Pre-treatment with cell-wall targeting antibiotics and aminoglycosides does not confer cross protection to cell-wall targeting antibiotics in *Escherichia coli* despite upregulation of *rprA*

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SUMMARY The Rcs phosphorelay system is a signaling pathway in *Escherichia coli* involved in cell envelope stress response. RcsB, located in the cytoplasm, activates numerous downstream targets including *rprA* upon disruption of the peptidoglycan layer. *rprA* is believed to confer greater resistance to cell-wall targeting antibiotics in *Escherichia coli*. Previous studies observed *rprA* upregulation in wild-type *Escherichia coli* (DH300) following exposure to aminoglycosides and cell-wall targeting antibiotics, but not in *rcsB* deletion mutants (DH311). A correlation was also shown between *rprA* expression and susceptibility to cell-wall targeting antibiotics where the absence of *rprA* expression was found to coincide with increased susceptibility. It is unknown whether elevated expression of *rprA* results in increased resistance to antibiotics. We hypothesized that pre-treatment with cell-wall targeting antibiotics and aminoglycosides will result in *rcsB*-mediated upregulation of *rprA*, conferring increased resistance to subsequent treatments with cell wall-targeting antibiotics. A preliminary round of minimum inhibitory concentration (MIC) assays was conducted with penicillin, streptomycin and tetracycline to determine sub-MIC levels of each antibiotic for *E. coli* DH300 and DH311. Following this, *rprA* expression was quantified using a β-galactosidase activity assay based on expression of an *rprA-lacZ* promoter reporter fusion gene. Strains were then pre-treated at sub-MIC levels of each antibiotic, and another set of MIC assays was carried out to measure resistance to penicillin. Under all antibiotic pre-treatments, DH300 showed increased β-galactosidase activity indicating increased *rprA* expression compared to DH311. DH300 pre-treated with penicillin and tetracycline also showed increased *rprA* expression compared to untreated DH300. Regardless of *rprA* expression levels, no differences in penicillin resistance were seen between DH300 and DH311 in MIC assays. Similarly, pre-treatment with antibiotics did not result in any difference in resistance to penicillin between DH300 and DH311 in subsequent MIC assays.

INTRODUCTION

Gram-negative bacteria such as *Escherichia coli* are enclosed in cell envelopes that provide protection from environmental factors such as antibiotics (1). The peptidoglycan layer within the cell envelope maintains cell shape and resists lysis due to osmotic pressure (2). β-lactam antibiotics such as penicillin kill bacteria by binding to penicillin-binding proteins required for peptidoglycan synthesis (3). Upon experiencing peptidoglycan stress, bacteria express stress response genes that encode for proteins which preserve the integrity of the cellular envelope (1). It was shown through a series of experiments that inhibition of peptidoglycan synthesis using β-lactam antibiotics resulted in the induction of the regulator of capsule synthesis (Rcs) phosphorelay system (1).

The Rcs phosphorelay system has three main components: RcsC and RcsD located in the inner membrane and RcsB in the cytoplasm (4). RcsF is located in the outer membrane and acts as an envelope stress detector between environmental stimuli and the Rcs phosphorelay system (4). Upon phosphorylation by RcsD, RcsB promotes the transcription of many downstream genes, including *osmC, cps,* and the small non-coding RNA, *rprA* (4,5). The *rprA* gene has been identified as one of the candidates required for resistance

Published Online: 9 September 2019

Citation: Bowman J, Couput R, Pookpun A, Wong K. 2019. Pre-treatment with cell-wall targeting antibiotics and aminoglycosides does not confer cross protection to cell-wall targeting antibiotics in *Escherichia coli* despite upregulation of *rprA*. UJEMI 24:1-11

Editor: Julia Huggins, University of British Columbia

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against cell-wall targeting antibiotics (1,6). It is believed that *rprA* regulates downstream genes that are required for survival in the presence of cell-wall targeting antibiotics (1). Furthermore, elevated *rprA* expression has been observed in *E. coli* exhibiting resistance against cell wall targeting antibiotics (6).

Previous studies have performed experiments to investigate the relationship between *rprA* and antibiotic resistance. Jalalkamali *et al*. measured the MIC of DH300 and DH311 treated with penicillin and phosphomycin, antibiotics targeting cell wall synthesis (6). DH300 and DH311 are strains derived from their parental *E. coli* strain MG1655, but the *rprA* promoter in DH300 and DH311 has been fused to *lacZ*. Compared to the DH300 wild-type (WT) strain, DH311 has a kanamycin-resistance gene inserted into the *rcsB* region (Δ*rcsB*), rendering the transcribed mRNA non-functional (7). DH300 had an MIC two-fold higher than DH311 at 25.0 and 12.5 µg/mL for penicillin and 6.25 and 3.13 µg/mL for phosphomycin, respectively (6). These results suggest that DH311 was not able to utilize the Rcs phosphorelay system to induce upregulation of *rprA* (6). This is supported by Richter *et al*., who found a similar decrease in antibiotic resistance to β-lactams for the mutant strain and concluded that *rcsB* contributes to the intrinsic resistance of *E. coli* to antibiotics targeting peptidoglycan synthesis (8). The role of *rprA* has also been investigated with respect to antibiotic resistance linked to the Rcs phosphorelay system. Jalalkamali *et al.* used a β-galactosidase activity assay to measure the expression of an *rprA–lacZ* reporter fusion gene following antibiotic treatment (6). This experiment showed that following stress induction with antibiotics, *rprA* expression was significantly upregulated in DH300, but not in DH311 (6). Upregulation of *rprA* was also seen following treatment with aminoglycosides, such as streptomycin and tetracycline (6). This information suggests a correlation between higher *rprA* expression and the increase in MIC of cell wall-targeting antibiotics on DH300, compared to DH311. Overall, these studies support the conclusion that *rcsB* is necessary for the upregulation of *rprA*, which confers increased resistance to treatment with cell wall-targeting antibiotics (6, 8).

Although a lack of *rprA* expression has been shown to be involved with decreased resistance to cell-wall targeting antibiotics (6), it is unknown whether further elevating *rprA* expression may confer additional resistance towards subsequent cell-wall targeting antibiotic treatments. Given that antibiotic treatment will result in an *rcsB*-mediated upregulation of *rprA* (6, 8), the effect of elevated *rprA* expression on increased cell-wall targeting antibiotic resistance was evaluated through pre-treatment of *E. coli* with penicillin, tetracycline, or streptomycin. We hypothesized that following pre-treatment with cell wall-targeting antibiotics or aminoglycosides, DH300 would display increased resistance to cell wall-targeting antibiotics compared to DH311. Elevated levels of *rprA* were also expected in *E. coli* exhibiting increased resistance to subsequent treatments with cell wall-targeting antibiotics.

METHODS AND MATERIALS

Bacterial strains and preliminary strain verification. A summary of bacterial strains used in this study is shown in Table 1. DH300 and DH311 were obtained from the Ramey culture collection maintained by the Department of Microbiology and Immunology at the University of British Columbia. Isolated colonies from the DH300 and DH311 plates were streaked onto a fresh Lysogeny broth (LB)-agar plate and an LB-agar plate containing 50 µg/mL kanamycin, respectively.

Oligonucleotide primer design. Primers were designed to flank the *rcsB* region in *E. coli* MG1655, the parent strain used to produce DH300 and DH311 used in this study (7). The forward and reverse primers shown in Table 2 are designed to anneal 42 bp upstream and 24 bp downstream of the *rcsB* region. The annealing locations of these primers were chosen specifically to allow matching melting temperatures (Tm) (within 2 ^{o}C) as well as suitable GC content (40%-60%). The primers were designed using SnapGene and the Tm and GC content of each primer were assessed with the Integrated DNA Technologies website (https://www.idtdna.com/calc/analyzer/).

Colony PCR and gel electrophoresis for strain verification. DH300 and DH311 were grown overnight in LB broth. The DNeasy Blood and Tissue Kit (Qiagen Cat. #: 69504) was used to extract the genomic DNA of each sample. The total PCR mixture volume was 50 µL, comprising of 5 µL 10x PCR buffer, 1 µL 10 mM dNTPs, 1 µL 10 µM forward primer, 1 µL 10 µM reverse primer, 1.5 µL 50 mM $MgCl_2$, 1 µL (10 ng) template DNA, 0.2 µL platinum Taq DNA polymerase (Invitrogen) and 39.3 µL sterile dH_2O. The primer pair shown in Table 2 was used to amplify the *rcsB* region in both strains. PCR reactions were run on the BioRad T100^{TM} Thermal Cycler at an initial denaturation of 94^{o}C for 2 minutes, 35 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 53^{o}C for 30 seconds and extension at 72^{o}C for 2 minutes, and a final extension at 72^{o}C for 10 minutes. 1.0 % gel electrophoresis was performed to determine the amplicon size in each PCR reaction.

Sanger sequencing of colony PCR amplicons. The amplicons of DH300 and DH311 were purified using the PureLink PCR Purification Kit (Invitrogen Cat. #K3100-01). The forward and reverse sequencing primers were the same ones used in colony PCR (Table 2). According to the guidelines defined by GeneWiz, the forward and reverse sequencing primers were diluted to 5 µM and the concentration of each amplicon was diluted to 2 ng/µL in the final mixture (9).

Preparation of antibiotic stock solutions. Concentrated stock solutions were made for all antibiotics used in this experiment. Stocks of penicillin-G (Sigma-Aldrich 24C-2180), streptomycin sulfate salt (Sigma-Aldrich S6501-50G), and tetracycline hydrochloride (Sigma-Aldrich 31K1763) at 100 mg/mL were sterilized using a 0.22 µm filter. Stocks were diluted in dH_2O to 200 µg/mL, acting as the 2X working solution for the MIC assays.

Preliminary MIC assay to determine DH300 and DH311 sub-MIC for the subsequent pre-treatments. To culture DH300 and DH311, strains were inoculated in 5 mL of LB broth. Cultures were grown in a shaking incubator at 37 ^{o}C for 3-4 hours. The OD_{600} was measured and necessary dilutions were made in LB broth at a final volume of 10 mL to achieve a cell concentration of 10^4-10^5 cells/mL. To perform the MIC assay, 100 µL of sterile LB broth was added to each well (columns 1-11). 100 µL of the 2X penicillin antibiotic stock solution was added to the first well of row A, and 2-fold serial dilutions were made, up to the 10th column. Column 11 lacked antibiotics and was used as a positive control for bacterial growth. This same scheme was followed for row B. Row C and D were plated with 2X streptomycin stock, and row E and F with 2X tetracycline stock. Row G with an addition of 50 µL LB broth served as a blank. 50 µL of diluted DH300 was plated across the wells in columns 1 to 11 for row A, C, and E, while 50 µL of diluted DH311 was plated across the wells in columns 1 to 11 for row B, D, and F. A maximum concentration of 67 µg/ml was tested in the MIC assay based on the MICs reported by Jalalkamali *et al.* (6). After incubation for 18-19 hours at 37°C, OD_{600} readings were taken with the BioTek Synergy H1 plate reader to determine the MICs for each antibiotic. Sub-MIC values for subsequent pre-treatment were calculated as half of the mode MIC as determined from three trials of assays. (10)

β-galactosidase reporter assays to evaluate the expression of *rprA* in DH300 and DH311 after subculturing in sub-inhibitory concentrations of penicillin, streptomycin, or tetracycline. DH300 and DH311 have the *rprA-lacZ* promoter fusion gene allowing for the qualitative β-galactosidase reporter assay to be conducted. The *E. coli* MG1655 strain was used as a positive control, given its known LacZ activity while the *E. coli* DH5α strain

TABLE 1. *E. coli* K-12 strains used in this study

Strain	Designation	Relevant Genotype	Reference
DH300	WT	MG1655 Δ(*argF-lac*)*U169*; *rprA142-lacZ*	7
DH311	Δ*rcsB*	DH300 *rcsB*::Kan^{r}	7

was used as a negative control, given its deficiency in *lacZ*. DH300, DH311, MG1655, and DH5α were grown overnight in LB broth. Samples were sub-cultured by adding 50 µL of overnight culture to 5 mL of broth at sub-MICs of penicillin, streptomycin, or tetracycline determined by the preliminary MIC assay (8.33 µg/mL, 4.17 µg/mL, and 0.52 µg/mL respectively). Cultures were grown in a shaking incubator at 37 °C for 2 hours. The cultures were placed on ice for 20 minutes to prevent further growth. Absorbance was measured at 600 nm to normalize for cell density in β-galactosidase units calculations. 0.5 mL of culture was added to 0.5 mL of Z-buffer ($Na_2HPO_4{\cdot}7H_2O$, $NaH_2PO_4{\cdot}H_2O$, KCl, $MgSO_4{\cdot}7H_2O$, and β-Mercaptoethanol). 100 µL of chloroform and 50 µL of a 0.1% SDS solution were added to the assay mixture and vortexed. After incubating at 28°C for 5 minutes, 0.2 mL of ONPG was added to the assay until a yellow colour developed and the reaction was stopped by adding 0.5 mL 1 M Na_2CO_3 solution after 28 minutes. The absorbance of each assay was recorded at 420 nm to detect for o-nitrophenol and light scattering by the cell debris, followed by a measurement at 550 nm to correct for the light scattering. Enzyme units of β-galactosidase were calculated using the following equation (11):

$$\frac{1000 \times (OD_{420} - 1.75 \times OD_{550})}{\text{time of reaction (min)} \times \text{volume of culture (mL)} \times OD_{600}} = \text{units of β-galactosidase}$$

MIC assay to determine DH300 and DH311 resistance to penicillin following pre-treatment in untreated, penicillin, streptomycin and tetracycline conditions. DH300 and DH311 were grown overnight in LB broth. Samples were sub-cultured by adding 50 µL of overnight culture to 5 mL of broth at sub-MICs of penicillin, streptomycin, or tetracycline (8.33 µg/mL, 4.17 µg/mL, and 0.52 µg/mL respectively). An untreated control strain was also sub-cultured. All cultures were grown in a shaking incubator at 37 ^oC for two hours. The OD_{600} was measured and cultures were then diluted to 5×10^5 cells/mL for plating. Two sterile 96-well plates were used for each MIC assay. The first plate included one row each for DH300 and DH311 pre-treated with penicillin (rows A and B), streptomycin (rows C and D), and untreated samples (rows E and F), as well as a blank with no bacteria (row G). The second plate included one row each for DH300 and DH311 pre-treated with tetracycline (rows A and B) and untreated samples (rows C and D), as well as a blank with no bacteria (row E). 100 µL of sterile LB broth was added to each well being used (in columns 1-11). The MIC assay was set up through plating the 2X antibiotic stock at 2-fold serial dilutions following the same preliminary MIC assay plating scheme across rows. Column 11 lacked antibiotics and was used as a positive control for bacterial growth. Following this, each well was seeded with 50 µL of the 5×10^5 cells/mL solution, except for the last row on both plates, which received 50 µL of additional sterile LB broth to serve as a blank. A maximum concentration of 67 µg/ml was tested in the MIC assay based on the MICs reported by Jalalkamali *et al.* (6). Following incubation for 16-24 hours at 37°C, OD_{600} readings were taken to identify the concentration at which bacteria could no longer grow successfully. This procedure was performed for three biological replicates.

RESULTS

Strain verification of DH300 and DH311. To verify the identity of the strains used in this study, genomic DNA extraction followed by colony PCR was performed to amplify the putative *rcsB* region in each strain. Forward and reverse primers were designed such that they would flank the *rcsB* region in *E. coli* MG1655, the parental strain from which DH300 and DH311 were generated (7). After colony PCR was conducted, the resulting amplicons were run on a 1% agarose gel to determine the size of the amplified region. In DH300, the

TABLE 2. Oligonucleotides used in colony PCR

Description	Sequence (5'-3')	Tm (^oC)	% GC
rcsB-Forward	ACAGTTATGTCAAGAGCTTGCTGT	56.7	41.7
rcsB-Reverse	GCGTCTTATCTGGCCTACAGGT	58.6	54.5

rcsB region should be 651 bp in length (12); as the forward and reverse primers were placed 42 bp upstream and 24 bp downstream of the *rcsB* region, respectively, the expected amplicon length of DH300 is 717 bp. As shown in Figure S1, the amplicon of DH300 migrates at approximately 750 bp, which is consistent with the expected size. In contrast, the amplicon from DH311 appears larger than the DH300 band and has an approximate size of 900 bp. Since the genotype of DH311 (Table 1) suggests an insertion of a kanamycin-resistance gene into the *rcsB* region, it is expected that the amplification of the putative *rcsB* region in DH311 will result in a higher molecular weight PCR product compared to DH300. In addition, DH311 was able to grow in the presence of kanamycin whereas DH300 did not. Taken together, these results support the conclusion that the acquired strains differ in the region of the genome corresponding to *rcsB*.

Sanger sequencing of *rcsB* amplicons generated from DH300 and DH311 reveals the presence of an insertion in the mutant strain, resulting in a frameshift mutation. To further characterize the PCR amplicons of the *rcsB* region in DH300 and DH311, samples were sequenced. The forward and reverse reads of DH300 and DH311 amplicons were aligned to the sequence of *rcsB* obtained from the *E. coli* MG1655 genome using BLAST. Both the forward and reverse reads of DH300 amplicon are identical to the *rcsB* sequence. The alignments of DH311 amplicon reads to the *rcsB* sequence reveals an insert in the *rcsB* region of this strain. The insertion appears at the end of the forward read which expectably appears at the beginning of the reverse read (Figure S2). The online ExPASy translate tool (https://web.expasy.org/translate/) was used to analyze the effect of the insert. The translation of forward and reverse nucleotide sequences reveals that a premature stop codon is introduced through the insert, resulting in a frameshift mutation (Figure S3). Consequently, the transcribed *rcsB* from DH311 cannot be translated to a functional RcsB protein. Together, these results indicate that DH300 has the native sequence of *rcsB* while a frameshift mutation has been introduced to DH311 through an insert.

To characterize the insert, both sequences from the forward and reverse reads were aligned to a kanamycin resistance gene. Despite Majdalani *et al.* reporting the insert in DH311 to be a kanamycin-resistance gene, the alignment did not show matching results (7). Given that they also prepared DH311 using P1 transduction, it was speculated that the insert could be from a phage (7). No match was found when the insert was aligned with Enterobacteria phage P1 (taxid:10678). Finally, the insert sequence was aligned with the NCBI nucleotide database, but this resulted in over 110 BLAST hits. The top 100 hits with distinct identities all had 100% identity to the insert sequence. Therefore, it was not possible to assign a specific identity to the insert. The attempt to characterize the insert has illustrated that it is not a kanamycin-resistance gene and that its identity remains inconclusive.

DH300 and DH311 show equivalent MIC when treated with tetracycline, streptomycin, and penicillin. To determine the sub-MIC of each antibiotic for the pre-treatment, a preliminary MIC assay on DH300 and DH311 was performed using penicillin, streptomycin, and tetracycline. Cultures were grown up in a shaking incubator at 37 °C for

TABLE 3 Minimum inhibitory concentration (μg/mL) of DH300 and DH311 treated with penicillin, streptomycin and tetracycline. Strains were grown at 37 °C for 2 hours and diluted to a cell density of to a cell density of 10^{4}-10^{5} cells/mL. Strains were then treated with 2-fold serially diluted concentrations of penicillin, streptomycin, or tetracycline. Three biological replicates were performed for this experiment denoted as trial 1, trial 2 and trial 3.

Strain	Minimum Inhibitory Concentration (µg/mL)								
	Penicillin			Streptomycin			Tetracycline		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
DH300 (WT)	17	17	33	8	8	8	1	1	1
DH311 (Δ*rcsB*)	17	17	33	8	8	8	1	1	1

3-4 hours then diluted to 10^4-10^5 cells/mL using OD_{600} measurements. Following this, a MIC assay was performed to determine the MIC for each of these strains treated with penicillin, streptomycin, and tetracycline at various concentrations, using a 2-fold dilution scheme starting at a maximum concentration of 67 µg/mL. This procedure was repeated for 3 biological replicates. Table 3 shows the lowest concentration at which no growth was recorded for each of these antibiotics which defines the MIC. The MICs for DH300 and DH311 treated with tetracycline, streptomycin, and penicillin were 1 µg/mL, 8 µg/mL, and either 17 µg/mL or 33 µg/mL, respectively (Table 3). No difference in MIC between DH300 and DH311 in all of the antibiotic treatments were observed. Dividing the mode MIC value by 2, the sub-MICs for tetracycline, streptomycin, and penicillin were 0.52 µg/mL, 4.17 µg/mL, and 8.33 µg/mL respectively.

***rprA* expression occurs in an RcsB dependent manner and is upregulated under tetracycline and penicillin treatments, but not streptomycin.** In order to gauge whether antibiotic pre-treatment can upregulate *rprA* expression, the *rprA* expression was measured for DH300 and the DH311 following penicillin, streptomycin, and tetracycline treatments. DH300 and DH311 were grown at 37 °C for 2 hours under sub-MIC conditions of the different antibiotics as determined from the preliminary MIC (Table 3). Then, a β-galactosidase reporter assay was used to quantify the expression of the *rprA-lacZ* reporter fusion gene. *E. coli* strains MG1655 and DH5α were used as positive and negative controls because they are genotypically known to be lac^+ and lac^-, respectively. High levels of β-galactosidase activity were measured in MG1655 while no activity was detected in DH5α. For all the given antibiotic treatments, it was observed that DH300 exhibits significantly greater β-galactosidase activity, indicating greater *rprA* expression, compared to DH311 which exhibits expression levels comparable to the negative control (Figure 1). Additionally, when comparing between antibiotic treatments, tetracycline and penicillin yields increased β-galactosidase activity, while being unaffected with streptomycin treatment. These results indicate that *rprA* expression is induced in an RcsB dependent manner. In addition, streptomycin treatment fails to induce *rprA* expression compared to penicillin and tetracycline.

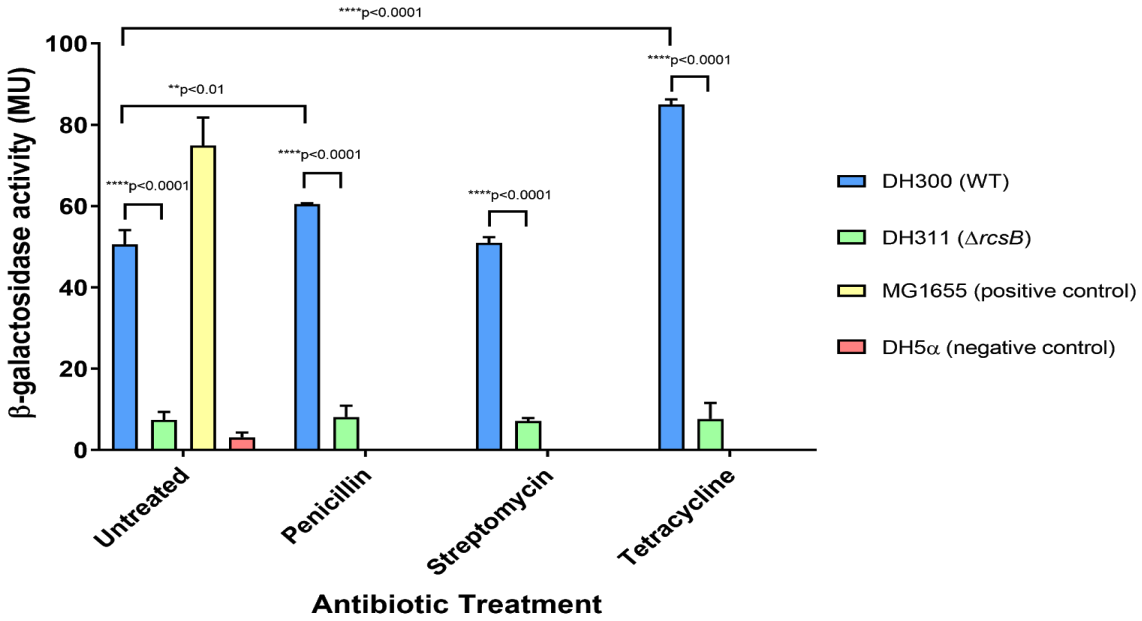

FIG. 1 β-galactosidase reporter assay shows *rprA* expression to occur in an RcsB dependent manner, with upregulation of *rprA* under tetracycline and penicillin, but not streptomycin. DH300 and DH311 were sub-cultured in sub-MIC concentrations of either no antibiotic, penicillin, streptomycin or tetracycline until OD_{600} reached 0.28 - 0.70. A β–galactosidase reporter assay was then performed on each sub-cultured strain (n=3), measuring the absorbance at 420 nm for detection of o-nitrophenol and light scattering by the cell debris. Absorbance measurements were corrected for light scattering through obtaining absorbance at 550 nm and normalized for cell density through measuring absorbance at 600 nm.

TABLE 4 Penicillin MIC (μg/mL) of DH300 and DH311 following pre-treatment with antibiotics. Strains were subcultured in sub-MICs of no antibiotic, penicillin, streptomycin, or tetracycline and incubated at 37 °C for 2 hours. After normalizing to a cell density of 5×10^5 cells/mL, strains were then treated with 2-fold serially diluted concentrations of penicillin. This procedure was repeated 3 times for biological replicates, denoted as trial 1, trial 2, and trial 3.

Strain	Minimum Inhibitory Concentration (µg/mL)											
	Pre-Treatment											
	Untreated			Penicillin (8.33 µg/mL)			Streptomycin (4.17 µg/mL)			Tetracycline (0.52 µg/mL)		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
DH300 (WT)	33	67	67	33	33	33	33	33	67	33	33	67
DH311 (Δ*rcsB*)	33	67	67	33	33	33	33	33	67	33	33	67

Antibiotic pre-treatment of *E. coli* does not result in an increased MIC in subsequent treatments with penicillin. In order to determine if pre-treatment with penicillin, streptomycin or tetracycline would confer increased resistance to subsequent treatments with cell wall-targeting antibiotics, MIC assays were performed following pre-treatment with the mentioned antibiotics. In order to subject these samples to pre-treatment by aminoglycosides and cell-wall targeting antibiotics, overnight DH300 and DH311 cultures were sub-cultured in sub-MICs of each antibiotic. Cultures were grown at 37 °C for 2 hours then diluted to 5×10^5 cells/mL. Following this, a MIC assay was conducted to determine the penicillin MIC of each of these pre-treated strains (Table 4). Untreated samples acted as a control to confirm whether antibiotic pre-treatment conferred increased antibiotic resistance to subsequent penicillin treatments. DH311 was included as a negative control to determine whether *rcsB* is involved in any observed increases in antibiotic resistance. Given the activation of the *rcsB* pathway, the pre-treated samples are hypothesized to display a higher MIC. The resulting data showed that for the untreated samples, both DH300 and DH311 had a MIC of 33 µg/mL in Trial 1, and 67 µg/mL in Trials 2 and 3. For pre-treatment with penicillin, both DH300 and DH311 had a MIC of 33 µg/mL in all three trials. With respect to the streptomycin and tetracycline pre-treatments, DH300 and DH311 both had a MIC of 33 µg/mL in Trials 1 and 2, followed by a 67 µg/mL in Trial 3. These data show that throughout all antibiotic pre-treatment conditions, no penicillin MIC differences were observed between the two strains within each trial. Furthermore, similar penicillin MIC values within a 2-fold difference were observed for the different antibiotic pre-treatment conditions. Taken together, these results indicate that antibiotic pre-treatment of DH300 does not increase the MIC in subsequent treatment with penicillin compared to the untreated. In addition, resistance to penicillin is likely not dependent on *rcsB* as DH300 and DH311 showed the same MICs.

DISCUSSION

The focus of the paper was to investigate if pre-treatment of *E. coli* with penicillin, streptomycin or tetracycline would result in increased resistance to subsequent treatments with cell wall-targeting antibiotics. Specifically, the paper aims to study whether *rprA* is the signaling factor that is involved in increased antibiotic resistance. Experiments done by Laubacher and Ades have demonstrated that RcsB activity is necessary for the survival of *E. coli* in the presence of cell-wall targeting antibiotics (1). Upon activation, RcsB can activate the transcription of downstream targets including *rprA* (4,5). Jalalkamali *et al.* conducted experiments that associate lack of *rprA* expression with increased susceptibility to cell-wall targeting antibiotics in *E. coli* (6). They concluded that *rprA* expression is likely involved in the cell-wall targeting antibiotic resistance of *E. coli* (6). Based on their

findings, we hypothesized that pre-treatments of DH300 with cell-wall targeting antibiotics and aminoglycosides would induce upregulation of *rprA*, resulting in increased resistance to subsequent antibiotic treatments. To answer these questions, a set of preliminary MIC assays with penicillin, streptomycin, and tetracycline were performed to determine the sub-MICs that would be used as pre-treatment conditions in the subsequent MIC assay. β-galactosidase assays were performed to investigate the effects of sub-MIC antibiotic treatments on *rprA* expression in both DH300 and DH311. Lastly, after pre-treating DH300 and DH311 with the antibiotics at sub-MIC, a penicillin MIC assay was performed on these strains to study the effects of pre-treatments on subsequent cell-wall targeting antibiotic resistance.

In *E. coli* strain K12, deletion of *rcsB* abrogates antibiotic-induced upregulation of *rprA* expression but is not sufficient to affect resistance to antibiotics in subsequent treatments. We observed no difference in the MICs within the same penicillin, streptomycin, and tetracycline treatments between DH300 and DH311 (Table 3). These findings are supported by Chen *et al.* and McDade *et al.* who performed similar experiments and found no differences in the MICs between DH300 and DH311 within a given antibiotic treatment (13, 14). Nonetheless, discrepancies in MIC existed between different trials for the penicillin treatment, where the MIC in trial 3 was 2-fold greater than trials 1 and 2. Despite the differences, 17 µg/mL, which was the most frequently observed MIC for both strains, was used in the sub-MIC calculation for penicillin. Given that only three trials of MIC assays were performed, using the mean MIC instead of the mode would render the sub-MIC calculation susceptible to the influence of outliers. For instance, 33 µg/mL could be an outlier MIC for penicillin, thus incorporating the value in calculations would skew the sub-MIC to a higher value.

Similar to Jalalkamali *et al.*, the effect of elevated *rprA* expression on resistance to cell-wall targeting antibiotics for DH300 and DH311 was investigated (6). DH300 showed increased β-galactosidase activity indicating increased *rprA* expression compared to the DH311, which showed minimal expression (Figure 1). The elevated *rprA* expression corresponds to the findings reported by Jalalkamali *et al.* and validates the loss of RcsB function in the mutant, given that *rprA* is a downstream target of RcsB (6, 7). Correlating these results with the preliminary MIC assay (Table 3), no differences were observed in MICs between the two strains despite the differences in *rprA* expression. This finding contradicts the MIC results and conclusions reached by Jalalkamali *et al.* which suggest that *rprA* is involved in intrinsic resistance to cell-wall targeting antibiotics in *E. coli* (6). Furthermore, a lack of upregulation in *rprA* expression was detected under streptomycin treatment while tetracycline and penicillin treatment caused for induction of *rprA* (Figure 1). In contrast, Jalalkamali *et al.* found that all antibiotic treatments resulted in upregulation of *rprA* (6). The unchanged *rprA* expression observed in our study could be due to insufficient concentrations used for antibiotic treatments to fully trigger for the *rcsB* stress response pathway.

We observed no difference in the MICs of penicillin between any of the pre-treatment conditions (Table 4). Furthermore, we observed no difference in terms of penicillin resistance between DH300 and DH311 within any trial (Table 4). It has also been demonstrated in the β-galactosidase assay that pre-treatments of DH300 with streptomycin and tetracycline result in elevated *rprA* expression. Taken together, these results indicate that elevated levels of *rprA* will not confer increased resistance to subsequent penicillin treatment in *E. coli*. This refutes our hypothesis that pre-treatments with cell-wall targeting antibiotics and aminoglycosides will result in increased resistance in *E. coli* to subsequent cell-wall targeting antibiotic treatments in an *rprA* dependent manner. The results from the MIC assays following pre-treatment also showed a 2 to 4-fold increase in MIC, compared to the MIC from the preliminary MIC assays under penicillin treatment (Table 3 and 4). However, this increase in MIC is likely not the result of pre-treatment, as the same 2-fold to 4-fold increase is also seen in the untreated DH300 and DH311 control in the MIC assay following pre-treatment (Table 4). Since the untreated DH300 and DH311 are the same as DH300 and DH311 in the preliminary MIC assay, where neither samples undergo pre-treatment with any antibiotics, their penicillin MIC should remain the same.

The discrepancies observed throughout the MIC assays either between trials or between the preliminary MIC assays and the MIC assays following pre-treatment (Table 3 and 4) are likely a result of some other variable. To probe this result further, we decided to investigate factors that could explain the MIC such as inoculum size or incubation time. Following the methods outlined in the supplementary materials, the effects of seeding different amounts of bacteria and the effects of different incubation times on MICs were evaluated. Penicillin MICs did not change regardless of incubation time over 24 hours (Figure S4), suggesting that differences in incubation time between MIC assays are not sufficient to cause for discrepancies in MIC. However, increased inoculum sizes with a lower starting dilution factor used in the MIC assays resulted in higher MICs obtained (Table S1). This indicates the ability for variations in inoculum size to change recorded MICs. As a result, this is a likely reason for the discrepancies observed throughout the MIC assays where there were slight differences in the amount of bacteria seeded between different trials or between the preliminary MIC and the MIC following pre-treatment.

Other pathways may be upregulated to account for deletion of Δ*rcsB*. DH300 and DH311 pre-treated with penicillin, streptomycin, and tetracycline did not show increased resistance to further penicillin treatments in MIC assays despite having increased *rprA* expression. Since DH311 did not display reduced penicillin resistance compared to DH300, we hypothesized that there could be another stress response pathway that is upregulated to compensate for the deletion of the *rcsB* gene. There are several other pathways that could be responsible for this (15,18,19). The Cpx system is one pathway known to respond to envelope stress (15). The Cpx pathway is a two-component pathway composed of a membrane sensor kinase *cpxA* and cytosolic response regulator *cpxR* (15). Huang *et al.* have shown that when treating *E. coli* with β-lactams such as penicillin, the resulting damage to the peptidoglycan layer induced overexpression of *cpxR* (16). The potential involvement of the *cpxAR* component was further supported by a study conducted by Hirakawa *et al.,* which found that overproduction of *cpxR* induced resistance to β-lactams in gram-negative bacteria such as *E. coli* (17). Overexpression of *cpxR* has also been linked to multidrug resistance (17). Due to this, the Cpx pathway is a likely candidate to compensate for the deletion of *rcsB*. Other factors such as *evgA* and *baeR*, two separate transcriptional regulators, have also been linked to multidrug resistance (17). EvgSA is another two-component signal transduction system with EvgS and EvgA functioning as a sensory kinase and a response regulator, respectively. Nishino and Yamaguchi have demonstrated that overexpression of EvgA can result in increased transcription of *yhiUV*, which is a gene for a multidrug transporter (18). Furthermore, another study by Baranova and Nikaido has shown that BaeSR, composed of a sensory kinase BaeS and response regulator BaeR, upregulates *mdtABC* following treatment with aminoglycosides. *mdtABC* is a gene for a multidrug efflux pump and its expression can provide multi-drug resistance to *E. coli* (19). Upregulation of one of these factors could explain why we did not see a change in resistance to penicillin in MIC assays following pre-treatments with penicillin, streptomycin, or tetracycline, despite having increased *rprA* expression in DH300 but not DH311.

Limitations The 2-fold dilution used for the MICs represented one of the limitations to this experiment. The use of a 2-fold dilution starting at 67 µg/mL resulted in a sudden drop to 33 µg/mL and so on. As a result, if the true MIC of one strain for a given antibiotic was 50 µg/mL, it would be represented as 33 µg/mL on the MIC assay, resulting in an inaccurate MIC determination. The use of another technique allowing for the evaluation of a tighter range of concentrations may be preferred to obtain more accurate and precise MIC values. Another limitation that existed with the experiments were the discrepancies in MICs between trials and between the preliminary MIC assays and the MIC assays following pre-treatment. Although our supplementary experiments suggest the differences to be due to varying inoculum sizes, it is unclear whether further efforts to control inoculum size will eliminate these discrepancies and whether inoculum size is the only variable responsible for these changes. Nonetheless, controlling for inoculum size may still be difficult and

imprecise given the use of absorbance in normalizing inoculum sizes when employing our method of MIC determination.

Conclusions After pre-treating with penicillin, streptomycin, and tetracycline at sub-MIC conditions, DH300 showed increased β-galactosidase activity compared to DH311, indicating the active expression of *rprA* in the strain. Under penicillin and tetracycline pre-treatments, DH300 also exhibited elevated levels of *rprA* compared to untreated DH300. Despite differences in *rprA* expression levels, no variations in penicillin resistance were observed between DH300 and DH311 in MIC assays conducted. Additionally, DH300 and DH311 pretreated with penicillin, streptomycin and tetracycline do not exhibit increased resistance to subsequent penicillin treatments in MIC assays. Overall, this suggests that pre-treatment with aminoglycosides and cell-wall targeting antibiotics does not alter resistance to subsequent treatments with cell-wall targeting antibiotics; however, it does upregulate *rprA* expression.

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Future Directions As stated in the discussion, there are multiple factors that could be upregulated to compensate for the *rscB* deletion. We suggest using double-knockout mutants to narrow down which pathway is upregulated to compensate for the deletion of *rcsB*. Using double-knockout Δ*cpxR* Δ*rcsB*, Δ*evgA* Δ*rcsB* and Δ*baeR* Δ*rcsB* mutants would demonstrate if *cpxR*, *evgA*, and *baeR*, respectively, are upregulated to compensate for the *rcsB* deletion. If the additional deletion of each mentioned gene results in the susceptibility of *E. coli* to cell-wall targeting antibiotics, this would allow us to narrow down on a response regulator gene that is upregulated to compensate for *rcsB* deletion. However, it is unclear whether these double mutations would produce viable bacterial strains. Therefore, before any experiments can be done, an attempt should be made to confirm the viability of the suggested double mutants.

ACKNOWLEDGEMENTS

We would like to thank the Department of Microbiology and Immunology for their generous funding and resource provisions. We are very appreciative of the media room staff for their aid in resource location and biohazardous waste disposal. We would also like to thank Aya Zakaria, Tina Liao, Samantha Feng and Maye Cheng for their collaborative efforts. Finally, we would like to express our gratitude to Dr. David Oliver and Mihai Cirstea for their support throughout the project.

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