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Review Article

Gentamicin-induced protective effects against T7 bacteriophage infection in *Escherichia coli*

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SUMMARY The rapid growth of antibiotic-resistant *Escherichia coli* globally highlights the need to develop novel antimicrobial treatments including phage therapy or combination therapies. To further understand the combined dynamics between bacteriophage and antibiotics on bacterial survival, past studies have found that *E. coli* exhibit an increased resistance to T7 bacteriophage when exposed to sub-inhibitory levels of gentamicin, a common antibiotic treatment for *E. coli* infections. However, the gentamicin-induced mechanism of resistance to phage remains to be elucidated. Here, we review current research and its limitations regarding this phenomenon and the potential cellular mechanisms by which it occurs, including the gentamicin-induced secretion of outer membrane vesicles, release of soluble lipopolysaccharides, RpoS-mediated stress responses, and increase in capsular polysaccharide formation. We conclude that soluble lipopolysaccharides and RpoS are likely not involved in gentamicin-induced phage resistance, while past studies have not conclusively supported the roles of outer membrane vesicles and capsular polysaccharides. The conflicting evidence for several extracellular mechanisms suggests that it may be useful to consider possible intracellular processes such as ribosomal inhibition or toxin-antitoxin systems. Moreover, limitations and variability in past studies should be addressed in follow-up experiments. With this retrospective, we emphasize the importance of future research in narrowing the scope of potential mechanisms and thus discuss methods to identify the subcellular localization of these protective factors.

INTRODUCTION

With the increasing emergence of antibiotic-resistant bacterial strains which cause more than 2.8 million infections and at least 35,000 deaths annually in the United States alone, according to the Centers for Disease Control and Prevention (https://www.cdc.gov/media/releases/2019/p1113-antibiotic-resistant.html), developing an alternative solution to tackle these microbes is of urgent need. As a result, the use of bacteriophage alone or combined with antibiotics as an alternative treatment has become of great interest (1). Bacteriophage treatment has many advantages. They are less disruptive to the normal microbiota due to their high single-species specificity, have reduced side effects compared to antibiotics, and are self-limiting in replication potential (1). On the other hand, bacteria can evolve resistance to multiple phages, thereby limiting the long-term sustainability of the use of phage therapies (2). To counter this, multiple studies have shown that combined antibiotic-bacteriophage therapy could produce a synergistic effect that would not only increase bacterial susceptibility but also reduce the emergence of bacterial strains resistant to antibiotic and/or phage (2, 3). As such, combined therapy may be a promising alternative to the sole use of antibiotics. However, current researches have shown that this synergistic action was dependent on essential factors such as the combination of phage, antibiotic, and bacterial strain used and antibiotic concentration (1).

Escherichia coli is a Gram-negative bacterium that colonizes human gastrointestinal systems as part of the normal microbiota. Some strains of *E. coli* possess virulence factors that give rise to severe diseases such as diarrhea, urinary tract infections, pneumonia, and meningitis (1). The development of antibiotic resistance among these strains is currently increasing and responsible for many serious infections in community environments (1).

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One previous study reported that the combined use of ciprofloxacin and ELY-1 phage to target *E. coli* TOP10 showed greater efficacy in reducing bacterial load compared to the use of single treatments (1). However, when other studies tested the combined effect of aminoglycoside antibiotics and T7 phage on *E. coli* UB1005, the opposite was observed - sub-lethal concentrations of aminoglycosides reduced *E. coli* UB1005's susceptibility to T7 phage (4).

The T7 phage, one of the more well-studied types, is a lytic bacteriophage that infects Gram-negative bacteria by adhering to lipopolysaccharide (LPS), injecting its genome into the cell, and self-replicating using host-cell machinery. The host is subsequently lysed by viral-encoded proteins, allowing for further propagation of the phage (5). Aminoglycosides are a broad-spectrum class of concentration-dependent bactericidal antibiotics, including streptomycin, kanamycin, and gentamicin, that are active against both Gram-positive and negative bacteria. This class of antibiotics is commonly used to treat *E. coli* due to its high potency against the species (6). For Gram-negative bacteria, the polycationic aminoglycosides bind to the negatively charged LPS to initiate cellular uptake. Once in the cell, they inhibit protein synthesis by binding to the 16S ribosomal RNA of the ribosome in bacteria with high affinity and altering its conformation; different members of the class bind to different regions of the A-site. As a result, incorrect amino acids are incorporated into the polypeptide, preventing essential proteins synthesis (6). Characterizing the specific mechanism by which aminoglycosides confer *E. coli* protection against T7 phage is important for the development of combined therapies by improving our understanding of antibiotic-phage interaction and determining effective antibiotic-phage pairings.

Many studies have been done to understand the protective effect of aminoglycoside pre-exposure to T7 phage infection in *E. coli*, with a focus on gentamicin due to its association with outer membrane vesicles (OMVs) and capsular polysaccharides (4, 9). These studies mainly focused on four proposed mechanisms, namely gentamicin-induced OMV secretion, release of soluble LPS, increase in RpoS response, and increase in capsular polysaccharide formation. The first proposal was based on a previous observation that gentamicin treatment increased the release of lipid vesicles from the outer membrane as OMVs, which could potentially act as decoys to inhibit T7 phage attachment to the *E. coli* membrane, thereby preventing its entry (4). The second proposal was of a similar theory, except it involved the induced production of free, soluble LPS instead of OMVs that would bind to T7 phage (7). The third proposal hypothesized that increased RpoS response, an immune regulon known to introduce resistance in *E. coli* in response to antibiotic stress, is responsible for reduced susceptibility to T7 phage (8). Lastly, the fourth proposal suggested that gentamicin-induced capsule formation prevents T7 phage-bacterium entry because colonic acid interferes with phage binding to LPS (9). This review will be exploring the details of the observed protective effect of gentamicin on phage infection as well as evaluating studies that had been done on the proposed mechanisms.

Treatment of *E. coli* with aminoglycoside antibiotics could confer T7 bacteriophage resistance. Initial observations on how exposure to sub-inhibitory levels of aminoglycosides confers increased resistance to T7 phage infection in *E. coli* were described in several studies, namely those by Bleackley et al., Gu et al., and Hardman et al. Studies done by Bleackley et al. and Gu et al. originally intended to investigate the effect of aminoglycoside treatment on *E. coli* capsule formation. Based on previous observations that aminoglycoside treatment induced an increase in extracellular polysaccharide production, they hypothesized that aminoglycoside-treated *E. coli* would have reduced T7 phage infectivity because of increased capsule formation. In this model, the capsular molecule colonic acid blocks the binding of T7 phage to its host receptor LPS, leading to failed phage entry into the cells (7, 9). Plaque assays were performed to quantify T7 phage infectivity, stationary phase *E. coli* cells were either treated or not treated with sub-inhibitory concentrations of kanamycin and streptomycin for various durations, followed by incubation with T7 phage culture. The mixtures were added to tubes of molten phage top agar (PTA) and poured onto phage bottom agar (PBA) plates. As the phage propagated, it lysed the bacteria and resulted in clearing zones on a confluent lawn of bacteria, termed plaques. Each plaque indicated a single cell lysis event and can be used to quantify phage

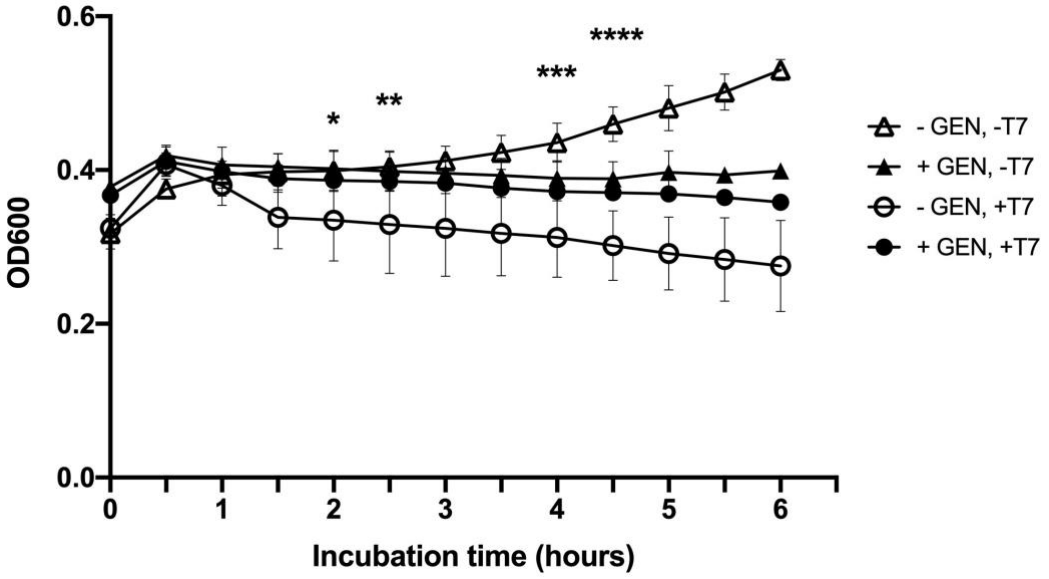

FIG. 1 Sub-inhibitory treatment of gentamicin reduces T7 phage infectivity. *E. coli* UB1005 was treated with 8 μg/mL gentamicin or a LB control for 1 hour and plated in triplicates on a 96-well plate. Phage and H2O control were added at an MOI of 10-2 and OD was measured every 0.5 hours for 6 hours. 2-way ANOVA test, $p < 0.05$; $n = 3$ for every time point in each group. * shows start time point of significant difference between +GEN, -T7 vs. -GEN, +T7; ** shows the start time point of significant difference between -GEN, -T7 vs. -GEN, +T7; *** shows start time point of difference between -GEN, -T7 vs. +GEN, +T7; **** shows start time point of difference between -GEN, -T7 vs. +GEN, -T7.

infectivity. Plates were incubated overnight, and plaques were enumerated the next day. Both studies observed a significantly lower number of plaques for *E. coli* that were treated with sub-inhibitory levels of kanamycin and streptomycin than those that were not treated (7, 9). Subsequent plaque assay experiments by Gu et al. involved the generation of *E. coli* mutants with defects in capsule formation to test whether the observed reduction in T7 phage infectivity in *E. coli* pre-exposed to streptomycin was indeed due to increased capsule production. They found no significant differences in plaque numbers between *E. coli* cells with and without capsules (9). Hardman et al.'s plaque assay experiments also observed the same effect on T7 phage infectivity when pre-exposing *E. coli* cells to gentamicin (10). Together, these results suggested that pre-exposure of *E. coli* to sub-inhibitory levels of aminoglycoside class of antibiotics, including kanamycin, streptomycin, and gentamicin confers *E. coli* increased resistance to T7 phage infection, and that the mechanism of action is likely not due to increased capsule formation.

Aminoglycoside-induced protection against T7 bacteriophage infection in *E. coli* is not due to a delayed onset of cell lysis mediated by phage. Three studies done by Beskrovnaya et al., Krystal et al., and Amanian et al. used a different approach to analyze the effect of exposing *E. coli* to sub-inhibitory concentrations of aminoglycosides prior to incubation with T7 phage. They hypothesized that pre-exposure to aminoglycosides would delay the onset of cell lysis events mediated by phage, which would contribute to an observed reduction in T7 phage infectivity in these cells. Consequently, plaque assays with these antibiotic-exposed cells would yield a lower number of plaques as seen in previous plaque assay experiments. As such, they performed lytic assays, where the absorbance readings of *E. coli* cells measured at different time points were plotted against the duration of time that had passed since incubation with T7 phage to generate a lytic curve. As incubation time with T7 phage increased, more cell lysis events would occur, and absorbance readings of *E. coli* cells would decrease. This allowed for observations that monitored how T7 phage mediate cell lysis would progress with time. For these studies, *E. coli* cells with and without pre-exposure to sub-inhibitory levels of aminoglycosides were cultured onto a 96-well plate. T7 phage were subsequently added and the OD600 absorbance readings of the cell culture were taken over time. If the tested hypothesis was

correct, *E. coli* cells pre-exposed to aminoglycosides would have had delayed onset of cell lysis by T7 phage compared to cells with no pre-exposure. Here, the onset of cell lysis was defined as a time point where absorbance values began to decrease (4, 8-9). Beskrovnaya et al.'s results showed no difference in time of the onset of cell lysis between cells with and without pre-exposure to kanamycin and gentamicin. However, the *E. coli* cells they used were in stationary phase, which could explain the lack of differences because later findings suggested that delayed onset of lysis was only seen in exponential phase *E. coli* (9). Similarly, Krystal et al. observed only minimal delay in lysis when cells were pre-exposed to gentamicin, although they did note the lack of confidence in this result due to inconsistency between trials. Lastly, even though Amanian et al. claimed to have observed differences in time points, their lytic curve contradicted their claim as the time by which the onset of cell lysis began was the same for *E. coli* with and without pre-exposure gentamicin. Moreover, they did not perform any statistical testing to evaluate whether there was a significant difference in time (4). To resolve these conflicting reports, we replicated the experiment performed by Amanian et al. Our results showed that there was no statistically significant difference in the onset of cell lysis between *E. coli* cells with and without pre-exposure to gentamicin, which began approximately 30 minutes after incubation with T7 phage (Fig. 1). Together, these findings suggested that the observed decrease in T7 phage infectivity after aminoglycoside pre-exposure was not due to delay in the onset of cell lysis. The speed by which T7 phage initiates cell lysis events was the same, and an alternative mechanism likely accounts for the later observed reduction in infectivity.

Decreased T7 bacteriophage infectivity of *E. coli* pre-exposed to gentamicin is dose dependent. Previous researchers reported that gentamicin treatment induced the formation of outer membrane vesicles (OMVs) in *E. coli* (4). Based on this observation, many of the later studies on how pre-exposure of aminoglycosides confer T7 phage protective effects in *E. coli were* performed exclusively with gentamicin; these studies had hypothesized that gentamicin-induced OMVs act as decoys by binding to T7 phage, thereby blocking its entry into cells by preventing its binding to host LPS (4). While no significant correlation between OMV production and reduced T7 phage infectivity was found, these studies

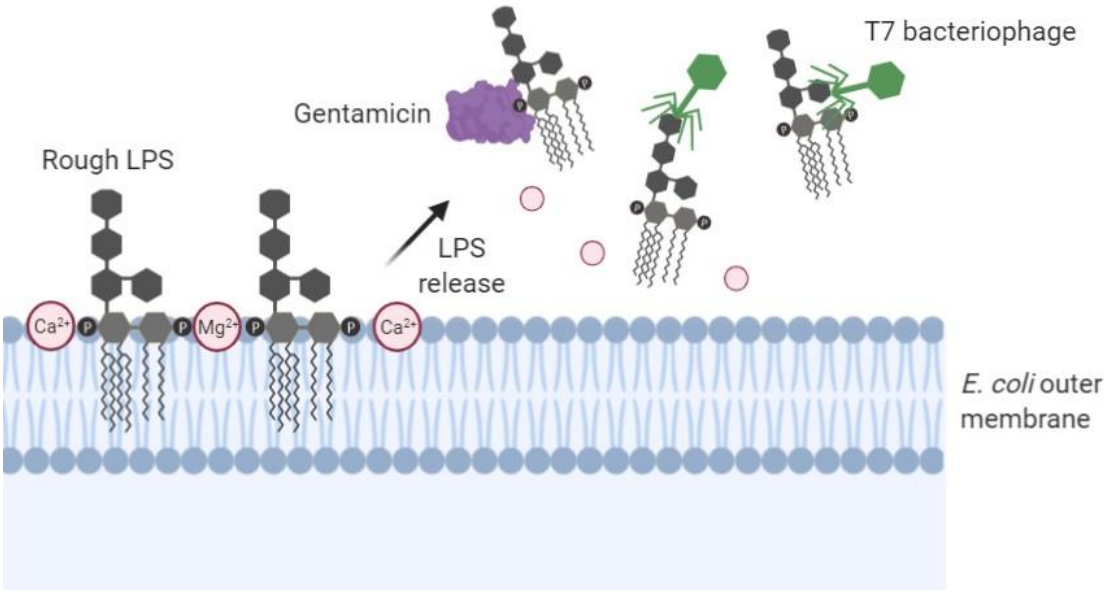

FIG. 2 Potential mechanism for T7 bacteriophage resistance in gentamicin-treated *E. coli*. Gentamicin has been proposed to confer bacteriophage resistance by binding to LPS and disrupting cation bridges, mediated Ca^{2+} and Mg^{2+}, that connect LPS to adjacent phospholipids in the *E. coli* outer membrane. This induces the release of LPS in the form of free, soluble polysaccharides that act as competitive receptors for T7 phage. The binding of T7 phages to extracellular LPS thus decreases the amount of phage that are able to attach to outer membrane-bound LPS for host entry, thereby reducing phage infectivity in *E. coli* cells.

identified that decreased T7 phage infectivity for *E. coli* pre-exposed to gentamicin was gentamicin-dose dependent. In a plaque assay done by Nagra et al., *E. coli* cells were exposed to various concentrations of gentamicin - 0, 0.5, 1.5, or 2.5μg/mL, prior to incubation with T7 phage. A similar number of plaques formed when *E. coli* was treated with 0, 0.5, and 1.5 μg/mL of gentamicin, but significantly fewer plaques were observed when *E. coli* was treated with 2.5μg/mL of gentamicin compared to the other 3 concentrations. Taken together, this suggested that a minimum sub-inhibitory concentration is required to observe gentamicin-conferred protective factor against T7 phage in *E. coli* (11). Amanian et al. observed similar results from a lytic curve, where the absorbance reading of cells incubated with 0, 4, or 8μg/mL decreased more with increasing concentrations of gentamicin used to treat *E. coli*. Ultimately, these results indicated that pre-exposing *E. coli* to increasing concentrations of gentamicin above a certain threshold may lead to decreasing T7 phage infectivity.

Protective effects of gentamicin on T7 phage infectivity is growth phase dependent. Gentamicin inhibits ribosomes, whereas T7 phage needs host translational machinery to synthesize essential proteins needed for its propagation (5, 6). Based on this, researchers hypothesized that gentamicin-induced inhibition of host ribosomal activity hinders T7 phage from using host ribosome to synthesize essential protein needed for its propagation (5, 6). As such, there would be reduced T7 phage infectivity for *E. coli* with previous exposure to gentamicin. To test this hypothesis, Amanian et al. compared the effect of gentamicin pre-exposure on T7 phage infectivity between stationary and exponential phase *E. coli* through a lytic curve. They predicted that stationary phase cells would show less T7 mediated cell lysis compared to exponential phase because they have lower rates of protein synthesis, thereby reducing T7 phage's ability to synthesize viral proteins essential for propagation. Results from the lytic curve contradicted their prediction, however, as stationary phase cells with and without pre-exposure to gentamicin both showed a more drastic decrease in absorbance than exponential phase cells with and without pre-exposure to gentamicin. T7 phage showed a higher infectivity against stationary than exponential phase *E. coli.* Furthermore, reduced T7 phage infectivity was only observed in exponential phase cells with pre-exposure to gentamicin when compared to exponential phase cells with no previous exposure. For stationary phase *E. coli*, no difference in absorbance reading was observed between cells treated with or without gentamicin at all time points (4). Together, these findings suggested that the protective effect of gentamicin was growth phase dependent and that lower ribosomal activity caused by gentamicin exposure was likely not the mechanism of action. However, there are some limitations given that the cells' stationary phase was used as a proxy for rather than as a direct measure of protein synthesis, although it has been made clear that gentamicin induced protective effects against T7 phage infection were not observed in stationary phase *E. coli.*

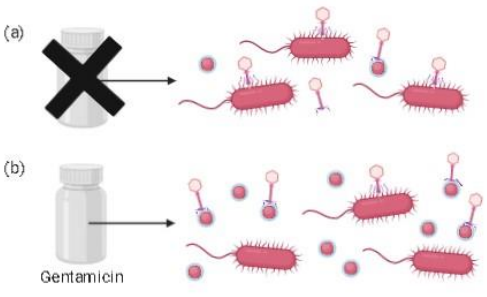

FIG. 3 Release of outer membrane vesicles as a mechanism for gentamicin-induced T7 bacteriophage resistance. (a) OMVs are constitutively secreted by Gram-negative bacteria, including *E. coli*. Basal production of OMVs is sufficiently low such that T7 bacteriophage retain high levels of infectivity. (b) Aminoglycosides (e.g., gentamicin) perturb membrane structure by displacing divalent cations, facilitating increased OMV production High concentrations of OMVs inhibit phage infectivity by acting as decoys for T7 binding. The presence of membrane co-receptors addresses limitations of earlier LPS models. Figure created in BioRender.

Free, soluble LPS is not sufficient to reduce T7 bacteriophage infectivity in *E. coli.* Numerous studies have shown that sub-lethal gentamicin treatment causes the release of extracellular free LPS and many have proposed that this effect was due to gentamicin destabilization of LPS on *E. coli* outer membranes (12-16). Cationic gentamicin binds to LPS and is suggested to trigger its release by competitively displacing Ca^{2+} and Mg^{2+} divalent cations, which form ion bridges between adjacent LPS and phospholipid molecules and are thus important for outer membrane stability (17). Although rough LPS is a main receptor for T7 phage attachment to *E. coli in vitro* and was speculated to be involved in gentamicin-induced resistance to T7 phage, Hardman et al. found evidence against the role of free, soluble LPS as a potential mechanism (7, 18). They initially believed that gentamicin exposure could induce the release of free, soluble LPS molecules that T7 phage could bind to as decoy receptors (Fig. 2). This would in turn reduce the amount of phage available to bind and attach to LPS within the outer membranes of *E. coli*, thereby decreasing its ability to infect host cells. To test this model, they added 10 and 100ug/mL purified, rough LPS isolated from *E. coli* UB1005 to fresh *E. coli* cultures of the same strain in minimal (M9) media, then measured T7 phage infectivity using a plaque assay. However, results showed that the added LPS did not lead to significant differences in *E. coli* UB1005 sensitivity to T7 phage across all treatments compared to wildtype *E. coli*. They also found that adding LPS to *E. coli* cells that were treated with 8 ug/mL of gentamicin and washed with M9 media did not impact their phage infectivity. The study concluded that LPS alone was likely not sufficient for granting *E. coli* phage resistance since both LPS concentrations did not impact *E. coli*'s susceptibility to T7 phage when added in either fresh or gentamicin-treated cells (7). Interestingly, they also found that the gentamicin-treated *E. coli* exhibited significantly greater susceptibility to phage after washing to remove extracellular factors, and were more sensitive to phage than washed, untreated *E. coli*. The authors concluded that alternative extracellular factors may be responsible for gentamicin-induced resistance to phage, since their removal after washing caused the gentamicin-treated cells to restore sensitivity to T7 phage. Contrary to these findings, Nagra et al. did not find a significant trend in infectivity after transferring various proportional volumes of supernatant from gentamicin-treated *E. coli* UB1005 in M9 media to untreated cultures. All supernatant treatments exhibited no significant difference in T7-mediated lysis except for the sample with 50% of transferred supernatant, which showed reduced lysis compared to samples 80% and 100% of transferred supernatant. Nagra et al. concluded that extracellular, soluble factors were not involved in phage resistance among gentamicin-exposed bacteria since the supernatant had minimal impact on infectivity, which contradicted the findings of Hardman et al. Potential explanations for the differences in these conflicting studies and methods to address experiment limitations are discussed in the latter portion of this review. These experiments altogether showed that while soluble LPS was likely not the main factor by which gentamicin-exposed *E. coli* confers phage resistance, alternative extracellular factors that could not remain intact after transferring supernatant could be at play (7, 11).

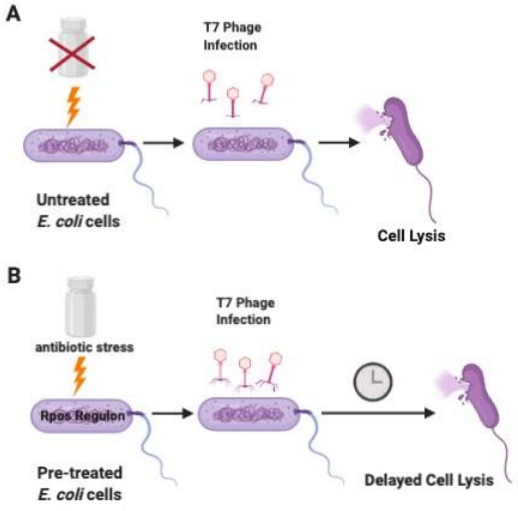

FIG. 4 . *E. coli* cells lyse after being directly infected with T7 phage. **B**. RpoS may confer cross protection against T7 phage infection during exponential phase of growth. Upregulation of RpoS regulon in pre-treated *E. coli* cells with sublethal levels of antibiotics may delay T7 phage-induced lysis.

Outer membrane vesicles may be a potential mechanism of bacteriophage resistance in *E. coli*. While it was concluded that free soluble LPS did not confer protection against T7 bacteriophage, Amanian et al. proposed that other extracellularly released factors may be involved including OMVs as a potential mechanism. In this model, Gram-negative bacteria constitutively extrude portions of their outer membrane, forming OMVs which serve as decoys for bacteriophage binding; this production of OMVs is further stimulated by gentamicin treatment (19). OMVs could subsequently lower T7 infectivity by inhibiting attachment and entry, resulting in the protective effects of gentamicin previously described (Fig. 3). This model was supported by findings that OMV production was upregulated in the presence of envelope-acting stressors (20, 21) including aminoglycosides which form fissures in the bacterial outer membrane (22). Specifically, these positively charged antibiotics displaced Mg^{2+} and Ca^{2+} cations bridging adjacent LPS molecules perturbing membrane structure, facilitating the release of OMVs. Moreover, it was found that the production of OMVs served as an innate defense mechanism in *E. coli* with potency against select antimicrobial peptides (AMPs) polymyxin B and colistin as well as bacteriophage T4 (20). The latter was observed to be a result of the rapid and irreversible formation of complexes between OMVs and T4, leading to reduced phage infectivity (20). Although specific interactions with T7 have not been described, the presence of LPS alongside coreceptors on OMVs was thought to facilitate similar formation of complexes, providing a plausible explanation for gentamicin's protective effect while addressing limitations of earlier LPS models.

Earlier studies by Nagra et al. found that the protective effects of gentamicin treatment against T7 phage were not transferable, contradicting findings by Hardman et al. However, this was later addressed as a limitation in the experiment whereby extracellular factors may have been pelleted due to centrifugation. In a follow-up study, Ahadzadeh et al. tested for extracellular protective factors once more by transferring cell filtrates from gentamicin-treated *E. coli* to non-treated cell cultures (23). 0.22 um filters were used, allowing for the passage of OMVs, but not entire cells. To determine whether filtrates conferred T7 phage protection, growth curves were used. Cells suspended in filtrate were normalized to an OD_{600} of 0.1 and transferred over to a microtiter plate. Again, it was found that the mechanism of protection was not due to extracellularly released factors, as *E. coli* receiving filtrates from 3 ug/mL and 4 ug/mL gentamicin treated cells did not yield growth curves that significantly differed from *E. coli* receiving non-treated cell filtrates after incubating with T7 phage (23). This was despite the observation that gentamicin concentrations of 3 ug/mL and 4 ug/mL were sufficient for inhibiting phage-mediated lysis. However, one limitation was the selection of the 0.22 um filter, which may have excluded larger OMVs from being transferred as OMVs typically range from 0.01 to 0.3 um in diameter (24). A 0.45 um syringe filter may have been more appropriate and should be used in future experiments to confirm these results. However, given the lack of evidence for the OMV model, alternative hypotheses should be explored, including the roles of RpoS-mediated cross protection, capsular production, as well as implications of ribosomal inhibition.

RpoS-mediated stress response does not delay T7-induced lysis in exponential phase *E. coli* treated with sub-inhibitory levels of antibiotics. RpoS is a primary regulator that plays an important role in the expression of various genes involved in cell permeability, metabolism, defense, and repair (25). Previous studies indicated that *E. coli* cells induced the RpoS regulon to obtain antibiotic resistance when treated with subinhibitory levels of antibiotics. The cells became more resistant to not only the antibiotic but also other further stresses. This phenomenon is described as cross protection (25, 26). As Li et al. reported minor delays in T7-induced onset of cell lysis and enhanced resistance in *E. coli* treated with subinhibitory levels of aminoglycoside antibiotics, they hypothesized that a RpoS-mediated stress response was the intracellular factor protecting against T7 phage infection (Fig. 4) (27).

Krystal et al. repeated the experiments with sub-inhibitory levels of gentamicin from 0.25 to 8 μg/mL. They reported a 10-minute delay in the wild-type cells at 0.25, 0.5, 2, 4 or 8 μg/mL. However, as the delayed lysis of wild-type cells treated with gentamicin was not

consistently observed in all trials, they were not able to conclude that the pre-treatment of *E. coli* cells with subinhibitory levels of gentamicin led to delayed T7-induced lysis (8). Moreover, the results from the rpoS knockout cells treated with gentamicin showed a different trend in the lytic curves. In the presence of T7 phage, the cells treated with 0.25, 0.5, 4 and 8 µg/mL of gentamicin did not show any delay in cell lysis compared to the controls with no gentamicin. The curves for cells treated with 0.125, 1 and 2 µg/mL of gentamicin did not show distinct peaks. This may be due to the slower growth rate of the knockout compared to the wild-type cells (8). As the results from both the wild-type and the knockout were inconclusive, the effect of RpoS on phage-induced lysis remains unclear in gentamicin-treated *E. coli* during the exponential phase of growth.

Recent studies found that sufficient levels of the RpoS general stress response may occur only in the stationary phase of growth, as cells are faced with increased stress from limiting nutrients and accumulation of waste products (29, 30). This may be one possible explanation for the lack of significant differences in T7 phage-induced lysis between the exponential phase wild type and mutant *E. coli* treated with ampicillin or gentamicin. To address the uncertainty in current results, more replicates are needed to confirm if there is a delay in the lysis when *E. coli* are pretreated with subinhibitory levels of gentamicin.

Antibiotic-induced upregulation of capsular polysaccharide may play a minor role in the reduction of T7 phage adsorption to *E. coli*. The capsule is an extracellular polysaccharide layer that can be found in many strains of *E. coli.* It functions as a physical barrier against phages that specifically recognize and bind to structures beneath it (31). Previous studies have shown that *E. coli* B23 cells treated with sub-inhibitory levels of antibiotics, such as kanamycin or streptomycin, increased capsular polysaccharide production through the *cps* operon (32, 33). Bleackley et al. also observed a correlation between reduced T7 phage adsorption and increased capsular production in *E. coli* B23 cells pretreated with sub-inhibitory levels of kanamycin (9). The capsule production induced by subinhibitory levels of aminoglycoside antibiotics have been thought to be an important defense factor interfering with T7 phage ability to bind to lipopolysaccharides on the outer membrane of *E. coli* cells (Fig. 5). However, Bleackley et al.'s results and experimental methods were not sufficient to support the hypothesis that increased capsule production reduces T7 phage adsorption, as they did not exclude other potential factors which may play a more significant role than the capsule production in their research.

To test the hypothesis, Gu et al. repeated Bleackley et al.'s experiments using a wild-type *E. coli* strain BW25133 and also a knockout strain, JW2034, which lacks part of the *cps* operon (10, 33). They performed an anthrone carbohydrate assay to verify the effect of subinhibitory levels of streptomycin on capsule growth for *E coli* cells with or without the *cps* operon. The results showed a threefold increase in carbohydrate concentration for the wild-type cells treated with streptomycin compared to the untreated control, and no

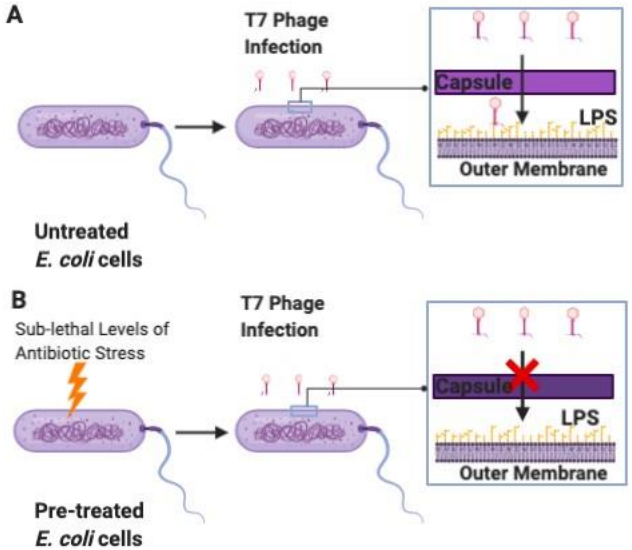

FIG. 5 A capsule as a physical barrier in response to T7 phage adsorption. **A**. In untreated *E. coli* cells, T7 phage still can pass through the capsule and attach to its receptors on the outer membrane. **B**. In antibiotic-treated cells, sub-lethal levels of antibiotic stress increases the production of capsular carbohydrates. This may be a potential mechanism that inhibits T7 phage adsorption.

significant change for the *cps* knockout strain (10). However, the results of their phage adsorption assay failed to support their hypothesis, as both strains showed a significant decrease in T7 phage adsorption when treated with sub-lethal concentrations of streptomycin. This suggested that capsule production alone had a minor effect on the reduction of phage adsorption (10). They attributed this unexpected decrease in phage adsorption to an alteration of the cellular membrane that down-regulates LPS and/or blocks T7 phage attachment to LPS (34).

From the results of these studies, it is suggested that capsular polysaccharide production likely occurs when *E. coli* cells encounter the stress from aminoglycosides, but it is unlikely the major factor involved in the reduction of T7 phage adsorption. Because the experiments were done with other aminoglycosides except for gentamicin, the effect of capsular formation induced by gentamicin on T7 phage adsorption remains unknown. For further research in this field, as Gu et al. failed to calculate the magnitude of reduction in T7 phage adsorption as they could not count the number of plaques in the adsorption assay, more replicates of the experiments are required to measure the effect and improve confidence in the results. Experiments with gentamicin treatment should also be done to investigate if gentamicin also gives the same results as other aminoglycosides.

DISCUSSION

Plaque and cell lysis assays have shown that *E. coli* cells exposed to sub-inhibitory levels of aminoglycosides, including kanamycin, streptomycin, and gentamicin prior to incubation with T7 phage, have reduced susceptibility to phage infection (4). Furthermore, this gentamicin-conferred protective effect was characterized to be growth phase dependent (4). Amanian et al. concluded that because stationary phase *E. coli* cells with low translational activity did not reduce T7 phage's ability to infect and induce cell lysis compared to exponential phase cells with high translational activity, gentamicin inhibition of ribosomal activity was not the mechanism that decreased T7 phage infectivity. However, such conclusions failed to consider potential physiological differences between stationary and exponential phase cells that might be responsible for this observation, such as varying permeability to gentamicin. Moreover, as later studies suggested the role of secreted factors in the reduction of T7 phage infectivity by acting as decoys that prevent T7 phage attachment to cells, stationary phase cells with low metabolic activity may not synthesize and release as many secreted factors compared to exponential phase cells (4). As such, to investigate whether gentamicin inhibition of ribosome is responsible for reduced T7 phage infectivity, further comparative studies should be done on identical *E. coli* cells with ribosomal activity level as the sole variation.

Plaque assay experiments performed by Hardman et al. revealed that the addition of extracellular LPS to *E. coli* cells did not result in reduced susceptibility to T7 phage (7). Hence, the induced production of extracellular LPS from exposure to gentamicin is likely not a sufficient protective factor to confer T7 phage resistance within *E. coli* (7). A limitation of the experiment by Hardman et al. was that since they were unable to measure the absolute concentration of LPS that was induced from *E. coli* UB1005 in response to 8ug/mL of gentamicin treatment, they could not ensure the 10 ug/mL and 100 ug/mL LPS treatments accurately represented conditions that conferred delayed T7 phage infectivity. Nonetheless, these treatment conditions were likely sufficient concentrations of LPS to confirm that it was not a protective factor, as Evans et al. found that the maximum gentamicin-induced release of LPS was 58.2% of gentamicin concentrations and Hardman et al. used tenfold this amount of LPS (13). Hardman et al. further solidified this conclusion because LPS did not impact phage infectivity even when added to washed, gentamicin-treated cells, which effectively controlled for the isolated effects of LPS as all other extracellular factors were removed and *E. coli* cells were treated under the same gentamicin conditions as those shown to confer phage resistance. In addition, it is important to note the fact that the study found washed, gentamicin-treated cells exhibited even greater susceptibility to phage compared to washed-untreated cells, suggesting that cells were more sensitive to T7 phage infection in the absence of extracellular factors. This could be explained by the fact that gentamicin disrupts *E. coli* outer membrane in its competitive displacement of metal cations that bridge LPS and phospholipids (17). Since outer

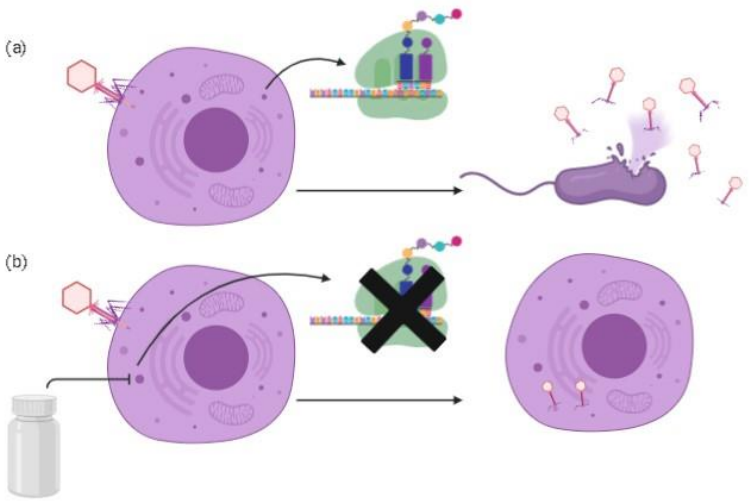

FIG. 6 Ribosomal inhibition by gentamicin reduces production of viral proteins required for replication and cell lysis. (a) Host ribosomes are needed to synthesize viral proteins. (b) Inhibition of ribosomes by gentamicin may result in insufficient production of viral proteins required for genome replication and cell lysis. Virus cannot propagate when cells do not lyse. Figure created in BioRender.

membrane degradation is important for phage lysis, gentamicin could cause host cell membranes to be more easily lysed by T7 phages compared to untreated cells when extracellular factors are removed (17). However, the confidence of these results is also decreased because the study lacked a control with washed, gentamicin-treated cells that were not infected by T7 phage and were unable to rule out whether the gentamicin treatment is contributing to cell death rather than T7 phage lysis. Follow-up studies with gentamicin-treated controls that lack T7 bacteriophage are needed to verify whether similar results to Hardman et al. can be found. If their results are reproducible, this could suggest that other extracellular factors may play a role in gentamicin-induced resistance to phage; they not only may be responsible for delayed phage infection, but may also protect *E. coli* cells that are more susceptible to phage lysis after antibiotic exposure.

Yet the follow-up experiment by Nagra et al. suggested that extracellular factors may not be involved, as supernatant from gentamicin-treated cells did not impact phage infectivity in both untreated and gentamicin-exposed *E. coli* (11). Their conflicting findings may be explained by the fact that Hardman et al. used a sub-lethal gentamicin concentration of 8 ug/mL compared 2.5 ug/mL used by Nagra et al., which could have been too low of a concentration to induce sufficient production of extracellular factors to impact phage infectivity. Both studies also likely treated cultures with differing amounts of soluble factors as they were unable to titrate the final concentration of factors in the supernatant before its removal from or transfer to *E. coli*. Moreover, the plaque assay by Nagra et al. likely had inaccurate results because untreated *E. coli* cells with 50% of transferred supernatant from gentamicin-treated cells showed significantly lower infectivity than 100% of transferred supernatant. Given that various studies supported that gentamicin-treated cells confer greater resistance to T7 phage, it is unexpected that less supernatant from these cells would increase phage resistance (4, 7, 11). This could be due to technical error during the plaque assay such as inaccurate pipetting when transferring supernatant or adding T7 phage to host cells. Hence, these studies showed that free, soluble LPS likely does not confer resistance to T7 phage among gentamicin-exposed *E. coli*, but it remains in question if the mechanism is extracellular as the findings by Hardman et al. have not been reproduced. Further research is needed to conclusively determine whether the gentamicin-induced resistance mechanism involves alternative extracellular factors as well as address limitations in experimental design and potential technical error from past studies.

Although Nagra et al.'s study determined that extracellular factors were likely unimportant, Ahadzadeh et al. reasoned that the experiments were not comprehensive enough as certain factors (e.g., OMVs) may have been pelleted during centrifugation (23). To address this, Ahadzadeh et al. performed a culture filtrate lysis assay. Filtrates were transferred from gentamicin-treated cells to non-gentamicin-treated cells using a 0.22 um syringe filter. This should have allowed for the passage of most OMVs, which typically range from 0.01 to 0.3 um in diameter but not entire cells. However, after incubating with

T7 bacteriophage over a six hour time course, it was found that the rates of cell lysis were not significantly different, once again refuting the hypothesis that extracellular factors are involved (23). One possible limitation is the choice of a 0.22 um filter which may exclude larger OMVs (i.e., 0.22 to 0.3 um) from being transferred (24). Regardless, these findings suggest that gentamicin-induced T7 resistance is not conferred by an extracellular factor, refuting the OMV model.

Among the studies investigating the potential mechanism of a RpoS-mediated stress response, Krystal et al. also performed Western blotting to determine the lowest concentration of gentamicin required to induce RpoS upregulation. The results from Western blotting confirmed the absence of RpoS in the knockout cells, but they were not able to quantify RpoS in the gentamicin-treated cells and identify the optimal concentration of gentamicin to induce RpoS regulation (8). Krystal et al. had to use a gradient of gentamicin concentrations in the lytic assay. One limitation was that the increased antibiotic stress may have induced not only RpoS but also some non-specific factors that have effects on the cell function (8). This may be one of the reasons why they observed the absence of delayed cell lysis in both wild-type and knockout cells. The results from the lytic curves suggested that the RpoS-mediated stress response did not provide cross protection against T7 infection in exponential phase E. coli. To evaluate their results, Krystal et al. reviewed several recent studies and found that the level of RpoS was not sufficient to confer cross protection against T7 infection until the E. coli cells reached stationary phase (29, 30). For continued research in this field, the optimal concentration of antibiotics should be determined to reduce other non-specific effects on cell function. Quantification of RpoS in *E. coli* cells during exponential phase and stationary phase of growth are required to identify the role of RpoS in response to T7 phage infection.

Another hypothesis related to T7 phage infection to antibiotic-treated *E. coli* was that capsular formation decreases T7 phage adsorption by physically preventing the attachment of the phage to its receptors on the outer membrane of *E. coli* (9-10, 33). Although the experiments were done with kanamycin and streptomycin, it was expected that gentamicin would elicit a similar response through a similar proposed mechanism. The results of plaque assay obtained from Gu et al. confirmed the upregulation of capsular carbohydrates during the exponential phase of cell growth. However, they also observed a significant decrease in T7 phage adsorption for both antibiotic-treated wild-type and knockout cells that could not produce capsules. This suggested that the reduction of T7 phage adsorption was not significantly impacted by increased capsule production (10). Hence, their results failed to support the hypothesis. Since the results from the plaque assay lacked countable numbers

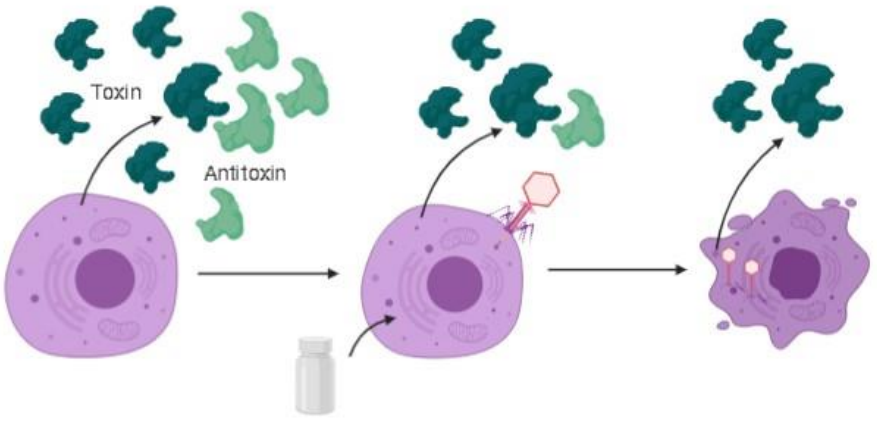

FIG. 7 Toxin/antitoxin systems trigger 'altruistic cell death' to protect against lytic infections. Gentamicin treatment inhibits synthesis of toxin/antitoxin proteins. Antitoxins are labile and degrade faster, allowing their toxin counterpart to take effect. Some toxin/antitoxin systems have been found to trigger 'altruistic cell death', protecting bacteria from viral propagation. Figure created in BioRender.

of plaques in the wild type and the knockout controls without antibiotic treatment, they had to approximate the magnitude of the adsorption reduction. They were not able to determine how much capsular formation could reduce T7 phage adsorption in the wild-type compared to the knockout cells. For future studies in this field, more replicates of the phage adsorption assay are required to calculate the magnitude of the reduction and compare data between the wild-type and the knockout strain after normalization.

Conclusions Taken altogether, studies have confirmed that pre-exposure of *E. coli* cells to sub-inhibitory levels of gentamicin reduces the cell susceptibility to T7 phage infection in a gentamicin dose-dependent manner (7, 9-11). Such gentamicin-induced protective effects against T7 phage were only seen in exponential but not stationary phase cells, and gentamicin pre-exposure did not delay the onset of cell lysis. Ribosomal inhibition by gentamicin is likely not the mechanism, as T7 phage showed greater infectivity for stationary phase cells with low protein synthesis activities compared to exponential phase cells (4, 8, 12). Induced production of different extracellular factors in response to gentamicin exposure, including free, soluble LPS and OMVs, are not sufficient to explain the observed reduction in T7 phage infectivity for *E. coli* (4, 7, 11, 23). The role of intracellular factor RpoS in T7 phage infectivity remains unclear. It is not involved in the delay of T7 phage-induced cell lysis during the exponential phase of cell growth (8, 28-30). Additionally, an increase in capsular formation plays a minor role in T7 phage adsorption to *E. coli* cells (9-10, 33).

Future Directions It has been repeatedly observed that sub-inhibitory levels of gentamicin induce T7 phage-protective effects in *E. coli* UB1005. However, the mechanism by which this occurs remains unclear. Several hypotheses have been proposed, with emphasis on the production of a ‘protective factor’ but these have largely been disproved. Given the contradictions in findings, it may be of benefit to revisit previous experiments using revised protocols to address their limitations (e.g., using 0.45 um filters for cell filtrate experiments, increasing number of replicates, including gentamicin treatment controls, etc.). It is also possible that past research has heavily focused on identifying an extracellular ‘protective factor’ when in fact there are none. It may be worthwhile then to consider the roles of other intracellular mechanisms such as dysregulation of protein synthesis by ribosomal inhibition. Being an aminoglycoside antibiotic, gentamicin inhibits protein synthesis by binding to the host’s 30S ribosomal subunit (6). As viruses depend on host ribosomes for protein synthesis, it is conceivable that the dysregulation of ribosomes by gentamicin inhibits: (a) the phage’s ability to replicate and/or (b) phage release via lytic enzymes. In either case, inhibiting the production of proteins required for the phage’s lytic cycle interferes with T7 propagation (Fig. 6). Importantly, this is consistent with previous observations that stationary phase *E. coli* retain similar levels of T7-susceptibility even after being exposed to subinhibitory concentrations of gentamicin (4). Because levels of protein synthesis are already low, further inhibition by gentamicin produces negligible effects on phage susceptibility compared to exponential phase cells where levels of protein production are initially high (4). Moreover, this model is consistent with the dose-effect that is observed with regards to gentamicin’s protective effect. To test this hypothesis, gentamicin-resistant strains may be used to determine whether phage resistance is conferred by some secondary effect, keeping in mind that the mechanism of resistance must not include inactivating or excluding the antibiotic from the cell. For instance, further studies may use the MRE600 strain as it becomes resistant to gentamicin after transformation with pAT780 (35). The production of aminoglycoside resistance methyltransferase A results in the methylation of ribosomes, which in turn confers resistance to a wide range of aminoglycosides (35). In general, resistant strains should show similar levels of T7 susceptibility, although reduced susceptibility in the absence of antibiotic treatment would suggest that the T7 phage requires access to machinery that is normally targeted by gentamicin. If the protective effect is indeed mediated by ribosomal inhibition, then resistant strains should demonstrate similar levels of susceptibility to T7 with or without exposure to gentamicin. In wildtype cells, subinhibitory concentrations of gentamicin result in reduced protein synthesis but maintain levels that are still sufficient for growth. However, during infection, this may lead to a

scarcity of viral proteins and hence interfere with viral replication and/or cell lysis. In gentamicin-resistant strains, cells maintain normal levels of protein production even in the presence of the antibiotic and thus, should remain phage-susceptible even after preliminary gentamicin exposure.

Alternatively, reduced susceptibility to T7 infection may be explained by factors that are synthesized by the cell and act on the phage intracellularly. This may be an avenue worth exploring as evidence suggests that the 'protective factor' is likely not secreted by the cell. While the RpoS-mediated general stress response has been discussed, it only accounts for the regulation of a select number of proteins. Of particular interest, are toxin-antitoxin systems which mediate phage resistance via abortive infection mechanisms. Each system is composed of a stable toxin protein and a corresponding labile antitoxin (36). One example is the ToxIN system previously described in *Pectobacterium atrosepticum* which confers phage protection by committing 'altrustic suicide', whereby infected cells are killed by high concentrations of the ribonuclease toxin, ToxN (37). Similar systems have been described in *E. coli* though their role in conferring phage protection remains to be elucidated (38). Previous studies have found that when bacteria encounter stress (e.g., antibiotics), synthesis of toxins/antitoxins ceases and because the antitoxin is degraded more quickly, the toxin is able to take effect (36, 39). Importantly, toxin-antitoxin systems interfere with phage propagation by inducing programmed cell death, preventing the release of viral progeny (37) (Fig. 7). Other ways intracellular factors may reduce phage infectivity include binding of/degradation of phage genome although bacteriophage have evolved various mechanisms to overcome this (40). Cell fractionation experiments may be performed to test for the subcellular location of protective factors, including the potential presence of intracellular processes. Specifically, untreated cell cultures may be resuspended in gentamicin-treated cell lysates and incubated with T7 phage. If a protective factor is found intracellularly, these samples will show decreased susceptibility to infection. However, it is important to consider whether these factors are able to enter the cell after resuspension and whether or not they remain stable over the course of the experiment. Using non-ionic detergents will help minimize the number of proteins denatured during fractionation while performing a heat shock step prior to resuspension will help ensure that sufficient amounts of protein will enter the cell. Membrane components and secreted factors in the supernatant are also isolated during the fractionation process. Treating cells with each of these fractions simultaneously with T7 bacteriophage will provide a more comprehensive understanding of the localization of the 'protective factor' and whether such a factor exists.

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CONTRIBUTIONS

All authors contributed equally to laboratory work and written sections of this paper. CT and BL were primarily responsible for plaque assays and YC and ML were responsible for lysis curve experiments in the lab. For this manuscript, the literature review and discussion were evenly divided among the authors and CT completed the Abstract, YC completed the Introduction, ML completed the Conclusion, and BL completed Future Directions.

ANNOTATED REFERENCES

Amanian et al. (4)

The authors conducted plaque assays and bacterial growth curve assays to determine whether exponential and stationary-phase *E. coli* UB1005 that are exposed to sub-inhibitory treatment of gentamicin would have reduced infectivity and cell lysis by T7 bacteriophage. Results verified this hypothesis and indicated that *E. coli* cultures in the exponential phase were more susceptible to gentamicin-induced T7 bacteriophage resistance compared to

those in the stationary phase. Amanian et al. suggested the potential role of outer membrane vesicles as protective factors against T7 phage infection.

Hardman et al. (7)
To investigate the mechanism behind gentamicin-induced resistance to T7 bacteriophage in *E. coli*, Hardman et al. added various concentrations of purified LPS isolated from *E. coli* UB1005 to untreated cells and found it had no significant impact on phage infectivity. They concluded that LPS did not sufficiently confer resistance to T7 phage during gentamicin treatment of *E. coli*. Additionally, they observed that gentamicin-treated cultures had restored infectivity by T7 bacteriophage after washing and proposed that released, soluble factors are likely involved in this mechanism of resistance.

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