



## Gentamicin-induced resistance to T7 bacteriophage lysis in *Escherichia coli* is not due to an extracellularly released factor

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**SUMMARY** The worldwide emergence of antibiotic resistant bacteria necessitates the search for alternative treatments for bacterial infections. A promising potential therapeutic is the use of bacteriophages that can lyse bacteria such as *Escherichia coli*. However, some antibiotics such as gentamicin have been shown to decrease the lysis rate of bacteriophages in *E. coli*. This ‘protective factor’ has been theorized to be released from the bacteria, such as outer membrane vesicles that competitively bind phage particles. To determine if the gentamicin-induced protective factor is extracellularly released, *E. coli* UB1005 was treated with different sub-inhibitory concentrations of gentamicin and cell filtrates were tested for protective ability against T7 bacteriophage. We hypothesized that if we transferred filtrate from gentamicin-treated *E. coli* to untreated cultures, then the untreated cultures would obtain protection against bacteriophage-mediated lysis, indicating that the protective factor was extracellularly released. We observed that filtrate from gentamicin-treated cultures was not able to confer protection to untreated cultures, refuting our hypothesis and disproving the outer membrane vesicle model of protection.

### INTRODUCTION

Antibiotics have been widely and successfully used to treat different types of bacterial infections. However, the emergence of antibiotic resistant bacteria has endangered their efficacy, placing a major concern on public health (1). According to the Centers for Disease Control, around 35,000 Americans die each year due to antibiotic resistant bacterial infections (2), thus it has become increasingly necessary to seek an alternative approach.

Bacteriophages are viruses that can infect and lyse bacteria without harming human cells (3). In contrast to antibiotics, phages are selective towards specific bacteria (4). Due to these benefits, the use of bacteriophages in combination with antibiotics holds promising therapeutic potential.

The T7 bacteriophage is an obligately lytic phage that specifically infects Gram-negative bacteria such as *Escherichia coli* (5). Lipopolysaccharides on the bacterial outer membrane are the primary receptor for T7 phage, which starts its infection cycle by injecting its genome into the host cell. Using host cell machinery, the phage undergoes replication to produce new virions that are released through lysis of the bacterium following production of phage lysis enzymes (6). The constant presence of such phages in the environment has selected for bacteria that evolved strategies to prevent viral infection. These strategies include the prevention of bacteriophage attachment through mutation of the phage receptor, the production of extracellular matrices that bind up phage particles, and the expression of phage inhibitors (7). Considering the therapeutic potential that phages have for multi-drug resistant bacteria, it is important to investigate phage resistance.

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Multiple previous studies have shown that when *E. coli* UB1005 is pre-treated with sub-inhibitory concentrations of aminoglycoside antibiotics such as gentamicin, the rate of lysis for subsequent T7 phage infection is decreased (8-10). Hardman *et al.* found that *E. coli* treated with gentamicin regained their susceptibility to the phage after washing, suggesting that a released protective factor was responsible for the observed bacteriophage resistance (9). This led to the proposal of a model involving a gentamicin-induced cellular stress response releasing outer membrane vesicles (OMVs) into the environment. OMVs are 0.1  $\mu\text{m}$  vesicles that consist of bacterial membrane components, such as outer membrane lipids, proteins, and periplasmic components (11-13). Employed as a defense mechanism, OMVs protect bacteria against external stressors, such as bacteriophages, by binding the viral particles to themselves and stopping phage replication (13).

As a follow-up to the Hardman *et al.* study, Nagra *et al.* transferred the supernatant of gentamicin-treated *E. coli* to untreated cultures. They found that the protective factor could not be transferred to untreated cultures via the supernatant (14). This suggested that the factor was not extracellularly released, which contradicted Hardman *et al.*'s experiment. However, Nagra *et al.* proposed that this discrepancy could be due to the extracellular factor having been pelleted during centrifugation (14).

To further investigate whether or not the protective factor is released extracellularly, we hypothesized that the transfer of cell filtrate from *E. coli* UB1005 cultures treated with sub-inhibitory concentrations of gentamicin would provide protection to untreated cultures, reducing T7 bacteriophage lysis. Transfer of protection would indicate that the protective factor is released extracellularly. Use of a 0.22  $\mu\text{m}$  filter for whole cells would avoid potentially pelleting the protective factor and unnecessary stress for the cells. Our phage infection assays showed that, contrary to our hypothesis, filtrate transfer from gentamicin treated cultures did not confer phage protection to untreated cells. This suggests that the protective factor is not released extracellularly. Instead, we propose an alternative model where gentamicin's mechanism of action, binding to the 30S ribosomal subunit and inhibition of protein synthesis (15), is preventing phage from replicating due to diminished host resources.

## METHODS AND MATERIALS

**Bacterial strains and culture conditions.** Bacterial strain *E. coli* UB1005 was obtained from lab stocks in the Microbiology and Immunology Department at the University of British Columbia. *E. coli* UB1005 liquid cultures were grown in Lysogeny broth (LB) overnight at 37°C on a shaker at 200 rpm. LB was prepared with 5 g of tryptone, 5 g of NaCl, and 2.5 g of yeast extract per 500 ml of dH<sub>2</sub>O. 7.5 g of agar was added for solid LB plates.

**Propagation of T7 bacteriophage stock.** Bacteriophage strain T7 was obtained from lab stocks in the Microbiology and Immunology Department at the University of British Columbia. *E. coli* UB1005 liquid subculture was grown in LB, shaking at 37°C to an OD<sub>600</sub> of 0.5. Liquid T7 bacteriophage lab stock was then stabbed and used to inoculate the *E. coli* subculture, which was incubated shaking at 37°C until clearing was observed. Lysed culture was treated with 1% chloroform and centrifuged at 5000 rpm for 5 min. Supernatant was drawn and stored at 4°C.

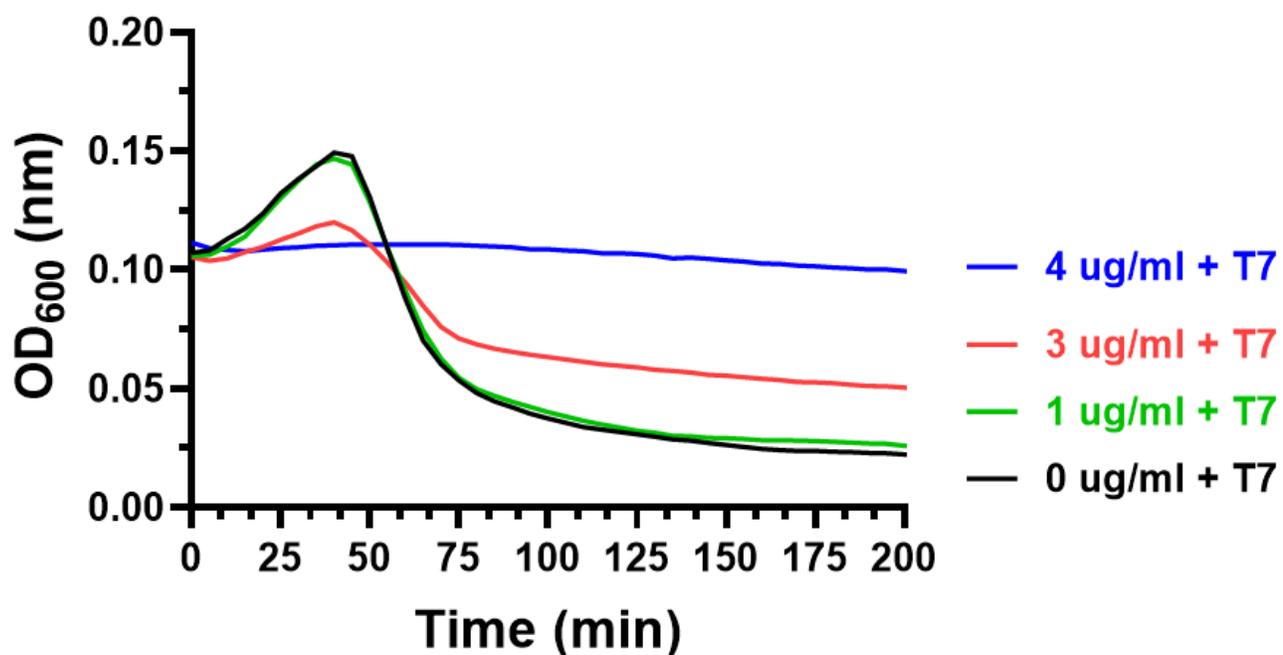
**TABLE 1. PCR primer sequences for confirmation of phage purity.**

Gene Target	Orientation	Sequence
T7 <i>rpol</i>	Forward	5'- CGAGGGCTTAGGTACTGC -3'
	Reverse	5'- GGTGAGGTGCGGAAGTTC -3'
T4 <i>gp23</i>	Forward	5'- GCCATTACTGGAAGGTGAAGG -3'
	Reverse	5'- TTGGGTGGAATGCTTCTTTAG -3'

**Determination of phage stock titer.** Phage titer was determined using a double agar overlay plaque assay using LB agar. *E. coli* UB1005 liquid subculture was grown in LB, shaking at 37°C to an OD<sub>600</sub> of 0.5. Serial dilutions of T7 bacteriophage stock were prepared ranging from 10<sup>-6</sup> to 10<sup>-10</sup>. 1 ml of *E. coli* subculture was inoculated with 100 µL of each phage dilution and incubated shaking at 37°C for 10 min. Inoculated culture was centrifuged at 5000 rpm for 2 min, the supernatant was discarded, and the culture was resuspended in LB. Cultures were added to phage top agar and poured onto phage bottom agar plate. Dilutions were plated in duplicate, with no phage, no *E. coli*, and agar controls. Overlay plates were incubated at 37°C for 8 hours, and phage titer was calculated by counting the number of plaques formed.

**Polymerase chain reaction (PCR) confirmation of phage purity.** Phage purity was confirmed through PCR amplification and gel electrophoresis using primers flanking T7 rpol and T4 gp23 (Table 1). An aliquot of phage stock was heated to 95°C for 10 min, and PCR was performed with Platinum Taq DNA polymerase (Invitrogen), using a Bio-Rad T100 Thermal Cycler. PCR was run for 30 cycles, with 5 min at 95°C, 30 sec at 52°C, 30s at 72°C, and 5 min at 72°C. PCR product was analysed through gel electrophoresis in a 1% agarose gel in TRIS/Borate/EDTA (TBE) buffer containing SYBR SAFE (Invitrogen). Gel was imaged on a ChemiDoc imaging system (Bio-Rad).

**Minimum inhibitory concentration (MIC) of gentamicin for *E. coli* UB1005.** Stock solution of gentamicin (1 mg/ml) was prepared by dissolving gentamicin sulfate (MilliporeSigma) in distilled H<sub>2</sub>O. Solution was filter sterilized through a 0.22 µm syringe filter (VWR) and stored at -4°C. *E. coli* UB1005 liquid subculture was grown in LB, shaking at 37°C to an OD<sub>600</sub> of 0.5. Serial dilutions of gentamicin were prepared ranging from 1 µg/ml to 32 µg/ml. *E. coli* subcultures were treated with the gentamicin dilutions, along with LB and no gentamicin controls, and incubated shaking overnight at 37°C. Cultures were analyzed visually for turbidity, and the MIC was determined to be 8 µg/ml. Sub-inhibitory concentrations used in downstream experiments included any concentration below 8 µg/ml.

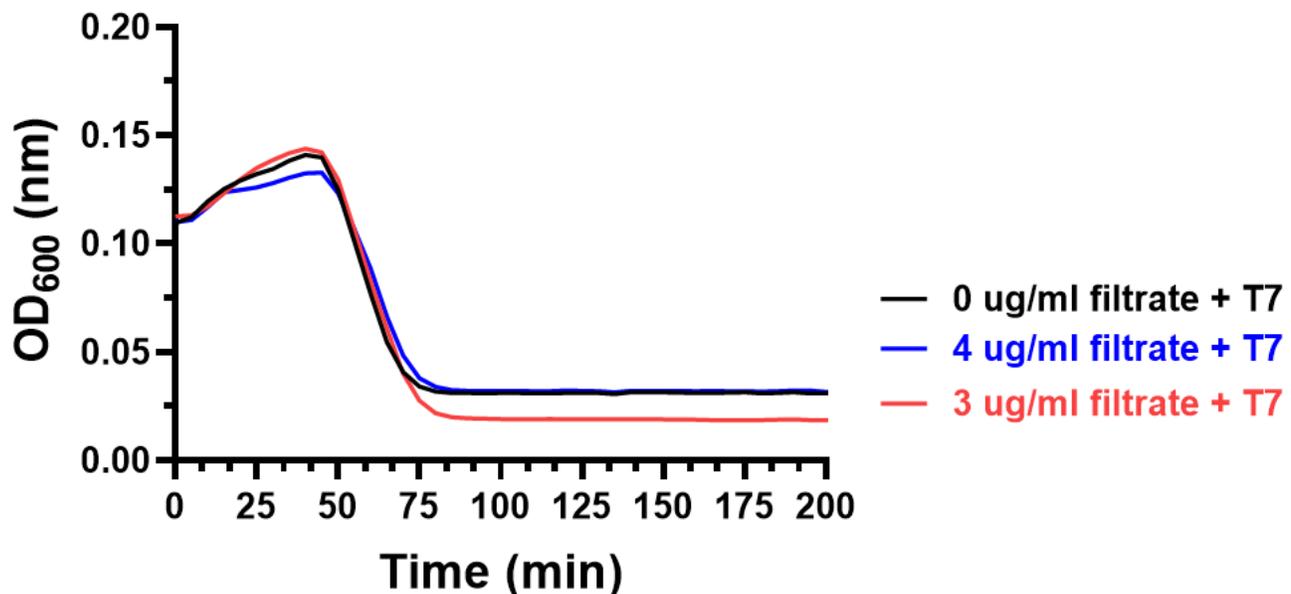


**FIG. 1 Growth curve of gentamicin-treated *E. coli* UB1005 incubated with T7 bacteriophage.** Subcultures were treated with gentamicin or an LB control until they reached an OD of 0.5. T7 phage was subsequently added at an MOI of 0.01. OD readings of samples in triplicate were taken every 5 minutes at 37°C in a 96-well plate, and the mean of these readings was compared at n=3.

**Phage lysis assay for gentamicin-induced protective effect.** *E. coli* UB1005 liquid subculture was treated with gentamicin concentrations ranging from 1 ug/ml to 4 ug/ml, using untreated subculture as a control. Subcultures were grown in LB, shaking at 37°C to an OD<sub>600</sub> of 0.5, and plated on a microtiter plate. T7 bacteriophage at a MOI of 10<sup>-2</sup> or an LB “no-phage” control was added to the wells. LB was used as blanks. Plate was immediately incubated at 37°C on a BioTek Plate Reader for 6 hours. OD<sub>600</sub> was measured every 5 minutes, with 5 seconds of shaking before each reading.

**Culture filtrate transfer lysis assay for transfer of gentamicin-induced protective effect.** *E. coli* UB1005 liquid subculture was treated with gentamicin concentrations ranging from 1 ug/ml to 4 ug/ml, with untreated subculture as a control. Subcultures were grown in LB, shaking at 37°C to an OD<sub>600</sub> of 0.5, and plated on a microtiter plate. Aliquots of all subcultures were run through a 0.22 um syringe filter (VWR) to remove cells, and gentamicin treated culture filtrates were transferred to untreated wells (with transfer of untreated filtrate as a control). T7 bacteriophage at a MOI of 10<sup>-2</sup> or LB vehicle control was added to the wells. LB was used as blank. Plate was immediately incubated at 37°C on a BioTek Plate Reader for 6 hours. OD<sub>600</sub> was measured every 5 minutes, with 5 seconds of shaking before readings.

**Growth curve for the effect of sub-inhibitory concentrations of gentamicin on *E. coli* growth.** *E. coli* UB1005 liquid subculture was grown in LB, shaking at 37°C to an OD<sub>600</sub> of 0.5. Subcultures were treated with gentamicin concentrations ranging from 1 ug/ml to 8 ug/ml and immediately plated on a microtiter plate, with untreated and just LB controls. Plate was incubated at 37°C on a BioTek Plate Reader for 6 hours. OD<sub>600</sub> was measured every 5 minutes, with 5 seconds of shaking before each reading.

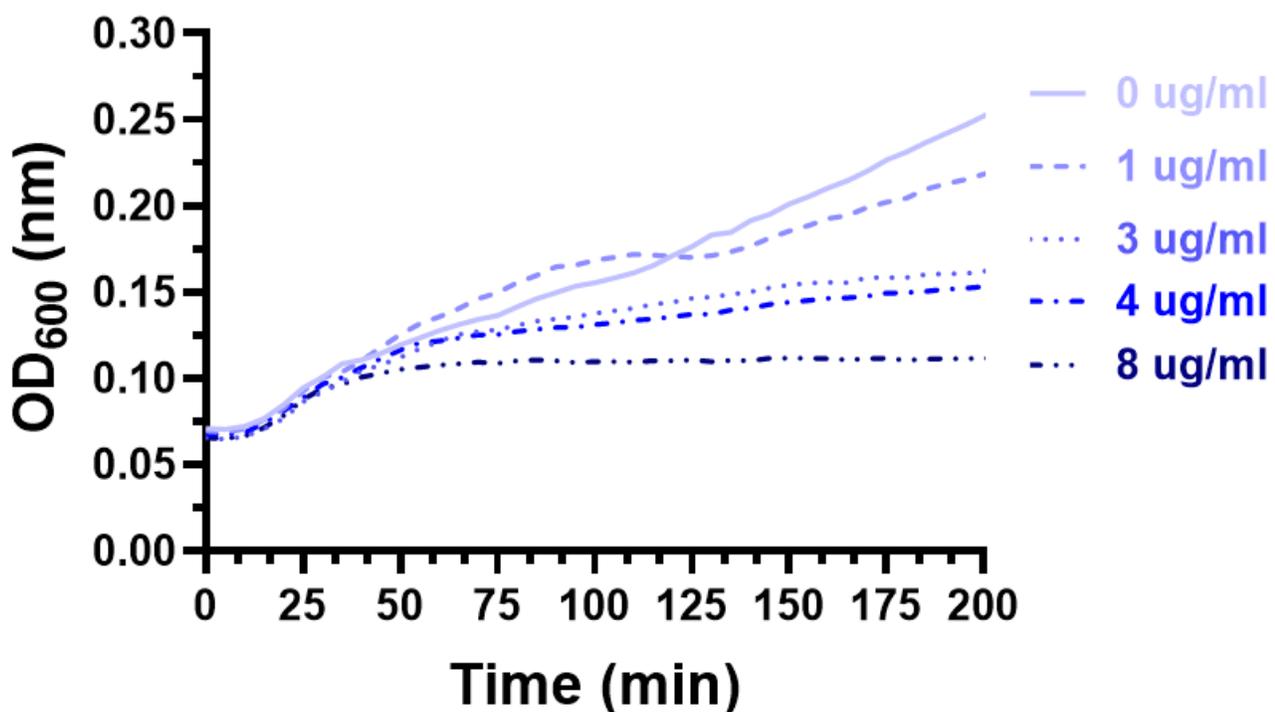


**FIG. 2 Growth curve of *E. coli* UB1005 incubated with filtrate from gentamicin-treated *E. coli* UB1005 and T7 bacteriophage.** Subcultures were treated with gentamicin or an LB control until they reached an OD of 0.5. Filtrate was subsequently transferred to untreated *E. coli* and T7 phage was added at an MOI of 0.01. OD readings of samples in triplicate were taken every 5 minutes at 37°C in a 96-well plate, and the mean of these readings was compared at n=3.

## RESULTS

**Decreased rate of T7 phage-mediated lysis observed in *E. coli* UB1005 with a 3 ug/ml sub-inhibitory gentamicin concentration.** To determine if gentamicin reduces the rate of lysis in response to T7 phage infection as previously shown (9, 10, 14), we used varying concentrations of gentamicin treatment (1 ug/ml, 3 ug/ml, and 8 ug/ml) to construct a bacterial growth curve. *E. coli* was treated with gentamicin prior to T7 infection. Figure 1 shows that treatment with 1 ug/ml of gentamicin produced a curve that closely follows the gentamicin untreated curve. At the other extreme, while 4 ug/ml of gentamicin did not show decreases in OD due to T7 lysis, it did show plateauing of bacterial growth over the time course. A gentamicin concentration of 3 ug/ml allowed a peak in growth at 35 minutes before T7 lysis resulted in a reduced rate of OD decrease compared to untreated cells. As a control, growth of *E. coli* with only LB responded to T7 phage challenge with the expected peak in growth before subsequent lysis. This data indicates that a sub-inhibitory concentration of gentamicin of 3 ug/ml reduces T7 phage-mediated lysis but still maintains cell growth, similar to the protective phenomenon observed in previous papers.

**Filtrate from gentamicin-treated *E. coli* UB1005 does not confer protection against T7 phage-mediated lysis.** To investigate if a decrease in T7 phage lysis is due to the release of an extracellular factor as previously suggested (9), we constructed a bacterial growth curve using the concentrations of gentamicin previously found to decrease T7 lysis (3 ug/ml and 4 ug/ml). Filtrates from *E. coli* treated with gentamicin were transferred to untreated *E. coli*, which were then infected with T7 phage. Figure 2 demonstrates that *E. coli* treated with filtrates from 3 ug/ml and 4 ug/ml gentamicin treated cells both still exhibited a drop in OD readings when incubated with T7 phage. The *E. coli* given gentamicin treated filtrates all followed the same lysis pattern with very similar OD values to those that received gentamicin untreated filtrate. For the control, filtrate from untreated cells led to the characteristic T7 phage lysis curve. The results indicate that T7 phage-mediated lysis is not protected against by the presence of filtrate from gentamicin-treated *E. coli*.



**FIG. 3 Growth curve of *E. coli* UB1005 treated with various concentrations of gentamicin antibiotic.** Subcultures were allowed to reach an OD of 0.5 before gentamicin or an LB control was added. OD readings of samples in triplicate were taken every 5 minutes at 37°C in a 96-well plate, and the mean of these readings was compared at n=3.

**Sub-inhibitory concentrations of gentamicin impair *E. coli* UB1005 growth over time.**

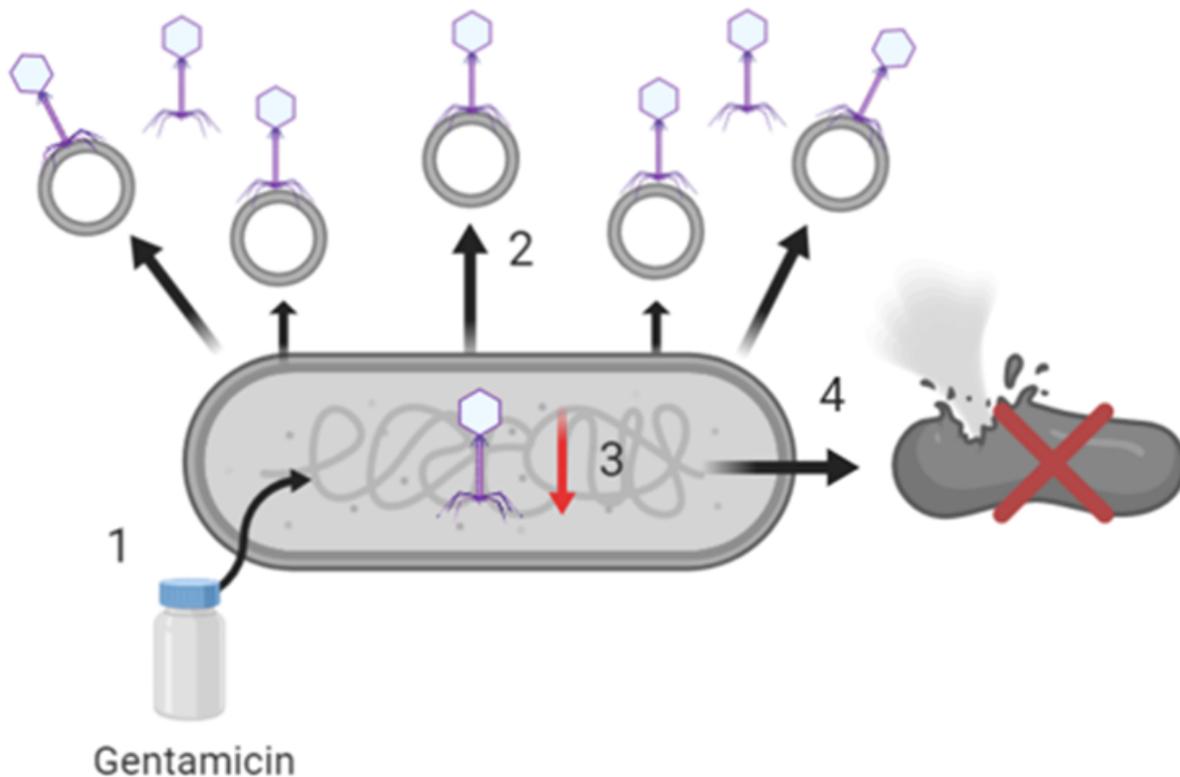
Finally, we wanted to determine whether the growth of *E. coli* is hindered over time even at sub-inhibitory gentamicin concentrations. To do this we measured *E. coli* growth with varying sub-inhibitory gentamicin concentrations (1 ug/ml to 8 ug/ml) to construct a bacterial growth curve. Growth was immediately tracked following gentamicin exposure. Figure 3 shows similar growth between gentamicin treatments for the first 30 minutes, but all the gentamicin curves diverged with increasing time. Over time, a gentamicin concentration of 1 ug/ml mimicked the growth of untreated *E. coli* most closely, but still showed a curve that lagged behind the untreated cells in OD readings. Concentrations of 3 ug/ml and 4 ug/ml led to curves showing even further lagging in growth with 4 ug/ml exhibiting slower growth between the two. Treating *E. coli* with a gentamicin concentration of 8 ug/ml (the MIC) led to an absence of growth relative to the other concentrations after 30 minutes. The LB control shows *E. coli* growing uninhibited. Together, this suggests that even sub-inhibitory gentamicin concentrations impair the growth of *E. coli* over time.

**DISCUSSION**

The emerging threat of antibiotic resistance has compelled research into alternative methods of treating bacterial infections (16). In this regard, bacteriophages may provide a viable means of augmenting current antibacterial therapies. However, the efficacy of these treatments may be reduced if the antibiotic also interferes with phage activity (17). Three previous studies have observed a decrease in the phage-mediated lysis of *E. coli* following sub-inhibitory gentamicin treatment (9, 10, 14). In the first, Hardman *et al.* conclude that the resistance to phage was the result of an extracellularly-released protective factor (9). In contrast, Nagra *et al.* found that this resistance was not transferable via cell supernatants, suggesting that the protective factor was not active extracellularly (14). While differing on the mechanism by which gentamicin treatment confers resistance to phage-mediated lysis, both studies agree that antibiotic treatment adversely affected phage infectivity. This was further confirmed via a separate assay based on OD measurements by Amanian *et al.*, who proposed that the effect could also be due to the inhibition of ribosomes by gentamicin, thus preventing viral replication (10). To resolve these conflicting observations, and to determine the mechanism by which gentamicin treatment confers protection against T7 phage infection, we measured changes in bacterial growth rate following infection for *E. coli* treated with cell-filtrate from gentamicin-dosed cells.

We first wanted to define the optimal concentration of gentamicin with which to dose the bacterial cells and to replicate the results shown in the previous studies. We began by visually determining the MIC of gentamicin for *E. coli* to be 8 ug/ml, as was done by Amanian *et al.* and Hardman *et al.* (9, 10). Thus, any concentration of gentamicin below 8 ug/ml was initially determined to be ‘sub-inhibitory’. However, when we then performed lysis assays with *E. coli* cells treated with 1 ug/ml, 3 ug/ml, and 4 ug/ml of gentamicin (Fig. 1), we saw that while samples treated with 4 ug/ml of gentamicin showed a decreased rate of phage-mediated lysis, there was also a lack of significant cell growth. Rather, the cells ‘flat-lined’ (Fig. 1), suggesting that even at half the initial MIC, gentamicin was inhibiting cell growth and thus masking any potential phage-resistive effect. Furthermore, samples treated with 1 ug/ml of gentamicin showed no change in their rate of phage-mediated lysis, indicating that such concentration was too low to induce protection. However, with 3 ug/ml of gentamicin, we saw that a peak in bacterial growth could be observed at 35 minutes (Fig. 1) into the lysis assay, suggesting that the gentamicin had less of an inhibitory effect. At this concentration, we were also able to observe a decrease in the rate of phage lysis (Fig. 1). This confirmed that the protection from phage-mediated lysis was dependent on the concentration of gentamicin with which the cells were treated, and was optimal at concentrations that were low enough to prevent inhibition of the cell growth (i.e. lower than 4 ug/ml), but high enough to prevent phage lysis, such as 3 ug/ml.

We additionally measured the rate of lysis following infection with phage-stock that had been treated with gentamicin, as well as following simultaneous phage infection and gentamicin dosage. No changes in the rate of phage-mediated lysis were observed, confirming that gentamicin did not directly affect phage activity (Fig. S1).



**FIG. 4 Proposed model for gentamicin-induced extracellular OMV production and subsequent reduction in T7 bacteriophage infectivity.** Treatment with sub-inhibitory gentamicin concentrations (1) causes a stress response in *E. coli* which leads to the release of OMVs into the extracellular environment (2). These OMVs serve as decoys that T7 phage binds to effectively reducing the amount of T7 phage entering *E. coli* (3). This results in less overall *E. coli* lysis (4).

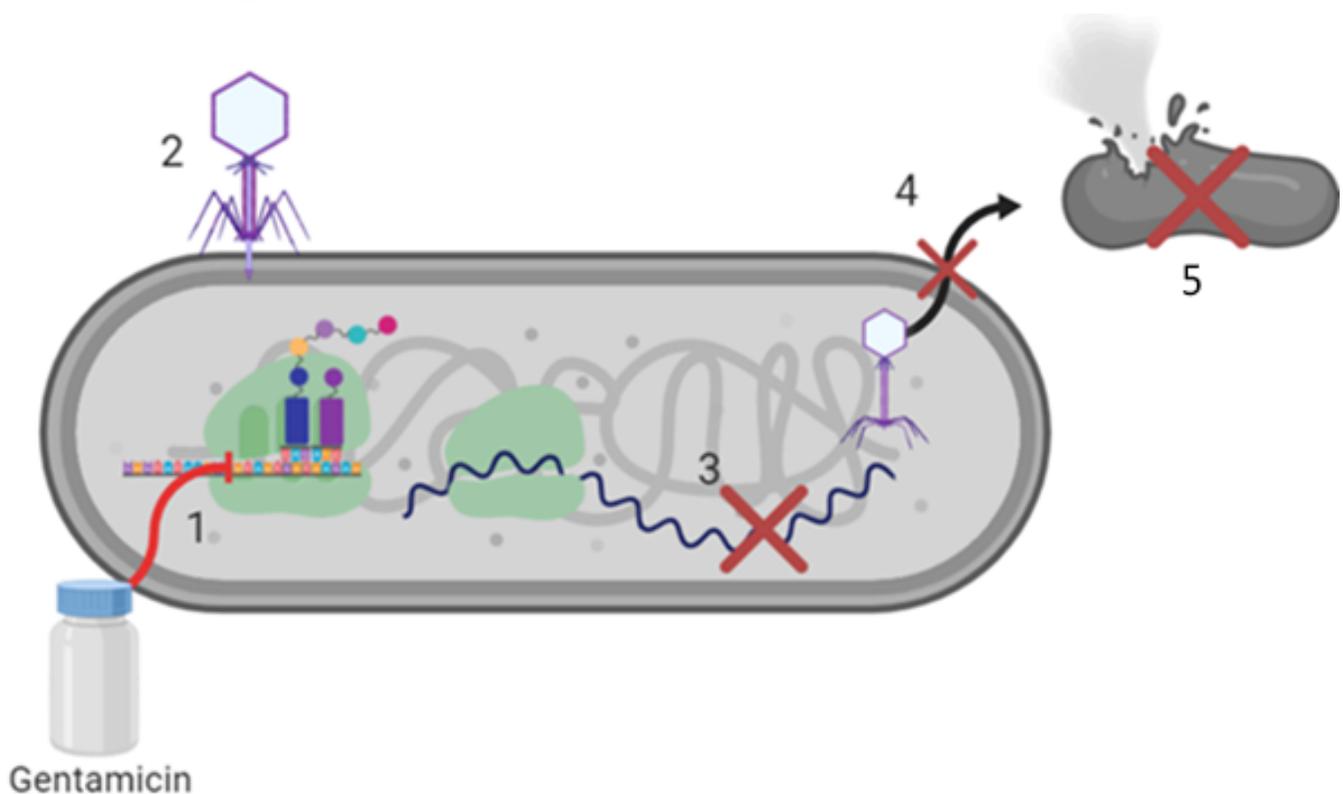
Next, we wanted to determine if this phage-protective effect was transferable through cell-filtrate. This would enable us to see if the possible resistance factor was active extracellularly. We found that while cells treated with gentamicin showed a similar trend to the previous experiment (the rate of phage-mediated lysis was reduced), *E. coli* treated with filtrate from gentamicin-dosed cells showed no such resistance to T7 phage infection (Fig. 2), suggesting that the phage-protective effect was not due to an extracellularly-released factor. As the cell samples were passed through a 0.22  $\mu\text{m}$  filter which would eliminate whole cells but allow OMVs (0.1  $\mu\text{m}$ ), we were able to rule out OMVs as the possible cause of phage-resistance (Fig. 4) (13).

Thus, we postulated that the gentamicin-induced resistance to phage might not be due to any particular 'protective factor'. Instead, it is likely the result of the effect of the antibiotic on some intracellular mechanism, such as a loss of ribosome function (Fig. 5). This was supported by the observation that sub-inhibitory concentrations of gentamicin still inhibited the growth of the treated *E. coli* (Fig. 3). Even at the optimal concentration of 3  $\mu\text{g}/\text{ml}$ , at which the phage-protective effect was seen, the peak observed corresponded to a lower cell density than that of gentamicin-untreated cells at the same timepoint (Fig. 1). While these sub-inhibitory concentrations may not wholly prevent cell growth, they likely prevent the phage from fully utilizing the host cell's resources, thus reducing phage infectivity.

**Conclusion.** In conclusion, this study has confirmed that treatment of *E. coli* UB1005 with sub-inhibitory concentrations of gentamicin results in protection against subsequent T7 bacteriophage-mediated lysis (Fig. 1). Through filtrate transfer lysis assays, we determined that the protective factor is not extracellularly released, as the factor could not be successfully transferred through cell filtrates (Fig. 2). Our data does not support the OMV model of protection (Fig. 4).

Instead, we consider an alternate model. Our growth curves and lysis curves suggest that ribosomal inhibition by gentamicin could possibly be the correct model (Fig. 5), due to gentamicin's inhibition of *E. coli* growth (Fig. 3). This experiment is a further step in characterizing this phenomenon as a consequence of the mechanism of growth inhibition by the antibiotic itself, and not as any novel produced 'protective factor' that can be isolated and studied on its own.

**Future directions.** Firstly, there is the possibility that any OMVs or other extracellularly released protective factors could have stuck to the membrane of the filter, thus removing them and their effect from the filtrate. Thus, the experiment could be repeated using differential centrifugation to separate the supernatant from the whole cells by mass, which avoids pelleting or filtering out any possible extracellularly released factor. Our experiment also relied on the use of a microtiter plate reader to measure *E. coli* growth (or lack thereof). As this was a different device from the spectrophotometer that was used to initially measure the OD, and as it also involved much smaller volumes of *E. coli*, we observed a difference in the initial OD measured and the starting OD when measured with the plate reader. While we considered this a minor issue as all replicates and test conditions still had the same starting OD (0.1) when measured with the plate reader (making them comparable with each other), future studies may avoid it altogether by using a different method to determine the initial and starting ODs. As it was only necessary to confirm that the bacterial concentrations remained the same across all the test conditions (which can be done with OD alone), we chose to measure our bacterial concentrations in terms of OD rather than in CFU/ml. This was also done in the three previous papers that we were focused on replicating. However, future studies should also determine the corresponding cell density in CFU/ml as it would account for the variance in the OD readings between different machines.



**FIG. 5 Proposed model for gentamicin-induced protein synthesis inhibition and subsequent reduction in T7 phage-mediated lysis.** Treatment with sub-inhibitory gentamicin concentrations has a bacteriostatic effect on *E. coli* and inhibits the *E. coli* ribosome (1) leading to reduced potential for protein synthesis and a more inactive cell state. Although T7 phage is able to enter *E. coli* (2), the lack of ribosome activity disrupts the phage replication cycle leading to less production of viral proteins (3) and subsequently less new virions (4). This decreased virion concentration in the *E. coli* reduces likelihood of cell lysis (5).

While our results suggest that the phage-protective effect observed arises from the effect of gentamicin on bacterial processes, the mechanisms by which they may affect viral infection remains unclear. It is likely that resistance arises from the gentamicin-mediated inhibition of the 30S ribosomal subunit, but it may also be due to some secondary effect the antibiotic has within the cells. Furthermore, the antibiotic may affect phage infectivity at any point during its replication cycle: phage replication may be affected due to ribosome inhibition, and phage release may be prevented by the inability to synthesize phage lysis enzymes.

To confirm that the resistance to phage induced by gentamicin treatment is the result of ribosomal inhibition by the antibiotic, rather than some other intracellular effects, future studies should test for the protective effect in *E. coli* strains resistant to gentamicin. The ribosomes of these cells would not be inhibited by the antibiotic, allowing phage-mediated lysis to occur at a rate similar to the untreated control. However, if the protective effect is instead due to some other intracellular effect of the antibiotic, a decrease in the rate of phage-mediated lysis would still be observed. When testing for this possibility, it is important that the mechanism by which the new *E. coli* strains are resistant to the antibiotic be considered. For example, gentamicin resistance via drug-efflux, exclusion, or inactivation would also prevent any secondary activities the antibiotic may have within the cell. Ideally, the resistance mechanism should only affect ribosomal activity (18).

Furthermore, previous studies have observed a reduction in phage adsorption as a result of streptomycin and kanamycin treatment (8, 19). Both of these drugs, as well as gentamicin, are aminoglycosides, which inhibit the activity of the 30S ribosome as their mechanism of action (20, 21), potentially suggesting that ribosomal inhibition may account for the reduction in this phage-protective effect. Thus, it would be useful to test antibiotics outside of the aminoglycoside class to see if the effect is dependent on inhibiting the 30S ribosome. Additionally, environmental stimuli, such as nutrient availability in the growth medium, may also affect phage infectivity (10). Therefore, it may be helpful to also investigate if the phage-protective effect is seen with other antibiotics, with the relevant environmental factors being accounted for. Ultimately, furthering our understanding of phage/antibiotic interactions will help us improve the approach we take towards the development of combination therapies for bacterial infections.

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## CONTRIBUTIONS

All authors contributed equally to design and experimentation, to the analysis and discussion of results, and to the final manuscript.

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