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Sub-Lethal Gentamicin Treatment of *Escherichia coli* UB1005 Induces the Release of Soluble Factors that Reduce Susceptibility to T7 Bacteriophage Infection

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***Escherichia coli* have evolved numerous mechanisms to resist infection by bacteriophages. Here, we investigate if resistance to phage T7 infection involves the gentamicin-induced release of soluble factors, such as lipopolysaccharide (LPS). We show that treatment with sub-lethal levels of gentamicin results in a decreased susceptibility to T7 phage infection in *E. coli* UB1005. Our data suggest that the mechanism of resistance is not explained by the release of soluble LPS since the addition of soluble LPS from *E. coli* UB1005 does not increase resistance to T7 infection. Surprisingly, washing cells by performing buffer changes with repeated centrifugation after gentamicin treatment heavily sensitized UB1005 to infection. This data suggests that a factor may be released upon gentamicin treatment that is responsible for resistance to T7 bacteriophage. Our results may be useful in industrial and biotechnological applications where bacteriophage contamination of bacterial cultures has proven to be a persistent and pertinent challenge.**

In order to ensure their survival, bacteria have developed many mechanisms of resistance against bacteriophage infection (1-3). Several adsorption inhibition strategies have been characterized including mutations in phage receptors, production of extracellular matrix, and exploitation of competitive inhibitors that bind to phage receptors (1-3). Through coevolution, phages in turn develop counter-adaptations to overcome these antiviral mechanisms (1).

Bacteriophage T7, a member of the *Podoviridae* family, infects most rough strains of *E. coli*, and employs a lytic life cycle (4, 5). The T7 phage has an icosahedral capsid containing a 40kbp DNA genome and a tail fibre required for cell surface recognition and adherence to the viral receptor on the host surface (6-8). Rough lipopolysaccharide (LPS) is thought to be the primary phage T7 receptor on the bacterial surface of *E. coli* (6-8). Previous literature has proposed that T7 adherence and entry is potentially mediated by interactions with secondary receptors (FhuA and TonB) that induce irreversible binding (4, 17). Porins (OmpA and OmpF) and other outer membrane proteins have been suggested to assist in viral entry (9). T7 phage binds to several sugar moieties of LPS, including the first glucose, the penultimate glucose, or the terminal heptose to initiate adherence and entry (1). Upon irreversibly binding to LPS, it is thought that proteins gp13 and gp7.3 of the phage tail are degraded, shaping a pathway for proteins gp14-16 to access the cell wall (17). Attachment is then followed by penetration of the peptidoglycan layer and inner membrane and subsequent DNA ejection into the cytoplasm (17).

Previous research has shown that *E. coli* treated with kanamycin or streptomycin, both aminoglycoside antibiotics, were found to be resistant to T7 bacteriophage adsorption (10, 11). In addition, antibiotic treatment can lead to the release of LPS, representing one potential mechanism of viral resistance (12-14). In particular, LPS has been shown to be released in a free, soluble form and also bound to outer membrane vesicles (OMVs) upon treatment with aminoglycosides (13, 14). Several proposed mechanisms for the induction of LPS release following antibiotic treatment exist in the literature. Kadurugamuwa *et al* proposed that cationic antibiotics, such as gentamicin, could destabilize cell membranes by perturbing salt-bridges formed between anionic sites on LPS and exogenous cations like Mg^{2+} and Ca^{2+} (15). Furthermore, T7 phage can bind to soluble rough LPS *in vitro* (non membrane-bound), inducing the ejection of the phage genome (8). The addition of soluble LPS prevented P22 phage adsorption to *Salmonella enterica* cells by blocking binding sites necessary for host cell receptor interactions (16). The culmination of literature is suggestive of a biological mechanism for inhibiting the adsorption of bacteriophage that involves the release of soluble LPS. In particular, it brings forth the question of whether or not soluble LPS released upon treatment with antibiotics can reduce phage

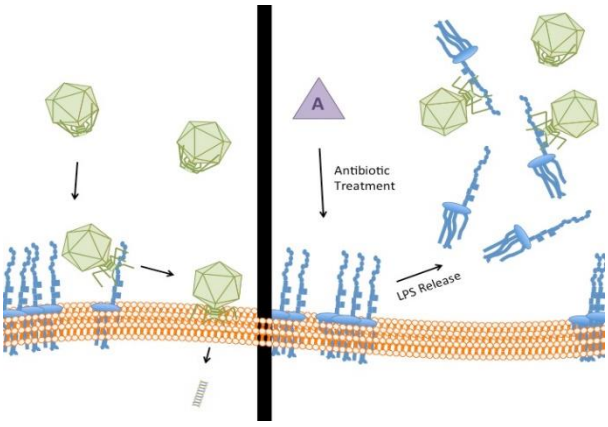

FIG. 1 Proposed Mechanism for Antibiotic-Induced Resistance to T7 Phage Infection. The left-hand side illustrates a normal infection, where T7 phage binds to its receptor, LPS, on the outer membrane of Gram-negative bacteria. This results in DNA ejection into the cell. The right-hand side illustrates LPS release as a result of antibiotic treatment. The T7 phage binds to a soluble form of LPS, reducing infection frequency.

infection by acting as a decoy receptor to the natural membrane-bound viral receptor.

Here, we investigated LPS release as a mechanism for the antibiotic-induced resistance to T7 phage infection (Fig. 1). In this mechanism, antibiotic treatment results in the release of soluble LPS into the environment which then acts as a soluble decoy receptor to T7 infection. Phage binding to soluble LPS decreases the frequency of phage binding to membrane-bound LPS, thus reducing phage adherence and infectivity.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *E. coli* UB1005 was donated by the Hancock lab from the University of British Columbia. Cultures were propagated in M9 minimal media liquid broth in a shaker at 37°C overnight. M9 minimal media was made with 100 mL of 10X M9 Salts, 50 mL of 10% glucose/L, 10 mL of 0.1M $MgSO_4$/L, 50 mL of 20% casamino acids/L, 2 mL of 10 mg/mL vitamin B1/L, 2 mL of 10 mg/mL tryptophan/L and 790 mL of deionized water. 10X M9 Salts were made by adding 60g Na_2HPO_4, 30g KH_2PO_4, 5g NaCl, 10g NH_4Cl to 999mL of dH_2O and autoclaved. 1mL of 1M filter-sterilized $CaCl_2$ was added to the 10X M9 salts after autoclaving.

Propagation and Purification of T7 Bacteriophage. The protocol used to propagate and purify bacteriophage was adapted from Chiang *et al*. and Barrick *et al.*(18, 19). T7 phage stock solution (unquantified) received from the Department of Microbiology and Immunology at the University of British Columbia was prepared in 2.5 ml of molten 42°C phage top agar (PTA) (M9 minimal media with 0.75% agar) with 200µl of *E. coli* UB1005 and plated on phage bottom agar (PBA) (M9 minimal media with 1.5% agar). PTA was allowed to solidify on a shaker in order to ensure even spreading of PTA and incubated overnight at 37°C. After incubation, plates that were observed to have numerous plaques were scraped with a sterile glass rod to collect the PTA layer in a single 50mL Falcon™ tube. For each 2.5mL of PTA added to the Falcon™ tube an aliquot of 2mL of M9 and 100µL of chloroform was added to the tube. This mixture was incubated for two days at 4°C and centrifuged at 5000 x g for 7 minutes at 4°C. The aqueous phase was carefully transferred to a new centrifuge tube and centrifuged again at 5000 x g for 7 minutes at 4°C to remove residual chloroform. The aqueous phase containing T7 was removed and stored at 4°C.

Confirmation of T7 Stock Purity. 1ml of phage stock was heated to 95˚C for 10 minutes to denature the viral capsid. Primers specific to the T7 *rpol* and T4 *gp23* genes yielding PCR products of 517 bp and 398 bp respectively were used in a PCR reaction with a 52˚C annealing temperature and 30 second extension time for 30 cycles. Presence of T7 and any contamination of T4 was visualized through gel electrophoresis in a 1% agarose gel with SYBR safe run for 60 minutes at 100 volts.

Primers:

T7 *rpol*: Fwd: CGA GGG CTT AGG TAC TGC
Rev: GGT GAG GTG CGG AAC TTC

T4 *gp23*: Fwd: GCC ATT ACT GGA AGG TGA AGG
Rev: TTG GGT GGA ATG CTT CTT TAG

Minimal Inhibitory Concentration (MIC) Assay of *E. coli* UB1005 Under Gentamicin Treatment. A culture of *E. coli* UB1005 was grown in M9 broth overnight at 37ºC. ½ serial dilutions of gentamicin from 8 µg/ml to 32 µg/ml were made in Eppendorf Tubes® and 100µl of these solutions were mixed in duplicates with 100µl of *E. coli* UB1005 culture in a 96 well plate for final concentrations of 4 µg/ml to 16 µg/ml. The plate was incubated overnight and MIC was determined by visualization of increased, decreased, or unchanged turbidity.

T7 Phage Plaque Assay. 100µl of T7 was added to 900µL *E. coli* UB1005 (OD of 0.5) at an MOI of $7.0x10^{-4}$ and incubated for 10 min. Cells were spun at 5000 x g for 1 minute and the supernatant was aspirated to remove any un-adhered phage. The pelleted cells were gently resuspended in 1mL of sterile water. 200uL aliquots of the resuspended mixture were added to test tubes containing 2.5 ml of molten 42°C PTA. Tubes were gently mixed to ensure even distribution while avoiding damaging phage or bacteria and plated on PBA. Plates were allowed to solidify on a shaker for 15 minutes. Solidified plates were incubated overnight at 37°C and plaques were enumerated visually by counting plates with more than 3 plaques and less than 200 to ensure no plaques were eclipsed by overgrowth.

Lipopolysaccharide Treatment of E coli UB1005. The propagated T7 stock solution was diluted to 10^{-5} PFU/mL. Lyophilized LPS derived from *E.coli* strain UB1005 was obtained from the Hancock lab in the Department of Microbiology and Immunology at the University of British Columbia and was resuspended and diluted in sterile water to a final concentration of 10µg/mL or 100µg/mL in an Eppendorf Tube® containing 900µL of *E. coli* UB1005 at OD 0.5. 100µl of the diluted T7 stock was added and tubes were incubated for 10 minutes and treated as previously described.

Removal of Extracellular Soluble Factors by Washing. Eppendorf Tubes® containing *E. coli* UB1005 at OD 0.5 were spun at 5000 x g for 1 minute. The supernatant containing soluble factors was removed and cells were gently resuspended in 1 mL of warm M9 broth to avoid shearing. The wash was repeated once more.

RESULTS

Sub-lethal gentamicin treatment decreases T7 bacteriophage infection in *E. coli* UB1005. A preliminary experiment suggested that gentamicin treatment resulted in a greater decrease in infection compared to kanamycin, tetracycline, or ciprofloxacin (data not shown). Additionally, Evans and Pollack demonstrated that sub-lethal gentamicin treatment releases a relatively large amount of LPS compared to other antibiotics (14). Therefore, we chose to focus on gentamicin.

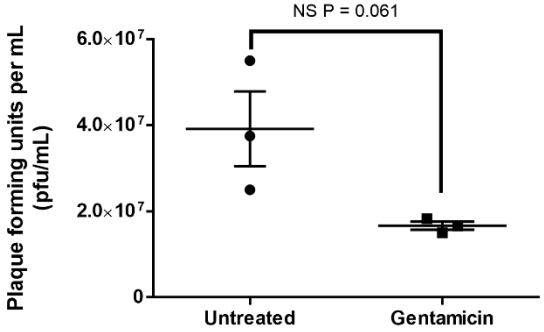

FIG. 2 Sub-lethal gentamicin treatment decreases T7 bacteriophage infection in *E. coli* UB1005. Each data point represents a single biological replicate of *E. coli*. Biological replicates were plated in triplicate and the pfu/mL was averaged between technical replicates. The line between data points indicates mean pfu/mL. Error bars represent the standard error of the mean. Gentamicin was added at a concentration of 8 µg/ml.

E. coli UB1005 treated with the highest concentration of gentamicin that remained sub-lethal (8μg/ml) or untreated were incubated at 37ºC for 1 hour. After treatment, a plaque assay was conducted to enumerate the number of adhered phage. A negative control, which included gentamicin-treated and untreated cells without T7 bacteriophage, resulted in a lack of plaques (data not shown). Sub-lethal gentamicin treatment decreased T7 bacteriophage infection in *E. coli* UB1005 (Fig. 2).

Soluble lipopolysaccharide treatment of *E. coli* UB1005 does not decrease phage infection. To distinguish between the induction of LPS release and other gentamicin-induced effects, we set out to determine whether soluble, rough LPS is sufficient to decrease T7 phage infection in *E. coli* UB1005. Purified rough LPS from *E. coli* UB1005 was added to cultures of *E. coli* at concentrations of 10 and 100 μg/mL and a plaque assay was performed. A plaque assay was also performed on LPS- treated *E. coli* without adding T7 bacteriophage, as well as M9 media-treated *E. coli* UB1005 prior to adding T7 bacteriophage (0 μg/ml LPS) as controls. Addition of 10 and 100 μg/mL of soluble rough LPS showed no significant change in observed plaques compared to controls (Fig. 3).

Soluble factors released by *E. coli* after gentamicin treatment protect against T7 bacteriophage infection. Based on our results showing that soluble LPS addition to bacterial cultures does not decrease T7 bacteriophage infection, we set out to determine if soluble factors produced by gentamicin treatment had a role in decreasing infection. Additionally, we wanted to see if LPS addition to a relatively LPS-free culture could modulate T7 bacteriophage infection. We used a plaque assay to measure infectivity. A sample not treated with gentamicin, but washed and resuspended in fresh M9 media was used as a control to determine the baseline level of infection after resuspending in fresh media. Figure 4 highlights that washing cells after gentamicin treatment to remove soluble factors prior to adding bacteriophage significantly

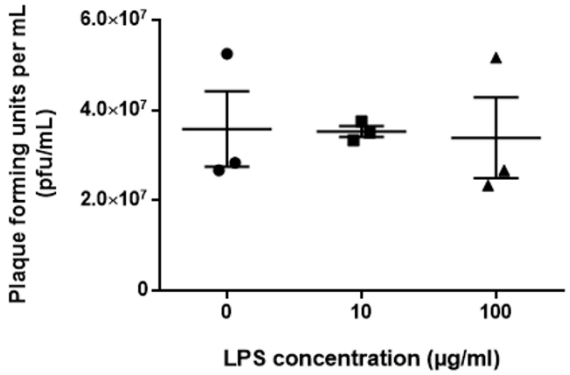

FIG. 3 Soluble lipopolysaccharide treatment of *E. coli* UB1005 does not decrease T7 bacteriophage infection. Each data point represents a single biological replicate of *E. coli*. Biological replicates were plated in triplicate and the pfu/mL was averaged between technical replicates. The large line between data points is the mean pfu/mL, and the error bars represent the standard error of the mean. No significant difference between treatments.

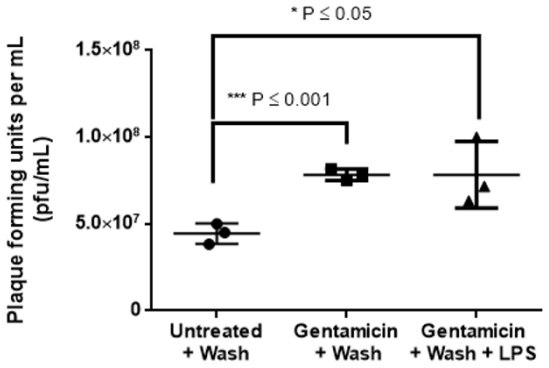

FIG. 4 Soluble factors released by *E. coli* UB1005 after sub-lethal gentamicin treatment protect against T7 bacteriophage infection. Each point represents an average of three technical replicates of a single biological replicate. The large line in between the data points is the mean pfu/mL, and the smaller error bars represent the standard error of the mean. An unpaired student's t test was used to evaluate the difference between groups. Gentamicin was added to bacterial cultures at a concentration of 8 μg/mL and LPS was added at a concentration of 100 μg/mL.

increases infection ($p \leq 0.001$). Additionally, adding LPS after gentamicin treatment and removing soluble factors does not decrease infection, further emphasizing the inability of soluble LPS to prevent T7 phage infection.

DISCUSSION

We set out to determine if gentamicin decreases phage infection through the release of T7 bacteriophage-neutralizing soluble LPS. Previous studies showed that sub-lethal gentamicin treatment causes LPS release in *E. coli*, and that T7 bacteriophage can bind to soluble, non-membrane-bound rough LPS (8, 14). These two observations guided our research question of understanding why sub-lethal aminoglycoside treatment decreases T7 bacteriophage infection in *E. coli*.

Here, we describe another aminoglycoside (gentamicin) that is able to decrease T7 bacteriophage infection in *E. coli* UB1005 (Fig. 2). This contributes to the findings by Gu *et al.* and Bleackley *et al.* that describe these phenomena with streptomycin and kanamycin, respectively (10, 11). Although we were not able to produce a significant result ($p = 0.061$), our data trends towards a gentamicin-induced reduction in infection suggesting that aminoglycosides play a physiologically relevant role in preventing phage infection.

Contrary to our model's prediction, adding soluble rough LPS does not decrease T7 bacteriophage infection in *E. coli* UB1005 (Fig. 3). This supports the null hypothesis that gentamicin does not decrease phage infection through a mechanism that involves soluble LPS. A limitation of this conclusion is that we were not able to quantify the absolute amount of LPS released in response to gentamicin treatment. Only relative LPS release in response to antibiotics, including gentamicin, has been described. As a result, 10 and 100 μg/mL

treatments of rough LPS as described in this study may not be representative of the amount of LPS released by *E. coli* in response to gentamicin treatment. Quantifying the amount of LPS released after gentamicin treatment will be imperative to draw absolute confirmation that soluble LPS release in response to antibiotic treatment does not act to decrease infection.

After observing that soluble LPS addition to gentamicin-treated cultures did not decrease infection, we conducted experiments to remove soluble factors present in the culture after gentamicin-treatment and prior to infection with phage (Fig. 4). Gentamicin-treated cells that were washed to remove soluble factors showed a significant increase in T7 bacteriophage infection compared to cells that were only washed ($p \leq 0.001$). Additionally, adding soluble LPS to washed-gentamicin-treated cells had no effect on reducing infection compared to washed-gentamicin-treated samples without added LPS (Fig. 4). This further suggests that soluble rough LPS does not have a role in decreasing T7 phage infection in *E. coli* UB1005. Interestingly, this experiment suggests that gentamicin causes the release of soluble factors that prevent T7 bacteriophage infection. Additionally, it appears in the absence of released soluble factors, gentamicin-treatment causes *E. coli* to become more susceptible to T7 bacteriophage infection.

Given our data, an attractive potential soluble factor may be outer membrane vesicles (OMVs) which are constitutively released by gram negative bacteria (20). Gentamicin has been described to cause a several fold increase in the release of outer membrane vesicles (OMVs) in gram negative bacteria, and OMVs are capable of blocking phage infection (15, 20). These observations may account for the decreased infection we see in gentamicin-treated cells (Fig. 2). It is important to note that OMVs are spherical shaped and are composed of outer membrane (OM) proteins in addition to LPS (20). Because we did not see a decrease in T7 phage infection following treatment with purified rough LPS (Fig. 3), this suggests that soluble rough LPS on its own does not have the same ability to block phage infection as has been reported with OMVs (20). This may be due to the presence of OM co-receptors in OMVs increasing the binding affinity of T7 phage, allowing for the successful inhibition of infection. TonB and FhuA are two OM receptors for T7 bacteriophage (17), however we could not find any evidence in the literature that they are included in *E. coli* OMVs.

We did not investigate why gentamicin-treated cells with removed soluble factors have increased T7 infection, however many conjectures can be put forth that may explain this phenomenon. Gentamicin treatment changes the outer membrane landscape of gram negative bacteria by disrupting the packing order of LPS, creating holes in the OM and releasing OMVs (15, 20, 21). OMVs and the surface of gram negative bacteria contain different concentrations of OM proteins; certain OM proteins are excluded from OMVs while others are enriched (20). The increase in infection we see after gentamicin treatment and removal of soluble factors may be due to a difference in T7 bacteriophage receptor densities on the OM of gentamicin treated cells. In addition, it has been well described that gentamicin treatment inhibits protein synthesis by binding to the 30S subunit of the ribosome (22). As a result, the restriction modification systems employed by bacteria in response to the detection of phage DNA may be unable to respond due to the gentamicin-induced downregulation of protein synthesis (1). Lastly, some strains of *E. coli* are known to produce secreted peptides, such as microcin J25, which can block phage infection through the binding of FhuA, a putative OM receptor for T7 bacteriophage (17, 23). It is unclear whether microcin J25 is produced in *E. coli* UB1005, however if it is, the gentamicin-induced inhibition of protein synthesis, as well as the removal of soluble factors will likely nullify the effects of this anti-phage system.

In conclusion, we describe sub-lethal gentamicin treatment decreasing T7 bacteriophage infection in *E. coli* UB1005. Rough, soluble LPS in the concentration tested is not involved in blocking T7 infection and gentamicin treatment induces the release of non-LPS, soluble factors that protect against T7 infection. We suspect that OMVs may have a role in conferring this gentamicin-induced protection towards T7 bacteriophage. In the absence of these soluble factors, gentamicin-treatment increases the susceptibility of *E. coli* UB1005 to T7 infection. This mechanism is not fully understood, however it may involve the formation of an OM landscape that is more favorable for infection or through the down regulation of anti-phage protein systems.

FUTURE DIRECTIONS

It would be valuable to quantify the absolute amount of physiological LPS and LPS released in response to sub-lethal gentamicin treatment in order to determine if the concentrations we used to treat cells were biologically relevant. This would confirm our conclusion that soluble rough LPS does not have a role in inhibiting T7 bacteriophage infection in *E. coli* UB1005. We attempted to confirm this using an LAL chromogenic endotoxin quantitation kit (Pierce ™), however we were not able to remove the background LPS. Future groups may attempt to use endotoxin-free supplies and media to avoid this problem.

Future experiments involving the removal of supernatant (Fig. 4) should include an internal control of an unwashed culture treated with gentamicin. This would allow a comparison between washed and unwashed samples while reducing the number of variables, increasing the robustness of the data.

In order to confirm the presence and nature of the soluble factor conferring resistance towards T7 bacteriophage in response to gentamicin treatment, future groups may wish to remove the supernatant of gentamicin-treated cells and treat the supernatant with heat, proteases, lipases or perform mass spectrometry and ultracentrifugation to determine the size of the soluble factors in the supernatant. This supernatant then may be used to resuspend untreated cells and T7 infectivity can be compared between treatments. This may shed insight into the chemical composition of the protective, soluble factors released in response to gentamicin treatment.

To implicate the role of OMVs in gentamicin-induced T7 resistance, an evaluation of the activity of regulator of capsule synthesis (Rcs) mutants in resisting T7 infection is required. RcsB of Rcs systems are believed to play a role in OMV production and knockout strains have been shown to not exhibit an increase in OMV production in response to cellular stress (24). It is important to note that these effects have not been observed in *E. coli*, however confirming these observations in *E. coli* may provide a means of identifying the role of OMVs in T7 phage resistance.

Finally, understanding why gentamicin-treated *E. coli* UB1005 exhibits a lower susceptibility to T7 bacteriophage infection may be evaluated by looking at restriction enzyme concentrations following gentamicin treatment, or at the relative concentrations of OM proteins that are known receptors for T7 (FhuA and TonB). It may be worthwhile to determine if microcin J25 is expressed in *E. coli* UB1005, and if this expression is downregulated as a result of gentamicin treatment. We suspect more than one anti-phage system may be inhibited as a result of the effects of gentamicin.

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