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The Rcs Phosphorelay System and RcsB Regulated *rpr*A Contribute to Intrinsic Antibiotic Resistance in *Escherichia coli* Exposed to Antibiotics Targeting the Cell Wall

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Bacteria orchestrate gene expression to cope with stressful conditions. Physical and antibiotic stressors induce regulators of stress response pathways to allow enhanced bacterial survival. The Rcs phosphorelay system regulates *rpr*A, a small non-coding RNA which in turn regulates translation of RpoS, in addition to genes important for the production of capsule. RpoS is a central regulator of the general stress response. Prior studies have shown that *Escherichia coli* exhibits enhanced bacterial survival upon exposure to β-lactam antibiotics with the activation of the Rcs pathway using a mechanism independent of capsule synthesis. This mechanism of Rcs-mediated antibiotic resistance is not well understood. It is also not known whether this resistance is linked to specific classes of antibiotics. The objective of this study was to investigate the role of the Rcs regulon in response to treatment with antibiotics targeting either peptidoglycan synthesis or protein synthesis. We hypothesized that an *rcs*B deletion mutant *E. coli* K-12 strain would exhibit an increased antibiotic sensitivity phenotype when exposed to antibiotics inhibiting cell wall synthesis. Antibiotics inhibiting protein synthesis were not expected to elicit different antibiotic sensitivity phenotypes in wild-type or Δ*rcs*B strains because they were not anticipated to induce the Rcs phosphorelay response. We show that the presence of RcsB increases antibiotic tolerance to penicillin and phosphomycin but not to tetracycline or streptomycin. The wild-type and Δ*rcs*B strains showed no difference in susceptibility to classes of antibiotics inhibiting protein synthesis, yet exhibited different antibiotic sensitivity phenotypes to classes of antibiotics inhibiting cell wall synthesis. The Δ*rcs*B mutant showed a 4-fold decrease in penicillin resistance and a 2-fold decrease in phosphomycin resistance compared to the wild-type strain. We postulate that *rpr*A functions downstream of RcsB, since RcsB is necessary to achieve full expression of *rpr*A in the presence of penicillin. Taken together, this study suggests that RcsB contributes to intrinsic resistance of *E. coli* to antibiotic stressors perturbing the peptidoglycan synthesis. Moreover, RcsB appears to regulate *rpr*A expression in response to treatment with penicillin. This study provides insight into intrinsic stress response mechanisms contributing to bacterial multidrug resistance.

The Rcs (regulator of capsule synthesis) phosphorelay system is a two-component pathway conserved in *Enterobacteriaceae* (1, 2). It is composed of the sensor kinase RcsC and the response regulator RcsB. The Rcs regulon is responsible for the regulation of the *cps* operon that encodes proteins required for the production of the capsular polysaccharide colonic acid (1, 2). It is debated whether capsules confer antibiotic resistance and whether this resistance is directed at specific classes of antibiotics (3, 4).

In the presence of β-lactam antibiotics, *Escherichia coli* exhibits enhanced bacterial survival with the activation of the Rcs pathway (5). The mechanism by which the Rcs phosphorelay contributes to β-lactam antibiotic resistance is not well understood, but it is proposed to function independently of capsule synthesis. Using an *E. coli* strain which disrupted expression of several enzymes required for capsule synthesis, Laubacher and Ades showed that inhibition of capsule had no effect on the survival of cells treated with β-lactam antibiotics. Further, expression in four genes (*rpr*A, *ydh*A, *ymg*G and *osm*B) of the Rcs regulon that are not involved with capsule synthesis increased in the presence of β-lactam antibiotics (5). *rpr*A is a small non-coding RNA that is activated by the RcsC-RcsB phosphorelay system (6). It assists the translation of RpoS, which is involved in the general stress response, by

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altering the secondary structure of *rpo*S mRNA to increase its half-life (7, 8). Overexpression of *rpr*A has been shown to increase tolerance to kanamycin after exposure to osmotic stress in RcsB-deficient *E. coli* K-12 (9).

It has been proposed that the Rcs phosphorelay system may contribute to an intrinsic resistance mechanism (5). Typical acquired mechanisms lead to resistance to only specific antibiotics. In contrast, intrinsic mechanisms take advantage of the general stress response to provide resistance to multiple antibiotics initiated by common stressors (10, 11). The Rcs phosphorelay may naturally respond to peptidoglycan (PG) damage by inducing cell envelope stress response genes, such as *rpr*A, to enhance survival. Activation of the Rcs pathway increases resistance to β-lactams (5); however, it is not known whether it contributes to resistance to other classes of antibiotics that disrupt bacterial cell wall synthesis, such as phosphoric acids, or to classes of antibiotics that target bacterial protein synthesis, such as aminoglycosides and tetracyclines (5, 12, 13).

The objective of this study was to investigate the role of the Rcs phosphorelay system of *E. coli* in response to treatment with antibiotics targeting either PG synthesis or protein synthesis. We hypothesized that a Δ*rcs*B mutant would exhibit an increased antibiotic sensitivity phenotype when exposed to antibiotics inhibiting cell wall synthesis. Antibiotics inhibiting protein synthesis were not expected to elicit different antibiotic sensitivity phenotypes in wild-type (WT) or Δ*rcs*B strains because they do not stress the PG layer and were not anticipated to induce the Rcs phosphorelay response. To test this hypothesis, we used an *rcs*B deletion mutant *E. coli* K-12 strain in a minimal inhibitory concentration (MIC) assay to measure antibiotic resistance. We found that RcsB is an important player of the Rcs phosphorelay system that leads to different antibiotic sensitivity phenotypes. The WT and Δ*rcs*B strains showed no difference in susceptibility to classes of antibiotics inhibiting protein synthesis, yet exhibited different antibiotic sensitivity phenotypes to classes of antibiotics inhibiting cell wall synthesis. The mutant strain showed a 4-fold decrease in penicillin resistance and a 2-fold decrease in phosphomycin resistance compared to the WT. In order to test whether β-lactam induced resistance mediated through RcsB involves *rpr*A, we conducted a β-galactosidase assay to measure *rpr*A transcription in WT and mutant treated with penicillin. We expected the expression of *rpr*A to be modulated by exposure to antibiotics inhibiting PG synthesis. Our data showed that expression of *rpr*A is significantly upregulated upon exposure to a PG targeting antibiotic. Taken together, our data supports a model in which cell stress induced by inhibitors of PG synthesis leads to intrinsic antibiotic resistance via RcsB, the non-coding RNA *rpr*A and possibly RpoS.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are described in Table 1. *E. coli* K-12 BW28357 (4) is referred to as wild-type (WT) and *E. coli* K-12 BW3009 (4) is designated as the isogenic *rcs*B deletion mutant (Δ*rcs*B). Two *rpr*A-*lac*Z genomic reporter fusion *E. coli* strains were used in this study as well. *E. coli* K-12 DH300 (5) is denoted as WT *rpr*A-*lac*Z, and *E. coli* K-12 DH311 (5) has the *rcs*B gene knocked out using a kanamycin resistance cassette and is denoted as Δ*rcs*B *rpr*A-*lac*Z.

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

Strain	Designation	Relevant Genotype	Reference
BW28357	WT	F-; Δ(*ara*D-*ara*B)567; Δ*lac*Z4787(::*rrn*B-3); λ-; Δ(*rha*D-*rha*B)568; *hsd*R514	(4)
BW30009	Δ*rcs*B	F-; Δ(*ara*D-*ara*B)567; Δ*lac*Z4787(::rrnB-3); λ-; Δ *rcs*B1320; Δ(*rha*D-*rha*B)568; *hsd*R514	(4)
DH300	WT *rpr*A-*lac*Z	MG1655 (*arg*F-*lac*)U169; *rpr*A142-*lac*Z	(5)
DH311	Δ*rcs*B *rpr*A-*lac*Z	DH300 *rcs*B::Kan^r	(5)

Growth conditions and preparation of antibiotics. All strains were grown in sterile Luria Bertani (LB) broth at 37°C with agitation. Concentrated stock solutions of kanamycin sulfate, penicillin-G, phosphomycin, tetracycline hydrochloride and streptomycin sulfate purchased from Sigma-Aldrich® were prepared. All antibiotic stock solutions were prepared by dilution in dH_20 and sterilized using a 0.22 µm nitrocellulose filter. Antibiotic stock solutions were stored at -20°C and thawed on ice prior to use.

Oligonucleotide primer design. The oligonucleotide polymerase chain reaction (PCR) primers were designed to amplify an 817 bp DNA fragment containing *rcs*B (Table 2). These primers were designed to be complementary to the regions upstream and downstream of the *E. coli* K-12 *rcs*B gene. Both forward and reverse primers were designed using software by Integrated DNA Technologies (IDT Inc. Coralville, IA).

TABLE 2. Oligonucleotides used for amplification of *rcsB* in this study.

Description	Sequence (5'-3')	T_m (ºC)	% GC Content
2ep715-Primer1	GAATTCTAAATCT GGTACCCGGCAAG	57.6	50
2ep715-Primer2	AAGCTTAACGCGTC TCTTATCTGGCCTA	61.0	50

Colony PCR amplification of *rcs*B and agarose gel electrophoresis to verify the *rcs*B deletion in *E. coli* K-12 BW3009. Primers were reconstituted in sterile water to a concentration of 100 µM. In each PCR reaction, a final concentration of 0.2 µM of each primer, 1X PCR Buffer (Invitrogen, Cat no. 18067-017), 0.2 mM dNTP (Invitrogen, Cat. No. 10297-018), 1.5 mM $MgCl_2$ (Invitrogen), 0.2 µl Platinum Taq (Invitrogen, Cat no. 10966-034) and sterile water to 50 µl were included. Colony PCR was performed by touching isolated colonies of WT or Δ*rcs*B with a sterile tip and adding cells directly to PCR tubes containing PCR reagents. Thermocycler parameters included an initial denaturation for 3 minutes at 94°C followed by

30 cycles of denaturation at 94°C for 30 seconds, annealing for 30 seconds at 52.6°C and extension at 72°C for 60 seconds. PCR products were stored at -20°C until ran in 1X TBE buffer at 100V for 1 hour on a 2% agarose gel with SYBR® Safe DNA Gel Stain in 0.5X TBE (ThermoFisher, Cat no. S33100). PCR products were later sequenced by the NAPS Unit at the University of British Columbia (NAPS Unit. Vancouver, BC).

MIC assays to determine antibiotic resistance of WT and Δ*rcs*B to penicillin, phosphomycin, tetracycline and streptomycin. OD_{600} measurements of overnight cultures of WT and Δ*rcs*B were obtained using an ultra-spectrophotometer 3000. Cultures were diluted in LB to approximately 10^{-4}-10^{-5} cfu/ml. 100 µl of LB was dispensed into all wells of a sterile 96-well microtitre plate. 100 µl of 2X antibiotics (penicillin to 800 µg/ml, phosphomycin to 800 µg/ml, tetracycline and streptomycin to 50 µg/ml) was pipetted into the first column and serially diluted 1:2 through the first 10 of 12 columns of the plate. 5 µl of the diluted bacteria of either WT or Δ*rcs*B was dispensed in each well. The last column was left free of bacteria and acted as a sterility control while the second to last column was left free of antibiotics and acted as a positive control. The plates were incubated overnight at 37°C and assessed visually for growth after 19 hours.

Kanamycin plating to confirm the deletion of *rcs*B in *E. coli* K-12 DH311. The *rcs*B gene in DH311 was previously knocked out by disruption of the gene with a kanamycin resistance cassette (see Table 1). To confirm the deletion of *rcs*B in DH311, cells were streaked on LB plates containing 100 µg/ml kanamycin and colonies were isolated from the LB-kanamycin plates for downstream experiments. DH300 was also streaked on LB-kanamycin to ensure that there was no contamination from DH311.

β-galactosidase reporter assay to evaluate expression of *rpr*A in WT and Δ*rcs*B cells upon incubation in sub-lethal concentrations of penicillin. 6 ml of overnight culture of each DH300 and DH311 were centrifuged at 6000 x g for 5 minutes. Cell pellets were re-suspended in 6 ml LB or LB containing 3.1 µg/ml of penicillin. 2 ml aliquots of re-suspended cell suspensions were incubated in penicillin for 20, 40 or 60 minutes shaking at 37°C. β-Galactosidase assays were performed according to standard methods (14).

RESULTS

Characterization of the Δ*rcs*B strain reveals a 648 nucleotide deletion of *rcs*B gene sequence. Given that the exact deletion in the *E. coli* K-12 BW3009 mutant has not yet been described, we needed to verify and characterize the *rcs*B deletion in the Δ*rcs*B strain. In order to do this, we chose to perform colony PCR on both WT and mutant strains. We created oligonucleotide primers that flanked the translated region of the *rcs*B gene (Table 2). Figure 1 shows that the PCR product of the WT strain resulted in a band around 900 bp, and the PCR product of the Δ*rcs*B mutant strain resulted in a band around 350 bp. As expected, the mutant has a smaller PCR product due to the gene deletion. As a negative control, dH_2O instead of DNA was used and displayed no band on the gel (Fig. 1). Figure 2 shows the sequencing data from the PCR products of WT and Δ*rcs*B. Compared to the WT, Δ*rcs*B presents a 648 nucleotide deletion of the entire translated region of the

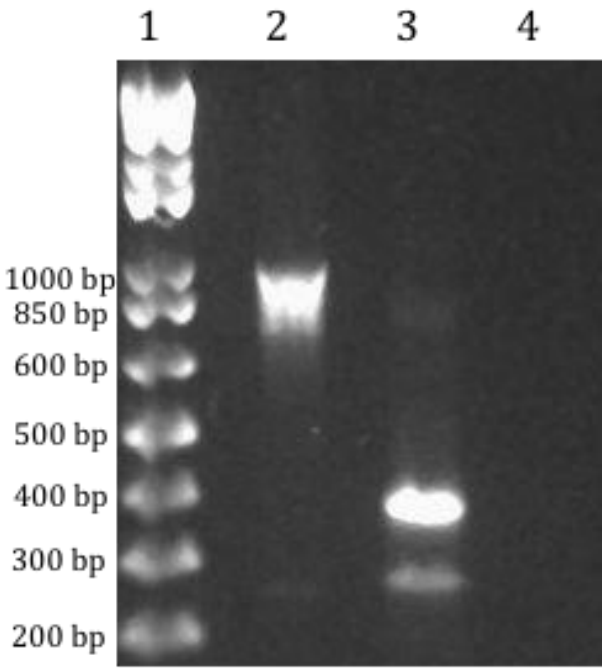

FIG 1. 2% agarose gel containing PCR products amplifying *rcs*B gene run at 100V for 1 hour. Lane 1, 1000 bp Plus Molecular Weight Ladder. Lane 2, WT PCR product. Lane 3, Δ*rcs*B PCR product. Lane 4, dH_2O negative control PCR product.

*rcs*B gene excluding the stop codon and includes a FLP recombinase target site that was used for the recombination to create the deletion (Fig. 2). These results indicate that the Δ*rcs*B strain used in this study contains the deletion of the *rcs*B gene sequence resulting in a 350 bp PCR product.

RcsB-deficient *E. coli* shows decreased resistance to penicillin and phosphomycin but not to tetracycline or streptomycin. To determine whether the Rcs phosphorelay system plays a role in antibiotic resistance to inhibitors of PG synthesis, we used a MIC assay to test survival of WT and Δ*rcs*B strains treated with penicillin and phosphomycin. Further, we tested survival of these strains treated with antibiotics that target protein synthesis: streptomycin and tetracycline. In all replicates, growth was observed in the positive control left free of antibiotic which indicates strain viability, and no growth was observed in the sterility control left free of bacteria which indicates no contamination from the environment or the growth media. Table 3 illustrates the highest antibiotic concentration with cell growth of the various antibiotics comparing WT and Δ*rcs*B strains. No difference in antibiotic sensitivity to streptomycin or tetracycline was observed between WT and Δ*rcs*B cells (Table 3). A 4-fold decrease in antibiotic resistance in Δ*rcs*B compared to WT was observed with growth at penicillin concentrations of 3.1 and 12.5 µg/ml, respectively (Table 3). A 2-fold decrease in antibiotic resistance in Δ*rcs*B compared to WT was observed with

FIG 2. Sequence alignment of a section of the WT and Δ*rcs*B PCR products. Underlined nucleotides represent the FLP recognition target site used to create the deletion.

growth at phosphomycin concentrations of 6.3 and 12.5 µg/ml, respectively (Table 3). The observed results with tetracycline, streptomycin, penicillin and phosphomycin support the hypothesis that RcsB is required for antibiotic resistance in response to treatment with antibiotics that specifically target PG synthesis. The difference in antibiotic resistance in WT and Δ*rcs*B growth in penicillin and phosphomycin, and the similarity of antibiotic susceptibility in growth in tetracycline and streptomycin, indicate the important role of RcsB in increasing the MIC of certain cell wall targeting antibiotics.

RcsB is required for penicillin-induced expression of *rpr*A. Finally, we examined whether the RcsB regulated non-coding RNA, *rpr*A, is involved in the penicillin resistance linked to RcsB. In order to determine whether *rpr*A expression is modulated by exposure to penicillin, we incubated WT and Δ*rcs*B strains for 20, 40, and 60 minutes in the presence or absence of sub-MIC penicillin (3.1 µg/ml). Each strain was transformed with a plasmid carrying an *rpr*A-*lac*Z transcriptional reporter. β-galactosidase assays were conducted to measure transcription from the *rpr*A promoter. Figure 3 shows a significant increase in β-galactosidase activity in WT cells after exposure to penicillin at each time point. Δ*rcs*B showed an increase in beta-galactosidase activity upon exposure to penicillin compared to the control LB condition after a 20-minute incubation time but not after 40 or 60 minutes (Fig. 3). At all time points, Δ*rcs*B exhibits 2-fold or greater decrease in β-galactosidase activity compared to WT when incubated with penicillin (Fig. 3). These data suggest that *rpr*A expression is modulated by RcsB in response to treatment with a PG synthesis inhibitor such as penicillin.

TABLE 3. Highest antibiotic concentration with observed cell growth in MIC assay based on 4 replicates of WT and Δ*rcs*B grown for 19 hours at 37°C in the presence of varied concentrations of penicillin, phosphomycin, streptomycin or tetracycline.

	Concentration (µg/ml)			
Cell Type	**Penicillin**	**Phosphomycin**	**Streptomycin**	**Tetracycline**
WT	12.5	12.5	3.1	0.8
Δ*rcs*B	3.1	6.3	3.1	0.8

DISCUSSION

The Rcs phosphorelay system of *E. coli* may play a key role to confer antibiotic resistance directed at specific classes of antibiotics. In this study, we investigated whether RcsB is required for intrinsic resistance to antibiotics targeting cell wall synthesis. Further, we aimed to understand whether the expression of *rpr*A, a potential downstream target of RcsB, is modulated following exposure to inhibitors of PG synthesis.

A Δ*rcs*B mutant strain of *E. coli* displayed a lower MIC than WT upon exposure to penicillin and phosphomycin (Table 3). This observation parallels the findings by Laubacher and Ades, which showed reduced survival in a Δ*rcs*B strain of *E. coli* when grown in the presence of the β-lactams amdinocillin and cefsulodin (5). Penicillin and phosphomycin both target cell wall synthesis using different mechanisms. Penicillin is a β-lactam that targets pencillin-binding proteins in the final step of PG synthesis (12). Phosphomycin is a phosphonic acid antibiotic that inhibits MurA, a cell wall synthesizing enzyme, which stops catalysis of the first committed step of PG synthesis (12). Penicillin and phosphomycin are structurally distinct (12). This suggests that the Rcs phosphorelay mediated antibiotic resistance involves a mechanism that detects peptidoglycan damage rather than recognizing a specific antibiotic. Intrinsic mechanisms of resistance operate through the general stress response and result in resistance to multiple antibiotics (5, 10, 11). Any antibiotic or stressor that inhibits peptidoglycan synthesis may induce the Rcs pathway. The regulon may then respond through activation of cell envelope stress response genes to enhance survival.

Applying this intrinsic resistance model, it is expected that antibiotics targeting protein synthesis would not

elicit a similar stress response. This hypothesis was tested using a MIC assay with tetracycline and streptomycin, antibiotics that target protein synthesis (13). As shown in Table 3, no difference in MIC was observed between WT and Δ*rcs*B cell types. Since these antibiotics do not damage the cell wall and therefore, according to our model, the Rcs system is not induced, and cells lacking the RcsB protein did not display a lower MIC than WT. Laubacher and Ades support the intrinsic resistance model of the Rcs pathway in response to β-lactam antibiotics specifically but do not elaborate upon alternative antibiotic classes (5). Our study expands this model beyond β-lactams and shows a resistance response to additional classes of cell wall targeting antibiotics and no changes in sensitivity to classes of antibiotics targeting protein synthesis.

The RcsB regulated non-coding RNA *rpr*A may provide the answer to how the Rcs phosphorelay system may be responding to antibiotic PG damage to prompt increased resistance. In the reporter assay depicted in Figure 3, expression of *rpr*A in WT and Δ*rcs*B strains containing the *lac*Z gene fused to the *rpr*A promoter results in expression of β-galactosidase, which hydrolyzes ONPG to galactose and o-Nitrophenol of which the absorbance is measured (14). The results indicated that expression of *rpr*A was significantly upregulated upon exposure to penicillin in WT (Fig. 3). Laubacher and Ades similarly found increased levels of expression of *rpr*A in the presence of other β-lactam antibiotics (5). *rpr*A may be important in antibiotic resistance. This may be possible because *rpr*A translationally stabilizes the *rpo*S mRNA, which increases RpoS concentrations (8, 9). In a study by Majdalani *et al*., it was shown that Rcs phosphorelay activation resulted in a significant increase in the expression of *rpr*A which subsequently resulted in a 32- to 35-fold increase in the amount of RpoS protein found in the cell (7). In an additional study by Battesti *et al*., increases in RpoS levels were found to be due to either changes in translation of *rpo*S or changes in RpoS degradation - the former is known to be mediated by *rpr*A (15). Since RpoS is a central regulator of the general stress response, significant increases of the protein can then lead to increased transcription of stress response associated genes that may be required for the cell to survive in the presence of antibiotics. It has been shown that RpoS-dependent gene expression under stressful conditions leads to general stress resistance and enhanced survival of *E. coli* (16). Comparing WT to Δ*rcs*B strains reveals that RcsB is necessary to achieve full expression of *rpr*A in the presence of penicillin (Fig. 3). Penicillin may trigger RcsB-dependent *rpr*A

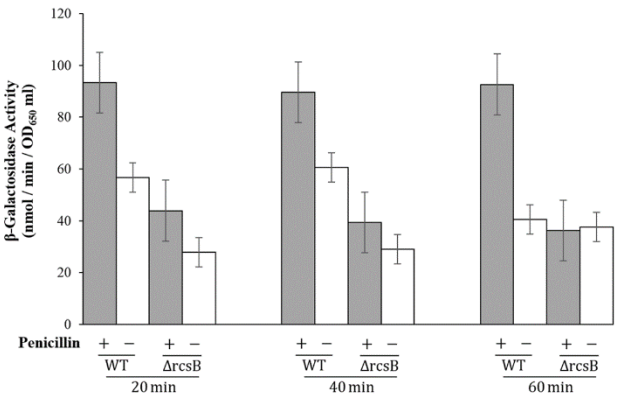

FIG 3. Average β-galactosidase activity from 3 replicates of β-galactosidase reporter assay of *rpr*A in WT and Δ*rcs*B stimulated with or without 3.1 μg/ml penicillin for 20, 40 or 60 minutes. Error bars represent the standard error of the mean.

expression by perturbing the PG layer which may lead to activation of the Rcs regulon. Once upregulated, *rpr*A can proceed to alter the secondary structure of *rpo*S mRNA, increasing its half-life so more RpoS can be translated per transcript leading to considerable induction of the general stress response. It is possible that the upregulated *rpr*A expression may largely contribute to the intrinsic resistance to the cell wall targeting antibiotics. Our proposed model of this intrinsic resistance mechanism is summarized in Figure 4.

The implications of this study are important to understanding intrinsic resistance of *E. coli*. Fundamental cell wall stress responses may not dramatically increase resistance, yet they may allow bacteria to survive longer upon antibiotic exposure or exhibit resistance to a variety of antibiotics. Insight into intrinsic stress response mechanisms potentially contributing to bacterial multidrug resistance may be gained by testing if the Rcs phosphorelay system confers resistance as a natural response to stochastic stressors of the PG.

In conclusion, we found that *E. coli* WT and Δ*rcs*B strains show different antibiotic sensitivity phenotypes upon exposure to penicillin or phosphomycin and no difference when treated with tetracycline or streptomycin. We also found that expression of *rpr*A is RcsB-dependent and significantly upregulated upon exposure to penicillin. This study suggests that the Rcs phosphorelay system contributes to increased resistance of *E. coli* to classes of antibiotics inhibiting cell wall synthesis and has no effect on classes of antibiotics inhibiting protein synthesis.

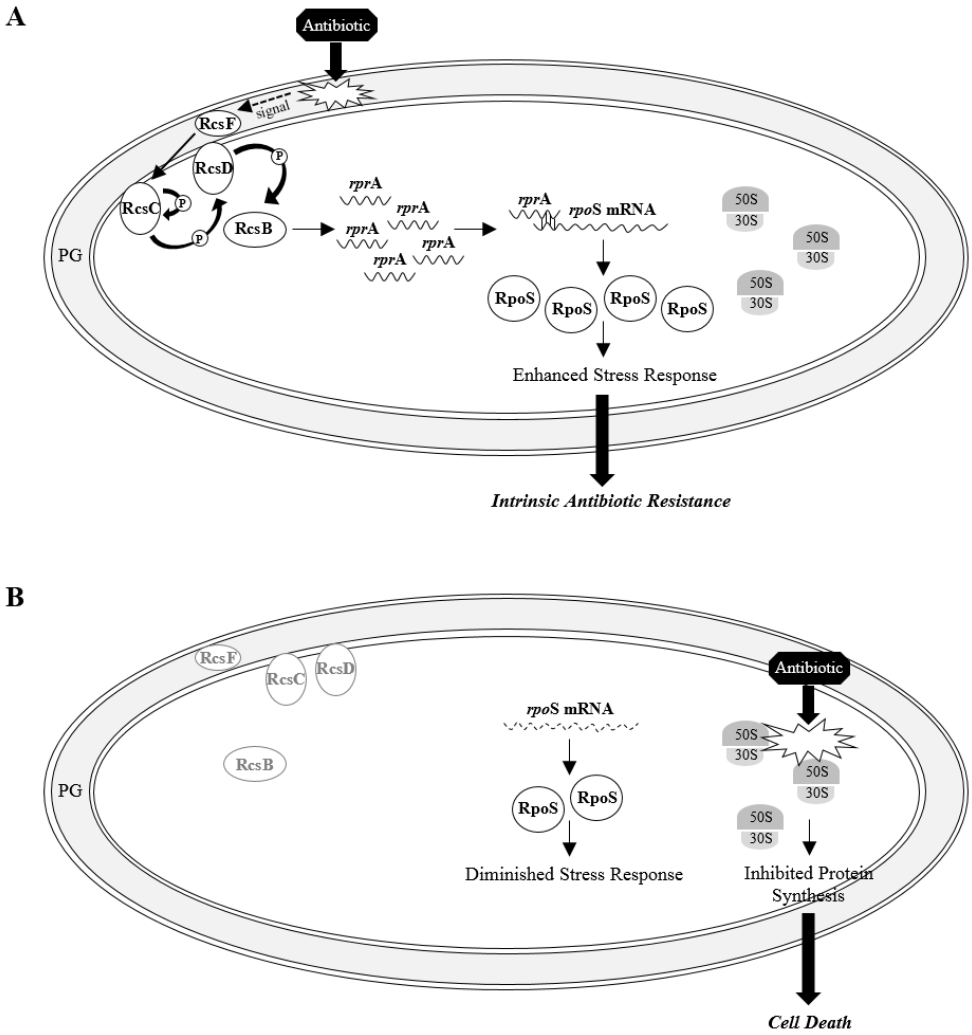

FIG 4. Proposed model of Rcs phosphorelay-mediated intrinsic antibiotic resistance in *E. coli*. Upon exposure to antibiotics targeting peptidoglycan synthesis (A), peptidoglycan damage is sensed and triggers the Rcs pathway. Upregulation of RcsB-dependent *rpr*A expression allows more of the non-coding RNA *rpr*A to translationally stabilize *rpo*S mRNA leading to increased RpoS concentration and a considerable induction of the general stress response. Upon exposure to antibiotics targeting protein synthesis (B), the Rcs phosphorelay is not induced, and without *rpr*A stabilization, *rpo*S mRNA is more rapidly degraded leading to a diminished stress response. Antibiotic inhibition of protein synthesis results in cell death.

Future studies may address whether the overexpression of *rpr*A in Δ*rcs*B is capable of rescuing, or partially rescuing, the penicillin antibiotic sensitivity phenotype we have observed. *E. coli* strain NM25508 contains a pBAD24 derivative called pNM12 (9). One of the plasmids has an empty pNM12 and the other contains the *rpr*A gene inserted downstream of the arabinose inducible promoter. The plasmids could be transformed into competent WT and Δ*rcs*B to create inducible strains capable of overexpressing *rpr*A. However, the pNM12 vector containing the *rpr*A gene used in previous studies (9) also contains an ampicillin resistance cassette encoding a β-lactamase. Due to the similarity in structure between ampicillin and penicillin, the β-lactamase produced from the pNM12 vector in transformed cells is capable of recognizing penicillin, therefore causing resistance to penicillin. To circumvent this problem in the future, an approach may be to remove the ampicillin resistance cassette in the pNM12 vector and replace it with a non-β-lactamase resistance cassette for selection. Alternatively, other cell wall targeting antibiotics that work against Gram-negative bacteria and that are not inactivated by β-lactamases, such as phosphomycin, could be tested using this method without the need to replace the ampicillin resistance cassette. It would also be interesting to determine whether any, or a combination of the three other three Rcs regulon genes (*ydh*A, *ymg*G

and *osm*B) that are not involved in capsule synthesis, yet are known to be upregulated upon the exposure to β-lactam antibiotics, are responsible for the antibiotic sensitivity phenotype.

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