Chloramphenicol reduces phage resistance evolution by suppressing bacterial cell surface mutants

Authors

Lavisha Parab*[1], Jordan Romeyer Dherbey[1], Norma Rivera[1], Michael Schwarz[1] and Frederic Bertels*[1]

[1]Department Microbial Population Biology, Max Planck Institute for Evolutionary Biology, Plön, Germany.

*Corresponding authors:

Lavisha Parab. Address: Max Planck Institute for Evolutionary Biology, August-Thienemann-Straße 2, 24306 Plön, Germany; email: parab@evolbio.mpg.de; phone: + 49 4522 763-229. ORCID 0000-0002-7626-3216.

Frederic Bertels. Address: Max Planck Institute for Evolutionary Biology, August-Thienemann-Straße 2, 24306 Plön, Germany; email: bertels@evolbio.mpg.de; phone: + 49 4522 763-222. ORCID 0000-0001-6222-4139.

Classification: Biological Sciences, Evolution

Keywords: antibiotic resistance, phage resistance, evolutionary rescue, fluctuation assay

Abstract

Bacteriophages infect Gram-negative bacteria by attaching to molecules present on the bacterial outer membrane, often lipopolysaccharides (LPS). Modification of the LPS can lead to phage resistance. LPS modifications also impact antibiotic susceptibility, allowing for phage-antibiotic synergism. The mechanism for these synergistic interactions is unclear. Here, we show that antibiotics affect the evolution of phage resistance using phage ΦX174 and *Escherichia coli* C wildtype. We use a collection of *E. coli* C LPS mutants, each of which is resistant to ΦX174, and has either a “rough” or “deep-rough” LPS phenotype. Growth of deep rough mutants is inhibited at subinhibitory chloramphenicol concentrations. In contrast, gentamicin has no major effect on growth. Treating *E. coli* C wildtype with ΦX174 and chloramphenicol eliminates deep rough mutants, and reduces phage resistance evolution. Our results show that differential survival of phage resistant mutants with antibiotics explains phage-antibiotic synergism in our model system.

Introduction

The rise of antibiotic resistant bacteria is a global threat to public health and food security (World Health Organization, 2014). To tackle this issue, many alternatives to antibiotics have been proposed (Czaplewski et al., 2016). Bacteriophages (phages) are among the most promising treatments and have been successfully applied to antibiotic resistant infections (Schooley et al., 2017; Chan et al., 2018; Abedon, 2019b; Pirnay et al., 2022). The efficacy of phage treatment can be improved by combining them with antibiotics (Lu and Collins, 2009; Kirby, 2012; Coulter et al., 2014; Kamal and Dennis, 2015; Chaudhry et al., 2017; Berryhill et al., 2021; Gordillo Altamirano et al., 2022; Pirnay et al., 2022). However, as with antibiotics the main reason for treatment failure is the evolution of resistance.

Specific combinations of phages and antibiotics can reduce resistance evolution (Coulter et al., 2014; Torres-Barceló et al., 2014; Chaudhry et al., 2017; Kebriaei et al., 2022). For example, phages NP1 and NP3 displayed synergistic effects with ceftazidime and ciprofloxacin when killing *P. aeruginosa* in biofilms (Chaudhry et al., 2017). Phages combined with antibiotics led to 100 to 1000 times higher killing of bacteria in biofilms than with phages alone. In a different study, combining phage ECA2 with a subinhibitory concentration of ciprofloxacin led to synergistic effects in both, eliminating *Escherichia coli* cells and reducing resistance evolution (Valério et al., 2017). But combinations with ampicillin, kanamycin, piperacillin, tetracycline and chloramphenicol were worse or comparable to phage or antibiotic monotherapy in liquid *in vitro* experiments. These effects strongly depend on antibiotic dosage among other factors. A lethal concentration of

ciprofloxacin prevented phage replication in a combination treatment, and no synergistic effects were observed (Valério et al., 2017; Tagliaferri et al., 2019).

Some combinations of antibiotics and phages can also lead to increased levels of resistance to treatment (Cairns et al., 2017). Similar observations to the antagonistic interaction between ciprofloxacin and phage ECA2 were made in an experiment with phage SBW25Φ2 infecting *Pseudomonas fluorescens* SBW25. SBW25Φ2combined with subinhibitory concentrations of streptomycin led to both, increased rates of phage resistance and extinction of the phage population (Cairns et al., 2017). None of these studies have explored the mechanism behind the evolution of phage resistance and how it is affected by antibiotics.

One common mechanism of phage resistance evolution is surface receptor modification (Labrie et al., 2010; Egido et al., 2021). Surface receptors can be used by phages to infect the cell. Common surface receptors include drug efflux pumps that help the cell to expel antibiotics and allow bacteria to become resistant to antibiotics (Chan et al., 2018; Burmeister et al., 2020). A deactivation of such a pump prevents phage infection but also compromises the cell's ability to expel antibiotics and hence makes the cell susceptible to the antibiotic (Burmeister et al., 2020). Conversely, if a phage uses an antibiotic uptake channel to infect a cell, for example a porin, then the loss of such a receptor leads to increased resistance to both the antibiotic and the phage (Kortright et al., 2021).

Apart from efflux pumps and porins, phages also infect the cell by attaching to lipopolysaccharides (LPS) (Hancock and Reeves, 1976; Mutalik et al., 2020; Romeyer Dherbey et al., 2023). LPS molecules are found at the outer membrane of all Gram-negative bacteria and are the most common phage receptor of Gram-negative infecting phages (Bertozzi Silva et al., 2016; Bio-Conversion Databank Foundation, 2021). In *E. coli*, most strains produce a "smooth" LPS phenotype, consisting of a lipid A domain, a core oligosaccharide (consisting of an inner and outer core) and a polysaccharide called the O-antigen (Frirdich and Whitfield, 2005). Some strains, such as *E. coli* C, lack the O-antigen, and produce a "rough" phenotype (Yethon et al., 1998). A deletion of both O-antigen and the outer core oligosaccharide leads to a "deep rough" phenotype (Nikaido and Vaara, 1985).

Deep rough LPS phenotypes can affect antibiotic susceptibility. For example, in *E. coli* deep rough mutants are more susceptible to membrane-targeting antibiotics such as colistin (Ebbensgaard et al., 2018; Burmeister et al., 2020). LPS modifications also change membrane permeability (Nikaido and Vaara, 1985; Nikaido, 2003), thereby affecting resistance to many antibiotics that do not target the membrane. For example, *E. coli* K-12 strains carrying a deep rough LPS structure are more susceptible to hydrophobic molecules such as novobiocin and spiramycin (Nikaido and Vaara, 1985; Nikaido, 2003). In contrast, deep rough mutants caused by mutations in *galU* have led to increased antibiotic resistance

in *P. aeruginosa* (El'Garch et al., 2007; Dötsch et al., 2009). Combining an LPS-targeting phage with an appropriate antibiotic and the appropriate target pathogen, could potentially reduce the evolution of resistance.

Phage ΦX174 also uses the LPS structure to infect *E. coli* C (Michel et al., 2010). ΦX174 is one of the oldest and best-known phage model systems (Sinsheimer, 1959; Sanger et al., 1977; Smith et al., 2003). Surprisingly though, the resistance mechanism and how to overcome phage resistance has only been explored recently (Romeyer Dherbey et al., 2023). We have shown that *E. coli* C easily becomes resistant to phage infection by modifying its LPS structure, and that ΦX174 can overcome this type of resistance. Yet, how phage resistance evolution is affected by subinhibitory levels of antibiotics was not explored.

Here, we investigate the effects of antibiotics on ΦX174 resistance evolution in *Escherichia coli* C. We first measure antibiotic susceptibility of 33 *E. coli* C strains resistant to ΦX174 (Romeyer Dherbey et al., 2023), 32 of which have LPS modifications. We chose two antibiotics: chloramphenicol and gentamicin. Chloramphenicol is a hydrophobic bacteriostatic antibiotic without significant use in clinical settings and gentamicin is a more hydrophilic bactericidal antibiotic, both inhibiting protein synthesis. We find that deep rough LPS mutants do not grow at chloramphenicol concentrations where the wildtype grows densely. However, there are only few deep rough mutants that cannot grow at subinhibitory concentration of gentamicin. These results suggest that the phage-chloramphenicol combination treatment should prevent the emergence of deep rough mutants but not the phage-gentamicin treatment. We predict that without the emergence of deep rough mutants, chloramphenicol will reduce the likelihood of phage resistance. Subsequent fluctuation experiments confirm that deep rough mutants are completely absent from the phage-chloramphenicol treatment. In contrast, most phage resistant mutants are deep rough in the phage-only treatment. Our results show that we can predict phage resistance rates surprisingly accurately in combination with subinhibitory levels of antibiotics.

Results

Deep rough LPS phenotypes are more susceptible to chloramphenicol

We studied how LPS modifications change antibiotic susceptibility using 33 *Escherichia coli* C strains resistant to phage ΦX174 infection. The strains were generated in a previous study through a fluctuation experiment (Romeyer Dherbey et al., 2023). Briefly, 33 independent overnight cultures of *E. coli* C wildtype (WT) were plated together with ΦX174 high-titre lysates on solid LB plates. After one day, a single colony was isolated at random from each plate. Thirty-two of the 33 phage resistant strains carry at least one mutation in genes involved in the LPS biosynthesis, assembly or regulation. Based on which LPS gene was

impacted and current knowledge on LPS biology (Yethon et al., 1998; Whitfield et al., 1999; Amor et al., 2000; Frirdich and Whitfield, 2005), we predicted the LPS structure of each *E. coli* C phage resistant strain. While we have previously shown that the exact predictions of the LPS structure may be inaccurate, the categorisation into “rough” or “deep-rough” may be useful (**Figure 1, Supplementary Table S1**).

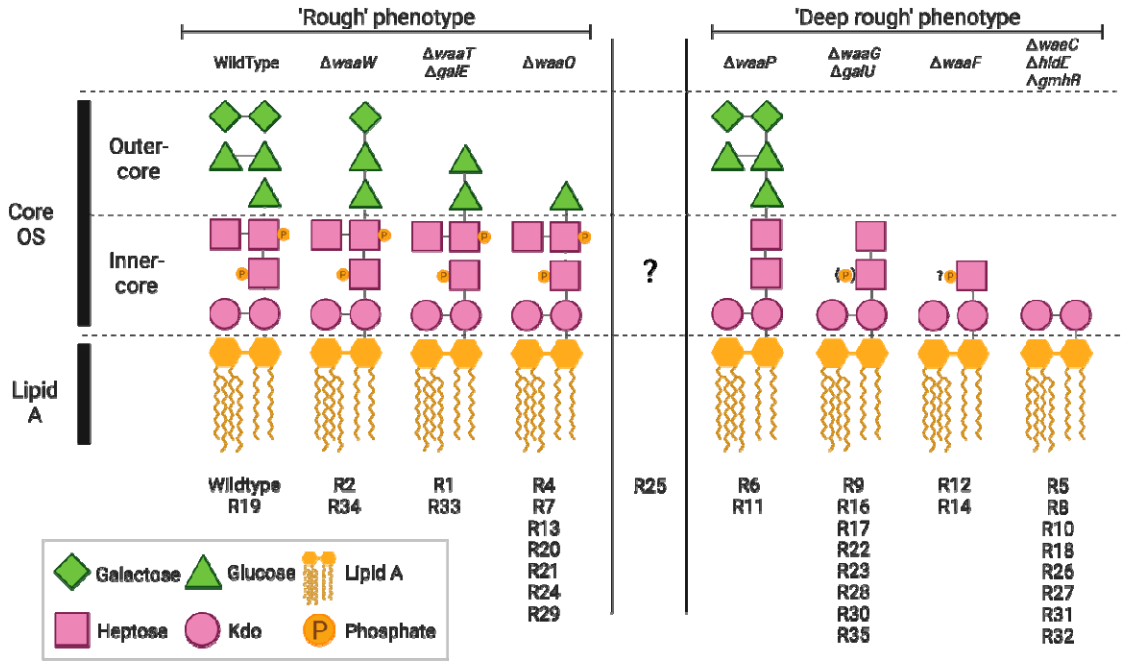

Figure 1. Predicted LPS phenotypes of 33 phage resistant *E. coli* C strains. Strains are categorised into “deep-rough” (outer core absent or inner core modified) and “rough” (outer core present but modified and inner core unmodified) based on which gene the mutation is located in even if the mutation does not delete or deactivate the gene (predicted structures are likely incorrect). The vertical solid lines separate LPS categories. LPS phenotype for strain R25 cannot be predicted as the mutated gene, *rfaH* controls the expression of the entire *waa* LPS operon (Klein and Raina, 2019). While the presence or absence of LPS outer core is commonly used to categorise LPS types, previous studies with gene deletion mutants show that absence of phosphate molecules in the inner core also leads to a “deep rough” phenotype, as in R6 and R11 (Yethon et al., 1998). Kdo: 3-deoxy-D-*manno*-octulosonic acid; “?P”: No information was found on the phosphorylation of Hep(I) in the absence of *waaF*; (P): only 40% of the hexose phosphorylation was observed (Yethon et al., 2000); R#: the number given to each *E. coli* C phage resistant strain; OS: oligosaccharide. Figure adapted from Romeyer Dherbey et al., 2023.

LPS phenotypes may affect antibiotic susceptibility differently for different antibiotics. In our first experiment we determined antibiotic susceptibility of LPS mutants for one bacteriostatic

antibiotic (chloramphenicol) and one bactericidal antibiotic (gentamicin). Both antibiotics inhibit protein synthesis. Chloramphenicol binds to the 50S while gentamicin binds to the 30S ribosomal subunit (Lin et al., 2018). To measure changes in antibiotic susceptibility, we compared growth of phage resistant strains to growth of *E. coli* C wildtype in the presence of these two antibiotics on solid agar plates.

With chloramphenicol, *E. coli* C WT and all rough mutants are inhibited at similar levels (4 to 6 µg/mL) with R2 and R7 being slightly more resistant than the wildtype. Whereas deep rough mutants are inhibited at significantly lower levels (0.5 to 2 µg/mL, **Figure 2, Supplementary Table S2, Supplementary Figure S1**). Among deep rough mutants, a subset of predicted heptoseless LPS mutants (R8, R10, R18, R26) – possessing the most truncated LPS structure – are the strains most susceptible to chloramphenicol. R25 (*rfaH* mutant) has a susceptibility similar to rough LPS mutants, indicating that R25 indeed displays rough LPS molecules. The higher chloramphenicol susceptibility of deep rough mutants may be caused by different levels of membrane permeability. Previous studies show deep rough LPS phenotypes have increased susceptibility to small hydrophobic molecules such as chloramphenicol (Nikaido and Vaara, 1985; Chang et al., 2010; Wang et al., 2015; Pagnout et al., 2019). Thus, deep rough mutants are (i) more susceptible to chloramphenicol than rough mutants, and (ii) completely inhibited at a chloramphenicol concentration (2 µg/mL) that is subinhibitory for wildtype *E. coli* C.

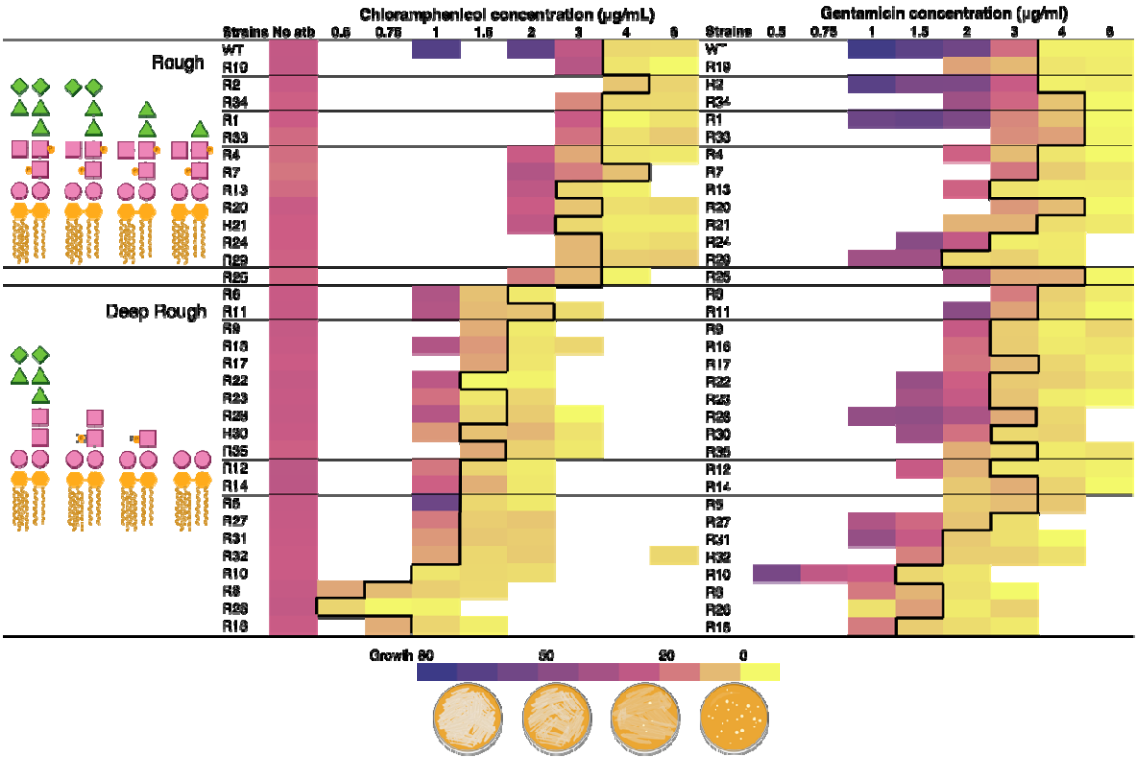

Figure 2. Deep rough LPS mutants are more susceptible to chloramphenicol. LPS modifications do not affect gentamicin susceptibility much. The heatmaps show growth of *E. coli* C WT and phage resistant strains (R#) at different concentrations of chloramphenicol and gentamicin. Growth is measured in 8-bit pixel grayscale values from 0 to 255 (values in **Supplementary Table S2**), and decreases from violet (uniform lawn) to yellow (no lawn, colonies might be visible). Average grey scale values of larger than eight are defined as growth (left of the bold black border) and values of less than eight are defined as no growth (see **Methods** for details). Bacterial strains are ordered by their predicted LPS phenotype and grouped as rough and deep rough LPS. White spaces are shown where we did not measure bacterial growth. Wildtype minimum inhibitory concentration (MIC) is 4 µg/mL for chloramphenicol, and 4 µg/mL for gentamicin. Higher bacterial yields in the presence of subinhibitory concentrations of antibiotics in comparison to no antibiotics when grown on plates and in liquid have been described previously (Frost et al., 2018; Mahrt et al., 2021; Farr et al., 2023; Aduru et al., 2024).

Gentamicin susceptibility does not change much across different LPS phenotypes (**Figure 2**). Most phage resistant strains grow roughly similar to the wildtype (inhibited at 4 µg/mL to 6 µg/mL). Four rough LPS strains (R34, R1, R33, R20) and *E. coli* C R25 are inhibited at a slightly higher gentamicin concentration (6 µg/mL) than the wildtype. Hence, one would expect LPS mutants to occur in gentamicin resistance screens, which is indeed the case for moderate levels of gentamicin (El'Garch et al., 2007; Ibacache-Quiroga et al., 2018). However, some severely truncated heptoseless LPS and only one rough LPS strain (R29) show increased susceptibility to gentamicin. A group of six *hldE*/*waaC* (heptose-less) mutants (R8, R10, R18, R26, R31, R32) with the shortest LPS are inhibited at a lower concentration (1.5 µg/mL to 2 µg/mL) than the wildtype MIC, but the difference to the wildtype MIC is smaller than for chloramphenicol (**Figure 2, Supplementary Table S2, Supplementary Note S1)**.

Subinhibitory concentrations of chloramphenicol should reduce ΦX174 resistance evolution

Our susceptibility assay suggests that subinhibitory levels of chloramphenicol should affect phage resistance evolution, by reducing the emergence of deep rough mutants. We can test our hypothesis by performing a fluctuation experiment with wildtype *E. coli* C grown with phage alone and phage with antibiotics. A fluctuation experiment allows the measurement of the resistance rate from the distribution of the number of phage and antibiotic resistant mutants across replicates (Luria and Delbrück, 1943). If there are no interactions between antibiotics and phages then we should be able to predict the outcome of the experiment accurately, based only on our susceptibility data.

In our first model we assume that mutation rates do not differ significantly between treatments. While this assumption is likely inaccurate as mutational bias can significantly affect evolutionary outcomes (Lind et al., 2019), it is currently impossible to predict mutational biases from the sequence alone. Furthermore, while the model may not be as close to reality as a more complex model, the comparison between simple and complex models provides insight into how much more understanding added complexity provides. Additional experiments later on in the manuscript will enable us to take mutational biases in our second model into account.

If mutation rates are constant across the genome, then mutational target size is proportional to gene length. The larger the mutational target the likelier it is that a random mutation leads to a resistant phenotype. Almost all LPS mutations we observed are deactivating mutations that lead to resistance by introducing stop codons through frame shifts or nonsense mutations. Because we do not know where exactly mutations that deactivate genes are clustered, we assume a uniform distribution across genes.

The mutational target size can be inferred from the genes that contain mutations in the 33 phage resistant strains. Mutations in a total of 13 LPS genes can lead to phage resistant mutants, with a combined length of 11,736 bp (**Table 1**). This mutational target size is only available in the phage only treatment. In treatments with antibiotics the number of genes that are available for the acquisition of resistance mutations is reduced because certain LPS mutants are not viable in the presence of low levels of antibiotics (**Figure 2**). We can now subtract the genes from the total length of the mutational target size to estimate the reduced phage resistance rate (**Table 1**).

Table 1. Model to predict resistance rates for phage-antibiotic combinations

		Mutant survival, if mutation in that gene[$]					Mutation probability (proportional to gene length)				
	Genes (previously observed)	CL 1 µg/mL	CL 2 µg/mL	GM 1 µg/mL	GM 2 µg/mL	Gene coding region (bp)	Φ (entire gene)	Φ + CL 1 µg/mL	Φ + CL 2 µg/mL	Φ + GM 1 µg/mL	Φ + GM 2 µg/mL
ROUGH	*yajC*[§]	Y[§]	Y	Y	Y	333	333	333	333	333	333
	waaW	Y	Y	Y	Y	1026	1026	1026	1026	1026	1026
	galE	Y	Y	Y	Y	1017	1017	1017	1017	1017	1017
	waaT	Y	Y	Y	Y	996	996	996	996	996	996
	waaO	Y	Y	Y	M (6/7)	1017	1017	1017	1017	1017	872
	rfaH	Y	Y	Y	Y	489	489	489	489	489	489
DEEP ROUG	*waaP*	Y	M (1/2)	Y	Y	798	798	798	399	798	798
	waaG	Y	N	Y	Y	1125	1125	1125	0	1125	1125
	galU	Y	M (1/5)	Y	Y	909	909	909	182	909	909
	waaF	Y	N	Y	Y	1047	1047	1047	0	1047	1047
	gmhB	Y	N	Y	Y	573	573	573	0	573	573

H										
hldE	M (3/6)	N	Y	M (1/6)	1434	1434	717	0	1434	244
waaC	N	N	Y	N	972	972	0	0	972	0
Total					11736	11736	10047	5459	11736	9428
				Relative resistance rate[#]		1.00	0.86	0.47	1.00	0.80

[$]Model is based on antibiotic susceptibilities of phage resistant strains. [§]"Y" indicates all strains with a mutation in that gene grow at that antibiotic concentration. "M (x/n)" indicates that x strains grow out of n strains with a mutation in that gene. "N" indicates none of the strains with a mutation in that gene grow at that concentration. The model assumes that mutations occur in genes with a probability proportional to their coding region length. Thus, in genes with a "Y", the mutational target size is the coding region length. For "M (x/n)", the mutational target size is (x/n) * (coding region length). For "N", the mutational target size is zero. [#]The predicted relative resistance rate is the ratio of the mutational target size of the treatment to that of ΦX174 alone. CL: chloramphenicol, GM: gentamicin. [§]*YajC* is the only gene that has no direct link with LPS assembly or synthesis.

The predicted resistance rate for chloramphenicol at 2 µg/mL is less than half (47%) of what is expected when treating the bacteria only with ΦX174 (**Table 1,** red dashes in **Figure 3B**). The predicted reduction in resistance rate is mostly the result of a lack of observed growth of deep rough mutants. Mutations in *waaG*, *waaF*, *gmhB*, *hldE* and *waaC* genes lead to complete inhibition of growth, while mutations in *waaP* and *galU* lead to a complete lack of growth in only some of the mutants (**Table 1**). In contrast, combination treatment with gentamicin reduces the predicted resistance by only 20% at the highest subinhibitory concentration (2 µg/mL). In gentamicin combination treatment only mutations in *waaC* are expected to completely inhibit growth and partial inhibition is expected for mutations in *hldE* and *waaO* (**Table 1**).

Fluctuation experiments qualitatively agree with predictions

In order to test whether our predictions hold, we performed a classic fluctuation experiment (Luria and Delbrück, 1943; Rosche and Foster, 2000). We treated *E. coli* C WT with ΦX174 in combination with either chloramphenicol or gentamicin at two subinhibitory concentrations or inhibitory concentration (**Figure 3A**, **Methods**). We counted colonies from 50 biological replicates for each treatment, and calculated the resistance rate from the distribution of colony numbers (Zheng, 2017).

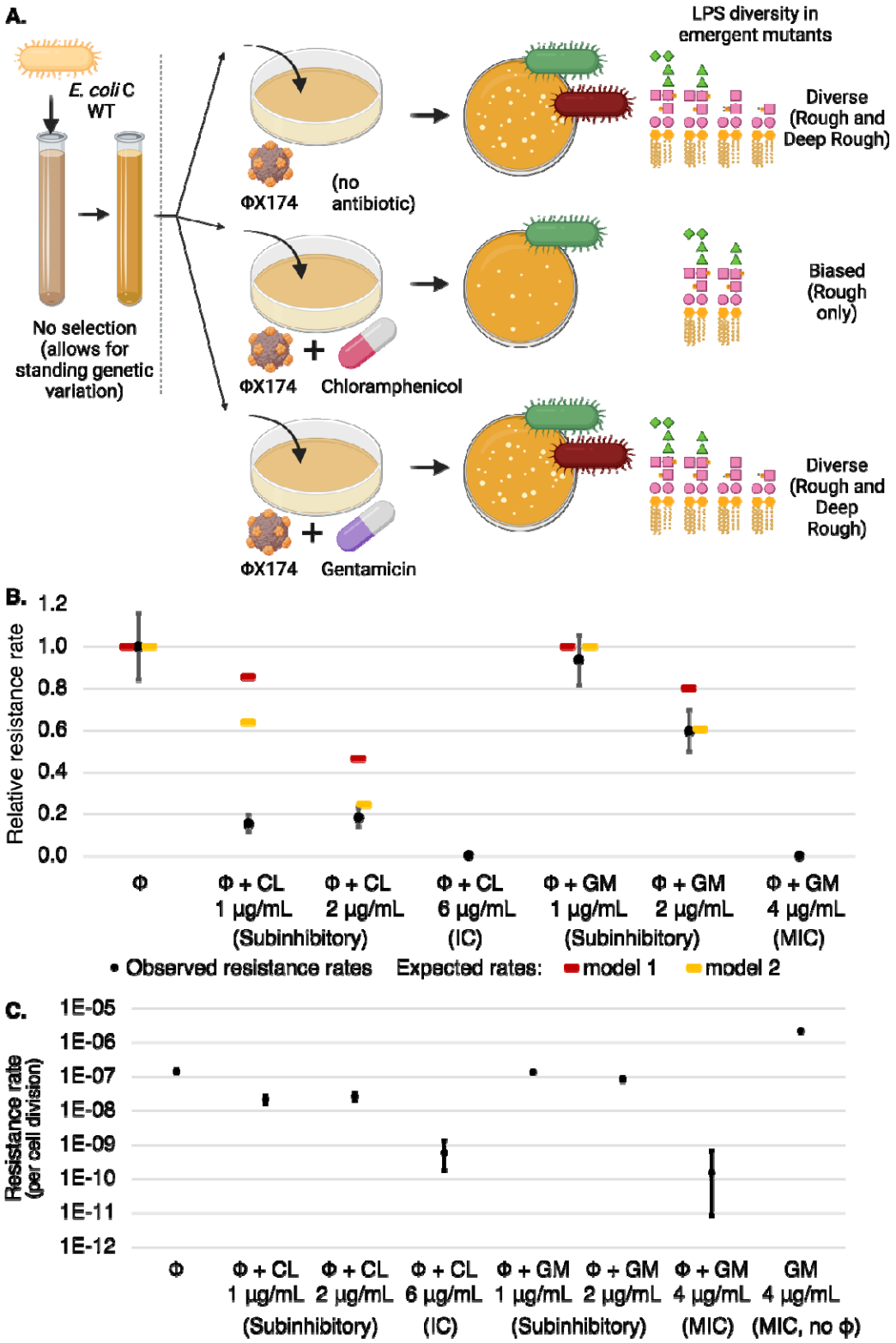

Figure 3. Chloramphenicol reduces the rate of ΦX174 resistance. (A) Schematic of the fluctuation experiment and expected results. Adding chloramphenicol at a subinhibitory concentration is predicted to reduce spontaneous emergence of resistance when treating *E. coli* C with ΦX174 due to inhibition of deep rough LPS phenotypes. Adding gentamicin,

however, is predicted to have little to no effect. **(B)** Resistance rates for each phage antibiotic combination relative to the resistance rate of ΦX174 alone. Observed rates (black) are qualitatively in line with the predicted rates from model 1 (gene length model, red, derived in **Table 1**). Rates predicted by model 2 (gene proportion model inferred from the occurrence of mutations in a phage-only fluctuation experiment (see below), yellow, derived in **Supplementary Table S6**) are within 95% confidence intervals for gentamicin, and 2% away from the 95% confidence interval in 2 µg/mL chloramphenicol. 1 µg/mL and 2 µg/mL are subinhibitory concentrations for chloramphenicol (CL) and gentamicin (GM). Resistance rates are not predicted at antibiotic concentrations inhibitory for wildtype (inhibitory concentration (IC): 6 µg/mL for CL and 4 µg/mL for GM). **(C)** Absolute resistance rates (per cell division), including gentamicin at wildtype MIC without ΦX174. Rates of ΦX174 with antibiotic at inhibitory concentrations seem to be zero in (B) but are actually ~400-1000X lower, as can be seen in (C). (Values are in **Supplementary Table S3**) Error bars correspond to 95% confidence intervals. Resistance rate to chloramphenicol treatment without ΦX174 could not be measured (see **Supplementary Note S2**).

Resistance rate of phage-chloramphenicol combination treatment is significantly lower than phage alone treatment (Likelihood ratio test (LRT), p-value $<10^{-5}$ at both 1 µg/mL and 2 µg/mL, **Supplementary table S3**). The resistance rate of phage alone treatment is about 10^{-7} per cell division. Once chloramphenicol was added at a concentration of 1 µg/mL, the resistance rate decreased to 2×10^{-8}, or 15% (95%CI: 12% to 20%) of the phage alone treatment (baseline, **Figure 3B, Supplementary table S3**). This rate is about four times lower than what we predicted with our simple gene model (model 1), where we predicted the resistance rate to only decrease to 86%. At twice the chloramphenicol concentration of 2 µg/mL, the rate is predicted to decrease to 47% of our baseline, which is about twice of our observation of 18% (95%CI: 14% to 23%).

Treating bacteria with ΦX174 and gentamicin at subinhibitory concentrations does not affect phage resistance rate much. At 1 µg/mL gentamicin, the emergence of resistance is not significantly different from the baseline rate (LRT, p-value ~ 0.53), as expected. The resistance rate was predicted to be the same as the baseline (100%), which is inside the 95% confidence interval of the observed rate of 94% (95% CI: 81% to 106%). Adding 2 µg/mL gentamicin to ΦX174 significantly reduces resistance rate to 60% of the baseline (LRT p-value ~ 10^{-5}, 95% CI: 50% to 70%), which is slightly lower than the predicted rate of 80% (**Figure 3B, Supplementary table S3**).

We also measured resistance rate for gentamicin treatment at wildtype MIC without ΦX174, which is ten times higher than any treatment with ΦX174 (**Figure 3C, Supplementary table S3**). Resistance rates to chloramphenicol treatment without ΦX174 could not be measured

due to the tiny size of most colonies (**Supplementary Note S2**). Any treatment with phage only is at least ten times better at reducing resistance evolution than antibiotics at MIC. Of course, at higher antibiotic concentrations we expect lower rates of antibiotic resistance.

We also combined phage treatment with inhibitory concentration of antibiotic and found significantly lower rates of resistance than any individual treatment. When ΦX174 is combined with either antibiotic at a concentration inhibitory for the wildtype (IC), resistance rates decrease 1000 times below phage treatment alone (**Figure 3C, Supplementary table S3**). Compared to gentamicin alone, resistance rates reduce 10,000-fold, hence, combining phages and gentamicin synergistically (more than additive) reduces resistance rates.

In our first model, we predicted that the reduction in phage resistance rates is due to a reduced mutational target size. We next explored whether the mutational target size, that is, the genomic distribution of mutations, changes across different treatments.

Chloramphenicol skews mutations towards genes causing a rough LPS phenotype

Adding antibiotics to phage treatment reduces the available mutational target size for resistance, as expected. Across five fluctuation experiments we genome sequenced a total of 217 phage/antibiotic resistant mutants. Only four of these mutants (1.8%) did not contain mutations in LPS related genes **(Supplementary Note S4, Supplementary Table S5)**. Without antibiotic, a majority (86%) of the mutations are found in deep rough genes – genes responsible for the assembly or modification of the inner core LPS structure (**Figure 4A and 4B**). In the following sections we will refer to these mutants as deep rough mutants. At 2 µg/mL of either antibiotic, this trend shifts towards rough genes – genes responsible for the assembly and modification of the outer core, which we will refer to as rough mutants. At 2 µg/mL gentamicin, mutations are absent in four deep rough LPS genes (*gmhA*, *hldE*, *hldD* and *waaC*). At 2 µg/mL chloramphenicol, mutations are not observed in any deep rough genes or *rfaH* (**Figure 4A and 4B**).

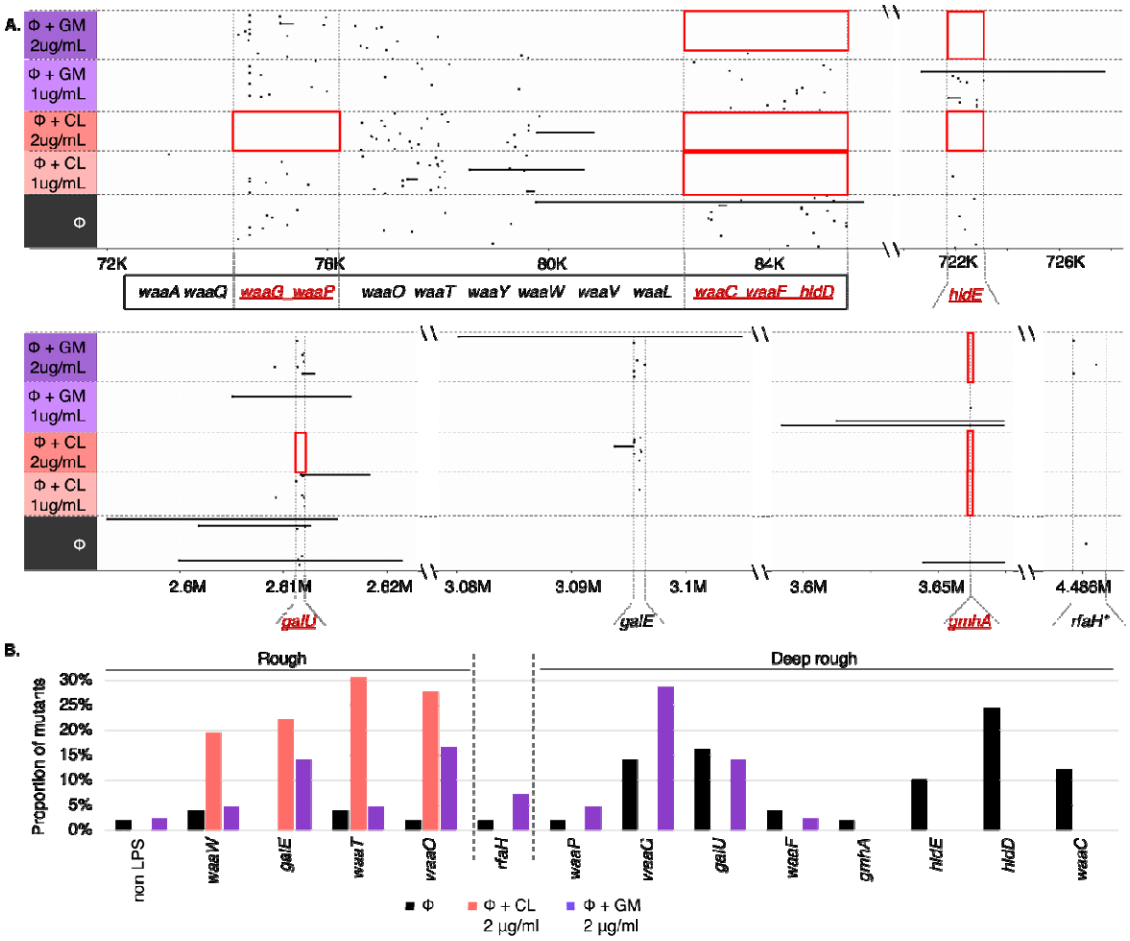

Figure 4: Subinhibitory concentrations of antibiotics reduce the mutational target size. (A) Distribution of mutations across LPS genes. Red boxes show absence/rarity of mutations in a treatment where other treatments have many mutations. Genes that contribute to inner core synthesis are underlined and in red; loss of function mutations in these genes lead to a deep rough phenotype. *Gene *rfaH* is responsible for regulation of the entire LPS operon, so mutations in *rfaH* can lead to a mixture of rough and deep rough types (Pradel and Schnaitman, 1991; Klein and Raina, 2019). Large deletions (>100 bp) are indicated as solid horizontal lines. (Details about types of mutations and parallelism in **Supplementary Figure S2** and **Supplementary Note S3**) **(B)** Distribution of mutated LPS genes. We whole-genome sequenced mutants from each selective condition and identified the mutated genes. Graph shows proportion of mutants with a mutation in a given gene (n = 49 for phage only, n = 39 for phage with 1 µg/mL CL, n = 36 for phage with 2 µg/mL CL, n = 46 for phage with 1 µg/mL GM, n = 42 for phage with 2 µg/mL GM; CL: chloramphenicol, GM: gentamicin). Details of mutations are in **Supplementary Table S5**.

Combining phage treatment with antibiotics biases mutations towards rough LPS genes (**Figure 5A**). Mutations in strains resistant to phage treatment alone are predominantly found

in deep rough LPS genes (~86%), and only about ~12% are in rough LPS genes. As expected from our predictions, adding chloramphenicol to phages enriches for rough mutants. At 1 µg/mL chloramphenicol the proportion of rough mutants increases from 12% to ~62%, and the proportion of deep rough mutants decreases from 86% to ~36%. At a concentration of 2 µg/mL chloramphenicol, all mutants are rough mutants. This result was surprising, since we predicted to observe at least some deep rough mutants (~11%) (**Figure 5A**). Gentamicin treatment also leads to a smaller proportion of deep rough mutants, but as expected from our predictions, the effect is much smaller. At a concentration of 1 µg/mL gentamicin deep rough mutants remain largely stable at ~85% and about 15% of the mutants are rough. At 2 µg/mL gentamicin, the ratio becomes more skewed towards a rough LPS with about 50% deep rough mutants and about 43% rough mutants (**Figure 5A**).

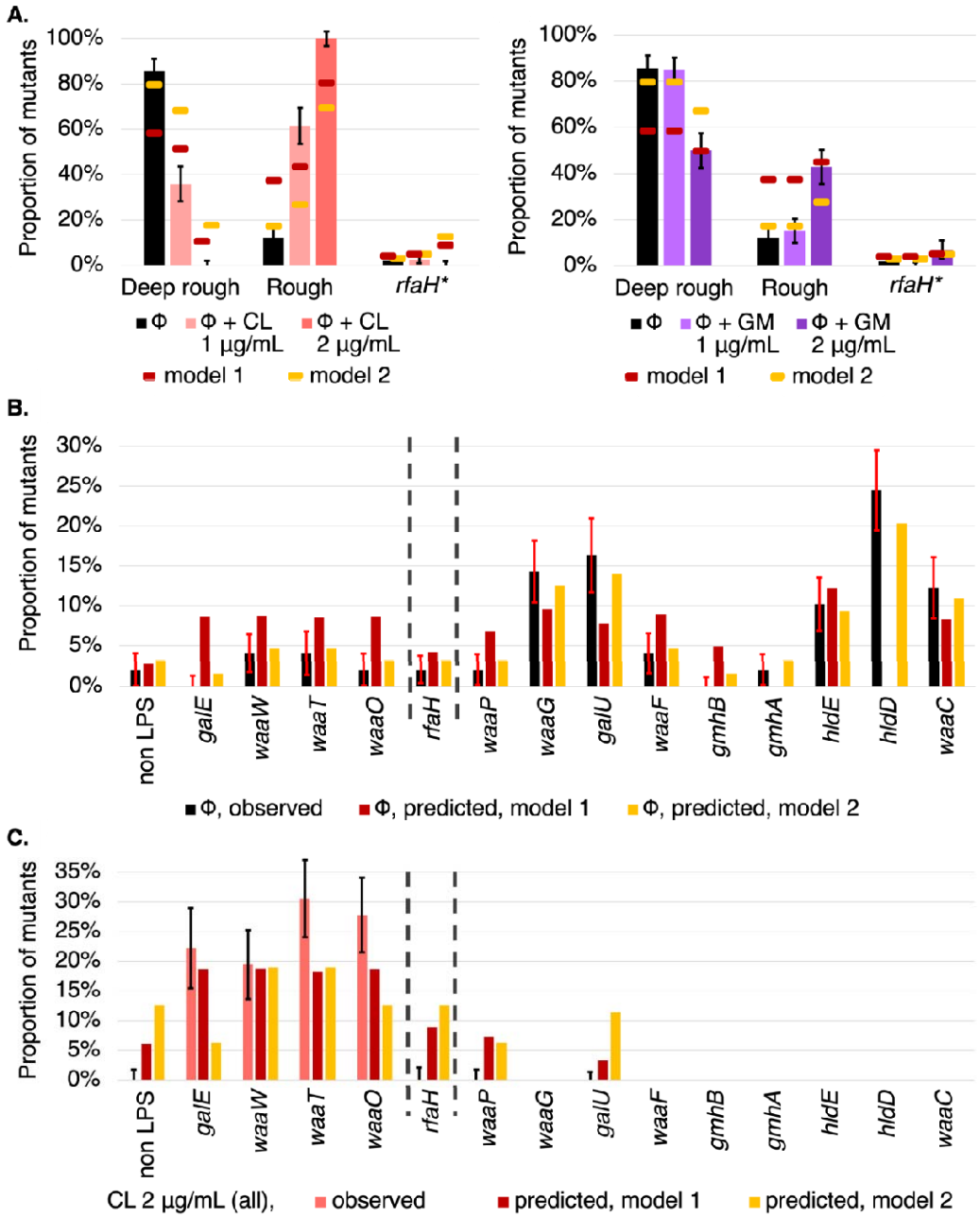

Figure 5. Chloramphenicol skews mutations towards rough LPS genes. (A) Graphs show proportion of mutants that possess a mutation in either rough or deep rough LPS genes. Model 1 predictions (red, applies to all figures) assume that the probability of a mutation in a gene is proportional to the gene length (**Table 1**). In model 2 (yellow), the probability of a mutation occurring in a gene is based on the observed proportions in the phage only treatment (**Supplementary Table S6**). Predictions for antibiotic treatments are based on the susceptibility data. Mutations in *rfaH* or promoter regions of the *waa* operon can affect any number of inner or outer core genes, hence are in a separate category "*rfaH**" (values in **Supplementary Table S8**). Error bars show the standard deviation for each LPS

type of 100 bootstrap replicates (resampling with replacement, see **Methods**). **(B)** Gene-wise distribution of mutants resistant to phage treatment without antibiotics. In model 1, *hldD* and *gmhA* were not accounted for because they were not observed by Romeyer Dherbey et al., 2023. The proportions in model 2 differ from the proportions in the phage only treatment because we add a pseudo-count of 1 to counts of each of the genes to account for the absence of *gmhB* and *galE* from the phage only treatment (see **Methods**). Genes where mutations are likely to lead to a rough or deep rough LPS are on the left and right side of the dotted lines, respectively. "Non LPS" refers to genes that are not known to be involved in LPS biosynthesis, assembly or regulation (four genes across all experiments, **Supplementary Note S4**). Error bars show the standard deviation 100 bootstrap replicates for each gene (**B** and **C**). **(C)** As per Model 2, in phage treatment with 2 µg/mL chloramphenicol, mutants of *rfaH, galU* and *waaP* are predicted to survive but none are observed. (See **Supplementary Figure S3** for model 3).

The discrepancies between predicted and observed mutation distributions across LPS genes could be due to an ill fitted model. Hence, we examined whether discrepancies between prediction and observation could be reduced by adjusting our model. If these adjustments do not improve the predictions then it is possible that second order effects such as phage-antibiotic interactions (Comeau et al., 2007) contribute to the discrepancies.

Before attempting to predict outcomes of combination treatments, our model should be able to predict the phage alone treatment accurately. In the phage only treatment deep rough mutants are significantly more common (86%) than we would predict from the mutational target size of deep rough genes (58%) (**Figure 5A, Supplementary Table S8**). Perhaps, the distribution of mutations in genes is not proportional to gene lengths, as we had previously assumed, for example, due to mutational bias (Lind et al., 2019). Instead of using gene length as proxy for mutational target size we simply used the proportion of mutants that we observed in the phage only treatment as baseline. We also added a pseudo-count of 1 to each gene, since two LPS genes which are potential targets for phage resistance were not observed in the phage only treatment (*gmhB* and *galE*) but are present in combination treatments (**Supplementary Table S6**). The adjusted proportions are shown in yellow in **Figure 5B** (model 2). Adjusting the baseline predictions should in theory improve the predictions for the combination treatments.

Adjusted model improves predictions and highlights potential phage-antibiotic interactions

Using model 2 improves predicted resistance rates (**Figure 3B**). For combinations with 2 µg/mL antibiotic, the predicted rates (25% for chloramphenicol, 61% for gentamicin) are now almost within the 95% confidence interval for chloramphenicol and almost perfect for

gentamicin (18% and 60% respectively). However, the predicted distribution of rough and deep rough mutants is worse than for model 1 (**Figure 5A**). The only treatment where the LPS mutant prediction improved is at 1 µg/mL gentamicin, presumably because this treatment is closest to the phage only treatment based on our initial susceptibility assay (**Figure 5A**).

Model 2 qualitatively predicts the shift towards rough mutants, but not accurately (**Figure 5A**). Since some *waaP* and *galU* mutants grow at 2 µg/mL chloramphenicol in our susceptibility assays, deep rough *waaP* and *galU* mutants are predicted to occur at a rate of 6% and 11% leading to a non-zero deep rough prediction (**Figure 5A and C, Supplementary Table S6**). *RfaH* mutants are predicted to occur at a rate of 13%, because they also grow well in our susceptibility assay. Yet, *waaP, galU* and *rfaH* mutants are never observed at 2 µg/mL chloramphenicol (**Figure 5C**). Similarly, at 1 µg/mL chloramphenicol, mutations are predicted in *rfaH*, *waaF*, *gmhB*, and *gmhA* but none are observed (**Supplementary Figure S3C**). At 2 µg/mL gentamicin mutations are predicted in *hldE*, *gmhA and gmhB,* all deep rough genes, but none are observed (**Supplementary Figure S3D**). There are at least three possible reasons for these discrepancies between observation and prediction. First, sampling only 40-50 mutants per treatment could under-sample the proportion of mutated LPS genes. Sampling more mutants in the phage only treatment may allow us to estimate the proportion of mutated LPS genes more accurately and prevent us from using pseudo-counts in our models, leading to more accurate predictions. Second, biological interactions between antibiotics and phages (Comeau et al., 2007; Gu Liu et al., 2020) may lead to the overrepresentation of different LPS genes compared to the phage only treatment. Third, it is possible that what we consider growth in our susceptibility assay is not sufficient to be detected in the fluctuation experiment.

We are planning to test hypotheses one and two in future studies, hypothesis three can be tested by adjusting the threshold for growth in our susceptibility assay. When we increase the threshold for growth from 8 to 12 average greyscale value, phage resistance rate predictions remain largely unchanged from model 2 to the new model 3, except for the 2 µg/ml gentamicin treatment where the prediction becomes worse (**Supplementary Figure S3A**). Predicted distributions across LPS genes do not change significantly for most treatments (**Supplementary Figure S3B, Supplementary Table S7-S8**), but model 3 does indeed improve the prediction for *waaG* at 2 µg/mL of chloramphenicol (**Supplementary Figure S3E**). Interestingly, with the higher threshold *gmhB*, *gmhA* and *hldE* are not predicted to grow anymore at 2 µg/mL gentamicin (**Supplementary Figure S3D**), which is also what we observe in our experiment. However, none of the model adjustments can explain the discrepancy between the predicted and observed phage resistance rates at 1 µg/mL chloramphenicol or the discrepancy in the proportions of deep rough and rough mutants at

that concentration (**Supplementary Figure S3A-B**). This effect is likely the result of direct or indirect interactions between phages and chloramphenicol.

Interactions between phages and chloramphenicol are also indicated by statistical comparisons between observations and expectations according to different models (**Table 2**). If there is no statistical difference between model and observation, we assume that the model performs well. As the difference between model and observation becomes more significant, the model becomes worse. Model 1 is better at predicting gene-wise distributions than the other two models, except for the phage with 1 µg/mL gentamicin treatment, indicating that gene length is a good approximation for the likelihood of observing a mutation in a certain gene (top of **Table 2**). Models 2 and 3 are better at predicting rough and deep rough proportions (**Table 2** bottom) and phage resistance rates (**Figure 3B, Supplementary Figure S3**). Hence, adjusting the mutation proportions to the phage only treatment leads to greater errors for gene predictions but fewer errors for resistance rate and LPS type predictions.

Table 2. Chi-squared tests to check goodness of fit between observed and predicted mutant proportions

Gene-wise distribution (expected and observed proportions of mutations in each gene)				
Treatment	Model 1 (added genes)^	Model 2	Model 3	
Phage	6.20E-05**	0.9981	0.9981	
Phage + 1 ug/mL CL	1.69E-05	7.15E-07	7.15E-07	
Phage + 2 ug/mL CL	0.0553	3.16E-08	3.51E-06	
Phage + 1 ug/mL GM	0.0091	0.01022	0.0102	
Phage + 2 ug/mL GM	0.0051	7.60E-05	0.0005	
LPS type (expected and observed proportions of deep rough and rough mutants, Figure 5A)				
Treatment	Model 1 (added genes)	Model 2	Model 3	Model 1
Phage	0.0046	0.576	0.576	0.0005
Phage + 1 ug/mL CL	0.0368	6.35E-06	6.35E-06	0.0779
Phage + 2 ug/mL CL	0.0124	0.000388	0.01052	0.0124
Phage + 1 ug/mL GM	0.0084	0.429	0.429	0.0012
Phage + 2 ug/mL GM	0.7795	0.059	0.555	0.838

**Values shown are p-values obtained by comparing the observed distribution of mutants (gene-wise or LPS type) with the distribution predicted by the given model. ^Before comparing model 1, two missing genes *hldD* and *gmhA* were added to resistance genes based on the underlying gene length assumption, to avoid zero expectation for any category.

Discussion

Here we find that phage-antibiotic combinations can reduce the evolution of phage resistance by preventing the growth of certain mutants. We start by measuring the effect of chloramphenicol and gentamicin on previously isolated phage resistant mutants. From these measurements, we predict that certain classes of mutations will not emerge in a combination

treatment, and hence will reduce the rate of phage resistance evolution. We test these predictions by performing an experiment where we expose *E. coli* C wildtype to antibiotics and phage ΦX174. Phage resistance rates generally agree with predictions. Whole-genome sequencing of phage resistant mutants shows that our predictions based on antibiotic susceptibility assays also largely agree with the observed mutation distributions. Hence, we show that antibiotics can reduce phage resistance evolution by preventing the growth of deep rough resistance types. This effect is often referred to as an evolutionary trap (Baym et al., 2016; Barbosa et al., 2017). Interestingly, first order effects – effects which do not take interactions between phage and antibiotic into account – are largely sufficient to explain reduced phage resistance evolution in combination treatments. Our predictions are based on several assumptions, one of which may be violated.

The original model assumption for the fluctuation assay posits that the outcome of the assay only depends on two parameters: the mutation rate and the mutational target size (Luria and Delbrück, 1943). The mutation rate should be identical across all treatments, since mutations occur during exponential growth in liquid culture in the absence of antibiotic and phages. The second parameter, the mutational target size, is very close to our expectations for 1 µg/mL chloramphenicol, and similar to the target size observed for 2 µg/mL gentamicin (**Figure 4A**). Yet, for gentamicin the predicted phage resistance rate (61%) fits well with the observed resistance rate (60%), while for chloramphenicol the rates differ significantly (64% predicted vs. 15% observed, **Figure 3B**). Hence, our assumption on the target size is correct. Because mutation rates are also identical between treatments there must be other factors that explain our observations.

One possible explanation is that mutations do not guarantee resistance. It is possible that for 1 µg/mL chloramphenicol the mutations only provide a certain survival chance rather than guaranteed survival. Previous studies have shown that subinhibitory concentrations of chloramphenicol do not lead to stochastic population extinctions, but the combination with bactericidal antibiotics does (Coates et al., 2018). Because phages can be considered bactericidal agents stochastic population extinctions could play a role in our experiment. If the stochastic extinction chance is about 50% then the expected resistance rate would correspond to our observed resistance rate. This survival chance may be caused by local fluctuation of chloramphenicol concentrations, the cell state or physiological interactions between phage and chloramphenicol on the plate (Akiyama and Kim, 2021).

The combination between a bactericidal agent (phage) and a bacteriostatic antibiotic may lead to a trade-off explaining why phage resistance rates at 1 µg/mL and 2 µg/mL chloramphenicol are similar. At high subinhibitory chloramphenicol concentrations the antibiotic can efficiently prevent the emergence of deep rough mutants but the efficiency of

killing by the phage may be compromised because the bacteria grow significantly lower at subinhibitory chloramphenicol concentrations and phage infection is inhibited by slower growing bacteria (Hadas et al., 1997; Coates et al., 2018). At slightly lower chloramphenicol concentrations chloramphenicol may inhibit growth less, which will make phage infection more efficient. These effects may balance each other out leading to similar phage resistance rates at 1 µg/mL and 2 µg/mL chloramphenicol concentration.

Mutants resistant to phage only treatment are biased towards a deep rough LPS

In treatment with phage only, mutants with a deep rough LPS emerge at a higher proportion than expected based on the assumption that mutations occur proportional to gene lengths. We observed that 86% of all mutants were deep rough (compared to 58% expected based on gene length, **Figure 5B**). It is unclear why deep rough mutants are overrepresented, but one reason could be that mutation rates are not completely uniform throughout the genome. Deep rough genes could have more loci with high mutation rates (mutational bias) (Moxon et al., 1994; Lind et al., 2019; Bertels et al., 2021). Similarly, deep rough candidate genes might have more mutational targets, that is, loci which produce a phage resistant LPS phenotype, than in rough candidate genes. Alternatively, mutations in deep rough genes may be more likely to produce robust resistance in the presence of phages alone. Robust resistance means that the mutation almost guarantees the establishment of a colony, while for rough mutations it is less likely that a resistant colony is successfully established.

Deep rough mutants are more abundant in phage resistant mutants, yet at least in *E. coli* are more susceptible to certain antibiotics (Nikaido and Vaara, 1985; Wang et al., 2015). This characteristic can be exploited by complementing phage therapy with antibiotics that specifically affect bacterial strains with a deep rough LPS structure.

LPS modifications affect susceptibility to chloramphenicol and, to a lesser degree, gentamicin

Chloramphenicol and gentamicin are similar in mechanism of action (Lin et al., 2018) and resistance (Burns et al., 1989; Recht and Puglisi, 2001; Kehrenberg et al., 2005; El'Garch et al., 2007), thus the reason chloramphenicol affects deep rough mutants differently may hinge on its physicochemical properties. The antibiotics differ in their type of inhibition, hydrophobicity and molecular size: chloramphenicol is a bacteriostatic, hydrophobic molecule of 323 Da, gentamicin is a bactericidal, hydrophilic molecule of 477 Da. The key differences are probably hydrophobicity and size, because small hydrophobic molecules can diffuse through the LPS membrane, while hydrophilic molecules generally require active transport for cell entry (Nikaido, 2003).

The increased susceptibility of deep rough LPS mutants to hydrophobic antibiotics is in line with previous studies. Deleting deep rough LPS genes (*waaC*, *waaF*, *waaG* or *waaP*) increases membrane hydrophobicity and permeability (Wang et al., 2015), can destabilise the membrane, and increase the entry of small hydrophobic molecules (Nikaido and Vaara, 1985; Chang et al., 2010; Pagnout et al., 2019). Testing more antibiotics could reveal whether it is molecular size, hydrophobicity or whether the antibiotic is bacteriostatic/bactericidal that defines the change in susceptibility.

Combining phages with antibiotics to reduce the evolution of treatment resistance

Phage-antibiotic treatment success depends on the types of phage and antibiotic applied and which bacterial species is targeted (Valério et al., 2017; Lopes et al., 2018; Abedon, 2019a; Chang et al., 2019; Henriksen et al., 2019). For example, synergistic effects were observed for chloramphenicol, gentamicin and tetracycline for some phage-bacteria combinations but not others (Comeau et al., 2007; Kirby, 2012; Kamal and Dennis, 2015). Gu Liu and colleagues tested many different antibiotics with two phages to test whether combinations were synergistic, antagonistic, additive or dominated by one agent (Gu Liu et al., 2020). Synergistic combinations were only observed with one of the two phages tested. Antibiotic choice is key for treatment outcome, not only should they be more efficacious together (not lead to phage extinction) but also reduce resistance evolution. Our results show varying levels of synergetic effects for all antibiotic phage combinations in reducing phage resistance evolution. It is possible that combining LPS targeting phages with antibiotics is more likely to lead to synergetic effects than phages targeting specific cell membrane proteins like efflux pumps or porins.

Implications for phage therapy research

ΦX174 is very specific in its receptor recognition: it only infects about 0.8% out of 783 *E. coli* human and animal commensal strains (Michel et al., 2010), which currently makes ΦX174 unsuitable for therapeutic purposes. However, the host range of ΦX174 can be expanded through evolution experiments (Romeyer Dherbey et al., 2023; Romeyer Dherbey and Bertels, 2024) or genetic engineering (Yehl et al., 2019). Once the limitation of phage entry is overcome, ΦX174 can replicate its DNA and lyse hosts successfully in different *E. coli* strains and other species such as *Salmonella typhimurium* and *Enterobacter aerogenes* (Suzuki et al., 1974; Mayr et al., 2005; Orta et al., 2023). If the host range of ΦX174 can be extended then the knowledge of ΦX174 biology accumulated over the last decades could be utilized to turn ΦX174 into an efficient therapeutic agent (Romeyer Dherbey and Bertels, 2024).

More generally our results show that phages in combination with certain antibiotics at subinhibitory levels can reduce the evolution of phage resistance. This result indicates that

the continued use of antibiotics may be beneficial even when the target bacteria are antibiotic resistant. Of course, further studies are required to test whether our results also hold for higher, more clinically relevant antibiotic concentrations and resistance rates.

LPS modifications may also reduce pathogen virulence when treating with LPS-targeting phages. LPS can increase virulence (Burns and Hull, 1998; Raetz and Whitfield, 2002; Pier, 2007) and elicit immune response (Alexander and Rietschel, 2001). If the evolution of phage resistance leads to the truncation of LPS structures then by becoming resistant to phage infection the pathogen may also become less virulent. Future experiments with clinically relevant antibiotics and phages, should be conducted to allow us to understand, which LPS targeting phage-antibiotic combinations are most beneficial in therapeutic settings.

Methods

Bacterial and phage strains

Ancestral *E. coli* C and coliphage ΦX174 (GenBank accession number AF176034) were provided by H.A. Wichman (Program in Bioinformatics and Computational Biology, University of Idaho, Moscow, Idaho 83844). The ancestral *Escherichia coli* strain C (wildtype) used in this study differs from the NCBI *E. coli* C strain (GenBank accession number CP020543.1) by 9 insertions and two nucleotide replacements, (details in Romeyer Dherbey et al., 2023). All resistant strains used or generated in this study were derived from the same ancestral *E. coli* C strain. *E. coli* C R1 to R35, used for antibiotic susceptibility measurements were generated in fluctuation experiments in a previous study (Romeyer Dherbey et al., 2023), while the new resistant strains have been generated in this study. The genomic sequence of CP020543.1 was manually modified using Geneious Prime (version 2020.1.2) in a previous study (Romeyer Dherbey et al., 2023). See **Data availability** for whole-genome sequences and mutation details.

Media and growth conditions

Bacteria and phages were grown in 2.5% LB broth (Luria-Bertani Broth, Miller, Sigma-Aldrich) supplemented with $CaCl_2$ and $MgCl_2$ at a final concentration of 5 mM and 10 mM respectively, henceforth referred to as supplemented LB. Solid LB agar (2.5% Sigma LB broth, 1.5% agar) was used to plate phages and bacteria. For bacterial overnight cultures, 5 mL supplemented LB in a 13 mL culture tube (Sarstedt) was inoculated with a single isolated colony from a quadrant-streaked plate and incubated for 17 hours at 37°C with shaking at 250 rpm (New Brunswick™ Innova® 44, Eppendorf). For antibiotic agar plates, liquid LB agar (2.5% Sigma LB broth, 1.5% agar) was supplemented with $CaCl_2$ and $MgCl_2$ at a final concentration of 5 mM and 10 mM respectively, and chloramphenicol or gentamicin at

desired concentration (0.5 µg/mL, 0.75 µg/mL, 1 µg/mL, 1.5 µg/mL, 2 µg/mL, 3 µg/mL, 4 µg/mL or 6 µg/mL) before pouring plates inside a laminar flow hood. Plates were dried for 30 min before behind stored at 4˚C for a maximum of two weeks. Chloramphenicol and gentamicin stocks were made at 20 mg/mL in ethanol and Milli-Q water respectively and stored at -20°C. ΦX174 stocks were prepared fresh every week and stored at 4°C

Determination of antibiotic susceptibility of bacterial mutants

Preliminary measurements of antibiotic susceptibility of *E. coli* C R1 to R35 were done with E-test strips (bioMérieux, France, data not shown) on LB agar plates to narrow down the range of concentrations to be tested for MIC determination. Overnight cultures of *E. coli* C wildtype and phage resistant strains were diluted 10 times in Ringer's solution (Sigma). 50 µL of each 10 times diluted overnight culture (5 x 10^6 cells) was spread, using 15 glass beads (4 mm), on a 90mm LB agar plate (supplemented with CaCl2 and MgCl2 at a final concentration of 5mM and10mM respectively) containing chloramphenicol or gentamicin at the concentration to be tested (0.5 µg/mL, 0.75 µg/mL, 1 µg/mL, 1.5 µg/mL, 2 µg/mL, 3 µg/mL, 4 µg/mL or 6 µg/mL). LB agar plates (without antibiotic or supplement) were used as a control. Plates were incubated inverted at 37°C overnight (17 hours). The next day, growth was assessed by imaging plates after they cooled to room temperature. If bacteria grew at all the concentrations tested, new rounds of experiments were performed to determine the MIC. Increasing antibiotic concentrations were tested until there was no detectable growth in three replicates (CLSI, 2019).

Agar plates were imaged using Chemidoc imaging system (Bio-Rad) at the colorimetric setting with an exposure of 1 second. Plate images were analysed using OMERO and OMERO-imageJ plugin to quantify growth as lawn formation (Allan et al., 2012). For uniform measurements, rectangular regions of interest with the same area (1157.85 mm^2) were marked on the agar plate images (without zooming or cropping). Growth values were obtained by subtracting average grayscale value of a blank plate from the average grayscale value of the sample plate. Growth was measured in 8-bit pixel grayscale values from 0 to 255. Uniform bacterial lawns corresponded to grayscale values of 30 to 80. No visible growth or only a slight haze corresponded with grayscale values of 0 to 8. Average grayscale values of larger than 8 are defined as growth and values of less than 8 are defined as no growth. Median of growth values across 3-5 replicates was used to make **Figure 2**. The sensitivity of the assay to the growth cut-off was tested by changing the growth cut-off to 12 in Model 3.

Fluctuation experiment and mutant isolation.

Mutation rates were measured using a revised Luria Delbrück fluctuation experiment (Luria and Delbrück, 1943; Rosche and Foster, 2000) in nine treatments. Treatments were: (a)

ΦX174 with chloramphenicol at 1 µg/mL, 2 µg/mL or 6 µg/mL; (b) ΦX174 with gentamicin at 1 µg/mL, 2 µg/mL or 4 µg/mL; (c) ΦX174 only; (d) chloramphenicol only, at 6 µg/mL; (e) gentamicin only, at 4 µg/mL. All treatment plates were made with LB agar supplemented with 5 mM CaCl2 and 10 mM MgCl2, and when needed, with chloramphenicol or gentamicin at 1 µg/mL, 2 µg/mL, 4 µg/mL or 6 µg/mL.

For each treatment, 50 complete independent single colonies of *E. coli* C WT, each started from a single cell were inoculated into 100 µL of supplemented LB without antibiotic or phage, in wells of a 96-well plate. Plates were incubated for 5 hours at 37°C (Eppendorf Innova plate shaker) shaking at 750 rpm. Cultures were resuspended by pipetting at the 3-hour and 4-hour timepoints to avoid settling from bacterial aggregation. After incubation, 50 µL of bacterial culture (cell density as below) was spread on the corresponding LB agar plates using sterile glass beads. For treatments involving ΦX174, bacterial cultures were first mixed with 50 µL of a high-titer lysate (3 x 10^8 PFU/mL) and immediately plated with sterile beads on the corresponding LB agar plates. Plates were incubated for 17 hours at 37°C. The plates with chloramphenicol at 6 µg/ml (with and without phage) were incubated further for 16 hours to account for small pinpricks which might turn into distinct colonies.

We altered the total number of cells spread on a selection plate according to the expected resistance rate, spreading ten times more cells if the expected resistance rate was ten times lower. This allowed us to measure rates ranging from 10^{-6} per cell division to 10^{-10} per cell division. For treatments (a) and (b), 50 µL of undiluted bacterial culture (3 x 10^8 CFU/mL) was mixed with 50µL of ΦX174 lysate; for (c), 50 µL of twice diluted bacterial culture was mixed with 50 µL of ΦX174 lysate; for (d) and (e), 50 µL of 10 times diluted bacterial culture was taken; the entire mixture was then plated on agar plates with the corresponding selective condition using 15 glass beads. For obtaining total CFU counts, cultures were serially diluted and plated on non-selective LB agar plates.

The next day, colonies were counted and one colony was randomly picked from each of the 50 plates for all treatments. Spontaneous resistant colonies were purified by re-streaking on non-selective LB agar plates. Again, one colony was randomly picked from each plate and was used to inoculate a fresh overnight culture in supplemented LB. One mL of each culture was mixed with glycerol saline solution and frozen at -80˚C.

Mutation rate calculation and statistical analysis

Resistant mutant frequencies, i.e., the number of resistant colonies across 50 independent replicates were used to calculate mutation rate. Mutation rates were calculated by the Lea-Coulson model following the method of Foster (Foster, 2006). We used this method because our bacterial population all started from a single cell. The model assumes that growth rates of

mutants and nonmutants are the same in the absence of selection, and allows for partial plating of cultures (Foster, 2006). Mutation rate and its 95% confidence interval were estimated by Lea-Coulson (ε<1.0, i.e., partial plating) method of *webSalvador*, the web-application of R package *rSalvador* (Zheng, 2017). Mutation rates were compared between different selective conditions by Lea-Coulson (ε<1.0) likelihood ratio test from the package *rSalvador* on R (Rstudio version 2022.02.1) (RStudio Team, 2019; R Core Team, 2020).

DNA extraction and whole-genome sequencing

Samples were prepared for whole genome resequencing from 500 µl of stationary-phase culture. Genomic DNA of *E. coli* C mutants was extracted using DNeasy Blood & Tissue kit (Qiagen). Quality of extracted DNA was confirmed before sequencing. Samples were prepared as previously described (Picelli et al., 2014) and sequenced at the Max-Planck Institute for Evolutionary Biology (Plön, Germany) with the NextSeq 500, using the NextSeq 500/550 High Output Kit v2.5 (300 Cycles) kit (cat. no. 20024908) (Illumina Inc.). The quality of the sequencing outputs was controlled using *FastQC* version 0.11.9 (Simon, 2010), read-trimmed using *Trimmomatic* (Bolger et al., 2014), mapped to the reference and analysed by *breseq* (Deatherage and Barrick, 2014). All mutations other than SNPs were confirmed by mapping reads to their corresponding modified genomes reconstructed using Geneious Prime 2023.1.1 (https://www.geneious.com).

Predicting mutation rates

Model 1. The probability of a ΦX174 resistance mutation to occur in a particular gene is assumed to be proportional to the length of the coding region of a resistance conferring gene (**Table 1**). We assumed that only genes which were previously observed in Romeyer Dherbey et al., 2023 can confer resistance to treatment with ΦX174. In case a strain possessed more than one mutation, only the mutation affecting LPS structure was considered, e.g. *waaO* for R29, *galE* for R1.

The ability of phage resistant strains to grow with antibiotics was used to predict resistance rates and the genes that confer resistance in phage-antibiotic combination treatments(**Figure 2** and values in **Supplementary Table S2**). If out of “n” strains with a mutation in a gene, only “x” strains grew with an antibiotic, (x/n) was assumed to be the probability that this gene confers resistance in a combination treatment. Hence, resistance caused by a mutation in this gene is proportional to (x/n)* gene length, which we call probability weight. For example, out of five *galU* mutants, only one can grow at 2 µg/mL chloramphenicol. Thus, resistance caused by a mutation in *galU* has a probability weight of (1/5*gene length) at 2 µg/mL chloramphenicol.

Resistance rate is proportional to the sum of all probability weights of genes conferring resistance. The predicted relative resistance rate for combination treatment is inferred as a ratio of the total probability weights at each selective condition to that of ΦX174 alone (**Table 1**).

Model 2. The probability weights assigned to each gene were changed to the observed number of mutations in the phage only treatment (**Supplementary Table S6**). Mutations in two genes (*gmhB* and *galE*) are not observed in the phage only treatment, meaning a zero frequency, and zero probability weight in model 2. A weight of zero incorrectly classifies these genes as not conferring resistance to phage (Romeyer Dherbey et al., 2023) or combination treatments (**Figure 2**) and will also predict zero probabilities for combination treatments. We solved the issue by adding a pseudo-count. A count of one was added to all genes including *galE* and *gmhB* (green highlight in **Supplementary Table S6**).

The inverse problem exists for *hldD* and *gmhA.* These genes have not been observed by Romeyer Dherbey et al. and hence their antibiotic susceptibility has not been measured. *HldD* is a bifunctional gene and mutations can cause two different LPS phenotypes, *waaC* or *waaF* like (Coleman, 1983; Schnaitman and Klena, 1993). Resistance rates and LPS distributions were calculated for both phenotypes. For final analyses, the model closest to observations was used. *GmhA* mutants were assumed to grow like *gmhB* mutants, that is, they can grow in all treatments except phage with 2 µg/mL chloramphenicol.

Model 3. The growth cut-off was increased from 8 to 12 (see **Determination of antibiotic susceptibility of bacterial mutants**). Candidate genes and probability weights of the baseline were the same as in model 2 (**Supplementary Table S7**).

Statistical Testing

T-test and Wilcoxon rank sum test were used to determine if antibiotic susceptibility for rough and deep rough strains are significantly different. T-test was performed using the stats package of R (R version 4.2.0, Rstudio version 2023.12.1) (RStudio Team, 2019; R Core Team, 2020). A T-test can often be incorrect for discrete numerical variables, which is why a Wilcoxon rank sum test was also performed to confirm the significance. Wilcoxon rank sum test with an exact solution for tied datasets was performed using the EDISON WMW calculator (Marx et al., 2016). Chi-squared tests to check goodness of fit of models were performed using R (R version 4.2.0, Rstudio version 2023.12.1) (RStudio Team, 2019; R Core Team, 2020). Observed distributions of mutations by gene and by LPS type were compared with the distributions predicted by each model. Before comparing model 1, two missing genes *hldD* and *gmhA* were added to resistance genes based on the underlying gene length assumption, to avoid zero expectation for any category.

Bootstrap analysis

Mutants were resampled from the original observations with replacement 100 times for each treatment to calculate the expected variation in the sampled observation. To resample genes that have not been observed but are expected to provide resistance, a pseudo-count of one was added to each gene that could provide resistance to treatment. The standard deviation of the 100 samples was used to show the error bars in both gene-wise and LPS type distributions in **Figure 5** and **Supplementary Figure S3**.

Data visualisation

The heatmap (**Figure 2**) and histograms (**Figures 3B-C, 4B, 5, S2, S3**) were generated using Excel (version 16.75.2). All schematic drawings (**Figures 1, 2 and 3A**) were created with BioRender.com. Genomic distribution of mutation loci across treatments (**Figure 4A**) and boxplot of antibiotic susceptibilities (**Figure S1**) were visualised using R (Rstudio version 2022.02.1) (RStudio Team, 2019; R Core Team, 2020).

Data availability

Antibiotic susceptibility. Agar plate images and raw grayscale values used to generate **Figure 2** and **Supplementary Table S2** have been deposited in Edmond (doi: 10.17617/3.PIDVUT).

Whole-genome sequences. Complete genomes of *E. coli* C WT used as reference and ΦX174 WT, and raw sequencing reads of resistant strains isolated from the fluctuation experiments in this study (**Supplementary Table S5**) have been deposited in Edmond (doi: 10.17617/3.PIDVUT). Raw sequencing reads of phage resistant strains *E. coli* C R1 to R35 used for antibiotic susceptibility testing (**Supplementary Table S1**) have been deposited in Zenodo (doi: 10.5281/zenodo.6952399).

Fluctuation experiments. Statistical test results and raw colony counts are in **Supplementary Table S3 and S4.**

Acknowledgements

This work was generously supported by funds from the Max Planck Society (L.P.-F.B.). L.P. was supported by the International Max-Planck Research School for Evolutionary Biology (IMPRS EvolBio). The authors also thank the J. Barrick Lab for their fluctuation test protocols, Prof. Qi Zheng for his help with rSalvador, Dr. Carsten Fortmann-Grote for his help with image analysis and all members of the Microbial Population Biology department for their help with experiments.

Author Information

Affiliations: Max Planck Institute for Evolutionary Biology, Plön, Germany.

Contributions: L.P. and F.B conceived the study. L.P. and F.B. planned the experiments. L.P., N.R., J.R.D. and M.S. carried out the experiments. L.P. conducted the sequence analyses. L.P. and F.B interpreted the results. L.P. and F.B. wrote the original draft of manuscript, L.P., J.R.D., and F.B. reviewed and edited the manuscript. L.P. prepared all figures and tables. All authors reviewed and approved the manuscript.

Ethics declaration

The authors declare no competing interests.

References

Abedon, S. T. (2019a). Phage-Antibiotic Combination Treatments: Antagonistic Impacts of Antibiotics on the Pharmacodynamics of Phage Therapy? *Antibiotics* 8, 182. doi: 10.3390/antibiotics8040182

Abedon, S. T. (2019b). Use of phage therapy to treat long-standing, persistent, or chronic bacterial infections. *Adv. Drug Deliv. Rev.* 145, 18–39. doi: 10.1016/j.addr.2018.06.018

Aduru, S. V., Szenkiel, K., Rahman, A., Ahmad, M., Fabozzi, M., Smith, R. P., et al. (2024). Sub-inhibitory antibiotic treatment selects for enhanced metabolic efficiency. *Microbiol. Spectr.* 0, e03241-23. doi: 10.1128/spectrum.03241-23

Akiyama, T., and Kim, M. (2021). Stochastic response of bacterial cells to antibiotics: its mechanisms and implications for population and evolutionary dynamics. *Curr. Opin. Microbiol.* 63, 104–108.

Alexander, C., and Rietschel, E. T. (2001). Bacterial lipopolysaccharides and innate immunity. *J. Endotoxin Res.* 7, 167–202.

Allan, C., Burel, J.-M., Moore, J., Blackburn, C., Linkert, M., Loynton, S., et al. (2012). OMERO: flexible, model-driven data management for experimental biology. *Nat. Methods* 9, 245–253. doi: 10.1038/nmeth.1896

Amor, K., Heinrichs, D. E., Frirdich, E., Ziebell, K., Johnson, R. P., and Whitfield, C. (2000). Distribution of Core Oligosaccharide Types in Lipopolysaccharides from Escherichia coli. *Infect. Immun.* 68, 1116–1124.

Barbosa, C., Trebosc, V., Kemmer, C., Rosenstiel, P., Beardmore, R., Schulenburg, H., et al. (2017). Alternative Evolutionary Paths to Bacterial Antibiotic Resistance Cause Distinct Collateral Effects. *Mol. Biol. Evol.* 34, 2229–2244. doi: 10.1093/molbev/msx158

Baym, M., Stone, L. K., and Kishony, R. (2016). Multidrug evolutionary strategies to reverse antibiotic resistance. *Science* 351, aad3292. doi: 10.1126/science.aad3292

Berryhill, B. A., Huseby, D. L., McCall, I. C., Hughes, D., and Levin, B. R. (2021). Evaluating the potential efficacy and limitations of a phage for joint antibiotic and phage therapy of Staphylococcus aureus infections. *Proc. Natl. Acad. Sci.* 118, e2008007118. doi: 10.1073/pnas.2008007118

Bertels, F., Metzner, K. J., and Regoes, R. (2021). Convergent evolution as an indicator for selection during acute HIV-1 infection. *Peer Community J.* 1, e4. doi: 10.24072/pcjournal.6

Bertozzi Silva, J., Storms, Z., and Sauvageau, D. (2016). Host receptors for bacteriophage adsorption. *FEMS Microbiol. Lett.* 363. doi: 10.1093/femsle/fnw002

Bio-Conversion Databank Foundation (2021). *Bio-Convers. Databank Found. Portal*. Available at: https://portal.bio-conversion.org/ (Accessed March 3, 2023).

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Burmeister, A. R., Fortier, A., Roush, C., Lessing, A. J., Bender, R. G., Barahman, R., et al. (2020). Pleiotropy complicates a trade-off between phage resistance and antibiotic resistance. *Proc. Natl. Acad. Sci.* 117, 11207–11216. doi: 10.1073/pnas.1919888117

Burns, J. L., Hedin, L. A., and Lien, D. M. (1989). Chloramphenicol resistance in Pseudomonas cepacia because of decreased permeability. *Antimicrob. Agents Chemother.* 33, 136–141.

Burns, S. M., and Hull, S. I. (1998). Comparison of Loss of Serum Resistance by Defined Lipopolysaccharide Mutants and an Acapsular Mutant of Uropathogenic Escherichia coli O75:K5. *Infect. Immun.* 66, 4244–4253.

Cairns, J., Becks, L., Jalasvuori, M., and Hiltunen, T. (2017). Sublethal streptomycin concentrations and lytic bacteriophage together promote resistance evolution. *Philos. Trans. R. Soc. B Biol. Sci.* 372, 20160040. doi: 10.1098/rstb.2016.0040

Chan, B. K., Turner, P. E., Kim, S., Mojibian, H. R., Elefteriades, J. A., and Narayan, D. (2018). Phage treatment of an aortic graft infected with Pseudomonas aeruginosa. *Evol. Med. Public Health* 2018, 60–66. doi: 10.1093/emph/eoy005

Chang, R. Y. K., Das, T., Manos, J., Kutter, E., Morales, S., and Chan, H.-K. (2019). Bacteriophage PEV20 and Ciprofloxacin Combination Treatment Enhances Removal of Pseudomonas aeruginosa Biofilm Isolated from Cystic Fibrosis and Wound Patients. *AAPS J.* 21, 49. doi: 10.1208/s12248-019-0315-0

Chang, V., Chen, L.-Y., Wang, A., and Yuan, X. (2010). The Effect of Lipopolysaccharide Core Structure Defects on Transformation Efficiency in Isogenic Escherichia coli BW25113 rfaG, rfaP, and rfaC Mutants. 14, 7.

Chaudhry, W. N., Concepción-Acevedo, J., Park, T., Andleeb, S., Bull, J. J., and Levin, B. R. (2017). Synergy and Order Effects of Antibiotics and Phages in Killing Pseudomonas aeruginosa Biofilms. *PLOS ONE* 12, e0168615. doi: 10.1371/journal.pone.0168615

CLSI (2019). *Performance Standards for Antimicrobial Susceptibility Testing*. 29th ed. Wayne, PA: Clinical and Laboratory Standards Institute.

Coates, J., Park, B. R., Le, D., Şimşek, E., Chaudhry, W., and Kim, M. (2018). Antibiotic-induced population fluctuations and stochastic clearance of bacteria. *eLife* 7, e32976. doi: 10.7554/eLife.32976

Coleman, W. G. (1983). The rfaD gene codes for ADP-L-glycero-D-mannoheptose-6-epimerase. An enzyme required for lipopolysaccharide core biosynthesis. *J. Biol. Chem.* 258, 1985–1990.

Comeau, A. M., Tétart, F., Trojet, S. N., Prère, M.-F., and Krisch, H. M. (2007). Phage-Antibiotic Synergy (PAS): β-Lactam and Quinolone Antibiotics Stimulate Virulent Phage Growth. *PLoS ONE* 2, e799. doi: 10.1371/journal.pone.0000799

Coulter, L. B., McLean, R. J. C., Rohde, R. E., and Aron, G. M. (2014). Effect of Bacteriophage Infection in Combination with Tobramycin on the Emergence of Resistance in Escherichia coli and Pseudomonas aeruginosa Biofilms. *Viruses* 6, 3778–3786. doi: 10.3390/v6103778

Czaplewski, L., Bax, R., Clokie, M., Dawson, M., Fairhead, H., Fischetti, V. A., et al. (2016). Alternatives to antibiotics—a pipeline portfolio review. *Lancet Infect. Dis.* 16, 239–251. doi: 10.1016/S1473-3099(15)00466-1

Deatherage, D. E., and Barrick, J. E. (2014). Identification of mutations in laboratory evolved microbes from next-generation sequencing data using breseq. *Methods Mol. Biol. Clifton NJ* 1151, 165–188. doi: 10.1007/978-1-4939-0554-6_12

Dötsch, A., Becker, T., Pommerenke, C., Magnowska, Z., Jänsch, L., and Häussler, S. (2009). Genomewide Identification of Genetic Determinants of Antimicrobial Drug Resistance in Pseudomonas aeruginosa. *Antimicrob. Agents Chemother.* 53, 2522–2531. doi: 10.1128/aac.00035-09

Ebbensgaard, A., Mordhorst, H., Aarestrup, F. M., and Hansen, E. B. (2018). The Role of Outer Membrane Proteins and Lipopolysaccharides for the Sensitivity of Escherichia coli to Antimicrobial Peptides. *Front. Microbiol.* 9, 2153. doi: 10.3389/fmicb.2018.02153

Egido, J. E., Costa, A. R., Aparicio-Maldonado, C., Haas, P.-J., and Brouns, S. J. J. (2021). Mechanisms and clinical importance of bacteriophage resistance. *FEMS Microbiol. Rev.* 46, fuab048. doi: 10.1093/femsre/fuab048

El'Garch, F., Jeannot, K., Hocquet, D., Llanes-Barakat, C., and Plésiat, P. (2007). Cumulative Effects of Several Nonenzymatic Mechanisms on the Resistance of Pseudomonas aeruginosa to Aminoglycosides. *Antimicrob. Agents Chemother.* 51, 1016–1021. doi: 10.1128/AAC.00704-06

Farr, A. D., Pesce, D., Das, S. G., Zwart, M. P., and de Visser, J. A. G. M. (2023). The Fitness of Beta-Lactamase Mutants Depends Nonlinearly on Resistance Level at Sublethal Antibiotic Concentrations. *mBio* 14, e00098-23. doi: 10.1128/mbio.00098-23

Foster, P. L. (2006). Methods for Determining Spontaneous Mutation Rates. *Methods Enzymol.* 409, 195–213. doi: 10.1016/S0076-6879(05)09012-9

Frirdich, E., and Whitfield, C. (2005). Lipopolysaccharide inner core oligosaccharide structure and outer membrane stability in human pathogens belonging to the Enterobacteriaceae. *J. Endotoxin Res.* 11, 133–144. doi: 10.1179/096805105X46592

Frost, I., Smith, W. P. J., Mitri, S., Millan, A. S., Davit, Y., Osborne, J. M., et al. (2018). Cooperation, competition and antibiotic resistance in bacterial colonies. *ISME J.* 12, 1582–1593. doi: 10.1038/s41396-018-0090-4

Gordillo Altamirano, F. L., Kostoulias, X., Subedi, D., Korneev, D., Peleg, A. Y., and Barr, J. J. (2022). Phage-antibiotic combination is a superior treatment against Acinetobacter baumannii in a preclinical study. *eBioMedicine* 80, 104045. doi: 10.1016/j.ebiom.2022.104045

Gu Liu, C., Green, S. I., Min, L., Clark, J. R., Salazar, K. C., Terwilliger, A. L., et al. (2020). Phage-Antibiotic Synergy Is Driven by a Unique Combination of Antibacterial Mechanism of Action and Stoichiometry. *mBio* 11, e01462-20. doi: 10.1128/mBio.01462-20

Hadas, H., Einav, M., Fishov, I., and Zaritsky, A. (1997). Bacteriophage T4 Development Depends on the Physiology of its Host Escherichia Coli. *Microbiology* 143, 179–185. doi: 10.1099/00221287-143-1-179

Hancock, R. E., and Reeves, P. (1976). Lipopolysaccharide-deficient, bacteriophage-resistant mutants of Escherichia coli K-12. *J. Bacteriol.* 127, 98–108.

Henriksen, K., Rørbo, N., Rybtke, M. L., Martinet, M. G., Tolker-Nielsen, T., Høiby, N., et al. (2019). P. aeruginosa flow-cell biofilms are enhanced by repeated phage treatments but can be eradicated by phage-ciprofloxacin combination. *Pathog. Dis.* 77, ftz011. doi: 10.1093/femspd/ftz011

Ibacache-Quiroga, C., Oliveros, J. C., Couce, A., and Blázquez, J. (2018). Parallel Evolution of High-Level Aminoglycoside Resistance in Escherichia coli Under Low and High Mutation Supply Rates. *Front. Microbiol.* 9, 427. doi: 10.3389/fmicb.2018.00427

Kamal, F., and Dennis, J. J. (2015). Burkholderia cepacia Complex Phage-Antibiotic Synergy (PAS): Antibiotics Stimulate Lytic Phage Activity. *Appl. Environ. Microbiol.* 81, 1132–1138. doi: 10.1128/AEM.02850-14

Kebriaei, R., Lev, K. L., Shah, R. M., Stamper, K. C., Holger, D. J., Morrisette, T., et al. (2022). Eradication of Biofilm-Mediated Methicillin-Resistant Staphylococcus aureus Infections In Vitro: Bacteriophage-Antibiotic Combination. *Microbiol. Spectr.* 10, e00411-22. doi: 10.1128/spectrum.00411-22

Kehrenberg, C., Schwarz, S., Jacobsen, L., Hansen, L. H., and Vester, B. (2005). A new mechanism for chloramphenicol, florfenicol and clindamycin resistance: methylation of 23S ribosomal RNA at A2503. *Mol. Microbiol.* 57, 1064–1073. doi: 10.1111/j.1365-2958.2005.04754.x

Kirby, A. E. (2012). Synergistic Action of Gentamicin and Bacteriophage in a Continuous Culture Population of Staphylococcus aureus. *PLoS ONE* 7, e51017. doi: 10.1371/journal.pone.0051017

Klein, G., and Raina, S. (2019). Regulated Assembly of LPS, Its Structural Alterations and Cellular Response to LPS Defects. *Int. J. Mol. Sci.* 20, 356. doi: 10.3390/ijms20020356

Kortright, K. E., Doss-Gollin, S., Chan, B. K., and Turner, P. E. (2021). Evolution of Bacterial Cross-Resistance to Lytic Phages and Albicidin Antibiotic. *Front. Microbiol.* 12, 1423. doi: 10.3389/fmicb.2021.658374

Labrie, S. J., Samson, J. E., and Moineau, S. (2010). Bacteriophage resistance mechanisms. *Nat. Rev. Microbiol.* 8, 317–327. doi: 10.1038/nrmicro2315

Lin, J., Zhou, D., Steitz, T. A., Polikanov, Y. S., and Gagnon, M. G. (2018). Ribosome-Targeting Antibiotics: Modes of Action, Mechanisms of Resistance, and Implications

for Drug Design. *Annu. Rev. Biochem.* 87, 451–478. doi: 10.1146/annurev-biochem-062917-011942

Lind, P. A., Libby, E., Herzog, J., and Rainey, P. B. (2019). Predicting mutational routes to new adaptive phenotypes. *eLife* 8, e38822. doi: 10.7554/eLife.38822

Lopes, A., Pereira, C., and Almeida, A. (2018). Sequential Combined Effect of Phages and Antibiotics on the Inactivation of Escherichia coli. *Microorganisms* 6, 125. doi: 10.3390/microorganisms6040125

Lu, T. K., and Collins, J. J. (2009). Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy. *Proc. Natl. Acad. Sci.* 106, 4629–4634. doi: 10.1073/pnas.0800442106

Luria, S. E., and Delbrück, M. (1943). Mutations of Bacteria from Virus Sensitivity to Virus Resistance. *Genetics* 28, 491–511.

Mahrt, N., Tietze, A., Künzel, S., Franzenburg, S., Barbosa, C., Jansen, G., et al. (2021). Bottleneck size and selection level reproducibly impact evolution of antibiotic resistance. *Nat. Ecol. Evol.* 5, 1233–1242. doi: 10.1038/s41559-021-01511-2

Marx, A., Backes, C., Meese, E., Lenhof, H.-P., and Keller, A. (2016). EDISON-WMW: Exact Dynamic Programing Solution of the Wilcoxon–Mann–Whitney Test. *Genomics Proteomics Bioinformatics* 14, 55–61. doi: 10.1016/j.gpb.2015.11.004

Mayr, U. B., Walcher, P., Azimpour, C., Riedmann, E., Haller, C., and Lubitz, W. (2005). Bacterial ghosts as antigen delivery vehicles. *Adv. Drug Deliv. Rev.* 57, 1381–1391. doi: 10.1016/j.addr.2005.01.027

Michel, A., Clermont, O., Denamur, E., and Tenaillon, O. (2010). Bacteriophage PhiX174's Ecological Niche and the Flexibility of Its Escherichia coli Lipopolysaccharide Receptor. *Appl. Environ. Microbiol.* 76, 7310–7313. doi: 10.1128/AEM.02721-09

Moxon, E. R., Rainey, P. B., Nowak, M. A., and Lenski, R. E. (1994). Adaptive evolution of highly mutable loci in pathogenic bacteria. *Curr. Biol.* 4, 24–33. doi: 10.1016/S0960-9822(00)00005-1

Mutalik, V. K., Adler, B. A., Rishi, H. S., Piya, D., Zhong, C., Koskella, B., et al. (2020). High-throughput mapping of the phage resistance landscape in E. coli. *PLOS Biol.* 18, e3000877. doi: 10.1371/journal.pbio.3000877

Nikaido, H. (2003). Molecular Basis of Bacterial Outer Membrane Permeability Revisited. *Microbiol. Mol. Biol. Rev.* 67, 593–656. doi: 10.1128/MMBR.67.4.593-656.2003

Nikaido, H., and Vaara, M. (1985). Molecular Basis of Bacterial Outer Membrane Permeability. *MICROBIOL REV* 49, 32. doi: 10.1128/mr.49.1.1-32.1985

Orta, A. K., Riera, N., Li, Y. E., Tanaka, S., Yun, H. G., Klaic, L., et al. (2023). The mechanism of the phage-encoded protein antibiotic from ΦX174. *Science* 381, eadg9091. doi: 10.1126/science.adg9091

Pagnout, C., Sohm, B., Razafitianamaharavo, A., Caillet, C., Offroy, M., Leduc, M., et al. (2019). Pleiotropic effects of rfa-gene mutations on Escherichia coli envelope properties. *Sci. Rep.* 9, 9696. doi: 10.1038/s41598-019-46100-3

Picelli, S., Björklund, A. K., Reinius, B., Sagasser, S., Winberg, G., and Sandberg, R. (2014). Tn5 transposase and tagmentation procedures for massively scaled sequencing projects. *Genome Res.* 24, 2033–2040. doi: 10.1101/gr.177881.114

Pier, G. B. (2007). Pseudomonas aeruginosa lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity. *Int. J. Med. Microbiol. IJMM* 297, 277–295. doi: 10.1016/j.ijmm.2007.03.012

Pirnay, J.-P., Ferry, T., and Resch, G. (2022). Recent progress toward the implementation of phage therapy in Western medicine. *FEMS Microbiol. Rev.* 46, fuab040. doi: 10.1093/femsre/fuab040

Pradel, E., and Schnaitman, C. A. (1991). Effect of rfaH (sfrB) and temperature on expression of rfa genes of Escherichia coli K-12. *J. Bacteriol.* 173, 6428–6431.

R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing. Available at: https://www.R-project.org/

Raetz, C. R. H., and Whitfield, C. (2002). Lipopolysaccharide Endotoxins. *Annu. Rev. Biochem.* 71, 635–700. doi: 10.1146/annurev.biochem.71.110601.135414

Recht, M. I., and Puglisi, J. D. (2001). Aminoglycoside Resistance with Homogeneous and Heterogeneous Populations of Antibiotic-Resistant Ribosomes. *Antimicrob. Agents Chemother.* 45, 2414–2419. doi: 10.1128/AAC.45.9.2414-2419.2001

Romeyer Dherbey, J., and Bertels, F. (2024). The untapped potential of phage model systems as therapeutic agents. *Virus Evol.* 10, veae007. doi: 10.1093/ve/veae007

Romeyer Dherbey, J., Parab, L., Gallie, J., and Bertels, F. (2023). Stepwise Evolution of E. coli C and ΦX174 Reveals Unexpected Lipopolysaccharide (LPS) Diversity. *Mol. Biol. Evol.* 40, msad154. doi: 10.1093/molbev/msad154

Rosche, W. A., and Foster, P. L. (2000). Determining Mutation Rates in Bacterial Populations. *Methods San Diego Calif* 20, 4–17. doi: 10.1006/meth.1999.0901

RStudio Team (2019). *RStudio: Integrated Development Environment for R*. Boston, MA: RStudio, Inc. Available at: http://www.rstudio.com/

Sanger, F., Air, G. M., Barrell, B. G., Brown, N. L., Coulson, A. R., Fiddes, J. C., et al. (1977). Nucleotide sequence of bacteriophage φX174 DNA. *Nature* 265, 687–695. doi: 10.1038/265687a0

Schnaitman, C. A., and Klena, J. D. (1993). Genetics of lipopolysaccharide biosynthesis in enteric bacteria. *Microbiol. Rev.* 57, 655–682. doi: 10.1128/mr.57.3.655-682.1993

Schooley, R. T., Biswas, B., Gill, J. J., Hernandez-Morales, A., Lancaster, J., Lessor, L., et al. (2017). Development and Use of Personalized Bacteriophage-Based Therapeutic Cocktails To Treat a Patient with a Disseminated Resistant Acinetobacter baumannii Infection. *Antimicrob. Agents Chemother.* 61, e00954-17. doi: 10.1128/AAC.00954-17

Simon, A. (2010). FastQC A Quality Control tool for High Throughput Sequence Data. Available at: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (Accessed April 19, 2023).

Sinsheimer, R. L. (1959). Purification and properties of bacteriophage φX174. *J. Mol. Biol.* 1, 37-IN5. doi: 10.1016/S0022-2836(59)80005-X

Smith, H. O., Hutchison, C. A., Pfannkoch, C., and Venter, J. C. (2003). Generating a synthetic genome by whole genome assembly: φX174 bacteriophage from synthetic oligonucleotides. *Proc. Natl. Acad. Sci.* 100, 15440–15445. doi: 10.1073/pnas.2237126100

Suzuki, M., Kaneko-Tanaka, Y., and Azegami, M. (1974). Transfection of non-host bacterial spheroplasts with bacteriophage ΦX174 DNA. *Nature* 252, 319–321. doi: 10.1038/252319a0

Tagliaferri, T. L., Jansen, M., and Horz, H.-P. (2019). Fighting Pathogenic Bacteria on Two Fronts: Phages and Antibiotics as Combined Strategy. *Front. Cell. Infect. Microbiol.* 9, 22. doi: 10.3389/fcimb.2019.00022

Torres-Barceló, C., Arias-Sánchez, F. I., Vasse, M., Ramsayer, J., Kaltz, O., and Hochberg, M. E. (2014). A Window of Opportunity to Control the Bacterial Pathogen Pseudomonas aeruginosa Combining Antibiotics and Phages. *PLoS ONE* 9, e106628. doi: 10.1371/journal.pone.0106628

Valério, N., Oliveira, C., Jesus, V., Branco, T., Pereira, C., Moreirinha, C., et al. (2017). Effects of single and combined use of bacteriophages and antibiotics to inactivate Escherichia coli. *Virus Res.* 240, 8–17. doi: 10.1016/j.virusres.2017.07.015

Wang, Z., Wang, J., Ren, G., Li, Y., and Wang, X. (2015). Influence of Core Oligosaccharide of Lipopolysaccharide to Outer Membrane Behavior of Escherichia coli. *Mar. Drugs* 13, 3325–3339. doi: 10.3390/md13063325

Whitfield, C., Heinrichs, D. E., Yethon, J. A., Amor, K. L., Monteiro, M. A., and Perry, M. B. (1999). Assembly of the R1-type core oligosaccharide of Escherichia coli lipopolysaccharide. *J. Endotoxin Res.* 5, 151–156. doi: 10.1177/09680519990050030901

World Health Organization (2014). *Antimicrobial resistance: global report on surveillance*. World Health Organization. Available at: https://apps.who.int/iris/handle/10665/112642 (Accessed October 18, 2021).

Yehl, K., Lemire, S., Yang, A. C., Ando, H., Mimee, M., Torres, M. D. T., et al. (2019). Engineering Phage Host-Range and Suppressing Bacterial Resistance through Phage Tail Fiber Mutagenesis. *Cell* 179, 459-469.e9. doi: 10.1016/j.cell.2019.09.015

Yethon, J. A., Heinrichs, D. E., Monteiro, M. A., Perry, M. B., and Whitfield, C. (1998). Involvement of waaY, waaQ, and waaP in the modification of Escherichia coli lipopolysaccharide and their role in the formation of a stable outer membrane. *J. Biol. Chem.* 273, 26310–26316. doi: 10.1074/jbc.273.41.26310

Yethon, J. A., Vinogradov, E., Perry, M. B., and Whitfield, C. (2000). Mutation of the lipopolysaccharide core glycosyltransferase encoded by waaG destabilizes the outer membrane of Escherichia coli by interfering with core phosphorylation. *J. Bacteriol.* 182, 5620–5623. doi: 10.1128/JB.182.19.5620-5623.2000

Zheng, Q. (2017). rSalvador: An R Package for the Fluctuation Experiment. *G3 GenesGenomesGenetics* 7, 3849–3856. doi: 10.1534/g3.117.300120