RcsB Does Not Have a Temperature-Dependent Protective Role for *Escherichia coli* K-12 Against T7 Bacteriophage Lysis

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SUMMARY The Regulator of Capsule Synthesis (Rcs) phosphorelay system in *Escherichia coli* is a complex thermally regulated two component signal transduction system involved in many cellular functions including capsule production. An overproduction of capsule could prevent phage absorption and offer protection to *E. coli* from bacteriophage lysis. In this study, we aimed to examine the role of *rcsB*, a response regulator of the Rcs pathway, in protecting *E. coli* K-12 against T7 bacteriophage lysis by comparing the growth curves of a wild type strain (DH300) and a *rcsB* knockout strain (DH311) at 25°C, 30°C and 37°C. Growth measurements showed no differences in lysis rates between the wild type and *rcsB* knockout strains at 37°C. However, the *rcsB* KO strain showed delayed T7 bacteriophage lysis compared to the wild type strain at 25°C and 30°C, with a greater effect observed at 25°C. These data suggest that *rcsB* may not have a temperature-dependent protective role in *Escherichia coli* K-12 against T7 bacteriophage lysis.

INTRODUCTION

The Regulator of Capsule Synthesis (Rcs) phosphorelay system is a complex two component signal transduction system in many *Enterobacteriaceae* species of Gram-negative bacteria. The Rcs pathway is thermally regulated and is involved in many cellular functions including the expression of the capsular polysaccharide synthesis (*cps*) operon (1, 2). The *cps* operon is responsible for the production of colanic acid, a major component of *E. coli* K-12 capsule (2). It has been reported that with the overexpression of *rcsA*, a member of the Rcs phosphorelay system, *E. coli* strain K-12 becomes resistant to various T7 bacteriophage strains including D104/LG37, LG30, HS33, and WT T7 (3). This is presumably because the overproduction of the host capsule prevents phage absorption (3). Other members of the Rcs phosphorelay include *rcsB* (a response regulator), *rcsC* (a histidine kinase), *rcsD* (a phospho-transfer protein) and *rcsF* (a signal transducer) as displayed in **Figure 1** (2). To date, there have not been any studies that specifically examine the potential roles of these other *rcs* genes in T7 bacteriophage infection.

Among these *rcs* genes, *rcsB* plays a critical role as a regulator for gene expression. RcsB forms a homodimer or a heterodimer with RcsA and then binds to the RcsAB box to enhance *cps* transcription (2). It has been reported that deletion of *rcsB* in *E.coli* K-92 strain (an *E. coli* strain capable of capsule synthesis) results in reduction of colanic acid synthesis at 19°C. This study also showed that RcsB controls the expression of other genes (*dsrA*, *rfaH*, *h-ns* and *slyA*) involved in thermoregulation of colanic acid production (2). Since expression of *rcsA* impacts production of colanic acid in both K-12 and K-92 strains at 19°C (2, 3), and can influence lysis rate against T7 bacteriophage, similar expectation is held for *rcsB* in K-12. Therefore, in this study, we aimed to investigate whether *rcsB* has a protective role in *E. coli* K-12 against T7 bacteriophage lysis by infecting both wild type strain and *ΔrcsB E. coli* strain at different temperatures (25°C, 30°C and 37°C). We hypothesize that there will be no difference in T7 lysis rate between the strains at 37°C, since the pathway should be inactive; and as temperature decreases, pathway will be activated to produce more colanic acid, leading to delayed lysis in WT strain as compared to *rcsB* KO strain. We expect to see greater delay at 25°C as compared with 30°C. Our model is visualized in **Figure 2.**

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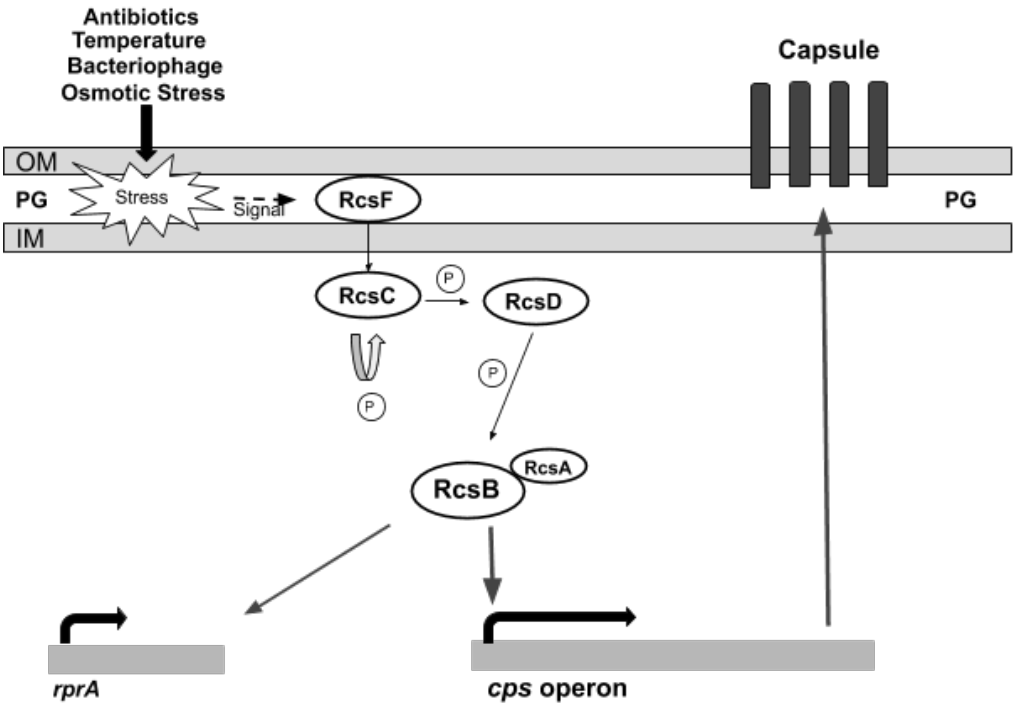

FIG. 1 Proposed model of capsule synthesis in the Rcs Phosphorelay Pathway in *E. coli* K-12 (1). Sensor kinase RcsF senses stress and damage to the peptidoglycan layer which initiates the phosphorelay pathway. Downstream signalling results in phosphorylation of the response regulator RcsB which binds to the promoter region of the *cps* operon and upregulates expression of the operon. Activated RcsB also binds to the *rprA* promoter which upregulates *rprA* synthesis.

METHODS AND MATERIALS

Bacterial strains and source. The two bacterial strains used in this study are summarized in Table 1. The wild-type *E.coli* K-12 reporter strain DH300 (WT) and the Δ*rcsB* strain DH311 were obtained from the MICB 421 course stock collection of the UBC Department of Microbiology and Immunology. The *rcsB* gene in the mutant strain was knocked out using a kanamycin cassette. The strains were maintained at their appropriate growth conditions, sterile lysogeny broth (LB) for WT strain and sterile LB with Kanamycin (50 ug/mL) for KO strain at various temperatures (25°C, 30°C or 37°C) with shaking.

Preparation of media, agar and antibiotic. Lysogeny broth (LB) was prepared with 1% (10.0 g/L) trypticase peptone, 0.5% (5.0 g/L) yeast extract (AMRESCO, Cat. J850), and 0.5% (5.0 g/L) NaCl (VWR, Cat. 7647-14-5) dissolved in distilled water and autoclaved (6). LB agar was made with LB and an addition of 1.5% (15.0 g/L) of Select Agar (Invitrogen, Cat. 30391023) and autoclaved (6). Concentrated stock solution of Kanamycin was prepared by dissolving 50 mg/mL of Kanamycin sulfate (gibco®, Cat. 11815-032) in distilled water then filter sterilized through a 0.22 µm polyethersulfone filter (VWR, Cat. 28145-501) and stored at -20°C.

Table. 1 *E.coli* K-12 strains used in this study (5).

Name	Designation	Genotype and/or description
DH300	Wildtype (WT)	MG1655 Δ(*argF-lac*)U169; *rprA*142-*lacZ*
DH311	Δ*rcsB* (KO)	DH300 *rcsB*::Kan

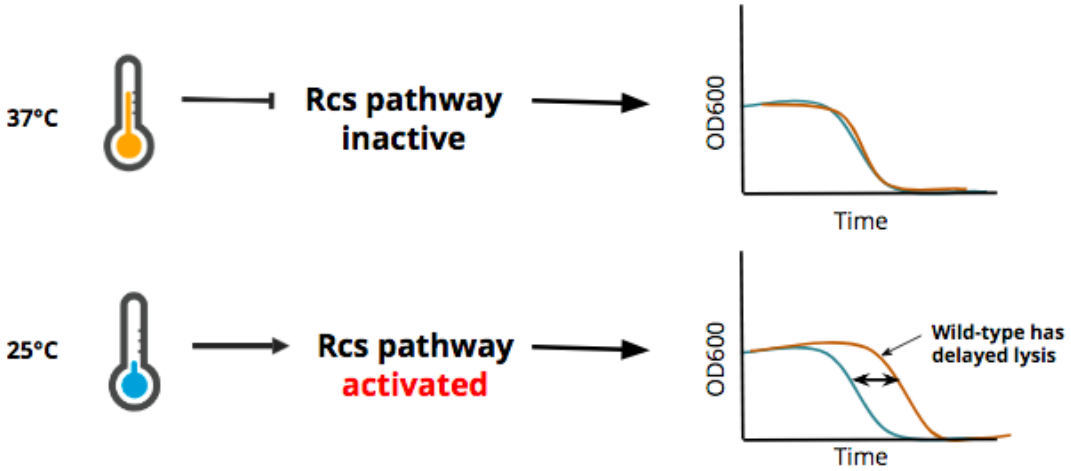

FIG. 2 Model of proposed hypothesis: No difference in T7 bacteriophage lysis rate seen in the strains at 37°C since the Rcs pathway would be inactive. Temperature decrease would delay lysis in WT strain due to Rcs activation.

Primers and PCR. The identities of the bacterial strains and the purity of the T7 bacteriophage stock used in this study were verified using PCR with primers summarized in **Table 2**. PCR was performed using Platinum^{TM} Taq DNA polymerase (Invitrogen, Cat. 10966-018) in a 25µL reaction and the components are outlined in the manufacturer's User Guide with alternation of forward and reverse primers to 0.5 µM each. For DNA template, a single colony was used for colony PCR and 2 µL of bacteriophage stock was used for the virus PCR. The PCR was performed using BioRad T100^{TM} thermal cycler with the following conditions: initial denaturation at 95°C for 10 minutes; 35 cycles of denaturation at 95°C for 1 minute, followed by annealing at 51°C (for virus PCR) or 56°C (for colony PCR) for 1 minute, and extension at 72°C for 1 minute; final extension at 72°C for 5 minutes; infinite hold at 4°C. PCR results were visualized with 1% agarose gel electrophoresis prepared using 1X TAE Buffer and SYBR Safe DNA Gel Stain (Invitrogen, S33102). Gel pictures were taken using Bio-Rad ChemiDoc^{TM} Touch Imaging System. Selected PCR reactions were purified using GeneJet PCR Purification Kit (ThermoFisher, Cat. K0701) and sent to GENEWIZ for Sanger Sequencing.

Bacteriophage Propagation. T7 bacteriophage was propagated using the host *E. coli* DH300. A host culture with optical density at 600 nm wavelength (OD_{600}) between 0.2-0.4 was obtained by diluting an overnight culture. T7 bacteriophage was then added to the DH300 culture at a multiplicity of infection (MOI) of 5 and was incubated at 37°C in a shaker for approximately 5-8 hours, until the lysate became clear. The lysate was centrifuged at 4000 x g for 20 minutes at 4°C for the collection of the supernatant. The phage supernatant was sterilized using a 0.22 µm PES filter. Further cleanup of the phage supernatant was achieved via adding 0.01 volumes of chloroform (Fisher Scientific, Cat. C298), incubating at room temperature for 10 minutes and centrifuging at 4000 x g for 5 minutes at 4°C. The supernatant containing T7 bacteriophage was collected and stored at 4°C for future use (8).

Double Plaque Assay for Titre Determination. A bacterial culture of *E. coli* DH300 with an OD_{600} of approximately 0.5 was prepared by diluting an overnight culture by 100-fold and allowed for growth for about 4 hours on the day of experiment. The phage stock was diluted to achieve the following concentrations: 10^{-3}, 10^{-5},10^{-6},10^{-7}, 10^{-9} before being diluted 10-fold more via mixing with the host culture. The host-phage cultures were incubated for 8 minutes at room temperature and then mixed with warmed top-agar (0.75% LB agar). The mixtures were poured onto bottom agar (1.5% LB agar) and were incubated at 37°C for overnight growth. Plaques were counted the next day and titre was determined with the equation (9): Titre = (Number of Plaques) x (10) x (Dilution Factor).

Table. 2 Primer sequences used in this study for strain verifications (6, 7).

Name	Sequence (5' to 3')	T_m (°C)
rcsB Forward	TGAGAGGACTTGCTAATGAACAATATG	59.0
rcsB Reverse	TTAGTCTTTATCTGCCGGACTTAAGGT	59.4
T7 *gp10* Forward	CGAGGGCTTAGGTACTGC	55.0
T7 *gp10* Reverse	GGTGAGGTGCGGAACTTC	56.1
T4 *gp23* Forward	GCCATTACTGGAAGGTGAAGG	55.7
T4 *gp23* Reverse	TTGGGTGGAATGCTTCTTTAG	52.9
pUC19ΔSKM Forward	CTACATACCTCGCTCTGCTAATC	54.5
pUC19ΔSKM Reverse	CACGCTGTAGGTATCTCAGTTC	54.8

β-galactosidase Assay. 2 sets of 5 mL overnight cultures of DH300 and DH311 were prepared with one set incubated at 20°C and the other set at 37°C. On the day of the experiment, all the overnight cultures from both temperatures were placed on ice for 20 minutes to prevent further bacteria growth prior to obtaining OD_{600} readings. 0.5 mL of each culture was mixed with 0.5 mL of Z-buffer (60mM $Na_2HPO_4•2H_2O$, 40mM $NaH_2PO_4•H_2O$, 10mM KCl, 1mM $MgSO_4$, and 50mM β-mercaptoethanol) (10). 100 µL of chloroform and 50µL of 0.1% SDS solution were added to the culture-Z-buffer mixture and then incubated at room temperature for 5 minutes. 200 µL of ortho-Nitrophenyl-β-galactoside (ONPG) (Sigma-Aldrich, Cat. N1127), a colorimetric and spectrophotometric substrate for detecting the activity of β-galactosidase, was added to each mixture, and incubated in the dark until a yellow colour developed. 0.5 mL of 1M Na_2CO_3 (Fisher Scientific, Cat. S263) was added to stop the reaction with the duration of ONPG incubation recorded. OD readings of wavelengths at 420 nm and 550 nm were obtained and the enzyme unit of β-galactosidase activity was calculated using the following formula (11):
Miller Unit of β-galactosidase activity=(1000*OD_{420}-1.75*OD_{550})/(minutes of reaction*volume (mL) of culture*OD_{600})

Infectivity Assay. Bacteria cultures of *E. coli* DH300 and DH311 at OD_{600} of about 0.5 were obtained by back diluting from an overnight culture and incubated at the desired temperature (25°C, 30°C or 37°C) for the infectivity assay. In a 96-well flat bottom plate (Corning, Cat. 25860), set up technical triplicate of each of the following 4 conditions: DH300, DH300 with T7, DH311, DH311 with T7. 100 µL of culture was pipetted into each well of the corresponding conditions. Using MOI of 0.5, calculated volumes of T7 bacteriophage were then added to DH300 and DH311. OD_{600} readings were collected at 10-minute intervals and recorded using the BioTek Synergy H1 Hybrid Multi-Mode Reader. The averaged OD_{600} of the triplicate for each condition at each time point were used to generate lysis curves (12).

RESULTS

PCR analysis verifies of T7 bacteriophage purity and double overlay plaque assay determines titre. We performed PCR to confirm the purity of our propagated T7 bacteriophage stock. Using primer sets specifically for the T7 bacteriophage (*gp10*) and the T4 bacteriophage (*gp23*), we demonstrated that our bacteriophage stock could only be amplified with T7 specific primers with the expected band size of about 295 bp (**Figure 3**). This verified that T4 bacteriophage is not present in our T7 bacteriophage stock. For the positive control we used the pUC19ΔSKM plasmid as the template and the PCR reaction was completed with *ori* primers which resulted in the appropriate band size at about 189 bp. The positive control confirmed that the PCR setup conditions can successfully produce

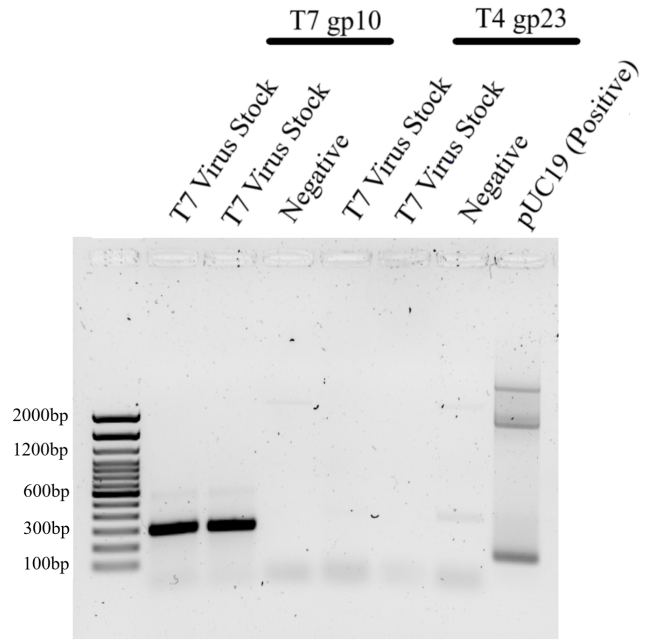

FIG. 3 Amplified PCR products of T7 bacteriophage stock on a 1% agarose gel run at 100V for 1 hour. The PCR analysis yielded a band at 295 bp for the reaction using the T7 primer set. There are no visible bands for the reaction using the T4 primer set and the negative control. The positive control yielded a band at 189 bp.

products. For the negative control we used double distilled water as the template and used the T7 bacteriophage specific primer set (*gp10*) to confirm the absence of non-specific products and primer dimers. No bands are seen for the negative control in **Figure 3**. We performed a double overlay plaque assay to determine the titer of our T7 bacteriophage stock. We calculated our bacteriophage titer to be 3.94 x 10^{10} PFU/mL.

PCR analysis and Sanger sequencing confirms identity of *E. coli* WT and Δ*rcsB* strains. Colony PCR was performed on the wild type (DH300) and the Δ*rcsB* (DH311) strains using primers flanking *rcsB*. PCR amplified products were visualized on a 1% agarose gel (**Figure 4**). Bands of about 650 bp were obtained for both DH300 colonies, as expected, and bands of about 700 bp were obtained for both DH311 colonies. For the positive control, we used the pUC19ΔSKM plasmid as the template with *ori* primers and the PCR reaction resulted in the expected band size at about 189 bp. For the negative control

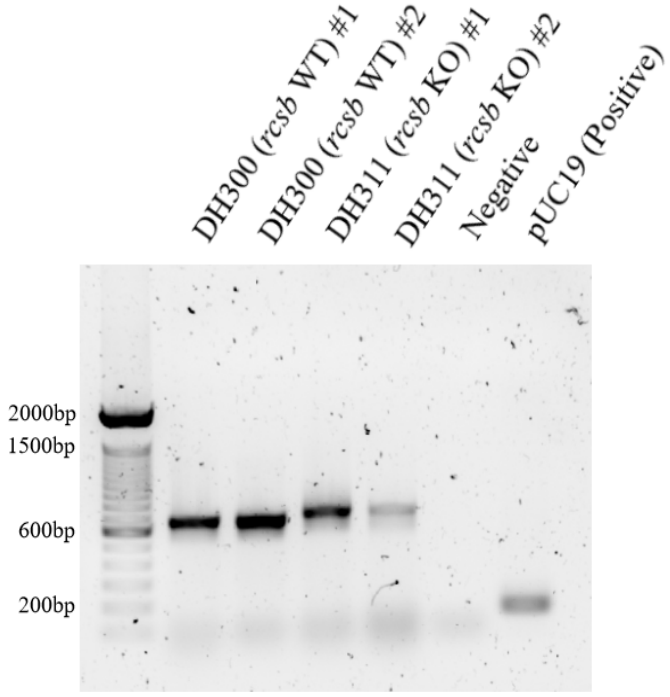

FIG. 4 PCR products of *E. coli* DH300 and DH311 using *rcsB* primers on a 1% agarose gel run at 100V for 1 hour. Lane 1 contains 1000 bp Plus Molecular Weight Ladder. Lanes 2 and 3 contain bands at approximately 650 base pairs. Lane 4 and 5 contain bands around 700 base pairs. Negative control shows no visible bands.

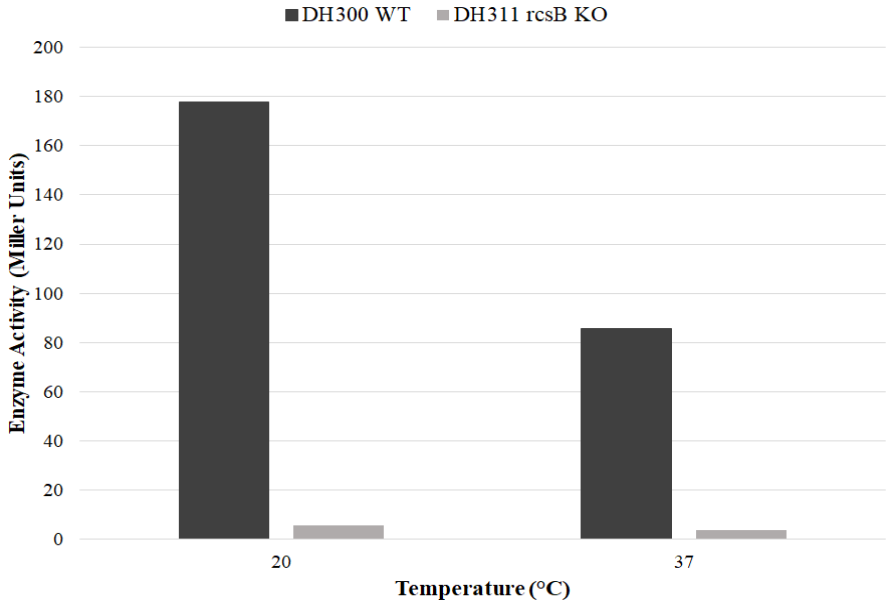

FIG. 5 β-galactosidase assay showing enzyme activity in both WT and *rcsB* strains at 20°C and 37°C (N=1).

we used double distilled water as the template and the primer set flanking *rcsB*. No visible bands were seen in this reaction confirming no primer-dimers and non-specific amplification. The PCR amplicons of both wildtype and knockout strains were sequenced using Sanger sequencing. The results indicated that *rcsB* within the Δ*rcsB* strain was indeed interrupted (data not shown).

β-galactosidase assay functionally verified the knockout in the Δ*rcsB* strain and demonstrated a temperature dependence on the pathway. Since *rprA* expression is downstream of the Rcs pathway, the Δ*rcsB* strain should have no *rprA* synthesis due to the absence of functional *rcsB*. This has been previously determined and reported (13). Our *E. coli* strains (DH300, DH311) have the *rprA* promoter fused with the *lacZ* (13) reporter, thus we conducted a β-galactosidase assay to functionally verify the knockout of *rcsB* in the Δ*rcsB* strain (DH311) used in this study. As expected, we observed low β-galactosidase activity corresponding to low *rprA* promoter activity in the Δ*rcsB* strain but much higher β-galactosidase activity corresponding to higher *rprA* promoter activity in the wildtype strain (**Figure 5**). We also observed that the wildtype strain had two-fold greater β-galactosidase activity at 20°C compared to 37°C. This confirms that the Rcs pathway is upregulated at lower temperatures, as suggested in literature (1, 2).

***E. coli rcsB* strain DH311 shows a delay in lysis compared to wild type DH300 when treated with T7 bacteriophage**. To investigate if *rcsB* has a role in protecting *E. coli* K-12 against T7 bacteriophage lysis and to determine if that role is temperature mediated, we completed infectivity assays. These assays were completed at the established optimal MOI of 0.5 (**Supplementary Figure 1**). At 37°C, both the WT strain and the Δ*rcsB* strain displayed cell lysis at around 40 minutes (**Figure 6C**). Therefore, no delay is seen between the two lysis curves at 37°C. At 30°C, the WT strain displays cell lysis at around 40 minutes while the Δ*rcsB* strain displays cell lysis at around 50 minutes. Here we start to see a delay in cell lysis between the two curves with increased delay for the Δ*rcsB* strain. At 25°C, the WT strain displays cell lysis at around 90 minutes while the Δ*rcsB* strain displays cell lysis at around 120 minutes. At the lowest temperature tested, we found the greatest delay in cell lysis in the Δ*rcsB* strain.

DISCUSSION

With the continuous emergence of antibiotic resistant bacteria strains, using bacteriophage is an attractive method to treat infections. Thus, it is important to study how bacterial pathways interact differently under different environmental conditions and how these changes impact the efficiency of phage-mediated killing. Among these, the Rcs pathway is

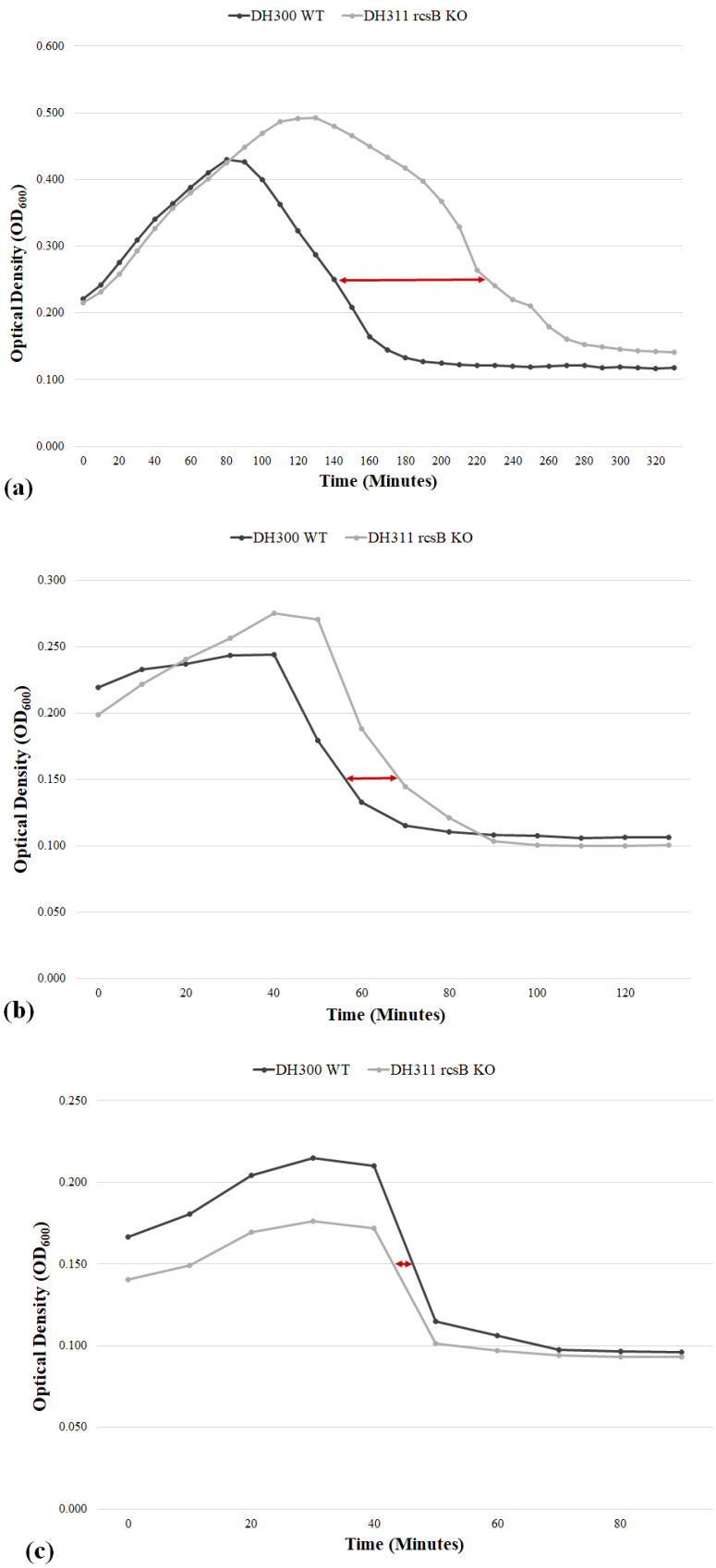

FIG. 6 T7 Infectivity Lysis Curves. Cultures were infected with T7 bacteriophage at a MOI of 0.5 and incubated at the following temperatures (a) 25°C, (b) 30°C, and (c) 37°C. N=1 at each temperature. Black curve indicates DH300 (WT) and light grey curve indicates DH311 (*rcsB* KO). Red arrows to indicate delay between the lysis curves.

for colonic acid production, which is involved in forming bacterial capsules that provide protection to bacteria against phage induced lysis. The pathway is thermally regulated and is strongly induced at lower temperatures (2). Previous studies have shown that overexpression of *rcsA* led to phage resistance, and deletion of *rcsB* led to decreased colanic acid production at lower temperatures (2, 3). However, the exact correlation between whether decreased colanic acid formation in *rcsB* KO leads to increased susceptibility to bacteriophage at lower temperatures compared to WT is unclear.

In this series of experiments we aimed to examine the temperature dependent role of *rcsB* in providing a protective response for *E. coli* against bacteriophage induced lysis. We hypothesized that DH311, an *rcsB* knockout, will be more susceptible to bacteriophage lysis than DH300, the wild type strain, at lower temperatures due to its inability to upregulate the Rcs pathway and increase capsule formation. This difference would be reflected in delayed lysis time of DH300 at 25°C and no difference seen in the lysis rates at 37°C .

First, we wanted to conduct a preliminary study to assess the thermal regulation of the Rcs pathway and functionally verify our bacterial strains WT DH300 and *rcsB* KO DH311. *rprA* is a gene downstream to *rcsB*; In DH300 and DH311, *rprA* is linked to a *lacZ* reporter. Therefore, we used a β-galactosidase assay to examine the activity of *rprA* in DH300 and DH311 under two temperatures, 20°C and 37°C (**Figure 3**). In DH300, we saw that there was a 2-fold increase in *rprA* activity at 20°C compared to 37°C, which showed that *rcsB* and Rcs pathway was indeed upregulated at 20°C due to temperature stress. In addition, we noticed that even at 37°C, *rprA* activity was still present, which could suggest that the Rcs pathway is always turned on, albeit at different levels depending on the temperature. This observation was consistent with previous research that showed activation of the Rcs pathway in normal conditions (14). Consistent with our expectations, DH311 showed negligible *rprA* activity at both temperatures, which suggests that *rcsB* was successfully knocked out and the Rcs pathway was interrupted as a result. Additionally, DH311 was kanamycin resistant and was grown on media with kanamycin during all steps of the study, which further strengthens our confidence in its identity.

We then aimed to determine an optimal multiplicity of infection (MOI) to use for our study. Using an OD-based lysis assay, we inoculated DH300 and DH311 with MOIs ranging from 0.1 to 10 (**Supplementary Figure 1**). Our aim was to identify an MOI that is sufficient to lyse the bacteria within a reasonable time period but avoid overwhelming the cells, and show distinct lysis rates between DH300 and DH311. An MOI of 0.5 gave rise to a clear difference in lysis times between DH300 and DH311 at 25°C, and was used in subsequent experiments.

Using the same OD-based lysis assay, we inoculated log phase DH300 and DH311 with T7 bacteriophage at 0.5 MOI at three different temperatures: 25°C, 30°C, and 37°C. As mentioned earlier, we expected no difference in lysis rates at 37°C between the two strains, and a slower lysis rate in DH300 at 25°C due to the upregulation of the Rcs pathway and increased capsule formation. At 37°C, consistent with our expectation, we observed similar lysis curves between DH300 and DH311, both beginning the lysis at around 40 minutes with similar slopes (**Figure 6C**). At 30°C, DH300 looked similar as 37°C with the drop still occurring at 40 minutes (**Figure 6B**). DH311, on the other hand, showed a delay in lysis rate of around 10 minutes, suggesting more protection of the bacteria against phage attachment and lysis. At 25°C, lysing of DH300 began at 90 minutes while DH311 was delayed to 120 minutes (**Figure 6A**). The lysis curve in DH311 also had a less steep slope compared to that of DH300. All together, the data suggests that at 37°C, DH300 and DH311 have similar susceptibility to T7 bacteriophage. At 25°C, both strains have greater protection against bacteriophage compared to 37 and 30°C, but DH311 has an even greater protection shown by the delay in lysis time compared to DH300 and a flatter slope in the lysis curve. Surprisingly, the results at 30°C and 25°C were almost exactly opposite of what we hypothesized.

Putting together the data from the β-galactosidase assay and the infectivity assay, it seems that the Rcs pathway is indeed upregulated and conferred protection against the WT strain at lower temperatures. However, contrary to our expectation, the absence of the pathway resulted in an even greater protection. In previous research, it has been reported that suppression of Rcs pathway stops thermosensitive growth defect in mutants that cannot

synthesize phospholipids (15). The growth defect was also exclusively observed at higher temperatures, which agrees with our data showing the strains experiencing more protection at 20°C. Although the authors did not know of the mechanism, this data further suggests that Rcs could in fact cause some compromise to bacterial survival in certain conditions (15). In addition, past studies also showed no difference in *rcsB* deletion on antibiotic resistance, but the experiments were only done at 37°C (5, 16). Overall, *rcsB* seems to have conflicting roles in helping bacterial survival; however, temperature does seem to play an important role in both the pathway and the impact on bacterial response to stress.

Conclusions To conclude our study, we confirmed that the Rcs pathway is indeed thermally dependent and upregulated at lower temperatures. Knocking out the Rcs pathway, although hypothesized to render *E. coli* more susceptible to T7 bacteriophage, actually provided more protection against phage-induced lysis. Our results indicated that *rcsB* probably does not have a protective role in *E.coli* K-12 against T7 bacteriophage lysis at lower temperatures.

Future Directions To confirm if the Rcs pathway is upregulated at lower temperatures, qPCR can be performed to detect differential expression of the *cps* operon in the WT *E. coli* strain and the Δ*rcsB* strain at different temperatures. Higher expression of the operon at lower temperatures will further validate the proposed model of increased *E. coli* protection at lower temperatures due to increased capsule synthesis. An alternative method to visualize and compare colanic acid levels would be to perform L-Fucose assay for colanic acid in WT *E. coli* strain and the Δ*rcsB* strain at different temperatures (17).

In addition, studies from previous years examining the Δ*rcsB* strains did not show a difference from WT in protecting *E. coli* against stresses such as antibiotics. However, those studies were only done at 37°C (5, 16). Since the differences between the two strains in our study occurred at lower temperatures, it would be worth pursuing the previous experiments using lower temperatures as well, given that the Rcs pathway does behave differently at lower temperatures and seems to give rise to unexpected phenotypes.

Also, the range of temperatures we used was from 25°C to 37°C. However, in previous literature, there was even greater induction of RcsB at 19°C (2). If equipment permits, it would be ideal to perform the infectivity assay at 20°C to observe whether there is an even more drastic difference in T7 bacteriophage induced lysis.

Lastly, the results show that Δ*rcsB* strain has delayed phage-induced lysis compared to the WT strain at lower temperatures, which is not what we expected. There may be other pathways that compensate for the failure to induce the Rcs pathway, which in turn increases *E. coli* fitness. Conducting a microarray for comparing difference in gene expression of WT and Δ*rcsB* at lower temperatures may help us identify potential pathways that could be involved in this process.

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