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The RcsB-dependent Upregulation of *rpr*A Contributes to the Intrinsic Antibiotic Resistance of *Escherichia coli* Exposed to Antibiotics Targeting Cell Wall Synthesis but not Protein Synthesis

Yasaman Jalalkamali, Niknaz Malekafzali, Raisa Shabbir, Tianna Sihota

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

SUMMARY The regulation of capsular synthesis (Rcs) phosphorelay system allows *Escherichia coli* to respond to stress resulting from disruption of the peptidoglycan layer. The cytosolic response regulator, RcsB, regulates the expression of various downstream genes including *rpr*A. *rpr*A is a small non-coding RNA that stabilizes the translation of RpoS, a regulator of general stress response in *E. coli*. Previous studies have shown that an *E. coli* K-12 strain bearing a deletion of *rcs*B displays enhanced susceptibility to β-lactams, which target peptidoglycan synthesis. This result correlated with a decrease in *rpr*A expression. It is not known whether induction of *rpr*A expression is limited to β-lactams. The objective of this study was to determine *rpr*A expression levels using β-galactosidase assays in both wild-type and Δ*rcs*B, following treatment with sub-inhibitory concentrations of antibiotics targeting either cell wall or protein synthesis. We hypothesized that following treatment with antibiotics targeting cell wall synthesis, *rpr*A expression would be lower in a strain bearing a deletion of *rcs*B in comparison to the wild-type strain. Furthermore, within a given strain, we expected *rpr*A expression to be upregulated in response to cell wall synthesis stress and unchanged in response to protein synthesis stress in comparison to unstressed (no antibiotic) conditions. The antibiotics penicillin and phosphomycin target cell wall synthesis and would induce the Rcs phosphorelay system while tetracycline and streptomycin target protein synthesis and would not activate Rcs via peptidoglycan disruption. We observed that in the absence of *rcs*B, the minimum inhibitory concentration decreased two-fold with respect to treatment with penicillin and phosphomycin, but remained unchanged when treated with tetracycline and streptomycin. *rpr*A expression was upregulated in wild-type cells in the presence of antibiotics targeting cell wall synthesis or protein synthesis and was suppressed in the absence of *rcs*B when treated with either type of antibiotic. This study provides insight into the intrinsic Rcs-mediated stress response mechanisms used by *E. coli* through the expression of RcsB, which appears to regulate *rpr*A expression independently of the mechanistic class of antibiotic stressor used.

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Address correspondence to:
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INTRODUCTION

The cell envelope of Gram-negative bacteria is comprised of the outer membrane, inner membrane, as well as a thin layer of peptidoglycan (PG) that provides structural support to the cell wall (1). The regulator of capsule synthesis (Rcs) phosphorelay system is a two-component signal transduction system found widely in Enterobacteriaceae, including *Escherichia coli* (2). The Rcs pathway is composed of an inner membrane sensor kinase RcsC and an intracellular response regulator termed RcsB. Two other proteins involved in regulating the activity of the Rcs pathway include the intracellular intermediate component RcsD, and an outer membrane signaling component, RcsF. In *E. coli*, sensing stress

associated with PG damage activates the Rcs regulon, which modulates the expression of various genes including those that control production of the capsular polysaccharide, colonic acid (2,3).

The Rcs two-component system induces a general stress response upon exposure to multiple antibiotics targeting cell wall synthesis mediated via the activity of the *rpr*A-regulated RpoS (1). Once phosphorylated, RcsB triggers a regulatory cascade that influences the expression of various downstream genes (2), such as *ydhA* and *osmB*, which contribute to intrinsic resistance to antibiotics (2,3). RpoS regulatory RNA A (*rpr*A) is one target regulated by RcsB (4). *rpr*A is a small noncoding RNA (ncRNA) which increases translation of RpoS by altering the secondary structure of *rpoS* mRNA to increase its half-life (3). In turn, RpoS, a stationary-phase sigma 2 factor, regulates several genes involved in growth under stress conditions in *E. coli* (5). A previous study by Laubacher and Ades demonstrated the importance of the Rcs pathway for responding to PG damage by measuring expression levels of various components of the Rcs pathway in response to β-lactam antibiotics (1). β-lactams act by inhibiting penicillin binding proteins (PBPs), enzymes involved in PG synthesis, thus disrupting cell wall synthesis during growth and causing envelope stress to the bacteria. It was observed, using global gene expression analysis, that the Rcs phosphorelay pathway was activated by inhibition of PBPs (1). The activation of the Rcs phosphorelay system in response to treatment with other classes of antibiotics was not tested.

To test whether activation of the Rcs phosphorelay system is a non-specific consequence of antibiotic-induced cell death or growth inhibition, experiments were conducted in which changes in Rcs regulon expression were analyzed following treatment with antibiotics that have a target other than PG (1). *rpr*A, *ydh*A, *ymg*G and *osm*B, were induced in response to treatment with cell-envelope targeting antibiotics, cefsulodin and amdinocillin (1). Further studies compared the minimum inhibitory concentrations (MIC) of *rsc*B deletion mutants and wild-type cells, and observed that in the absence of RcsB, there was a significant decrease in penicillin and phosphomycin resistance, which are antibiotics that interfere with cell wall synthesis (4). In contrast, no change in antibiotic resistance was observed in *rcs*B deletion mutant cells treated with aminoglycosides, which are antibiotics that interfere with protein synthesis (4). Taken together these data suggest that *rcs*B is required for cell wall induced antibiotic resistance. Whether *rpr*A expression is linked to cell wall induced antibiotic resistance is not known. As RprA is a regulatory ncRNA that acts upstream of RpoS, a key sigma factor in response to envelope stress, there may be a relationship between *rpr*A levels and susceptibility to certain antibiotics over others.

The mechanism of uptake of streptomycin, an aminoglycoside, and tetracycline, an antibiotic that targets protein synthesis, involves the antibiotic competitively displacing divalent cations essential for the integrity of the outer membrane (6). Upon entry, the antibiotic binds to the 30S ribosomal subunit, induces codon misreading, and inhibits translocation, resulting in protein synthesis inhibition (6). Penicillin, a β-lactam, targets penicillin-binding proteins (PBPs) by acylating their serine residues and inhibiting their essential function in cell wall synthesis (7). Phosphomycin is a phosphonic acid antibiotic that blocks cell wall synthesis by inhibiting the MurA enzyme, which catalyzes the first committed step of peptidoglycan synthesis (7). Since the specific antibacterial activity mechanism of penicillin and phosphomycin is different, this suggests that the induction of the Rcs phosphorelay system is dependent on detecting PG damage rather than specific antibiotics (3).

The objective of this study was to investigate whether the mechanism of action of an antibiotic affects the expression level of *rpr*A, and the role of the *rpr*A expression in Rcs-mediated stress response conferring resistance in *E. coli*. Because *rpr*A is upregulated by RcsB via the Rcs phosphorelay system in response to PG damage by β-lactams, we predicted *rpr*A to be overexpressed in cells exposed to antibiotics targeting cell wall synthesis (5). Treatment with antibiotics that inhibit protein synthesis was not expected to induce Rcs-mediated antibiotic resistance.

Using a transcriptional reporter assay to measure *rpr*A expression and a broth dilution minimum inhibitory concentration assay, we observed a two-fold decrease in resistance to antibiotics targeting cell wall synthesis in a strain deficient in *rcs*B when compared side-by-

side with its isogenic wild-type (WT) strain. We also observed a decrease in *rpr*A expression in response to antibiotic stress compared to the WT strain. Similar changes in *rpr*A expression were measured when cells were treated with antibiotics targeting cell wall synthesis or protein synthesis. The upregulation of *rpr*A alongside the increase in resistance to penicillin and phosphomycin in WT cells provides evidence for the participation of the Rcs phosphorelay system in acquiring intrinsic antibiotic resistance to antibiotics targeting cell wall synthesis (3). The contradiction observed between antibiotic resistance to tetracycline and streptomycin and *rpr*A expression in WT cells suggests a model in which the Rcs phosphorelay system is indirectly induced by stress resulting from the interactions and impaired protein synthesis effects of these antibiotics on the composition of cell wall-associated proteins, but the cell's intrinsic antibiotic resistance to antibiotics targeting protein synthesis is independent of the downstream stress response proteins mediated by the Rcs phosphorelay system (6,8).

METHODS AND MATERIALS

Bacterial strains. The strains used in this study are described in Table 1. *E. coli.* K-12 DH300 (4) is denoted as wild-type (WT) and *E. coli* K-12 DH311 (4) is denoted as the *rcs*B deletion mutant (Δ*rcs*B). For all experiments involving cell growth, the strains were grown overnight in their appropriate growth conditions (see Table 1; sterile LB for DH300 and 50μg/mL kanamycin in sterile LB for DH311) at 37°C with agitation. Overnight cultures were then diluted 1:5 and grown at 37°C with agitation.

Preparation of Antibiotic stock solutions. Concentrated stock solutions of kanamycin sulfate (Sigma-Aldrich® 10106801001), penicillin-G (IGN 100543), phosphomycin (Sigma® P-5396), tetracycline hydrochloride (OnmiPur® 8990), and streptomycin sulfate (Sigma® S6501-50G) were prepared by dilution in dH_2O and filter sterilized using a 0.22 μm nitrocellulose filter.

Kanamycin plating to confirm deletion of *rcs*B in Δ*rcs*B. DH311 previously had the *rcs*B gene knocked out using a kanamycin resistance cassette (Table 1). To confirm deletion of *rcs*B in DH311, cells were streaked on fresh LB plates that contained 50 μg/ml kanamycin, grown overnight at 37°C, and observed for colonies. DH300 cells were also streaked on a LB-kanamycin plate to ensure no contamination from DH311.

Qualitative β-galactosidase reporter assay to confirm *lac*Z promoter activity in WT and Δ*rcs*B. Overnight cultures were diluted and grown until an optical density at 600 nm (OD_{600}) of 0.4-0.8 was achieved. 2 ml of cells were pelleted and resuspended in 2 ml of chilled Z-buffer (60mM Na_2HPO_4•$2H_2O$, 40mM NaH_2PO_4•H_2O, 10mM KCl, 1mM $MgSO_4$, and 50mM β-mercaptoethanol) and placed on ice. 0.5 ml of cell suspension and 0.5 ml of Z-buffer were combined. 50 μl of 0.1% SDS and 100 μl of chloroform were added and the solution was incubated in a water bath at 28°C for 5 minutes. 200 μl of 1X 2-Nitrophenyl-β-D-Galactopyranoside (ONPG) (Sigma® N1127-5G) was added and samples were incubated in a water bath at 28°C until a yellow color developed.

Antibiotic microdilution MIC assays to determine resistance of WT and Δ*rcs*B to penicillin, phosphomycin, tetracycline and streptomycin. Overnight cultures were diluted and grown for 2 hours. OD_{600} measurements were obtained and cultures were diluted in sterile LB broth to approximately 10^4-10^5 cfu/ml. 100 μl of sterile LB was dispensed into each well of a sterile 96-well microtitre plate (Costar® 3596). 100 μl of 2X antibiotic stocks (penicillin

TABLE 1 Summary of *E. coli* K-12 strains used in this study (4).

Strain	Designation	Genotype	Growth Condition
DH300	WT	MG1655 (*arg*F-*lac*)U169; *rpr*A142-*lac*Z	None
DH311	Δ*rcs*B	DH300 *rcs*B::Kan^r	Kanamycin

and phosphomycin to 800 µg/ml, tetracycline and streptomycin to 50 µg/ml) were added to column 1 and 1:2 serial dilutions were made across each row through to column 10. 5 µl of the diluted bacterial cultures were dispensed into the appropriately labeled wells. Column 11 received 5 µl of the diluted cultures and no antibiotics. Column 12 had no bacteria or antibiotics added to it. The 96-well plate was covered, incubated overnight for 18-20 hours at 37°C, and visually assessed for growth. MIC was determined by the lowest concentration of a given antibiotic where growth was not observed.

β-galactosidase reporter assays to evaluate the expression of *rpr*A in WT and Δ*rcs*B after 60-minute incubation in sub-inhibitory concentrations of penicillin, phosphomycin, tetracycline and streptomycin. Overnight cultures were diluted and grown until an OD_{600} of 0.4-0.8 was achieved. Cultures were then diluted 1:10 in sterile LB and grown in either a condition of no antibiotic, penicillin, phosphomycin, tetracycline or streptomycin antibiotic stress at sub-inhibitory concentrations (as determined from the MIC results) for 60-minutes at 37°C with agitation. β-galactosidase reporter assay was performed, as described previously, until a yellow color developed. 500 µl of 1M Na_2CO_3 was added to stop the reaction and cell debris were pelleted. OD_{420} and OD_{550} measurements were taken from the supernatant and used in the calculation for β-galactosidase enzyme activity in Miller Units (MU), where 1 MU = 1000 x (OD_{420} - (1.75 x OD_{550})/(time of reaction in minutes x volume of culture in µl x OD_{600}). Statistical significance was determined using the t-test, where $p<0.05$. A portion of the DH300 and DH311 log phase bacterial cultures prior to treatment conditions were diluted for use in complementary MIC plate assays to ensure consistency of the antibiotic resistance phenotype.

RESULTS

Characterization of WT and Δ*rcs*B strains reveals LacZ activity. To confirm the identity of our *E. coli* strains, we conducted qualitative β-galactosidase assays to verify the presence of the *lac*Z promoter fusion gene in the wild-type (DH300) and the *rcs*B deletion mutant (DH311) strains (see Table 1 for genotype) (4). MG1655 with known LacZ activity was used as positive control while LacZ-deficient DH5α was used as a negative control. DH300 and DH311 displayed β-galactosidase activity, which was observed through the development of intense yellow colour upon addition of ONPG to the lysed cells, confirming the presence of a functional LacZ in both strains.

Characterization of Δ*rcs*B displays resistance to kanamycin. Given that *rcs*B deletion in DH311 was acquired through the insertion of a kanamycin resistance cassette, LB-kanamycin plating was used as an additional method of characterization (4). Colony growth was observed on DH311-streaked LB-kanamycin plates, indicating deletion of *rcs*B. No growth was observed on DH300 plates which ensured that there was no contamination from DH311.

Δ*rcs*B shows a two-fold decrease in resistance to penicillin and phosphomycin and no change in resistance to tetracycline or streptomycin when compared to WT. To determine the role of RcsB in the Rcs phosphorelay-mediated response triggered through

TABLE 2 MIC assay results of the lowest antibiotic concentration (µg/ml) with no observable growth for WT (DH300) and Δ*rcs*B (DH311). Strains were grown for 18-20 hours at 37°C and serially treated with different concentrations of penicillin, phosphomycin, streptomycin or tetracycline. Most common MIC of three independent experiments is represented. One replicate per experiment.

	Minimum Inhibitory Concentration (µg/ml)			
Strain	Penicillin	Phosphomycin	Tetracycline	Streptomycin
WT	25.0	6.25	0.78	3.13
Δ*rcs*B	12.5	3.13	0.78	3.13

disruption of the cell wall, we compared the resistance of WT and *rcs*B deletion mutant strains following treatment with two classes of antibiotics. To target cell wall synthesis in bacteria we used penicillin and phosphomycin. In order to determine whether Rcs phosphorelay-mediated stress response is a PG-specific mechanism or a general response to stress stimuli, we also compared resistance of the WT and *rcs*B deletion mutant strains against tetracycline and streptomycin, which inhibit protein synthesis.

To compare antibacterial resistance, we conducted three independent experiments to measure the MIC of each strain. Growth was observed in positive control wells (containing no antibiotic) and no growth was observed in negative control wells (containing no bacteria). Table 2 summarizes the results of our MIC assays. For the *rcs*B deletion mutant a two-fold decrease in penicillin MIC was observed compared to the WT strain. The *rcs*B deletion mutant also showed a two-fold decrease in phosphomycin MIC. The MICs for the *rcs*B deletion mutant and WT strain were equivalent when treated with tetracycline or streptomycin (Table 2). These results are consistent with the conclusion that RcsB is involved in response to antibiotics that target cell wall synthesis (e.g. penicillin and phosphomycin) but not protein synthesis (e.g. tetracycline and streptomycin) in *E. coli.*

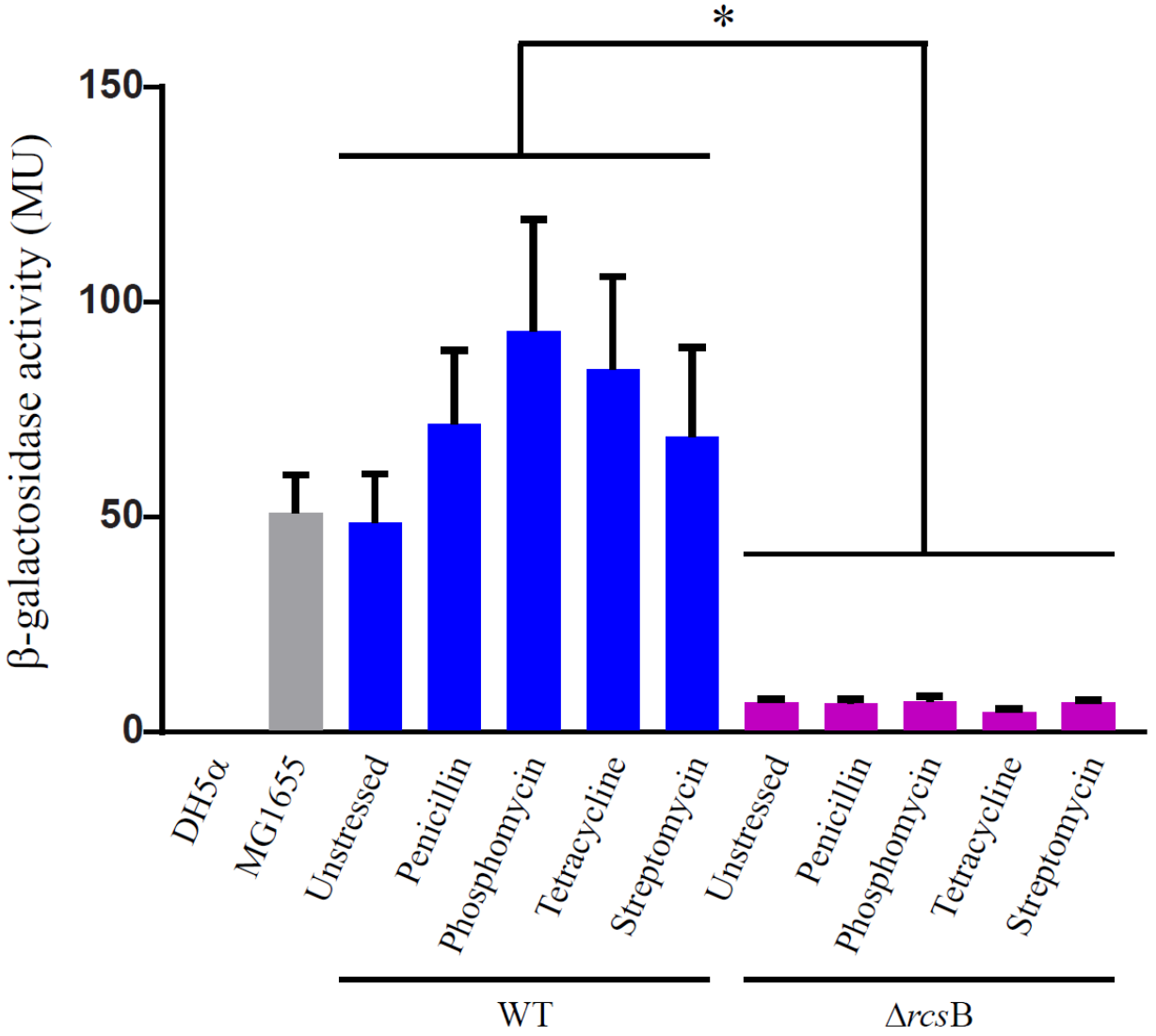

FIG. 1 β-galactosidase activity (MU) of WT (DH300) and Δ*rcs*B (DH311) showing *rpr*A promoter activity following 60-minutes of no antibiotic, penicillin, phosphomycin, tetracycline or streptomycin antibiotic stress conditions at sub-inhibitory concentrations. Average β-galactosidase activity from three independent experiments is represented. Error bars show standard error of the mean. Statistical significance was determined using the t-test, where * = $p<0.05$.

rpr*A expression is upregulated in response to penicillin, phosphomycin, tetracycline and streptomycin antibiotic stress in WT but not in Δ*rcs*B strains of *E. coli. To investigate the role of *rpr*A in cell wall-damage stress response we compared the changes in *rpr*A expression following 60-minute treatment with sub-inhibitory concentrations of penicillin, phosphomycin, tetracycline, or streptomycin. β-galactosidase assay was used to monitor the transcription of *rpr*A-*lac*Z reporter fusion gene in both DH311 and DH300. Sub-inhibitory concentrations of antibiotics were determined by dividing the MIC results by half for a given antibiotic (refer to Table 2).

E. coli strains MG1655 and DH5α were used as positive and negative controls for β-galactosidase activity. β-galactosidase activity was detected in *E. coli* strain MG1655. β-galactosidase was not detected in *E. coli* strain DH5α. Parallel to the β-galactosidase assays, MIC assays of the WT and *rcs*B deletion mutant log phase cultures from the β-galactosidase assay experiments showed similar trends in resistance to antibiotics that target protein synthesis and cell wall synthesis. We previously observed a two-fold decrease in resistance to penicillin and phosphomycin in the *rcs*B deletion mutant strain compared to the WT strain and no difference in resistance to tetracycline and streptomycin between the two strains (Table 2). The consistency of these observations serve as an additional control to confirm the intrinsic antibiotic resistances of these strains to the antibiotics that were used to determine the sub-inhibitory concentrations of the antibiotics for use in the β-galactosidase assay treatments.

Figure 1 shows β-galactosidase activity of *rpr*A-*lac*Z reporter fusion gene in WT and *rcs*B deletion mutant under no antibiotic treatment condition, penicillin, phosphomycin antibiotic, tetracycline or streptomycin antibiotic treatment stress conditions. We observed a statistically significant decrease in *rpr*A expression in the *rcs*B deletion mutant strain compared to in the WT strain. *rpr*A expression levels in antibiotic-treated and -untreated conditions were significantly higher for the wild-type *E. coli* strain than the *rcs*B deletion mutant strain. We observed an increase in *rpr*A expression under treatment with antibiotics targeting cell wall synthesis and protein synthesis in comparison to the no antibiotic treatment condition in the wild-type *E. coli* strain. We did not observe a statistically significant difference in *rpr*A expression in the *rcs*B deletion mutant *E. coli* strain between treatment with antibiotics that target cell wall synthesis, protein synthesis, or no antibiotic treatment. These observations suggest that *rpr*A expression is upregulated in the presence of RcsB when treated with either antibiotics that target cell wall synthesis or protein synthesis, and that the mechanism of action of antibiotics may not play a role in RcsB-mediated regulation of *rpr*A expression.

DISCUSSION

The two-component Rcs phosphorelay system triggers a regulatory cascade in response to stress resulting from the disruption of the PG layer in *E. coli* (2). Upon phosphorylation, the response regulator RcsB has been shown to influence the expression of various downstream genes, including *rpr*A (2,4). *rpr*A increases the translation of RpoS in order to promote cell growth under stress conditions (5). Previous studies have shown increased survival of *E. coli* with activation of the Rcs phosphorelay system following β-lactam treatment (1). Under β-lactam treatment conditions *rpr*A expression increases (1). In this study, we investigated a *rcs*B deletion mutant strain of *E. coli* to understand how the Rcs phosphorelay system responds to antibiotics targeting cell wall synthesis or protein synthesis. We also measured the expression of *rpr*A, downstream of *rcs*B, in wild-type and a *rcs*B deficient strain of *E. coli* under antibiotic treatments that target cell wall synthesis or protein synthesis. Based on the results of Laubacher and Ades, we hypothesized that following treatment with antibiotics that target cell wall synthesis, *rpr*A expression and antibiotic resistance would increase in the WT strain but not in the *rcs*B deficient strain (1). By comparison, we expected that following treatment with antibiotics targeting protein synthesis, *rpr*A expression and antibiotic resistance would remain unchanged.

To test this hypothesis, MIC assays were used to directly measure the inhibition of bacterial growth in the presence of cell wall synthesis inhibitory antibiotics, penicillin and

phosphomycin, and protein synthesis inhibitory antibiotics, tetracycline and streptomycin. As expected, *E. coli* strain DH311 which is a *rcs*B deletion mutant, showed a two-fold lower MIC than the wild-type strain DH300 for penicillin and phosphomycin (refer to Table 2) (6). By comparison, similar MICs were observed for the WT (DH300) and *rcs*B deletion mutant (DH311) following exposure to tetracycline or streptomycin (6). The results are consistent with findings of Richter *et al.*, who observed reduced survival in a *rcs*B deletion mutant strain of *E. coli* when grown in the presence of penicillin or phosphomycin, but not tetracycline or streptomycin (3). In addition, Laubacher and Ades showed enhanced susceptibility of the *rcs*B deletion mutant strain following exposure to β-lactams, amdinocillin and cefsulodin, however, resistance to aminoglycosides was not tested (1).

The expression of *rpr*A, a non-coding RNA that regulates the translation and stability of a key regulator in *E. coli* general stress response, Rpos, was indirectly measured in the presence of antibiotics targeting cell wall or protein synthesis using the β-galactosidase assay. β-galactosidase hydrolyzes ONPG to galactose and o-Nitrophenol, the product of which can be measured using absorbance at a wavelength of 420 nm (9). DH300 and DH311 encode a *rpr*A::*lac*Z reporter gene (10). Our findings show an upregulation of *rpr*A expression in the WT strain in the presence of cell wall synthesis inhibitory antibiotics or protein synthesis inhibitory antibiotics, suggesting no difference in *rpr*A expression between different antibiotic targets. We did not detect significant β-galactosidase activity in the *rcs*B deletion mutant cells treated with cell wall synthesis inhibitory antibiotics or protein synthesis inhibitory antibiotics. These observations support the model in which *rpr*A functions downstream of RcsB and parallel the findings of Majdalani *et al.*, who showed no *rpr*A expression in the absence of *rcs*B and restoration of *rpr*A promoter activity upon

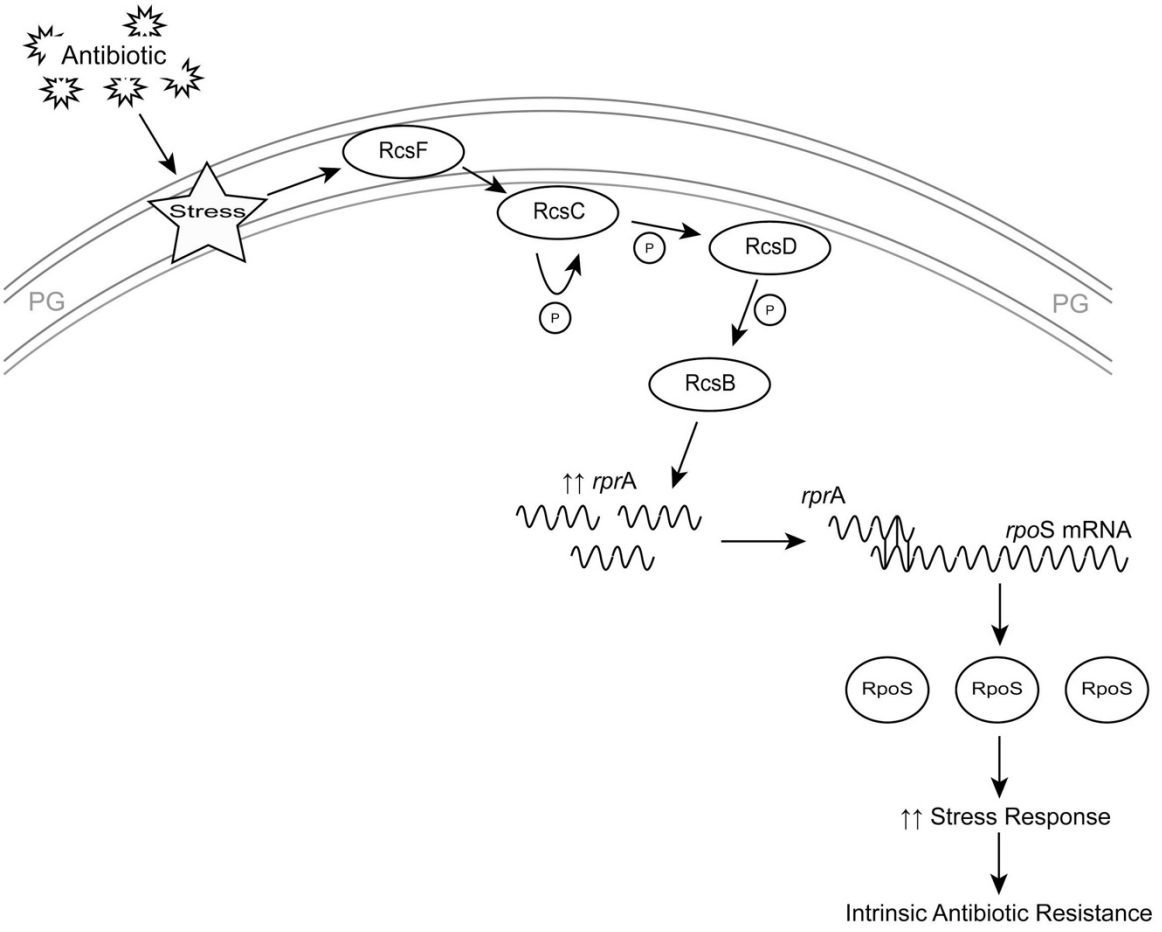

FIG. 2 Proposed model of intrinsic antibiotic resistance to antibiotics targeting cell wall synthesis mediated by the Rcs phosphorelay system in *E. coli* K-12.

complementation with a plasmid bearing the *rcs*B gene (1,4). In addition, Laubacher and Ades similarly showed upregulation of *rpr*A expression in the presence of different β-lactam antibiotics: cefsulodin and amdinocillin (1). Richter *et al.* also showed upregulation of *rpr*A expression through β-galactosidase assays after treatment with penicillin in WT but not in *rcs*B deletion mutant cells, which is consistent with our results (3). Our study extends this research by measuring *rpr*A expression following not only cell wall synthesis inhibitory antibiotic treatments but also protein synthesis inhibitory antibiotic treatments in order to provide additional insight into whether the Rcs phosphorelay system activation is antibiotic-class sensitive. The changes in expression of *rpr*A may be key to understanding the Rcs phosphorelay system-mediated intrinsic resistance response in *E. coli*. Majdalani *et al.* showed that upregulation of *rpr*A expression following activation of the Rcs phosphorelay system results in higher amounts of RpoS protein in the cell, a central regulator of the general stress response (3,4). Therefore, increase in RpoS levels can lead to an increase in the transcription of genes required for survival in the presence of antibiotics (3).

We propose a possible model based on our MIC and β-galactosidase assay results presented here (Table 2 and Figure 1). The increase in resistance to penicillin and phosphomycin in WT cells compared to the *rsc*B deletion mutant cells corresponds with the upregulation of *rpr*A in response to penicillin and phosphomycin treatment. This confirms a previously proposed model by Richter *et al.* in which intrinsic antibiotic resistance to antibiotics targeting cell wall synthesis is mediated by the activation of the Rcs phosphorelay system (Figure 2) (3). Despite the upregulation of *rpr*A in response to tetracycline and streptomycin treatment in WT cells, we did not observe a difference in resistance to tetracycline and streptomycin. This suggests that the intrinsic antibiotic resistance of *E. coli* K-12 to antibiotics targeting protein synthesis is acquired independently of the Rcs

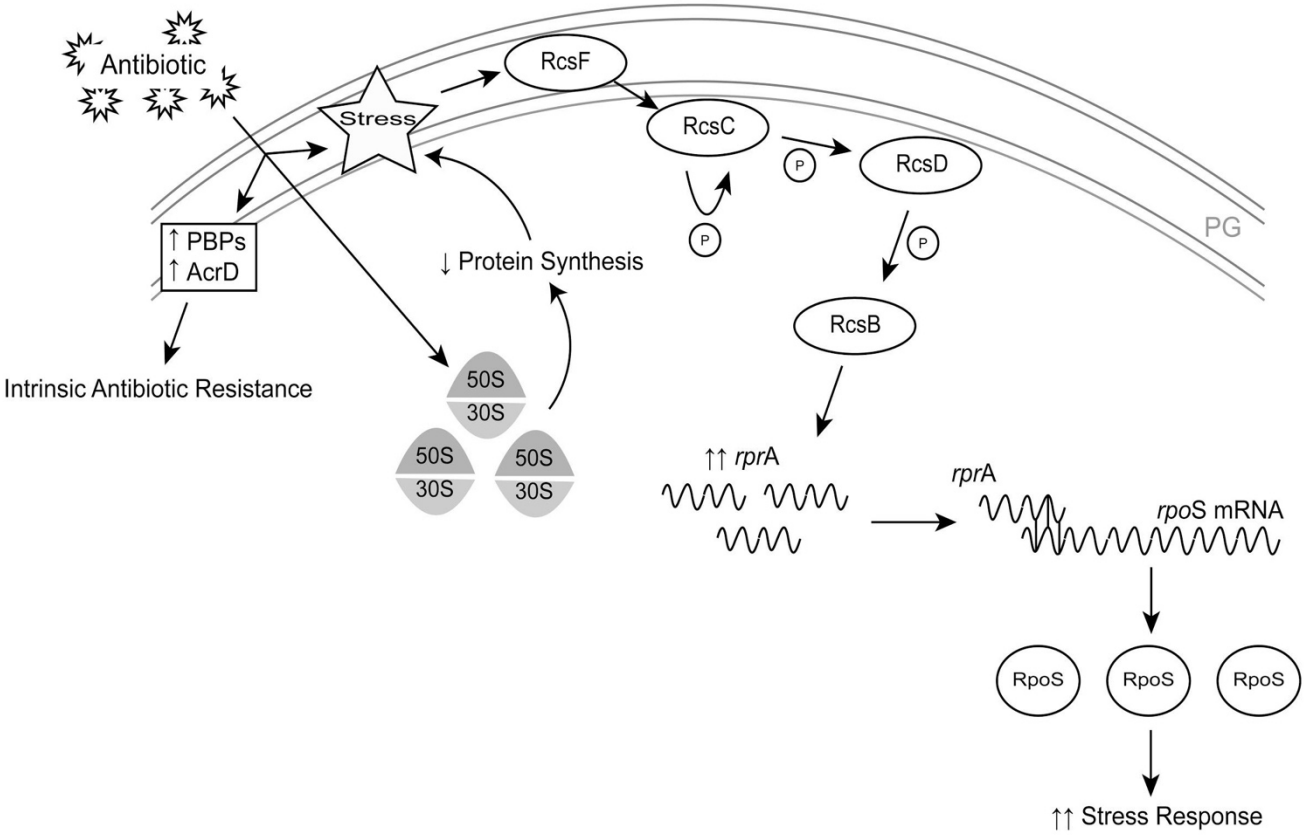

FIG. 3 Proposed model of indirect Rcs phosphorelay system activation via the interaction and action of antibiotics that target protein synthesis on the composition of cell wall-associated proteins. Rcs phosphorelay system is independent of proteins involved in intrinsic antibiotic resistance to tetracycline and streptomycin.

phosphorelay system (Figure 3). A study done by Hancock *et al*. previously showed that the interaction of aminoglycosides with the outer membrane of the cell leads to initial aggregation of the porin OmpF, contributing to the uptake mechanism of aminoglycosides across the *E. coli* outer membrane (8). Beliavskaia *et al.* have shown that pre-incubation of *E. coli* with aminoglycosides - gentamicin and streptomycin - increases the binding of β-lactams to PBPs, allowing for perturbation of the cell wall (11). In accordance, pre-treatment of *E. coli* with kanamycin has been shown to alter stress response systems that upregulate the aminoglycoside efflux pump, AcrD (12). Aminoglycosides are taken up from the periplasm and cytoplasm by AcrD and effluxed out of the cell to confer resistance (13). We propose that antibiotics targeting protein synthesis interact with cell wall-associated proteins, indirectly activating the Rcs phosphorelay system (Figure 3). It is also possible that the mechanism of action of tetracycline and streptomycin , which inhibit protein synthesis, alters the protein content around the cell wall and thus indirectly activates the Rcs phosphorelay system (Figure 3) (6). This is supported by our β-galactosidase assay results, where we see an increase in *rpr*A expression after aminoglycoside antibiotic incubation in WT cells (Figure 1). Furthermore, we propose that the resulting upregulation of downstream stress response genes by the Rcs phosphorelay system does not contribute to the cell's intrinsic aminoglycoside resistance. Instead, the upregulation of other response proteins, such as PBPs and efflux pumps, after antibiotic treatment independently contributes to intrinsic antibiotic resistance to antibiotics targeting protein synthesis (Figure 3) (11,12). This is supported by our results of the MIC assays, where we saw no difference in resistance to tetracycline and streptomycin between the wild-type and *rcs*B deletion mutant (Table 2).

Limitations Two classes of antibiotics were used to test for differences in MIC and *rpr*A expression in the wild-type and *rcs*B deletion mutant strains. The testing of additional classes of antibiotics with varying mechanisms of action or the expansion of our testing to other mutants that may regulate *rpr*A would have strengthened our results and our proposed model on the participation of the Rcs phosphorelay system in conferring intrinsic antibiotic resistance to various classes of antibiotics.

Conclusions In conclusion, we found that the *rcs*B deletion mutant *E. coli* strain DH311 shows reduced resistance to penicillin or phosphomycin but not to tetracycline or streptomycin when compared to the isogenic wild-type strain DH300. We also found that *rpr*A expression is dependent on RcsB and is upregulated in WT strain after treatment with penicillin, phosphomycin, tetracycline, or streptomycin. Our data suggests that the Rcs phosphorelay system contributes to *E. coli* enhanced intrinsic antibiotic resistance responses after treatment with antibiotics targeting cell wall synthesis but not protein synthesis, even though the expression of *rpr*A is upregulated following treatment with all of these antibiotics in the wild-type strain.

Future Directions To further confirm the results obtained from the β-galactosidase assay, qPCR could be performed as an alternate method of analysis for *rpr*A expression. Evaluating *rpr*A expression at various time points following induction of antibiotic stress may help to investigate the course of action of the Rcs phosphorelay response. Future studies may also address our proposed model (Figure 3) by investigating whether the increase in *rpr*A expression in response to tetracycline and streptomycin can be used as a pre-treatment to confer increased resistance to penicillin and phosphomycin . Furthermore, it would be interesting to perform similar expression analysis experiments on a strain that is lacking the Rcs pathway in its entirety. This would better allow for the determination of whether resistance changes are based on the mechanism of action of the antibiotic as opposed to the activation of the Rcs phosphorelay system.

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