Vol 4:1-15

The Journal of Experimental Microbiology & Immunology+

RpoS-Mediated Stress Response Does Not Impact the Rate of T7 Bacteriophage-Induced Lysis of *Escherichia coli*

Kate Huang, Erika Jang, Emily Kamma, Annie Wu

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY The alternative sigma factor RpoS is proposed to confer cross-protection against bacteriophage T7-mediated lysis in *Escherichia coli* after exposure to antibiotics. In *E. coli*, RpoS is a regulatory protein that modulates the expression of general stress response genes upon exposure to environmental stress. This response may prime a response against secondary environmental stressors. Previous studies found that pre-treatment with sub-lethal concentrations of antibiotics as a primary stressor can delay T7 bacteriophage-induced lysis. We hypothesized that the delayed lysis observed in *E. coli* pre-treated with antibiotics is the result of cross-protection conferred by RpoS activation, with T7 bacteriophage infection as the secondary stressor. We used minimum inhibitory concentration assays to determine the sub-lethal kanamycin and ampicillin concentrations for the wild-type and *rpoS* knockout mutant (EEKA18-1) *E. coli*. After pre-treating the cells with sub-lethal concentrations of antibiotics, we performed an OD-based real-time T7 bacteriophage lysis assay to generate lysis curves of each treatment (n=3). The EEKA18-1 *rpoS* knockout mutant exhibited similar lysis curves to the wild-type cells. Contrary to previous findings, our results suggest that pre-treatment with sub-lethal concentrations of kanamycin and ampicillin does not delay T7 bacteriophage-induced lysis in wild-type cells. This indicates that the RpoS-mediated general stress response may not confer cross-protection against T7 bacteriophage infection.

INTRODUCTION

Bacteriophage T7 is a lytic virus from the *Podoviridae* family that specifically infects rough strains of *Escherichia coli* (1). The bacteriophage T7 tail adsorbs to the surface LPS of *E. coli* and facilitates the ejection of its genome into the cytoplasm (1). T7 phage infection ends with lysis of the host cell to release the newly replicated progeny into the medium for another infection cycle. With the contemporary rise of antibiotic resistance, bacteriophages present a promising therapeutic avenue for treating pathogenic bacterial infections in healthcare and in agriculture (1). Aside from replacing antibiotic treatments completely, phage therapies could be used as a secondary treatment when antibiotics fail to eliminate pathogenic bacteria (2). In these cases, the pathogens would have been exposed to sub-lethal levels of antibiotics prior to phage treatment. As a result, there is a renewed interest in the interactions between bacteria, antibiotics, and bacteriophages, as well as the development of bacteriophage resistance in bacteria.

Previous studies indicated that treating *E. coli* with subinhibitory levels of aminoglycoside and β-lactam antibiotics as a primary stressor conferred resistance to T7 infection (3-6). Primary stressors can activate the alternative sigma factor RpoS and induce the general stress response (7). RpoS modulates the expression of hundreds of genes involved in cell permeability, metabolism, defense, and repair (7). Due to the broad spectrum of protective genes downstream of RpoS, activation of the general stress response by a primary stressor may confer cross-protection against a variety of secondary environmental stressors including heat, starvation, osmotic, and acid stress (7-9). RpoS induction also upregulates the

Submitted: 13 May 2018
Accepted: 15 Aug 2018
Published Online : 24 August 2018

Citation: Huang K, Jang E, Kamma E, Wu A. 2018. RpoS-Mediated Stress Response Does Not Impact the Rate of T7 Bacteriophage-Induced Lysis of *Escherichia coli*. JEMI+ 4:1-15

Editor: Julia Huggins, University of British Columbia

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Address correspondence to: https://jemi.microbiology.ubc.ca/

expression of mismatch repair genes, which may cause mutagenesis that results in antibiotic resistance (7, 9).

A previous investigation demonstrated that treating bacteria with sub-lethal levels of antibiotics induced the RpoS regulon (7). Another study found that overexpression of RpoS in *E. coli* conferred tetracycline, but not streptomycin or chloramphenicol resistance (10). These papers suggest that RpoS plays an important role in resistance to some antibiotics. To determine if exposure to different classes of antibiotics results in resistance against bacteriophage T7, Li *et al.* compared T7-mediated lysis curves after pre-treating wild-type *E. coli* with sub-minimum inhibitory concentrations (MIC) of aminoglycoside and β-lactam antibiotics (6). They found that antibiotic-treated cells have a later OD peak and a longer time to reach baseline after the peak compared to untreated cells (6). While the delayed OD peak may also indicate slower growth, Li *et al.* concluded that antibiotic treatment delayed cell lysis following T7 bacteriophage infection. Although the study concluded that antibiotic pre-treatment reduces *E. coli* susceptibility to bacteriophage T7 lysis, it remains unknown whether the delay in lysis involves the RpoS-induced general stress response (6).

Due to its involvement in antibiotic resistance and cross-protection, we hypothesized that RpoS plays a role in cross-protection against bacteriophage T7-mediated cell lysis following antibiotic exposure. In this experiment, we aimed to compare the T7-mediated lysis in wild-type and *rpoS* knockout (EEKA18-1) mutant *E. coli* after pre-treatment with sub-lethal concentrations of antibiotics. We used a 96-well plate OD-based MIC assay with serial dilutions of ampicillin and kanamycin to determine the sub-lethal antibiotic concentrations (SLC) for the strains. By using low antibiotic concentrations, we aimed to induce stress while minimizing cell death to keep growth rates of cultures with and without antibiotics comparable. By keeping growth rates comparable between treatments, differences in resistance against T7 phage-induced lysis between treatments would be more apparent. We predicted that the *rpoS* knockout mutant would exhibit lower sub-lethal antibiotic concentrations than the WT strain due to the lack of RpoS expression that normally induces the general stress response. Next, we compared the lysis curves of WT and EEKA18-1 pre-treated with sub-lethal concentrations of each antibiotic using a real time 96-well plate OD-based lysis assay developed by Cho *et al.* (11).

We hypothesised that pre-treatment with antibiotics would confer delayed bacteriophage T7-mediated lysis to WT, but not *E. coli* EEKA18-1. This result would imply that cross-protection by RpoS in WT *E. coli* contributed to greater phage resistance. However, we were unable to replicate the delayed bacteriophage T7-mediated lysis reported by Li *et al*. The EEKA18-1 mutant also exhibited similar lysis curves to the wild-type cells, which suggested that the RpoS-mediated general stress response may not confer cross-protection against bacteriophage T7 infection.

METHODS AND MATERIALS

Bacterial strains and culture conditions *E. coli* strains BW25113 (wild-type), JW5437 (Δ*rpoS::kan*), and BT340 (containing pCP20) were obtained from the Ramey Database from the Microbiology and Immunology Department at the University of British Columbia. Unless stated otherwise, cells were grown in Luria Bertani (LB) medium, LB medium supplemented with 50 μg/mL kanamycin, or LB medium supplemented with 100 μg/mL ampicillin. All strains used are summarized in Table 1.

Preparation of antibiotic stock solutions. Kanamycin (50 mg/mL) and ampicillin (100 mg/mL) stock solutions were prepared by dissolving kanamycin monosulfate (Alfa Aesar) or ampicillin sodium salt (Bio Basic) in distilled water, and then filter sterilizing through a 0.22 μm filter (VWR). Stock solutions were stored at -20°C.

T7 phage propagation and confirmation of purity. Bacteriophage T7 was obtained from the Microbiology and Immunology Department at the University of British Columbia. Phage was added to a log-phase *E. coli* BW25113 culture and incubated (200 RPM, 37°C) until the cells lysed completely. Chloroform (1%) was added (Fisher Scientific), the tube was centrifuged (2750 RCF, 10 min, 4°C), and the supernatant was stored at 4°C. A double agar overlay plaque assay was conducted to determine phage titer (12). Serial dilution factors of

TABLE 1 *E. coli* strains used in the study

Name of Strain	Description	Experiments
BW25113	Wild-type	• T7 phage propagation and confirmation of purity • Minimum inhibitory concentration assay to determine sub-lethal antibiotic concentrations • 96-well OD_{600}-based lysis assay to determine susceptibility to T7
JW5437	*ΔrpoS::kan* derivative of BW25113	• Removal of kanamycin resistance cassette from *E. coli* JW5437 • Minimum inhibitory concentration assay to determine sub-lethal antibiotic concentrations • 96-well OD_{600}-based lysis assay to determine susceptibility to T7
BT340	E. coli carrying pCP20 for excision of kan^r cassette	• Removal of kanamycin resistance cassette from *E. coli* JW5437
EEKA18-1	*ΔrpoS* derivative of JW5437 after removal of kan^r cassette	• Confirmation of kanamycin knockout by PCR and gel electrophoresis • 96-well OD_{600}-based lysis assay to determine susceptibility to T7 • Minimum inhibitory concentration assay to determine sub-lethal antibiotic concentrations • Sanger sequencing of EEKA18-1 amplicon

10^6, 10^7, 10^8, 10^9, and 10^{10} of the phage stock were incubated with 100 µL of *E. coli* at 37°C for 30 minutes. Three controls were prepared: *E. coli* in LB broth (no phage), phage in LB broth (no *E. coli*), and LB broth alone. Samples were mixed in 3 mL top agar and plated on top of LB agar, and then incubated at 37°C overnight. Phage titer was determined by the following calculation:

$$Titer = (Number\ of\ Plaques) \times (10) \times (Dilution\ Factor)$$

To confirm the purity of the working stock, PCR amplification and gel electrophoresis were performed with primers flanking T7 *gp10a* and T4 *gp23*. pUC19ΔSKM was used as a PCR positive control. The PCR reaction was carried out by the method of Fettig *et al.* with the Bio-Rad T100 Thermal Cycler using Platinum Taq DNA polymerase (Invitrogen) (13). Conditions and primers are outlined in Tables 2 and 3. The PCR products and a 100 bp DNA marker (Invitrogen) were electrophoresed on a 2% (w/v) agarose gel containing SYBR Safe DNA Stain in 0.5X TBE (Invitrogen) at 110V in 1X TBE. The gel was then visualized under UV light with the Alpha Innotech MultiImage light cabinet.

Removal of kanamycin resistance cassette from *E. coli* JW5437. Chemically competent cells were generated according to the procedure outlined by Chang *et al.* (14). An overnight JW5437 culture was added to LB broth with kanamycin and incubated (37°C, 200 RPM) until the optical density at 600 nm reached 0.3-0.6. The culture was centrifuged using a Beckman Coulter JA-20 Fixed-Angle Rotor (4°C, 4000 RPM, 10 min) and the pellet was resuspended in 10 mL of pre-chilled $CaCl_2$ (0.1 M). After a 30-minute incubation on ice, the cells were centrifuged (4°C, 4000 RPM, 10 min), and the pellet was resuspended in 0.1 M $CaCl_2$ with 15% glycerol. Cells were snap frozen and stored at -70°C. Concurrently, the pCP20 plasmid carrying an ampicillin resistance gene was isolated from *E. coli* BT340 using the Bio Basic EZ-10 Spin Column Plasmid DNA Miniprep Kit. Competent cells were transformed with pCP20 using the heat-shock method as described by Chang *et al.* (14). DNA (100 ng and 1 µg) was added to 50 µL cell aliquots and incubated on ice for 30 min. The cells were heat-shocked at 42°C for exactly 30 seconds and then placed on ice for 2 minutes. The samples were incubated in LB broth at 30°C for 1 hour to allow for outgrowth, and then plated on LB agar with ampicillin for overnight incubation at 30°C. Individual colonies were inoculated in LB broth with ampicillin and grown overnight at 43°C to induce FLP-FRT recombinase expression to excise the kanamycin resistance cassette. The cultures were spread on LB agar

TABLE 2 PCR primer sets and expected amplicon size

Gene	Primer Sequence (5'-3')
T7 *gp10a*	Forward: CGAGGGCTTAGGTACTGC Reverse: GGTGAGGTGCGGAACTTC
T4 *gp23*	Forward: GCCATTACTGGAAGGTGAAGG Reverse: TTGGGTGGAATGCTTCTTTAG
pUC19ΔSKM *ori*	Forward: CTACATACCTCGCTCTGCTAATC Reverse: CACGCTGTAGGTATCTCAGTTC
rpoS	Forward: TGGTGCGTATGGGCGGTAAT Reverse: TGCCGCAGCGATAAATCGG

and incubated overnight at 30°C. Resulting colonies were re-streaked three times to obtain single cell colonies. Cells were then patched on LB agar with kanamycin, LB agar with ampicillin, and LB agar to select for the loss of pCP20 and knockout of the kanamycin resistance cassette. The resulting cells with knockout of the kanamycin resistance cassette were referred to as strain EEKA18-1.

Confirmation of kanamycin knockout by PCR and gel electrophoresis. The gDNA from 1 mL of an overnight culture of EEKA18-1 was isolated with the Invitrogen PureLink Genomic DNA Mini Kit. PCR amplification and gel electrophoresis were performed with primers flanking the kanamycin resistance gene. pUC19ΔSKM was used as a PCR positive control. The PCR reaction was carried out with the method by Fettig *et al.* with the Bio-Rad T100 Thermal Cycler using Platinum Taq DNA polymerase (Invitrogen) (13). Conditions and primers are outlined in Tables 2 and 4. A 2% (w/v) agarose gel containing SYBR Safe DNA Stain in 0.5X TBE (Invitrogen) was prepared. Products and a DNA marker (Invitrogen) were electrophoresed at 110V in 1X TBE, and then visualized with the Alpha Innotech MultiImage light cabinet.

Minimum inhibitory concentration assay to determine sub-lethal antibiotic concentrations. Overnight cultures of BW25113, JW5437, and EEKA18-1 were subcultured in LB broth and grown until the optical density at 600 nm reached 0.3-0.6. Cultures were diluted to 10^5 cfu/mL using the estimate that an OD_{600} of 1 ≈ $8x10^8$ cfu/mL. On a 96-well

TABLE 3 Phage PCR reaction times and temperatures

PCR Step	Cycles	Temperature	Time
Initial Denaturation	1	95°C	10 min
Denaturation	33	95°C	30 sec
Annealing	33	50°C	30 sec
Extension	33	72°C	30 sec
Final Extension	1	72°C	5 min

TABLE 4 Kanamycin cassette removal PCR reaction times and temperatures

PCR Step	Cycles	Temperature	Time
Initial Denaturation	1	94°C	3 min
Denaturation	33	94°C	45 sec
Annealing	33	55°C	30 sec
Extension	33	72°C	30 sec
Final Extension	1	72°C	10 min

plate, 100 µL of LB broth was added to each well. Two-fold serial dilutions of 32 to 0.03125 µg/mL ampicillin or kanamycin diluted in LB broth were added to wells in triplicate before plating 5 µL of the normalized cell culture to each well. Negative controls were plated in triplicate with LB broth only. Positive controls were plated in triplicate and contained cells, LB broth, and no antibiotics. The plate was incubated at 37°C overnight before reading the OD_{600} on the BioTek Epoch Microplate Spectrophotometer. Sub-lethal concentration was defined as the concentration of antibiotic needed to inhibit a percentage of growth. Sub-lethal $concentration_{10}$ (SLC_{10}) was defined as the concentration of antibiotics that inhibited 10% of growth. To calculate SLC_{10}, the optical density at 600 nm which corresponded to 90% of the positive control OD_{600} was first calculated. This value was used to extrapolate the antibiotic concentration from the linear portion of an OD_{600} vs. antibiotic concentration plot. To follow the convention of using half of MIC, the sub-lethal concentrations which were used in the lysis assay were defined as half of the SLC_{10} value (SLC_5).

96-well OD_{600}-based lysis assay to determine susceptibility to T7. Overnight cultures of BW25113, JW5437, and EEKA18-1 grown in sub-lethal concentrations of each of kanamycin and ampicillin were subcultured and grown to log phase (37°C, 200 RPM). Cultures grown without antibiotics were used as a control. All cultures were normalized to an OD_{600} of 0.3, then 90 µL was plated in triplicate on a 96-well plate. Bacteriophage T7 was diluted to a multiplicity of infection (MOI) of 0.05, according to the following equation:

$$MOI = \frac{\left(\frac{pfu}{mL}\right) \times (mL\ Phage)}{Number\ of\ Cells}$$

10 µL of diluted T7 phage was added to each well containing cells. Negative controls were prepared by plating 90 µL of the normalized cell cultures in triplicate, and then adding 10 µL of LB broth instead of phage. LB broth (100 µL) was used as the blank. The optical density at 600 nm was read on the BioTek Epoch plate reader in 10-minute intervals until OD_{600} reached the starting value. The plate was incubated at 37°C between reads. The plate set-up is shown in Figure S1.

Sanger sequencing of EEKA18-1 amplicon. The gDNA from EEKA18-1 was extracted with the Invitrogen PureLink Genomic DNA Mini Kit. PCR and gel electrophoresis were performed with the procedure outlined above for EEKA18-1. The PCR product was purified using the Bio Basic EZ-10 Spin Column PCR Products Purification Kit, then the absorbance and concentration were read on the Nanodrop spectrophotometer. The purified PCR product and the forward primer were sent to GeneWiz for Sanger sequencing to confirm knockout of the kanamycin resistance cassette. Sequencing results were aligned to the *E. coli* BW25113 genome (accession number: CP009273.1) using the NCBI Nucleotide Basic Local Alignment Search Tool (BLASTN) 2.8.0+ (15, 16).

RESULTS

Confirming bacteriophage T7 titer and purity. Bacteriophage T7 propagated for the OD-based lysis assay was found to have a titer of 1.68 x 10^{13} pfu/mL in the double agar overlay plaque assay. Figure 1 shows the agarose gel of the PCR products from PCR performed on T7 phage using primers flanking the genes *gp23* (for T4) and *gp10a* (for T7). Amplification using the *gp10a* primers showed a single band at 295bp, which corresponded to the T7 *gp10a* amplicon. The absence of a band from the PCR reaction with the T4 *gp23* primers at 398 bp indicated that the bacteriophage T7 stock was not contaminated with bacteriophage T4 (Figure 1). The positive control performed with pUC19ΔSKM plasmid and *ori* primers yielded a band at the expected amplicon size of 189 bp, which indicated that the PCR reaction successfully amplified a known product. The negative control with distilled water instead of template DNA and T7 *gp10a* primers did not result in a band, which showed that the primers did not form primer-dimers or amplify other non-specific products. Overall, PCR analysis showed that the propagated bacteriophage T7 stock had a high titer sufficient for the lysis assay and was not contaminated with bacteriophage T4.

PCR analysis and Sanger sequencing confirms the removal of the kanamycin resistance cassette from *E. coli* JW5437 to generate *E. coli* EEKA18-1. *E. coli* JW5437 harbours a kanamycin resistance cassette in place of the *rpoS* gene. The cassette must be removed to investigate the effects of sub-lethal kanamycin treatment on bacteriophage T7-induced lysis in the *rpoS* KO mutant (EEKA18-1). We used the FLP recombinase encoded by the pCP20 plasmid to excise the cassette via FLP-FRT recombination with FRT sites within the kanamycin resistance cassette. Transformants sensitive to kanamycin and ampicillin were selected, as this indicated the removal of the kanamycin resistance cassette and the absence of the pCP20 plasmid.

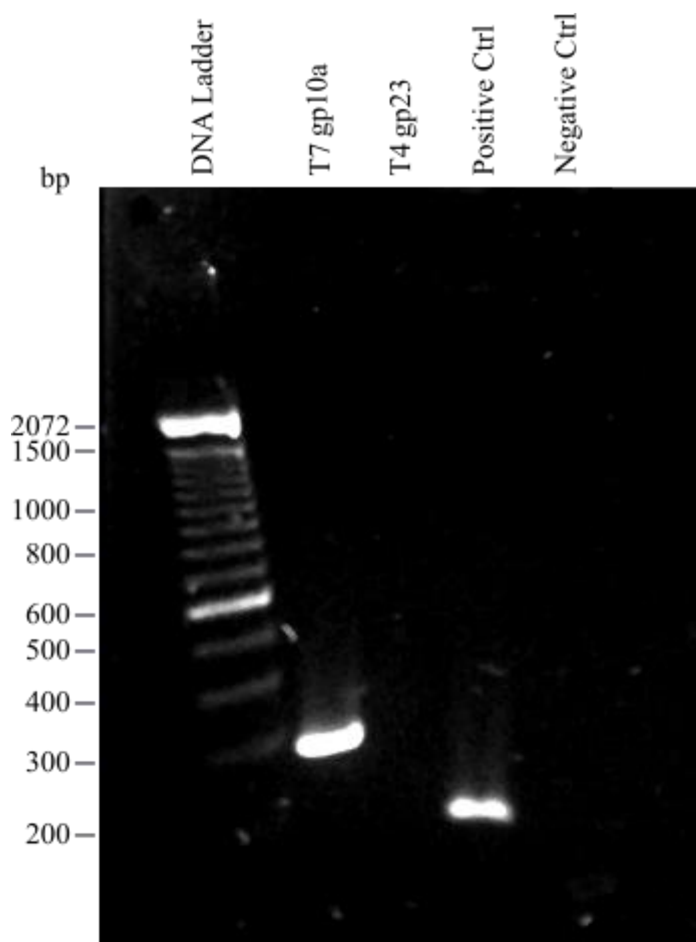

FIG. 1 Propagated bacteriophage T7 does not have bacteriophage T4 contamination. PCR on the propagated T7 bacteriophage was performed using T7 primers flanking *gp10a* and T4 primers flanking *gp23*, and the PCR products were run on a 2% agarose gel at 110V. The 295 bp amplicon of the T7 *gp10a* gene was observed while the 398 bp amplicon of the T4 *gp23* gene was not observed. The no template, H_2O negative control using T7 primers did not show a band, and the pUC19ΔSKM positive control showed a 189 bp band.

To confirm cassette removal, we selected five transformants after colony purification, performed PCR using primers flanking the *rpoS* gene, and resolved the amplicons via agarose gel electrophoresis (Figure 2). Successful removal of the cassette should yield a band approximately 298 bp in length containing sequences that correspond to the start codon, a 'scar' sequence 81 nucleotides in length, 18 nucleotides in the 3' region of the *rpoS* gene, and the stop codon (17). *E. coli* JW5437 was expected to have the kanamycin resistance cassette and yield a band around 1521 bp. The WT strain *E. coli* BW25113 was expected to have the *rpoS* gene and to yield a band around 1186 bp. pUC19ΔSKM was included in the PCR reaction as a positive control, and a no-template control containing *rpoS*-specific primers was included as a negative control. As expected, *E. coli* JW5437 showed a band near 1500 bp and BW25113 showed a band near 1200 bp. We observed a band slightly greater than 400 bp for all five transformants.

We used Sanger sequencing with the *rpoS* forward primer to characterize the amplicon from colony #1. We observed nucleotide sequences that correspond to the start codon, 117 extra nucleotides that align to the 5' region of *rpoS*, 4 extra nucleotides that do not align to *rpoS* or the scar sequence, a partial 'scar' sequence (75/81 nucleotides present), the expected 18 nucleotides in the 3' region, and the stop codon sequence (Figure S2). While we expected an amplicon approximately 298 bp in size, the presence of the additional 121 nucleotides likely contributed to the larger band size observed in the gel.

Together, the results from PCR and Sanger sequencing indicated that the kanamycin resistance cassette was removed from *E. coli* JW5437 Δ*rpoS::kan*. We renamed this Δ*rpoS*Δ*kan* mutant *E. coli* EEKA18-1 and used EEKA18-1 in downstream experiments.

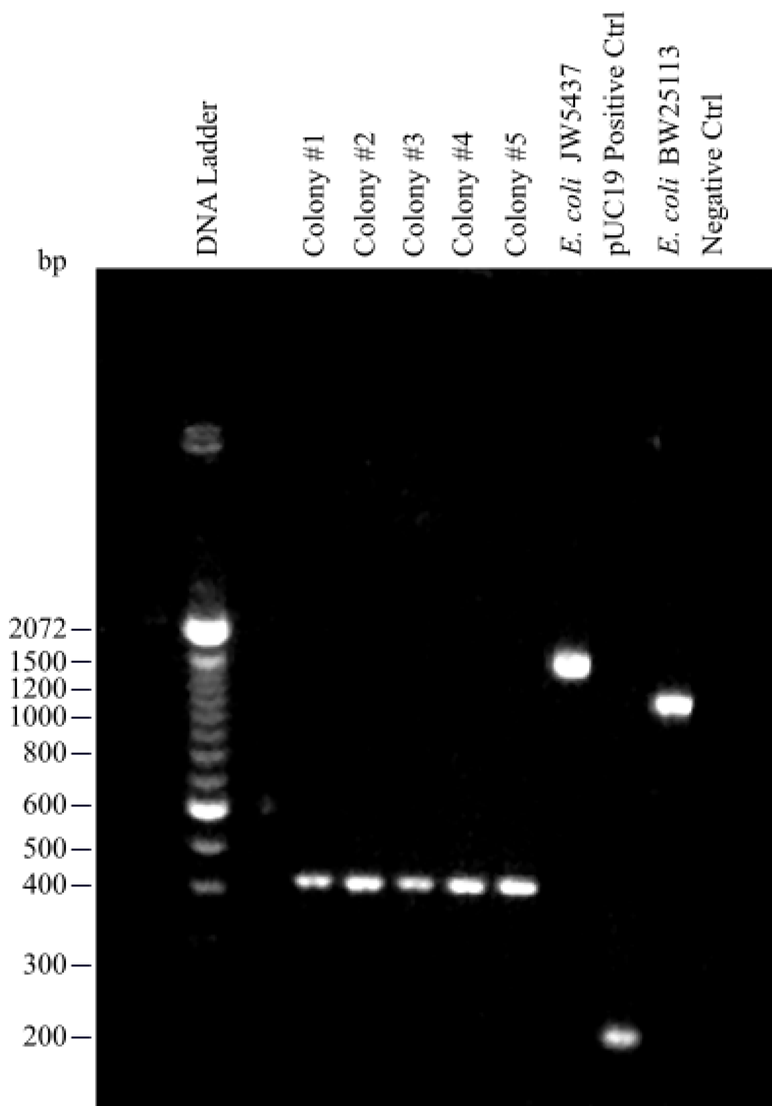

FIG. 2 PCR analysis confirms the kanamycin resistance cassette was removed from *E. coli* JW5437 to create *E. coli* EEKA18. PCR was performed on the gDNA of five purified transformants (colony #1 - #5) using *rpoS* primers flanking the kanamycin resistance cassette. Amplicons were resolved on a 2% agarose gel at 110V. Successful removal of the cassette should yield a small 298 bp amplicon compared to a 1521 bp amplicon from *E. coli* JW5437 with the intact resistance cassette. *E. coli* BW25113 should yield a 1186 bp amplicon. pUC19ΔSKM and primers flanking the *ori* were used in the positive control and should yield a 189 bp amplicon. *rpoS* primers were used in the no-template negative control.

Determining sub-lethal concentrations of antibiotics using a MIC assay. We performed a MIC assay to determine the sub-lethal concentrations of antibiotics for *E. coli* BW25113, JW5437, and EEKA18-1. The sub-lethal concentrations (SLC_{10} and SLC_5) of ampicillin and kanamycin for the *E. coli* strains are displayed in Table 5. As expected, all three strains were sensitive to ampicillin, but JW5437 had an approximately 10-fold increase in sensitivity to ampicillin compared to EEKA18-1 and BW25113. The increased sensitivity was unexpected since the kanamycin resistance cassette was the only difference and should not impact sensitivity to ampicillin. EEKA18-1 and BW25113 were more susceptible to kanamycin compared to ampicillin, and, as predicted, EEKA18-1 showed lower sub-lethal concentrations for both antibiotics compared to BW25113. The knockout of the kanamycin resistance cassette in EEKA18-1 was further confirmed by the low MIC of kanamycin required to inhibit cell growth. JW5437 was not inhibited by kanamycin because it contained the kanamycin resistance cassette.

Sub-lethal antibiotic treatment does not alter the growth rate of *E. coli* BW25113, JW5437, or EEKA18-1. The physiological state of the host cell can affect bacteriophage infection, and a faster host growth rate has been shown to support faster bacteriophage T7 growth and host lysis (18). To determine if sub-lethal concentrations of ampicillin or kanamycin (Table 5) will slow the growth rate of *E. coli* BW25113, JW5437, or EEKA18-1, we cultured these strains in the presence or absence of antibiotics and monitored growth by measuring OD_{600} readings over time. We observed similar growth rates regardless of the presence of antibiotics in *E. coli* BW25113, JW5437 and EEKA18-1 (Figure 3, Figure 4, Figure S5). The same results were observed in two additional biological replicates (Figure S3, Figure S4, Figure S5), indicating that the sub-lethal antibiotic concentrations established through the MIC assay do not impede cell growth. As such, growth rates of cultures with and without antibiotic treatment remained comparable in the lysis assay.

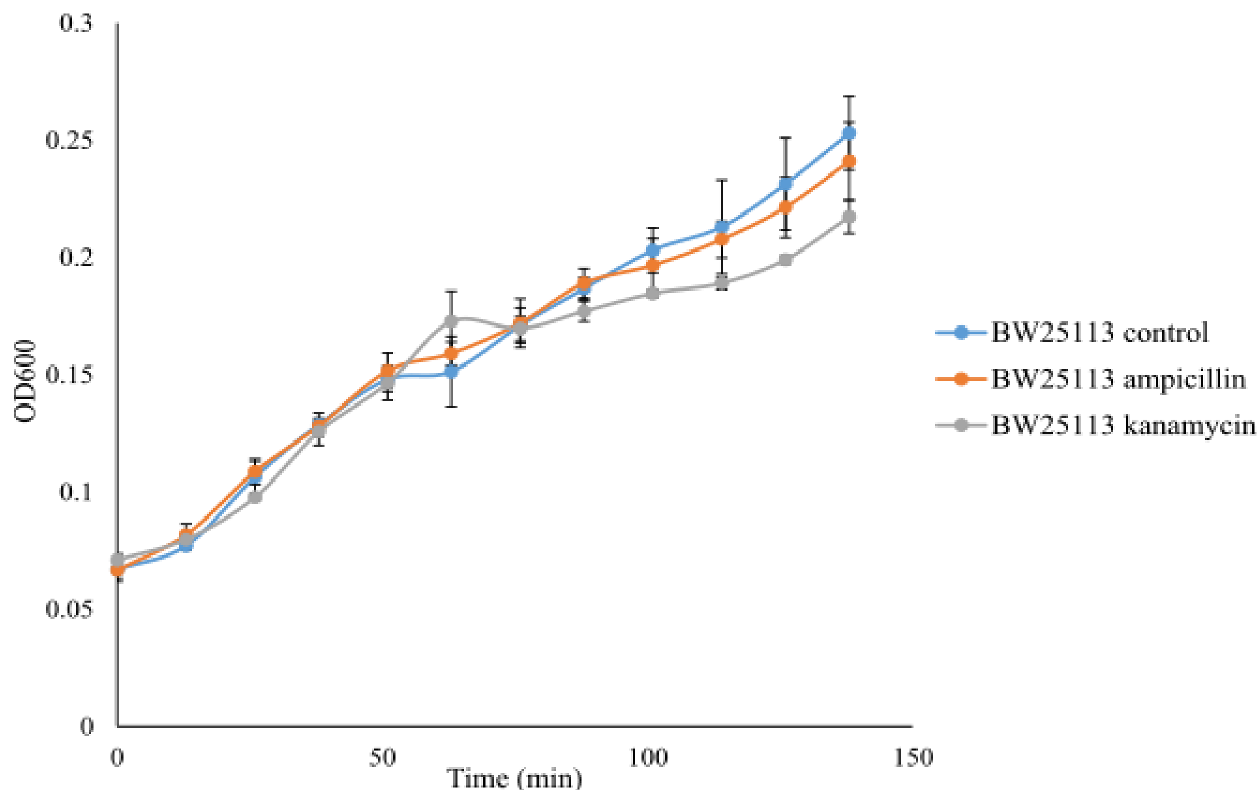

FIG. 3 The growth rate of *E. coli* BW25113 pre-treated with sub-lethal concentrations of ampicillin or kanamycin is similar to untreated BW25113. *E. coli* BW25113 was cultured overnight in three media conditions: LB control, LB with sub-lethal concentrations of kanamycin, or LB with sub-lethal concentrations of ampicillin. The cells were subcultured to obtain log-phase cells. The cultures were normalized to an OD_{600} of 0.3, plated in triplicates on a 96-well plate, and incubated at 37°C. OD_{600} measurements were taken in 10-minute intervals using a BioTek Epoch Microplate Spectrophotometer to generate the growth curves. Error bars represent standard deviations.

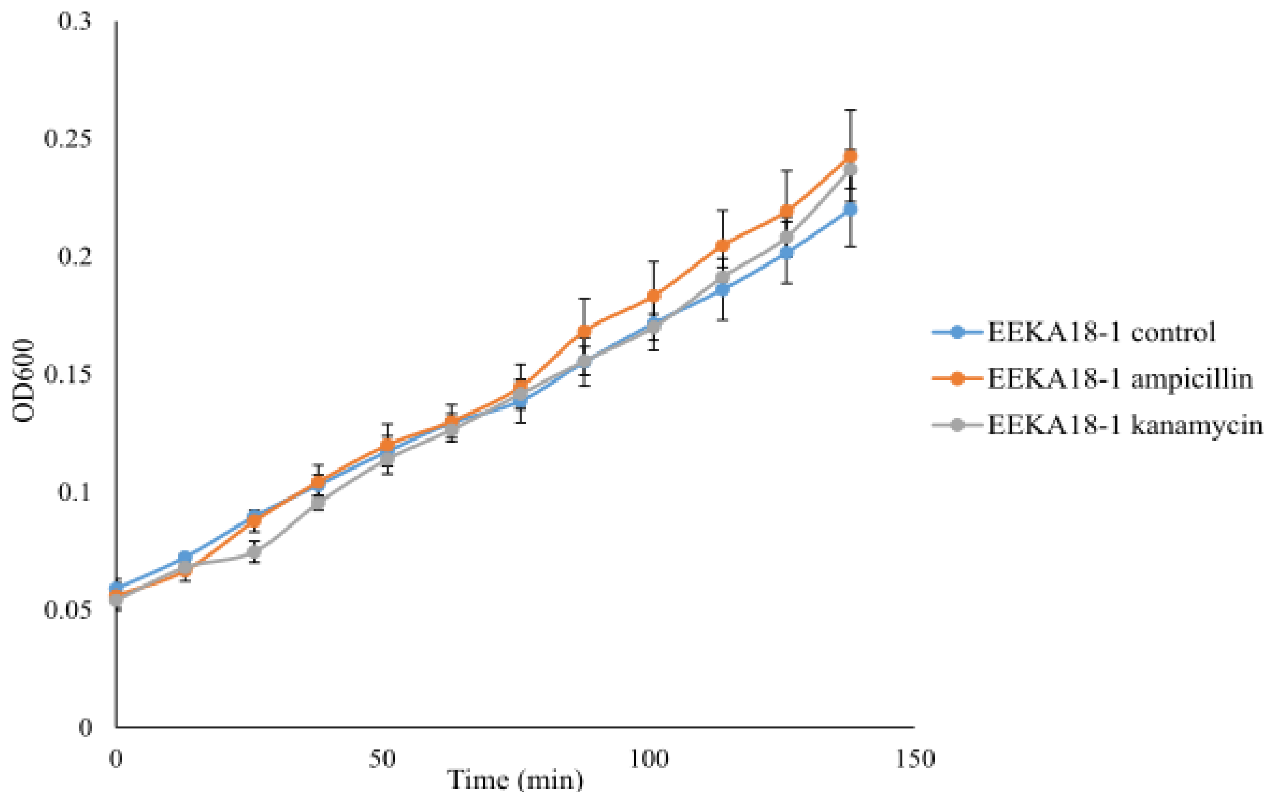

FIG. 4 The growth rate of *E. coli* EEKA18-1 pre-treated with sub-lethal concentrations of ampicillin or kanamycin is similar to untreated EEKA18-1. *E. coli* EEKA18-1 was cultured overnight in three media conditions: LB control, LB with sub-lethal concentrations of kanamycin, or LB with sub-lethal concentrations of ampicillin. The cells were subcultured to obtain log-phase cells. The cultures were normalized to an OD_{600} of 0.3, plated in triplicates on a 96-well plate, and incubated at 37°C. OD_{600} measurements were taken in 10 minute intervals using a BioTek Epoch Microplate Spectrophotometer to generate the growth curves. Error bars represent standard deviations.

Pre-treating *E. coli* BW25113, JW5437, or EEKA18-1 with sub-lethal concentrations of antibiotics does not delay T7 bacteriophage-mediated lysis. We conducted 96-well OD_{600}-based lysis assays to generate lysis curves for each strain. OD_{600} readings should increase over time and peak when the rate of cell growth equals the rate of cell lysis. OD_{600} should decline as the rate of lysis exceeds the rate of growth and eventually reach values below the initial reading. As seen in Figure 5, the lysis curves for the *E. coli* BW25113 control peaked between t = 70 min to t = 80 min in all three replicates, and sub-lethal antibiotic treatment did not result in delayed lysis compared to the no antibiotic control. As the OD_{600} values decreased, the lysis curves showed nearly identical slopes which suggested that the rate of lysis was similar between the conditions. Likewise, *E. coli* JW5437 did not exhibit delayed

TABLE 5 Minimum inhibitory and sub-lethal concentrations of ampicillin and kanamycin for *E. coli* EEKA18-1, BW25113, and JW5437.

E. coli strain	Ampicillin		Kanamycin	
	MIC (µg/mL)	Sub-lethal (µg/mL)	MIC (µg/mL)	Sub-lethal (µg/mL)
EEKA18-1	0.262	0.131	0.0233	0.0116
BW25113	0.526	0.263	0.0931	0.0465
JW5437	0.0349	0.0174	--	--

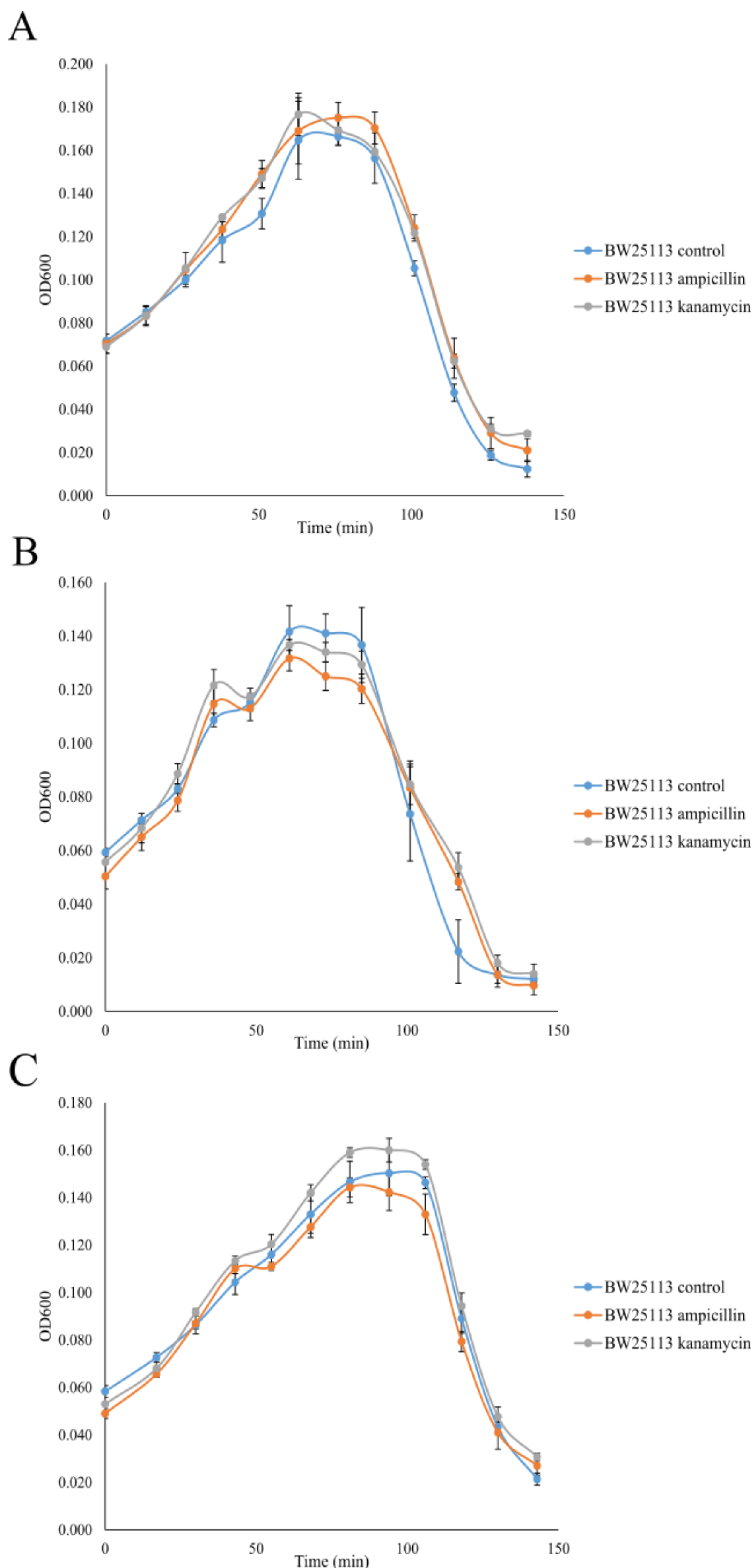

FIG. 5 Pre-treatment of *E. coli* BW25113 with sub-lethal concentrations of ampicillin and kanamycin does not delay bacteriophage T7-mediated cell lysis. Individual graphs in A, B, and C represent lysis curves from three biological replicates. *E. coli* BW25113 was grown overnight in three media conditions: LB broth, LB broth with sub-lethal concentrations of kanamycin, or LB broth with sub-lethal concentrations of ampicillin. The cells were subcultured the next day and grown to log phase. The cultures were normalized to an OD_{600} of 0.3, and 90 µL of each culture was plated in triplicates in a 96-well plate. A total of 10 µL of diluted T7 bacteriophage was added to the cells to obtain a MOI of 0.05. Negative controls were plated in triplicates using 10 µL of LB broth instead of bacteriophage. The plated cells were incubated at 37°C, and the OD_{600} was read in a plate reader in 10 minute intervals until the OD_{600} dipped below the starting values. Error bars represent standard deviations.

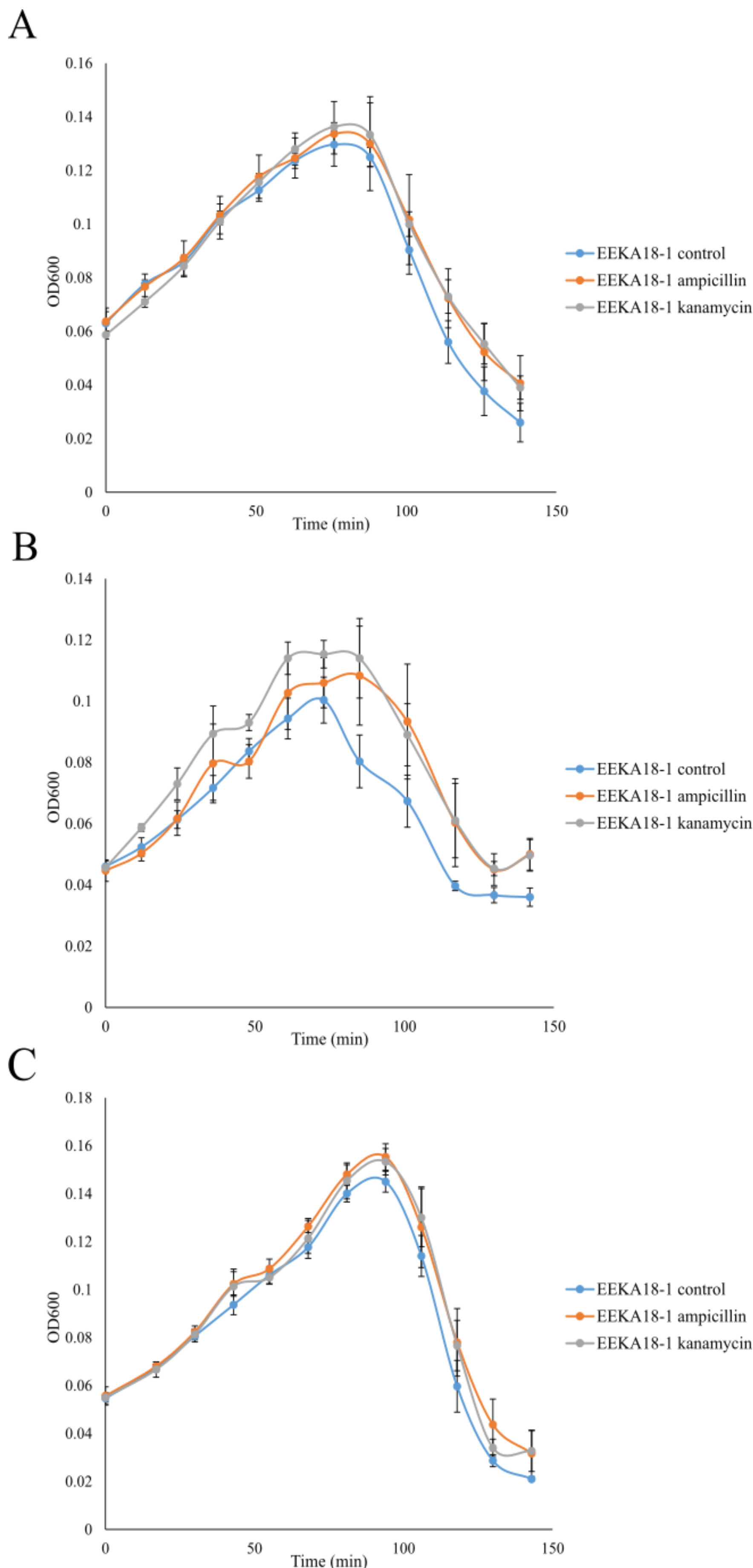

FIG. 6 Pre-treatment of *E. coli* EEKA18-1 with sub-lethal concentrations of ampicillin and kanamycin does not delay bacteriophage T7-mediated cell lysis. Individual graphs in A, B, and C represent lysis curves from three biological replicates. *E. coli* EEKA18-1 was grown overnight in three media conditions: LB broth, LB broth with sub-lethal concentrations of kanamycin, or LB broth with sub-lethal concentrations of ampicillin. The cells were subcultured the next day and grown to log phase. The cultures were normalized to an OD_{600} of 0.3, and 90 µL of each culture was plated in triplicates in a 96-well plate. A total of 10 µL of diluted T7 bacteriophage was added to the cells to obtain a MOI of 0.05. Negative controls were plated in triplicates using 10 µL of LB broth instead of bacteriophage. The plated cells were incubated at 37°C, and the OD_{600} was read in a plate reader in 10 minute intervals until the OD_{600} dipped below the starting values. Error bars represent standard deviations.

lysis when pre-treated with sub-lethal concentration of ampicillin (Figure S6), and *E. coli* EEKA18-1 also yielded similar results. The lysis curves for EEKA18-1 peaked at similar time points regardless of antibiotic treatment (Figure 6). These results indicated that sub-lethal antibiotic treatment does not result in delayed lysis of WT *E. coli* BW25113 or JW5437 and knocking out *rpoS* does not expedite or delay the onset of T7 bacteriophage-induced lysis.

DISCUSSION

Phage therapy is a promising therapeutic approach to treat pathogenic bacterial infections in a variety of settings. With the rise of antibiotic-resistant bacteria there is a renewed interest in studying the interactions between *E. coli*, antibiotics, and bacteriophages. In this study, we examined the impact of sub-lethal concentrations of antibiotics on delayed lysis at a cellular level. Previous studies indicate that pre-treatment of *E. coli* with subinhibitory concentrations of antibiotics as a primary stressor may also confer cross-protection against infection and delay bacteriophage T7-induced cell lysis (3-6). This phenomenon was observed across various *E. coli* strains, such as *E. coli* UB1005, *E. coli* B23, and *E. coli* BW25113, as well as different antibiotic classes such as β-lactams or aminoglycosides (3-6, 19). Many mechanisms have been proposed and examined including the upregulation of capsular polysaccharide expression after antibiotic treatment which impedes T7 phage adsorption (19), the secretion of extracellular factors such as LPS that act as decoys (4, 5), and the overproduction of outer-membrane vesicles (OMVs) that irreversibly bind T7 phage to prevent infection of the host cell (11). Many proposed mechanisms suggest that antibiotic treatment leads to the upregulation or release of extracellular factors that reduce the frequency of phage adsorption. Gu *et al.* examined the role of capsular polysaccharide or carbohydrate production which increases after subinhibitory antibiotic treatment (19). They observed that preventing capsular polysaccharide production does not impede phage adsorption (19). Another potential mechanism involves the release of soluble, aqueous factors such as LPS into the liquid medium in response to antibiotic stress. Hardman *et al*. found that adding soluble LPS does not block T7 phage from infecting host cells, and Nagra *et al*. showed that secreted factors in the culture supernatant does not reduce T7 infection (4, 5). Cho *et al*. investigated the overproduction of OMVs, which were shown to irreversibly bind T4 bacteriophage. However, the impact of OMVs in preventing T7 phage infection remains inconclusive (11).

Another model proposed that antibiotic treatment may alter the expression profile of *E. coli* and induce the upregulation of the RpoS transcription factor (7, 20). The RpoS regulon regulates the expression of hundreds of general stress response genes (7, 20). These RpoS-dependent genes regulate a variety of cell processes including permeability, metabolism, repair, and defense (7). This change in gene expression confers cross-protection to the cells and increases cell resistance to secondary environmental stressors such as heat, starvation, and osmotic stress (7-9).

We hypothesized that sub-lethal antibiotic treatment upregulates *rpoS* expression which contributes to delayed T7-bacteriophage mediated lysis, and the absence of *rpoS* expression should prevent delayed lysis from occurring. To investigate this, we treated *E. coli* BW25113 and the EEKA18-1 *rpoS* KO mutant with sub-lethal concentrations of antibiotics and used an OD-based lysis assay to monitor the rate of lysis following T7 bacteriophage infection. Our findings indicate that sub-lethal ampicillin or kanamycin treatment does not lead to delayed cell lysis. The lysis curves peaked at similar time points regardless of antibiotic treatment and OD_{600} values declined with similar slopes, indicating a similar rate of cell lysis.

We first determined the sub-lethal antibiotic concentrations for the *E. coli* BW25113 WT strain and the *E. coli rpoS* KO mutants, JW5437 and EEKA18-1, using an OD-based MIC assay. The MIC is the lowest concentration required to exhibit a clear, macroscopic inhibition in growth during the MIC assay (6). The subinhibitory concentration was used in previous studies and is defined as half of the MIC (6). In preliminary studies, we found that this definition of subinhibitory antibiotic concentration resulted in different growth rates compared to the culture without antibiotic treatment. After 1.5 hour incubation of the subculture from turbid overnight cultures, BW25113 without antibiotics had an OD_{600} of 0.548, while BW25113 with a subinhibitory concentration of kanamycin (2 μg/ml) had an OD_{600} of 0.295. Due to different growth rates, we would not be able to compare the effects of

these treatments on resisting T7 bacteriophage-induced lysis. Thus, we opted to use a sub-lethal concentration of antibiotics rather than the subinhibitory concentration to treat cells prior to T7 bacteriophage infection. We defined the sub-lethal concentration as half the concentration of antibiotics that resulted in a 10% OD_{600} reduction in the antibiotic treated cells compared to the untreated cells for each strain. We selected ampicillin and kanamycin as representatives of the β-lactam and aminoglycoside classes of antibiotics, respectively.

The MIC assay results indicate that *E. coli* EEKA18-1 is more susceptible to ampicillin or kanamycin treatment than the WT strain BW25113. The absence of *rpoS* expression in EEKA18-1 may be limiting stress responses, resulting in higher susceptibility to antibiotic treatment compared to the WT. Interestingly, *E. coli* JW5437 was more sensitive to ampicillin treatment than *E. coli* EEKA18-1 and the WT strain BW25113. Expression of the kanamycin resistance gene and protein in *E. coli* JW5437 may have hindered its response against ampicillin treatment by utilizing protein synthesis machinery intended to produce normal proteins. Existing literature shows that overexpression of proteins which do not have a function for the cells under the assay conditions leads to rRNA degradation and decreased protein synthesis capacity (21). This may be occurring when *E. coli* JW5437 is treated with ampicillin. More replicates are required to test the reproducibility of this observation. Experiments analyzing growth inhibition or ribosome degradation may also provide insight.

Through an OD-based lysis assay, we demonstrate that sub-lethal antibiotic treatment does not lead to a delay in T7 phage-induced lysis in *E. coli* BW25113 or in *E. coli* EEKA18-1. This finding is supported by the similar peak times and rate of decrease in the slopes of the lysis curves, and delayed lysis could not be consistently replicated for both strains across three separate replicates. The growth curves constructed for *E. coli* BW25113 and EEKA18-1 indicate that the sub-lethal concentrations of antibiotics used in this study does not substantially alter the growth rate of these strains. This eliminates the host growth rate as a variable that impacted the rate of T7 bacteriophage-induced lysis in the lysis assay. Since we did not confirm activation of RpoS using qPCR, the comparable growth rates between treatments may also suggest very little stress on the bacterial culture.

Our results contradict the findings of Li *et al.*, who observed that sub-lethal concentrations of β-lactams and aminoglycosides, including ampicillin and kanamycin, delayed the onset of cell lysis or results in a slower rate of lysis in *E. coli* B23 (6). One explanation for these conflicting findings is that we treated cells with much lower antibiotic concentrations. Gutierrez *et al.* demonstrated that a subinhibitory ampicillin concentration of 1 µg/mL can upregulate *rpoS* mRNA and protein expression and induce the general stress response (7). The sub-lethal antibiotic concentrations used in this study may be too low to induce the general stress response or other mechanisms that contributed to delayed lysis in previous studies. Under optimal growth conditions, RpoS levels are low during exponential phase and targeted proteolysis by ClpXP prevents the accumulation of RpoS (9). The absence of sufficient antibiotic stress may be limiting the number of active RpoS and consequently the general stress response.

In conclusion, the EEKA18-1 *rpoS* knockout exhibited similar lysis curves to the BW25113 wild-type *E. coli.* In contrast to previous findings, our results suggest that pre-treatment with sub-lethal concentrations of kanamycin and ampicillin does not delay T7 bacteriophage-induced lysis in wild-type cells. This indicates that the induction of the RpoS-mediated general stress response using sub-lethal concentrations of antibiotics may not confer cross-protection against T7 bacteriophage infection.

The treatment of bacterial infections with antibiotics has declined in efficacy due to antibiotic resistance. As a result, there is increased interest in therapeutics that use bacteriophage to control infections that are resistant to multiple drugs (22). These findings support the usage of antibiotic and phage combinational therapy since phage infectivity was not dampened in the presence of ampicillin and kanamycin.

Future Directions We aimed to probe the role of RpoS in delayed bacteriophage T7-mediated lysis through the comparison of wild-type to a Δ*rpoS* strain. However, we did not confirm the activation and upregulation RpoS in the wild-type after treatment with sub-lethal concentrations of antibiotics. Since it is an alternative sigma factor, the expression of RpoS is tightly regulated to prevent competition with RpoD, the vegetative sigma factor (9).

Quantification of RpoS expression at the transcript level using qRT-PCR and at the protein level using a western blot would confirm activation of the RpoS regulon in the wild-type.

We chose sub-lethal antibiotic concentrations that ensured comparability of growth curves between each treatment, rather than optimizing delayed lysis. We expected an antibiotic concentration-dependent increase in RpoS activation. If RpoS plays a role in delaying bacteriophage T7-mediated lysis, then there may be an optimal antibiotic concentration where the protection against T7 lysis balances with the reduction in growth due to antibiotics. Comparing lysis curves across different concentrations of antibiotics, especially when coupled to qRT-PCR or western blot to assess RpoS expression, may further elucidate any involvement of RpoS in cross-protection against T7-mediated lysis.

We found *E. coli* JW5437, which carried a kanamycin resistance cassette, to have much greater ampicillin sensitivity than EEKA18-1, its derivative after removal of the kanamycin resistance cassette. We proposed that overexpression of the kanamycin resistance cassette may lead to decreased protein synthesis capabilities and, thus, a decreased capacity to resist other antibiotics. Future studies can investigate this phenomenon with more replicates to compare the MIC of JW5437 to EEKA18-1. In addition, future researchers can compare the antibiotic sensitivity of other knockout strains from the Keio Collection and its counterparts after kanamycin resistance cassette removal. If this effect is reproducible, then future researchers can use western blots to compare the expression of the kanamycin resistance gene to overall protein production and determine if overexpression is the underlying mechanism.

Prior to the lysis assay, we pre-treated the *E. coli* strains with the sub-lethal concentrations of antibiotics overnight and during the re-growth to exponential phase. The bacteria may have acclimatized to the antibiotics during the long pre-treatment duration. As a result, the RpoS-mediated stress response may have already subsided after generating mutations to persist in the sub-lethal antibiotic concentrations. Using different durations of antibiotic pre-treatment prior to the qRT-PCR or western blot can clarify how long the RpoS activation persists. The results can provide the optimal pre-treatment duration prior to the lysis assay.

To further confirm our lysis assay results, future researchers can attempt to further minimize errors. Due to our plate layout, we tended to add phage to the control wells prior to the antibiotic-treated wells. Randomization of the order of the phage would reduce resulting biases in the results. In addition, we used a plate reader without any incubation function. This step may have introduced errors in the timing between each read as well, due to temperature fluctuations between the incubator and the plate reader. Using an incubating plate reader would ensure less variability between biological replicates. Also, we performed three biological replicates with samples plated in triplicates. Performing more biological replicates would help further demonstrate any trends in the lysis curves.

Since there is renewed interest in medical and agricultural applications of bacteriophage T7, it may be helpful to simulate these conditions in the *in vitro* lysis assay. Future researchers can use antibiotic concentrations that would be encountered by *E. coli* at the site of infection. Since bacteriophages have a narrow host range, polyphage cocktails may treat infections more effectively. Characterization of the role of RpoS in protection against other strains of bacteriophage individually, as well as in combination with bacteriophage T7, would help assess the feasibility of bacteriophages as antibacterial therapies.

ACKNOWLEDGEMENTS

We thank the Department of Microbiology and Immunology at the University of British Columbia for funding this project. We thank Dr. David Oliver, Gyles Ifill, and Ashley Arnold for their expertise and guidance. We also thank the UBC Wesbrook Building media room staff for their support.

CONTRIBUTIONS

All authors performed experiments, wrote the abstract and edited the manuscript. EJ wrote the materials and methods of the manuscript. EK and KH wrote the results and discussion, as well as prepared the figures and tables, of the manuscript. AW wrote the introduction and future directions of the manuscript.

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