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The Rcs-Phosphorelay Pathway Is Not Essential for Intrinsic Antibiotic Resistance to β-lactam Antibiotics in *Escherichia coli*

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The Rcs phosphorelay pathway in *Escherichia coli* is a response system that senses outer membrane stress conditions and regulating capsule and biofilm formation which can result in antibiotic resistance. The Rcs phosphorelay system consists of the outer membrane sensor protein RcsF, a sensor kinase RcsC, a response regulator RcsB, and a non-coding RNA *rprA*. RcsF signals downstream of the Rcs pathway to regulate expression of the Rcs regulon, including *rprA*, which induces the activity of the general stress response regulator RpoS. The role of RcsB and RcsF in regulating intrinsic antibiotic resistance remains unclear. Here we address this question by testing the minimum inhibitory antibiotic concentrations to four β-lactam antibiotics in *E. coli* strains DH311 and BW25113 which bear chromosomal deletions of *rcsB* and *rcsF*, respectively. We hypothesized that the disruption of the Rcs pathway would decrease RpoS expression, which in turn increases the antibiotic susceptibility in the mutant strains compared to wild type *E. coli.* We also predicted a correlation between increased *rprA* expression levels and antibiotic resistance. *E. coli* strains designed with an *rprA*::*lacZ* fusion were tested via β-galactosidase assay to measure *rprA* expression after treatment with β-lactam antibiotics. We found that the wild type and the mutant strains of RcsB displayed the identical levels of intrinsic antibiotic resistance against cefsulodin, ampicillin, phosphomycin, and penicillin. Wild type *E. coli* strain DH300 showed 5 times higher *rprA* expression compared to an *rcsB* deletion mutant when treated with antibiotics and a 1.5 times higher level of *rprA* expression compared to the untreated sample. These data suggest that RcsB directly contributes to *rprA* expression but deletion of RcsB or RcsF does not result in a detectable difference in the intrinsic antibiotic susceptibility of *E. coli* to β-lactams. Our data provides information on further understanding the contributions of the Rcs pathway to the regulation of *E. coli* stress response regulator RpoS.

The Rcs (Regulator of Capsule Synthesis) phosphorelay pathway is a two-component signal transduction system conserved in *Enterobacteriaceae* which regulates *cps* gene important for capsule synthesis (1,2). The Rcs pathway comprises of an inner membrane sensor kinase RcsC, a response regulator RcsB, an intermediate phosphorelay protein RcsD, and an outer membrane sensor kinase RcsF (3). Previous studies have shown RcsF to be oriented towards the periplasm and located upstream of RcsC which suggested that RcsF may sense cell surface perturbation and transmit the stress signal to RcsC (4). Rcs signaling is transmitted through an ordered cascade RcsF→RcsC→RcsD→RcsB (Fig. 1); however, the mechanism by which RcsF transmits the signal to RcsC is not well understood (3). Rcs system induces a general stress response to multiple β-lactam antibiotics mediated through the action of the *rprA*-regulated RpoS (5).

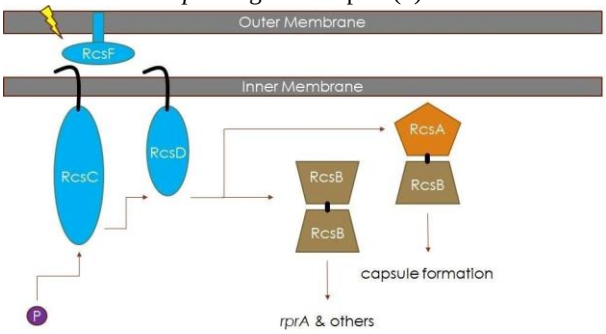

FIG. 1 The Rcs phosphorelay pathway as shown from the stress signal (lightning bolt) to the formation of capsule or upregulation of *rprA*.

Gram-negative bacteria have adapted system to respond and adapt to cell envelope stress such as osmotic shock or antibiotic inhibition of peptidoglycan synthesis (5). The Rcs phosphorelay pathway is activated as a stress response to peptidoglycan disruption, which can lead to osmotic shock and cell lysis (5). Using β-lactam antibiotics cefsulodin and amdinocillin to induce peptidoglycan stress in *E. coli*, Laubacher and Ades found elevated RNA levels of Rcs-regulated genes including *rprA* and *osmB* (5). RcsB regulates the expression of *rprA*, a small noncoding RNA, which in turn regulates RpoS, a general stress response regulator (4). *rprA* regulates the translation of RpoS by protecting *rpoS* mRNA from degradation by ribonuclease RNase E (6). RpoS associates with a core DNA polymerase enzyme to promote the transcription of various genes required for cell survival, such as biofilm formation genes, which can enhance the stress survival of *E. coli* K-12 (7). Richter *et al*. have previously shown that RcsB-dependent *rprA* expression is involved in intrinsic antibiotic resistance to penicillin in *E. coli* K-12 substrains by measuring the increase in β-galactosidase activity of ΔrcsB mutant strain possessing *rprA:lacZ* reporter upon exposure to penicillin (1).

The objective of this study is to investigate the role of the Rcs phosphorelay system of *E. coli* in response to β-lactam antibiotics. We hypothesized that *E. coli ΔrcsB* and *ΔrcsF* mutants, which exhibit nonfunctional Rcs pathways, would show elevated β-lactam antibiotic susceptibility phenotypes due to a decrease in the expression of *rprA*-regulated RpoS. We did not observe differences in β-lactam resistance between the wild type (WT) strain and the *ΔrcsB* and *ΔrcsF*

mutant strains. However, the *ΔrcsB* strains resulted in a much lower level of *rprA* expression compared to the WT in the presence of β-lactam induction. The results suggest that there are alternate regulatory mechanisms for the expression of RpoS independent of the Rcs pathway.

MATERIALS AND METHODS

Strains used in this study. *E. coli* DH300 and DH311 were obtained from the MICB 447/421 culture collection from the Department of Microbiology and Immunology at the University of British Columbia. *E. coli* BW25113 *ΔrcsF* was supplied by the Coli Genetic Stock Centre (CGSC). The cells were grown overnight in Luria broth (LB) (pH7.0, 1% w/v trypton, 0.5 w/v yeast extract, 1% w/v NaCl) in 1:100 ratio at 37°C with shaking.

Plasmids. *E. coli* BW25113 containing pKD46 (9) and *E. coli* BW25141 containing pKD3 (9) were supplied by the Department of Microbiology and Immunology at the University of British Columbia. pKD46 is a temperature-sensitive, 6329 bp plasmid carrying the λ-Red recombinase genes (9), and pKD3 is a 2804 bp plasmid carrying the chloramphenicol acetyl-transferase gene (*cat*) (9). *E. coli* BW25113 carrying pKD46 was grown overnight at 30°C with shaking in LB containing 100 μg/ml ampicillin, and *E. coli* BW25141 carrying pKD3 was grown overnight at 37°C with shaking in LB containing 100 μg/ml chloramphenicol.

Plasmid isolation. The plasmids pKD46 and pKD3 were extracted from *E. coli* BW25113 and *E. coli* BW25141, respectively. Plasmid extraction was performed using Invitrogen's PureLink® Quick Plasmid Miniprep Kit (Cat# K210010). DNA concentration and purity were determined using the Thermo Scientific NanoDrop 2000c Spectrophotometer at absorbance of 260 nm and 280 nm.

Minimum inhibitory concentration (MIC) assay to determine the sub-inhibitory concentration of cefsulodin for subsequent β-galactosidase assay. Colonies of *E. coli* DH300 wild type and DH311 *ΔrcsB* were each inoculated into 5 ml of LB and grown separately overnight at 37°C with shaking. Overnight cultures were standardized based on OD_{600} readings and diluted to 5 x 10^6 CFU/ml. A two-fold serial dilution of the antibiotic cefsulodin was performed in a 96-well microtitre plate and the following varying concentrations of each column of wells were obtained: 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml, 6.3 μg/ml, 3.1 μg/ml, 1.6 μg/ml. A positive control with no cefsulodin added and a negative sterility control were also included. The plate was sealed with aluminum foil to prevent drying before incubating overnight at 37°C. Minimum inhibitory concentration was determined by the well that had the lowest concentration of antibiotic added with no visible growth as detected by the unaided eyes. Sub-inhibitory concentration was determined to be half of MIC.

Minimum inhibitory concentration assay to compare antibiotic susceptibility. A colony of *E. coli* DH300 WT, DH311 *ΔrcsB*, and BW25113 *ΔrcsF* were each inoculated in 5 ml of LB and grown separately overnight at 37°C with shaking. Overnight cultures were standardized based on OD_{600} readings and diluted to 5 x 10^6 CFU/ml. A two-fold serial dilution of the antibiotic cefsulodin, ampicillin, phosphomycin and penicillin was performed separately in four 96-well microtitre plates. 2 experiments were performed, and each experiment had 2 replicates. The following varying concentrations of each column of wells were obtained: 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml, 6.5 μg/ml, 3.1 μg/ml, 1.6 μg/ml. All overnight cultures were set up in duplicates in each plate. A positive control with no antibiotics added and a negative sterility control were also included. The plates were sealed with aluminum foil to prevent drying before incubating overnight at 37°C. Minimum inhibitory concentration was determined by the well that had the lowest concentration of antibiotic added with no visible growth as detected by a micropipette spectrophotometer.

β-galactosidase assay for detection of LacZ activity in *E. coli* DH300. Overnight cultures of *E. coli* DH300 and DH311 were obtained by inoculating an isolated colony from *E. coli* DH300/DH311 plates respectively. Each strain was grown overnight in 2 ml of LB containing 2 μl of 1000X kanamycin stock solution at 37°C. 100 μl of each fresh overnight culture was diluted 10^{-3}-fold with LB and grown at 37°C until an OD_{600} reading of 0.4 - 0.8 were obtained. Sub-inhibitory concentration of cefsulodin was added to one of the diluted culture to induce *rprA* activity while the other culture was left untreated. 2 ml of cells from each culture were centrifuged at 6000 g for 10 minutes at 4°C. The supernatant was discarded before resuspension in 2 ml of chilled Z buffer (60 mM Na2HPO4 * 2H2O, 40 mM NaH2PO4 * H2O, 10 mM KCl, 1mM MgSO4, and 50 mM β- mercaptoethanol). Using Z buffer as the blank, OD_{600} reading for each sample was measured and recorded. Next, 0.5 ml of the cell suspension from each culture were added to 0.5 ml of Z buffer to dilute the samples to 1 ml. 100 μl of chloroform and 50 μl of 0.1% SDS were then added to permeabilize the cells. Samples were then vortexed and incubated in 28°C water bath for 5 minutes. To induce activity of the LacZ promoter, 200 μl of 1X *o*-nitrophenyl- β-D-galactoside were added to each sample. Samples were incubated in 28°C water bath until sufficient yellow colour had developed. 0.5 ml of 1M Na_2CO_3 was added to each sample to stop the reaction. Samples were vortexed and spun at 16,000 g for 5 minutes to remove debris and chloroform. The supernatant from each sample was transferred into a cuvette, and absorbance readings were measured at A_{420} and A_{550} using a Biochrom Ultrospec 3000 spectrometer. The absorbance values were converted to Miller Units according to the following formula: 1 Miller Unit = 1000 × (A_{420} - (1.75 x A_{550}) / (time of reaction in minutes x volume of culture in μl x OD_{600})).

Transformation of pKD46 encoding the λ-Red recombinase gene into *E. coli* DH300. 300 μl of overnight *E. coli* DH300 culture is inoculated into 30 ml of LB, and the diluted culture was grown at 37°C with shaking until 0.3 - 0.4 OD_{600} was achieved. The cells were centrifuged at 3000 g for 15 minutes and supernatant was discarded. Cell pellets were resuspended in 8 ml of chilled 10 mM $MgCl_2$, spun at 2000 g for 15 minutes, and supernatants was discarded. Then the cell pellets was resuspended in 16 ml of chilled 100 mM $CaCl_2$ and chilled on ice for 20 minutes before being spun at 2000 g for 15 minutes. Supernatants was removed, cell pellets was resuspended in 4 ml of chilled 85 mM $CaCl_2$ with 15% glycerol and spun at 1000 g for 15 minutes. Supernatants was discarded and the cell pellets was resuspended in 2 ml of chilled 85 mM $CaCl_2$ with 15% glycerol. 100 μl of the DH300 competent cells were added to each Eppendorf tubes containing the following amount of pKD46 plasmid DNA: 0 ng, 1 ng, 10 ng, 100 ng and 500 ng. Tubes were chilled on ice before being subjected to heat shock at 42°C for 30 seconds in water bath. The transformants were kept on ice for 2 minutes before being plated onto LB plates containing 100 μg/ml ampicillin. The plates are incubated at 37°C for 24 hours.

Gradient PCR amplification of chloramphenicol acetyl-transferase (*cat)* in pKD3. The sequence of forward primer (pKD46-F-rcsF) used was 5'-AACGCCTATTTGCTCGAACTGGAAACTGCTCATTTCGCC GGTGTAGGCTGGAGCTGCTTC-3,' and the sequence for reverse primers (pKD46-R-rcsF) 5'-GCTCCTGATTCAATATTGACGTTTTGATCATACATTGAG GATGGGAATTAGCCATGGTCC-3'. The primers were ordered from Integrated DNA Technologies (IDT). All PCR reactions were carried out in volumes of 50 μl, and each reaction contained 1 μl of pKD3 plasmid DNA template at 50 ng/μl, 5 μl of 10X Pfx

amplification buffer, 1 µl of 50 mM $MgSO_4$, 1.5 µl of 10 mM dNTPs, 1.5 µl of 10 µM forward primer, 1.5 µl of 10 µM reverse primer, 38.1 µl dH_2O, 0.4 µl Invitrogen's Platinum® Pfx DNA Polymerase (Cat# 10966018). Gradient PCR thermocycling conditions were 5 minutes of initial denaturation at 95°C followed by 35 cycles of 15 seconds of denaturation at 94°C, 30 seconds of annealing at 55°C, 90 seconds of extension at 72°C, and a final extension at 72°C for 5 minutes. To eliminate unwanted nonspecific PCR products, the target band was extracted from the agarose gel and PCR was repeated using the gel extracted DNA as template. PCR products were purified using Invitrogen's PureLink® PCR Purification Kit (Cat# K3100001), and DNA concentration and purity were measured with the Thermo Scientific NanoDrop 2000c Spectrophotometer at absorbance of 260 nm and 280 nm. PCR products were run on 1% agarose gel in 1X TAE buffer at 110V for 1 hour. Bands were visualized using Invitrogen's SYBR® Safe DNA Gel Stain (Cat# S33102).

Transformation of Chloramphenicol cassette into DH300 transformants carrying pKD46. Colonies of the *E. coli* DH300 transformants carrying pKD46 were grown in two tubes of 2 ml LB containing 50 µg/ml ampicillin at 30°C with shaking until 0.1 OD_{600} was achieved. 20 µl of 1 M L-arabinose (10 mM) was added to one tube while the other tube was left untreated. The two tubes were grown at 30°C with shaking until 0.4 OD_{600} was achieved. 1 ml of each samples were transferred into 1.5 ml microfuge tubes and chilled on ice for 10 minutes before being spun at 4000 g for 10 minutes. Supernatants was discarded and cell pellets was resuspended in 1 ml ice-cold dH_2O. Resuspended cells were kept on ice for 10 minutes before being spun at 4000 g for 10 minutes. Supernatants was discarded and cell pellets containing the competent DH300 cells expressing λ-Red recombinase was resuspended in 50 µl of ice-cold dH_2O. Transformation of the competent cells was done via electroporation and the transformants were plated on LB plates with 5, 10, and 25 µg/ml of chloramphenicol.

Colony PCR amplification of *rcsF* region to verify *rcsF* deletion. The sequence of forward primer (rcsF-F) was 5'-CTATTTGCTCGAACTGGAAAC-3', and the sequence for reverse primer (rcsF-R) was 5'-GCTCCTGATTCAATATTGACG-3'. The primers were ordered from the IDT. Colony PCR reactions were carried out in volumes of 20 µl. A colony of transformed *E. coli* DH300 was resuspended with 20 µl of dH_2O and1 µl of resuspended culture was mixed with 2 µl of 10X Taq reaction buffer, 0.5 µl of 10 mM dNTPs, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 14 µl of dH_2O, and 0.5 ul Taq polymerase. PCR conditions were 6 minutes of initial denaturation at 95°C followed by 30 cycles of 30 seconds of denaturation at 95°C, 30 seconds of annealing at 60°C, 45 seconds of extension at 72°C and a final extension at 72°C for 20 minutes. PCR products were run on 1% agarose gel in 1X TAE buffer at 100V for 1 hour. Bands were visualized using Invitrogen's SYBR® Safe DNA Gel Stain (Cat# S33102).

RESULTS

***E. coli* DH300 WT and DH311 Δ*rcsB* had the same minimum inhibitory concentraion of cefsulodin.** We wanted to verify that our experimental strain DH300 had a functional *lacZ*::*rprA* fusion by inducing *rprA* expression using sub-inhibitory concentration of antibiotic and measuring the level of LacZ activity. We selected cefsulodin as the inducting antibiotic as Laubacher and Ades have shown cefsulodin is able to induce expression of Rcs pathway (5). To determine the sub-inhibitory concentration of cefsulodin required to induce *lacZ*::*rprA* expression, we performed preliminary cefsulodin MIC assay on the WT and the Δ*rcsB* mutant in duplicates We standardized overnight cultures of the WT and the Δ*rcsB* mutant to 5 x 10^5 CFU/ml. We performed two-fold serial dilutions in a 96-well plate to set up varying concentration range of cefsulodin from 0 µg/ml to 100 µg/ml. The negative control was treated antibiotic-free, and showed growth. The positive control was treated uninoculated and showed no growth. Both *E. coli* DH300 WT and DH311 Δ*rcsB* had the same minimum inhibitory concentration of 25.0 µg/ml for cefsulodin. We set the sub-inhibitory concentration for both WT and the mutant strains to be the half of the MIC, 12.5 µg/ml.

***E. coli* DH300 shows the increased *rprA* expression upon treatment with sub-inhibitory cefsulodin.** In order to measure the LacZ activity and quantify the level of *rprA* expression, we performed a β-galactosidase assay. Overnight cultures of WT and the Δ*rcsB* mutant were diluted and grown to OD_{600} 0.8. We then added 12.5 µg/ml of cefsulodin. After 14-minutes ONPG was added to the samples, the absorbance was measured at 420 nm and 550 nm to calculate the Miller Units (10). We hypothesized that the *E. coli* DH311 bearing a deletion of *rcsB* would not be able to induce *rprA* expression upon antibiotic treatment since *rprA* is activated by the RcsB-RcsC phosphorelay system (11). As shown in Figure 2, the induced and uninduced cultures of DH311 Δ*rcsB* showed similar levels of Lac Z activity, which were significantly lower than those from the wild type DH300 strain. The induced DH300 culture showed 1.5x higher LacZ activity than the uninduced DH300 culture. Still, the uninduced DH300 culture showed 4.0x higher LacZ activity than the DH311 cultures, indicating that that both cultures of DH300 could induce *rprA* expression (Fig. 2). The results show that the *E. coli* DH300 expresses β-galactosidase only, and the β-galactosidase expression was upregulated following treatment with sub-inhibitory levels of cefsulodin.

***E. coli* mutant strains DH311 Δ*rcsB* and BW25113 Δ*rcsF* did not show antibiotic susceptibility to β-lactam antibiotics than DH300 WT.** In order to investigate whether *rcsF* plays a role in increasing antibiotic resistance to antibiotics that disrupt PG synthesis, we measured the MIC of WT, the Δ*rcsB* mutant and the Δ*rcsF* strains treated with cefsulodin, ampicillin, phosphomycin, and penicillin.

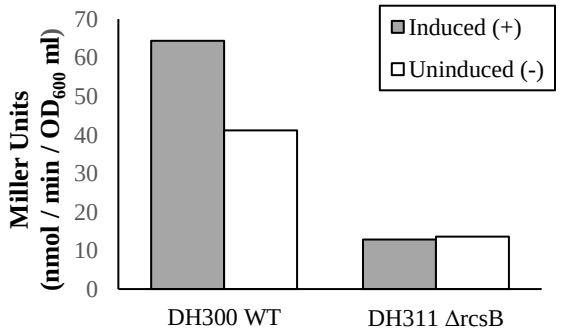

FIG. 2 β-galactosidase assay results of *rprA* reporter in *E. coli* DH300 WT and DH311 Δ*rcsB* induced with 25.0 µg/ml cefsulodin and reacted with ONPG for 14 minutes, calculated in Miller Units.

TABLE 1 The effect of LPS on the minimum inhibitory concentration of Polymyxin B for E. coli strain CWG655

Strain	MIC (µg/ml)			
	Cefsul-odin	Ampicillin	Phospho-mycin	Penicil-lin
DH300	25.0	6.3	1.6	50.0
DH311 Δ*rcsB*	25.0	6.3	1.6	50.0
BW251 13 Δ*rcsF*	25.0	6.3	1.6	50.0

We hypothesized that the mutant *rcsF* strain would show decreased resistance against the PG-synthesis inhibitors since RcsF is an outermembrane lipoprotein that senses cell surface disruption (12). We included DH311 Δ*rcsB* as our negative control strain since it has previously been shown to have increased susceptibility to phosphomycin and penicillin compared to the WT (1). Overnight cultures of the WT and the mutant strains were standardized to 5 x 10^5 CFU/ml. We performed two-fold serial dilutions in a 96-well plate to set up varying concentration range of antibiotics from 0 µg/ml to 100 µg/ml. The negative control was set up with inoculum only, and the positive control only contained the antibiotics. The negative control showed growth and the positive control showed no growth, confirming that the MIC assay had no contaminants, and the results were valid. We observed no difference in antibiotic resistance between the WT and the *ΔrcsB* and *ΔrcsF* strains in the MIC assay with all four antibiotics (Table 1). The results suggest that the deletion of *rcsB* gene and *rcsF* gene was not sufficient to decrease resistance to antibiotics targeting PG synthesis.

pKD46 encoding the λ-Red recombinase gene was transformed into *E. coli* DH300. In order to test the correlations between the Rcs pathway to the expression of *rprA* in *E. coli*, we decided to create an Δ*rcsF* mutant from *E. coli* DH300 which contains a *rprA::lacZ* fusion reporter gene. We obtained one isolated chloramphenicol resistant colony. The negative control plate showed no growth. The single transformant colony pinked and grown overnight in LB broth containing chloramphenicol. Genomic DNA was extracted. Bands were not observed in the lane containing the PCR reaction mixture from the putative *rcsF* mutant strain of DH300. In contrast, PCR of genomic DNA from wild type strain DH300 did show a band at the expected size of 400 base pairs. We cannot conclude from these data that the deletion was made since we did not see amplification in the putative *rcsF* mutant nor did we run positive controls reactions using this template DNA. However, the observation that PCR of template DNA isolated from strain DH300 showed a 400 base pair band, which suggests that the putative mutant colony is worth further investigation.

DISCUSSION

Previous studies have shown increased levels of *rprA* expression upon treatment with β-lactam antibiotic cefsulodin (5). RcsB-dependent rprA expression contributes to increase in the intrinsic resistance to penicillin of *E. coli* (1). We hypothesized that *E. coli* DH311 Δ*rcsB* and BW25113 Δ*rcsF* would be more susceptible to β-lactam antibiotics as they both disrupt key regulatory steps in the Rcs phosphorelay pathway, which ultimately lead to the induction of the stress regulator RpoS. Two approaches for comparing the intrinsic antibiotic resistance of *E. coli* to β-lactams are used in this study. The first approach is through direct measurement of bacterial growth inhibition by antibiotics which prevent PG synthesis. The MIC assay performed in this study compares the susceptibility of *E. coli* DH300 WT, DH311 Δ*rcsB* and BW25113 Δ*rcsF* to four different β-lactam antibiotics. The second approach measures the expression levels of *rprA* which regulates sigma factor RpoS, a key regulator of general bacterial stress response. The β-galactosidase assay indirectly measures *rprA* expression in *E. coli* strains conferring a *rprA*::*lacZ* reporter gene.

The MIC of strain DH311 Δ*rcsB* and DH300 WT using cefsulodin was the same. This result is inconsistent with the findings reported by Laubacher and Ades which showed reduced survival of the Δ*rcsB* mutant strain *of E. coli* upon treatment with amdinocillin (mecillinam) and cefsulodin (5). Cefsulodin inhibits peptidoglycan-binding proteins (PBP) 1a and 1b, a set of enzymes involved in PG synthesis (5), causing a bacteriolysis. MICs of strain DH300 WT, DH311 ΔrcsB and BW25113 ΔrcsF were the same when tested with four different antibiotics. The regulation of *rpoS* expression is known to be complex and controlled by multiple interacting factors (18). It is possible that other gens are involved in *rpoS* linked antibiotic resistance (23). Laubacher and Ades suggested in their findings that cefsulodin treatment induced the expression of 20 different genes in *E. coli* MG1655 strain (5), it is possible these upregulated genes had a compensatory effect on the reduced *rpoS* expression hence resulting in no observable reduction in antibiotic resistance in the mutant strains.

Although both the DH300 WT and the DH311 Δ*rcsB* mutant displayed the same MIC for cefsulodin, we observed a significant difference in the levels of expression of *rprA*::*lacZ* fusion gene between the two strains in β-galactosidase assay using sub-inhibitory concentrations of cefsulodin as an antibiotic stress signal. As expected, both the induced and uninduced cultures of the *ΔrcsB* mutant displayed noticeably lower expression of LacZ. This observation is consistent with findings reported by Majdalani *et al.* which showed no *rprA* expression in *E. coli* DH311 bearing a deletion of *rcsB* (13). We show that the expression of *rprA* is directly dependent on RcsB. The cefsulodin-treated culture of the WT showed 1.5-times higher LacZ activity compared to the untreated culture, suggesting that the Rcs pathway of

the WT was activated in response to the cefsulodin treatment and upregulated the *rprA*::*lacZ* fusion gene. This observation agrees with the findings of Laubacher and Ades in that *rprA* is directly regulated by the Rcs pathway (5). The DH311 *ΔrcsB* mutant did not show a difference in LacZ activity between the induced and uninduced samples. Using β-galactosidase assay, we show that upregulation of *rprA* is dependent on a functional Rcs pathway in the presence of β-lactam antibiotic stress.

In attempts to create an Δ*rcsF* mutant of DH300 WT, we faced several challenges. The PCR amplification of the linear *cat* cassette insert resulted in persistent primer dimer formations. Through primer design analysis programs (IDT OligoAnalyzer 3.1), the primer sequences do not appear likely to form secondary structures or dimerize with each other. PCR reactions with only pKD46-F-rcsF or pKD46-R-rcsF primers are tested as controls. The dimers are observed in the reactions containing both primers, only pKD46-F-rcsF but not only the pKD46-R-rcsF (Fig. 3). This suggests that the forward primer likely forms homodimers with itself.

How can we explain the observation that Rcs-linked antibiotic resistance did not change in our study but did in previous studies? RpoS is a major stationary phase regulator of nearly 300 genes in *E. coli* (14). While RpoS levels are normally low during the log phase of an organism, *rpoS* has been also demonstrated to be triggered by different stress conditions (18). At the transcriptional level, RpoS is mainly regulated by a single rpoSp promoter where guanosine 3',5'-bispyrophosphate (ppGpp) acts as the main positive regulator and cyclic Adenosine monophosphate receptor proteins (cAMP-CRP) as the main negative regulator (15, 19). The transcriptional regulation is mainly associated with the organism's entry into stationary phase. The stress-induced upregulation of *rpoS* is instead regulated by small noncoding ribonucleic acids (sRNAs). The different sRNAs react to different environmental signals. For example, DsrA upregulates RpoS at low temperatures, OxyS senses oxidative shock, and RprA senses cell surface stress signals (18). RpoS is the central part of a very complex and integrated regulatory network with many lower level secondary regulators that could receive specific signals (18). The complexity of the regulatory network requires that the regulation of RpoS involve a large number of signals and parameters such as pH, temperature, osmolarity and even cell density (18, 19). The initial hypothesis of this study is based on the restriction that RprA is the main regulator of RpoS activity. The observed results suggest that *E. coli* is able to compensate for a nonfunctional Rcs pathway and maintain RpoS expression despite having non-inducible RprA expression. Other potential mechanisms of intrinsic β-lactam resistance also exist. It is found in recent years that Gram-negative bacterial β-lactamase activity may be directly induced by free murein fragments as a result of peptidoglycan disruption (17). Stoebel *et al.* has also found that through a rapid and repeatable movement of a mobile genetic element, *E. coli* is even able to evolve new mechanisms of gene regulation to compensate for the lack of RpoS in Δ*rpoS* strains (16).

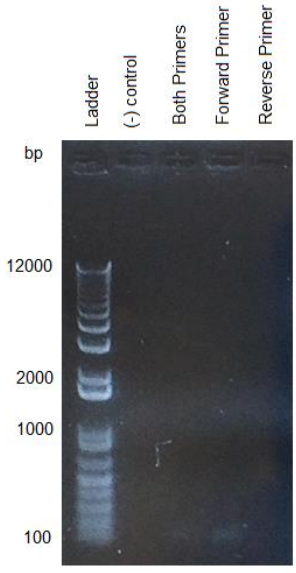

FIG. 3 PCR amplification of no-template primers control reactions visualized on 1.0% agarose gel, ran in 1X TAE buffer at 110 V for 60 minutes.

In conclusion, we found that *E. coli* WT and *ΔrcsB* and *ΔrscF* strains show the identical intrinsic antibiotic resistance to cefsulodin, phosphomycin, ampicillin, and penicillin, suggesting Rcs-phosphorelay pathway is not essential for intrinsic antibiotic resistance. We also found that expression of *rprA* is directly dependent on RcsB upon exposure to cefsulodin. but deletion of RcsB or RcsF alone does not result in a detectable increase in the intrinsic antibiotic susceptibility of *E. coli* to β-lactams.

FUTURE DIRECTIONS

This study laid the foundation for understanding several aspects of how the Rcs pathway in *E. coli* interacts with RpoS expression through the induction of sRNA *rprA* to maintain its resistance to antibiotics of the β-lactam class. One potential explanation for this is that RpoS regulation is complex and other sRNA signals such as *dsrA* and *oxyS* could still positively regulate RpoS expression to compensate for the lack of *rprA* signal (18, 20). Our study did not consider other potential stress signals that could trigger such sRNAs. The growth phase of the organism during these experiments should also be considered as *rpoS* transcription is heavily dependent on the organism's entry into stationary phase. Previous studies have also shown that the over expression of cold shock proteins CspC and CspE in *E. coli* K-12 resulted in an upregulation of various RpoS-mediated proteins (21). It may be

interesting to investigate the role of the Csp family genes in the regulation of RpoS expression.

To further understand the individual roles of the Rcs pathway components such as *rcsF*, it would be valuable to continue the creation of single gene knockouts. This study provides a good basis for using the λ-Red recombination method. One key limitation on our experiment was the amplification of the *cat* cassette insert. The primers used were not optimal and resulted in nonspecific amplification as well as primer dimers forming. Future studies should look to improve on the primer designs to help obtain high quality linear inserts for recombination. The transformation of our linear insert lacked a more stringent set of controls to help characterize the identity of the isolated colonies post-transformation. Other approaches to creating single knockouts could also be considered. For example, the CRISPR/cas system has been shown to improve *E. coli* chromosomal gene replacement when coupled with λ-Red recombineering (22).

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