Chloramphenicol reduces phage resistance evolution by suppressing bacterial cell surface mutants

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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. Hypothesis testing shows that 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; Pirnay et al., 2022). However, as with antibiotics the main reason for treatment failure is the evolution of resistance.

Resistance evolution has been shown to reduce in specific combinations of phages and antibiotics (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). However, these effects strongly depend on antibiotic dosage among other factors (Valério et al., 2017; Tagliaferri et al., 2019). A lethal concentration of ciprofloxacin prevented phage replication in a combination treatment, and no synergistic effects were observed.

Some combinations of antibiotics and phages can also lead to increased levels of resistance to treatment (Cairns et al., 2017). Valério and colleagues show that apart from ciprofloxacin, all other phage-antibiotic combination treatments were worse or comparable to phage or antibiotic monotherapy (Valério et al., 2017). Similar observations were made in an experiment with a phage infecting *Pseudomonas fluorescens* SBW25. Phage SBW25Φ2 combined with subinhibitory concentrations of streptomycin led to both, increased rates of phage resistance as well as 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 allows 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 cells 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 destruction of such a receptor would lead to the resistance to both the antibiotic and the phage, potentially increasing phage resistance evolution (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 is 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).

Apart from granting phage resistance, a modification from a “rough” to “deep rough” LPS phenotype can decrease resistance to multiple antibiotics. Deep rough mutants for example become 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). Combining an LPS-targeting phage with an appropriate antibiotic, therefore, 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 infection by modifying its LPS structure, and that ΦX174 can overcome this type of resistance. Yet, how 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 changes in antibiotic susceptibility of 33 *E. coli* C strains resistant to ΦX174 (Romeyer Dherbey et al., 2023), 32 of which have LPS modifications. We find that deep rough LPS mutants cannot grow at concentrations of chloramphenicol where the wildtype is not inhibited. However, deep rough mutants are not significantly affected by gentamicin. These results suggest that only a phage-chloramphenicol combination treatment should prevent the emergence of deep rough mutants. 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, the majority of resistant mutants are deep rough in the phage-only treatment. Our results show that we can predict the likelihood of phage resistance accurately without taking into account interactions between phages and antibiotics, when chloramphenicol levels are high but still subinhibitory for the wildtype. However, this is not possible for intermediate levels of chloramphenicol, suggesting that, in this case, interactions may play a larger role in shaping resistance evolution and need further investigation.

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 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 resistant strain. According to their predicted LPS structures, we categorized the strains as “rough” or “deep-rough” (**Figure 1, Supplementary Table S1**).

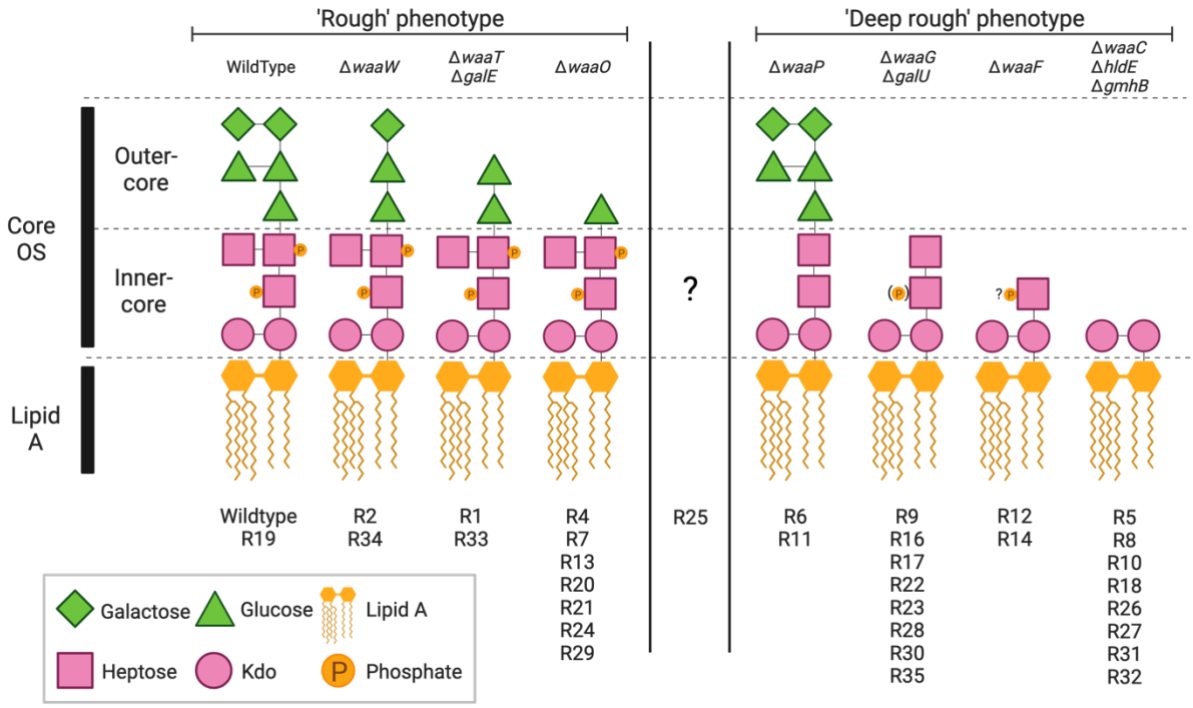

Figure 1. Predicted LPS phenotypes of 33 phage-resistant *E. coli* C strains. Strains are categorised into “deep-rough” (outer core absent or inner core modified) and “rough” (outer core present but modified and inner core unmodified). The vertical solid lines separate LPS categories. LPS phenotype for 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 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 resistant strains to growth of *E. coli* C wildtype in the presence of these two antibiotics on solid agar plates.

In chloramphenicol, *E. coli* C WT and all rough mutants are inhibited at similar levels (4 to 6 µg/mL), whereas deep rough mutants are inhibited at lower levels (0.75 to 2 µg/mL, **Figure 2, Supplementary Table S2**). 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.

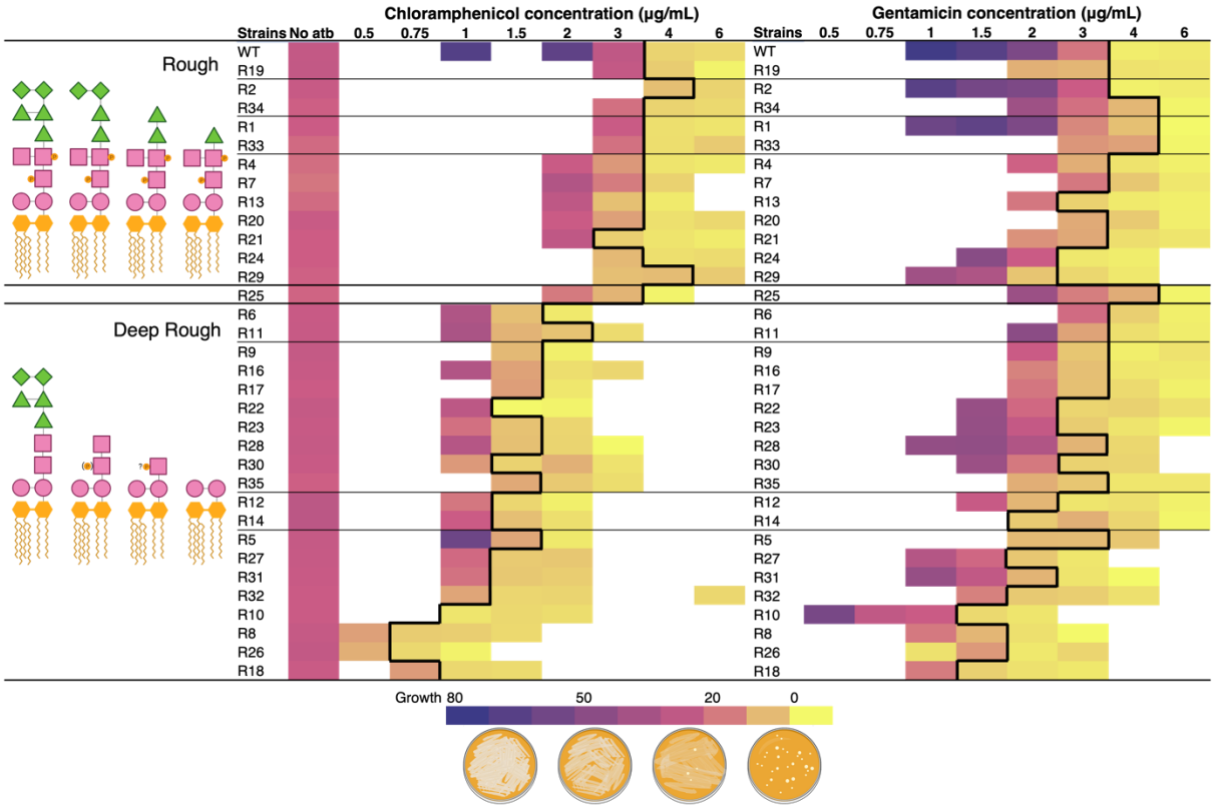

Figure 2. Deep rough LPS mutants are more susceptible to chloramphenicol. LPS modifications do not affect gentamicin susceptibility much. The heatmaps show growth of *E. coli* C WT and phage-resistant strains (R#) at different concentrations of chloramphenicol and gentamicin. Growth is measured in 8-bit 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.

Surprisingly, 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). Three rough LPS strains (R34, R1, R33) 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 show increased susceptibility to gentamicin. A group of six *hldE*/*waaC* (heptose-less) mutants (R8, R10, R18, R26, R27, 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 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.

If we assume that mutation rates do not differ significantly between treatments, then the only parameter that affects phage resistance rate is the mutational target size. 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, mutations that lead to resistance by introducing stop codons through frame shifts or nonsense mutations. Mutations that deactivate genes are possible at any locus in the coding region, thus should be distributed similarly across genes. Hence, we assume that the mutational target size should be proportional to the gene length.

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 genes lead to resistant LPS 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	Y	1017	1017	1017	1017	1017	1017
	rfaH	Y	Y	Y	Y	489	489	489	489	489	489
DeepRough	*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	N	Y	Y	909	909	909	0	909	909
	waaF	Y	N	Y	M (1/2)	1047	1047	1047	0	1047	524
	gmhB	Y	N	Y	Y	573	573	573	0	573	573
	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	5277	11736	9050.28
						Relative resistance rate[#]	1.00	0.86	0.45	1.00	0.77

[$]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.

The predicted resistance rate for chloramphenicol at 2 µg/mL is less than half (45%) 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*, *galU*, *waaF*, *gmhB*, *hldE* and *waaC* genes lead to complete inhibition of growth, while mutations in *waaP* 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 23% 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 *waaF* (**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).

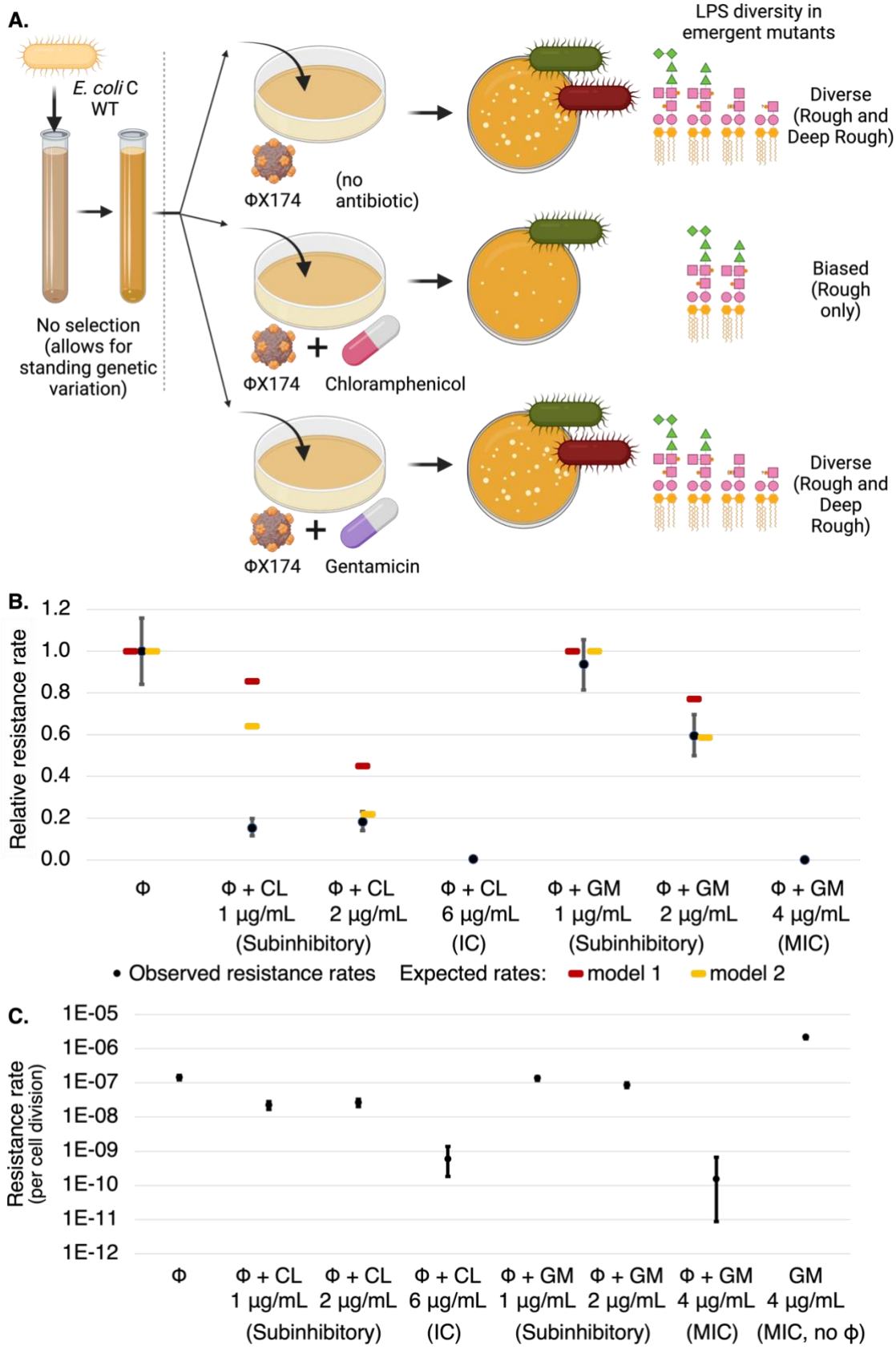

Figure 3. Chloramphenicol reduces the rate of ΦX174 resistance. (A) Schematic of the fluctuation experiment and expected results. Adding chloramphenicol at a subinhibitory concentration is predicted to reduce spontaneous emergence of resistance when treating *E. coli* C with ΦX174 due to inhibition of 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, yellow, derived in **Supplementary Table S6**) are within 95% confidence intervals for all except 1 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 (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 45% 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 77% (**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.

When ΦX174 is combined with either antibiotic at a concentration inhibitory for the wildtype (IC), resistance rates reduce up to 1000 times below phage treatment alone and up to 10^4 times below antibiotic treatment alone (**Figure 3C, Supplementary table S3**). These results could suggest that growth of ΦX174 is not significantly negatively impacted by either of the antibiotics, since combinations are better than monotherapy (Chaudhry et al., 2017; Valério et al., 2017). Alternatively, these observations are simply specific to the fluctuation experiment.

In our first model, we predicted that the reduction in phage resistance rates is due to a reduced mutational target size. Hence, 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. 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**). At 2 µg/mL of either antibiotic, this trend shifts towards rough genes – genes responsible for the assembly and modification of the outer core. 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**).

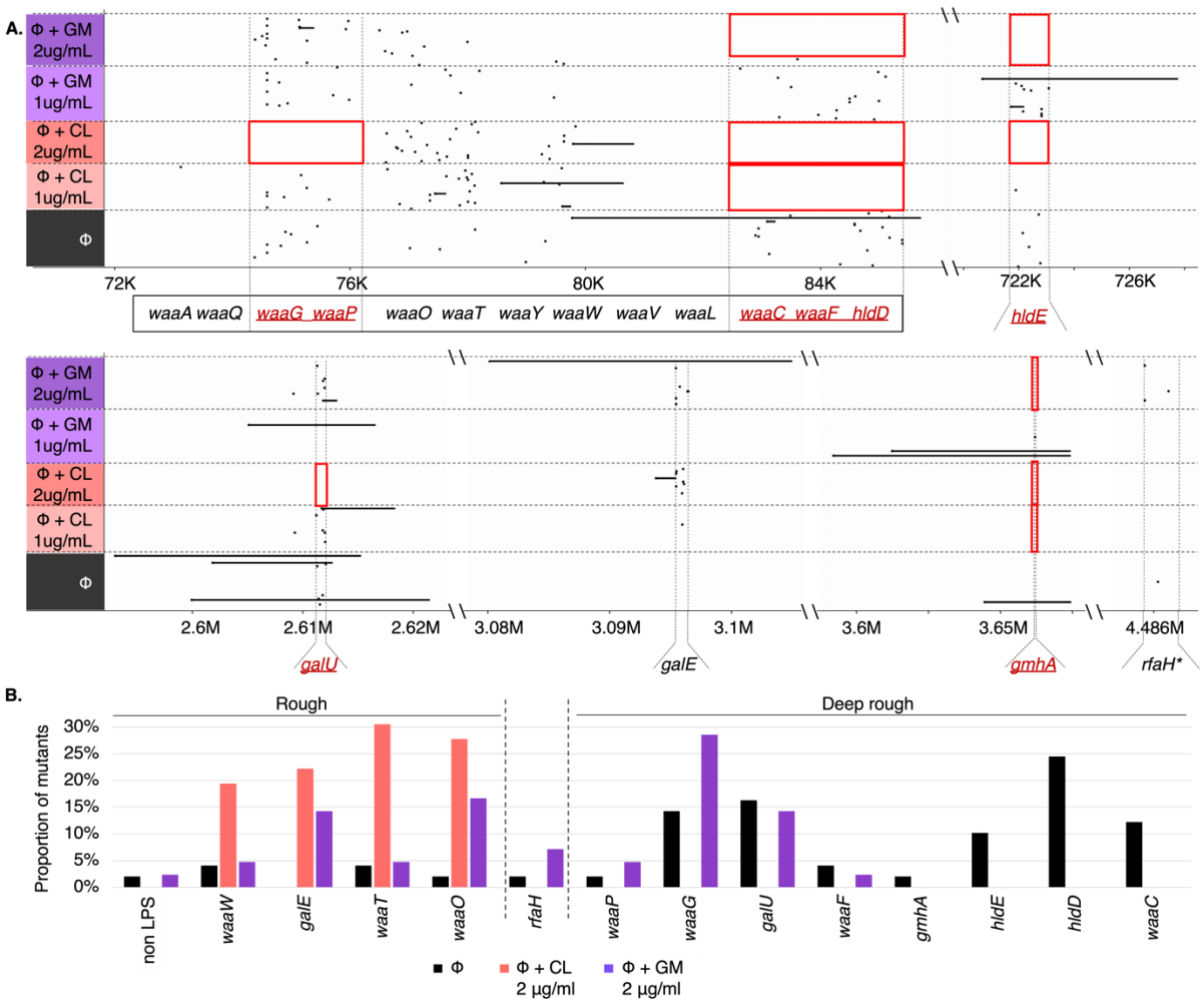

Figure 4: Subinhibitory concentrations of antibiotics reduce the mutational target size. (A) Distribution of mutations across LPS genes. Red boxes show absence/rarity of mutations in a treatment where other treatments have many mutations. Genes that contribute to inner core synthesis are underlined and 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 S1** 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 mutants with a predicted rough LPS. 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 of the mutants possess a rough LPS. This result was surprising, since we expected to observe at least some deep rough mutants (~8%) according to our prediction (**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**).

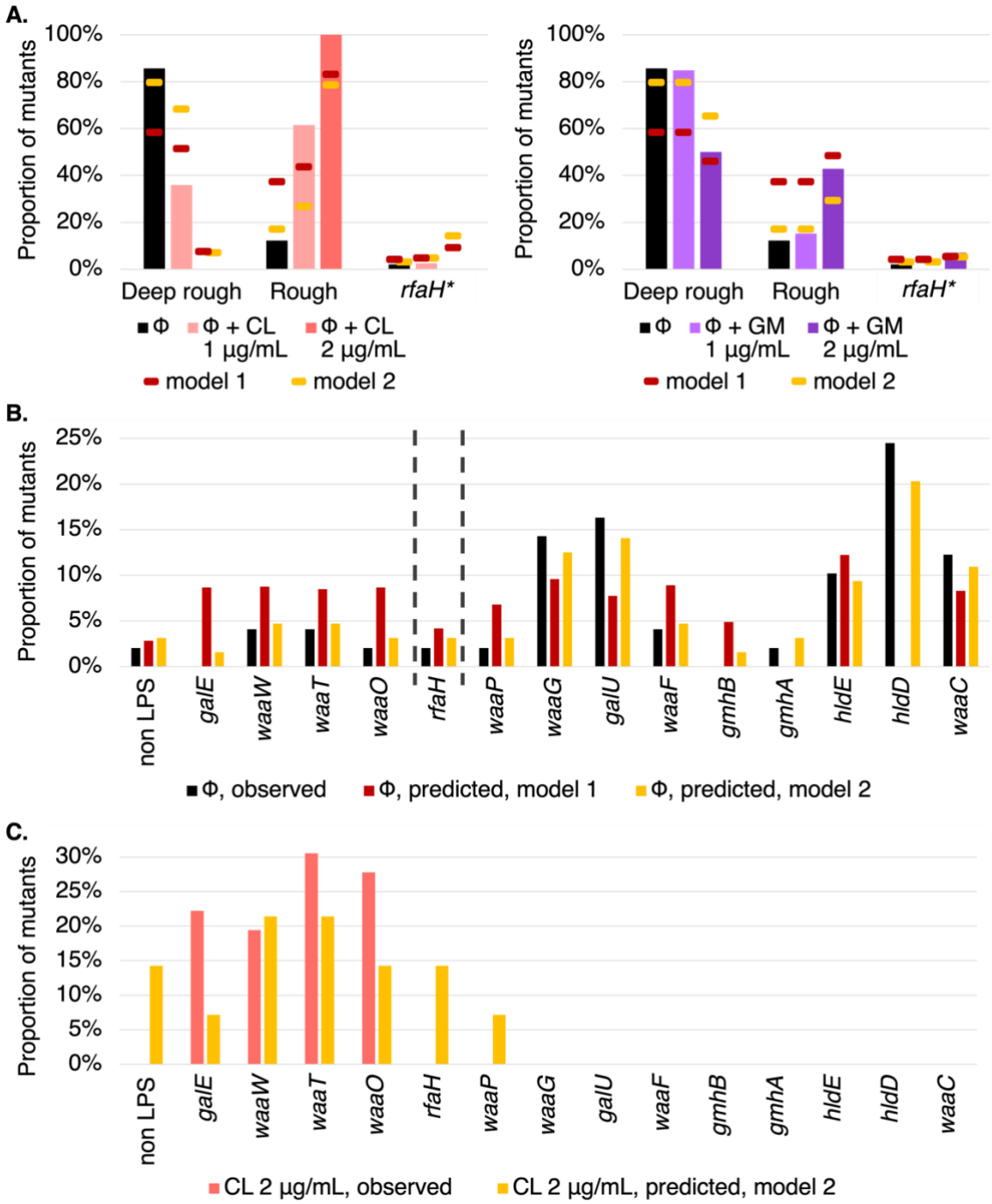

Figure 5. Chloramphenicol skews mutations towards rough LPS genes. (A) LPS distribution in mutants isolated in the fluctuation experiment. Graphs show proportion of mutants with a certain LPS phenotype. Model 1 predictions (red, applies to all figures) are based on the assumption that probability of mutation in a gene is proportional to the gene length (**Table 1**). In model 2 (yellow), probability of mutations occurring in a gene was 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**". **(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. Model 2 makes predictions based on the proportions observed in the phage only treatment. The proportions in model 2 differ from the proportions in the phage only treatment because we add a pseudocount of 1 to counts of each of the genes to account for the absence of *gmhB* and *galE* from the phage only treatment. Genes leading to a rough and deep rough LPS are on the left and right side of the dotted lines respectively. “Non LPS” refers to genes that are not involved in LPS biosynthesis, assembly or regulation (four genes across all experiments, **Supplementary Note S4**). **(C)** As per Model 2, in phage treatment with 2 µg/mL chloramphenicol, mutants of *rfaH* and *waaP* are predicted to survive but none are observed. (See **Supplementary Figure S2** 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 model adjustment does not lead to significantly better predictions, then the assumption that all effects we see are due to the combination of effects observed for treatments in isolation must be challenged. Second order effects such as phage-antibiotic interactions (Comeau et al., 2007) may 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**). 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 pseudocount 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 combinations (**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 (22% for chloramphenicol, 59% for gentamicin) are now within the 95% confidence intervals of the observed rates (18% and 60% respectively). However, the predicted distribution of mutations among rough and deep rough genes is worse than for model 1 for combination treatments (**Figure 5A**). The LPS distribution predictions

improve only for 1 µg/mL gentamicin, but became worse for both levels of chloramphenicol and 2 µg/mL gentamicin (**Figure 5A**).

Model 2 qualitatively predicts the shift towards rough LPS, but not accurately (**Figure 5A**). Since *waaP* mutants grow at 2 µg/mL chloramphenicol in our susceptibility assays, deep rough *waaP* mutants are predicted to occur at a rate of 7% leading to a non-zero deep rough prediction (**Figure 5A and C**). *RfaH* mutants are predicted to occur at a rate of 14%, because they also grow well in our susceptibility assay but are indeed 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 S2C**). At 2 µg/mL gentamicin mutations are predicted in *hldE*, a deep rough gene, but none are observed (**Supplementary Figure S2D**). There are at least three possible reasons for these discrepancies between observation and prediction. First, sampling only 40-50 mutants per treatment could bias the proportion of mutated LPS genes. Sampling more mutants in the phage only treatment may allow us to more accurately estimate the proportion of mutated LPS genes and prevent us from using pseudocounts 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.

While we will not address possibility one and two here, we have adjusted the threshold for growth in our susceptibility assay to assess the impact on the predictions. When we increase the threshold for growth from 8 to 12 average greyscale value, phage resistance rate predictions do not change significantly from model 2 to the new model 3 (**Supplementary Figure S2A**). Predicted distributions across LPS genes do not change significantly for most treatments (**Supplementary Figure S2B**), but model 3 does indeed change the prediction for *waaP*, the only deep rough gene still predicted to grow at 2 µg/mL of chloramphenicol (**Supplementary Figure S2E**). Interestingly, with the higher threshold *gmhB*, *gmhA* and *hldE* are also not predicted to grow at 2 µg/mL gentamicin (**Supplementary Figure S2D**), 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 S2A**). This effect is likely the result of direct or indirect interactions between phages and chloramphenicol.

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.

Complex evolutionary outcomes can be predicted from simple growth and susceptibility measurements

The high predictive power of our models shows that it is possible to predict resistance rates for phage-antibiotic combinations simply by measuring antibiotic susceptibilities of a range of phage resistant mutants. However, there are a few exceptions. For example, at 1 µg/mL chloramphenicol the final model predicts a resistance rate three times higher (64%) than the observed rate (~18%, 95% CI: 14% to 23%). This indicates the possibility of second order interactions between phage and chloramphenicol.

There is evidence for second order effects is in our data. For example, the observed target size of the 1 µg/mL chloramphenicol treatment is similar to that of the 2 µg/mL gentamicin treatment (**Figure 4A**). The target size observations for these treatments are also very similar to the predictions. Yet, for gentamicin the predicted phage resistance rate (52%) fits well with the observed resistance rate (60%), while for chloramphenicol the rates differ significantly (64% predicted vs. 15% observed, **Figure 3B**). Hence, the observed reduction in target size for chloramphenicol does not explain the much more significant reduction in phage resistance rate. In our opinion, a possible explanation for this discrepancy is an interaction between ΦX174 and chloramphenicol.

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). Alternatively, mutations in rough LPS genes might be more likely to result in a non-viable phenotype than in deep rough genes. Some rough LPS genes are involved in glucose or galactose metabolism (Frey, 1996), which may be more central to cell function than heptose metabolism genes (deep rough genes) (Kneidinger et al., 2002). Hence, heptose metabolism genes may be mutated in more loci without fatally affecting the cell. This could explain the higher abundance of deep rough mutants. Another possibility is that deep rough candidate genes might have more mutational targets, that is, loci which produce a robust phage-resistant LPS phenotype, than in rough candidate genes.

Deep rough mutants are more abundant in phage resistant mutants, yet 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.

ΦX174 is very specific in its receptor recognition: it could infect only 0.8% out of 783 *E. coli* human and animal commensal strains (Michel et al., 2010). However, the host range of ΦX174 can be expanded through evolution experiments (Romeyer Dherbey et al., 2023) 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). ΦX174 has been extensively studied and proven to be safe in humans in studies approved by the FDA (Bearden et al., 2005; Smith et al., 2014). All these qualities make ΦX174 an ideal candidate for efficient combination therapies (Romeyer Dherbey and Bertels, 2023). Further research should focus on which antibiotics can be combined with ΦX174 and other LPS-targeting phages to avoid antagonistic combinations. For example, ΦX174 and other phages might not be compatible with antibiotics which target RNA synthesis, e.g., rifampicin, (Higashi and Komano, 1977) or target DNA gyrase, e.g., nalidixic acid and novobiocin (Sarachu et al., 1980; Hamatake et al., 1981) because the antibiotic action also stops phage replication.

Implications for phage therapy research

Our results show that ΦX174 can be used in conjunction with certain antibiotics at subinhibitory levels to reduce the evolution of phage resistance. The use of subinhibitory concentrations indicates that the combinations can be used even when the target bacteria are antibiotic resistant. Clinicians often face decisions of whether to continue antibiotic treatment when starting phage treatment. Since phage-antibiotic combinations can be more effective than

phage alone, antibiotic treatment should be continued if such an effect exists. Moreover, since LPS mediate virulence (Burns and Hull, 1998; Raetz and Whitfield, 2002; Pier, 2007) and elicit immune response (Alexander and Rietschel, 2001), LPS modifications may also reduce pathogen virulence due to treatment with LPS-targeting phages. To make informed decisions, clinicians need to know which phage-antibiotic combinations are beneficial when administered together and more research is required to obtain this information.

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 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 plate (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. Average grey scale values of larger than 8 are defined as growth and values of less than 8 are defined as no growth. Mean of growth values across 3-5 replicates was used to make Figure 2.

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 independent single colonies of *E. coli* C WT were each 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) with 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

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). 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 ($\varepsilon<1.0$, 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 ($\varepsilon<1.0$) 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. We assumed that only genes which were previously observed in Romeyer Dherbey et al., 2023 can confer resistance to treatment with ΦX174 only (without presence of antibiotic). In case a strain had more than one gene mutated, the gene which impacts LPS the most was considered as the gene conferring ΦX174 resistance, e.g. *waaO* for R29, *galE* for R1. We also assumed that only mutations occurring in the gene coding region have an effect. Thus, the probability of a mutation to occur in a particular gene was assumed to be proportional to length of the coding region of a gene. That is, when treating with ΦX174 alone, probability weight for a gene is equal to its length (**Table 1**).

Growth of the phage-resistant strains with antibiotic was used to predict which mutants will grow in phage-antibiotic combination treatments. It was assumed that mutants will only survive if they possess mutations in genes that confer phage resistance while also sustaining growth at a particular antibiotic concentration, as observed in **Figure 2** (values in **Supplementary Table 2**). That is, if all strains with a mutation in gene A grew at that concentration (e.g., 2 µg/mL chloramphenicol), it was assumed that gene A will form mutants in the combination treatment (in this example, phage combined with 2 µg/mL chloramphenicol). Then, the entire coding region length was assumed as the probability weight for this concentration. If none of the strains with a mutation in a gene grew at that concentration, probability weight was assumed to be zero. If out of “n” strains with a mutation in a gene, only “x” strains grew, (x/n)*coding region length was assumed as probability weight. For example, out of five *galU* mutants, two were able to grow at 2 µg/mL chloramphenicol. Thus, *galU* has a weight of (2/5*gene length) at 2 µg/mL chloramphenicol.

The cutoff for defining growth as enough to grow in the fluctuation experiment was set at an average greyscale value of 8 (see **Determination of antibiotic susceptibility of bacterial mutants**). That is, the lowest concentration when the growth of a strain drops lower than 8 was considered as the lowest inhibiting concentration. For example, for both R12 and R14 (*waaF* mutants), growth drops below 8 at 2 µg/mL chloramphenicol. Based on our predictions,

we assumed that *waaF* mutants would not form colonies in the presence of both phage and 2 µg/mL chloramphenicol, or that the colonies will not be visible.

When treating with ΦX174 alone, resistance rate would be 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. The proportion of deep rough and rough mutants were found from the mutated genes according to Romeyer Dherbey et al., 2023.

Model 2. Genes *hldD* and *gmhA* were added to to the list of previously known genes conferring phage resistance (Romeyer Dherbey et al., 2023). Based on current knowledge of LPS biosynthesis pathways, mutations identified in *hldD* were assumed to lead to a deep rough phenotype like *waaC* or *waaF* (Coleman, 1983; Schnaitman and Klena, 1993), and mutations in *gmhA* to be like *gmhB*. LPS distributions and rates were predicted considering both phenotypes of *hldD*. A phenotype like *waaF* would lead to *hldD* mutations occurring in all treatments except phage and 2 µg/mL chloramphenicol, while a phenotype like *waaC* would mean *hldD* mutations occur only in phage only treatment and phage and 1 µg/mL gentamicin. Since *hldD* mutations were only observed in phage only treatment and phage and 1 µg/mL gentamicin, models assuming a phenotype like *waaC* were used for further analysis.

All genes observed in the fluctuation experiment were included with a weight of their observed frequency in the phage only treatment (**Supplementary Table S6**). Other genes mutated in the 33 phage-resistant strains isolated in Romeyer Dherbey et al., 2023 but not observed in the phage-resistant mutants isolated from the fluctuation experiment in this study were set at a baseline weight of 1 (pseudocount). The baseline weight was added to all gene weights.

Model 3. The cutoff for defining growth as enough to grow in the fluctuation experiment 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.

Data visualisation

The heatmap (**Figure 2**) and histograms (**Figures 3B-C, 4B, 5, S1, S2**) 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**) was 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 the 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.**

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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.

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979