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Arias-Sánchez, Flor I.; Allen, Richard C.; [Hall, Alex](#) 

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Effects of prior exposure to antibiotics on bacterial adaptation to phages

F. I. ARIAS-SÁNCHEZ , R. C. ALLEN & A. R. HALL

Institute of Integrative Biology, ETH Zürich, Zürich, Switzerland

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Abstract

Understanding adaptation to complex environments requires information about how exposure to one selection pressure affects adaptation to others. For bacteria, antibiotics and viral parasites (phages) are two of the most common selection pressures and are both relevant for treatment of bacterial infections: increasing antibiotic resistance is generating significant interest in using phages in addition or as an alternative to antibiotics. However, we lack knowledge of how exposure to antibiotics affects bacterial responses to phages. Specifically, it is unclear how the negative effects of antibiotics on bacterial population growth combine with any possible mutagenic effects or physiological responses to influence adaptation to other stressors such as phages, and how this net effect varies with antibiotic concentration. Here, we experimentally addressed the effect of pre-exposure to a wide range of antibiotic concentrations on bacterial responses to phages. Across 10 antibiotics, we found a strong association between their effects on bacterial population size and subsequent population growth in the presence of phages (which in these conditions indicates phage-resistance evolution). We detected some evidence of mutagenesis among populations treated with fluoroquinolones and β -lactams at sublethal doses, but these effects were small and not consistent across phage treatments. These results show that, although stressors such as antibiotics can boost adaptation to other stressors at low concentrations, these effects are weak compared to the effect of reduced population growth at inhibitory concentrations, which in our experiments strongly reduced the likelihood of subsequent phage-resistance evolution.

Introduction

Organisms in nature encounter various stressors. The effects of exposure to one stressor on population size, physiology or the rate at which genetic variation is produced potentially influence subsequent adaptation to other stressors. For bacteria, one of the most common stressors in natural and clinical environments is exposure to antibiotics (Aminov, 2009; Martinez, 2009), for which there is independent support for all three types of effects on adaptation to other stressors. (i) All antibiotics reduce the supply of genetic variation at inhibitory

concentrations by decreasing bacterial replication. However, (ii) antibiotics can also alter bacterial physiology by inducing stress responses (Guest & Raivio, 2016; El-Halfawy *et al.*, 2017) or influencing biofilm formation (Kuczyńska-Wisnik *et al.*, 2010; Kaplan *et al.*, 2012), which may alter the selective effects of other stressors. (iii) Some responses are also associated with altered mutation rates (Ysern *et al.*, 1990; Thi *et al.*, 2011; Jee *et al.*, 2016), such as SOS responses induced by fluoroquinolones and quinolones (Ysern *et al.*, 1990; Butala *et al.*, 2009; Charpentier *et al.*, 2012) or DNA damage due to β -lactams and aminoglycosides (Dwyer *et al.*, 2007; Kohanski *et al.*, 2010a). Therefore, antibiotics potentially inhibit or accelerate subsequent adaptation to other selection pressures (Couce & Blázquez, 2009; Gillings & Stokes, 2012). Although it is not yet clear how these different effects combine, we can expect the

Correspondence: Flor I. Arias-Sánchez, Institute of Integrative Biology, ETH Zürich, 8092 Zürich, Switzerland.
Tel. +41 44 632 56 05; fax: +41 44 632 12 71;
e-mail: flor.arias@env.ethz.ch

net effect to be concentration-dependent (Bernier & Surette, 2013; Sengupta *et al.*, 2013; Ter Kuile *et al.*, 2016). Reductions in population size, and therefore mutation supply, are more likely at higher concentrations, but effects arising from stress responses or mutagenesis may be more likely at lower concentrations that do not completely kill bacteria or prevent growth. If positive effects on genetic variation or physiology outweigh the negative effects of growth inhibition at some antibiotic concentrations, we may observe a net positive effect on subsequent adaptation to other stressors.

Here, we aimed to determine how the net effect of exposure to antibiotics on subsequent adaptation and population growth upon exposure to another stressor varies with antibiotic concentration. Specifically, we tested whether antibiotics promote or inhibit adaptation to viral parasites of bacteria (phages). We focus on phages for two reasons. First, phages are highly abundant in nature (Breitbart & Rohwer, 2005; Suttle, 2007), so information about interactions between bacterial responses to antibiotics and subsequent adaptation to phages is important for our basic understanding of how antibiotics influence bacterial ecology and evolution (Sengupta *et al.*, 2013). Second, there is currently much interest in potential applications of phages as an alternative or complement to antibacterial therapy (Torres-Barceló & Hochberg, 2016). If antibiotics promote adaptation (Gillings & Stokes, 2012) to other stressors including phages, then using phages against pathogen populations previously exposed to antibiotics may be less effective than we would otherwise expect. By contrast, if prior exposure to antibiotics inhibits adaptation to phages, then treatment options that maximize this effect may be less rapidly overcome by bacterial resistance evolution.

To test this, we quantified the change in bacterial population density in the presence of phages (which in the conditions of our experiment is strongly dependent on the emergence of phage-resistant mutants) and in the absence of phages across replicated populations of *Escherichia coli* that had been previously exposed to either no antibiotics or one of seven antibiotic concentrations. Because different antibiotics have different effects on bacterial physiology and genetic variation (Kohanski *et al.*, 2010b), we predicted that their effects on adaptation to phages would also vary. We therefore did the same experiment for each of 10 antibiotics that target bacterial physiology through different mechanisms, including damage of DNA repair systems (Table 1). Crucially, if some antibiotics have effects on adaptation to phages that cannot be explained by changes in mutation supply rate resulting from inhibition of bacterial population growth (e.g. mutagenesis or beneficial stress responses) and others do not, the association between growth inhibition and subsequent adaptation to phages will vary among antibiotics. We tested this using a linear modelling approach. We then

isolated the effect of variable population size on bacterial responses to phages by manipulating it without adding antibiotics. We also tested separately for possible changes in mutation rate due to antibiotics by fluctuation assays. We found that adaptation to phages was positively correlated with initial population size across all antibiotics, and although we detected some evidence of mutagenesis after exposure to subinhibitory doses of certain antibiotics, the strength of these effects was small compared to the major effect of population growth inhibition on adaptation.

Materials and methods

Organisms and culture conditions

We used *E. coli* K12-MG1655 and two lytic bacteriophages T4 (*Myoviridae*) and T7 (*Podoviridae*). All experiments were performed at 37°C in lysogeny broth (LB) medium supplemented with 10 mM MgSO₄ and 10 mM Tris HCl, hereafter referred to as LBMT.

Antibiotic–phage experiment

Our 48-h antibiotic–phage full-factorial experiment consisted of two phases. In Phase 1 (antibiotic phase), for each antibiotic ($n = 10$), we exposed 24 independent bacterial populations to each of seven concentrations or antibiotic-free conditions for 24 h. We started each bacterial population (0 h) ($n = 1920$) by 10⁻⁶ dilution from an independent overnight culture into a microcosm containing 99 µL of antibiotic-supplemented or antibiotic-free medium. After 24 h, we measured bacterial biomass (OD_{24 h}) using an M2 Spectramax spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). We then began Phase 2 (phage phase) by sampling each population three times, transferring each 1 µL sample with a pin replicator to a microplate well containing either 99 µL of LBMT with a high concentration (~5 × 10⁷ PFU) of phage T4 or phage T7 or phage-free LBMT (control). We then measured bacterial biomass of each population after 24 h of phage

Table 1 Antibiotics used in the main experiment.

Antibiotic	Class	Target
Ciprofloxacin	Fluoroquinolones	DNA gyrase
Levofloxacin	Fluoroquinolones	DNA gyrase
Nalidixic acid	Quinolones	DNA gyrase
Ampicillin	β-Lactam	Cell wall
Rifampicin	Rifamycin	RNA polymerase
Streptomycin	Aminoglycosides	Protein synthesis, 30S
Kanamycin	Aminoglycosides	Protein synthesis, 30S
Tetracycline	Tetracycline	Protein synthesis, 30S
Azithromycin	Macrolide	Protein synthesis, 50S
Trimethoprim	Reductase inhibitor	Folic acid biosynthesis

exposure (end of Phase 2, OD48 h), using a Tecan Infinite F200 Spectrophotometer (Tecan, Männedorf, Switzerland). All measurements were corrected by subtracting the mean score of sterile medium (OD = 0.045).

To make meaningful comparisons between antibiotics, we standardized the scale for each antibiotic so that concentrations are given relative to the concentration required to inhibit bacterial growth by 50% (IC₅₀). This was estimated by fitting Hill function dose-response curves (Regoes *et al.*, 2004) to the data for bacterial growth (OD24 h) as a function of antibiotic concentration (*C*), for each of the 10 antibiotics (Fig. S1). First, we estimated coefficients for asymptote (*a*), dissociation constant (*k_a*) and Hill coefficient (*n*), using an in-house R script. We then used these as starting values for the nonlinear least squares (nls) function in R with the equation ($Growth = a \cdot k_a^n / [k_a^n + C^n]$). The IC₅₀ is then equivalent to *k_a*. Finally, we transformed the standardized concentrations to improve the scale's linearity by calculating $\log_2((\text{concentration}/IC_{50})+1/31)$ prior to analysis (Figs 1 and S2).

We then analysed the effects of antibiotic pre-exposure on average bacterial population densities at the end of Phase 2 (OD48 h), by fitting ANCOVA (analysis of covariance) models for each phage treatment. Each model included average OD48 h (averaged across all replicate populations within each antibiotic × concentration combination) as the response variable. We used the average here rather than including all values because of the skewed distribution within each set of replicates, which we discuss further below. We included antibiotic and concentration (with concentration as linear and quadratic terms; standardized and transformed as above) as explanatory variables. We also included an antibiotic × concentration interaction term, which would indicate that the effect of increasing

antibiotic concentration on subsequent adaptation to phages varies among antibiotics. We then simplified each model by removing nonsignificant ($P > 0.05$) higher-order terms using *F*-tests comparing full and reduced models. We excluded 37 populations that reached low OD48 h in our phage-free treatment from all analyses (average OD48 h for these populations = 0.11). These populations came from different antibiotic treatments but were clustered in the same experimental plate, suggesting they were false-inoculated, probably due to high temperature of the pin replicator during this inoculation step.

We found that population densities after exposure to phages had a skewed distribution (Fig. S3), with many populations reaching only very low OD48 h scores. Previous work in this system showed that this distribution is caused by some populations evolving phage resistance and others not, with only those populations that contain phage-resistant mutants attaining viable growth scores (Arias-Sánchez & Hall, 2016). We therefore analysed the fraction of populations in each phage treatment that attained viable growth by imposing a cut-off value for viable growth in the presence of phages of OD48 h > 0.05, which approximates the minimum score for populations where resistance to phages has evolved (Arias-Sánchez & Hall, 2016). We calculated the fraction of OD48 h scores above this threshold for each antibiotic at each concentration in each of the phage treatments, before testing whether a higher fraction of viable populations (and therefore more frequent resistance evolution) was associated with higher average population size at the beginning of phage treatment (OD24 h). To do this, we used ANCOVA models as above but with the proportion of viable populations as the response variable, average OD24 h as a continuous predictor (linear and quadratic terms) and antibiotic as a factor, including the interactions between antibiotic

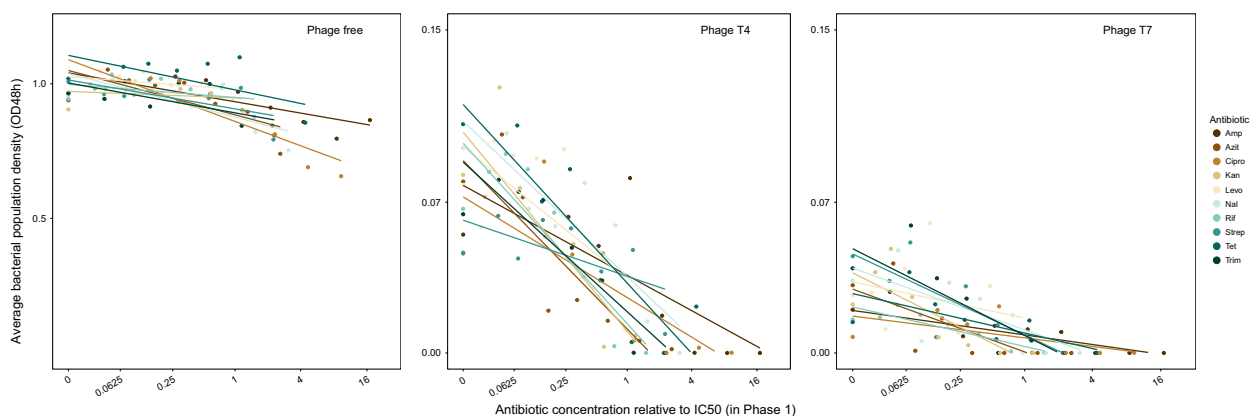


Fig. 1 Average population densities at the end of Phase 2 as a function of antibiotic concentrations in Phase 1 for each of ten antibiotics. x-axis shows antibiotic concentrations relative to the IC₅₀. Panels represent phage treatments. Note that the y-axis scale for the phage-free treatments is different.

and average OD₂₄ h. This model tests whether population density at the end of Phase 1 (after antibiotic exposure) determines the fraction of populations that evolve resistance and attain viable growth upon exposure to phages (in Phase 2), and whether that association varies among antibiotics. Crucially, by including OD₂₄ h as a predictor but not antibiotic concentration, this model assumes that the only effect of varying the concentration of each antibiotic is to change OD₂₄ h. Therefore, if some antibiotics have other effects on bacterial growth in the presence of phages that are not reflected by a change in OD₂₄ h, we would expect a significant antibiotic × OD₂₄ h interaction term.

Testing the effect of initial population size without using antibiotics

To further separate the effects that antibiotic exposure and variable population size have on bacterial responses to phages, we performed an experiment under similar conditions as above, but manipulated population size at the beginning of Phase 2 by dilution rather than growth inhibition by antibiotics. First, we grew independent bacterial populations ($n = 384$) in media without antibiotics. After 24 h of growth, we transferred aliquots to phage treatments as above, but transferring variable numbers of bacterial cells by varying the dilution factor upon transfer (24 populations at each of seven dilution factors plus a control treatment containing no bacteria). We then measured optical density (OD₂₄ h) after dilution and started Phase 2 by adding $\sim 5 \times 10^7$ PFU of either phage T4 or T7. We then incubated the populations and measured OD₄₈ h as above. To test whether bacterial population size at the beginning of phage exposure in this experiment was associated with viable growth (indicating resistance evolution as above) at the end of phage exposure, we regressed the proportion of viable populations (OD₄₈ h > 0.05) after phage exposure against the average population size at each dilution factor used at the end of Phase 1 (OD₂₄ h).

Testing for changes in mutation rate due to antibiotic exposure

To test whether sublethal doses of antibiotics increase mutation rates (μ) to phage resistance, we performed fluctuation assays. From a diluted overnight culture, we transferred 30–300 cells (N_0) into microcosms containing 100 μ L of sterile LBMT medium (control) or LBMT medium containing subinhibitory doses (3% and 10% MIC) of four of the antibiotics used above (ampicillin, ciprofloxacin, nalidixic acid and tetracycline), giving a total of 18 independent populations per antibiotic per concentration. After incubation for 24 h, we transferred a sample of each population to LB-agar plates containing a concentration of phages

where only resistant bacteria can grow ($\sim 5 \times 10^7$ PFU of either T4 or T7). Additionally, we randomly selected six populations from each antibiotic treatment and the control after 24 h and plated them on phage-free LB-agar plates after serial dilution. After a further 24 h of incubation, we estimated the number of phage-resistant mutants (r) and the number of viable cells (N_t) in each fluctuation assay (treatment) by scoring the number of colony-forming units (CFUs) on phage-agar and phage-free plates. We then used rSalvador (Zheng, 2017) to estimate mutation rate (μ) to phage resistance in each treatment, accounting for plating efficiency (T4 treatments: $e = 0.05$ and T7 treatments: $e = 0.5$) and using the likelihood ratio test with partial plating when comparing estimates from different treatments. This test uses a Newton–Raphson type algorithm and accounts for differences in population size (Zheng, 2015, 2016). This and all other analyses were conducted in R 3.1.1 (R Core Team, 2016).

Results

Prior exposure to high antibiotic concentrations inhibits bacterial population growth in the presence of phages, but not by killing entire populations

Both average population density and the proportion of surviving populations after phage exposure were affected by antibiotics. After model simplification, we found that average population density at the end of phage treatment (average OD₄₈ h in T4 and T7 treatments) was negatively associated with increasing antibiotic concentration in Phase 1 (T4 treatment: $F_{1,69} = 121.91$, $P < 0.0001$, T7 treatment: $F_{1,69} = 52.50$, $P < 0.0001$; Fig. 1), and this association was approximately linear ($P > 0.05$ for all quadratic terms). We found no evidence that the effect of increasing concentration on subsequent population growth in the presence of phages varied among antibiotics ($P > 0.05$ for all interaction terms for both phages). We also observed a negative effect of increasing antibiotic concentration (Phase 1) on average final population density (Phase 2) in the control treatment (linear term: $F_{1,59} = 135.88$, $P < 0.0001$). However, in the control (phage-free) treatment the overall association was nonlinear (quadratic term: $F_{1,59} = 58.25$, $P < 0.0001$), with only the highest concentrations being associated with reduced growth in Phase 2. Because the range of inhibitory effects in Phase 1 varied among antibiotics ($F_{9,59} = 5.69$, $P < 0.0001$; Fig. S1), this resulted in variation of the association between final population density (Phase 2) and antibiotic concentration (Phase 1) among antibiotics in the phage-free treatment (concentration × antibiotic interaction: $F_{9,59} = 2.52$, $P = 0.016$; Fig. 1).

The effects of antibiotic exposure (Phase 1) on population growth in the presence of phages (Phase 2) were

also evident from a decline in the proportion of populations in each treatment attaining viable growth ($OD_{48\text{ h}} > 0.05$) after prior exposure to higher antibiotic concentrations (Figs S2 and S4). Given that viable growth in the presence of phages is strongly linked to the emergence of phage-resistant mutants (Arias-Sánchez & Hall, 2016), we attribute this to a declining fraction of populations that evolved phage resistance after exposure to higher antibiotic concentrations. We observed viable growth in only 8% of T7-treated populations and 26% of T4-treated populations (Fig. S3). However, the lack of viable growth in most phage-treated populations was not due to those populations being completely killed off by antibiotics in Phase 1: the vast majority of populations in the phage-free treatment, which were inoculated from the same Phase 1 populations, attained viable growth in Phase 2 (98.7% of populations; Fig. S3).

Stronger growth inhibition by antibiotics reduces the likelihood of viable growth in the presence of phages

We next asked whether variation in the fraction of viable populations in Phase 2 (indicating the fraction of populations that evolved phage resistance) was explained by the effects of antibiotics on bacterial population growth in Phase 1, hypothesizing that smaller populations at the end of Phase 1 are less likely to contain a phage-resistant mutant and therefore less likely to attain viable growth in Phase 2. For both phage treatments, we found a positive association between the proportion of viable populations at the end of Phase 2 and population density at the end of Phase 1 (T4 treatment: $F_{1,68} = 153.43$, $P < 0.0001$; T7 treatment: $F_{1,68} = 68.63$, $P < 0.0001$; Fig. 2). We also found a significant quadratic term in both treatments (T4 treatment: $F_{1,68} = 16.58$, $P = 0.0001$; T7 treatment: $F_{1,68} = 16.55$, $P = 0.0001$; Fig. 2), and a main effect of antibiotic (T4 treatment: $F_{9,68} = 2.63$, $P = 0.01$; T7 treatment: $F_{9,68} = 4.29$, $P = 0.0002$), which results from variation of average population size among antibiotic treatments. However, we found no significant interactions between linear or quadratic population density terms with antibiotic, providing no evidence that some antibiotics have effects on subsequent responses to phages that are not explained by their effects on population density. Despite this, for some antibiotics the fraction of populations showing viable growth was highest at intermediate antibiotic concentrations (Fig. 2), and these were concentrations that had marginal effects on population density. In eight of these cases (three in the T4 treatment; five in the T7 treatment), the proportion of populations attaining viable growth upon phage exposure was higher than in any of the control treatments (the ten sets of populations that were never exposed to

antibiotics and used as independent controls for each antibiotic).

The effect of initial population size on adaptation to phages does not require antibiotics

The above results indicate that bacterial population size at the end of antibiotic exposure was the main driver of the likelihood of viable population growth upon subsequent exposure to phages. Nevertheless, variation in population density at the end of Phase 1 was always due to addition of antibiotics, so variation of population size was confounded with the presence of antibiotics. To test the effect of population density at the end of Phase 1, but excluding any other effects of antibiotics, we diluted populations to varying degrees in a separate experiment, before inoculating them into phage treatments as above. We found that population size before phage exposure was a strong linear predictor of the fraction of populations attaining viable growth in the presence of phages (T4: $r^2 = 0.84$, $P = 0.0009$; T7: $r^2 = 0.93$, $P < 0.0001$; Fig. 3).

Mutagenic effects were small and not consistent

Despite the clear overall decline in the proportion of viable populations in phage treatments with decreasing population density at the end of Phase 1, in some cases both average $OD_{48\text{ h}}$ and the frequency of viable populations were highest at intermediate antibiotic concentrations (Fig. 2). We therefore asked whether subinhibitory concentrations of four antibiotics were associated with altered mutation rates towards phage resistance in these conditions. We selected antibiotics either showing greater growth or survival after exposure to intermediate concentrations (nalidixic acid and tetracycline) or associated in other studies with mutagenic effects on other types of resistance (Jee *et al.*, 2016; Mo *et al.*, 2016) (ciprofloxacin and ampicillin). All our estimates for mutation rate to phage resistance were within approximately one order of magnitude of each other (Fig. 4). Some of the differences among treatments were statistically significant, although these differences were not consistent across phage treatments (Fig. 4). We note that during the fluctuation assays, growth in the presence of antibiotics was associated with altered population density prior to plating on phage-agar (ANOVA: T4 treatment: $F_{8,51} = 7.51$, $P < 0.001$; T7 treatment: $F_{8,51} = 2.04$, $P = 0.06$). Thus, both our main experiment and our fluctuation assays offer only weak evidence for mutagenic effects of antibiotics influencing adaptation to phages.

Discussion

We addressed two of the key ways by which exposure to one stressor or selection pressure can influence

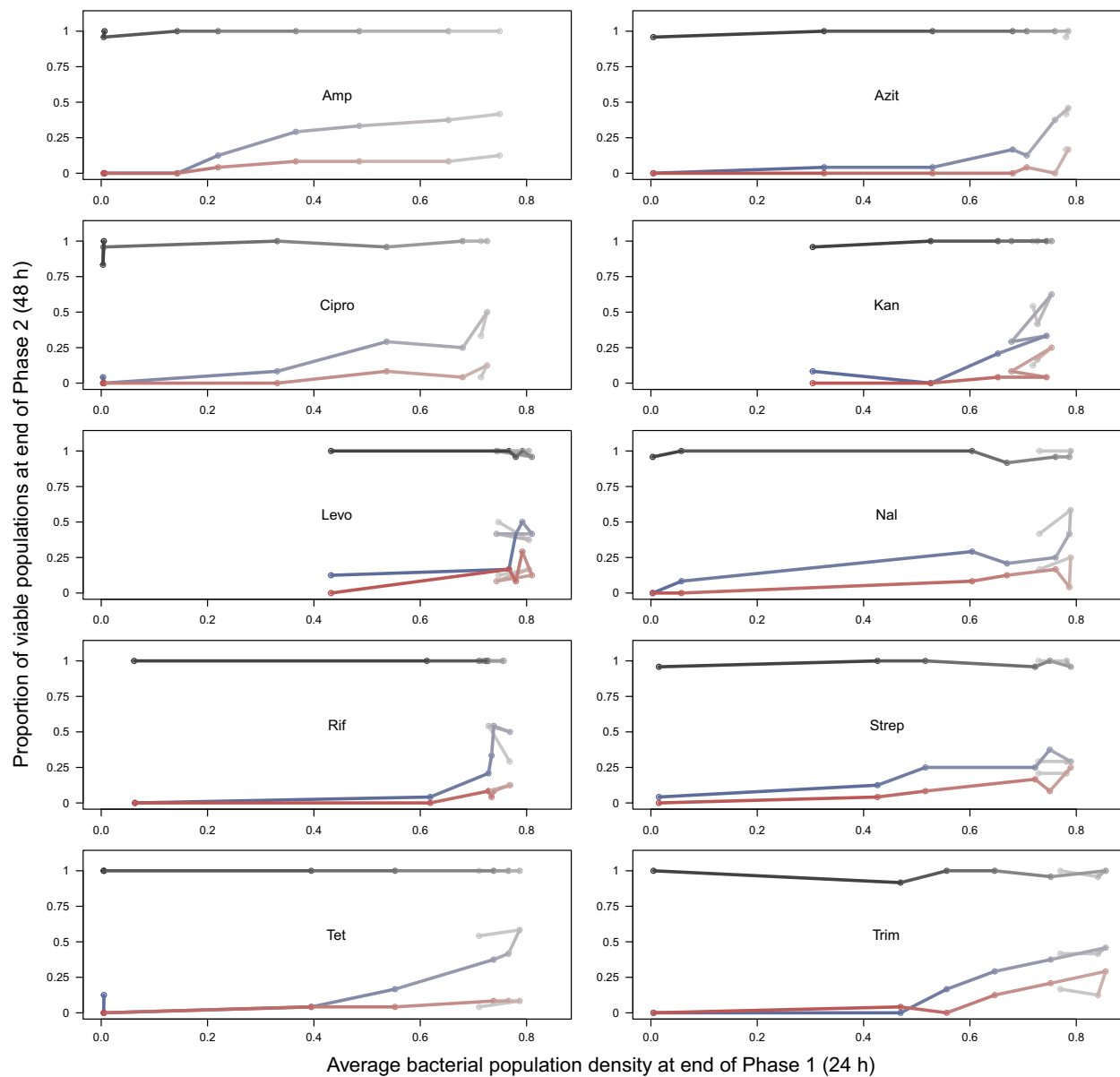


Fig. 2 Proportion of viable populations after exposure to phages (end of Phase 2; OD_{48 h} > 0.05) as a function of average population density for the same populations at the end of Phase 1 (OD_{24 h}). Each panel shows one antibiotic treatment, and each point shows a single antibiotic concentration from Phase 1. Points are connected in the same sequence as descending antibiotic concentrations in each treatment, with the colour gradient of each line showing lower (light grey) to higher (dark colour) concentrations. Each line represents one phage treatment (T4 = blue, T7 = red and no phage = black).

adaptation to others (changes in population growth and changes in per capita mutation rate). Although these effects are well studied independently, our study provides several new insights. (i) We compared the net impact of these counteracting effects across a concentration gradient for a range of different antibiotics, a key stressor for bacteria in nature. (ii) We included an additional experiment uncoupling the effects of population growth inhibition from other effects of antibiotics

(by manipulating population size in the absence of antibiotics). (iii) We tested the downstream effects of antibiotic exposure on bacterial adaptation to bacteriophages, which are extremely common in nature (Breitbart & Rohwer, 2005; Suttle, 2007; Hatfull, 2015) and a potential alternative or complement to antibiotics in treating bacterial infections of humans or animals (Merril *et al.*, 2003; Meyer *et al.*, 2012; Reardon, 2014; Torres-Barceló & Hochberg, 2016). As predicted, increasing

Fig. 3 Average population density at the end of phage exposure (48 h) as a function of average population density at the beginning of phage exposure (24 h). Here, bacterial population densities were manipulated through dilution in sterile media, rather than by adding antibiotics as in the main experiment.

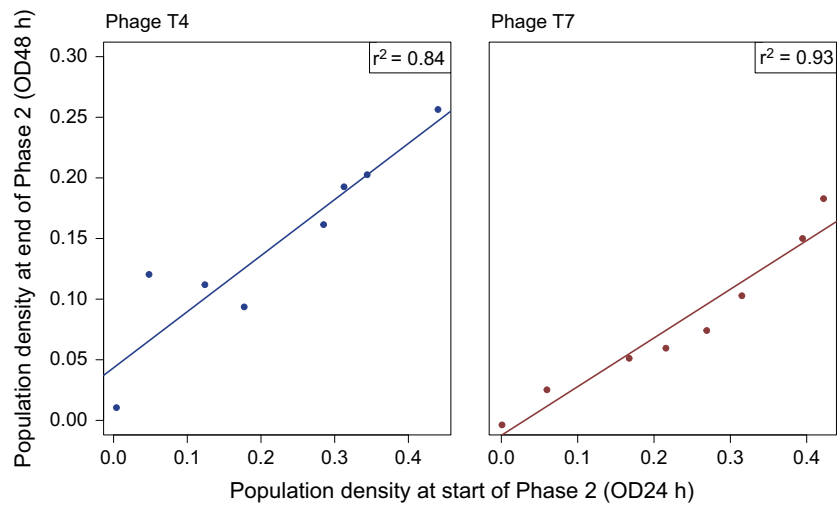
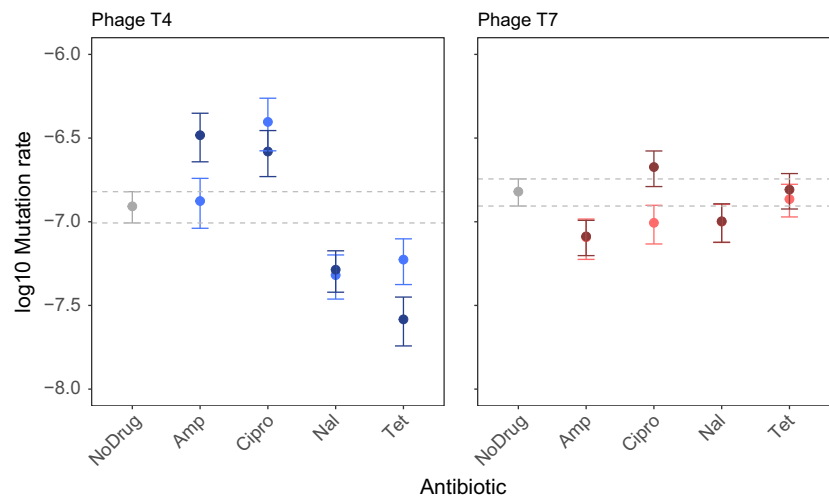


Fig. 4 Mutation rates to phage resistance after exposure to subinhibitory doses of antibiotics. Exposure to phage T4 (blue) and T7 (red) occurred after exposure to sub-MIC doses of antibiotics (light blue and light red = $0.03 \times \text{MIC}$; dark blue and dark red = $0.1 \times \text{MIC}$; grey = no antibiotic). Error bars are 95% confidence intervals estimated with rSalvador.



antibiotic concentration had a negative effect on bacterial population size in Phase 1 and this was associated with a reduced likelihood of viable growth upon subsequent exposure to bacteriophages in Phase 2, which in these conditions reflects a reduced frequency of populations evolving phage resistance (Arias-Sánchez & Hall, 2016). Surprisingly, this was not due to antibiotics killing off entire populations: the vast majority of populations were still viable at the start of Phase 2, even after exposure to the highest antibiotic concentrations. Crucially, the effects of growth inhibition in Phase 1 on adaptation in Phase 2 were much stronger than those of any antibiotic-associated mutagenesis at lower doses, which we also tested for directly in a separate experiment.

We attribute the effects of antibiotics in Phase 1 on adaptation to phages in Phase 2 to a reduced supply of phage-resistance mutations, because larger populations are more likely to contain mutants (Fisher, 1930;

Maynard-Smith, 1976; Kimura, 1979), assuming they have undergone more replications during Phase 1. An alternative explanation is that larger populations are somehow less susceptible to viruses, for example if a lower phages-to-bacteria ratio permits some sensitive cells to attain a viable population size without being infected (Delbruck, 1940). However, this is unlikely to explain our results for two reasons. First, even at the highest bacterial densities at the start of Phase 2, there was an excess of phages (assuming $\sim 10^7$ cells/mL bacteria after 100-fold dilution, equivalent to $\sim 10^6$ cells in $100 \mu\text{L}$, exposed to $\sim 5 \times 10^7$ PFUs). Second, we observed a skewed distribution of final growth scores among replicate populations exposed to phages, both within and across treatments (Figs S3 and S4), consistent with the stochastic appearance of resistant mutants in some populations but not others (Mani & Clarke, 1990). Nevertheless, we do not rule out variable infection dynamics depending on absolute bacterial and

phage densities. An additional possibility is that antibiotic-resistance alleles spread in some populations during Phase 1 and these conferred some cross-protection to phages. However, this is unlikely because (i) the distribution of OD_{24 h} scores within each group of replicates is not consistent with the stochastic appearance of resistance mutations in some populations and not others, with the possible exception of three intermediate doses of antibiotics and (ii) past work testing directly for effects of defined antibiotic-resistance alleles on sensitivity to these phages in this system found no such interactions (Arias-Sánchez & Hall, 2016).

Our finding that the vast majority of populations were still viable at the start of Phase 2, even after exposure to the highest antibiotic concentrations, likely reflects the emergence of persister cells in these cultures (Lewis, 2010), observed previously for this strain in other experiments (Balaban *et al.*, 2004; Dörr *et al.*, 2010). This suggests that although antibiotics can dramatically reduce the likelihood of adaptation to other stressors, they will only do so by completely eliminating the bacterial population when it is too small to contain a significant fraction of persisters. Our data also suggest any other phenotypic changes that are specific to particular types of antibiotics do not significantly influence subsequent adaptation to phages, in that the association between growth inhibition and phage-resistance evolution did not vary among antibiotics (i.e. there were no effects of individual antibiotics that could not be explained by growth inhibition.).

One possible limitation of our study is that the range of growth-inhibitory effects observed in Phase 1 varied among antibiotics (although it overlapped considerably and included the IC₅₀ in all cases except levofloxacin, where it was just above the range used). This explains our finding that the effect of increasing concentration (standardized relative to the IC₅₀) on growth in Phase 2 varied among antibiotics in the control treatment only: here, the overall association was nonlinear, and *post hoc* contrasts showed that this resulted from only the highest antibiotic concentrations reducing population growth in Phase 2. Thus, the decline in average Phase 2 growth with increasing concentration was steepest for antibiotics with stronger maximum inhibitory effects in Phase 1 in the control treatment (Fig. 1A). However, because the overall association between growth inhibition in Phase 1 and growth/adaptation in Phase 2 was approximately linear in the phage treatments, the minor variation in the range of inhibitory effects among antibiotics did not produce an interaction here, and moreover, this cannot explain our key finding that this association does not vary among the antibiotics tested here.

Despite past work showing that some antibiotics are mutagenic at sublethal concentrations (Piddock & Wise, 1987; Ysern *et al.*, 1990; Couce & Blázquez, 2009; Jee *et al.*, 2016), we found only weak evidence for

mutagenesis caused by antibiotics accelerating phage-resistance evolution. There are multiple possible reasons for this. First, although we accounted for variation of population size across our fluctuation assays, treatment effects could potentially also be influenced by any effect of changes in population density due to antibiotics on the tendency to under- or overestimate mutation rates, and by any other effects of antibiotics on bacterial growth dynamics not reflected in population density prior to plating, such as an altered relationship between final population size (N_t) and the number of genome replications during the growth phase of the assay (Frenoy & Bonhoeffer, 2017). Second, we tested a limited number of antibiotic concentrations and although there is evidence of mutagenesis at similar doses (Mo *et al.*, 2016), it may be that mutagenic effects occur only over a narrow range, where mechanisms such as SOS-induced mutagenesis (Torres-Barceló *et al.*, 2015) are triggered but cells remain viable (Couce & Blázquez, 2009). Third, the impact of any mutagenic effects will depend on the type of genetic changes driving adaptation to a new stress. It has been shown that mutagenic effects of antibiotics and other stressors are uneven across different types of genetic changes (Jee *et al.*, 2016; Maharjan & Ferenci, 2017). For some types of phage resistance, including those important in this system (Wielgoss *et al.*, 2015), various types of genetic changes such as large deletions and insertion sequence movements can be involved and might not be affected by antibiotics in the same way as, for example, single nucleotide polymorphisms (Song *et al.*, 2016). Fourth, mutagenic effects at the concentrations we tested may have simply been too small for us to detect, although this would still be consistent with our conclusion that they are small relative to the effects of growth inhibition.

Our results suggest a relatively small risk of mutagenesis caused by past exposure to antibiotics contributing to resistance evolution during subsequent exposure to phages, compared to the strong negative effect of exposure to inhibitory concentrations on population growth and mutation supply. This suggests combining phages with antibiotic treatments (Torres-Barceló & Hochberg, 2016) using them sequentially (antibiotics to decrease bacterial population size before applying phages) may benefit from a similarly reduced likelihood of resistance evolution as observed in our experiments. We note however that the latter effect relies on the size of the bacterial population and will therefore depend on the timing of exposure to each stressor, being weaker if there is an interval between them that allows regrowth. The timescale and concentration of antibiotic exposure will also vary in other ways in nature and in clinical settings that our experiment did not capture. In particular, over longer timescales antibiotic concentrations that had little effect in our experiment may promote resistance evolution to either antibiotics (Gullberg *et al.*,

2011; Long *et al.*, 2016) or phages (Cairns *et al.*, 2017). Consistent with our conclusion that any mutagenic effects would have weak effects on adaptation to phages relative to the effects of growth inhibition, a long-term experiment with *Pseudomonas aeruginosa* found no evidence that SOS-induced mutagenesis accelerated the rate of adaptation to antibiotics over 200 generations (Torres-Barceló *et al.*, 2015). Thus, despite strong evidence for mutagenic effects of antibiotics on some phenotypes (Kohanski *et al.*, 2010a; Thi *et al.*, 2011; Mo *et al.*, 2016), the downstream effects of this for adaptation can be weak relative to other effects on physiology or growth, as they were in our experiment.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article: **Figure S1** Bacterial population densities (OD₂₄ h; end of Phase 1) after exposure to antibiotics (given by the abbreviation at top-right in each panel) at different concentrations (x -axis).

Figure S2 Proportion of populations attaining viable growth scores in Phase 2 as a function of antibiotic concentration they were exposed to for each of ten antibiotics.

Figure S3 Histograms of OD₄₈ h scores after phage exposure (end of Phase 2) in each treatment.

Figure S4 Bacterial population densities after phage exposure (OD₄₈ h) in each treatment.

Data deposited at Dryad: <https://doi.org/10.5061/dryad.60h3r>

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