this particular mutant. However, the generation of our *acrAacrE* double-knockout would have remained unaffected because the base of this mutant was the *ΔacrE* mutant, in which we deleted *acrA*.

TABLE. 3 Same MIC levels against kanamycin observed in the wild-type, single-knockout strains and double-knockout strain of *acrAacrE*. Growth inhibition was assessed as wells that did not reach optical density values greater than 0.1.

Strain ID	Genotype	MIC (µg/mL)	Biological Replicates (n)
BW25113	Wild-type	12.5	1
JW3232-1	*ΔacrS*	12.5	1
ASQ12W-2	*ΔacrA*	12.5	1
JW3233-2	*ΔacrE*	12.5	1
JACS220	*ΔacrAΔacrE*	12.5	3

Conclusions Here we successfully generated an *acrAacrE* double-knockout using λ-Red recombination technology. Successful verification of the deletion was done via agarose gel electrophoresis and Sanger sequencing. Although the MIC assay results indicated the same kanamycin susceptibility for the wild-type, the three single-knockout strains and the double-knockout we generated, this cannot be concluded definitively due to the lack of biological replicates. Hence, we were unable to address our experimental question regarding the effects of the double-knockout on kanamycin resistance. We were unable to conclude whether the double-knockout has higher or lower levels of kanamycin resistance compared to any of the single-knockout strains or the wild-type. Another MIC assay needs to be conducted, with at least 3-5 biological replicates for each strain to determine if the data for the MIC assay is consistent and of high confidence.

Future Directions In this study, we have presented methods and preliminary data pertaining to the generation of a *ΔacrAΔacrE* mutant and MIC assays comparing the kanamycin susceptibilities of other mutants/strains of *E. coli*. Since the results do not support our initial hypothesis regarding the MIC assay and instead showed no difference in kanamycin resistance among the strains, we suggest that future studies repeat the MIC assays with at least three biological replicates for each strain. The main issue with the MIC assay conducted in our study was that we only performed one biological replicate of the wild-type and single-knockout mutants, which hindered us from acquiring sufficient information to confidently deduce differences in kanamycin resistance between the strains. Therefore, to increase confidence in the results and to gain more statistically significant MICs, repetition of the MIC assay in biological triplicates is imperative. Future MIC assays may also explore the use of smaller kanamycin concentration ranges with more discrete concentrations to obtain a more definitive and precise MIC for each mutant. This may yield a more precise MIC for each strain.

Another avenue to investigate is the difference in growth among the strains and mutants. A future study could investigate the differences in the viability and growth patterns among all the strains. Plotting the various phases of a bacterial growth curve for each mutant would show the effect of certain gene deletions on the growth of each mutant compared to the wild-type. Determining whether or not the strains have any growth defects or viability issues is important because they have the potential to affect downstream assays. According to previous studies by Hay *et al*. and Belmans *et al*., the wild-type strain along with the mutants *ΔacrA*, *ΔacrE*, and *ΔacrS* should follow similar growth patterns (6, 15). However, the growth curve of a *ΔacrAΔacrE* mutant has not been plotted and it would be interesting to note whether deviations in its viability and growth pattern are observed.

Lastly, the *ΔacrAΔacrE* mutant generated in this study could be used to investigate how the *acr* system operates under different types of stress conditions, such as exposure to other aminoglycosides. Additionally, a MIC assay could be performed using streptomycin or gentamicin to determine whether the *acr* system plays a role in conferring resistance to other antibiotics of the same class as kanamycin.

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CONTRIBUTIONS

Ada Ang: Co-performed some experiments of the λ-Red recombination, such as plasmid DNA extraction, PCR, gel electrophoresis and preparation for plasmid transformations. Co-performed the kanamycin MIC assay and analyzed results. Wrote the Introduction, Discussion, Conclusion and generated figures and captions (Fig 1, Fig 2, Table 3).

Cathy Park: Co-performed some experiments of λ-Red recombination, such as plasmid DNA extraction, genomic DNA extraction, PCR, purification of PCR products, and preparation of samples for sequencing. Wrote the Materials and Methods, Results, Future Directions sections of the paper.

Joshua De Guzman: Co-performed some experiments of the λ-Red recombination, such as plasmid DNA extraction, preparation of competent *ΔacrE* cells for transformation, PCR, electrocompetent transformation of pKD3 into *ΔacrE* pKD46$^+$ cells, and genomic DNA extraction. Designed primers and made the table of primers (Table 1). Analyzed and denoted the Sanger sequencing data (Fig S1). Wrote the Abstract, Materials and Methods, Results, and Supplemental Data sections.

Shaneel Kumar: Co-performed the experiments that generated the double-knockout, such as the $CaCl_2$ transformation of pKD46 into *ΔacrE* cells and the electrocompetent transformation of pKD3 into *ΔacrE* pKD46$^+$ cells. Ran both gels and generated their respective figures and captions (Fig 3 and Fig 4). Co-performed the kanamycin MIC assay. Wrote majority of Introduction, majority of Materials and Methods, majority of Discussion, small portion of Results, majority of Future Directions, and References sections of this paper.

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