Sublethal Concentrations of Antibiotics Do Not Markedly Delay T7 Bacteriophage-Mediated Lysis Between Wild-Type and *rpoS*, and *oxyR* Mutants of *Escherichia coli*

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SUMMARY T7 bacteriophages are obligatory lytic phages that infect bacterial cells, and hijack host cell machinery to replicate their genome. Bacteriophage infection acts as an environmental stressor for *E. coli*, like other known factors such as antibiotics, and nutrient deprivation. In response to stressors, *E. coli* has been shown to increase the production of the regulatory proteins RpoS and OxyR. Both regulators may play a role in protective response against oxidative stress, which is induced by exposure to sublethal antibiotic concentrations. Previous studies have suggested that increased production of RpoS and OxyR following sublethal concentration of antibiotics may play an important role in delaying T7 bacteriophage-mediated cell lysis. However, this cross-protection phenomenon is not well understood. The aim of our project is to investigate the role of RpoS and OxyR in delaying T7 bacteriophage-mediated cell lysis following exposure to sublethal antibiotic concentrations by comparing the onset of lysis in *E. coli* wild-type (WT) strains to that of *rpoS* and *oxyR* single knockout mutant strains. We hypothesized that both RpoS and OxyR are necessary for the delayed T7 bacteriophage-mediated cell lysis in *E. coli* strain BW25113 following exposure to sublethal antibiotic concentrations. Validation of each strain was achieved via PCR and sublethal concentrations of ampicillin, gentamicin, and kanamycin were determined using a minimum inhibitory concentration (MIC) assay. Infectivity assay of the two mutant *E. coli* strains treated with a range of sublethal antibiotic concentrations did not show a change in the onset or the rate of lysis compared to the WT.

INTRODUCTION

Bacteriophages are ubiquitous viruses that infect and kill bacteria via cell lysis (1). T7 bacteriophages belong to the *Podoviridae* family, consisting of short non-contractile tails and a capsid head (1, 2). The double-stranded DNA genetic material of the T7 bacteriophage is stored in the capsid head (1, 2). T7 bacteriophages are obligatory lytic phages, which attach to lipopolysaccharide (LPS) on the outer membrane (OM) of an *E. coli* host cell and then inject their DNA into the host cell's cytoplasm (2-4). The phage takes over the host's cellular machinery to replicate the viral genome and translate viral proteins, eventually producing viral progeny (5-6). When sufficient viable phage progeny has been assembled, the *E. coli* host cell is lysed through the action of the viral proteins holin and endolysin resulting in the release of the viral progeny (7).

Ampicillin and gentamicin are bactericidal antibiotics that have different killing mechanisms (21). Ampicillin is a β-lactam antibiotic, which inhibits the last stage of bacterial cell wall synthesis by acting as an irreversible inhibitor of the transpeptidase enzyme (21, 23). This is followed by bacterial cell lysis, which is mediated by bacterial cell wall autolytic enzymes (24). Gentamicin belongs to the aminoglycoside class of antibiotics (25). It inhibits bacterial protein synthesis by irreversibly binding to four nucleotides of the 16S ribosomal RNA (rRNA) and one amino acid of S12 in the bacterial ribosome complex (25). This results in the ribosomes becoming nonfunctional and the bacteria are no longer capable of synthesizing proteins necessary for growth and survival (25).

Bacteriophage infection, antibiotics, heat, extreme pH and nutrient deprivation often act as environmental stressors for *E. coli* (8-9). In the presence of sublethal doses of antibiotics, such as ampicillin and gentamicin, *E. coli* and other related bacteria increase the production of RpoS, a RNA polymerase sigma factor responsible for regulating genes necessary for the

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general stress response (10-11, 13, 22). Specifically, the stress response increases *rpoS* transcription and translation while inhibiting RpoS degradation. In addition, exposure of *E. coli* cells to sublethal concentrations of antibiotics can lead to disruption of molecular processes and affect cell growth by generating toxic reactive oxygen species (ROS) (12-13, 22). Accumulation of ROS such as peroxide and superoxide can lead to oxidative stress in bacterial cells. Two of the primary regulators activated during oxidative stress include OxyR and RpoS (14). OxyR, a 34 kDa protein, is the principal regulator for hydrogen peroxide detoxification in *E. coli* cells (14). Under oxidative stress conditions, OxyR controls a regulon of almost 40 genes which protect *E. coli* cells from hydrogen peroxide toxicity, heat stress, and UV radiation (14). Furthermore, OxyR activity is increased in the presence of ampicillin, gentamicin, and norfloxacin (15). This is found to have downstream effects on the promoters involved in the oxidative stress response pathway and results in changes to the redox state of the intracellular environment, which protects the cell against oxidative damage (12). The regulons controlled by RpoS and OxyR are important for the general stress response and protection against oxidative stress in *E. coli*. Although there may be overlapping regulation of certain genes, RpoS and OxyR can function independently of each other as well (14).

Previous studies have suggested that both RpoS and OxyR may play important roles in delaying T7 bacteriophage-mediated cell lysis following exposure to sublethal concentrations of antibiotics by triggering protective changes in the cell physiology and metabolic activities (8, 13). The role of RpoS and OxyR in enhancing phage resistance of *E. coli* following sublethal treatment with antibiotics is not well understood and needs further investigation.

Both RpoS and OxyR are stress response regulators which may induce the activation and expression of downstream genes involved in membrane synthesis upon exposure to environmental stressors (13). These downstream genes may allow the cells to maintain a high level of membrane integrity during a T7 bacteriophage infection. Some of these activated genes are also involved in energy metabolism and translational capacity (13). This provides the cells with the appropriate energy and enzymatic resources necessary to carry out protective functions under the stress response. Sublethal concentrations of antibiotics have been shown to increase *rpoS* and *oxyR* expression in *E. coli*, which can regulate the production of the colanic acid capsule and outer membrane vesicles (OMVs) (26, 27, 28). Increased capsule and OMV production in *E. coli* following exposure to sublethal concentration of antibiotics can potentially protect against T7 bacteriophage infection by shielding host cell LPS, which is needed for viral attachment. These observations can potentially explain the ability of *E. coli* cells treated with sublethal doses of antibiotics to better withstand the lysis attempts of the bacteriophage as compared to an untreated control (8, 29, 31).

We hypothesized that both RpoS and OxyR are necessary for the delayed T7 bacteriophage-mediated cell lysis in *E. coli* strain BW25113 following exposure to sublethal concentrations of antibiotics. We expected that mutant strains of *rpoS* and *oxyR* infected with T7 bacteriophage after exposure to sublethal concentrations of antibiotics will lyse earlier as compared to the WT BW25113 *E. coli* strain. The aim of our project is to investigate the role of RpoS and OxyR in delaying T7 bacteriophage-mediated cell lysis following exposure to sublethal concentrations of antibiotics by analyzing the time required for the onset of lysis of *E. coli* BW25113 wild-type (WT) strain and mutant single-knockout *ΔrpoS* and *ΔoxyR* strains.

METHODS AND MATERIALS

Bacterial Strains and Growth Condition. *E. coli* BW25113 wild-type strain, JW5437-1 *rpoS* knockout (*ΔrpoS)*, and JW3933-3 *oxyR* knockout (*ΔoxyR*) strains were obtained from the Microbiology and Immunology Department at the University of British Columbia. Both mutant strains contained a kanamycin resistance gene in their genome replacing the corresponding knockout genes. The Lysogeny Broth (LB) media was prepared using the Hancock protocol (19). All three strains were grown on LB agar plate or in liquid LB at 37℃. The LB media used to initially propagate the knockout strains was supplemented with 50 ug/mL of kanamycin.

Primer Design, *ΔrpoS and ΔoxyR* Confirmation, and T7 Bacteriophage Validation. Bacteriophages T7 and T4 stocks were obtained from the Microbiology and Immunology

Department at the University of British Columbia. The T7 was the bacteriophage of interest, whereas the T4 bacteriophage was used as a control to check for contamination in the T7 stock. Validation of T7 and the two mutant bacterial strains was achieved using polymerase chain reaction (PCR). The *rpoS* and *oxyR* gene sequences (along with 1 upstream and 1 downstream gene sequence) were downloaded from NCBI. Primer pairs (forward and reverse) were designed to flank the start and stop codons of *rpoS* and *oxyR,* amplifying the region containing the kanamycin resistance cassette. Primers were designed using the SnapGene® software (Table S2) and were subsequently acquired from Integrated DNA Technologies® (IDT). Primer pairs for T7 and T4 bacteriophage were obtained from the Microbiology and Immunology Department.

Genomic DNA of the *ΔrpoS* and *ΔoxyR* strains were extracted using PureLink™ Genomic DNA Mini Kit (Invitrogen™ Cat. K182001). Each 50uL PCR reaction contained final concentrations of 1X PCR Buffer, 1.5mM of $MgCl_2$, 0.2mM of dNTPs, 0.4uM of forward and reverse primers, 5 units/reaction of Platinum™ Taq DNA polymerase (Invitrogen™ Cat. 10966018). Each of the PCR reactions contained 5uL of either bacterial genomic DNA (<1000ng) or 5uL bacteriophage stock solution. pUC19 plasmid was used as positive control to ensure that the PCR conditions were working. Negative controls without DNA templates were also included in the PCR experiment. Reaction component concentrations were adapted from New England Biolabs® (20).

The T100™ thermocycler (Bio-Rad Laboratories) was programmed to begin at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 68°C for 1 minute (final extension at 68ºC for 5 minutes), and hold indefinitely at 4-10ºC. The PCR products (18uL) were loaded on a 1.5% agarose gel containing SYBR® Safe DNA gel stain in 0.5X TBE buffer (Invitrogen) with 2uL of 10X BlueJuice™ Gel Loading Buffer (Invitrogen). DNA gel electrophoresis was run in 1X TBE buffer at 120V for 45 minutes. Amplicons were visualized and imaged with UV light using AlphaImager® (ProteinSimple).

Growth Curve Generation. Overnight cultures (in 5 mL of LB) of *E. coli* WT, *ΔrpoS*, and *ΔoxyR* strains were each diluted 1:100 in an Erlenmeyer flask containing 50 mL of pre-warmed LB and incubated at 37°C on a shaking platform at 200 rpm. Optical density (OD_{600}) of each culture was measured at 30-minute intervals until three consecutive readings had approximately the same OD_{600} values. The OD readings were taken using a Beckman spectrophotometer with a cuvette containing 1 mL of the bacterial culture.

T7 Bacteriophage Propagation and Titer Determination. An overnight culture of *E. coli* wild-type strain was diluted 1:100 in an Erlenmeyer flask containing 100 mL of LB media and was incubated at 37℃ until the OD_{600} reading was over 0.3. The culture was then inoculated with 100 uL of T7 bacteriophage stock and re-incubated until clearing was observed. The culture was centrifuged at 2,750 x g at 4℃ for 10 minutes and the supernatant was filtered through a 0.22 um syringe filter to remove contamination and to create the new T7 bacteriophage stock solution.

The titer of the stock solution was determined via the double-overlay plaque assay (17,18). The stock was diluted to final dilutions of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} in 5mM $CaCl_2$. Before adding 3 mL of soft agar, a 100 uL aliquot of stationary *E. coli* wild-type culture was added to each of the 100 uL of bacteriophage dilutions. The equilibrating temperature for the soft agar was maintained at 60ºC to prevent its solidification prior to its use instead of the 46ºC suggested in the protocol adapted from Ryken et al. (18) which resulted in premature solidification of the agar. Solidified double-layer agar cultures were incubated overnight at 37℃, and the plaques were counted.

Minimum Inhibitory Concentration (MIC) assays of ampicillin, kanamycin, and gentamicin for *E. coli* WT, *ΔrpoS*, and *ΔoxyR* strains. The MIC assay protocol was adapted from Lu et al. (16). *E. coli* wild-type, *ΔrpoS*, and *ΔoxyR* strains were grown overnight in 5 mL antibiotics-free LB media, followed by a 1:100 dilution in a new 5 mL antibiotics-free LB media until an OD_{600} of 0.3-0.6 were reached.

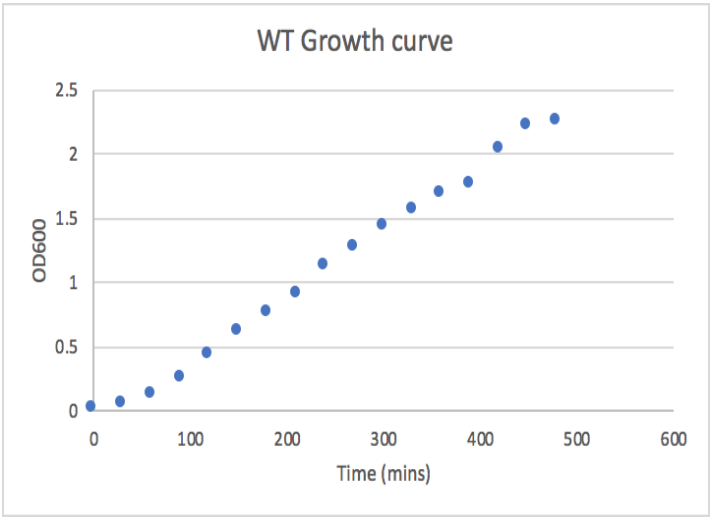
Eleven serial dilutions from stock ampicillin, kanamycin, and gentamicin solutions (100mg/mL) ranging from 0.125 ug/mL to 128 ug/mL, as well as a zero-concentration were used to determine the MIC for each antibiotic in all three strains of *E. coli*. Following the antibiotics dilution (100 uL) in a 96-well plate, 5 uL of diluted cell culture (10^5 CFU/mL) of each *E. coli* strain were added to the corresponding wells, and the plates were incubated overnight at 37℃ for 24h. The cell growth in each plate was measured by determining the OD_{600} using the Epoch™ Microplate Spectrophotometer (BioTek®). The MIC of each antibiotic was determined by using the lowest concentration of antibiotic that had an equal or lesser OD_{600} value compared to the OD_{600} of the wells with the negative control. The sublethal concentration of each antibiotic is defined as half of the MICs.

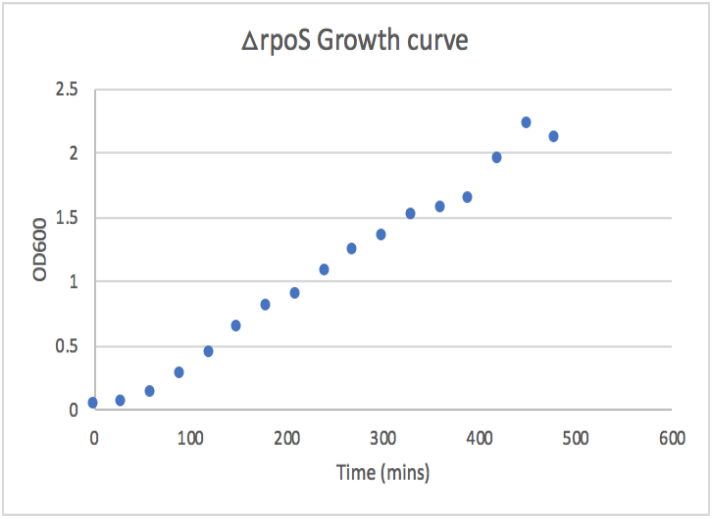
T7 Phage infectivity assay. A 5 mL O/N culture was prepared for each of the WT, *ΔrpoS,* and *ΔoxyR* strains. The next morning, fresh LB was inoculated with a 1/10 dilution (500 uL) of the O/N cultures and sublethal concentrations of ampicillin or gentamicin. These cultures were grown for 2 hours to mid-log phase and normalized to an OD_{600} of 0.3 using LB. Three 96-well plates were used to perform nine replicates with each replicate containing 90 uL of the WT, *ΔrpoS,* and *ΔoxyR* outgrowth cultures plus 10 uL of T7 phage. The phage stock was diluted such that a multiplicity of infection of 0.1 was achieved when adding the 10 uL of T7 phage. The WT cultures were grown with 0.5, 1, 2, 4, and 8 ug/mL of ampicillin. Reduced concentrations of 0.25, 0.5, 1, 2, and 4 ug/mL ampicillin were used for the *ΔrpoS* strain and were further reduced to 0.25, 0.5, 0.75, 1, and 1.25 ug/mL for the *ΔoxyR* strain. These ranges were chosen based on the values obtained during the ampicillin MIC assay for these strains. A limited range of 0.25, 0.5, and 1 ug/mL of gentamicin was used for all 3 strains. This was determined based on the MIC assay results as well as preliminary ampicillin data showing that higher concentrations of antibiotics inhibited growth. Various controls were included in each plate with each well containing a final volume of 100 uL (Figure S1). The full set-up of one such 96-well plate is shown in Figure S1. Finally, the Epoch™ microplate reader was used to take a t = 0 mins OD_{600} reading. Readings were taken every 10 mins and the plates were incubated at 37℃.

RESULTS

Growth curves of *E. coli* BW25113 WT, *ΔrpoS*, and *ΔoxyR* strains. The growth curves for the *E. coli* BW25113 WT, *ΔrpoS*, and *ΔoxyR* strains were generated to allow for the approximation of growth stage time points in future infectivity assay experiments. The growth curves obtained for all 3 strains were quite similar in both shape and values at each time point (Figure 1). All three strains were in exponential phase from 0 to 420 minutes and reached stationary phase after 450 minutes. The WT and *ΔoxyR* strains both grew to an OD_{600} value of 2.2 after 480 minutes while the *ΔrpoS* strain reached an OD_{600} value of 2.1 within the same time period. However, the OD_{600} readings of *ΔoxyR* strain seemed to lag behind the WT and *ΔrpoS* strains by about 30 mins until about 270 mins into the growth time points. There was a large increase in OD_{600} values from 1.772 to 2.040, 1.820 to 2.152, and 1.634 to 1.956 for the WT, *ΔoxyR* and *ΔrpoS* strains, respectively, between the 390 and 420 min readings. The OD_{600} readings for all three strains increased at approximately the same rate throughout the duration of the assay.

PCR analysis of *E. coli* BW25113 WT, *ΔrpoS, ΔoxyR* strains and T7 bacteriophage. A PCR analysis of *E. coli* BW25113 WT, *ΔrpoS, ΔoxyR* strains and T7 bacteriophage was performed to verify that the mutations were present in the single knockout strains and to confirm the purity of the T7 bacteriophage prior to its use in future experiments. The T7 lane contained both the T7 and T4 primers to validate the presence of T7 DNA, while also detecting for contamination from T4 DNA (Figure 2). The T4 lane had a similar set up to the T7 lane. One band was observed on each of the T7 and the T4 lanes, at 295 bp and 398 bp, respectively. The *ΔoxyR* and *ΔrpoS* lanes contained the appropriate primers and the DNA template from the respective mutant stains. One band was observed in each of the *ΔoxyR* and *ΔrpoS* lanes, at 918 bp and 993 bp, respectively. The pUC19 was used as a positive control to ensure that the PCR conditions and reagents were working. Two bands were observed in pUC19 lane, at ~100 bp and 2686 bp, respectively. The (-)T7 and (-)T4 lanes contained all





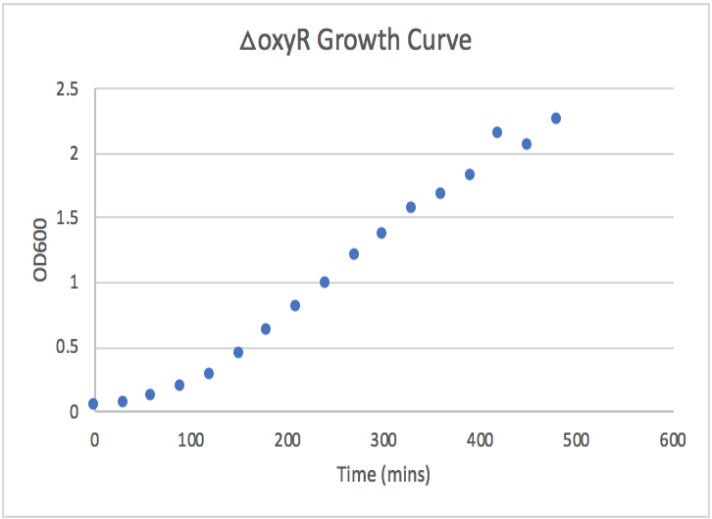


FIG. 1 Growth curves for the BW25113 WT, *ΔrpoS*, and *ΔoxyR* strains in LB media at 37°C (n=1).

the PCR reagents and the primers, but no T7 and T4 bacteriophage DNA. Faint bands were observed in the (-)T7 and (-)T4 lanes, at ~300 bp and ~400 bp respectively. The (-)*ΔrpoS*, and (-)*ΔoxyR* lanes contained the appropriate primers but no DNA template from the respective strains to check for contamination and primer dimer formation. Faint bands, at <100bp, were observed in each of the (-)*ΔrpoS*, and (-)*ΔoxyR* lanes were most likely due to the presence of DNA loading buffer. The absence of the band representing T4 in the T7 sample lane indicates that T7 is free from T4 contamination. The PCR result validates the two mutant strains which contain the kanamycin resistance cassettes in place of the *rpoS* and *oxyR* genes, respectively.

Phage titer for T7 bacteriophage was determined. The phage titer for T7 bacteriophage was performed to determine the plaque forming unit (PFU) per mL of the working stock, which allowed for inoculation of *E. coli* at the desired multiplicity of infection (MOI). All of the lower dilution plates ($>10^{-5}$) displayed complete clearing due to high number of viruses as such no plaque counts were obtained for these plates. The plate with 10^{-5} dilution contained 210 plaques and had a 2.1×10^{8} PFU/mL (Table 1). There were 38 plaques and 3 plaques counted in the plates with 10^{-6} and 10^{-7} dilutions, respectively. The average PFU/mL was calculated for each dilution to determine the T7 bacteriophage titer. The determined T7 bacteriophage titer was 3×10^{8} PFU/mL, which was within the satisfactory range needed to perform future experiments.

TABLE 1 Determining phage titer for T7 bacteriophage

Plaques Counted	Dilution Factor	Volume Plated (uL)	PFU/mL (x10^8)
210	10^{-5}	100	2.1
38	10^{-6}	100	3.8
3	10^{-7}	100	3
0	10^{-8}	100	N/A
			Average: 2.9

Sublethal concentrations of ampicillin, kanamycin, and gentamicin were determined for the BW25113 WT, *ΔoxyR* and *ΔrpoS* strains. Using a 96-well plate assay, the MICs of kanamycin, ampicillin, and gentamicin for the BW25113 WT, *ΔoxyR* and *ΔrpoS* strains were observed to determine the sublethal concentration of antibiotics to use in the phage infectivity assays. The MIC was defined as being the lowest concentration of antibiotics that resulted in an OD_{600} reading of equal to or less than that of the negative control (LB media only). The MIC values were determined by selecting wells where marked increases in readings were observed that were unlikely due to variations in the plate reader. For the WT strain, the MIC values for kanamycin, ampicillin, and gentamicin were 1, 2, and 1 ug/mL, respectively (Table 2). The *ΔrpoS* strain had MIC values of 128, 2, and 2 ug/mL for kanamycin, ampicillin, and gentamicin, respectively (Table 2). For the *ΔoxyR* strain, the MIC values were 128, 1, and 2 ug/mL for kanamycin, ampicillin, and gentamicin, respectively (Table 2).

The sublethal concentration of each antibiotic was defined as half of the MIC. For the WT strain, the sublethal concentrations for kanamycin, ampicillin, and gentamicin were 0.5, 1, and 0.5 ug/mL, respectively (Table 2). The *ΔrpoS* strain had sublethal concentration values of 64, 1, and 1 ug/mL for kanamycin, ampicillin, and gentamicin, respectively (Table 2). Finally, the sublethal concentration values for the *ΔoxyR* strain, were 64, 0.5, and 1 ug/mL for kanamycin, ampicillin, and gentamicin, respectively (Table 2). The sublethal concentrations of antibiotics for each strain were determined and used to grow overnight bacterial cultures for the phage infectivity assay.

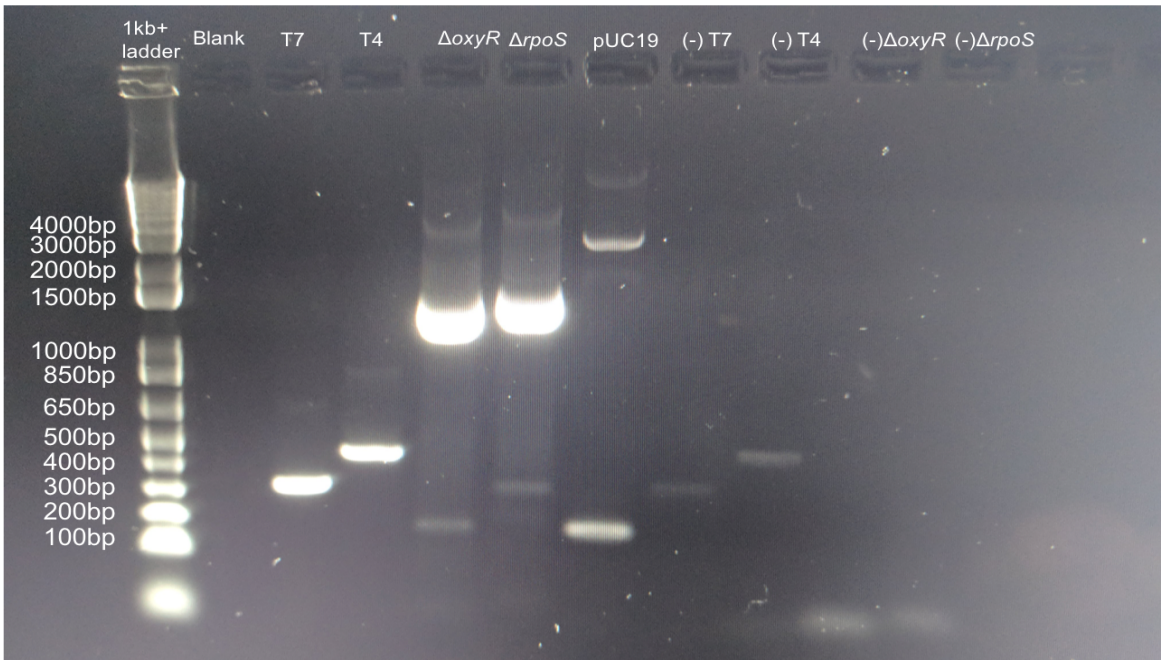


FIG. 2 Agarose gel electrophoresis analysis of PCR products from T7 and T4 bacteriophages, *ΔoxyR* and *ΔrpoS* strains.

TABLE 2 Minimum inhibitory concentrations (MIC) and sublethal concentrations of kanamycin, ampicillin, and gentamicin

	MIC (ug/mL)			Sublethal concentrations (ug/mL)		
	Kan	**Amp**	**Gent**	**Kan**	**Amp**	**Gent**
WT	1	2	1	0.5	1	0.5
ΔrpoS	128	2	2	64	1	1
ΔoxyR	128	1	2	64	0.5	1

***ΔrpoS* and *ΔoxyR* strains did not show a change in the onset or the rate of lysis compared to the WT following treatment with sublethal concentrations of antibiotics.** Phage infectivity assays were performed to determine the time taken for T7 bacteriophage to lyse *E. coli* BW25113 WT, *rpoS* mutant, and *oxyR* mutant strains following exposure to sublethal concentration of antibiotics. The positive control cultures containing only *E. coli* (no antibiotics and no phage) served to show the expected growth for all 3 strains during both the ampicillin and gentamicin infectivity assays (Figure 3 and 4). All cultures grown with phage at an MOI of 0.1 displayed a decrease in OD_{600} readings over time. For both, ampicillin and gentamicin, the OD_{600} readings of all three strains decreased when exposed to increasing concentrations of antibiotics (Figure 3). Compared to the control (phage and no antibiotics), the WT strain showed lower OD_{600} readings at ampicillin concentrations greater than 1 ug/mL (Figure 3). The *ΔrpoS* and *ΔoxyR* strains displayed this trend at ampicillin concentrations greater than 0.5 ug/mL (Figure 3). Increasing the concentration of ampicillin had no effects on the onset of lysis, or the rate of lysis in the WT and *ΔrpoS* strains (Figure 3). A delay in the onset of lysis was observed in the *ΔoxyR* strain when grown in 0.25 ug/mL of ampicillin (Figure 3). The onset of lysis was observed at approximately 30, 40, and 50 mins for the *ΔoxyR, ΔrpoS,* and WT strains respectively during the ampicillin infectivity assay.

Based on the data from the MIC and ampicillin infectivity assays, the gentamicin infectivity assay was conducted using 0.25, 0.5, and 1 ug/mL of gentamicin for all three strains. However, growth of the WT and *ΔoxyR* strains were not negatively affected when cultured at 0.5 ug/mL of gentamicin. Data could not be obtained for the *ΔrpoS* strain with gentamicin since growth was not observed during the outgrowth stage prior to the assay. For the gentamicin infectivity assay, both the WT and *ΔoxyR* strains displayed an onset of lysis around 50 minutes when grown in 0.5 ug/mL gentamicin and phage (Figure 4).

In conclusion, no marked difference in the onset of lysis was observed between the WT, *ΔrpoS,* and *ΔoxyR* strains during the ampicillin infectivity assay, and similar results were obtained for the WT and *ΔoxyR* strains in the gentamicin infectivity assay.

DISCUSSION

Previous studies have found that the treatment of *E. coli* UB1005 strain with sublethal concentrations of gentamicin delayed lysis of *E. coli* by T7 bacteriophage (29, 31). It has also been observed that treatment of *E. coli* B23 strain with sublethal concentrations of penicillin, ampicillin, gentamicin, and kanamycin delayed T7 bacteriophage mediated lysis in the host cell (8). Mathieu et al. demonstrated that sublethal antibiotic treatments induce a general stress response in *E. coli*, which protects the bacteria against subsequent exposure to other stressors (13). Furthermore, it has been shown that sublethal concentrations of ampicillin and gentamicin increase the expression of *rpoS* and *oxyR*, which are key regulators of the bacterial stress response (13). Based on these observations, the focus of this research study is to investigate the role of RpoS and OxyR in delaying T7 bacteriophage-mediated cell lysis in *E. coli* BW25113 strain following exposure to sublethal concentrations of ampicillin and gentamicin.

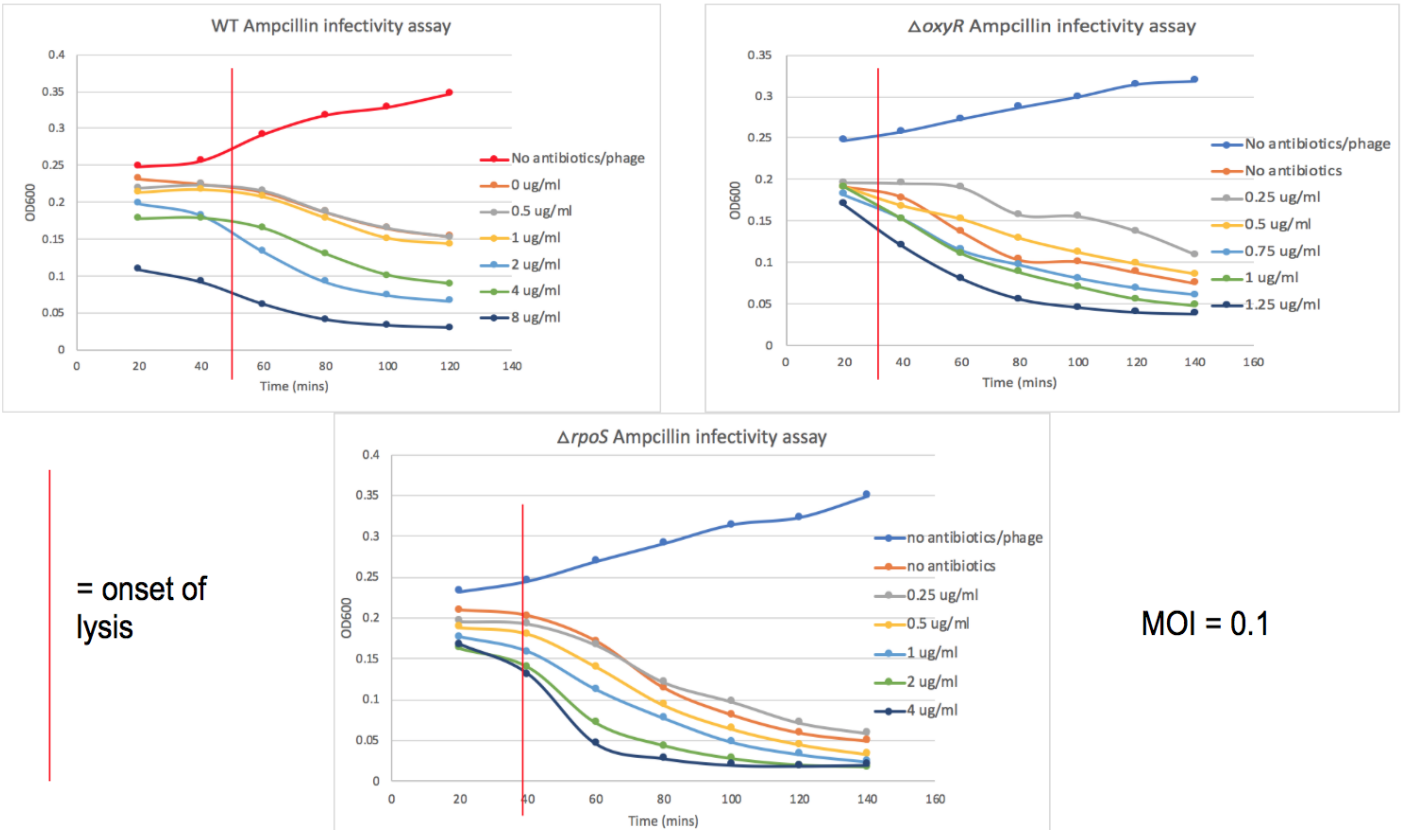


FIG. 3 96-well plate infectivity assay for *E. coli* WT, *ΔrpoS*, and *ΔoxyR* strains with increasing sublethal concentrations of ampicillin. Onset of T7 phage lysis was defined as a negative slope in the growth curves.

E. coli have developed different mechanisms to protect against bacteriophage infection and lysis following exposure to sublethal concentration of antibiotics (30). We hypothesized that both RpoS and OxyR are necessary for the delayed T7 bacteriophage-mediated cell lysis in *E. coli* strain BW25113 following exposure to sublethal concentrations of antibiotics. To test our hypothesis, growth curves for both the WT and mutants strains were generated to allow for the approximation of growth stage time points in future experiments. All three strains of *E. coli*, including the WT and two mutant strains, had growth curves similar in shape and comparable OD_{600} values at given time points. This indicates that growth of the mutant strains was not affected compared to the wild-type *E. coli*. The increase in OD_{600} readings for all 3 strains between the 390 and 420 mins readings was likely due to intrinsic variations in readings from the spectrophotometer. This was further supported by a similar pattern of fluctuation in the final three time points for all three strains. These data points did not affect the results of our downstream experiments as they occurred around the stationary phase of the growth curves.

The ampicillin infectivity assay showed that there was no delay in lysis in the *ΔrpoS,* and *ΔoxyR* mutant strains as the concentration of antibiotics was increased. This result was consistent with our hypothesis since the *ΔrpoS* and *ΔoxyR* mutants do not express RpoS and OxyR, respectively, which are thought to play a role in producing the stress response and therefore, delayed lysis. The *ΔoxyR* strain grown with 0.25 ug/mL of ampicillin was the one divergence from this pattern. We propose this is possibly due to the expression of RpoS compensating for the absence of OxyR in this strain and possibly resulting in delayed lysis. Ivanova et al. have previously shown evidence of this compensation effect occurring in *E. coli* (33). Downstream genes regulated by OxyR during the stress response may also be activated by RpoS, which protects the *ΔoxyR* strain against oxidative stress and contributes to delayed lysis (14). However, during the gentamicin infectivity assay, the *ΔoxyR* strain did not show delayed lysis compared to WT strain following exposure to 0.5 ug/mL of

gentamicin. Our data suggests that the mechanism of ampicillin action, not gentamicin, may be involved in activating RpoS pathways, which offers protection to the ΔoxyR strain against T7 bacteriophage lysis. A western blot can be performed to determine whether RpoS is being expressed in the *ΔoxyR* strain at sublethal concentrations of ampicillin and gentamicin.

In addition, the absence of delayed lysis in the WT strain with increasing concentrations of antibiotics was unexpected. This may be due to pre-existing basal expression levels of OxyR and RpoS causing delayed lysis in the absence of antibiotics. Thus, additional stress from the antibiotics did not induce the stress response enough to further delay lysis. A western blot can be performed on the WT strain to determine if this is indeed the case. When comparing the onset of lysis in the WT strain to that in the *ΔrpoS* and *ΔoxyR* strains, a promising trend was observed. Lysis was delayed in the WT strain by 10 and 20 mins when compared to the *ΔrpoS* and *ΔoxyR* strains, respectively. This supports our hypothesis as it suggests that the RpoS and OxyR proteins both share a role in delaying lysis by T7 phage in the *E. coli* BW25113 strain. Unfortunately, due to an error in the plating of samples, plates containing the *ΔrpoS* and *ΔoxyR* strains were incubated with the phage prior to the initial t = 0 mins reading while the WT plate was still being set up. This error may have led to the false positive results indicating delayed lysis in WT strain when compared to the *ΔrpoS* and *ΔoxyR* strains. Furthermore, the error also explains the lack of peaks observed when plotting the data. Cells from all three strains were already seen to be in lysis stage from the first point of reading (at time = 20 mins) instead of first increasing in number as was expected from the start of the incubation period. However, it is also possible that delayed lysis was not seen in the *ΔrpoS* strain at various ampicillin concentrations due to the lack of colanic acid capsule production, which is regulated by RpoS and is thought to contribute to delayed lysis (26, 30). A repeat ampicillin infectivity assay was attempted; however, the outgrowth cultures were unable to grow in the sublethal concentrations that they had previously been able to grow in. These outgrowths were repeated a second time with a fresh batch of LB media and ampicillin, however they did not grow a second time either. It is unclear why these outgrowths were inhibited, but it is possible that the ampicillin stock solution was an incorrect concentration.

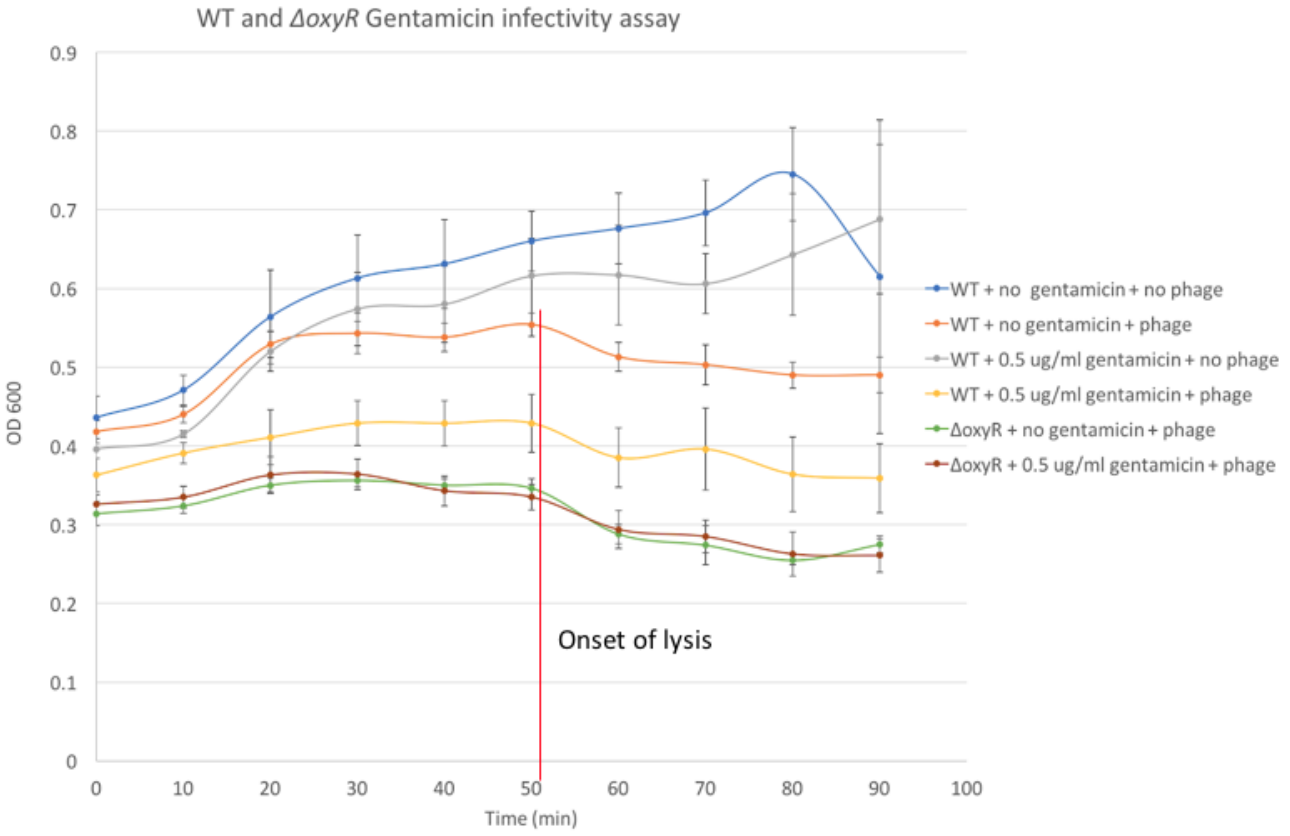


FIG. 4 96-well plate infectivity assay for *E. coli* WT, and *ΔoxyR* strains with 0.5 ug/mL of gentamicin. Onset of T7 phage lysis was defined as a negative slope in the growth curves. Multiplicity of infection (MOI) = 0.1

Both the WT and *ΔoxyR* strains began lysing at near exact times at ~50 mins during the gentamicin infectivity assay. The rate of lysis (seen by comparing the relative slopes of the graphs) in both of these strains was also near identical. No data was obtained for the *ΔrpoS* strain with gentamicin as the cells never reached the mid-log phase during the outgrowth stage. Multiple explanations are possible for why this occurred and include but are not limited to: erroneous dilution of antibiotic stock, *ΔrpoS* cell stock was too old, and an error in the determined MIC values for this strain with gentamicin. Nonetheless, the data for the WT and *ΔoxyR* strains supports the idea that the results seen during the initial ampicillin infectivity assay are a false positive and in fact, there is no delayed lysis seen in WT cells due to exposure to sublethal concentrations of antibiotics. Further repetitions of these experiments need to be erformed in order to validate the results obtained as there were large variations in the OD_{600} readings between replicates. Additionally, gentamicin infectivity assay data needs to be obtained for the *ΔrpoS* mutant.

Various studies in the past have demonstrated the presence of delayed lysis in *E. coli* cells after exposure to sublethal concentrations of antibiotics (8, 29, 31). Although different models have been proposed to explain the delayed lysis, there is no consensus in the literature on the exact mechanisms that underlie this phenomenon (29, 31). Through the use of *ΔrpoS* and *ΔoxyR* mutants, we aimed to elucidate the role of these stress response regulators in delaying T7 bacteriophage mediated cell lysis following exposure to sublethal concentrations of antibiotics. The WT and *ΔrpoS* strains grown under a range of sublethal ampicillin concentrations showed no evidence of delayed lysis. However, the *ΔoxyR* mutant grown under 0.25 ug/mL of ampicillin displayed a late onset of lysis and a slower rate of lysis. This could be due to the activity of RpoS compensating for the lack of OxyR and warrants further investigation. Finally, the WT and *ΔoxyR* mutants grown under sublethal concentrations of gentamicin displayed similar times for onset of lysis and rate of lysis thus implying a lack of delayed lysis under these conditions.

Future Directions. The large variation in OD_{600} readings between replicates warrants that the infectivity assays for both ampicillin and gentamicin are repeated in order to obtain a larger sample size. The larger sample size will allow for a more robust analysis of the data, from which more reliable conclusions can be drawn in regards to the presence of delayed lysis. It will also help determine if the variation in OD_{600} readings are due to experimental and instrumental errors or if the lack of *rpoS* and *oxyR* expression are responsible for the differences in the onset of lysis. Different stressors, such as lack of nutrients, low levels of O_2, temperature, and pH, can activate both the general and oxidative stress responses through RpoS and OxyR, respectively (8, 9). These stressors can be hard to control for during bacterial growth in the WT strain. Thus, it is possible that *rpoS* and *oxyR* may be constitutively expressed even in the absence of sublethal doses of antibiotics. This constitutive expression can contribute to delayed lysis in the control condition of *E. coli* grown with phage in the absence of antibiotics. This theory can be tested by performing a western blot to determine the amount of RpoS and OxyR present in the WT strain pre and post exposure to sublethal concentrations of antibiotics. Based on previous findings, it is expected that the expression of RpoS and OxyR will increase as sublethal concentration of antibiotics is introduced (13). Ivanova et al. have shown that RpoS is capable of protecting *E. coli* against oxidative damage by compensating for the lack of OxyR in a *ΔoxyR* mutant (33). This warrants further investigation and can potentially explain why the *ΔoxyR* mutant strain did not show delayed lysis during the gentamicin infectivity assay. This can be elucidated by performing ampicillin and gentamicin infectivity assays on a double knockout (*ΔrpoS* and *ΔoxyR*) mutant. This will help determine whether RpoS and OxyR share similar pathways that might play a role in delaying T7 bacteriophage-mediated cell lysis. Our experiments were performed with *E. coli* BW25113 strain, which is different from the *E. coli* B23 and UB1005 strains for which reduced T7 bacteriophage infectivity following antibiotic exposure was observed (8, 29, 31). Future experiments should be conducted using *rpoS* and *oxyR* mutants for the *E. coli* B23 and UB1005 strains.

Different models have been proposed in how exposure to sublethal concentrations of antibiotics can increase resistance to phage infectivity and lysis in *E. coli* cells (8, 29, 31).

Hardman et al. found that the release of soluble LPS did not decrease T7 bacteriophage infectivity following exposure to sublethal concentration of antibiotics (29). However, it is hypothesized that a factor may be released from the host cell which decreases its susceptibility to T7 bacteriophage infection following treatment with sublethal doses of gentamicin (29). OxyR is a transcriptional activator of three genes associated with OMV phenotypes (28). Formation of OMVs can prevent T7 bacteriophage from attaching to *E. coli* host cells. Future experiments can investigate whether sublethal concentration of antibiotics increase *oxyR* expression and how this affects OMV formation. RpoS has been involved in the transcription of genes needed for colanic acid capsule formation in *E. coli* (26). Increased capsule formation can interfere with T7 bacteriophage binding to host cells and thereby contribute to delayed lysis (30). Future studies should investigate whether sublethal doses of antibiotics increased colanic acid capsule formation in *E. coli* cells and whether this contributes to decreased T7 bacteriophage infection.

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