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Treatment of *Escherichia coli* K-12 with sub-inhibitory concentrations of antimicrobial agents does not induce RpoS-mediated cross-protection to T7 bacteriophage infection

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SUMMARY The Rcs-phosphorelay pathway is used by *Escherichia coli* to express the alternative sigma factor RpoS in response to extracellular stressors. It has been proposed that RpoS may mediate cross-protection from both antibiotics and bacteriophage infection. Here, we hypothesize that, upon pre-treatment with sub-inhibitory antibiotic concentrations, RpoS induction in *E. coli* can confer additional protection from T7 bacteriophage-mediated lysis. Using Minimum Inhibitory Concentration (MIC) assays, we established the sub-lethal antibiotic concentrations for wild-type (WT), RpoS- and Rcs-deficient strains of *E. coli* K-12. Then, bacteriophage-mediated lysis assays were conducted in *E. coli* K-12, pre-treated with sub-inhibitory antibiotic concentrations. However, we observed no significant delay in bacteriophage T7-mediated cell lysis. Further, using the MIC assay method, we also observed that RpoS deletion results in impaired growth in presence of antibiotics, suggesting that this protein is partially responsible for the intrinsic antibiotic resistance mechanisms. Additionally, we found that prolonged treatment with beta-lactam antibiotics restored cell growth, indicating utilization of beta-lactam-specific resistance mechanisms by *E. coli* K-12. Altogether, our findings suggest that while RpoS is partially responsible for intrinsic resistance to antibiotics in *E. coli* K-12, defence pathways regulated by this protein are not required to induce cross-protection from bacteriophage-mediated cell lysis.

INTRODUCTION

Escherichia coli possesses multiple systems dedicated to stress response and adaptation. Among the numerous strategies, it utilizes the Rcs-phosphorelay pathway to quickly sense extracellular stresses and respond to them by regulating capsule and biofilm synthesis, as well as expression of an alternative sigma factor RpoS (1, 2). RpoS is responsible for regulating gene expression during stationary phase, starvation, as well as exposure to various killing agents, including antibiotics, and previous research demonstrates that RpoS-deficient *E. coli* strains exhibit impaired stress tolerance (2, 3). In addition to RpoS, *E. coli* utilizes other alternative sigma factors, such as RpoH and RpoE, which are essential to induction of stress response pathways upon sensing heat shock and extracellular disruption, respectively (4). Despite the apparent specialization among these proteins, previous studies suggest the possibility of cross-talk between multiple stress response mechanisms, resulting in elevated tolerance towards many seemingly unrelated stressors (5). Additionally, studies by Hardman *et al.* and Li *et al.* indicated that exposure to sub-lethal levels of aminoglycoside and beta-lactam antibiotics can lead to increased tolerance to T7 bacteriophage infection in *E. coli* (6, 7). Another study by Chen *et al.* attempted to elucidate the connection between intrinsic antibiotic resistance in *E. coli* and its regulation by the Rcs-phosphorelay pathway via RpoS, but concluded that the Rcs pathway is non-essential to antibiotic resistance due to the possibility of other cellular mechanisms upstream of RpoS (8).

Here, we hypothesized that the RpoS-mediated stress response of *E. coli*, upregulated upon exposure to antibiotics, can induce cross-protection from bacteriophage infection and, consequently, delay cell lysis. To investigate this, we assessed WT and RpoS-deficient *E. coli* K-12 for discrepancies in times of T7 bacteriophage-mediated lysis onset, following pre-treatment of cells with sub-lethal concentrations of beta-lactam, aminoglycoside and macrolide antibiotics. Further, given the findings of Chen *et al.*, which showed that *E. coli*

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lacking the Rcs-phosphorelay components did not display increased sensitivity to antibiotics, we also hypothesized that Rcs-phosphorelay pathway would not be essential to the cross-protection effect (8). With this, we expected that the two Rcs-deficient strains incorporated in this study – knockouts of the sensor kinase component RcsD, as well as the DNA-binding domain RcsB – would display phenotypes that are identical to the WT strain. Since all previous work involving phage-mediated lysis of cells pre-treated with antimicrobials was performed in an *E. coli* B strain, we designed this study so that we could verify the redundancy of Rcs-phosphorelay in RpoS-induced stress response in *E. coli* K-12, by ensuring that all chosen mutations were present in the same genetic background (9).

METHODS AND MATERIALS

Strains and growth conditions. The following *E. coli* K-12 strains were obtained from The Ramey Culture Collection from the Department of Microbiology and Immunology, University of British Columbia: BW28357(WT), BW28465 (ΔRpoS), BW30009 (ΔRcsB). *E. coli* strain BW29856 (ΔRcsD) was obtained from the Coli Genetic Stock Center, Yale. Cells were routinely cultured using liquid and solid Luria-Bertani (LB) media at 37°C.

Propagation of T4 and T7 bacteriophages. T4 and T7 bacteriophage stocks resuspended in LB broth were obtained from The Ramey Culture Collection from the Department of Microbiology and Immunology, University of British Columbia. Propagation was performed as described (10). In short, WT *E. coli* K-12 was grown in LB broth at 37°C with shaking, until the optical density at a wavelength of 600 nm (OD_{600}) of 1 was achieved. Cells were then inoculated with 1 mL of appropriate phage stock and incubated overnight at 37°C with shaking, as no clearing was observed after 2 hours. The next day, chloroform was added to a final concentration of 1% (v/v) to lyse the cells and release bacteriophage into the media. Cultures were then centrifuged at 2750 g at 4°C for 10 minutes. Supernatant was collected, filtered through 0.22 µm syringe filter and stored at 4°C in Falcon tubes.

Double agar overlay plaque assays. Bacteriophage titer was determined in plaque forming units (PFU) per mL, as previously described (10). In short, 100 µL of T4 and T7 phage stocks serially diluted (10^{-1} through 10^{-10}) in 5 mM $CaCl_2$ were mixed with 100 µL WT *E. coli* K-12, resuspended in LB to OD_{600} of 1, vortexed and incubated at 37°C for 15 minutes without agitation. 3 mL of warm 0.75% (w/v) LB agar was then added to the mixture and poured over the underlay 1.5% (w/v) solidified LB agar. Plates were incubated overnight at 37°C, and plaques were counted the next day.

PCR and agarose gel electrophoresis to confirm T7 bacteriophage purity. Polymerase Chain Reaction (PCR) analysis of phage stock purity was performed as described by Li *et al.* and Fettig *et al.* (7, 10). T4- and T7-specific primers were designed to amplify the *gp23* gene of T4 and the *gp10A* gene of T7 phage, respectively. Exact primers and associated sequences are summarized in Table S1. The PCR reaction consisted of a 1/100 dilution of T7 bacteriophage stock, forward and reverse primers at 0.4 µM each, 1x Taq polymerase (Invitrogen), 1x Taq polymerase buffer (Invitrogen), 1.5 mM $MgCl_2$ and 5% (v/v) DMSO. The Bio-Rad T100™ Thermal Cycler (Bio-Rad Laboratories, Inc.) was programed to run denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds, and repeat for 33 cycles, followed by a final extension at 72°C for 5 minutes. PCR products were then held in the Thermocycler at 4°C overnight. All PCR products and a 100 bp molecular weight ladder were run on a 1.5% (v/w) agarose gel in Tris/Borate/EDTA (TBE) buffer containing SYBR™ Safe DNA Gel Stain (Invitrogen) at 100V for 40 minutes. Bands were visualized using the Gel Doc UV imager.

T4 phage infectivity analysis. Infectivity of T4 bacteriophage was assessed by streaking a lawn of WT *E. coli* K-12 and B23 on solid LB media, which was then inoculated with appropriate phage stock using a stab needle. Plates were incubated overnight at 37°C, and infectivity was confirmed if plaques were observed in the bacterial lawn.

Minimum inhibitory concentrations (MIC) assay. Minimal inhibitory concentration (MIC) assays were conducted to test the following antibiotics: beta-lactams (penicillin, ampicillin), aminoglycosides (kanamycin, gentamicin) and a macrolide (azithromycin). The assays were conducted in 96-well plates, where each well was divided into sections, representing 2-3 different strains by columns, and varying concentrations of antibiotic by row, with 3 technical replicates per each sample. Each horizontal row represented a serial dilution, beginning with the highest concentration for the first row, and ending with the lowest concentration for the last row. Specific antibiotic concentrations were based on MIC values determined by Li *et al.*, where the established MIC value fell within the centre of the dilution series (7). All wells then received 30 µL of an appropriate *E. coli* strain taken from a log phase culture grown in LB at 37°C with agitation for 2-3 hours. Appropriate dilutions of antibiotic stocks, resuspended in LB, were added to each well and topped with sterile LB to a total volume of 100 µL per well. No-antibiotic positive control wells and a no-*E. coli* negative control well were also included. Plate was incubated at 37°C with shaking using the Infinite 200 PRO microplate reader (Tecan), and OD_{600} was measured at regular intervals for a total incubation time of up to 24 hours. Each mutant strain was tested at least once in each antibiotic, and the WT strain was tested at least 3 times in each antibiotic. Data analysis was based on average values for the WT strain and the relative growth patterns for each mutant relative to WT. MIC was taken as the highest antibiotic concentration that produced a relatively flat growth curve with minimal killing or culture growth. Sub-inhibitory concentrations were taken as half of MIC, and low sub-inhibitory concentrations were taken as the concentration of antibiotic at which growth was most similar to no-antibiotic control.

96-well plate phage infectivity assay. Assays were performed as described previously, with slight modifications (7). In short, *E. coli* strains were incubated overnight in LB broth, supplemented with a sub-inhibitory or low sub-inhibitory concentration of one antibiotic, at 37°C with shaking. Overnight cultures were then diluted in plain LB to an OD_{600} of 1. The multiplicity of infection (MOI) of T7 bacteriophage was based on the assumption that an OD_{600} of 1 for *E. coli* is equivalent to $8x10^8$ cfu/mL. The assay was performed in 96 well-plates, where each well received appropriate volumes of *E. coli* and T7 phage to achieve MOI of 0.03 in the final volume of 100 µL. Uninfected and sterile control wells were also used. There were 3 technical replicates per each sample on the plate. Plate was incubated at 37°C with shaking using the Infinite 200 PRO microplate reader (Tecan), and OD_{600} was measured at regular intervals for a total incubation time of up to 4 hours. There was only one biological replicate for both sub-inhibitory and low sub-inhibitory antibiotic concentrations.

RESULTS

Measurement of viral titer of T7 bacteriophage stock. Double agar overlay assay was used to determine the titer of T7 phage, using WT *E. coli* K-12 as the host, based on plaque counts summarized in Table S2. T7 plates yielded a titer of 4.3 x 10^9 PFU/mL and plaques were large and uniform in size and shape, indicating that cross-contamination with T4 phage was unlikely (Fig. S1). However, no plaques were observed on plates containing *E. coli* and T4 bacteriophage, suggesting the possibility of either phage inactivation in the stock or lack of infectivity by T4 in the chosen K-12 strains. An infectivity assay was therefore subsequently performed for the T4 phage.

PCR and agarose gel analysis confirmed T7 bacteriophage identity. PCR was done to confirm the identity and purity of T7 bacteriophage stock. The reactions were run with primers specific for the *gp10A* gene of T7 phage, and the *gp23* gene of T4 bacteriophage, as well as a no-primer control was included. As illustrated in Fig. 1, only the reaction containing both T7 phage stock and the T7-specific primers yielded a bright band at 295 bp (Lane 4), corresponding to the expected size of the *gp10A* amplicon. PCR mixtures lacking primers, but containing T7 bacteriophage template (Lane 2), or the T4-specific primers (Lane 3) were designed as negative controls and produced no bands on the agarose gel. Altogether, these results verify the identity and purity of the T7 bacteriophage stock.

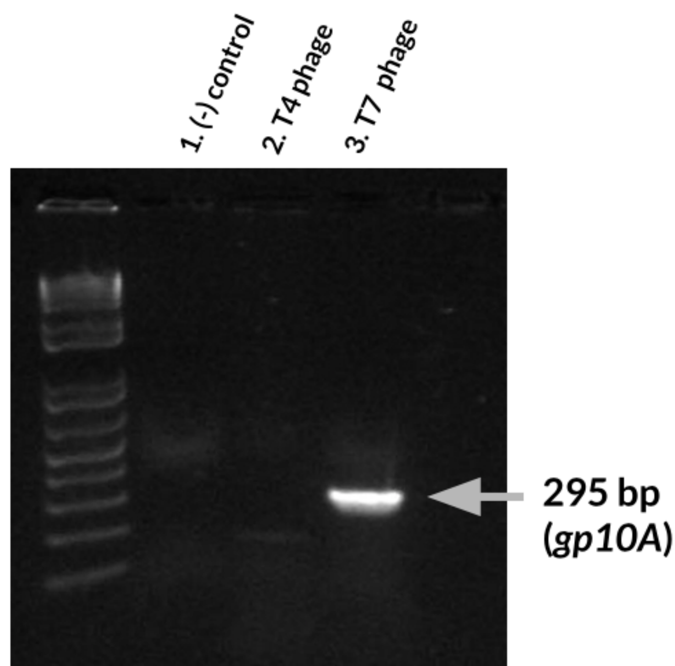

FIG. 1 A 1.5% agarose gel containing PCR products of amplified T7 bacteriophage. Lane 1 is the molecular weight ladder. Lane 2 has no bands and corresponds to the negative control containing T7 bacteriophage template with no primers added to the PCR. Lane 3 also has no bands and represents T4 primers with T7 phage stock as a control to confirm T7 bacteriophage identity. Finally, Lane 4 shows one band at approximately 295 bp corresponding to the targeted region of T7 *gp10A* gene and represents the PCR mixture containing both T7 primers and template DNA.

T4 bacteriophage poorly infects *E. coli* K-12. Since we did not observe clearing of liquid *E. coli* culture or plaques on solid media upon addition of T4 bacteriophage, we speculated that the observed phenomena could arise due to the inability of T4 phage to infect the chosen K-12 strains. To test this, we performed an infectivity assay using *E. coli* K-12 and *E. coli* B23 (a type B strain) with our T4 bacteriophage stock, on solid LB media. While we observed plaques on plates containing both strains, T4 produced much smaller, barely visible plaques in K-12 compared to B23. We therefore decided to exclude T4 phage from all future experiments, as it would complicate our assessment of phage-mediated cell lysis.

RpoS partially mediates intrinsic antibiotic resistance in *E. coli* K-12. A 96-well plate assay was performed to determine the sub-lethal concentrations of beta-lactams (penicillin and ampicillin), aminoglycosides (gentamicin and kanamycin) and a macrolide (azithromycin) for each genotype of *E. coli* K-12. MIC was determined as the concentration at which minimal growth or killing was observed throughout the course of the experiment, represented by a flattened growth curve, and the sub-lethal concentrations were calculated as half MIC. Low sub-inhibitory concentrations were taken as the ones at which growth was most similar to the no-antibiotic control. These established concentrations are further summarized in Table 1. However, azithromycin displayed relatively high working concentration ranges (over 100 ng/mL), compared to all other antibiotics, which complicated our analysis (Figure S2). The azithromycin MIC was therefore not based on an actual tested

Table 1. Summary of MIC and sub-inhibitory antibiotic concentrations established for WT *E. coli* K-12 using the MIC assay.

Antibiotic	MIC (µg/mL)	Half-MIC (µg/mL)	Minimal difference to LB control (µg/mL)
Ampicillin	2	1	1
Penicillin	25	12.5	10
Gentamicin	3	1.5	0.25
Kanamycin	8	4	0.5
Azithromycin	150	75	ND

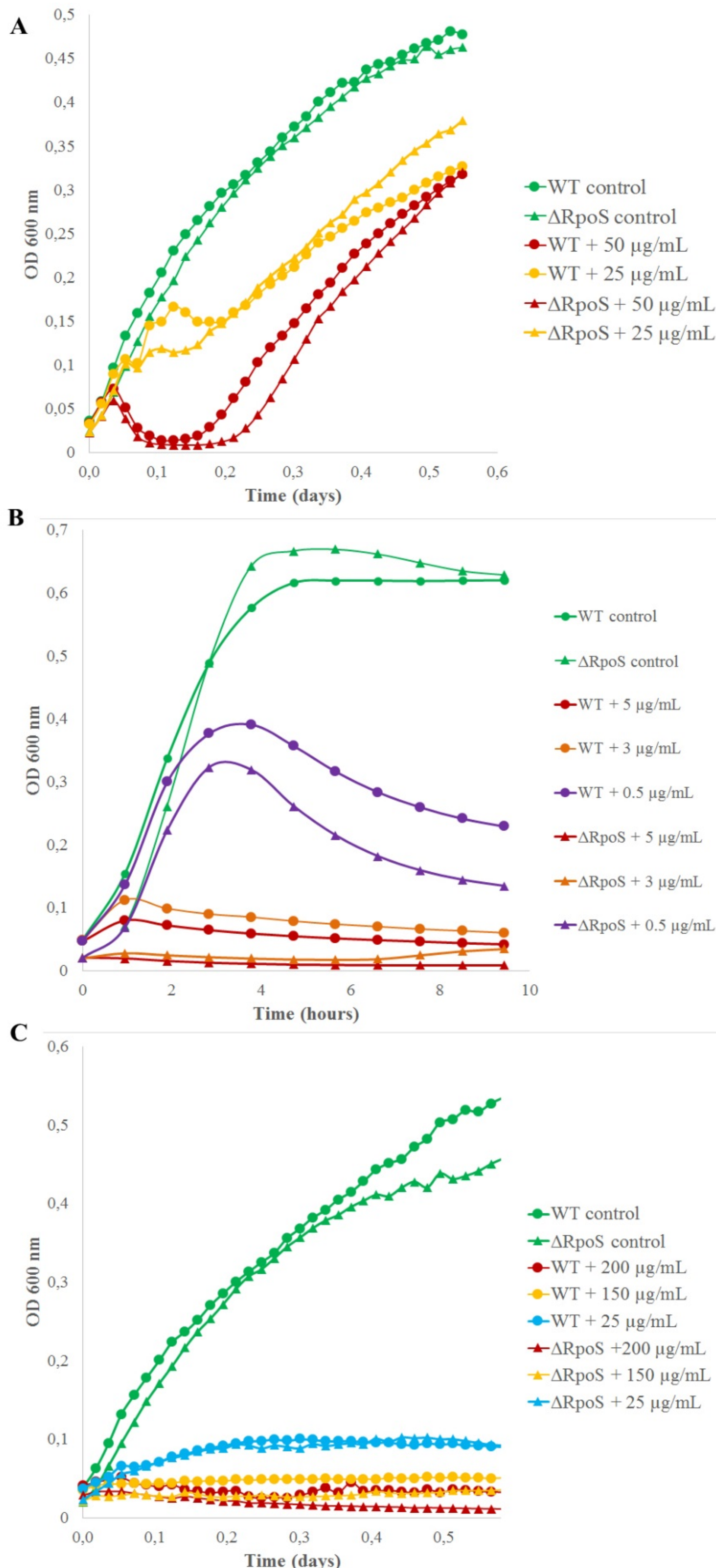

FIG. 2 RpoS partially mediates intrinsic resistance to antibiotics in *E. coli* K-12. Growth of WT and RpoS-deficient *E. coli* K-12 in LB media supplemented with varying concentrations of penicillin, a beta-lactam (A); gentamicin, an aminoglycoside (B); and azithromycin, a macrolide (C). In all cases, deletion of RpoS resulted in consistently impaired growth in presence of antibiotics, but not in plain LB control. Similar patterns were observed in ampicillin and kanamycin.

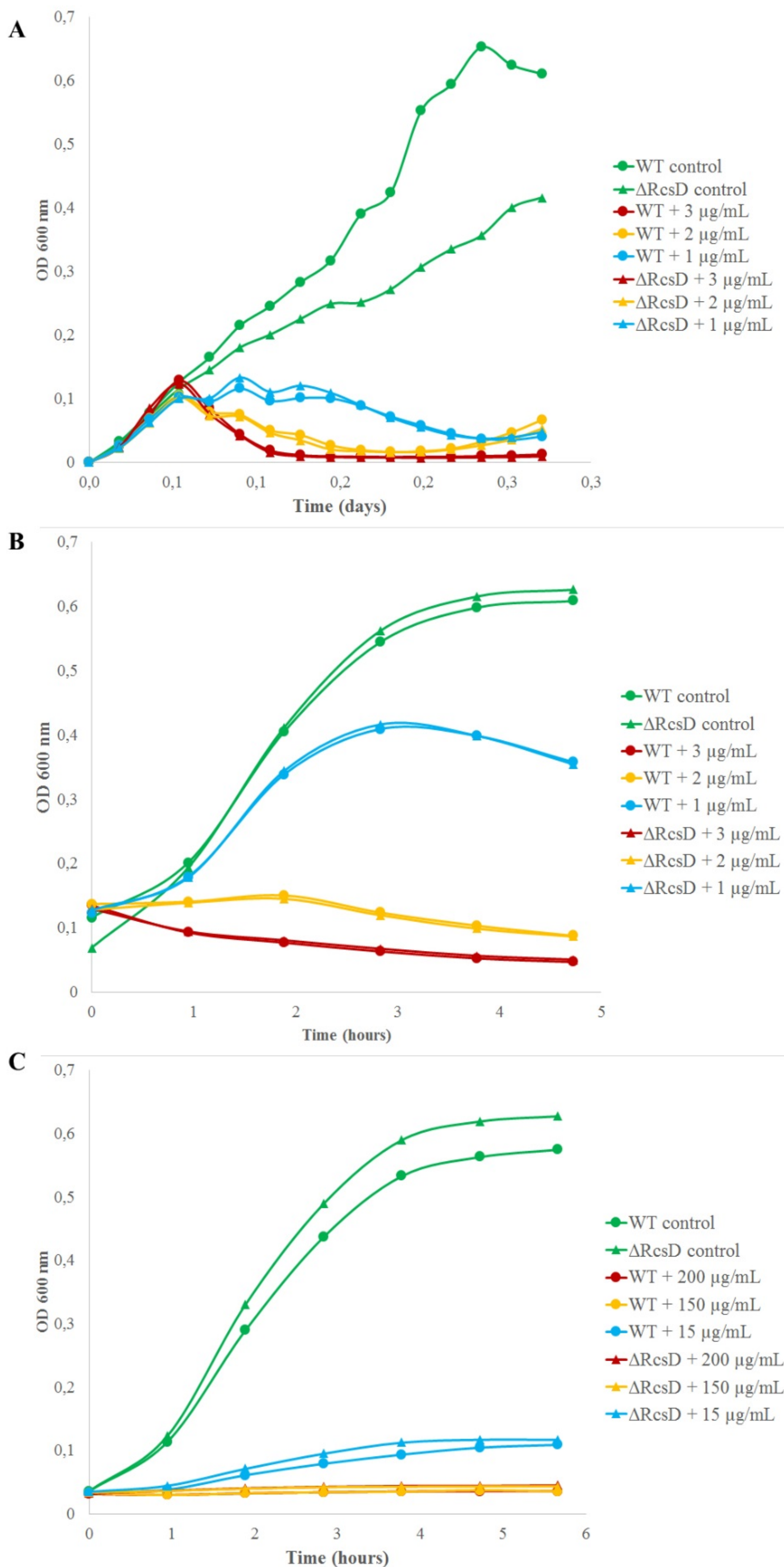

FIG. 3 Rcs-phosphorelay pathway is non-essential to intrinsic antibiotic resistance of *E. coli* K-12. Growth of WT and RcsD-deficient *E. coli* K-12 in LB media supplemented with varying concentrations of ampicillin, a beta-lactam (A); gentamicin, an aminoglycoside (B); and azithromycin, a macrolide (C). In all cases, deletion of the Rcs-phosphorelay response regulator RcsD did not result in any discrepancies in growth when compared to WT, regardless of media type. Same patterns were observed in penicillin and kanamycin, as well as for the RcsB sensor kinase component-deficient strain.

concentration, but was estimated from growth patterns observed at highest and lowest concentrations included in the assay. Since overnight exposure to half-MIC azithromycin resulted in complete killing of cells, suggesting that the chosen value was incorrect, we decided to not use this antibiotic in any future experiments.

Further, we observed that the RpoS-deficient strain of *E. coli* showed slower growth and reached a lower maximum OD_{600} under all antibiotic concentrations compared to WT, but had no observable growth defect in plain LB broth (Fig. 2). While the growth was only slightly impaired, this effect was consistent throughout all trials. Conversely, the Rcs-deficient strains exhibited no differences in growth under any antibiotic concentrations compared to WT (Fig. 3). Collectively, these data suggest that intrinsic antibiotic resistance of *E. coli* K-12 at least partially relies on the alternative sigma factor RpoS, and that the Rcs-phosphorelay pathway is not essential for the observed resistance.

Cells pre-treated with sub-MIC antibiotics showed no delay in lysis by T7 bacteriophage. In the initial experiment where *E. coli* K-12 strains were subject to exposure to half-MIC antibiotics prior to infection with T7 phage, we observed slight discrepancies in the onset of cell lysis, ranging between 5-10 minutes, particularly, in samples treated with kanamycin and gentamicin, as shown in Fig. 5. We hypothesized that the slight but prominent delay in gentamicin-treated cells was due to inhibiting action of the antibiotic, as growth of these cells prior to lysis was also impaired, compared to the other samples. We therefore decided to repeat the experiment using lower antibiotic concentrations at which growth was comparable to plain LB control, as reflected by the MIC assay data. Following this, we observed no growth differences prior to lysis between samples, and no consistent differences in the time of lysis onset (Fig. 5), suggesting that the slight growth impairment by the inhibiting concentration of gentamicin was partially responsible for the apparent initial delay of lysis. However, these delays do not appear significant due to their inconsistent nature throughout the experiments, as well as the fact that we loaded phage samples into the wells containing aminoglycoside-treated cells 5-10 minutes later than the no-antibiotic and beta-lactam-treated samples. Collectively, these data suggest that pre-treatment with sub-inhibitory concentrations of antibiotics has no effect on induction of cell lysis by the T7 bacteriophage.

We also observed impaired growth prior to lysis in all samples containing the RpoS-deficient strain (Fig. S3), further indicating that intrinsic antibiotic resistance mechanisms of *E. coli* K-12 rely on this alternative sigma factor. Further, no differences between cell lysis onset were observed for the RpoS-deficient strain, also suggesting that pretreatment with low concentrations antibiotics does not induce cross-protection from killing by phage.

DISCUSSION

RpoS is known to alter gene expression to confer protective changes in the cell in response to environmental stressors (2). MIC analysis confirmed that RpoS is indeed partially responsible for mediating intrinsic antibiotic resistance. As can be seen in Fig. 2, ΔRpoS strain grew comparably to WT only when cultured in LB control, and displayed impaired growth in presence of antibiotics. This pattern was consistent throughout the experiments and present in all antibiotic treatments, regardless of molecular structure. Additionally, the RcsB and RcsD knockouts displayed phenotypes identical to the WT strain, both in presence and absence of antibiotics (Fig. 3). This observation suggests that, while Rcs-phosphorelay is known to be upstream of RpoS-mediated stress response, it is not required for antibiotic resistance or tolerance (11).

As shown in Fig. 4, prolonged treatment with beta-lactams did not result in complete killing of cells. While the MIC curves showed an initial decrease in cell concentration that reached a plateau within 2-3 hours, growth appeared to resume 2-5 hours later even under the highest concentrations of both penicillin and ampicillin that were tested. While this effect was observable for both beta-lactams, it was more prominent with penicillin. One possible explanation for this could be the initial presence of a small resistant population. Alternatively, resistance may have arisen due to spontaneous mutations or gene duplications over the course of the experiment. Finally, it is possible that the antibiotics were subjected to gradual

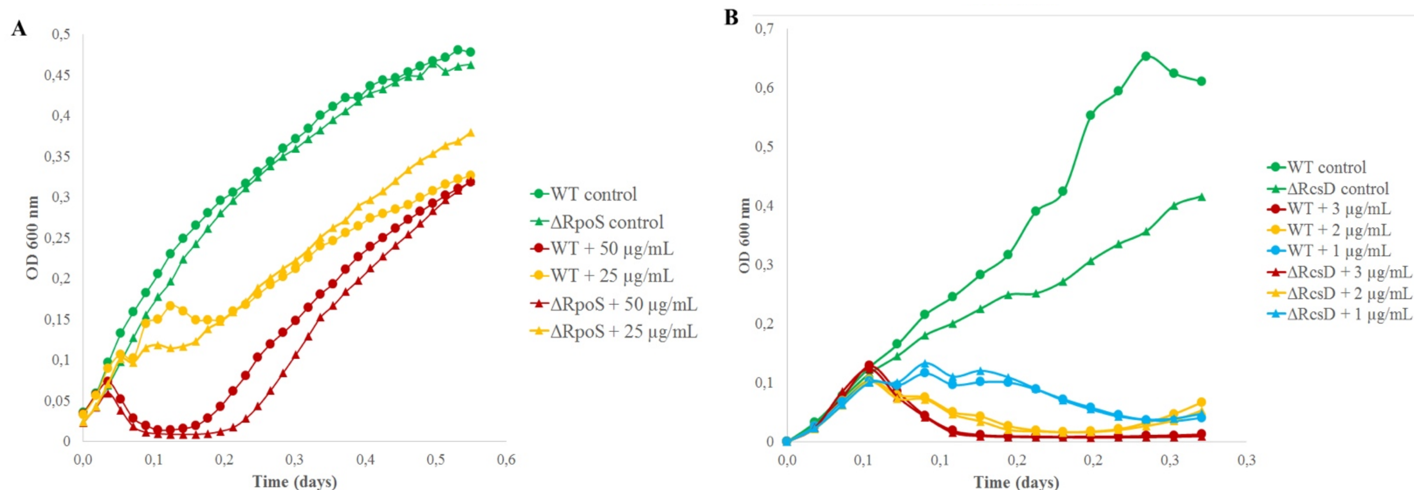

FIG. 4 Prolonged treatment with beta-lactam antibiotics does not result in complete killing of *E. coli* K-12. A) Growth of WT and RpoS-deficient *E. coli* K-12 in LB media supplemented with varying concentrations of penicillin. Following initial killing and a short lag phase, lasting in total for up to 5 hours, cells resumed growth at an exponential rate comparable to that of no-antibiotic control. **B)** Growth of WT and RcsD-deficient *E. coli* K-12 in LB media supplemented with varying concentrations of ampicillin. While some growth resumption was still observed after the initial killing and a brief lag phase, the effect was not as prominent as with penicillin treatments. Although both graphs represent only one biological replicate, the displayed patterns were consistent for all strains throughout the experiment.

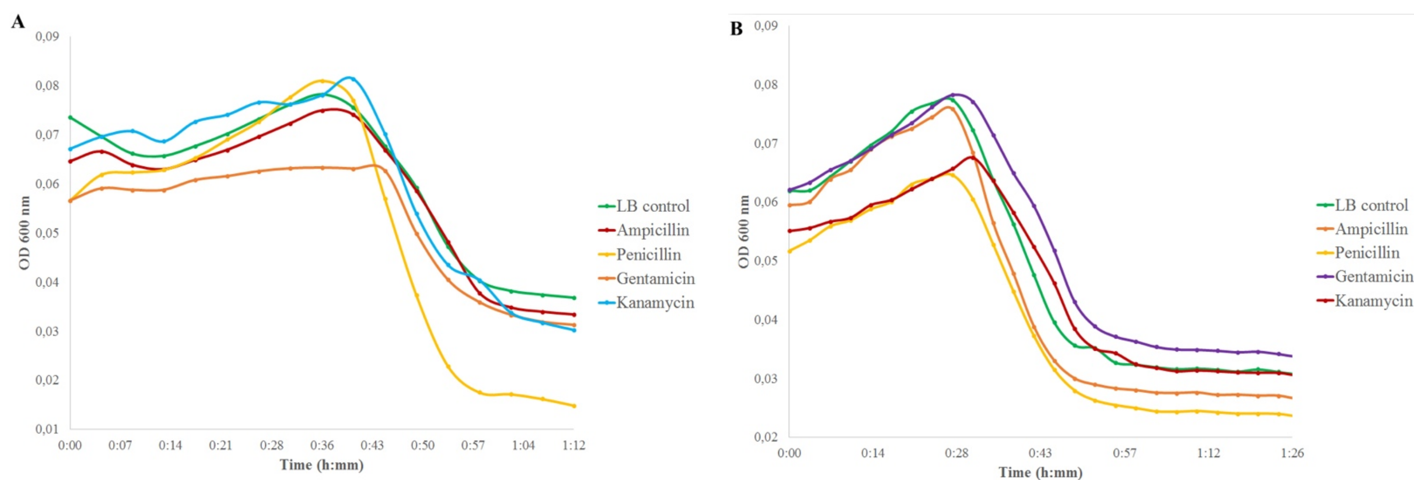

FIG. 5 Pre-treatment of *E. coli* K-12 with sub-inhibitory antibiotic concentrations does not stimulate cross-protection from T7 phage-induced cell lysis. A) T7-mediated cell lysis of WT *E. coli* K-12, grown overnight in presence of half-MIC antibiotics. The observed differences in time of lysis onset are negligible and range between 5-10 min. Due to impaired growth of culture prior to lysis, the chosen concentration of gentamicin appears too high and was corrected for in the subsequent experiment. **B)** T7-mediated cell lysis of WT *E. coli* K-12, grown overnight in presence of antibiotics at low sub-inhibitory concentrations determined by lack of growth impairment compared to the antibiotic-free controls in the MIC assay. No consistent or significant differences in time of lysis onset were observed. Each assay was only performed once.

degradation by beta-lactamases. Beta-lactamases are evolutionary ancient enzymes that are widely distributed among both Gram-negative and Gram-positive bacteria, and many strains of *E. coli* are known to encode and express these enzymes – especially, in presence of antibiotics (12). In this case, the enzymes secreted by *E. coli* would be more effective against penicillin, as resumption of growth was more dramatic in presence of this antibiotic (Fig. 4). The latter could be explained by the difference in structure between the two antibiotics. As such, ampicillin possesses an additional amino group, which allows for efficient penetration through pores in the outer membrane of Gram-negative bacteria (13). This way, ampicillin could penetrate the cell quicker, avoiding degradation by the beta-lactamases in the media.

Further, we found that T4 bacteriophage poorly infects the *E. coli* K-12 strains used in the study, resulting in very small plaques on solid media, and no observable clearance in liquid cultures. Thus, T4 was excluded from further analyses, which meant that there was no additional bacteriophage-driven lysis for comparison. Previous studies reveal examples of T4-resistant *E. coli* K-12, characterized by altered LPS expression. This could be one of the mechanisms behind the observed T4 phage resistance, as LPS is bound by phage tail fibers to facilitate entry (14, 15). However, additional studies are required to devise the exact mechanisms.

Previous studies have used phage-mediated lysis assay to show that exposure to sub-lethal levels of aminoglycoside and beta-lactam antibiotics can lead to increased tolerance to T7 bacteriophage infection in *E. coli* (7). Based on these observations, we explored the idea that reduced T7 phage infection could be attributed to a general response related to antibiotic treatment-induced stress, characterized by increased expression of RpoS (16). Here, we performed a T7 phage-mediated lysis assay using cells pre-treated with sub-MIC antibiotic concentrations. As illustrated in Fig. 5, we observed no effect of antibiotic treatment on susceptibility of *E. coli* to T7 infection.

Initially, upon treatment of cells with half-MIC antibiotics, we saw small discrepancies in the time of lysis onset (Fig. 5A). The roughly 10-minute delay was observed between strains grown in an LB control, and gentamicin and kanamycin. Due to the visibly impaired growth of gentamicin-treated cells prior to lysis, we speculated that slower cell division due to inhibiting action of gentamicin could result in slower progression of bacteriophage infection. Because of this, we performed another lysis assay using cells pre-treated with low sub-inhibitory antibiotic concentrations (Fig. 5B). As expected, we observed that gentamicin-treated cells were no longer impaired in growth and, as a result, showed no consistent or significant delays in time of phage-mediated lysis onset. This suggests that inhibition of cell growth and metabolism by antibiotic agents can indeed hinder phage infection through slower production of progeny viruses, and thus slower cell lysis. However, Li *et al.* observed delayed lysis – although these delays also ranged between 10-15 minutes – even upon treatment with sub-inhibitory concentrations of antibiotics, which were not impairing *E. coli* growth (7). Despite this, we believe that any small discrepancies in lysis onset likely occurred due to technical delays in the assay set-up – e.g. infection takes place over a 10-15-minute period. Further, the assay described in the study by Li *et al.* was only done once, so it is not clear whether their findings were significant or could be consistent (7). We speculate that the small 5-10-minute discrepancies observed in our study were due to the late addition of phage stocks to the gentamicin and kanamycin-treated cells in the 96-well plate as the time taken to load the samples parallels the lysis pattern observed (Fig. 5). Lastly, we did not observe any differences in the lysis onset patterns between the WT and RpoS-deficient strains (Fig. S3). Collectively, our findings suggest that RpoS does not stimulate cross-protection from phage-mediated lysis through induction of stress response under presence of antibiotics.

In this study, we show that, while RpoS is indeed at least partially responsible for the intrinsic mechanisms of antibiotic resistance in *E. coli*, it does not induce stress-response mechanisms that could delay or inhibit bacteriophage infection and the subsequent cell lysis. In addition, we confirmed the non-essential nature of Rcs-phosphorelay pathway in antibiotic resistance, indicating that there are indeed multiple triggers upstream of RpoS. Altogether, our findings demonstrate that treatment of *E. coli* with sub-inhibitory antibiotic concentrations does not stimulate cross-protection to infection and lysis by the T7 phage. While this is contrary to our expected results and hypothesis, our findings are nonetheless still informative. Future studies are required to confirm and elaborate on these results.

Future Directions. Future studies should focus on confirming the absence of delayed lysis by subjecting cultures to staggered phage inoculation, or randomizing order of samples in the 96-well plate to account for bias during the assay set-up. This will help to determine whether the small, but insignificant discrepancies in time of lysis onset could indeed be attributed to the late addition of phage stocks to the wells containing cells pre-treated with aminoglycosides. Moreover, the analyses conducted herein focused solely on use of liquid media for growth assessment, as well as phage-mediated lysis, so it could be beneficial to replicate the study using solid media. With this method, the success of phage infection would

be directly proportional to the size of plaques observed on plates, as suggested by previous studies (17). Furthermore, this study only analyzed T7 bacteriophage-mediated lysis. Despite our initial plan to also include T4 phage in this study, we decided against it, because T4 infection was less robust and thus less tractable in *E. coli* K-12. For this reason, protocol optimization would be necessary to replicate this study with the T4 phage. Some ways in which this could be done include calibration of media composition for pH; supplementation with $CaCl_2$, known to facilitate attachment in some phages; or substitution of media components to achieve optimal ionic strength (18, 19). Lastly, further analysis into the restoration of *E. coli* K-12 cell growth upon prolonged treatment with beta-lactam antibiotics could be performed. Additional assays could aim to establish whether the observed effects would be most likely attributed to initial presence of resistant populations, or gradual induction of secreted beta-lactamases.

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