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Assessing the contributions of the multidrug efflux pump components *acrE* and *acrA* in mediating resistance to kanamycin in *E. coli BW25113* : steps towards the generation of *acrA/acrE* double mutants using CRISPR/Cas9 system.

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SUMMARY Efflux pumps in *Escherichia coli* are responsible for their intrinsic resistance to antimicrobial compounds. AcrAB-TolC and AcrEF-TolC are structurally similar efflux pumps that have been proposed to be responsible for the export of kanamycin. AcrS is a repressor of *acrAB* and possibly *acrEF*. AcrS deletion was shown to increase the expression of *acrE* and confer an increase in kanamycin resistance. Therefore, it was suggested that AcrEF-TolC and possibly AcrAB-TolC were contributors to the development of kanamycin resistance. Since AcrAB-TolC is constitutively expressed in the cell and at higher levels than AcrEF-TolC, we proposed that it has a greater contribution to the resistant phenotype. Furthermore, the pumps have high structural similarity, so we hypothesized that the loss of one pump could be compensated by the other, thus a double mutant would exhibit a lowered resistance due to the loss of the two main pumps responsible for the efflux kanamycin. To test this hypothesis, we used the wild-type strain *E. coli* MG1655, an *ΔacrAΔkan* and a *ΔacrEΔkan* deletion mutant and attempted to construct a double mutant *ΔacrAΔacrEΔkan*. A minimum inhibitory concentration (MIC) assay comparing the single mutants and the wild-type strain resulted in a MIC of 8 µg/mL for all strains. This suggests that a single loss of *acrA* or *acrE* expression has no apparent effect in kanamycin resistance. In our attempt to generate a double mutant with CRISPR/Cas9 system, technical difficulties were faced in the cloning of the pCRISPR-SacB-gDNA. Therefore, this study also sought for and provided preliminary evidence in the characterization of the pCRISPR-SacB plasmid using restriction digest and partial genome sequencing.

INTRODUCTION

In Gram-negative bacteria, resistance nodulation division (RND) is one of the five main families of efflux pumps, and a major contributor to drug resistance (1). *Escherichia coli* RND efflux protein pumps are embedded in the cytoplasmic membrane and have a wide variety of substrates, which are exported from the periplasm and cytoplasm using the proton motive force (PMF) (2). RND pumps can function as a resistance mechanism against detergents, dyes and antibiotics (3). Two major RND members include the tripartite AcrAB-TolC (AcrAB) and AcrEF-TolC (AcrEF) complexes (4). AcrAB is constitutively expressed and contributes to the resistance phenotype with the export of hydrophilic and lipophilic antibiotics such as beta-lactams, quinolones, tetracyclines, and chloramphenicol (5). Relative to *acrAB*, *acrEF* expression is low under laboratory conditions (6). AcrEF has been shown to export quinolones and tigecyclines (5). AcrA shares 69.3% amino acid homology with AcrE, and AcrB shares 77.6% amino acid homology with AcrF (5). AcrEF would therefore be expected to be similar to AcrAB and have a similar broad substrate specificity. This idea was demonstrated by Kawamura-Sato *et al.* that found that the overexpression of the *acrEF* operon in an *acrAB* negative mutant allowed the hypersusceptible phenotype to be salvaged, for antibiotics such as erythromycin and novobiocin (7).

Even though contributions of AcrAB and AcrEF have been assessed for a wide variety of compounds, data was not generated for kanamycin. The antibiotic, kanamycin, is a hydrophilic aminoglycoside that inhibits bacterial protein translation and thus is lethal. It is of particular interest, as kanamycin is widely studied in experiments pertaining AcrS

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repression and its effects. Double mutants in both AcrAB and AcrEF have not yet been studied. We aim to assess the relative contributions of each pump in developing kanamycin resistance by comparing single and double mutant strains.

AcrS has been shown to repress both *acrAB* and *acrEF*, although the mechanism is not clearly understood (9). Belmans *et al.* found that a deletion in *acrS* increased the resistance to kanamycin (10). Hay *et al.* determined that AcrS deletion increases the expression of *acrE* and confers an increase in kanamycin resistance (9). Therefore, it was proposed that AcrEF and possibly and AcrAB would mediate kanamycin resistance.

Sulavik *et al.* found that an *acrEF* mutant showed no significant changes on the resistance phenotype for 20 other antimicrobial compounds (8). In the same experiment, the *acrAB* mutant had a decreased resistance compared to wild-type for the majority of the conditions tested. Following these results, and the low expression of *acrEF* expression under laboratory conditions, we would expect AcrAB to have a greater contribution in exporting kanamycin than AcrEF.

In Hay *et al.*'s study, AcrEF was suggested to play a role in kanamycin resistance, and AcrAB is a major contributor to the general resistant phenotype. Since AcrAB and AcrEF share a large percentage homology, it is proposed that the single mutants could compensate the loss of one pump for the expression of the other. Thus, a double mutant in both *acrEF* and *acrAB* would be more sensitive to kanamycin than the single mutants.

We hypothesize that knocking out both genes would result in a much lower kanamycin resistance. We postulate that *E. coli* strains from most to least resistant to kanamycin would be as follows: wild-type MG1655, *acrE* single mutant, *acrA* single mutant, and lastly *acrA/E* double mutant.

In this study, we took steps in creating the *ΔacrAΔacrEΔkan* double mutant strain using the recently developed CRISPR-Cas9 mediated genome editing technique. The RNA-guided endonuclease Cas9 can be engineered to target and cut specific sequences in the genome. This is done by constructing a guide DNA (gDNA) that matches the sequence of interest and binds Cas9. The introduction of mutagenic donor DNA (dDNA) with an in-frame stop codon results in homologous recombination and targeted gene knockout (11).

METHODS AND MATERIALS

Bacterial strains. *E. coli* MG1655, and mutants JW0452-3 (*ΔacrA::kan*) and JW3233-2 (*ΔacrEΔkan*) (hereinafter referred to as *E. coli ΔacrA::kan* and *ΔacrE,* respectively) were obtained from the Microbiology and Immunology Department at the University of British Columbia. *E. coli* MG1655, Δ*acrA*::*kan,* and Δ*acrE*Δ*kan* were streaked on LB agar with and without 50 μg/ml kanamycin to screen for kanamycin sensitivity. pCRISPR-SacB and pCasRed plasmids were obtained from Synthetic and Structural Vaccinology Unit, CIBIO, University of Trento, Italy, and transformed into commercially available competent *E. coli* DH5cells (Invitrogen).

Preparation of antibiotic 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. 25mg/ml chloramphenicol (Sigma-Aldrich®) stock solution was prepared by dissolving the antibiotic in 100% ethanol. The antibiotic stock solutions were then sterilized by filtering the solutions through 0.22 μm nitrocellulose filters (EMD Millipore) and stored at -20°C.

Removal of kanamycin resistance cassette from JW0452-3. The kanamycin cassette in the Keio collection strain, JW0452-3, was removed using pCP20 plasmids, with protocol described by Barrick Lab (12). The pCP20 plasmid (from BT340 cells, provided by the Microbiology and Immunology Department at the University of British Columbia), contained a flippase (FLP) recombinase and an ampicillin resistance gene used as a selection marker. FLP recombinase recognition targets (FRT) that flank the kanamycin cassette in *ΔacrA::kan* lead to site-directed recombination (13). $CaCl_2$ competent cells of JW0452-3 were prepared based on the protocol from Hancock Lab (14). Five ml of LB media with 100 μg/ml ampicillin (LB + Amp) was inoculated with an isolated colony of BT340 overnight shaking at 25˚C, and

pCP20 plasmid extraction was performed using Invitrogen™ PureLink® Quick Plasmid Miniprep Kit, as per the manufacturer's instructions. The plasmid concentration was quantified using Nanodrop3000 spectrophotometer, and 0.5 ng of pCP20 was transformed into 100 µl of $CaCl_2$ competent JW0452-3 cells, recovered for 1 hour at 25˚C shaking, and plated on LB + Amp overnight. A positive control was included by transforming competent JW0452-3 cells with pUC19 plasmid, which contained ampicillin resistance marker, and plated on LB + Amp, as a means to test the competency of the cells. An isolated colony from the pCP20 transformation was used to inoculate 5 ml of LB and grow overnight at 43˚C to cure the plasmids. A 50 µl of $1/10^6$ dilution of the overnight culture was plated on LB and grown overnight at 30˚C. Six individual colonies were patched from this plate onto LB with 50µg/ml (LB + Kan), LB+Amp, and LB plates and grown overnight at 37˚C for LB and LB + Kan, 30˚C for LB + Amp plates. Patches on LB plates that are sensitive to both antibiotics were inoculated in 5 ml LB and grown overnight shaking at 37˚C.

Genotypic confirmation. Removal of kanamycin cassette and whether *acrA* and *acrE* were present in their respective single mutant strains was assessed by polymerase chain reaction (PCR) and gel electrophoresis. Primers were designed using the genomic sequence of parent strain MG1655 (NCBI accession number NZ_CP027060.1) and Snapgene Viewer, and were ordered from Integrated DNA Technologies (Table S1). Genomic DNA was isolated from 1 mL overnight cultures of *E. coli* MG1655, Δ*acrA*Δ*kan,* and Δ*acrE*Δ*kan* with PureLink® Genomic DNA Mini Kit (Invitrogen) as per the manufacturer's instructions, and used to as templates to amplify the target sequence. DNA concentrations were quantified with a Nanodrop3000 spectrophotometer. See Table S2 for reagents used and volumes for each reaction. Positive control was performed using a primer set that amplifies 163bp in pUC19 plasmid. Negative control was performed with *acrE* and *acrA* primers and no template DNA, substituting template volume with sterile water. The T100 Thermocycler (Bio-Rad) were programmed for an initial denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 1 minute, with a final extension at 68°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 0.5X TBE buffer at 100 V for 40 minutes. Amplicons were visualized and imaged with UV light using ChemiDoc™ Touch Imaging System (Bio-Rad).

Cloning of guide DNA into pCRISPR-SacB. Two complementary single-stranded synthetic oligonucleotides encoding the guide DNA (gDNA), were designed according to the published protocol (11). A protospacer adjacent motif (PAM) sequence (5'-NGG-3') was identified near the 5' end of the *acrA* gene and 30 nucleotides upstream of the PAM region (protospacer) were selected. The forward oligonucleotide was consisted of the chosen protospacer, PAM sequence, and additional five base pairs that are recognition site for the restriction enzyme (RE) BsaI, to facilitate the ligation for the subsequent cloning of pCRISPR-SacB-gDNA. The reverse oligonucleotide of the gDNA is the reverse complement of the forward sequence (Table S1). Phosphorylation of both oligonucleotides was performed through a reaction containing 1 µl of 100 µM forward oligonucleotide, 1 µl of 100 µM reverse oligonucleotide, 5 µl of 10x T4 ligase buffer, 1 µl of T4 PNK and 42 µl of sterile water. After incubation for 30 min at 37 °C, 2.5 µl of 1 M NaCl was added to the reaction mix, heated to 95°C for 5 min using the T100 Thermocycler (Bio-Rad) and then allowed to cool down slowly to room temperature. pCRISPR-SacB was digested with BsaI in a reaction containing 10 µl pCRISPR-SacB (100 ng/µl, total amount 1-2 µg), 1 µl of *Bsa*I, 5 µl of 10x NEB CutSmart Buffer, 34 µl of MilliQ water, leading to a final volume of 50 µl. After incubation for 1 hr at 37°C, the digested product was purified with PureLink™ PCR Purification Kit (Invitrogen) and then quantified for DNA concentration with a Nanodrop3000 spectrophotometer. Dephosphorylation reaction using Calf Intestinal Alkaline Phosphatase (CIP) contained 5 µl 10x CutSmart Buffer, 1 µl CIP, 25 µl purified pCRISPR-SacB and 19 µl sterile water. After incubation for 2 hr at 37°C, the plasmid was purified once more with with PureLink™ PCR Purification Kit (Invitrogen) before the final ligation step, which contained 3 µl digested and purified pCRISPR-SacB (10 ng/µl, total amount of 30 ng plasmid), 2 µl phosphorylated, double-stranded 1 µM gDNA, 1 µl T4 ligase, 2 µl T4 ligase buffer and 12 µl sterile water.

Alternatively, PCR purification with ethanol precipitation following MRC-Holland protocol (15) was also performed in parallel with the PCR purification Kit on replicates. 10 µl of the ligation reaction was used to transform *E. coli* DH5α chemically competent cells and then plated on LB + Kan plates. A negative control without adding the ligase was included and plated on LB + Kan plates. A positive control transforming the *E. coli* DH5α chemically competent cells with pUC19 was also performed and plated on LB + Amp plates.

Colony PCR to assess insertion of gDNA in pCRISPR-SacB. Colony PCR was performed to determine if pCRISPR-SacB-gDNA was successfully cloned, using gDNA as the forward primer and Neo-R (Table S2). Addition of the template DNA was performed by inoculating an individual colony with a 100 µl micropipette tip and dipping the tip into the reaction mix. The colony was recovered by subsequently inoculating the same tip into 5 ml of LB broth supplemented with 50 µg/ml kanamycin. 30X amplification cycle was performed with the T100 Thermocycler (Bio-Rad). The resulting PCR products were visualized on a 1% agarose gel with 0.5x TBE.

PCR Amplification of double-stranded donor DNA. The double-stranded (ds) donor DNA (dDNA) is responsible for the repair and insertion of a stop codon of the double stranded break induced by Cas9 endonuclease. It was designed based on the protocol created by Grandi *et al.* (15). The forward and reverse dDNA was each 45 bp long and had 20 bp overlapping sequence that are complementary to each other (Table S1). An additional condition with 1 µl 5% DMSO was also performed (Table S2). A negative control was included with only one of the two dDNAs as template. The T100 Thermocycler (Bio-Rad) was programmed for an initial denaturation at 95°C for 30 seconds, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 1 minute, with a final extension at 68°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 0.5X TBE buffer at 100 V for 40 minutes. Amplicons were visualized and imaged with UV light using ChemiDoc™ Touch Imaging System (Bio-Rad).

Gene knockout using CRISPR/Cas9 system. Competent *ΔacrE* cells would be first transformed with Cas9 plasmid, followed by transformation with pCRISPR-gDNA-SacB and ds-dDNA, according to the published protocol (11). The gDNA containing sequence flanking the target *acrA* directs the endonuclease Cas9 to cut at the target gene locus. The ds-dDNAs are used as template for repairing the nick using homologous recombination under the assistance of lambda red machinery, which is expressed by pCas. The donor DNA includes an in-frame stop codon, leading to the knockout of *acrA* in *ΔacrE.* Colony PCR would be used to confirm the successful knockout of acrA. CRISPR-gDNA-SacB plasmids would need to be removed as its kanamycin resistance can interfere with the antibiotic screening. Using SacB as a selection marker, plasmids would be cured by growing in 5% sucrose solution.

Restriction enzyme digestion and gel electrophoresis. Restriction enzyme (RE) BsaI (New England BioLabs) as well as several REs with only one cut site on the pCRISPR-SacB plasmid including RsrII (New England BioLabs), SacI (New England BioLabs), XhoI (New England BioLabs), ApaLI (Fermentas), AatII (Fermentas), MscI (Invitrogen) were used for single and double digestions. Each reaction containing 1 µl of each RE, 5 µl of 10X Cutsmart buffer, 10 µl of pCRISPR-SacB (90-110 ng/µl) and sterile water to make up a final volume of 50µl. 10 µl of each digest mixture was loaded with 2 µl 6X loading buffer dye on a 1% agarose gel with SYBR® Safe and run in 0.5X TBE for 45 minutes at 90V. 2 µl of undigested pCRISPR-SacB (100ng/µl) was also run as a negative control.

Minimum inhibitory concentration of kanamycin. The protocol for the MIC assay was adapted from the Hancock lab (16). The 50 mg/ml kanamycin was diluted with LB into 32 µg/ml through two sequential dilutions. Two-fold serial dilution was done in the 96-well plate (Corning® TC-Treated Microplates) with the concentrations at 32-0.0625 µg/ml. The single mutant strains *ΔacrAΔkan* and *ΔacrEΔkan,* along with the wild-type MG1655 and *ΔacrA::kan* were inoculated in 5 ml LB and grown overnight shaking at 37°C. 1:200 dilution

in fresh LB was performed for each strain after 16 hrs and then left for growth until OD_{600} reaches 0.125. 5 µl of bacteria with OD_{600} of 0.125 was used to inoculate each well. Two biological replicates were performed for each strain with the first column being negative control without inoculum and the last column being positive control without antibiotic. The plate was grown overnight for 18 hours at 37˚C before the OD_{600}s were taken with Microplate Reader Model 3550 (Bio-Rad). A follow-up repeat experiment was performed with kanamycin concentrations at 8-3.5 µg/ml.

RESULTS

Induction of FLP recombinase removed the kanamycin cassette in *E. coli* JW0452-3. Each strain *E. coli* JW0452-3, Δ*acrA*Δkan, and Δ*acrE*Δkan matched the expected genotypes. To generate the *ΔacrAΔkan* single mutant, pCP20 was transformed and resulting transformants were patched onto selective media. From a total of 6 colonies patched, 3 of them displayed both kanamycin and ampicillin sensitivity (Figure 1), suggesting the successful removal of kanamycin resistance cassette as as well the pCP20 plasmids. This strain was renamed *E. coli* JW0452-3.1 to indicate the modification. To confirm the deletion of each gene in the corresponding strain, PCR was performed using custom-designed primers flanking *acrA* and *acrE*. The resulting amplicons were visualized by gel electrophoresis and had produced the expected results (Figure 2). The *ΔacrAΔkan* and *ΔacrEΔkan* amplicon sizes were expected to be approximately 677 bp and 710 bp, respectively. The *E. coli* JW0452-3 amplicon was expected to be around 2200 bp, since the kanamycin cassette insert is approximately 1500 bp in size. Our results indicated that *E. coli* JW0452-3, *ΔacrAΔkan* and *ΔacrEΔkan* confirmed removal of the kan resistance cassette.

Loss of *acrA* or *acrE* has no effect on MIC of kanamycin. Due to technical difficulties in creating the double mutant, we decided to use the single mutant to measure the effect of loss of efflux pump component on kanamycin resistance. MIC assays were performed on strains MG1655 (WT), Δ*acrA*Δkan, Δ*acrE*Δkan and *E. coli* JW0452-3 (Δ*acrA*::kan) (Figure 6). Growth inhibition was scored with an overnight OD_{600} value of 0.04 or less, comparable to the LB-only negative control. Growth appeared to be inhibited between 0.25 and 4 µg/mL. Our results suggested that strains MG1655, Δ*acrA*Δkan, and Δ*acrE*Δkan showed similar resistance with the same MIC value of 8 µg/mL (Figure S3). The strain *E. coli* JW0452-3 displayed the highest MIC value of 32 µg/mL, which was expected as this strain contained a kanamycin resistance cassette. For further investigation, a repeated experiment with kanamycin concentration ranging from 8 µg/mL to 3.5 µg/mL was performed and the same MIC value was observed (Figure S4). Lastly, OD_{600} value of all strains, in general, consistently increased when subjected to decreasing kanamycin concentrations (Figure S3).

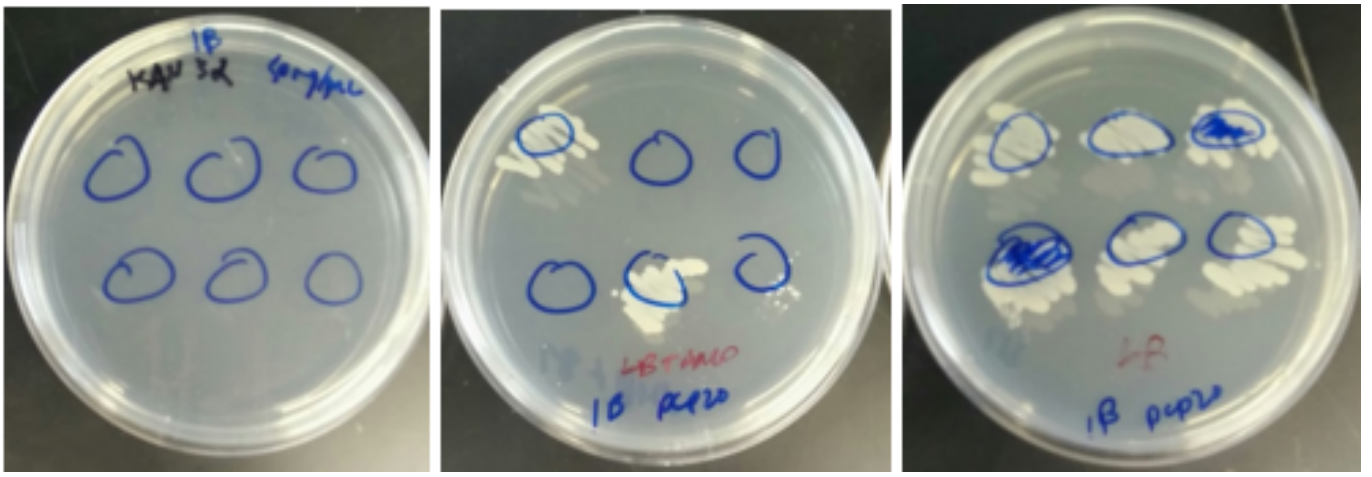

FIG. 1 Patching of transformants onto selective media after FLP-FRT recombination with pCP20. Kanamycin (left) verified the cassette had been removed, whereas ampicillin ensured pCP20 had been lost. LB-agar plates without antibiotics allowed identification of colonies that have met both conditions.

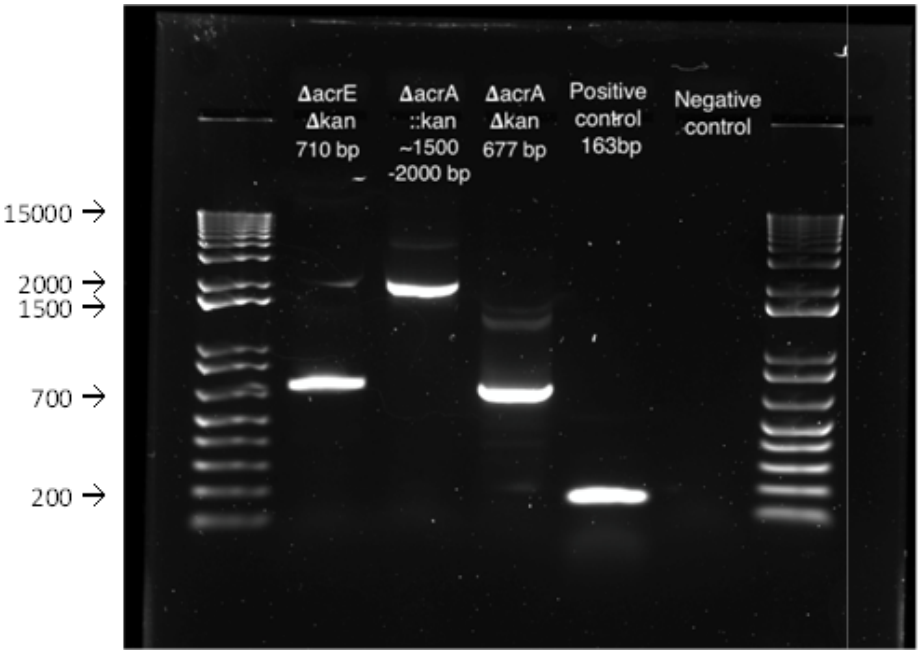

FIG. 2 Gel analysis of PCR products. Various strains were run on a 1% agarose gel stained with SYBR® Safe in 0.5X TBE for 45 minutes at 90V. The positive control was performed using a primer set that amplifies 163bp in pUC19 plasmid. The negative control was performed with *acrE* and *acrA* primers and no template DNA, substituting template volume with sterile dH_2O. The products of from each strain matched the expected band sizes as shown above.

Overall, this data suggested that loss of *acrA* and *acrE* expression had no apparent effect on kanamycin resistance when comparing to wild-type *E. coli*. Creating a double mutant would help elucidate difference in MIC between the various strains.

Construction of the mutagenic donor DNA. We generated a double-stranded oligonucleotide that contained an in-frame stop codon for subsequent transformation later on. Two 45 bp primers with a 20 bp overlap was extended via PCR amplification to create an expected 70 bp product (Figure S2). The products were resolved by gel electrophoresis and an unexpected amplicon size of approximately 120 bp was observed in both DMSO and no DMSO treatment (Figure 4). However, the negative controls, which only amplified a single primer, did not display any bands as expected. Our results suggested the correct dDNA was generated for subsequent transformation upon successful cloning.

FIG. 3 Nucleotide sequence analysis of pCRISPR-SacB. Sequencing was performed by GeneWiz, using the universal primer, Neo-R, to identify a short segment of pCRISPR-SacB containing the 4 Bsa1 cut sites and directed repeats. The sequencing data from our cloning experiment (bottom) was searched against the unmodified pCRISPR-SacB with no gDNA (top) using Snapgene software.

Construction of the double mutant yielded transformants with uncloned pCRISPR-SacB. To generate the double mutant, pCRISPR-SacB was digested with restriction enzyme, BsaI, specifically for our custom-designed gDNA to be cloned in. After a 48h incubation post-transformation, the resulting transformants were isolated and colony PCR was performed to validate oligonucleotide insertion. Gel analysis of 8 different colonies displayed small amplicon sizes of approximately 200 bp, which was far from the expected 450 bp product (Figure S1). These low molecular weight products were more indicative of primer dimer interactions. For further confirmation, 4 of the isolated colonies were sequenced using a universal primer, Neo-R, found on the pCRISPR-SacB vector. In addition, untreated pCRISPR-SacB was sequenced as a control for comparison. Sequencing data did not yield the 30 bp gDNA insert within the BsaI cut sites (Figure 3). Instead, the data revealed only two BsaI cut sites were present and the 30 bp sequence between these sites remained consistent with the untreated pCRISPR-SacB. We expected a total of four BsaI cut sites but observed the stretch of DNA from the second and fourth cut site was missing when comparing with the Addgene sequence from the Marraffini lab (17). Our findings indicated the transformants observed after cloning and transformation carried pCRISPR-SacB with no gDNA insert.

Characterization of pCRISPR-SacB. To investigate the features of pCRISPR-SacB in an attempt to understand difficulty with cloning, this vector was digested with various restriction enzymes. Uncut, single and double digest treatments were performed, and the products were resolved by gel electrophoresis (Figure 5). For a majority of the digests, an approximately 6000 bp product was observed and indicative of linearized pCRISPR-SacB, which was slightly higher than expected, as the gel electrophoresis performed by Zerbini *et al.* showed a band around 3000 bp (11). Additionally, the uncut pCRISPR-SacB, as well as the single digests with RsrII, SacI, and BsaI produced abnormally large bands of over 15,000 bp. This was indicative of incomplete digestion and possibly concatemerized plasmids. Above the linearized form, a thin band representing the relaxed, circular form of pCRISPR-SacB was observed. The double digest treatment also yielded two more products, 3000 bp and 1600 bp, as expected from single-cut restriction enzymes. Our findings did not support the literature about the size of the pCRISPR-SacB vector and were not conclusive of four Bsa1 cut sites.

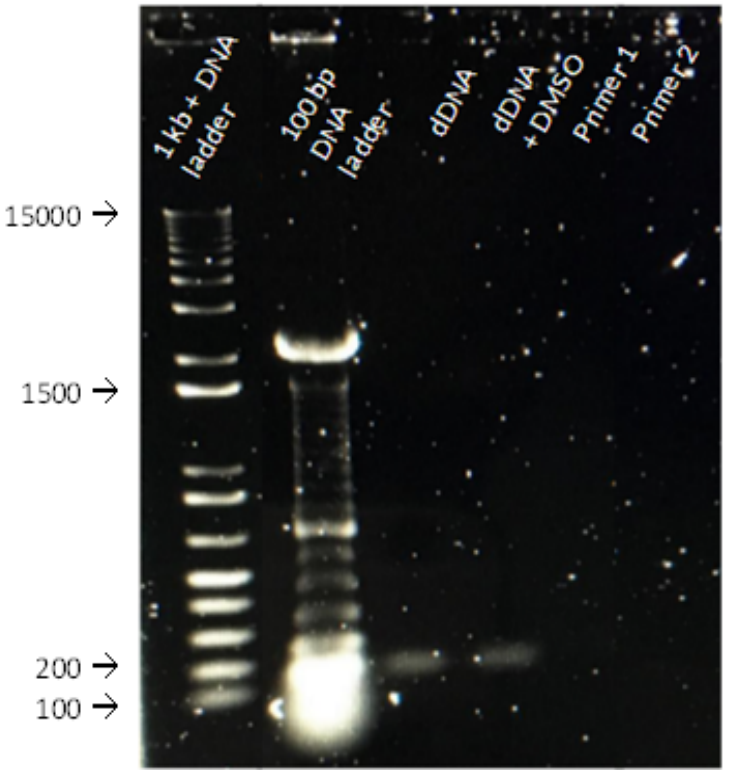

FIG. 4 Gel analysis of PCR products of dDNA. The PCR products were run on a 1% agarose gel stained with SYBR® Safe in 0.5X TBE for 45 minutes at 90V. The dDNA consisted of Primer 1 and Primer 2, which also served as negative controls when loaded individually. DMSO was added to reduce secondary structure interactions.

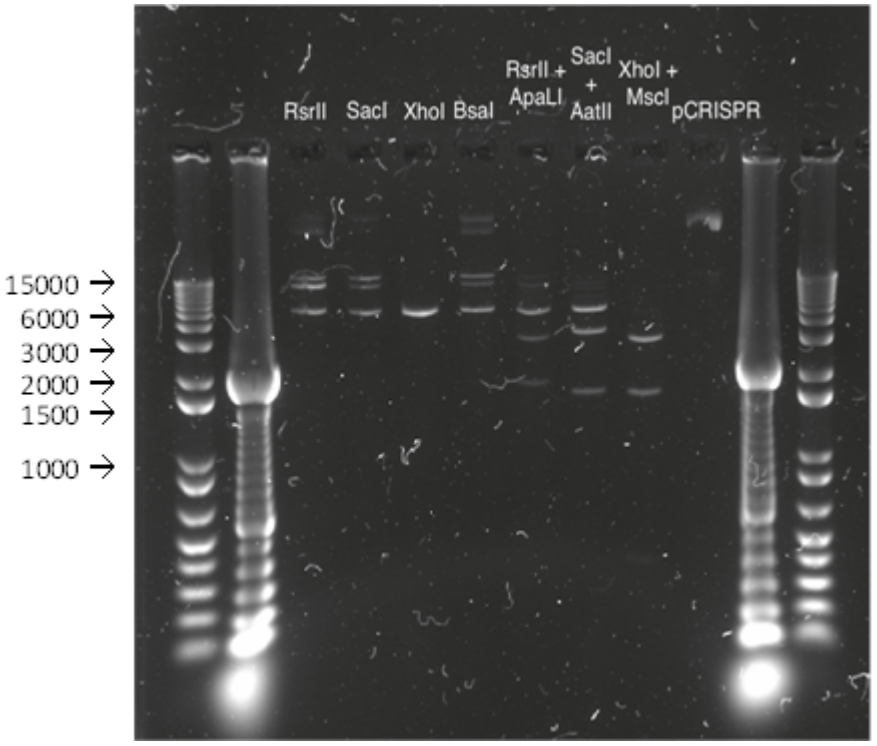

FIG. 5 Gel analysis of pCRISPR-SacB digested with various restriction enzymes. The PCR products from uncut and digest treatments of pCRISPR-SacB run on a 1% agarose gel stained with SYBR® Safe in 0.5X TBE for 45 minutes at 90V. Each digestion treatment was incubated for 1 hour at 37°C.

DISCUSSION

In this study, we aimed to determine whether AcrAB and AcrEF are the two main RND efflux pumps responsible for kanamycin exclusion in *E. coli* MG1655. This was done by first removing the kanamycin resistance cassette in the *ΔacrA::kan* strain using flp recombinase encoded on the pCP20 plasmids, followed by PCR confirmation to assess the removal of the kanamycin resistance cassette in both *ΔacrAΔkan* and *ΔacrEΔkan* strains. Generation of a *ΔacrAacrEΔkan* double mutant was attempted using pCRISPR/Cas9 machinery to insert an in-frame stop codon in *acrA* from the *ΔacrEΔkan* strain. MIC values for each single mutant were determined in comparison to wild-type MG1655 *E. coli.*

We hypothesized that *acrA* and *acrE* single deletions would have a lower resistance to kanamycin than the wild-type strain while a complete deletion of both pumps would render significant susceptibility to the antibiotic. Our findings indicate that deletion of AcrA and AcrE have similar kanamycin resistance in comparison to the WT strain. After encountering difficulties in the cloning of pCRISPR-SacB-gDNA, we led first insights into characterizing pCRISPR-SacB plasmid. As no plasmid map was available, this latter work consisted of performing restriction digests of pCRISPR-SacB with an appropriate set of restriction enzymes (RE) and sequencing parts of the plasmid.

Deletion of single RND pumps does not increase the susceptibility to kanamycin. Susceptibility to kanamycin was assessed by the MIC assay comparing the single mutants, *ΔacrAΔkan and ΔacrEΔkan,* the resistant strain *ΔacrA::kan* and the WT MG1655 strain. The minimum inhibitory concentration for each of susceptible strains was found to be close to 8 µg/ml while the resistant strain had higher MIC value (>16 µg/ml).

Resistance of the wild-type strain was similar to single mutants although we expected it to be significantly higher. It is worth noting that we used E. coli MG1655 rather than it's derivative BW25113 as the wild type strain in this assay. Strain BW25113 was originally created for construction of the Keio gene knock collection (reference Baba paper). BW25113 are MG1655 is isogenic with respect to genes coding for efflux pumps so we do not anticipate any difference in phenotype in our assay. Wild-type MIC is 2-fold lower than the *ΔacrA::kan* strain which is coherent as wild-type MG1655 should be more susceptible to kanamycin than a resistant strain. MIC values for *ΔacrEΔkan* and *ΔacrAΔkan* seem reasonable as exhibiting greater than twice as much susceptibility compared to the resistant strain. Similar MIC values

in the single mutants suggest that the expression of AcrEF pump is able to compensate for the loss of AcrAB and inversely. This result could also be explained by the expression of other RND pumps such as AcrD, which could compensate for the loss of AcrAB or AcrEF. AcrD specifically exports hydrophilic substrates, such as aminoglycosides (5). For AcrD, a previous study led by *Emami M. et al.* demonstrated that AcrS acted as an activator of *acrD* when cells were pretreated with sub-inhibitory kanamycin concentration (20). As we did not perform any antibiotic pretreatment, we would not expect AcrS to activate *acrD* to help compensate for loss of other RND pumps. A previous study performed by Belmans *et al.* exhibit MIC values of 20 µg/ml for *ΔacrAΔkan* and >20 µg/ml for the wild-type strain which is more than 2-fold higher than our findings. It was shown by Hay *et al.* that *ΔacrEΔkan* had a MIC value of 3.1 µg/ml which is closer to our results for this strain.

Comprehensive approach for cloning in pCRISPR-SacB Knocking out *acrA* in *ΔacrEΔkan* for the construction of a double mutant was attempted using pCRISPR-SacB and pCasRed plasmids. The cloning of the guide DNA (gDNA) in pCRISPR-SacB turned out to be a particularly challenging step among the mutagenesis process. Although some transformants were able to grow on selective media, sequencing results did not reveal any insertion of the gDNA in between the directed repeat (DR) sequences of pCRISPR-SacB. Transformants after first attempt at cloning also showed an unexpected phenotype. DH5α colonies transformed with cloned pCRISPR-SacB-gDNA were particularly small compared to DH5α colonies transformed with original pCRISPR-SacB and exhibited a slow growth rate. Colonies were only visible after 48 hours incubation. Colony streaking as well as overnight inoculation in LB media did not lead to any growth of the potential transformants. Whilst transformation with the cloned plasmid let to slow growth, the original pCRISPR-SacB had normal growth. This suggests that cloning is the source of the slow-growing phenotype. The cloned gene may have affected the expression of kanamycin resistance or somehow inhibit the growth of the cells

To better understand why cloning the gDNA in pCRISPR-SacB with BsaI was unsuccessful, we investigated the behaviour of the plasmid by digesting it with appropriate REs. RE digests of pCRISPR-SacB were not matching the expected results based on the original plasmid map of pCRISPR-SacB from Addgene (17). Moreover, the size of the

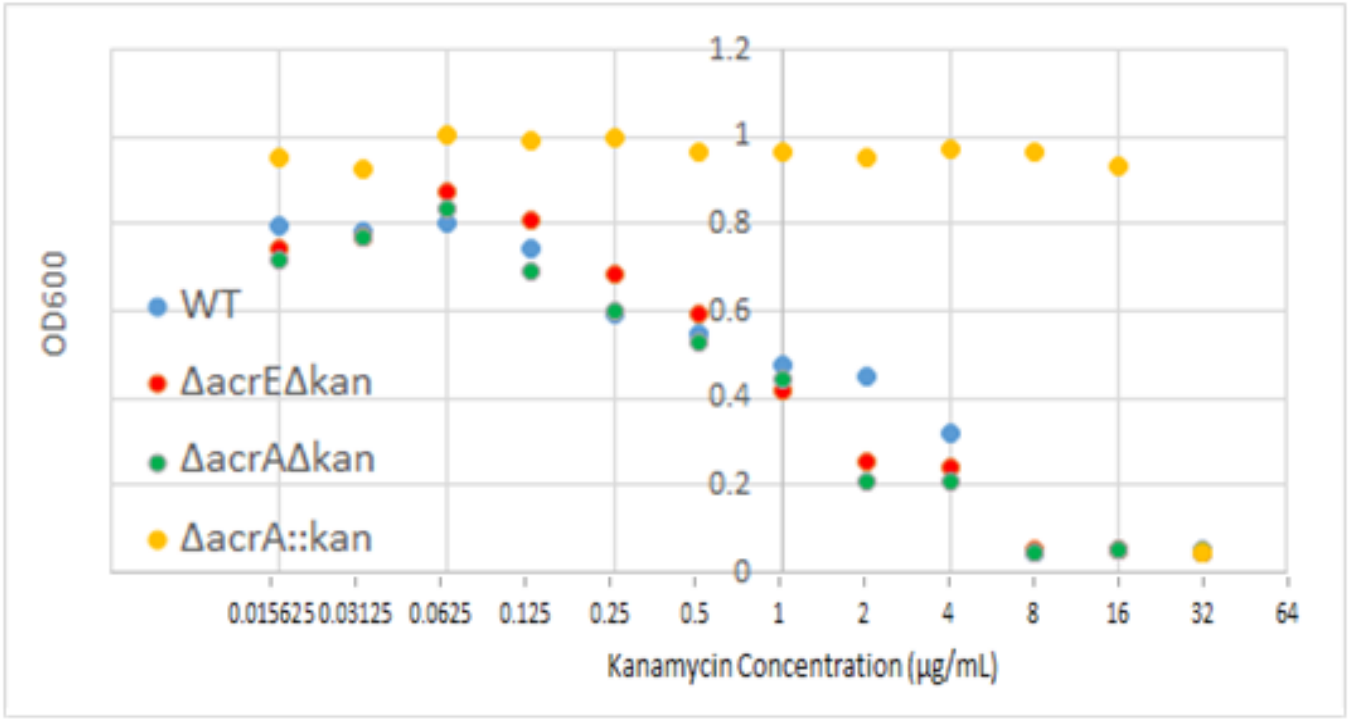

FIG. 6 Growth comparison of *E. coli* strains bearing deletion of *acrE* and *acrA*.

linearized plasmid was over 6 kb for every RE used, which is 3 kb over the size of the modified plasmid from Zerbini *et al.* (11) used for this experiment. Band pattern observed for BsaI was representative of a partial digestion. As the experimental conditions for digestion were similar in the cloning process, difficulties cloning might have arisen from partial digestion of the plasmid, possibly due to non-optimal conditions. We performed a sequencing of the direct repeat (DR) regions of the plasmid where the gDNA should have been inserted after BsaI digestion to gain more precision in the mechanism of cloning. Only two BsaI cut sites were observed from the sequencing results. This result is consistent with the band pattern observed for BsaI digestion of pCRISPR-SacB as no band corresponding to the sequence between the two pairs of BsaI cut sites were observed.

Several controls were performed during the cloning process (Figure 7). Effective transformation (step 5) was assessed by transforming pUC19 and pCRISPR-SacB without digesting the plasmids in commercially available DH5-α competent cells. This positive control yielded hundreds of colonies for pUC19 and around thirty colonies for pCRISPR for the same amount of DNA transformed. This result means that transformation efficiency for pCRISPR-SacB is much less than pUC19. As a control for the ligation step (step 4), positive control using pUC19 digested with HindIII without ligation of any template yielded as many colonies as when directly transforming pUC19 in competent cells. As HindIII generates compatible ends after cutting, plasmid was able to self-ligate, confirming that T4 ligase used in step 5 was properly closing pUC19. As the main challenge during the process was to retrieve high yields of DNA when purifying the plasmid after step 2 and 3, we performed a control without template DNA and without dephosphorylation of the plasmid. As BsaI digestion had incompatible ends, a full digestion of the plasmids should yield no or very few transformants after ligation. However, we observed approximately as many colonies as without digestion with the RE. This control means that digestion with BsaI was not efficient enough to properly digest the plasmid. The result is in agreement with the band pattern of the gel electrophoresis, which was characteristic of partial digestion.

These results indicate that cloning of pCRISPR-SacB-gDNA needs high purification yields as its transformation needs much more DNA to be successful compared to pUC19.

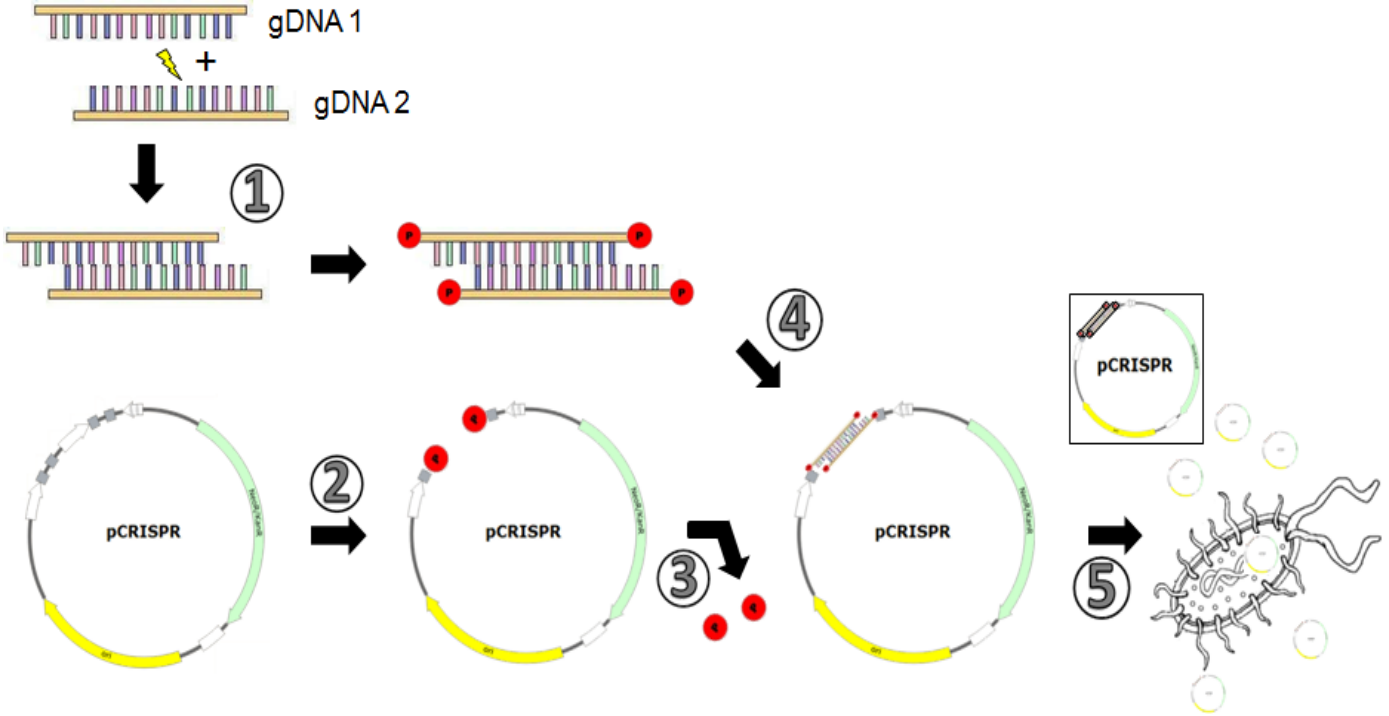

FIG. 7 Procedural framework of cloning gDNA into pCRISPR-SacB vector. Generation of the double mutant was initialized by the following steps: 1) Annealing two custom-designed oligonucleotides and phosphorylating with T4 PNK; 2) Digesting pCRISPR-SacB with Bsa1; 3) Dephosphorylating purified pCRISPR-SacB with CIP; 4) Ligating gDNA into pCRISPR-SacB with T4 Ligase; 5) Transforming cloned pCRISPR-SacB into competent *E. coli* DH5α cells.

Purification using ethanol precipitation or PureLink™ PCR Purification Kit (Invitrogen) did not show any significant difference in yields. However, the low amount of DNA to recover during ethanol precipitation made the process less efficient. Adding glycogen during the procedure should results in better yield. As glycogen is insoluble in ethanol, it precipitates and traps DNA. This results in formation of visible pellet after centrifugation while no pellet was observed without using glycogen (19). As it was observed that digestion with BsaI followed by ligation with no template yielded colonies, the dephosphorylation of the plasmid is necessary to ensure growing colonies are not products of self-ligation.

Conclusions. In conclusion, our results suggest that a deletion of *acrA* in *E. coli* BW25113 does not render the cell more susceptible to kanamycin than a bacterium lacking the *acrE* gene as both strains had a similar MIC of 8 µg/ml. This result could either suggest that AcrEF efflux pump is able to compensate for the loss of AcrAB and inversely or that other antibiotic resistance mechanisms are predominant. Generation of a double mutant for both efflux pumps will be able to differentiate these two hypotheses. MIC for the wild-type strain was also found to be 8 µg/ml, which is lower than expected as deleting one of the RND pumps should increase susceptibility to the antibiotic. Additionally, restriction digest performed on pCRISPR-SacB showed an unexpected plasmid length of 6kb instead of 3kb. However, even if it is possible that an incorrect plasmid has been used, cloning should have been successful as the BsaI sites are present. Complete sequencing of the plasmid would give better insights on its behaviour and how to improve the efficiency of the cloning procedure.

Future Directions. In this study, we have generated preliminary data suggesting that deletions of *acrA* or *acrE* alone in *E. coli* BW25113 strains do not render the bacteria significant susceptibility to kanamycin compared to the WT *E. coli* MG1655. However, more experimental replicates could be done in the MIC assay to help provide stronger evidence. Using the parent strain *E. coli* BW25113 as wild-type instead of *E. coli* MG1655 is also suggested for the consistency of the comparison.

Additional studies towards the other members of this efflux pump family as well as the phenotype of the double mutant in both AcrAB and AcrEF complexes would help elucidate the constancy of MIC values between the different strains. Indeed, a significantly lower MIC value for the double mutant in comparison to the single mutants would prove that AcrAB and AcrEF are the two main RND efflux pumps responsible for kanamycin exclusion. Otherwise, a similar MIC value would suggest that other pumps from the RND family or any other resistance mechanism can compensate for the loss of AcrAB or AcrEF.

Successful construction of *ΔacrAΔacrEΔkan* double mutant and MIC result would further help elucidate whether acrAB and acrEF are the two main RND efflux pumps that are responsible for kanamycin exclusion. In order to achieve that, more work needs to be done in determining the optimal condition for the action of RE BsaI. Complete sequencing of pCRISPR-SacB that characterizes the size of the plasmid and respective BsaI cut sites would provide more insights on how to optimize the cloning steps.

Alternatively, investigations on other RND pumps or regulatory genes such as *acrS* and *acrD* involved in the kanamycin resistance could be done in the future. As *acrD* is upregulated by AcrS, overexpressing *acrS* in a double mutant *ΔacrAΔacrEΔkan* and determining the kanamycin MIC could assess whether AcrD is involved in kanamycin export (20). This would help assess the contribution of different efflux pumps in mediating kanamycin resistance. Additionally, AcrB and AcrF are the other components of the RND pumps involved in substrate recognition, which could be investigated also.

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