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Aminoglycosides Antagonize Bacteriophage Proliferation, Attenuating Phage Suppression of Bacterial Growth, Biofilm Formation, and Antibiotic Resistance

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ABSTRACT The common cooccurrence of antibiotics and phages in both natural and engineered environments underscores the need to understand their interactions and implications for bacterial control and antibiotic resistance propagation. Here, aminoglycoside antibiotics that inhibit protein synthesis (e.g., kanamycin and neomycin) impeded the replication of coliphage T3 and *Bacillus* phage BSP, reducing their infection efficiency and mitigating their hindrance of bacterial growth, biofilm formation, and tolerance to antibiotics. For example, treatment with phage T3 reduced subsequent biofilm formation by *Escherichia coli* liquid cultures to 53% ± 5% of that of the no-phage control, but a smaller reduction of biofilm formation (89% ± 10%) was observed for combined exposure to phage T3 and kanamycin. Despite sharing a similar mode of action with aminoglycosides (i.e., inhibiting protein synthesis) and antagonizing phage replication, albeit to a lesser degree, tetracyclines did not inhibit bacterial control by phages. Phage T3 combined with tetracycline showed higher suppression of biofilm formation than when combined with aminoglycosides (25% ± 6% of the no-phage control). The addition of phage T3 to *E. coli* suspensions with tetracycline also suppressed the development of tolerance to tetracycline. However, this suppression of antibiotic tolerance development disappeared when tetracycline was replaced with 3 mg/liter kanamycin, corroborating the greater antagonism with aminoglycosides. Overall, this study highlights this overlooked antagonistic effect on phage proliferation, which may attenuate phage suppression of bacterial growth, biofilm formation, antibiotic tolerance, and maintenance of antibiotic resistance genes.

IMPORTANCE The coexistence of residual antibiotics and phages is common in many environments, which underscores the need to understand their interactive effects on bacteria and the implications for antibiotic resistance propagation. Here, aminoglycosides acting as bacterial protein synthesis inhibitors impeded the replication of various phages. This alleviated the suppressive effects of phages against bacterial growth and biofilm formation and diminished bacterial fitness costs that suppress the emergence of tolerance to antibiotics. We show that changes in bacteria caused by environmentally relevant concentrations of sublethal antibiotics can affect phage-host dynamics that are commonly overlooked *in vitro* but can result in unexpected environmental consequences.

KEYWORDS phage therapy, biofilms, phage-antibiotic combinations, antibiotic resistance, antagonism

Antibiotic-resistant bacteria (ARB) pose a major threat to global health (1). Residual antibiotics in the environment are widely recognized as critical effectors for the maintenance and propagation of ARB and associated antibiotic resistance genes (ARGs) (2). Thus, numerous studies have addressed the selective pressure exerted by different antibiotics and the associated bacterial adaptation and defense mechanisms (3, 4). In contrast, the influence of phages on antibiotic resistance dissemination is understudied despite their significant effect on bacterial populations and fitness (5). Phages are ubiquitous (6, 7), and residual

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antibiotics are widespread (2), making their incidental cooccurrence with bacteria common in many environments. Therefore, it is important to determine whether combined exposure to phages and antibiotics may result in generalizable but unintended consequences for bacterial control and selection for resistance.

Notwithstanding the potential for some phages to help disseminate antibiotic resistance through transduction, phages are promising candidates to combat antibiotic resistance through phage-antibiotic combination therapy, which can suppress the emergence of resistance through their additive or even synergistic bactericidal effects (8, 9). Combined exposure to phages and antibiotics may lead to smaller bacterial populations and less chance of mutations or horizontal gene transfer conferring resistance (1). Furthermore, phages can bind to multidrug efflux pumps conferring antibiotic resistance (10), exerting a trade-off cost where selective pressure from phages favors bacteria with fewer efflux pumps that, as a result, are more sensitive to antibiotics (11). Thus, interactions between antibiotics and bacteria can be indirectly but importantly affected by the presence of phages, even though such interactions may vary depending on the specific idiosyncrasies of the interacting phage and bacterial species as well as the antibiotic's mode of action.

Phage-antibiotic combinations are generally synergistic for microbial control, making them a promising alternative for controlling resistant pathogens (1), although there are exceptions where selective pressure from either phages (11) or antibiotics (12) drives changes in bacterial surface chemistry that may interfere with the antibacterial activity of the other. Our previous study demonstrated that antibiotics acting extracellularly may have an antagonistic effect on phage attachment and resulting infection (12). However, whether sublethal levels of antibiotics that act intracellularly can exert antagonistic effects on phage-based bacterial control remains largely undocumented.

Among the antibiotics acting intracellularly, aminoglycosides are widely used in medicine (13, 14) and agriculture (15), leading to their substantial accidental and incidental discharge to natural systems (16, 17). Furthermore, aminoglycosides have received renewed attention because of their ability to treat multidrug-resistant pathogenic bacteria (18, 19). Aminoglycosides target protein synthesis by binding to the A site on the 30S ribosome, altering its conformation and allowing the assembly of incorrect amino acids, which promotes mistranslation (20). As viruses, phages hijack the host's machinery to produce phage proteins and progeny. Thus, aminoglycosides such as kanamycin and neomycin, which damage the protein synthesis capacity of bacteria, may also interfere with the production of phage progeny in phage-infected bacteria. Furthermore, biofilms, which facilitate horizontal gene transfer and ARG propagation (21), are suppressed by phage predation (22), and aminoglycoside interference with phages may alleviate this mitigation mechanism.

When phages exert greater control of bacterium populations than antibiotics, sublethal levels of some antibiotics may even provide an incidental benefit to the antibiotic-sensitive bacteria by interfering with the production of phage progeny. Such conditions are plausible because antibiotic levels in the environment are frequently low (2), while phages are generally abundant, reaching over 10^9 viral particles/ml in wastewater treatment plants (23) and marine environments (5). Thus, it is important to discern whether such aminoglycoside-phage interactions can affect bacterial control and antibiotic resistance development.

In this work, we used three antibiotics (kanamycin, neomycin, and tetracycline) and three phages (coliphage T3 and two wild phages isolated from activated sludge) to examine their interactive effects when controlling the Gram-negative bacterium *Escherichia coli* and the Gram-positive bacterium *Bacillus cereus*. Different antibiotic-phage combinations were considered to discern whether their interactive effects on phage proliferation and bacterial growth followed a generalizable trend. Further effects on biofilm formation, the development of tolerance to antibiotics, and the ability to maintain ARGs were examined using the well-characterized *E. coli* and phage T3 to provide a more-in-depth study.

RESULTS

Aminoglycosides attenuate phage infections of Gram-negative *E. coli* K-12 and Gram-positive *B. cereus* bacteria. The efficiency of plating (EOP) of coliphage T3 and two wild sludge phages (phage WSP infecting *E. coli* and phage BSP infecting *B. cereus*),

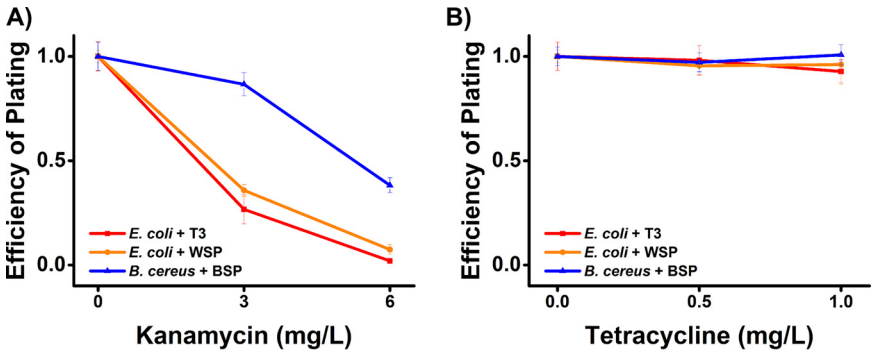

FIG 1 The efficiency of plating of three different phages was hindered by kanamycin but not tetracycline. (A) The EOP (measured as the number of PFU relative to the no-antibiotic control) decreased when 100 PFU of phage T3, WSP, or BSP were plated using LB agar on the respective host with 3 and 6 mg/liter of the aminoglycoside kanamycin. (B) The EOP of the three phages was not affected by 0.5 or 1 mg/liter tetracycline. Higher concentrations of either antibiotic interfered with uniform bacterial lawn formation, preventing accurate EOP measurements.

measured as the number of PFU relative to untreated controls, decreased in the presence of the aminoglycosides kanamycin and neomycin at sublethal levels that did not hinder uniform bacterial lawn formation. When phages T3, WSP, and BSP were plated on their respective hosts in the presence of 3 mg/liter kanamycin, the EOPs decreased to 0.27 ± 0.07, 0.36 ± 0.03, and 0.87 ± 0.06, respectively, compared to when no kanamycin was added (Fig. 1A). In the presence of 6 mg/liter kanamycin, the EOPs of phages T3, WSP, and BSP further decreased to 0.02 ± 0.01, 0.07 ± 0.02, and 0.38 ± 0.04, respectively, compared to the control. Similar trends were observed for neomycin. The EOPs of phages T3, WSP, and BSP decreased to 0.47 ± 0.07, 0.86 ± 0.04, and 0.73 ± 0.03, respectively, in the presence of 3 mg/liter neomycin and further decreased to 0.11 ± 0.03, 0.39 ± 0.01, and 0.47 ± 0.02, respectively, in the presence of 6 mg/liter neomycin compared to the no-neomycin control (see Fig. S1 in the supplemental material). This demonstrates that the ability to attenuate the overall phage infection process is not specific to kanamycin but rather shared with other aminoglycosides and is applicable to both Gram-negative *E. coli* and Gram-positive *B. cereus* bacteria.

In contrast to aminoglycosides, the EOP of T3 on *E. coli* K-12 in the presence of 0.5 or 1 mg/liter tetracycline was statistically indiscernible from 1.00 (i.e., 0.98 ± 0.07 or 0.93 ± 0.05, respectively) (Fig. 1B). This was also true for the EOPs of phage WSP on *E. coli* (0.95 ± 0.04 and 0.96 ± 0.10, respectively) and phage BSP on *B. cereus* (0.97 ± 0.05 and 1.01 ± 0.05, respectively) when treated with 0.5 and 1 mg/liter tetracycline (Fig. 1B).

Aminoglycosides decrease phage burst size more than tetracyclines, resulting in a difference in the ability to attenuate phage infections. Although both antibiotics inhibit protein synthesis, aminoglycosides decrease phage proliferation more than tetracyclines, resulting in their differential effects on the phage EOP. Adsorption of phage T3 to *E. coli* K-12 was not affected by treatment with either 3 mg/liter kanamycin or 1 mg/liter tetracycline (Fig. 2A). However, the amount of phage progeny produced greatly decreased from 303 ± 32 to 9 ± 2 normalized PFU/ml when *E. coli* K-12 was treated with 3 mg/liter kanamycin (Fig. 2B). Although not zero, this is sufficient to attenuate the overall phage infection process, resulting in no plaques when measuring the phage EOP from such infection events since not all phage particles can reach the host intact and result in a successful infection (24). In contrast, 1 mg/liter tetracycline decreased the phage progeny produced from 303 ± 32 to 91 ± 6 normalized PFU/ml. However, this inhibitory effect of tetracycline on phages was observed only in the phage one-step growth curves and was not sufficient to affect the overall phage infection process. This is evidenced by similar concentrations of tetracycline having no effect on the phage EOP (Fig. 1B) and tetracycline-phage combinations being more effective at controlling *E. coli* K-12 than either agent alone (Fig. 3B). This corroborates

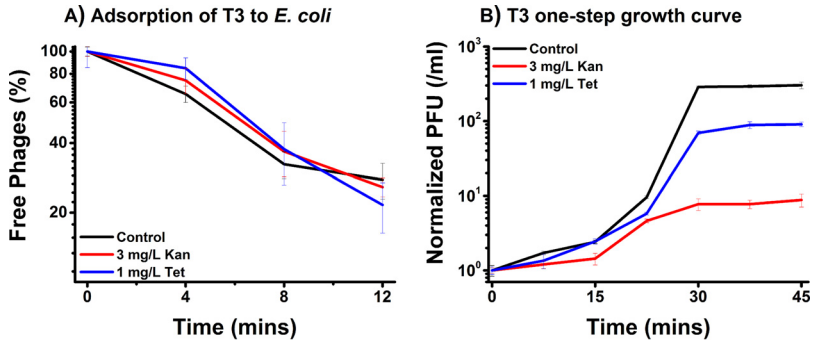

FIG 2 Kanamycin and tetracycline hinder phage proliferation intracellularly without affecting their adsorption to cell surfaces. (A) Phage T3 adsorption to *E. coli* was unaffected by prior treatment for 30 min of host bacteria with 3 mg/liter kanamycin (Kan) or 1 mg/liter tetracycline (Tet). (B) The burst size of phage T3 in host *E. coli* decreased by the addition of 3 mg/liter kanamycin or 1 mg/liter tetracycline, with kanamycin exerting greater phage suppression.

previous studies in which aureomycin, a chlortetracycline, similarly decreased phage burst size (25).

Attenuation of phage replication by aminoglycosides may increase bacterial populations and subsequent biofilm biomass that would otherwise be suppressed by phages. *E. coli* K-12 treated with both 0.25 mg/liter tetracycline and 50 PFU/ml phage T3 reached a final optical density at 600 nm (OD_{600}) of 0.03 ± 0.002 after 15 h, which is significantly lower ($P < 0.05$) than those of both the phage-only treatment (OD_{600} of 0.18 ± 0.03) and the tetracycline-only treatment (OD_{600} of 0.48 ± 0.06) (Fig. 3B). However, *E. coli* K-12 treated with 0.5 mg/liter kanamycin and 50 PFU/ml phage T3 reached a final OD_{600} of 0.35 ± 0.06 after 15 h, which was higher than that of the phage-only treatment (OD_{600} of 0.11 ± 0.02 after 15 h) and closer to that of the kanamycin-only treatment (OD_{600} of 0.41 ± 0.02 after 15 h) (Fig. 3A). Similar results were observed when *B. cereus* treated with both 5 mg/liter kanamycin and 2.5×10^7 PFU/ml phage BSP reached an OD_{600} of 0.58 ± 0.004 after 15 h, but an OD_{600} of just 0.36 ± 0.02 was reached when treated with phage BSP only (Fig. S2A). Likewise, *E. coli* K-12 reached a final OD_{600} of 0.40 ± 0.04 after 15 h when treated with both 0.75 mg/liter neomycin and 15 PFU/ml phage WSP but reached an OD_{600} of only 0.16 ± 0.002 after treatment with just phage WSP (Fig. S2B). Thus, interference with phage propagation by aminoglycosides provided a benefit to the bacteria, making aminoglycoside-phage combinations less effective at controlling bacterial populations than phage-only treatments.

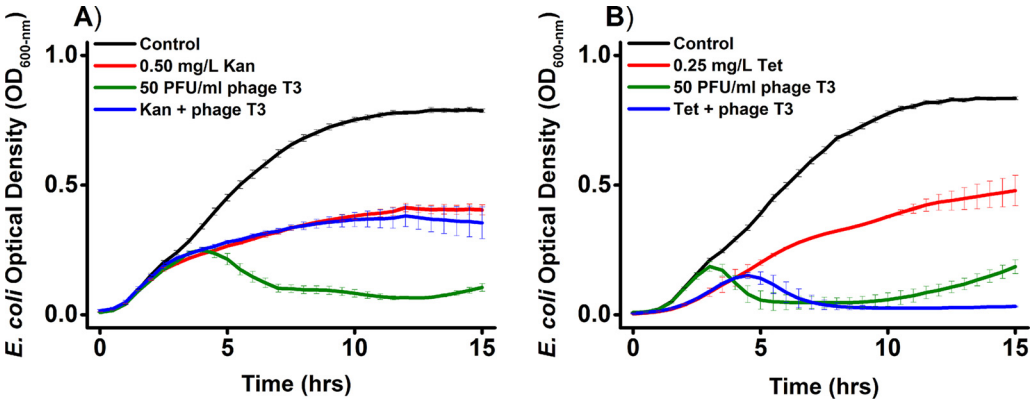

FIG 3 Aminoglycosides protect bacterial populations from phage infection. The combined effects of phage-antibiotic exposure were assessed with *E. coli* growth curves in LB medium. (A) The combination of kanamycin and phage T3 resulted in higher *E. coli* optical densities than the phage-only treatments. (B) This antagonism was not observed for combined exposure to phages with tetracycline, which showed greater suppression than either agent alone.

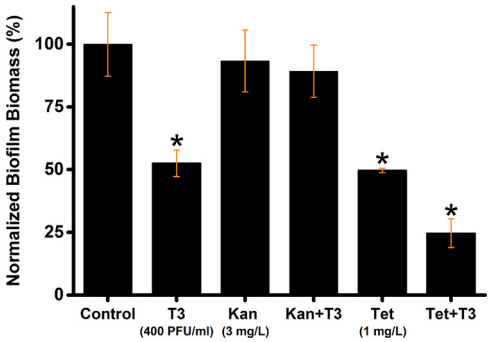

FIG 4 Kanamycin (but not tetracycline) prevented phage T3 from suppressing *E. coli* biofilm formation. *E. coli* biofilm formation after 48 h at 30°C in a 96-well plate was significantly decreased by the addition of 400 PFU/ml phage T3 to the initial liquid culture. Compared to the phage-only treatment, the resulting level of biofilm formation was high for combined exposure to phage T3 and kanamycin. Note that kanamycin alone had no effect on biofilm biomass, possibly due to its lower efficacy in TSBYE (see Fig. S3 in the supplemental material). In contrast, combined treatment with tetracycline and phage T3 suppressed biofilm formation more than either agent alone. Asterisks denote treatments that resulted in significantly less biofilm biomass than the control.

The amounts of biofilm biomass formed after 48 h at 30°C by *E. coli* K-12 in the biofilm formation medium, tryptic soy broth with 0.5% (wt/vol) yeast extract (TSBYE), were 50% ± 1% of that of the no-treatment control when 1 mg/liter tetracycline was added and only 25% ± 6% when both 1 mg/liter tetracycline and 400 PFU/ml phage T3 were added (Fig. 4). However, initial liquid cultures of planktonic *E. coli* K-12 treated with both phages and aminoglycosides had higher resulting biofilm formation than the phage-only treatments. The amount of biofilm biomass formed after 48 h was reduced to 53% ± 5% of that of the no-treatment control when the culture was treated with 400 PFU/ml phage T3. When treated with both 3 mg/liter kanamycin and 400 PFU/ml phage T3, biofilm biomass formation increased to 89% ± 10% of that of the no-treatment control (Fig. 4).

Aminoglycosides hinder phage suppression of antibiotic resistance development. Overnight-grown cultures of *E. coli* K-12 treated using 3 mg/liter kanamycin resulted in 144 ± 11, 94 ± 20, and 33 ± 5 CFU when equivalent volumes of each culture were streaked on base agar plates containing 0, 3, and 6 mg/liter kanamycin, while cultures grown overnight with 3 mg/liter kanamycin and 2,000 PFU/ml phage T3 had 143 ± 8, 105 ± 10, and 49 ± 11 CFU (Fig. 5A). Thus, the addition of phage T3 to the culture grown overnight resulted in slightly higher but not significant changes to the number of CFU formed under selective pressure from kanamycin. In contrast, overnight-grown cultures of *E. coli* K-12 treated using 1 mg/liter tetracycline had 82 ± 6, 29 ± 7, and 7 ± 0.4 CFU when equivalent volumes of each culture were streaked on base agar plates containing 0, 0.5, and 1 mg/liter tetracycline, while cultures grown overnight with 1 mg/liter tetracycline and 2,000 PFU/ml phage T3 had 37 ± 6, 6 ± 1, and 0 CFU (Fig. 5B). Furthermore, the numbers of CFU on base agar containing 0.5 and 1 mg/liter tetracycline were 36% ± 3% and 8% ± 0.4% of the number of CFU on base agar with no antibiotics for overnight-grown cultures of *E. coli* K-12 treated with only 1 mg/liter tetracycline but only 17% ± 3% and 0% for cultures grown overnight with 1 mg/liter tetracycline and phage T3. Thus, the addition of phages decreased both the total number of CFU formed on base agar containing tetracycline and their percentage compared to the no-antibiotic control. Here, phage T3 decreased the antibiotic tolerance of *E. coli* (i.e., the ability of bacteria to survive transient exposure to antibiotics) to tetracycline but not kanamycin. This has implications for the emergence of antibiotic resistance since tolerance precedes and is a key contributor to antibiotic resistance development (26).

Additional experiments were conducted to assess the effect of phage inhibition on ARG maintenance and propagation in a mixed culture of *E. coli* BL21 harboring plasmids with bla_{NDM-1} and *E. coli* K-12. Combination treatment with 400 PFU/ml phage T3

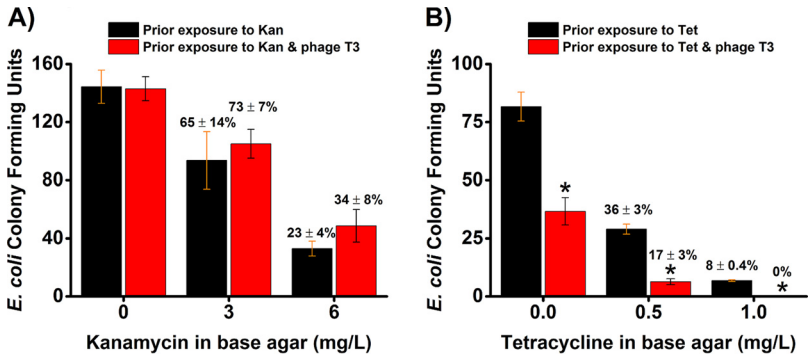

FIG 5 Phage T3 suppressed antibiotic tolerance to tetracycline, but not to kanamycin, in *E. coli*. Suspensions of *E. coli* grown overnight were treated with either 3 mg/liter kanamycin or 1 mg/liter tetracycline, with and without 2,000 PFU/ml phage T3, and then streaked onto base agar plates containing antibiotics to examine the effect of phages on antibiotic resistance development. Total CFU from equal dilutions and volumes are shown on the vertical axis, and CFU as percentages compared to CFU on base agar with no antibiotics are shown on top of the bars. Phage T3 decreased both the total number and percentage of CFU, denoting bacteria surviving tetracycline treatment. In contrast, phage T3 had no significant effect on the number of CFU when paired with kanamycin. Asterisks denote where the addition of T3 resulted in significantly fewer CFU.

and 6 mg/liter kanamycin for 15 h at 30°C resulted in a higher copy number of $bla_{\text{NDM-1}}$ genes than with the phage-only treatment (i.e., 4.63 ± 0.05 versus 3.79 ± 0.23 $\log_{10}$ gene copies/ml, respectively) (Fig. 6). Similar trends were found for the total *E. coli* population density measured by the optical density (Fig. S4) and by 16S rRNA gene copy numbers (Fig. 6). However, the ratios of $bla_{\text{NDM-1}}$ to 16S rRNA gene copy numbers for the combined and phage-only treatment groups were not significantly different (0.55 ± 0.03 versus 0.55 ± 0.02) since phage T3 does not differentiate between antibiotic-resistant and -susceptible *E. coli*. Although ARG propagation measured by the relative abundance of ARGs in the population was unaffected, the larger total population (due to kanamycin's ability to inhibit phage infections) led to a higher absolute abundance of ARGs. This illustrates how phage inhibition by aminoglycosides could contribute to ARG maintenance at the population level in the common presence of phages.

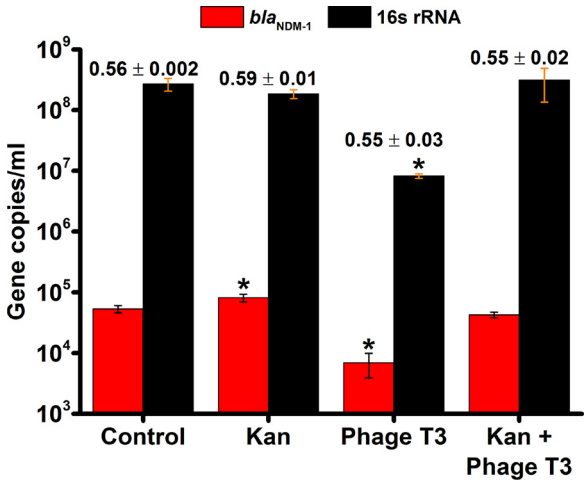

FIG 6 Combined treatment with kanamycin and phage T3 results in higher total abundances of $bla_{\text{NDM-1}}$ and bacteria than phage-only treatment. Overnight-grown cultures of *E. coli* BL21 harboring plasmids with $bla_{\text{NDM-1}}$ and *E. coli* K-12 were mixed (1/1,000 [vol/vol]). Triplicate wells containing 10 μl of the mixed culture and 190 μl of TSBYE were treated with 400 PFU/ml phage T3 and 6 mg/liter kanamycin. After growth for 15 h at 30°C, DNA was extracted from 200 μl of each well for qPCR analysis. In the presence of phage T3, the relative abundance (i.e., the ratio of $bla_{\text{NDM-1}}$ to 16S rRNA in $\log_{10}$ gene copy numbers) (shown above the bars) was not significantly affected by the addition of kanamycin. Asterisks denote significant differences of $bla_{\text{NDM-1}}$ and 16S rRNA gene copies from the untreated controls.

DISCUSSION

Some antibiotics indirectly interfere with the life cycle of phages due to their reliance on bacterial hosts to reproduce. Here, aminoglycosides attenuated phage infections in both Gram-negative *E. coli* and Gram-positive *B. cereus* bacteria (Fig. 1A), with greater attenuation corresponding to higher but still sublethal antibiotic concentrations (see Table S1 in the supplemental material). Thus, residual aminoglycosides may inadvertently hinder phages in suppressing biofilm formation and associated problems like biofouling (27), harboring opportunistic pathogens (28), and ARG propagation by enhanced horizontal gene transfer (21). Furthermore, this represents a caveat for antibiotic-phage combination therapy to preserve antibiotic efficacy (1) and an unintended consequence of the growing interest in aminoglycosides to combat multidrug resistance (18, 29).

Previously, we showed that *E. coli* can experience lipopolysaccharide (LPS) modifications following exposure to β-lactams. This conferred cross-resistance to phage T3, which binds to LPS phage receptors (12). Conversely, exposure to phages may also result in cross-resistance to antibiotics due to LPS mutations that confer not only phage resistance but also tetracycline resistance through a combination of decreased permeability and reduced expression of the tetracycline uptake porin OmpF, which is linked to the LPS (11). However, phages can utilize various membrane structures besides lipopolysaccharides as receptors, such as outer membrane proteins (30) that are unaffected by this extracellular mechanism. The intracellular inhibition mechanism discerned here may not be limited to certain species of bacteria or phages since phage-host specificity largely occurs extracellularly by the recognition of phage receptors on the host surface. Furthermore, phages can evolve to target alternative receptors (31) to bypass the extracellular inhibition mechanism, but there are no such alternatives for producing phage proteins since all phages need to hijack host protein synthesis to reproduce. While all phages should be broadly affected by their host's protein synthesis capacity, owing to their great biological diversity, some phages may experience less inhibition by aminoglycosides than the phages used in this study. For example, unlike phage T3, temperate phages can incorporate their DNA into the host genome (32), allowing them to enter a dormant phase and be better maintained without replicating than phage T3. Differences in how the dynamics between phage T3 and *E. coli* are affected by β-lactams and aminoglycosides highlight the complexity of phage-antibiotic combinations and underscore the need for further studies to understand the full intricacies.

At sublethal concentrations, aminoglycosides inhibit phage proliferation to a degree where the entire infection process is attenuated. This is not so for tetracycline, another protein synthesis-inhibiting antibiotic, which was less effective at suppressing phage proliferation. The exponential replication of phage progeny masks the partial inhibition of phage proliferation by tetracyclines, which have no discernible effects on the overall infection process, allowing tetracyclines to work in conjunction with phages to suppress bacterial growth despite having the same mode of action as aminoglycosides. This difference is possibly due to aminoglycosides having the additional function of inducing mistranslation. Aminoglycosides are known to cause mistranslation of various phage proteins such as phage coat proteins, maturation proteins, and lysozymes (33, 34). Apparently, mistranslation may have cascading effects downstream since the incorporation of mistranslated proteins might make an entire phage particle defective and jeopardize its infectivity. We demonstrate that while attenuation of phage replication can be caused by different aminoglycosides, this is not generalizable to all antibiotics that inhibit protein synthesis. In fact, the capacity to attenuate phage replication is likely determined more specifically by the protein synthesis-inhibiting mechanisms, whose effects on phages require further study.

It is widely recognized that phages can complement antibiotics to combat resistance (35–37). The likelihood of horizontal gene transfer conferring resistance is decreased by combined exposure to phages and antibiotics due to the resulting smaller bacterial populations (1). Additionally, bacterial evolution of phage resistance commonly involves hindering phage adsorption by cell surface receptor modifications (38). However, such

modifications may compromise antibiotic resistance. For example, phage resistance due to surface polysaccharide mutations (as reported for *Enterococcus faecalis*) increases its sensitivity to antibiotics that target the cell wall and cell membrane, such as vancomycin and daptomycin (39, 40). Thus, the ubiquitous presence of phages in the environment inherently suppresses to some extent the development of antibiotic resistance when sublethal concentrations of antibiotics are present. In this context, aminoglycosides are an exception since their ability to inhibit phage infections may provide an advantage to affected bacteria in the common presence of phages due to less hindrance to bacterial growth and antibiotic resistance development and maintenance at the total population level, as illustrated here for $bla_{\text{NDM-1}}$. Therefore, further research is needed to assess the extent and significance of this overlooked phenomenon.

MATERIALS AND METHODS

General approach. Two different aminoglycosides (kanamycin and neomycin) were tested to examine if phage inhibition is an effect generalizable to the aminoglycoside class of antibiotics. We also evaluated tetracyclines, another class of antibiotics that (similarly to aminoglycosides) bind to the 30S ribosome and inhibit protein synthesis, to determine if phage inhibition was a generalizable consequence of antibiotics that inhibit protein synthesis (41). The effects of combined treatment on the bacterial and phage populations were examined using EOP tests, growth curves for liquid cultures, and crystal violet assays for biofilm formation. The development of ARB was assessed through changes in the number of CFU when plated with antibiotics. The effect of the cooccurrence of antibiotics and phages on ARG maintenance and propagation was also considered by quantifying changes in the abundance of $bla_{\text{NDM-1}}$, coding for New Delhi metallo-β-lactamase in multidrug-resistant, NDM-1-producing *E. coli* since the cooccurrence of $bla_{\text{NDM-1}}$ and broad-spectrum aminoglycoside resistance genes (i.e., 16S rRNA methylase genes) is frequently detected (42).

Bacterial strains and phage stocks. The bacterial strains used in this study were *E. coli* K-12 (ATCC 10798), *E. coli* BL21 (catalog number BIO-85035; Bioline), and *B. cereus* NRS 248 (ATCC 10987). According to the manufacturer's instructions, plasmid pET-29a(+) containing $bla_{\text{NDM-1}}$ and additionally conferring kanamycin resistance was inserted into *E. coli* BL21, and 50 mg/liter kanamycin was used for selection after transformation. All cells were cultured in Luria-Bertani broth (LB) (BD Difco) at 30°C. To explore response variability and demonstrate that aminoglycosides broadly attenuate phage infections, three different phages were used in this study. These include *Podoviridae* coliphage T3 (ATCC 110303-B3) and two uncharacterized wild phages isolated from activated sludge, one infecting both *E. coli* K-12 and BL21 (phage WSP) and another infecting *B. cereus* (phage BSP); both were isolated from activated sludge as previously described (43). Phage stocks were suspended in SM buffer (50 mM Tris-HCl, 0.1 mM NaCl, 8 mM $MgSO_4$, 0.01% [wt/vol] gelatin) (44) and stored at 4°C. The numbers of bacteria and phages were enumerated by colony and plaque counting assays, respectively (45). For the double-layer plaque assay, the base layer was made with dextrose BD Difco plate count agar, and the top layer was LB agar (0.3% [wt/vol] agar).

Phage efficiency of plating, adsorption, and burst size. The EOP for the phages was determined using the ratio of PFU on double-agar-layer plates with antibiotics to that on plates with no antibiotics when ~100 PFU of each phage was plated with 100 μl from an overnight-grown culture (optical density at 600 nm [OD_{600}] of ~0.4) of their respective hosts (46). The antibiotics used were 3 and 6 mg/liter of the aminoglycoside kanamycin (catalog number BP906-5; Fisher BioReagents) or neomycin (catalog number 97061-908; VWR) or 0.5 and 1 mg/liter of the nonaminoglycoside antibiotic tetracycline (catalog number B21408-14; Alfa Aesar). These concentrations were below the MICs (optical density at 600 nm of <0.1 after incubation overnight at 30°C in LB) of ~8 mg/liter for kanamycin, ~10 mg/liter for neomycin, and ~2 mg/liter for tetracycline, which interfered with uniform bacterial lawn formation for plaque assays. Host bacteria, phages, and antibiotics were added to 5 ml of ~50°C LB agar, poured onto the base layer, inverted after solidifying, and incubated overnight at 30°C. Adsorption and one-step growth curve experiments were performed using cultures of *E. coli* K-12 grown overnight in LB at 37°C to determine which step of the phage life cycle aminoglycosides interfered with. Phage adsorption to bacteria in the presence of antibiotics was examined by pretreating bacteria with either 3 mg/liter kanamycin or 1 mg/liter tetracycline for 30 min prior to the addition of phage T3 to test adsorption, performed as described previously (47). The effect of 3 mg/liter kanamycin and 1 mg/liter tetracycline on phage proliferation was measured with the simultaneous addition of phage and antibiotic using a one-step growth curve as previously described (48). The concentrations of aminoglycoside were chosen to be sublethal but sufficient to decrease the phage EOP. At the same concentration as aminoglycosides, tetracycline was lethal; thus, lower concentrations of tetracycline were used.

Bacterial challenge tests in liquid medium. To test whether antibiotics could protect bacteria from phage attacks, growth curves were generated in tissue culture-treated Costar 96-well flat transparent plates. Each well contained 190 μl of LB medium and 10 μl of a bacterial culture grown overnight. The OD_{600} was measured with a Tecan Infinite 200 Pro plate reader, with measurements taken every 30 min for 15 h at 37°C. Due to their different efficacies, different concentrations of antibiotics were used to treat *E. coli* K-12 (0.5 mg/liter kanamycin, 0.75 mg/liter neomycin, and 0.25 mg/liter tetracycline) to have comparative effects of lowering the final OD_{600} values by about one-half, compared to those of the no-antibiotic controls. This experiment used a lower antibiotic concentration to ensure a detectable bacterial optical density. *E. coli* K-12 was also treated with either 50 PFU/ml phage T3 or 15 PFU/ml phage

TABLE 1 Primers for real-time qPCR analysis[a]

Target gene	Reagent	T_a (°C)	Primer sequence	Reference(s)
bla_{NDM-1}[b]	SYBR green	54	5′-CAA CTG GAT CAA GCA GGA GAT-3′ 5′-GTC GAT CCC AAC GGT GAT ATT-3′	54, 55
16S rRNA	SYBR green	57	5′-CGG TGA ATA CGT TCY CGG-3′ 5′-GGW TAC CTT GTT ACG ACT T-3′	56

[a]All primers were custom ordered from Integrated DNA Technologies (Coralville, IA). T_a, annealing temperature.
[b]Primer sequences for bla_{NDM-1} were designed as previously described (54, 55).

WSP, which decreased the OD_{600} more than aminoglycosides such that interference with phage proliferation by aminoglycosides would result in an increase in the optical density. *B. cereus* required higher concentrations of phage BSP (2.5×10^7 PFU/ml) and kanamycin (5 mg/liter) for a comparable effect.

Biofilm formation and characterization. To examine the effect of aminoglycoside interfering with phage infection of planktonic bacteria on subsequent biofilm formation, *E. coli* K-12 biofilms were formed in tissue culture-treated Costar 96-well flat transparent plates. For biofilm-related experiments, LB medium was replaced with tryptic soy broth (BD Difco) with 0.5% (wt/vol) Bacto yeast extract (TSBYE) (Thermo Fisher) due to better *E. coli* biofilm formation in TSBYE than in LB medium. Triplicate wells contained 190 μl of TSBYE and 10 μl of an overnight-grown *E. coli* K-12 culture diluted by a factor of 100 and 400 PFU/ml phage T3 with and without 3 mg/liter kanamycin or 1 mg/liter tetracycline. The multiplicity of infection here is 0.0001, the same as the one used in the assays described below. Plates were cultured at 30°C without shaking for 48 h. A crystal violet optical assay was used for biofilm characterization as previously described, with some modifications (49). Each well was washed three times with 200 μl of phosphate-buffered solution (PBS), 200 μl of 0.1% (wt/vol) crystal violet was then added, and the plate was incubated in the dark at room temperature for 30 min. The crystal violet solution was pipetted out from each well, each well was washed three times with 200 μl PBS again, 200 μl of 70% ethanol was then added to each well, and the plate was incubated for 30 min at room temperature in the dark again. The ethanol in each well was then transferred to a new plate, and a Tecan Infinite 200 Pro plate reader was used to measure the optical density at 595 nm, which was calculated as a percentage compared to the no-treatment control.

Selecting representative antibiotic concentrations is important, even though residual aminoglycoside concentrations in the environment are seldom reported due to a lack of suitable quantification methods (16). There are reports of neomycin concentrations of 0.0225 mg/liter in pond water (50) and kanamycin concentrations of 0.0982 ± 0.01 mg/liter in wastewater effluent (51), which are 1 order of magnitude lower than the concentrations used here. However, it is important to consider the total aminoglycoside concentration in the environment, especially when the modes of action are similar (as is the case for kanamycin and neomycin, both of which attenuate phage replication). Considering that at least 10 different antibiotics are often found in impacted environments (50), which increases the total antibiotic concentration, we conclude that the concentrations used in this study are environmentally relevant.

Development of tolerance to antibiotics. Overnight-grown cultures inoculated with 125 μl *E. coli* K-12 (from a culture grown overnight to an OD_{600} of ~0.4), 3 mg/liter kanamycin, and 2,000 PFU/ml of phage T3 in 5 ml of LB medium were streaked onto base agar plates with 3 and 6 mg/liter kanamycin. The resulting percentages of antibiotic-tolerant subpopulations were calculated as the numbers of colonies formed (from equal dilutions and volumes) on base agar containing antibiotics compared to those formed with no antibiotics. This experiment was repeated with the culture grown overnight containing 1 mg/liter tetracycline, and the culture was streaked onto base agar plates with 0.5 and 1 mg/liter tetracycline instead of kanamycin to assess their differential effects despite their similar modes of action.

Effect of antibiotic and phage cooccurrence on bla_{NDM-1} maintenance and propagation. Plasmid pET-29a(+) containing bla_{NDM-1} and additionally conferring kanamycin resistance was inserted into *E. coli* BL21. The effect of phage inhibition on ARG maintenance and propagation was assessed using overnight-grown cultures of *E. coli* BL21 harboring plasmids with bla_{NDM-1} (OD_{600} of ~0.2) and *E. coli* K-12 (OD_{600} of ~0.4) mixed at a ratio of 1/1,000 (vol/vol) in 96-well plates. Triplicate wells containing 10 μl of the mixed culture and 190 μl of TSBYE were treated with 400 PFU/ml phage T3 and 6 mg/liter kanamycin. After 15 h at 30°C, 200 μl of each well was processed with a DNeasy PowerSoil kit (Qiagen) to extract DNA for real-time quantitative PCR (RT-qPCR) to measure the abundance of bla_{NDM-1}. Real-time qPCR was performed as previously described, with modifications, using a reaction mixture containing 7.5 μl of PowerUp SYBR green master mix (Applied Biosystems, Foster City, CA), 5.5 μl nuclease-free water, 500 nM primers (Table 1), and 1 μl of the DNA sample, for a total volume of 15 μl (52). The RT-qPCR protocol used was 10 min at 95°C followed by 45 cycles of 95°C for 15 s for denaturation, 30 s at 54°C for annealing, and 30 s at 72°C for extension on the Bio-Rad CFX96 qPCR machine. The cycle time was converted to $\log_{10}$ gene copies per milliliter using standard curves generated from linear regression (53).

Statistical analysis. All the experiments were performed in triplicate (or more replicates), and Student's *t* test (two tailed) was used to determine if differences between treatments were significant at the 95% confidence level (i.e., $P < 0.05$).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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