

# Role of the Rcs Phosphorelay in Intrinsic Resistance to Penicillin, Phosphomycin, and Cefsulodin in *Escherichia coli* K12

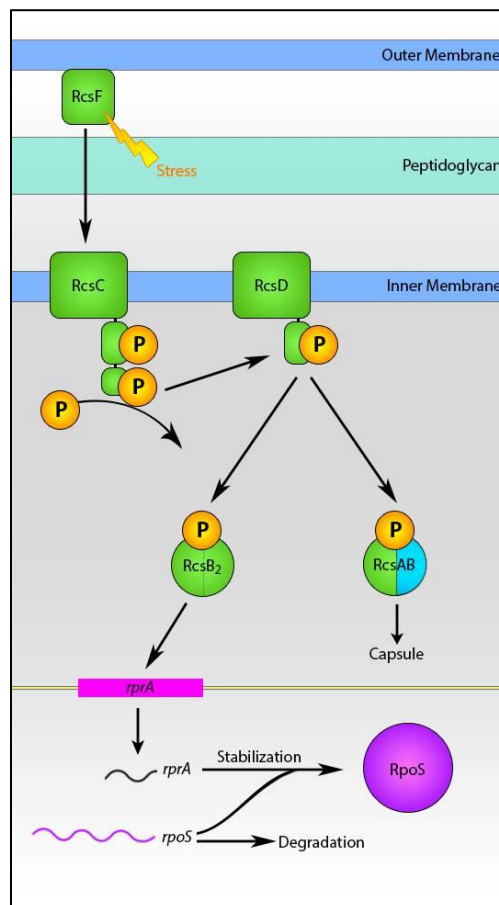
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**The Regulator of Capsule Synthesis (Rcs) of *Escherichia coli* detects cell wall stress and elicits a stress response. The Rcs phosphorelay system consists of the outer membrane kinase sensor RcsF, the inner membrane kinases RcsC and RcsD, and then response regulator RcsB. RcsB regulates the transcription of *rprA*, a non-coding RNA which affects the translation and stability of multiple mRNAs. Prior studies reported that RcsB is critical for resistance against cell wall antibiotics. Conversely, other studies found that a  $\Delta rcsB$  strain did not alter resistance to the tested cell wall antibiotics. In this study, we attempted to replicate the experiments that demonstrate RcsB as a critical factor for resistance against cell wall antibiotics such as penicillin, phosphomycin, and cefsulodin. Additionally, we utilized the more sensitive plating efficiency assay to discern any differences in strain sensitivity. We show that deletion of RcsB does not lead to decreased antibiotic resistance. Our findings suggest that RcsB is not essential for resistance to antibiotics targeting the cell wall. We further propose that the loss of Rcs function to cell wall stress may be compensated by other pathways such as the Cpx pathway, which also regulates *rprA*.**

The bacterial cell wall is the first-barrier located between the outside environment and the bacteria (1, 2). Due to its accessibility, the bacterial cell wall is the target to many antibiotics such as  $\beta$ -lactams, which cause cell toxicity (3). To survive, bacteria have evolved a variety of resistance mechanisms, controlled by envelope stress responses (ESR), that allow survival in the presence of antibiotics (4). For example, certain ESRs can upregulate the expression of chaperone proteins to refold affected molecules, proteins such as DegP that degrade other proteins, or efflux pumps such as SmeIJK to transport antibiotics outside the cell (1, 5-7). One of these cellular responses is the Regulator of Capsule Synthesis (Rcs) pathway (4). The Rcs pathway is a two-component system phosphorelay cascade that responds to cell wall stress in *Escherichia coli* (*E. coli*) (4, 8) (Fig. 1). The Rcs pathway consists of the outer membrane histidine kinase sensor RcsF, which is thought to act as a sensor of membrane perturbations (9). When left in the periplasm, RcsF instead phosphorylates inner membrane kinase RcsC (9). RcsC in turn phosphorylates the cytosolic protein RcsD (8). RcsD phosphorylates the downstream transcription factors RcsA and RcsB (8). The heterodimer of RcsA and B is necessary for the transcription of genes needed for colanic acid capsules (8). RcsB<sub>2</sub> homodimer upregulates the expression of *rprA* which is a small non-coding RNA. *rprA* is important to stabilize the major regulator of stress gene, *rpoS* (10) (Fig.1).

Previous studies have provided evidence that the Rcs pathway contributes to antibiotic resistance. Hirakawa *et al.* showed that RcsB protein overexpression promoted resistance to kanamycin and phosphomycin (11). Laubacher & Ades found that genes controlled by the Rcs pathway were upregulated in microarray analysis upon exposure to antibiotics (12). Other studies showed RcsB is involved in *rprA* expression to respond to penicillin stress by a genomic reporter assay (13, 14). In addition, the antibiotic sensitivity of  $\Delta rcsB$  mutant to cell wall antibiotics such as penicillin,



**FIG. 1 The Rcs phosphorelay pathway.** RcsF detects membrane perturbations through an unknown mechanism. This activates RcsC which autophosphorylates and in turn, phosphorylates RcsD. RcsD phosphorylates RcsB, which then dimerize with either another RcsB to form RcsB<sub>2</sub>, or RcsA to form RcsAB. RcsAB is involved in capsule synthesis. RcsB<sub>2</sub> activates transcription of *rprA*, and the *rprA* mRNA binds to the promoter region of *rpoS* mRNA to favor its translation.

**TABLE 1 MIC of penicillin, phosphomycin, and cefsulodin from this study and various past studies for WT and *ΔrcsB* strains**

Reference	Strain	Cell Type	Concentration (μg/mL)		
			Penicillin	Phosphomycin	Cefsulodin
This study	BW28357	Old WT	25	6.3	50
	BW30009	Old <i>ΔrcsB</i>	25	6.3	50
	BW28357	New WT	50	6.3	50
	BW30009	New <i>ΔrcsB</i>	25/50*	6.3	50
Richter <i>et al.</i>	BW28357	WT	12.5	12.5	-
	BW30009	<i>ΔrcsB</i>	3.1	6.3	-
McDade <i>et al.</i>	BW28357	WT	25	3.1	-
	BW30009	<i>ΔrcsB</i>	25	3.1	-
Chen <i>et al.</i>	DH300	WT	50	1.6	25
	DH311	<i>ΔrcsB</i>	50	1.6	25
Laubacher & Ades	DH300	WT	-	-	50
	DH311	<i>ΔrcsB</i>	-	-	50

cefsulodin and phosphomycin were tested (13-15). However, a consensus has not been reached for the effect of *ΔrcsB* and antibiotic resistance.

A summary of the previous studies is shown in Table 1. Richter *et al.* (14) found that BW28357 (Wild-Type) strain had 4-fold penicillin (12.5μg/mL to 3.1μg/mL) and 2-fold higher phosphomycin (12.5μg/mL to 6.3μg/mL) resistance compared to the BW30009 strain (*ΔrcsB*) in a 96 well minimum inhibitory concentration (MIC) assay. McDade *et al.* (15) repeated the MIC assay developed by Richter. They concluded that the BW28357 and BW30009 strains consistently showed the same MIC for penicillin (25 μg/mL) and phosphomycin (3.1 μg/mL) over multiple

replicates and thus failed to corroborate Richter's findings. Chen *et al.* (13) repeated the MIC assay with DH300 (WT), DH311 (*ΔrcsB*), and BW25113 (*ΔrcsF*) with 4 different cell wall targeted antibiotics, and found no difference in the MIC for any of the four (penicillin 50μg/mL, phosphomycin 1.6μg/mL, cefsulodin 25μg/mL, and ampicillin 6.3μg/mL). However, both Richter and Chen showed that an increase in *rprA* transcription that was dependent on RcsB following sub-MIC penicillin or cefsulodin treatment in the DH300/DH311 strains that contained a *rprA* genomic reporter. As Richter *et al.* and McDade *et al.* used the same strains and methodology, the discrepancy may be because of biological variation, a key mutation resulting in a

phenotypic shift, the lack of an antibiotic resistance marker

allowing contamination, and human error.

We hypothesized that the Rcs pathway is necessary for cell wall antibiotic resistance and that the inconsistent results may have been due to a genetic change that occurred during strain passaging in the laboratory. At some point, different groups may have been testing different

phenotypes. The different growth conditions can affect mutations rates of bacteria (16). For example, carbon starvation of bacteria causes an increase in the rate of mutations to rifampicin resistance (16). To test this, we

ordered a new strain stock and compared its antibiotic resistance phenotype to our laboratory strain stock that was studied previously (13-15). We found that the loss of RcsB did not affect either the MIC or plating efficiency. We propose that there are alternate pathways such as the Conjugative Pilus Expression (Cpx) pathway may enable cell survival in absence of Rcs signaling.

## MATERIALS AND METHODS

**Bacteria strains and source.** *E. coli* strains BW28357 (WT) and BW30009 ( $\Delta rcsB$ ) (Table 2) were from two strain collections. The first source was from the UBC Department of Microbiology collection, hereafter referred to as old strains that were studied by the Richter and McDade groups (14, 15). The second source was from the Coli Genome Stock Centre (Yale), referred to as new strains.

**Preparation of culture medium and antibiotic.** Lysogeny Broth (LB) was made of NaCl 10.0 g/L, yeast extract 5.0 g/L, tryptone 10.0 g/L. LB agar plates (broth with agar 15.0 g/L) were dissolved in water, mixed, and autoclaved. Benzylpenicillin (Sigma), phosphomycin (Sigma-Aldrich), and cefsulodin (Sigma-Aldrich) stocks were prepared at concentrations of 100 mg/mL and filtered through 0.22  $\mu$ m PES filters and stored at -20°C.

**Culture preparation.** All cultures of WT and  $\Delta rcsB$  were grown overnight at 37°C with shaking. Overnight cultures were diluted down, using a conversion of 1 OD<sub>600</sub> to 8x10<sup>8</sup> cells/mL, to approximately 1x10<sup>5</sup> CFU/mL for MIC assays, and 1x10<sup>3</sup> CFU/mL for spot plate assays.

**Primers and PCR.** Colony PCR was performed using the primers described in Table 3. PCR was performed with a thermocycler, with reagents thawed on ice before use. The lid temperature was 105°C and initiated with 3 minutes at 94°C (denaturation). Afterwards, 40 cycles were performed starting with 94°C for 30 seconds (denaturation), followed by 52.6°C for 30

**TABLE 2 Bacterial strains used**

Strain	Designation	Genotype	Resistance
BW28357	Old WT	F; $\Delta(araD-araB)567$ ;	None
	New WT	$\Delta lacZ4787(::rrnB-3)$ ; $\lambda$ ; $\Delta(rhaD-rhaB)568$ ; <i>hsdR514</i>	
BW30009	Old $\Delta rcsB$	F; $\Delta(araD-araB)567$ ;	None
	New $\Delta rcsB$	$\Delta lacZ4787(::rrnB-3)$ ; $\lambda$ ; $\Delta(rhaD-rhaB)568$ ; <i>hsdR514</i> ; <i>RcsB1320</i>	

**TABLE 3 Primers used for colony PCR.**

Name	Sequence	T <sub>m</sub> (°C)	% GC
2ep715-Primer1	GAATTCTAAATCTGGT ACCCGGCAAG	57.6	50
2ep715-Primer2	AAGCTTAACGCGTCTC TTATCTGGCCTA	61.0	50

seconds (annealing), and then 72.0°C for 60 seconds (elongation). The reaction was held at 4°C until frozen or run on a gel.

The PCR mixture components were at concentrations of 0.5  $\mu$ M Primer1, 0.5  $\mu$ M Primer2, 1x

Platinum Taq PCR buffer with no Mg (Invitrogen), 0.2  $\mu$ M dNTPs, 1.5 mM MgCl<sub>2</sub>, along with 0.2  $\mu$ L of Platinum Taq (Invitrogen). Colonies were obtained using an inoculation loop and added to an aliquot of PCR master mix. A pUC19 control PCR was conducted using the same concentrations (different primers) to ensure that reagents behaved in the expected manner.

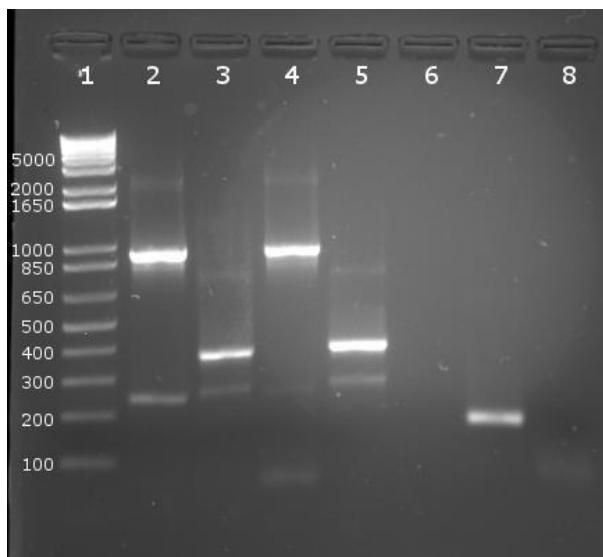
A 2% w/v agarose gel was used for optimal resolution between band sizes. 9  $\mu$ L of sample was mixed with 1  $\mu$ L of loading dye, loaded, and run at 150 voltage for 1 hour.

**MIC assays.** MIC assays were performed in triplicates on 96-well microtitre plates with benzylpenicillin, phosphomycin, or cefsulodin in each well. Antibiotic concentrations started at 100  $\mu$ g/mL in the first column, and serially diluted across each row in 2-fold steps until 0.05  $\mu$ g/mL is reached. 10<sup>3</sup> cells were inoculated into each well. The plates were then incubated overnight at 37°C and wrapped in aluminum foil with no agitation. After incubation, plates were shaken using a plate shaker to resuspend the cells. Each well was then evaluated for visible growth by eye, and read on a plate reader. The MIC is taken to be the lowest concentration of antibiotic necessary to inhibit visible growth and turbidity within a well.

**Plating efficiency spot assays.** LB plates with sub-MIC antibiotic concentrations were prepared as above. 20  $\mu$ L of cells diluted to 1x10<sup>3</sup> CFU/mL were spotted in quadruplicate on a plate. For LB control plates, 300  $\mu$ L of cells diluted to 1x10<sup>2</sup> CFU/mL were spotted in duplicate. Plates were grown overnight at 37°C and colonies counted the next day. Plating efficiency was calculated as the number of actual cells divided by the expected number of cells.

## RESULTS

**Confirmation of deletion of *rscB* coding region with PCR.** We first confirmed the *rscB* genotype of the strains using colony PCR with the same primers used in Richter *et al.* The expected band sizes of 900 bp for the BW28357 WT strain and 350 bp for the BW30009  $\Delta rcsB$  are present in both the new and old strains (Fig. 2). Smaller bands of 250 bp are seen, which may be non-specific products from colony PCR. This presence of 250 bp bands is consistent with previous studies (14, 15). The bands smaller than 100 bp are likely primer dimers, since this band is present in the primers-only control.



**FIG. 2** 2% agarose gel of PCR amplification of the *rcsB* gene in New and Old strains. Lane 1 = 1 Kb Plus DNA Ladder; Lane 2 = WT new; Lane 3 =  $\Delta rcsB$  new; Lane 4 = WT old; Lane 5 =  $\Delta rcsB$  old; Lane 6 = WT new, DNA-only control; Lane 7 = PCR reaction Positive control with pUC19; Lane 8 = primers-only control.

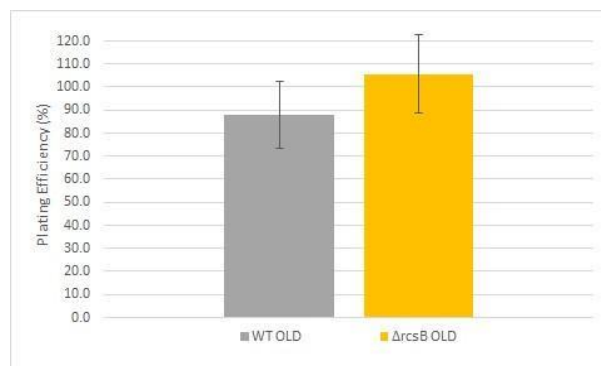
**RcsB is not involved in antibiotic sensitivity to penicillin, phosphomycin, and cefsulodin in MIC assays.**

The requirement of RcsB for antibiotic resistance to penicillin, phosphomycin and cefsulodin was tested using the MIC assay. Wild-type and  $\Delta rcsB$  *E. coli* from old and new strains were used. The observed MICs for penicillin, phosphomycin, and cefsulodin are summarized in Table 1. For both old and new wild-type and  $\Delta rcsB$  strains, no difference was observed for the MIC of phosphomycin (6.3 $\mu$ g/mL) or cefsulodin (50 $\mu$ g/mL). In the two old strains, the penicillin MIC was 25 $\mu$ g/mL. In the new wild-type, the penicillin MIC was 50 $\mu$ g and 25 $\mu$ g/mL in the  $\Delta rcsB$ . We did not observe the Richter *et al.* results of a 4-fold decrease in penicillin resistance and a 2-fold decrease in phosphomycin resistance. Instead, we observed a 2-fold increase in penicillin resistance for the WT new strain compared to the other strains. However, in WT new strain, 8 of 9 replicates agreed on a MIC of 50  $\mu$ g/mL. In the  $\Delta rcsB$  new strain, 5 of 9 replicates show a MIC of 25  $\mu$ g/mL, while 4 of 9 replicates show a MIC of 50  $\mu$ g/mL (Table 1). Due to this variability, the observed difference is not significant enough to conclusively report a loss of antibiotic resistance in the  $\Delta rcsB$  strain. Therefore, MIC results suggest that  $\Delta rcsB$  *E. coli* do not have increased sensitivity to penicillin, phosphomycin, and cefsulodin.

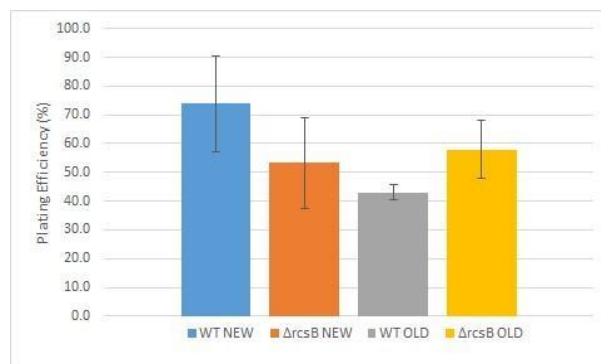
**RcsB is not involved in plating efficiency of sub-MIC penicillin, phosphomycin, and cefsulodin in spot assays.**

To use a more sensitive assay for antibiotic sensitivity, we selected the spot plate assay to test our strains. The advantage of a spot assay lies in its ability to monitor the growth or death of a single cell. In the turbidity based MIC assay, signal could be generated from a few surviving cells that are able to generate turbidity. By spotting known amounts of cells, the spot assay allows the determination of

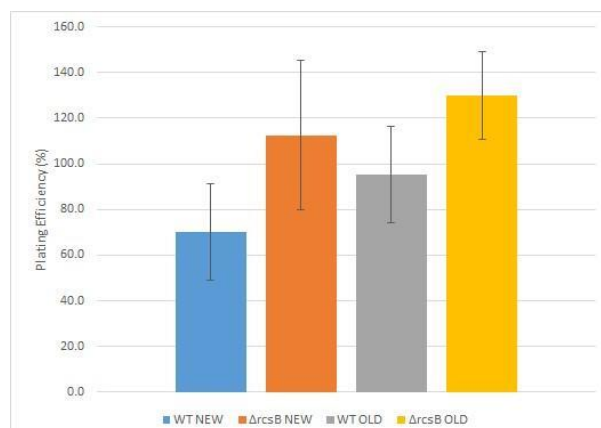
survival of the number of cells spotted, also known as the plating efficiency. Thus via the spot plate assay, the survival of single cells can be determined, whereas the turbidity based MIC method can only monitor entire populations of cells. The spot assay method compares the efficiency of cells plated on plates with sub-MIC levels of antibiotics compared to LB control plates. Results for penicillin, phosphomycin, and cefsulodin are shown in Figures 3-5 respectively. Plating efficiencies varied from 40-110% and



**FIG. 3** Plating efficiency of WT and  $\Delta rcsB$  strains on 25  $\mu$ g/mL of penicillin. n=4 replicates of 20 CFU spots. Error bars represent Standard Deviations.



**FIG. 4** Plating efficiency of WT and  $\Delta rcsB$  strains on 6.3  $\mu$ g/mL of phosphomycin. n=4 replicates of 20 CFU spots. Error bars represent Standard Deviations.



**FIG. 5** Plating efficiency of WT and  $\Delta rcsB$  strains on 45  $\mu$ g/mL of cefsulodin. n=4 replicates of 20 CFU spots. Error bars represent Standard Deviations.

the  $\Delta rcsB$  strains did not have consistently lower efficiencies. Instead, a trend of  $\Delta rcsB$  higher efficiencies than WT was seen in cefsulodin (Fig. 5). However, as indicated by the error bars, there were large standard deviations across our technical replicates, thus the differences between WT and  $\Delta rcsB$  are not reliable. This suggests that deletion of *rcsB* does not have a detrimental effect on the cells.

## DISCUSSION

The Rcs ESR has been shown to be activated in response to antibiotic stress and is thought to promote bacterial survival through intrinsic resistance mechanisms. We tested whether the loss of RcsB, the downstream transcription factor would increase bacterial susceptibility to penicillin, phosphomycin, and cefsulodin. Our results show there is no difference in resistance between the wild-type and  $\Delta rcsB$  in both MIC and spot assays. Possible explanations for our results include the formation of localized biofilms, differences in antibiotic effectiveness in liquid versus solids, and the potential compensatory effect provided by other ESRs such as the Cpx pathway.

**Limitation of MIC assay due to potential biofilm formation.** The MIC values for penicillin and phosphomycin were found to be 25-50  $\mu\text{g/mL}$  and 6.3  $\mu\text{g/mL}$ , respectively. These values are comparable to McDade *et al* and Chen *et al*, but not Richter *et al*. (Table 1). We observed in our MIC assays that wells containing phosphomycin can lack turbid growth yet still have localized spots of bacterial growth. These spots of growth were observed in both WT and  $\Delta rcsB$ . Without careful visual inspection, these spots are easy to miss. A plate reader would not detect the small colonies unless if it is directly in the light path. Rapidly shaking the plates on a plate shaker does not dislodge or shear these spots. This suggests that these spots of growth may be biofilms. However, to confirm if the cluster colonies represent biofilm, further examination via the crystal violet method for biofilm formation is required (17). Since this observation was not reported by Richter *et al.*, it is possible that their observed 2-fold decrease in antibiotic resistance was actually due to this localized biofilm formation causing the cell population to be undetected by the plate reader, and not due to the change in antibiotic sensitivity. During our initial observations, careful visual inspections of the plate were not performed, and a plate reader was used to measure the  $\text{OD}_{600}$  of each well. When measured using only a plate reader, we observed a possible 2-fold decrease in phosphomycin. However, after revisiting the assay we noticed that the plate reader fails to detect these spots of bacterial growth. We then corrected our data to reflect these spots, and found that there was no difference in phosphomycin resistance between WT and  $\Delta rcsB$ .

**MIC values are different and depends on the determination method.** In our plating efficiency assays we tested various antibiotic concentrations and found that an effective sub-MIC to use would be the MIC obtained from the earlier MIC assays. This suggests that the MIC between liquid and solid mediums are not the same. In other words, 25  $\mu\text{g/mL}$  of penicillin inhibits visible growth in liquid medium, but 25  $\mu\text{g/mL}$  on solid medium results in visible colonies after an overnight incubation at 37°C. 30  $\mu\text{g/mL}$  of penicillin was necessary to inhibit visible growth on a solid medium. The spot plate and a spread plate method, however, have been shown to be strongly correlated and precise to one another (18).

Another explanation may be difference in sensitivity between the MIC and spot plate assay. A spot plate assay detects total number of colonies grown relative to expected, while a MIC will only detect many or 0 cells, as the rapid replication of 1 surviving *E. coli* cell overnight in a well will cause turbidity. A MIC assay also only determines the MIC between 2-fold changes, whereas the spot plate assay requires an accurate sub-MIC for optimized results. Thus, spot plate assays can be more accurate in determining the MIC, more sensitive to differences in strain sensitivity, and be more robust to errors. The plate reader also only measures turbidity, not spots of growth. Thus the results of a MIC will be inaccurate if experimenters do not visually inspect and detect such errors.

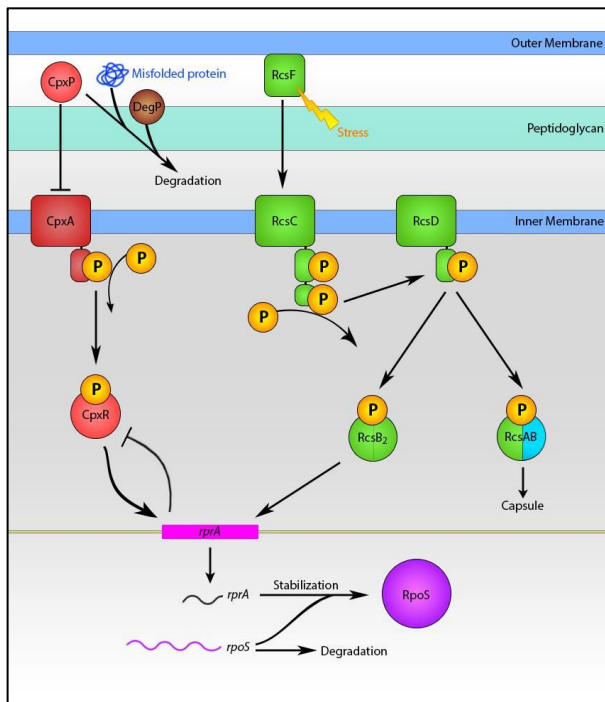
**Diversity of bacteria morphologies may reflect various intrinsic antibiotic resistances.** We noticed there were two distinct colony morphologies on LB plates with phosphomycin. The first phenotype had circular, raised, and opaque-white colonies of 2 mm diameter. The second phenotype had circular, flat, colourless colonies of 1 mm diameter. However, streaking out the colonies on plain LB plates revealed that it is not the result of contamination, but that they are in fact the same strain. A previous study has shown that more resistant cells would form larger opaque colonies and less resistant cells form smaller translucent colonies (19). The different colony morphologies found in our study might be associated to phosphomycin resistance variance between *E. coli* cells of the same strain, which requires further investigation.

**The Cpx pathway is proposed as a compensatory response to antibiotic resistance in  $\Delta rcsB$  strain.** As shown by Laubacher & Ades (12), the knockout of sigma factor *rpoS* gene, the major regulator of stress response genes, does not affect the sensitivity of *E. coli* to  $\beta$ -lactam antibiotics such as cefsulodin and amdinocillin. This result contrasts to the  $\Delta rcsB$  strain, which was found to be significantly more sensitive to the antibiotics by plating efficiency factors of over 100 times (12). Taken together, these findings suggest that *rprA* or another element signaling between RcsB and RpoS is linked to

antibiotic resistance. A pathway that interacts with *rprA* directly is the Conjugative Pilus Expression (Cpx) (20).

The CpxAR two-component system activates in response to a variety of stresses, including protein misfolding and cell wall stress (21) (Fig. 6). CpxA is a histidine kinase sensor that phosphorylates response regulator CpxR, which in turns upregulates the expression of chaperones and proteases (7). CpxP is a key component of the CpxAR system (22). One function is to negatively regulate CpxA at basal levels (22). The other function is to facilitate degradation of misfolded proteins by protein DegP (22). Thus at normal levels, CpxP serves as a checkpoint to turn off the CpxAR system. Since CpxP is degraded when stress is sensed, the system activates, via CpxA being uninhibited. Laubacher & Ades reported that amdinocillin increases *cpxP* expression (12). CpxR is also necessary for resistance to phosphomycin, a non-β-lactam cell wall synthesis inhibitor (6). Taken together, there is evidence of CpxAR playing a role in β-lactam resistance and other cell wall antibiotics.

There is evidence that supports that CpxAR is linked to the Rcs pathway (Fig. 6). The Cpx pathway has been shown to interact with *rprA* and it has also been shown that *rprA* levels modulate Cpx activity. This relationship



**FIG. 6 The Rcs and Cpx phosphorelay systems, and their link to *rprA*.** CpxP in the periplasmic space normally inhibits CpxA. If CpxP detects misfolded proteins, it will facilitate the degradation of the misfolded protein and itself via DegP. This activates CpxA, which autophosphorylates and phosphorylates CpxR. CpxR activates transcription of *rprA*, but at high levels of *rprA* it negatively regulates CpxR and inhibits CpxA activity. The *rprA* mRNA binds to the promoter region of *rpoS* mRNA to favor its translation.

with *rprA* thus positions the Cpx pathway as a compensatory mechanism for the Rcs pathway. Vogt *et al.* (20) showed through electrophoretic mobility shift assays (EMSA) that CpxR binds to the promoter of *rprA*. In addition, they showed that high levels of *rprA* negatively regulate the overall activity of the Cpx pathway, although the mechanism is unknown. *rprA* is known to be upregulated upon antibiotic stress (11, 12). Thus, the loss of *rscB* may initially decrease *rprA* levels and remove the inhibition of the Cpx pathway. The Cpx pathway then restores *rprA* expression and the wild-type levels of resistance. The WT BW28357 and BW30009  $\Delta rcsB$  strains used in both this study and McDade *et al.* (15), where *rprA* was silenced through loss of RcsB, may have enhanced the Cpx response, helping to restore resistance to near basal levels. *rprA* may act to prevent uncontrolled CpxAR activity by serving as a negative regulator (1, 7). Chen *et al.* (13) showed that using a  $\Delta rcsB$  *rprA::lacZ* reporter mutant will show increased levels of β-galactosidase activity when induced with cefsulodin. However, this result does not consider the fluctuations of *rprA* levels, which can have a half-life of 7 minutes (23). Thus high levels of *rprA* is likely not maintained throughout the stress response. *rprA* levels may fluctuate to adjust the CpxAR response to various antibiotic concentrations. Thus the Cpx pathway is proposed as a compensatory pathway for the Rcs pathway.

Our results demonstrated RcsB is not required for antibiotic resistance to penicillin, phosphomycin, or cefsulodin in BW28357 (WT) and BW30009 strains ( $\Delta rcsB$ ). We postulate that the CpxAR system plays a role in restoring β-lactam resistance and that *rprA* is involved. Future experimental findings with a  $\Delta rprA$  model will clarify the role of the Rcs and Cpx pathways in intrinsic antibiotic resistance and show whether *rprA* is a critical player.

## FUTURE DIRECTIONS

The key weakness of the spot plate method is that there is currently no standardized procedure with regards to factors such as statistical significance, dilutions, and replicates (24). To account for biological variation, the experiment must be repeated extensively, and many replicates of the spots should be plated. We have established a standard operating procedure for the plating efficiency assay however that needs further optimization. Several factors need to be determined, such as the effect of the amount of cells plated, volume plated, number of plated spots, incubation times for colony size, incubation temperature, and the type of culture media used. Especially important is the number of biological replicates that need to be performed to obtain low standard deviations and significant results. Each batch should be normalized by the actual amount of cells plated determined by the positive control, which should differ only by plating on a non-antibiotic plate. If there is a significant difference in the plating

efficiency between the strains, then amdinocillin, phosphomycin, and other antibiotics should be tested in order to further compare to Laubacher & Ades, Richter *et al.*, McDade *et al.*, and Chen *et al.* (12-15).

A useful experiment that will determine if accrued mutations over time is responsible for inconsistent results is to sequence the entire Rcs pathway/regulon for all tested strains. The generated genotypes can be compared to the reference *E. coli* genome to determine if any point mutations have occurred. This would generate more information than colony PCR for *rscB*, as the inconsistent results may be due to a mutation of a key residue in the wild type, such as one of the phospho-residues.

It will also be interesting to investigate the role of interaction of CpxP and *rprA* in antibiotic resistance. Since the regulator CpxR interacts with *rprA* (20), the next step is to determine the plating efficiency of a  $\Delta rprA$  strain compared to a wild-type when stressed with cefsulodin and amdinocillin. An increased efficiency in the mutant strain will support the idea that *rprA* is a negative regulator for antibiotic responses. qPCR measurement of the mRNA level of *cpxR* under  $\beta$ -lactam antibiotics treatment will investigate if the CpxAR pathway is involved in antibiotic stress response. Determining the RNA levels of *rprA* at different time points of  $\beta$ -lactam antibiotic stress by qPCR will be useful in understanding if *rprA* is a checkpoint negative regulator of the CpxAR system.

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