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Article

Bacteriophage TLS sensitizes Escherichia coli to antibiotics

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SUMMARY

There is a significant threat to health across the world due to the misuse of antibiotics, which yield resistant pathogens. Discovering new antibiotics or using bacteriophages—viruses that infect bacteria—to treat resistant infections are two possible solutions to antibiotic resistance. A third strategy is to use phages to make current antibiotics more effective. We hypothesized that targeting the TolC protein in *Escherichia coli* with a bacteriophage would increase *E. coli* sensitivity to antibiotics. TolC is the pore-forming protein in the AcrAB-TolC efflux pump and is involved in antibiotic resistance. We used TLS, a bacteriophage that binds TolC to initiate infection, to generate TLS-resistant *E. coli*. We tested these phage-resistant variants for their sensitivity to the antibiotics novobiocin and chloramphenicol and performed Sanger sequencing to identify genotypic changes in the *tolC* gene locus that could account for changes in sensitivity. The minimal inhibitory concentration for both novobiocin and chloramphenicol was lower in all phage-resistant variants except for one, which showed sensitivity only to novobiocin. This finding indicates that *E. coli* variants that survive infection by TLS are sensitized to antibiotics, illustrating an evolutionary tradeoff. Interestingly, we did not identify mutations in the *tolC* gene that could explain the phenotypic changes aside from a point mutation in the PhoP binding site in one variant. We hypothesize that resistance to novobiocin and chloramphenicol arises not from genetic changes to *tolC*, but from alterations in its level of expression. Alternatively, mutations that affect lipopolysaccharide synthesis could explain the phenotypes we observed.

INTRODUCTION

Antibiotics have played a crucial role in battling bacterial infections since their discovery in the 20th century (1). However, bacteria have evolved to resist these drugs due in part to increasing misuse of antibiotics (1). One of the contributing factors to increasing antibiotic resistance is cross-resistance, or the development of resistance to one antibiotic that transfers to similar drugs (1). For example, mutations in *Pseudomonas aeruginosa* leading to triclosan resistance result in resistance to other antibiotics (1). Rising antibiotic and pan-drug resistance in bacteria is causing a major threat worldwide (1). The ESKAPE pathogens, which include *Escherichia coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, and *Enterobacter* species, are the most common hospital-acquired bacterial infections and some of the most difficult to treat due to their extensive antibiotic resistance (2). ESKAPE pathogens cause 80% of the antimicrobial resistance-related deaths worldwide (2). An estimated 5 million fatalities were caused or exacerbated by antimicrobial resistance globally in 2019 (3). It is predicted that 39 million deaths will result directly from antibiotic-resistant bacteria between 2025 and 2050, and that an additional 169 million deaths will be indirectly caused by antibiotic-resistant bacteria, such as through the exacerbation of an underlying health condition (4).

Transporting antibiotics and antimicrobials out of cells through efflux pumps is one mechanism of antibiotic resistance (5). Efflux pumps exist as both an intrinsic and acquired mechanism of antibiotic resistance (5). They are found throughout the Eubacteria domain, including gram-positive and gram-negative bacteria (5). *E. coli* has several classes of efflux pumps, including the resistance-nodulation-division (RND) family, which are primarily found in gram-negative bacteria (5). The AcrAB-TolC system is an RND efflux pump in *E. coli* consisting of three polypeptides that function together to transport molecules, such as antibiotics, out of the cell (5, **Figure 1**). AcrB is a translocase that transports hydrogen ions (H^+) into the cell while moving molecules from inside the cell into the periplasm (6). The molecules are funneled from AcrB through the periplasm to the pore-forming protein TolC via AcrA (6, **Figure 1**). All components of the AcrAB-TolC system are required for resistance to antibiotics and antimicrobials such as novobiocin, chloramphenicol, sodium dodecyl sulfate (SDS), and crystal violet (7).

Outer membrane proteins, such as TolC, can be receptors that bacteriophages (phages) attach to when they initiate an infection. Phages are viruses that infect bacteria with high specificity, often infecting only one species or subspecies of bacteria (8). Other receptors for infection can include specific motifs or structures on flagellum and lipopolysaccharide (LPS) (8). After attachment, the phage injects its DNA into the cytoplasm and utilizes the host cell machinery to replicate itself (8). This infection causes the cell to burst open, killing the bacterium and spreading the phage particles to infect other bacterial cells (8). However, populations of bacteria can survive infection via mutations that affect the production or expression of the factors to which phages bind, but these mutations come at a cost (9). The relationship between the phage OMKO1 and the bacteria *P. aeruginosa* is an example of this “evolutionary tradeoff”. *P. aeruginosa* can be resistant to OMKO1 or antibiotics, but not both at the same time (9). OMKO1 initiates infection by binding the outer membrane protein OprM, which functions as part of an efflux pump system (9). *P. aeruginosa* variants that have mutations in

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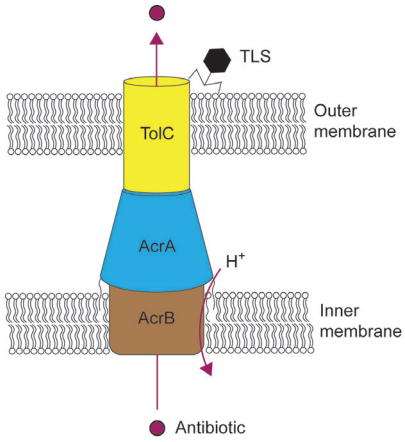

Figure 1: The AcrAB-TolC complex removes antibiotics from *Escherichia coli*. Substrates of the efflux pump system first bind AcrB and are funneled to TolC through AcrA. The AcrB translocase transports the substrate into the periplasm as it imports hydrogen ions (H+). The TolC outer membrane protein removes the antibiotic from the cell. Bacteriophage TLS binds to the extracellular portion of TolC and does not contact AcrA or AcrB.

oprM do not express the OprM protein and are therefore resistant to infection by OMKO1 (9). These variants are more susceptible to antibiotics than the parental population of *P. aeruginosa* because OprM no longer functions, thereby trapping antibiotic in the bacterium (9).

The tradeoff between antibiotic resistance and phage resistance can be exploited to treat infections caused by antibiotic-resistant bacteria. Phage therapy could potentially be a safer alternative to antibiotics because phages are highly specific. Phages can specifically target infectious bacteria and allow healthy, native bacteria in the human body to survive (10). For example, beneficial gut bacteria can be killed while someone is taking an antibiotic, but targeted phage therapy could prevent the disruption to healthy gut microbiomes (10). While the possibility of phage therapy has been considered for a hundred years, antibiotic resistant infections have led to renewed interest in alternative treatments to antibiotics (10). Phage therapy is used today to treat humans, particularly those impacted by bacterial infections resistant to antibiotics (10). The therapy has been used in Eastern European nations and Russia since the 1940s, while Western European countries opted to treat infections with antibiotics (10). Countries such as the United States and parts of Europe have recently resumed research, clinical trials, and case-by-case phage therapy due to the increasing concern for antibiotic resistance (10). Additionally, medical professionals in the United States, Europe, and Asia have successfully treated antibiotic-resistant infections with an approach called phage-antibiotic synergy, where a phage and antibiotic are delivered at the same time to treat an infection (11). An example of this synergy is the use of OMKO1 and ceftazidime to treat a recurrent infection of *P. aeruginosa* (12).

We set out to determine if a phage, TLS, could sensitize *E. coli* to antibiotics. TLS binds the TolC efflux pump in *E. coli,* and its attachment to TolC is the first step in an infection (13). We hypothesized that, after infecting wild-type *E. coli* with TLS, the surviving phage-resistant *E. coli* would exhibit sensitivity to the antibiotics novobiocin and chloramphenicol. We chose to use novobiocin because *E. coli* is considered to be naturally resistant to it, and previous research has shown a strain of *E. coli* lacking *tolC* (Δ*tolC E. coli*) is 100-fold more sensitive to novobiocin than wild-type *E. coli* (7). We used chloramphenicol because *E. coli* that do not express *tolC* are sensitive to it and because it is widely available (7). We also hypothesized these phenotypic changes would correspond with mutations in the *tolC* gene, resulting in a non-functional efflux pump. We observed TLS-resistant (TLSR) *E. coli* was sensitive to novobiocin and chloramphenicol, and that subinhibitory concentrations of novobiocin and TLS delivered at the same time inhibited the growth of wild-type *E. coli.* We did not consistently find mutations within the *tolC* promoter or protein coding region of TLSR *E. coli.* Collectively, these findings indicate that TLS can be used to sensitize *E. coli* to antibiotics, and that the mechanism of action may involve a change in the expression of TolC, or another mechanism we have yet to uncover.

RESULTS

We hypothesized that TLSR *E. coli* was antibiotic-sensitive, and generated TLSR *E. coli* to test the hypothesis. We exposed *E. coli* to TLS and allowed phage-resistant variants to grow. We confirmed variants to be resistant to TLS by their ability to grow on LB agar supplemented with the phage. We randomly selected five TLSR variants and tested them for antibiotic sensitivity. We used wild-type *E. coli* strain BW25113 as a negative control for sensitivity and Δ*tolC E. coli*, which cannot express the TolC protein, as a positive control for sensitivity to novobiocin. The average minimal inhibitory concentration (MIC) of novobiocin for wild-type and Δ*tolC E. coli* was 256±0 μg/mL and 6±10 μg/mL, respectively (**Figure 2A**). All five TLSR *E. coli* were more sensitive to novobiocin

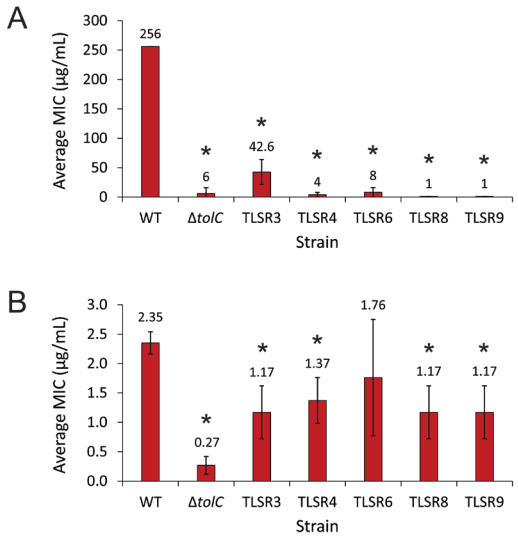

Figure 2: Average minimum inhibitory concentrations (MICs) of novobiocin and chloramphenicol in TLS-resistant (TLSR) strains of *Escherichia coli*. We tested five variants of TLSR E. coli for antibiotic sensitivity to novobiocin (A) or chloramphenicol (B). The bar graphs show the average minimum inhibitory concentration (MIC) of each antibiotic needed to inhibit the growth of the mutant strains in comparison to wild-type and Δ*tolC E. coli* (n=4). The error bars represent the 2x standard error of the mean (*: Student's T-test, $p<0.05$ compared to wild-type *E. coli*).

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than wild-type *E. coli* (**Figure 2A**, Student's T-test, p<0.05). TLSR4, 6, 8, and 9 were comparable to Δ*tolC E. coli,* while TLSR3 required an average of 42.6±21.3 µg/mL of novobiocin to inhibit its growth (**Figure 2A**).

We next assayed TLSR variants for their sensitivity to chloramphenicol. The MIC for wild-type *E. coli* was 2.35±0.19 µg/mL of chloramphenicol, and the MIC for Δ*tolC E. coli* was 0.27±0.15 µg/mL (**Figure 2B**). The MIC of chloramphenicol for TLSR3 (1.17±0.45 µg/mL), TLSR4 (1.37±0.39 µg/mL), TLSR8 (1.17±0.45 µg/mL), and TLSR9 (1.37±0.45 µg/mL) were roughly half that of wild-type *E. coli* (**Figure 2B**, Student's T-test, p<0.05). However, these variants were not as sensitive to chloramphenicol as Δ*tolC E. coli.* The MIC of chloramphenicol for TLSR6 was similar to the other TLSR variants, but the precision between replicates was too low to be statistically different from the wild-type strain (**Figure 2B**).

The decreases in the MICs suggested that TLSR *E. coli* variants were more sensitive to antibiotics than phage-sensitive *E. coli*. We wanted to know if subinhibitory concentrations of TLS and novobiocin could be used at the same time to inhibit the growth of wild-type *E. coli* (**Table 1**). Exposing wild-type *E. coli* to $1x10^7$ PFU/mL (MOI=1) of TLS allowed 24.7±13.9% of wells with wild-type *E. coli* to grow, while 99.7±0.7% of wells containing wild-type *E. coli* grew in the presence of $1x10^6$ PFU/mL (MOI=0.1) of TLS. All wells containing wild-type *E. coli* grew in LB supplemented with 32 µg/mL of novobiocin. However, the growth of wild-type *E. coli* was inhibited in the presence of TLS and novobiocin regardless of the TLS concentration (**Table 1**, Student's T-test, p<0.05 compared to TLS or novobiocin alone).

Our data showed TLSR *E. coli* were sensitive to novobiocin and chloramphenicol. We hypothesized the sensitivity could be explained by mutations in the *tolC* gene locus. We sequenced the *tolC* promoter and gene in TLSR *E. coli* variants and compared them to a reference sequence (NC_000913.3) to test if these phenotypic changes were due to mutations within the *tolC* gene locus. There were no genetic differences between wild-type *E. coli* and four of the five variants. We identified TLSR4 had a point mutation in the *tolC* promoter (**Figure 3**). These results indicate that the observed phenotypic changes are not caused by mutations within the promotor or gene of *tolC*, but they could be the result of other mutations or expression changes.

Treatment	$1x10^7$/mL TLS (MOI = 1)	$1x10^6$/mL TLS (MOI = 0.1)
TLS	24.7 ± 13.9%	99.7 ± 0.7%
NB	>99.0 ± 0.0%	>99.0 ± 0.0%
TLS + NB	<1.0 ± 0.0% *	<1.0 ± 0.0% *

Table 1: Inhibition of *Escherichia coli* growth by a cocktail of phage TLS and novobiocin. We cultured wild-type *E. coli* in wells of a 96-well plate with phage TLS at a concentration of $1x10^7$ or $1x10^6$ PFU/mL, novobiocin (NB), or TLS and NB. We calculated the percentage of wells that showed bacterial growth (n=3) and reported it as the average ± 2 standard error of the mean (*: Student's T-test, p<0.05 compared to the TLS and the novobiocin treatments).

DISCUSSION

In this study, we determined if a phage, TLS, could sensitize *E. coli* to antibiotics. We hypothesized that TLS would sensitize *E. coli* to novobiocin and chloramphenicol. TLS can initiate infection in *E. coli* by binding TolC—a component of the AcrAB-TolC efflux system—and Δ*tolC E. coli* is sensitive to a variety of antibiotics, including novobiocin and chloramphenicol. Our data showed that all five TLSR *E. coli* variants we generated were sensitive to novobiocin, and four of five were sensitive to chloramphenicol. *E. coli*, a gram-negative bacterium, is considered resistant to novobiocin, while gram-positive bacteria are sensitive (14–16). The MICs of novobiocin needed to inhibit gram-positive bacteria are similar to the MICs for the TLSR variants we have generated, indicating *E. coli* can be as sensitive to novobiocin as gram-positive bacteria are.

We hypothesized that TLS and novobiocin could work together to inhibit the growth of wild-type *E. coli*. Subinhibitory concentrations of TLS and novobiocin inhibited the growth of *E. coli*, while neither could do so alone. The combination of TLS and novobiocin allowed lower concentrations of both phage and antibiotics to inhibit growth, which could minimize side effects if used therapeutically and reduce the regrowth of bacteria resistant to both agents.

Not all TLSR variants exhibited the same level of sensitivity to novobiocin or chloramphenicol. This heterogeneity

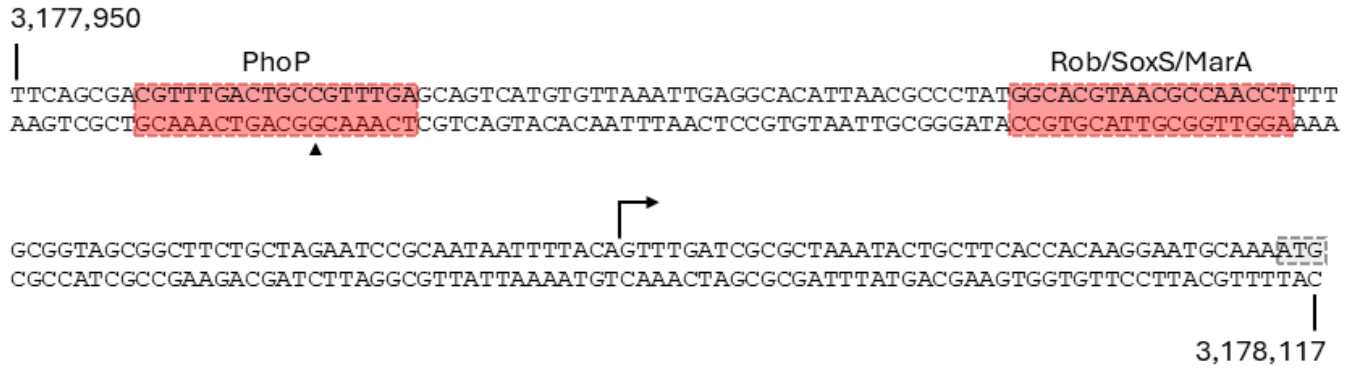

Figure 3: A map of the tolC promoter. Shown is the sequence of wild-type *E. coli* from base pair 3,177,950 to 3,178,117, which includes the *tolC* promoter and the TolC start codon. The transcriptional start site is indicated with an arrow and binding sites for transcriptional activators PhoP, Rob, SoxS, and MarA are indicated in boxes. The start codon for the TolC protein (ATG) is boxed. The sequence of TLSR3, TLSR6, TLSR8, and TLSR9 was identical to the sequence of wild-type *E. coli*. TLSR4 had a point mutation mapping to where PhoP binds the promoter. The location of the cytosine to guanine change is indicated by the arrowhead.

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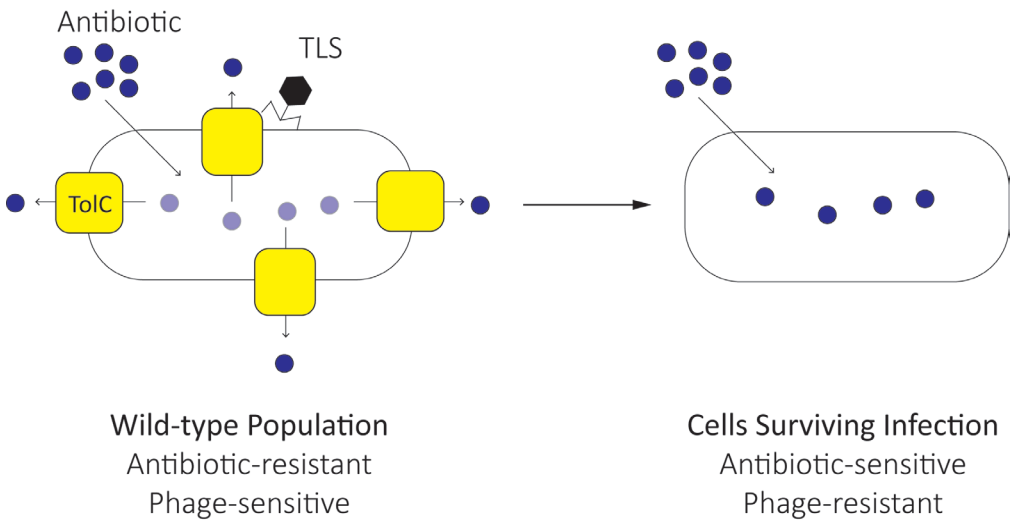

Figure 4: A hypothesis for how bacteriophage TLS renders *Escherichia coli* sensitive to antibiotics. The AcrAB-TolC efflux system uses active transport to remove antibiotics from the bacterial cell, which makes antibiotics less effective. TLS binds to the TolC protein to initiate infection in a population of wild-type *E. coli*. *E. coli* with TolC support the viral lifecycle and are killed. The TLSR variants that survive infection are sensitive to novobiocin and chloramphenicol. We hypothesize the TLSR variants that survived infection either do not express TolC or cannot synthesize lipopolysaccharide.

indicates the variants are not genetically identical and may have differences in the functionality or expression of TolC. Of the five TLSR variants we tested, only TLSR4 contained a mutation in the *tolC* promoter. The point mutation occurred in the PhoP binding sequence, and the mutation may disrupt the ability of PhoP to enhance the transcription of *tolC* (17). We hypothesize that at least four of our TLSR *E. coli* variants contain mutations not yet identified that contribute to their sensitivity.

It is possible that alterations to the expression of the TolC protein may explain antibiotic sensitivity and phage resistance without alterations to the *tolC* genetic locus. For example, the small regulatory RNA, *sdsR,* can inhibit translation of TolC by binding the *tolC* mRNA (18). Overexpression of *sdsR* in TLSR variants would reduce the expression of TolC and could explain both phage resistance and antibiotic sensitivity. Mutations affecting the structure of the AcrAB-TolC efflux system, such as in *acrA,* could contribute to antibiotic sensitivity, but we do not expect such mutations to explain phage resistance unless they affect the structure and function of the TolC protein.

Another possible explanation is that LPS biosynthesis is altered in TLSR variants. WaaP is a protein that contributes to the synthesis of the inner core polysaccharide of LPS (19). Deleting the *waaP* gene from *E. coli* renders *E. coli* sensitive to novobiocin, other lipophilic antibiotics, and SDS (19). We have observed that TLSR clones are sensitive to SDS (data not shown). In addition, Δ*waaP E. coli* is resistant to infection by TLS, indicating LPS is required for infection (20).

Our working hypothesis for how TLS sensitizes *E. coli* to antibiotics is that the phage selects TLSR variants with mutations that affect the expression of TolC (**Figure 4**). RT-PCR, SDS-PAGE, and Western blot analysis could reveal if TolC expression is affected transcriptionally or post-transcriptionally. Future studies can assess whether other phages that bind LPS can infect the TLSR variants and we can search for mutations in genes involved in the biosynthesis of LPS to determine if alterations to LPS can explain the observed antibiotic-sensitivity in TLSR variants.

Bacteria can acquire resistance to antimicrobials by overexpressing efflux pumps, allowing them to export antibiotics from the cell (5). Some bacteriophages bind these efflux pumps as part of their natural lifecycle and can kill the host cell. Cells that survive infection with phages often have mutations that affect the structure of the protein or its expression (9). In this way, phages force an evolutionary tradeoff in bacteria. While the surviving bacteria are phage resistant, the efflux pumps are nonfunctional and sensitive to antibiotics (9). Following a bacterial infection with a combination of phage therapy and antibiotics can effectively kill the bacteria (9,12).

While we found that TLS sensitizes *E. coli* to novobiocin and chloramphenicol, we could not detect genotypic changes that could account for the changes in sensitivity, aside from a point mutation within the promoter of TLSR4. TolC is a possible target for combination therapy for *E. coli* infections; together, phages that target the TolC efflux system and antibiotics can kill bacteria. *K. pneumoniae, Salmonella enterica, Enterobacter cloacae*, and the emerging human pathogen *Enterobacter bugandensis* all have homologs of TolC that are 85% or more identical to TolC in *E. coli* (21,22). Without *tolC*, *S. enterica* serovar Typhimurium strain ATCC 14028s is sensitive to both novobiocin and chloramphenicol (21). Given the species-specificity of phages, it is unlikely TLS will bind these TolC homologs. Yet, given their function and homology to TolC in *E. coli* and our findings, the homologs serve as attractive candidate targets for phage therapy. Our findings provide an intriguing possibility that TLS and novobiocin could be used in combination to reduce treatment time, the risk of side effects, and bacterial resistance for individuals suffering from *E. coli* infections.

MATERIALS AND METHODS

Culturing bacteria and bacteriophages

Wild-type *E. coli* strain BW25113, Δ*tolC* BW25113 (gifts from Jon Werner, Wisconsin Lutheran College), and phage-resistant variants of BW25113 that we generated (described

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below) were cultured in LB broth (BD Life Sciences, Cat # 240230) with shaking (300 rpm) at 30°C or on LB agar (BioRad, Cat # 1660472) at 30°C. We maintained phage-resistant variants without continual phage selection. A stock of TLS (a gift from Jon Werner, Wisconsin Lutheran College) was generated by inoculating a log-phase culture of *E. coli* with TLS and growing the phage overnight at 30°C with shaking (300 rpm). We centrifuged the culture to remove cellular debris, filtered it through a 0.45 µm cellulose acetate syringe filter (VWR, Cat # 76479-040) into a sterile test tube, and stored it at 4°C. We measured the concentration of the TLS stock by performing a serial dilution of the phage stock in sterile 0.9% (w/v) NaCl (Flinn Scientific, Cat # S0336), incubating the phage and an overnight culture of BW25113 at a 1:1 volume for 10 minutes, mixing the phage and bacteria with LB soft agar (1x LB broth and 0.6% bacteriological agar; VWR Life Sciences, Cat # J637) and pouring the mixture onto an LB agar plate. Plates were incubated at 30°C overnight, and plaques were counted.

Generating and isolating variants resistant to bacteriophages

We mixed TLS (1x10^8 plaque forming units, or PFU) with an overnight culture of wild-type *E. coli.* We measured the growth of cultures with a spectrophotometer by measuring the optical density of cultures at 600 nm (OD_{600}). We used a UV-1600PC spectrophotometer (VWR, Cat # 10037-436) for individual cultures, and a µQuant plate reader (BioTek, Cat # 7271000) for experiments performed in 96-well plates. The overnight culture of *E. coli* had an OD_{600} of 1.5, and it was diluted 1:2 in LB broth and incubated at room temperature for 10 minutes. The concentration of bacteria in these co-cultures was estimated to be 4x10^7 CFU/mL because a typical culture of *E. coli* has 8x10^8 CFU/mL at an OD_{600} of 1.0. Therefore, we estimated the multiplicity of infection (MOI) to be 2.5. We added this mix to LB soft agar and poured it onto an LB agar plate, which was incubated at 30°C overnight. TLS-resistant (TLSR) variants were isolated and patched on LB agar and LB agar supplemented with 100 µl of TLS (1x10^9 PFU/mL). Wild-type *E. coli* and Δ*tolC E. coli* were used as controls for phage activity. The plates were incubated overnight at 30°C and checked for growth the following day. TLSR variants grew equally well on both plates, while phage-sensitive mutants grew only on the LB agar plate. Five TLSR variants were randomly selected for further investigation.

Primer Name	Use	Sequence
tolCpro F	PCR, Sequencing	5'- TGTAACGGGCAGGTTGTCTG -3'
tolCpro R	PCR, Sequencing	5'- ATGGACTGCGCGCTTCATTA -3'
tolCgene F	PCR, Sequencing	5'- ACGTAACGCCAACCTTTTGC -3'
tolCgene R	PCR, Sequencing	5'- CTGGTTTTCTGGTGCCATGC -3'
tolCgene I1	Sequencing	5'- CACGTATCAGACCGATCAGCA -3'
tolCgene I2	Sequencing	5'- CTGACACCTCTTATAGCGGTTCG -3'

Table 2: Primers used for PCR amplification and DNA sequencing. We used primers to amplify the *tolC* gene or promoter and to sequence them in TLSR variants.

Broth dilution assays

We serially diluted antibiotics 1:2 in LB broth to concentration ranges of 1 µg/mL to 1024 µg/mL for novobiocin (Thermo Scientific Chemicals, Cat # J60928) and 0.02 µg/mL to 25 µg/mL for chloramphenicol (Thermo Scientific Chemicals, Cat # 22792). We performed broth dilution assays in 96-well plates (VWR, Cat # 10861-562) with LB supplemented with antibiotics in each well. We left one row uninoculated as a negative control to ensure the media was not contaminated. We measured the OD_{600} of *E. coli*, and diluted cultures in each well of the 96-well plate to a final OD_{600} of 0.0025. The plates were incubated at 30°C overnight, and we analyzed for growth the next day by measuring the OD_{600}. Growth of *E. coli* occurred when the OD_{600} was equal to or greater than 0.09. We defined the minimal inhibitory concentration (MIC) as the smallest concentration of the antibiotic that inhibited the growth of *E. coli*.

Phage and antibiotic cocktail

We supplemented LB broth with: 1) phage TLS at 1x10^6 or 1x10^7 PFU/mL, 2) novobiocin at a concentration of 32 µg/mL, or 3) phage TLS at 1x10^6 or 1x10^7 PFU/mL and novobiocin at a concentration of 32 µg/mL. We inoculated each broth at a concentration of 1:200 with an overnight culture (OD_{600} ~1.5) of the wild-type *E. coli*. We calculated the MOI of TLS to be 1 and 0.1 for 1x10^7 and 1x10^6 PFU/mL, respectively. Each culture was added to each well of separate 96-well plates at a volume of 200 µl per well. Plates were incubated overnight at 30°C and analyzed for growth by measuring the OD_{600}. Growth occurred when the OD_{600} was above 0.09. We calculated growth as the number of wells that showed growth divided by the total number of wells tested.

Polymerase chain reaction (PCR) and gel electrophoresis

We amplified the *tolC* gene from genomic DNA by PCR using the Phusion Hot Start Flex DNA Polymerase (New England Biolabs, Cat # M0535S) and primers (IDT DNA) specific to the *tolC* gene locus **(Table 2)**. We designed primers with Primer BLAST from NCBI (23). Primers needed to have a GC content of 45-55% and similar melting temperatures around 60°C. The *tolC* locus from *E. coli* K-12 MG1655 (NC_000913.3) was used as a template to create PCR primers. Each PCR reaction contained 1x Phusion Buffer, 0.2 mM dNTPs (Promega, Cat # U1330), 0.6 µM primer tolCgene F, 0.6 µM primer tolCgene R, 0.5 U Phusion Hot Start DNA Polymerase, and 4 ng/µL of genomic DNA. The cycling parameters in a thermal cycler (MiniPCR, Cat # QP-1000-01) were 98°C for 30 seconds and then 35 cycles of 98°C for 10 seconds, 53°C for 10 seconds, and 72°C for 90 seconds. The final extension ran for 10 minutes at 72°C.

We amplified the *tolC* promoter similarly to the amplification of the *tolC* gene, with the following exceptions: 1) the primers tolCproF and tolCproR were used, and 2) the cycling parameters were 98°C for 30 seconds and then 35 cycles of 98°C for 10 seconds, 57°C for 10 seconds, and 72°C for 30 seconds, and then a final extension for 30 seconds at 72°C.

We loaded PCR products into a 1% TAE agarose gel (VWR Life Sciences, Cat # 0710) supplemented with GelGreen nucleic acid stain (MiniPCR, Cat # RG-1550-01) and separated them by electrophoresis in 1x TAE (VWR Life Sciences, Cat # 0796) at 100 V for 30 minutes (MiniPCR, Cat # QP-1500-01).

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We visualized the PCR products with blue light and compared the products to a 1 kb ladder (New England Biolabs, Cat # N3232S) to verify if the PCR was successful and to determine the size of the products.

Sequencing Reactions

We mixed PCR products in strip tubes (VWR, Cat # 53509-304) at a concentration of 3.3 ng/mL for the *tolC* gene PCR product or 1.33 ng/mL for the *tolC* promoter PCR product with 0.6 µM of primer in nuclease-free water (Promega, Cat # MC1191). The primers were used to cover the full *tolC* gene locus **(Table 2)**. MCLab (San Francisco, CA) performed Sanger sequencing, and we analyzed the data with FinchTV (Geospiza, Inc.). Sequences were compared to the sequence of the *tolC* gene locus for *E. coli* K-12 MG1655 (NC_000913.3) using ClustalW, version 2.1(24,25).

Statistical analysis

We calculated averages from a minimum of three independent biological replicates and reported values as averages ± 2x standard error of the mean. Two-tailed Student's T-tests were performed in Excel to determine if differences were statistically significant. For the purposes of statistical testing, the broth dilution assays were compared to the wild-type *E. coli*, and we compared the effect of TLS and novobiocin to TLS alone and novobiocin alone. We assumed the TLSR variants were representative of a larger population.

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