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RcsB-Deficient *Escherichia coli* K-12 Do Not Exhibit Decreased Intrinsic Resistance Towards Antibiotics That Target the Cell Wall

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Understanding intrinsic antibiotic resistance is crucial in face of the many challenges arising in therapeutic antibiotic use. The protein RcsB in the regulator of capsule synthesis pathway has been suggested to be a key player in intrinsic resistance in *Escherichia coli* towards antibiotics that target cell wall synthesis. We compared the resistance of RcsB knockout strains versus wild type strains of *Escherichia coli* towards various antibiotics using minimum inhibitory concentration assays. Contrary to our expectations, no difference in antibiotic resistance was observed between the two strains, including towards penicillin. We further validated these results with plate assays and disk-diffusion assays. Additionally, we began preliminary investigation into why the RcsB knockout strain did not exhibit previously observed penicillin susceptibility. Through additional minimum inhibitory concentration assays, we determined that neither differing growth phases nor varying inoculum concentration explained our results. Other factors such as biofilm formation or compensatory signaling through alternative pathways may have contributed to resistance in the RcsB knockout strain instead.

Antibiotic resistance is a constantly growing challenge in the treatment of microbial infections. From the first successful treatment of a streptococcal infection with penicillin in 1942, antibiotics have been a crucial aspect of medicine (1). Two types of antibiotic resistance are acquired and intrinsic. Unlike acquired antibiotic resistance, intrinsic resistance is not acquired through horizontal gene transfer. It is instead universally found in the genome of an organism (3). Mechanisms of intrinsic resistance include efflux pumps that remove the antibiotics, or the production of enzymes which are able to inactivate specific antibiotics among many others (2).

The mechanism of interest, because of its possible involvement in intrinsic antibiotic resistance, is the regulator of capsule synthesis (Rcs) phosphorelay system that is found in most *Enterobacteriacaea*, including *E. coli* (5). The Rcs phosphorelay system is mainly involved in the production of colanic acid, which is vital for the formation of biofilms (5). The phosphorelay system is composed of multiple components beginning from the RcsF and RcsC sensor kinases. When the sensor kinases are activated and phosphorylated at their respective histidine residuals, RcsD functions as a phosphorelay that transfers the phosphate group to the aspartate residue of the response regulator RcsB. RcsB then proceeds to influence the expression of various downstream genes (5). RcsB is the protein of interest in the phosphorelay system as prior research suggests its involvement in intrinsic resistance towards multiple antibiotics such as penicillin, phosphomycin, cefsulodin and amdinocillin (6,7), which are all involved in PG-synthesis. Some of the downstream genes regulated by RcsB, such as *ydhA* and *osmB* may directly contribute to intrinsic resistance. YdhA is a membrane-bound lysozyme inhibitor of C-type lysozyme (4). As lysozyme actively disrupts peptidoglycan (PG) via hydrolysis, its inhibition may lead to increased cell wall stability (4). YdhA may therefore indirectly confer increased resistance towards antibiotics like penicillin, which also destabilize the PG layer. On the other hand, OsmB is an osmotically induced outer membrane lipoprotein that is also controlled by RcsB (21). Previous studies have indicated a possible role of OsmB in the cross-linking of the outer membrane to the PG in response to hyperosmotic conditions (22). Protoplasts, or cells lacking cell walls are highly susceptible to lysis under hyperosmotic conditions (23). As such, in cells which have penicillin-compromised cell walls, OsmB may provide protection against cell lysis and death due to osmotic conditions, thus increasing cell survivability. As RcsB regulates these proteins, it may have a significant role in the intrinsic resistance towards PG-targeting antibiotics.

In this study, we evaluated the importance of RcsB in *E. coli* intrinsic resistance towards various antibiotics, specifically ones targeting the peptidoglycan (PG). Our intention was to further investigate downstream genes that are regulated by RcsB. As we hypothesize that RcsB is a key player in *E. coli* intrinsic resistance, we expect the Δ*rcsB* strain of *E. coli* to demonstrate increased susceptibility to PG-targeting antibiotics. Antibiotic resistance was evaluated using minimum inhibitory concentration (MIC) assays, plating assays and disc-diffusion assays. Contrary to our expectations, our data show that there is no difference in antibiotic susceptibility between the wild-type and Δ*rcsB* strains of *E. coli*. The results suggest that RcsB is not essential for intrinsic resistance towards PG-targeting antibiotics like penicillin. These surprising observations prompt questions about the reliability and specificity of the assays used, as well as biological factors of *E. coli* that could have contributed to these results.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The two strains used in this study are *E. coli* K-12 BW23857 (7) and BW30009 (7).

TABLE 1 Summary of 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	(1)
BW30009	*ΔrcsB*	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)	(1)

BW23857 is referred to as wildtype (WT) and BW30009 is desginated as the isogenic *rcsB* deletion mutant (Δ*rcsB*). Both strains were grown overnight in Luria Bertani (LB) broth at 37°C with agitation before use. The relevant genotypes of these strains are summarized in Table 1.

Antibiotic stock solutions. Concentrated stock solutions of penicillin-G, phosphomycin, streptomycin, and tetracycline were made from powder provided by Sigma-Aldrich. All antibiotic stock solutions that were diluted in dH_2O were sterilized using a 0.22 µm nitrocellulose filter. The tetracycline stock solution was diluted in 70% ethanol. All stock solutions were stored at -20°C until use.

Oligonucleotide primer design. The oligonucleotide primers for polymerase chain reactions (PCR) used in this study (Table 2) were designed to be complementary to regions 100 bp upstream and downstream of the *E. coli* K-12 *rcsB* gene. Both forward and reverse primers were designed using Primer3 software (19).

Colony PCR and agarose gel electrophoresis. PCR thermocycling was carried out in the BioRad T100™ Thermal Cycler. After initial denaturation at 95°C for 3 minutes, the PCR was carried out with the following protocol: 105°C (lid temperature), 95°C for 1 minute (denaturation), 54.8°C for 30 seconds (annealing), and 72°C for 1 minute (extension) for 35 cycles, followed by a final extension at 72°C for 5 minutes. The PCR reaction mix was set up with 5 µL 10X Platinum *Taq* buffer -Mg (Invitrogen), 1 µL 10mM dNTP, 2 µL 50 mM $MgSO_4$, 0.25 µL Platinum *Taq* polymerase (Invitrogen), 38.75 µL of sterile dH_2O, and 1 µL each of *rcsB* forward and reverse primers. A single plate-isolated colony from each respective strain was transferred into their respective reaction mix with a sterile pipette tip. 2 µL of PCR products were mixed with 10 µL of 6X loading dye for a total of 12 µL, then loaded onto a 1% (w/v) agarose gel supplemented with 0.01% (v/v) SYBRSafe DNA Gel Stain (Invitrogen). Electrophoresis was ran at 175V for 1 hour in 1X TAE buffer and visualized with the Alpha Innotech AlphaImager.

TABLE 2 Primer oligonucleotides used to amplify *rcsB* in this study

Description	Sequence (5'-3')	Tm (°C)	% GC
rcsB-F	GCTGGAACATCT GATTCGTG	59.25	50.00
rcsB-R	GAATCGTAGGCC GGATAAGG	60.78	50.00

Growth curves. Overnight cultures of WT and Δ*rcsB* strains were diluted (1:100) with sterile LB in Klett flasks to a final volume of 50 mL. The flasks were incubated at 37°C on a rotator set at 150 RPM. The turbidity was measured in Klett units (K.U.) using the Klett-Summerson Photoelectric Colorimeter at 30-minute intervals from 0 to 1.5 hours, then at 15-minute intervals from 1.5 to 5 hours as the cultures entered log phase growth.

Antibiotic microdilution MIC assay. OD_{600} measurements were obtained for overnight cultures using the Ultrospec 3000 UV-Vis spectrophotometer. Overnight cultures were diluted in LB to a final concentration of 10^4 to 10^5 cfu/mL unless otherwise stated. 100 µL of LB was dispensed into all wells of a 96-well polypropylene plate. 100 µL of 2X antibiotics (penicillin = 1600 µg/mL, phosphomycin = 1600 µg/mL, streptomycin = 100 µg/mL, tetracycline = 100 µg/mL) was added to the first column of each plate and mixed with a multichannel pipette. Two-fold serial dilutions were created in columns 1 to 10 by pipetting 100 µL from the first column into the next. 100 µL were discarded after diluting the solution into column 10. Column 11 was the positive control and contained no antibiotics. 5 µL of diluted overnight cultures were pipetted into each well starting from the column with the lowest antibiotic concentration (column 11) to highest (column 1). Column 12 was the negative control and contained no bacteria. Plates were incubated at 37°C degrees overnight (18 to 24 hours) and inspected visually for bacterial growth. The minimum inhibitory concentration (MIC) was taken as the lowest concentration where growth was not observed.

Plating colonies at sub-MIC. Overnight cultures were diluted to approximately 10^4 cfu/mL and 10^3 cfu/mL. Diluted cultures were spread plated onto LB agar containing 0 µg/mL, 12.5 µg/mL or 20.0 µg/mL of penicillin. These concentrations are below the MIC as determined using the microtitre broth dilution assay, such that strains are able to grow on the plate though not as well when plated on LB. Plates were incubated overnight (18 to 24 hours) at 37°C degrees and were counted for colony forming units (CFU). An increase in the number of CFU indicates reduced susceptibility, and a decrease indicates increased susceptibility.

Disk diffusion assay. Disk diffusion assays to evaluate differences in antibiotic resistance in WT and *rcsB* strains were performed using a modified Kirby-Bauer protocol (20). Strains were grown overnight and were diluted to approximately 1 OD_{600}. 100 µL of diluted cultures were spread-plated onto LB-agar plates to obtain a confluent lawn. Antibiotic disks (7mm diameter) containing penicillin, ampicillin, erythromycin, and polymyxin B (AB-Biodisks) supplied by the Department of Microbiology and Immunology at UBC were placed onto LB-agar plates using sterile forceps. Plates were incubated at 37°C overnight and zones of inhibition were measured in millimeters. An increase in the zone of inhibition indicates increased susceptibility, and a decrease indicates reduced susceptibility.

RESULTS

Characterization of BW30009. PCR to amplify the *rcsB* gene was done to confirm the identity of BW28357 (WT) and BW30009 (Δ*rcsB*) strains used in this study (Fig. 1). Oligonucleotides were designed to flank the *rcsB* gene (Table 2). As expected, the PCR product from WT showed a band size around 800 to 900 kb. A smaller band at ~200 bp was observed in amplification products from Δ*rcsB*, indicating a ~600 bp gene deletion which is in agreement with previous findings (7). Laubacher and Ades have reported that Δ*rcsB* mutants grew as well as WT when plated on LB alone. Therefore, we expected similar growth rates when growing the strains in LB broth. When

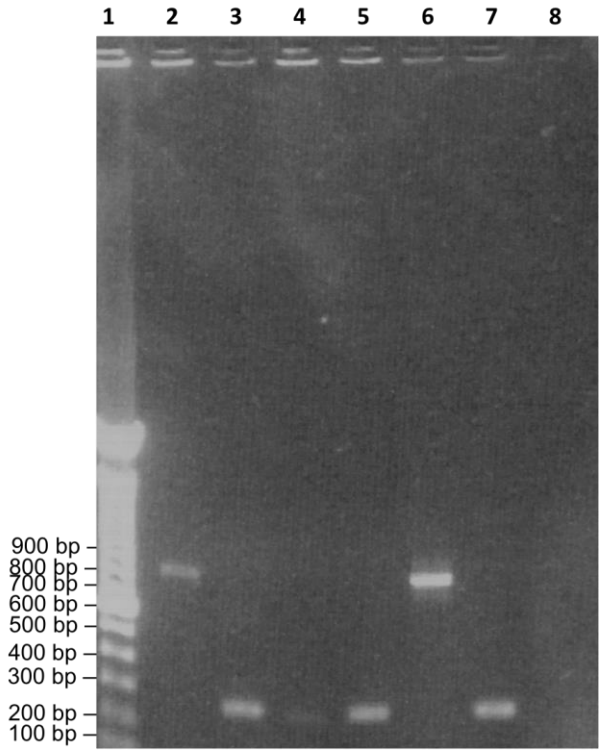

FIG. 1 1% agarose gel with PRC products of *rcsB* amplification ran at 175V for 60 minutes. Lane 1: GeneRuler 100 bp DNA Ladder. Lane 2: WT PCR product. Lane 3: WT PCR control. Lane 4: *ΔrcsB* PCR product. Lane 5: *ΔrcsB* PCR control. Lane 6-7: primer controls. Lane 8: negative control.

comparing the growth curves of WT and Δ*rcsB*, both strains entered the exponential growth phase at nearly the same time, and continued growth at comparable rates (Fig. 2). Based on these results, we concluded that the *E. coli* strains that we acquired were indeed WT and Δ*rcsB*.

Microbroth dilution assays reveal no difference in antibiotic resistance between WT and Δ*rcsB* strains. Previous findings reported that RcsB-deficient *E.* coli showed decreased antibiotic resistance towards penicillin and phosphomycin, but not to streptomycin and tetracycline (7). WT strains were able to grow up to 12.5 μg/mL of penicillin and phosphomycin, as compared to 3.1 μg/mL of penicillin and 6.5 μg/mL of phosphomycin in Δ*rcsB* strains

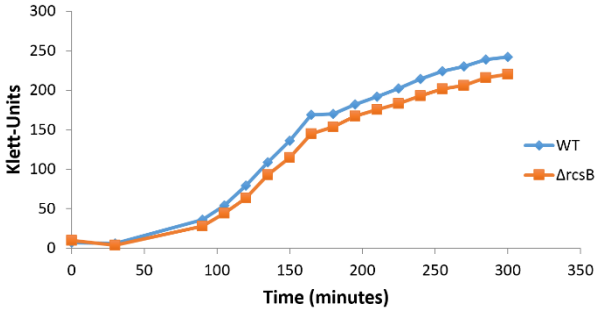

FIG. 2 Bacterial growth curves of WT and Δ*rcsB* strains in LB broth over 5 hours. Single cultures (n=1) of WT and Δ*rcsB* strains were incubated at 37°C with agitation and measured for turbidity using Klett units.

(7). We attempted to repeat the results reported by Richter *et al.* by using performing MIC assays using the microbroth dilution method in this study, and results are summarized in Table S1.

In our experiments, both WT and Δ*rcsB* strains were able to grow up to 25 μg/mL of penicillin, but were only able to grow in the positive control for phosphomycin and not at the lowest antibiotic concentration (1.6 μg/mL). Both strains grew up to 3.1 μg/mL of streptomycin and 0.8 μg/mL of tetracycline. Our results did not show the 4-fold decrease in penicillin resistance and 2-fold decrease in phosphomycin resistance, as observed by Richter *et al.*. These results contradict our hypothesis that RcsB is vital for intrinsic resistance towards PG-targeting antibiotics.

Although the precise activating signals are unknown, the Rcs phosphorelay system is involved in biofilm formation (6). The volume of overnight cultures may have allowed WT and Δ*rcsB* strains to reach stationary phase earlier and establish a biofilm at the bottom of the plate, thereby masking the differences in resistance. To investigate this, we inoculated 2 mL or 5 mL of LB for overnight cultures and compared MIC results between WT and Δ*rcsB* strains. Although both strains grew to a higher density in the 5 mL overnight culture, there were no differences in resistance towards penicillin, streptomycin, and tetracycline (Table S1). Furthermore, neither strain was able to grow even in the lowest concentration of phosphomycin (Table S1).

To confirm that growth phase had no effect in differential antibiotic resistance in the Δ*rcsB* strain, we inoculated MIC assays with either diluted overnight culture, or overnight culture that was first transferred into fresh LB media and incubated until the exponential growth phase. Again, there was no difference in MIC between WT and Δ*rcsB* strains, hence confirming that growth phase did not affect intrinsic antibiotic resistance (Table S1).

Increased inoculum can potentially lead to an increase in the MIC especially if the bacterium is capable of producing an enzyme that destroys the antibiotic (5). To interrogate the effects of inoculum size on the MIC assay results, we inoculated serial dilutions of the WT and Δ*rcsB* overnight cultures into wells containing 2-fold serial dilutions of penicillin. The MIC for WT and Δ*rcsB* were the same at all concentrations of inoculum tested (Fig. 3), demonstrating that it did not impact levels of intrinsic antibiotic resistance.

Collectively, our findings suggest that the growth phase that cultures are in have no effect on the MIC results, and that there is no difference between WT and Δ*rcsB* resistance towards penicillin, streptomycin, and tetracycline. Furthermore, these strains show high sensitivity towards phosphomycin.

There is no difference in the number of CFU between WT and Δ*rcsB* strains when plated with sub-MIC penicillin. The frequency of cells capable of surviving antibiotic treatment can be measured by spread-plating diluted cultures of WT and Δ*rcsB* onto LB-agar plates containing sub-MIC antibiotics. Previous MIC assays showed that WT and Δ*rcsB* were able to grow in up to 25 μg/mL of penicillin in most replicates (Table S1); therefore, we chose 20 μg/mL as the highest concentration since the

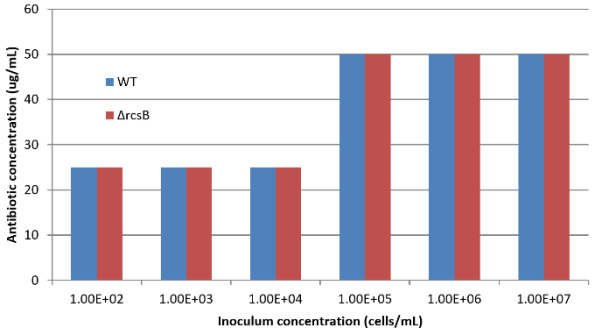

FIG. 3 Highest concentration of antibiotics with observed turbidity (n=2). WT or Δ*rcsB* were diluted to various concentrations, and then inoculated into an antibiotic microdilution MIC assay in duplicate (n=2). Plates were incubated overnight at 37°C. Turbidity was assessed visually.

effective antibiotic concentration in broth may be different in agar. We spread diluted cultures of WT and Δ*rcsB* on plates containing 0, 12.5 or 20 µg/mL of penicillin. After overnight incubation at 37°C, we observed no significant difference (LB: p=0.91, 12.5µg/mL: p=0.19, 25µg/mL: p=0.08) in the number of colony forming units between WT and Δ*rcsB* strains (Fig. 4), contrary to other studies that demonstrated a decrease in plating efficiency when cultures were grown at sub-MIC of cefsulodin (6). Interestingly, there did not appear to be a significant decrease in CFU when WT and Δ*rcsB* strains were plated in the presence of antibiotics (Fig. 4). These findings confirm our MIC assay results demonstrating no differential antibiotic resistance between the two strains.

There are no differences in zones of inhibition between WT and Δ*rcsB* strains. We also surveyed antibiotic resistance by comparing zones of inhibition towards a panel of antibiotics (penicillin, ampicillin, polymyxin B, and erythromycin) using a modified Kirby-Bauer protocol to compare zones of inhibition between WT and *ΔrcsB* strain. Consistent with our previous results using broth-based MIC assays and plating efficiency, there were no zones of inhibition around the penicillin disk for both WT and ΔrcsB (Fig. S1). The only two antibiotics that WT and *ΔrcsB* strains showed sensitivity towards were ampicillin (10 mm)

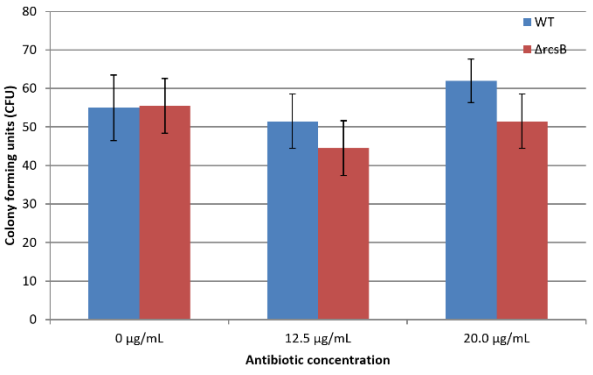

FIG. 4 Mean number (n=2) of colony forming units for WT and *ΔrcsB* strains when plated on various concentrations of penicillin. 100 µL of overnight cultures diluted to around 10^2 cells/mL were spread onto each plate.

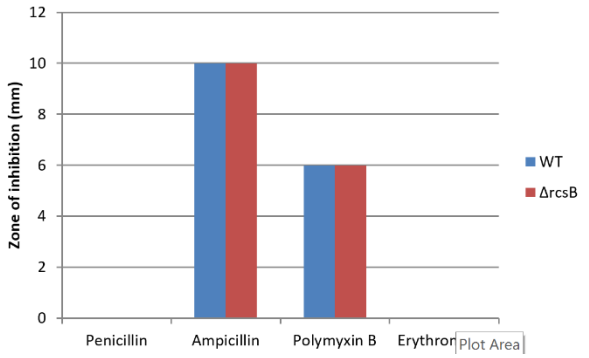

FIG. 5 Mean diameters of Inhibition zones (n=2) of WT and *ΔrcsB* strains when exposed to antibiotics.

and polymyxin B (6 mm), however there were no differences in the diameter of inhibition zones between the two strains (Fig. 5). These results provide further confirmation that intrinsic resistance towards PG-targeting antibiotics does not differ between the two strains.

DISCUSSION

The RcsB protein is a vital component of the Rcs phosphorelay system and is suspected to be a key player in *E. coli* intrinsic resistance towards PG-targeting antibiotics like penicillin. The study by Richter *et al.* supports RcsB's role in the aforementioned intrinsic resistance by demonstrating that Δ*rcsB* mutants were more susceptible to penicillin and phosphomycin through MIC assays (7). Surprisingly, we were unable to replicate the results of Richter *et al.*. Our MIC results instead show that there is no difference in antibiotic susceptibility to the same antibiotics.

A possible explanation for the lack of difference between the two strains is the stage of the growth curve that the bacteria are in when used for the minimum inhibitory concentration (MIC) assay. Antibiotic susceptibility has been correlated with the state of growth, namely exponential growth versus the stationary phase (8). In one study, *E. coli* K12 strain MG1655 was exposed to inhibitory concentrations of four classes of antibiotics in exponential or stationary phase of growth. Bacteria in the stationary phase were much less susceptible to the addition of antibiotics compared to their counterparts in the exponential growth phase (8). Although the Richter group also prepared overnight cultures for MIC assay use, the exact growth time was unspecified and may therefore differ from our study. To evaluate the possible impact of growth phase variability, we performed the MIC assay with inoculum from both exponential phase and stationary phase bacteria. However, the results from the assay demonstrated no difference in antibiotic resistance between the two groups. The growth phase of the *E. coli* does not seem to be the reason behind the contradicting data.

In broth-based methods, the presence of a single cell that can survive antibiotic treatment has the capacity to

colonize and cause turbidity. A larger inoculum size would have a greater chance of including bacteria that could survive antibiotic treatment, and can also increase the MIC if the bacterium produces enzymes that inactivates the antibiotic (18). On the other hand, an inoculum size that is too small can yield false-susceptible results (18). We made use of differing dilutions of our overnight inoculum in order to examine if the quantity of plated cells obscured any difference between the WT and Δ*rcsB* strains. However, the MIC results revealed no difference in antibiotic susceptibility between the two strains. As such, differences in inoculum concentration do not explain our MIC results either.

One factor in our study that was not thoroughly considered is the formation of biofilms. A biofilm is a structure composed of surface-associated bacterial cells enclosed in an extracellular matrix (ECM) made up of polysaccharides, proteins and DNA (9). Studies have shown that bacterial growth is stratified within the biofilm. The stratification occurs due to the different availabilities of oxygen at the surface compared to the center of the biofilm (11). Due to the slow growth rates at the center of the biofilm, classes of antibiotics such as β-lactams that target active, dividing cells are ineffective at killing the bacteria (10). The formation of biofilms in our experiments, especially in the Δ*rcsB* strain, may have compensated for its increased susceptibility to antibiotics. In all our antibiotic resistance assays, the possibility of biofilm formation is present. In the plate assays and disc-diffusion assays, the cells have been plated onto an agar surface and it is therefore possible for the cells to have formed a biofilm. In the broth based MIC assay, although the cells are grown in LB broth, the microtitre plate was not shaken during overnight incubation. The lack of shaking during overnight growth may have allowed cells to settle to the bottom of the wells and adhere to the plastic. However, as these conditions also apply to Richter et al.'s experiments, biofilm formation cannot be a definitive candidate for our differences in antibiotic susceptibility.

A more likely explanation is the inherent variability of broth based MIC assay results. The broth-based method not only allows the possibility of biofilm formations as discussed, but it is also prone to human error due to the extensive antibiotic and inoculum preparation required (17). However, the reproducibility of our results indicate that this is a minor, if not trivial, contribution to the lack of difference in antibiotic resistance between our control and subject samples. What we did omit to include is the greater variation in inoculum size, incubation time and growth medium best used to determine MIC values and the variations that exist between our MIC assay and Richter et al.'s. (17).

Our second MIC method, the agar disk-diffusion method, also has its limitation in testing changes in antibiotic resistance. For instance, there is no way of quantifying how much antibiotic has diffused into the agar (17). In addition, unless applying a second layer of lower percentage agar with bacteria uniformly mixed in, it is unlikely that any volume of inoculum can be spread equally across the surface of the plate. In this case, inoculum size, incubation time and growth medium also contribute to the accuracy and efficacy of the MIC test. Overall, it is also important to consider whether the antibiotics, regardless of methodology, have a bacteriostatic or bactericidal effect on our target strains.

Other than the Rcs phosphorelay pathway, there are other pathways known to be involved in envelope stress response including the Cpx two-component system. While the Rcs phosphorelay pathway is known to sense peptidoglycan stress, the Cpx pathway is responsible for envelope stress activated by heightened pH levels, alteration of inner membrane lipid composition and misfolded periplasmic proteins (12, 13). More recently, research has shown that the Cpx two-component system is also responsible for modification of proteins involved in peptidoglycan cross-linking (14). Under beta-lactam-induced stress, the expression of *cpxP*, a core precursor gene of the Cpx pathway, is induced. *cpxP* is regulated in a manner independent of the Rcs pathway; therefore, it is possible that antibiotic resistance to beta-lactams was compensated by the Cpx pathway despite deletion of *rcsB*.

The sigma E pathway and the BaeSR pathway are other forms of envelope stress response. Sigma E can be activated by elevated temperatures, ethanol and misfolding of proteins in the outer membrane while BaeSR is broadly activated by spheroplast formation, regulating the spheroplast protein Y (Spy) in conjunction with the Cpx pathway to trigger envelope biogenesis (12, 15, 16). As both stress response pathways are independent of the Rcs pathway, their activation could compensate for the loss of *rcsB* much like the Cpx pathway. For instance, overexpression is known to specifically activate the sigma E pathway (12). As OsmB is a downstream outer membrane protein of the Rcs regulon, deletion of *rcsB* will affect OsmB protein levels, leading to activation of the sigma E pathway. However, activation of sigma E via underexpression of proteins has not been examined and will require further exploration.

With pathways independent of Rcs that are able to respond to peptidoglycan disruption and consequently spheroplast formation, it can be speculated that these pathways are capable of conferring intrinsic resistance towards PG-targeting antibiotics. The activity of these pathways may serve to compensate for the loss of *rcsB* in our study to rescue intrinsic resistance. Their exact roles in the presence of antibiotic treatment require further elucidation.

In conclusion, we were unable to identify any differences in intrinsic resistance towards PG-targeting antibiotics between the Δ*rcsB* and wild-type strains of *E.*

coli. Variables such as overnight culture volume, bacterial growth phase, inoculum size, and inherent limitations of MIC assays were investigated and did not appear to contribute to our MIC results. Based on these observations, *rcsB* does not appear to be essential for *E. coli* intrinsic resistance towards penicillin and phosphomycin.

FUTURE DIRECTIONS

Following up on our study, further efforts to investigate the experimental setup should be made. For example, the sensitivity of the broth based MIC method is still in question. The sensitivity of the assay can be investigated by using certain *E. coli* strains with well-established MIC values such as those documented by Sulavik *et al.* (21). In our study, we used doubling increments of antibiotic concentration across the 96-well plate. However, the use of *E. coli* strains with known MICs allows for the use of smaller increments to investigate the sensitivity of the assay.

An interesting observation from our MIC assays was the very high susceptibility towards phosphomycin in both the wild-type and Δ*rcsB* strains (Table S1). This observation runs contrary to Richter *et al.*'s observation of intrinsic phosphomycin resistance up to 12.5 µg/mL for the wild-type and 6.3 µg/mL for the Δ*rcsB* mutant (7). We did not investigate this discrepancy in our study and focused mainly on penicillin. However, it would be interesting to determine the reason behind the difference in results.

As discussed previously, there are multiple pathways in *E. coli* such as the Cpx and sigma E pathways that may compensate for the loss of *rcsB* when challenged with a PG-targeting antibiotic. A subsequent study could investigate whether these pathways are upregulated when exposed to a PG-targeting antibiotic such as penicillin. Expression of proteins related to these pathways could be evaluated at the mRNA or protein level using RT-PCR or western blotting respectively. Furthermore, if a candidate gene is found, a double knockout using a Δ*rcsB* background could be produced to evaluate its importance in intrinsic resistance. Studying other pathways involved in intrinsic resistance as well as their cross-talk with each other allows for the development of a more complex and complete picture of the underlying mechanisms.

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