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Nilang Shah April 17, 2018

The susceptibility of non-replicating populations of bacteria to killing by bactericidal antibiotics

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a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Non-growing populations of bacteria are known to be phenotypically refractory to antibiotics to which they are genetically susceptible. Here, we consider three classes of non-growing planktonic bacteria: (1) Stationary phase, when the concentration of the limiting resource is too low to allow for replication; (2) Minority subpopulations of non- or slowly replicating cells, known as persisters; (3) Populations exposed to bacteriostatic antibiotics that prevent their replication and kill them slowly. Using experimental populations of Staphylococcus aureus Newman and Escherichia coli K12 and bactericidal antibiotics of 10 and 8 classes, respectively, we address the quantitative question of how refractory non-growing and slowly dying populations of these different types are susceptible to antibiotics and the rates at which different concentrations of these drugs kill them. Contrary to the common belief that non-growing bacteria are refractory to antibiotic-mediated killing, all three classes of non-growing populations of these Gram-positive and Gram-negative bacteria are killed by one or more bactericidal antibiotic and some of these drugs kill non-growing bacteria at relatively low concentrations. Lastly, we investigate the relationship between persistence and bacteriostasis by examining the ability of these non-growing states to support the replication of bacteriophage.

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The susceptibility of non-replicating populations of bacteria to killing by bactericidal antibiotics

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Abstract:

Non-growing populations of bacteria are known to be phenotypically refractory to antibiotics to which they are genetically susceptible. Here, we consider three classes of non-growing planktonic bacteria: (1) Stationary phase, when the concentration of the limiting resource is too low to allow for replication; (2) Minority subpopulations of non- or slowly replicating bacteria, known as persisters; (3) Populations exposed to bacteriostatic antibiotics that prevent their replication and kill them slowly. Using experimental populations of *Staphylococcus aureus* Newman and *Escherichia coli* K12 and bactericidal antibiotics of 10 and 8 classes, respectively, we address the quantitative question of how refractory non-growing and slowly dying populations of these different types are susceptible to antibiotics and the rates at which different concentrations of these drugs kill them. Contrary to the common belief that non-growing bacteria are refractory to antibiotic-mediated killing, all three classes of non-growing populations of these Gram-positive and Gram-negative bacteria are killed by one or more bactericidal antibiotics and some of these drugs kill non-growing bacteria at relatively low concentrations. Lastly, we investigate the relationship between persistence and bacteriostasis by examining the ability of these non-growing states to support the replication of bacteriophage.

Introduction:

In vitro, the relationship between the concentrations of antibiotics and the rates of growth and death of bacteria, pharmacodynamics, is almost exclusively studied under conditions that are considered optimal for the action of these drugs; cells growing exponentially in media and culture conditions where all members of the exposed population have equal access to these drugs, resources and other by-products and metabolites.

The minimum concentration of an antibiotic needed to prevent the growth of the bacteria, the MIC, is a prime example of a pharmacodynamic parameter the value of which is estimated under conditions that are optimal for the drug. MICs, the single most commonly employed pharmacodynamic parameter, are used both as the criteria for susceptibility/resistance of bacteria to antibiotics (see http://www.eucast.org/clinical_breakpoints/), and as the primary pharmacodynamic (PD) parameter of the PK/PD indices employed for the "rational" [1, 2] design of antibiotic treatment regimens [3-9]. In accord with the traditional and officially sanctioned

protocol, relatively low densities of bacteria are incubated in liquid media at a temperature in which they can grow at their maximum rate and are mixed with different concentrations of the antibiotic. After a defined period, the optical densities of the cultures are estimated. The MIC is then calculated as minimum concentration of the antibiotic in which the normalized optical density of the culture is that of the bacteria-free medium. Cells growing exponentially, in rich, liquid, media are also used to estimate the minimum concentration of the antibiotic needed to kill the bacteria, the minimum bactericidal concentration (MBC), and the concentration of the drug needed to kill a specified fraction of the exposed population [10]. Bacteria growing exponentially in liquid culture are also employed to estimate the parameters of more comprehensive pharmacodynamics function, like the Hill Function. [11]. While MICs can be estimated for bacteria on surfaces, E-tests [12, 13], and their values inferred from zones of inhibition of Kirby-Bauer disk diffusion assays, there as well, the bacteria are growing exponentially with an abundance of nutrients.

At some moments, in some sites and tissues in infected hosts, the target bacteria may be growing exponentially at their maximum rate and thereby under conditions that are optimal for the action of the antibiotic. This situation is almost certainly rare for established and host-responsive (symptomatic) infections. In some sites and tissues the infecting bacteria may have exhausted the available resources; their populations will be at stationary phase and thereby not replicating when nutrients are available [14, 15]. Furthermore, the target bacteria, which may be genetically sensitive to the drug, in some sites and tissues these bacteria may be not be replicating and, for that reason, are phenotypically, resistant to the antibiotic, e.g. the so called "persisters"[16-18]. While in some tissues, though there may be an abundance of resources for growth, the bacteria are not replicating or are dying slowly because of exposure to bacteriostatic or weakly bactericidal antibiotics. Finally, and perhaps most importantly, the infecting bacteria may be inside host cells (professional or non-professional phagocytosis), attached to surfaces by polysaccharide matrices known as biofilms [19] and are either not growing because of a dearth of nutrients [20, 21], non-optimal pH, or, as suggested, by [22] are persisters.

In this report, we explore the susceptibility of non-growing populations of *Staphylococcus aureus* and *E. coli* to killing by, respectively 10 and 8 bactericidal antibiotics. We consider three types of non-growing and slowly replicating states; (i) stationary phase, (ii) phenotypically resistant cells, persisters, and (iii) bacteria exposed to bacteriostatic antibiotics, which we will call bacteriostatic or BS-persisters. Contrary to the popular conception that antibiotics preferentially can kill replicating bacteria, [23-25] even at relatively low concentrations a number of existing bactericidal antibiotics kill non-replicating bacteria of all three states. They are also able to markedly increase the rate of kill of bacteria exposed to weakly bactericidal antibiotics.

Materials & Methods

Growth Media and Strains

The bacterial cultures were grown at 37°C in either Mueller-Hinton Broth (MHII)

(275710, BD™) or Lysogeny Broth (LB) (244620, Difco). The *E. coli* strains used in experiments were either wild type K-12 derivatives of the parent strain MG1655 (hipA7) or the parent strain itself. The hipA7 mutant strain used in the *E. coli* persister experiments was provided by Kyle Allison (Emory/GA-Tech. *S. aureus* Newman (ATCC 25904) was the sole strain of *S. aureus* used for all experiments. Phage lysates were prepared from single plaques at 37°C in LB medium alongside wild type MG1655. Chloroform was added to the lysates and the lysate was centrifuged to remove any remaining bacterial cells. The λ^{VIR} strain used in these experiments was obtained from Sylvain Moineau (Laval University) and all other phage were provided by James Bull (UT Austin).

Sampling Bacterial and Phage Densities

Bacteria and phage densities were estimated by serial dilution in 0.85% saline followed by plating. The total density of bacteria was estimated on Lysogeny Broth hard (1.6%) agar plates. To estimate the densities of free phage in the experimental cultures, chloroform was added to suspensions before serial dilution. These suspensions were mixed with 0.1 mL of overnight LB cultures of wild type MG1655 (~$5x10^8$ cells per mL) in 3 mL of LB soft (0.65%) agar and poured onto semi-hard (1%) LB agar plates. To see a complete list of phage used in this study, see Supplementary Table S1.

Estimating Minimum Inhibitory Concentration

The MICs of both *S. aureus* Newman and *E. coli* MG1655 to the antibiotics seen in Table 1 were estimated using the two-fold micro dilution procedure [13]. Various initial concentrations of antibiotics were used to obtain accurate measurements from the two-fold micro dilution procedure.

Time-kill Curves

Overnight cultures of the bacteria were diluted 1:1000 or 1:100 in fresh broth for *S. aureus* and *E. coli*, respectively, and incubated at 37°C for 2 hours. The cultures were divided into 10 mL aliquots in 50 mL borosilicate glass flasks. Antibiotics were subsequently added at the concentrations given in Figure 1. Cultures tested with bacteriostatic antibiotics as seen in Table 1 were incubated at 37°C, for 4 days with the viable cell densities estimated at 0, 24, 48, 72 and 96 hours. Cultures tested with bactericidal antibiotics were sampled over 8 hours with samples taken every 30 minutes for the first 2 hours followed by hourly sampling for an additional 6 hours. Final estimates of viable cell density at 24 hours for all treatments were taken (see Figure S1). Control cultures without the treatment of antibiotics were set up in parallel and sampled accordingly. Time-kill curves for hipA7 were set up almost identically to the *E. coli* experiments with small differences in the sampling times and antibiotic concentrations which were measured every 30 minutes for the first 2 hours followed by sampling every hour for another 3 hours, and 10X MIC for ciprofloxacin and ampicillin, respectively. Identical treatments for wild-type *E. coli* MG1655 were used as controls.

Stationary Phase Experiments

Cultures of *E. coli* and *S. aureus* were incubated at 37°C, for 48 hours. The cultures were divided into 5 mL aliquots in 6 well macro-titer plates. Super-MIC antibiotic treatments were added accordingly to each well at 50X MIC for *S. aureus* and *E. coli* save for the treatment of *E. coli* with the aminoglycosides and colistin, which were added at lower concentrations (see Figure 3A). The treated cultures were incubated at 37°C, for an additional 24 hours. Viable cell densities were estimated prior to the addition of antibiotic treatment and following the 24 hour incubation period, which we regarded as N(0) and N(24), respectively.

Persisters Experiments

Overnight cultures of *E. coli* hipA7 were diluted 1:100 into fresh LB and incubated for 2 hours at 37°C. The cultures were immediately treated with either 10X MIC of ciprofloxacin or 10X MIC of ampicillin and incubated under similar conditions for another 6 hours. The cultures were divided into 5 mL aliquots in 6 well micro-titer plates and treated with super-MIC concentrations of different antibiotics or phage. Viable cell and phage, when applicable, densities were estimated prior to and after the addition of the second antibiotic, N(0) and N(24), respectively.

Bacteriostatic (BS) Persisters Experiments

Overnight cultures of *E. coli* and *S. aureus* were diluted 1:100 into fresh broth and incubated for 2 hours at 37°C. The cultures were incubated with super-MIC concentrations of a bacteriostatic antibiotic for 24 hours at 37°C. The cultures were divided into 5 mL aliquots in 6 well macro-titer plates and treated with super-MIC concentrations of antibiotics, and in the case of *E. coli,* phage for an additional 24 hours at 37°C. Viable cell and phage densities were estimated immediately before and after the second antibiotic treatment, N(0) and N(24), respectively.

Results:

Pharmacodynamics in replicating populations of *E. coli* and *S. aureus*

To provide a baseline for this consideration of the susceptibility of non-growing bacteria to antibiotics, we open this report with the results of experiments on the pharmacodynamics of bacteriostatic and bactericidal antibiotics in replicating populations of *E. coli* and *S. aureus.* In Table 1, we list the minimum inhibitory concentrations (MICs) of the antibiotics used for *E. coli* MG1655 and *S. aureus* Newman estimated by two-fold serial dilution procedure [13]. In Figure 1 we present the results of experiments following the change in viable cell density of these bacteria exposed to these antibiotics, "time kill experiments".

Table 1 MICs of the bacteriostatic and bactericidal antibiotics used in this study in µg/ml.

Antibiotic	*E. coli* MIC	*S. aureus* MIC
Ampicillin (AMP)	4.7	4.7
Azithromycin (AZI)	19	N/A
Chloramphenicol (CAM)	4.7	13
Ciprofloxacin (CIP)	0.6	0.2
Colistin (COL)	1.1	N/A
Daptomycin (DAP)	N/A	1.6
Erythromicin (ERM)	N/A	0.6
Gentamicin (GEN)	9.3	0.8
Kanamycin (KAN)	4.7	2.3
Linezolid (LIN)	N/A	1.1
Meropenem (MER)	1.2	N/A
Oxacillin (OXA)	N/A	0.3
Rifampin (RIF)	9.4	0.002
Tetracyline (TET)	2.3	0.6
Tobramycin (TOB)	1.2	0.3
Vancomycin (VAN)	N/A	1.6

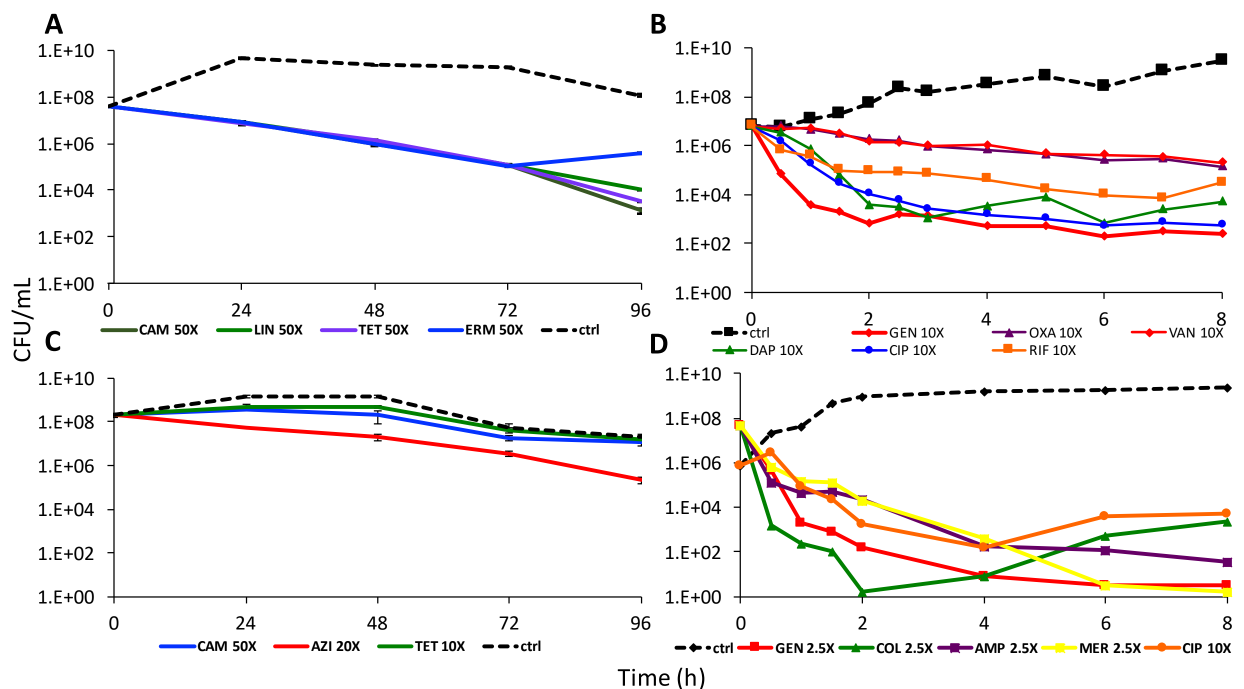

Figure 1—Time kill for exponentially growing bacteria. Change in viable cell density of exponentially growing broth (respectively LB and MHII) cultures *E. coli* and *S. aureus* exposed to super-MIC concentrations of bacteriostatic and bactericidal

antibiotics. The concentrations of the antibiotics used are noted as multiples of their respective MICs for these bacteria. In each figure, we include the change in the viable cell densities of the antibiotic-free controls, the black, broken lines. For the bacteriostatic antibiotics, the changes in viable cell density were estimated over 96 hours with samples taken every 24. A- *S. aureus* exposed to bacteriostatic antibiotics, B- *S. aureus* exposed to bactericidal antibiotics, C- *E. coli* exposed to bacteriostatic antibiotics. D- *E. coli* exposed to bactericidal antibiotics. Mean and standard error presented for A and C of three replicates per point.

The contrast between antibiotics designated bacteriostatic and bactericidal is very apparent. Over a period of four days, save for exposure to azithromycin, the viable cell density of *E. coli* confronted with bacteriostatic antibiotics declined less than an order of magnitude. For *S. aureus*, the viable cell density declined by 2 to 3 orders of magnitude, when exposed to the bacteriostatic antibiotics and less than 0.3 for the control.

While the bactericidal antibiotics varied the rate at which they killed *E. coli*, at the concentrations used, exposure to gentamicin and colistin, reduced the viable cell density by nearly 6 orders of magnitude within 2 hours. For all the drugs used, the rate of kill of *E. coli* declined with time with time. While there is the suggestion of the viable cell density leveling off and even increasing, save for colistin, the viable cell density was the below the detection limit, 10^2 cells per ml. For colistin the viable cell density at 24 hours was the same as that of the control and there was no evidence for an increase in MIC, resistance (Supplemental Figure S1A). For *S. aureus*, within 2 hours at the concentrations used gentamicin, daptomycin and ciprofloxacin reduced the viable cell density by 3 to 4 orders of magnitude in 3 hours, and rifampin by more than 2 orders of magnitude in that same amount of time. Oxacillin and vancomycin on the other hand, were substantially less bactericidal. For the more bactericidal antibiotics, the rate of kill declined substantially and there is little or no change in viable cell density between 3 and 8 hours; the phenomenon of persistence. In Supplemental Figure S1B, we present the viable cell densities at 24 hours, which ranged from 10^2, gentamicin and ciprofloxacin to 10^4 for oxacillin, vancomycin and daptomycin. For rifampin, the viable cell density at 24 hours was that of the control, which can be attributed to the ascent of resistant mutants.

The non-replicating and slowly dying populations considered in this study

(i) Stationary phase

For these experiments, we use cultures that have incubated under optimal growth conditions for 48 hours. To estimate the contribution of mortality in the absence of antibiotics, we estimate the ratio of the CFU estimate of the viable cells these cultures at 72 hours relative to that at 48, N(72)/N(48) for three cultures with three replicas for each sample. They are, respectively, $2.45 \times 10^{-1} \pm 0.65 \times 10^{-1}$ for *E. coli* and 3.27 ± 2.3 for *S. aureus*. To estimate the amount of unconsumed resource in these 48-hour stationary phase cultures, and thereby the capacity for additional growth we centrifuged and filtered (0.20 microns) four 48-hour cultures of these bacteria and added 10^5 cells from diluted fresh overnight and estimated the viable density at 24 hours for 3 samples of *E. coli* and *S. aureus*. Our results report that the supernatant can support, on average,

$4.8x10^6 \pm 7.8x10^5$ CFU/ml and $3.5x10^6 \pm 7.6x10^5$ CFU/ml for *E. coli* in LB and *S. aureus* in MHII, respectively.

ii) Persisters (phenotypically resistant and presumably non-growing bacteria surviving exposure to bactericidal antibiotics)

For *E. coli,* we exposed the growing cells to ciprofloxacin and ampicillin to generate phenotypic insusceptibility, while *S. aureus* persisters were only generated with ampicillin. As can be seen Figure 1 B for *E. coli*, even when concentration of ciprofloxacin and ampicillin were, respectively 10X MIC and 2.5X MIC, the density of surviving cells is low, too low to test for the susceptibility of these non-growing cells to other antibiotics. To address this issue, we restricted the *E. coli* experiments to a constructed strain, hipA7 (the Moyed mutant – [26] that produces 10^3 -10^4 times greater numbers of persisters due to an increase in the basal level of (p)ppGpp synthesis [27]. This is illustrated in Figure 2, where we compare the level of persisters for *E. coli* MG1655 and the hipA7 construct exposed to 10X MIC ciprofloxacin and ampicillin. The dynamics of persisters formation for *S. aureus* exposed to 25X MIC ampicillin can be seen in Figure 1D. To generate the *S. aureus* persisters, we exposed diluted 48-hour cultures of *S. aureus* 1/10 in fresh MHII broth and immediately add 25X MIC ciprofloxacin or ampicillin, what Balaban and colleagues call type I, or stationary phase, persisters [17]. These are produced during stationary phase and do not change in density during growth phase.

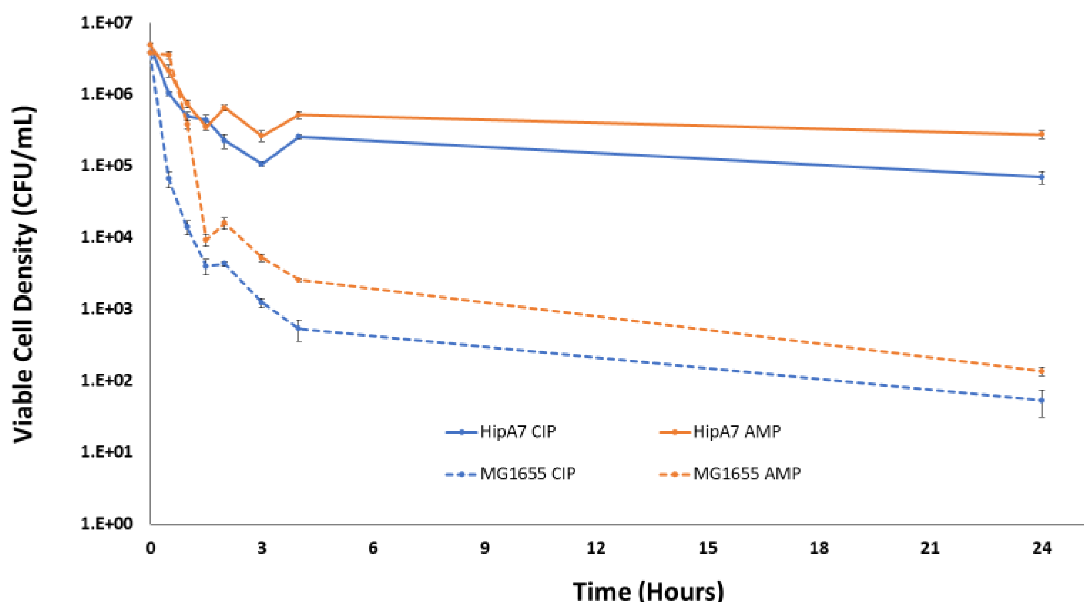

Figure 2—Dynamics of hipA7 persister formation. A single graph comparing persisters formed with MG1655 and a mutant hipA7 strain capable of forming ~10^4 fold more persisters when confronted with 10X MIC doses of CIP or AMP. *E. coli* K12 curves shown with dashed lines, whereas *E. coli* hipA7 curves are shown with solid

lines, with orange and blue representing either AMP or CIP treatment, respectively. All data points represent the average of three independent measurements.

iii) Bacteriostatic antibiotic-induced, BS-persisters

When exponentially growing populations of bacteria are treated with bacteriostatic antibiotics, the viable cell densities decline at a low rate, if at all. This can be seen in Figure 1A and 1C, respectively for *E. coli* and *S. aureus*. We are referring to these cells as persisters because like the bacteria surviving exposure to bactericidal antibiotics, they are not growing, and there is an abundance of resource present.

III. Susceptibility of non-replicating bacteria to killing by bactericidal antibiotics:

(i) Stationary phase

In Figure 3A and 3B we present the results of experiments with stationary phase *E. coli* and *S. aureus* exposed to high concentrations of antibiotics.

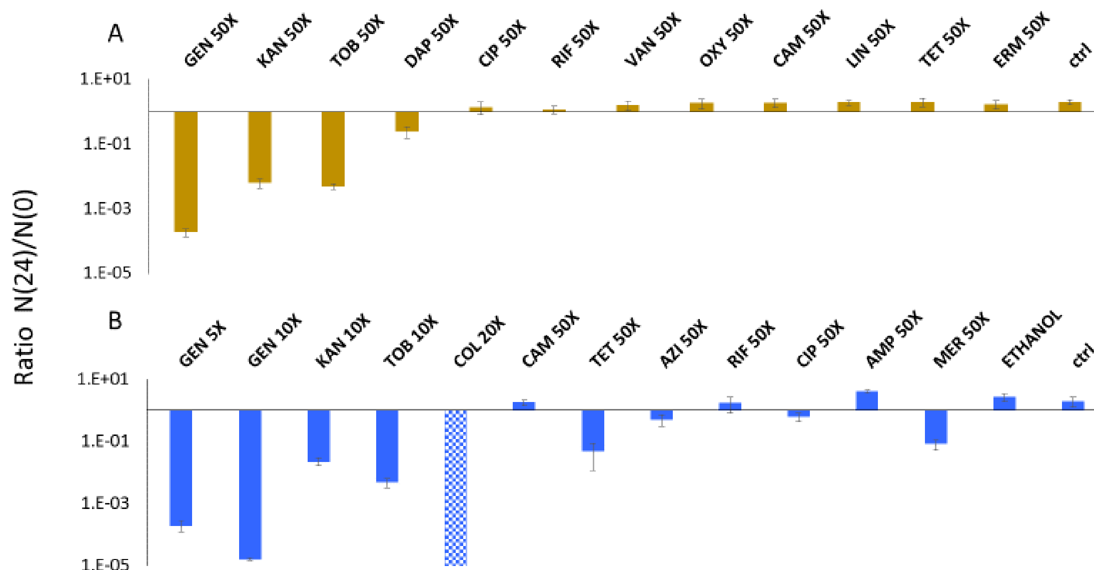

Figure 3—The effect of single dose antibiotic treatment on stationary phase cultures of *E. coli* and *S. aureus*. Stationary phase (~48-hour old) cultures of *S. aureus* (A) and *E. coli* (B) treated with the given antibiotic and antibiotic concentration for 24 hours. All cultures in A were treated with 50X the estimated MIC for each drug whereas the concentrations for each treatment are given in B. N(0) densities were considered the average of three samples after 48 hours of incubation and similar considerations were made for the estimation of N(24) after 24 hours of antibiotic exposure. Average viable N(0) densities were ~5.76E9 CFU/ml and ~4.08E8 CFU/ml for A and B, respectively. Hashed bars indicate that viable cell density measured at N(24) was below our detection limit of 10^2 CFU/mL. Mean and standard error of the N(24)/N(0) ratios of two independent experiments.

For stationary phase *E. coli*, the aminoglycosides, gentamicin, tobramycin and kanamycin, are effective in killing as is relatively high concentrations of colistin. Save possibly for high concentrations of meropenem and tetracycline, there is no evidence for the other antibiotics tested (ciprofloxacin, rifampin, azithromycin, ampicillin, chloramphenicol), killing stationary phase *E. coli*. For stationary phase *S. aureus*, only high concentrations of the aminoglycosides (gentamicin, tobramycin and kanamycin) and the cyclic peptide, daptomycin, are effective in reducing the viable density the exposed cells.

(ii) Persisters

In Figures 4 and 5, we follow the change in viable cell density of, respectively *E. coli* hipA7 and *S. aureus* Newman persisters following subsequent treatment with other antibiotics. For *E. coli*, the persisters were generated by exposure to ciprofloxacin and ampicillin at, respective 10X. *S. aureus* persisters, on the other hand, were generated using 25X ampicillin only. For *E. coli* hipA7, we use a protocol similar Keren et al. in [28], exponentially growing bacteria were exposed to ciprofloxacin and ampicillin for 4 hours, at which time they were treated with the noted antibiotic for an additional 24 hours. For the *S. aureus*, the overnight cultures were diluted 1/10 in fresh MHII and the ampicillin was added immediately. These antibiotic-treated cultures were maintained for 24 hours, at which time the second antibiotic was added.

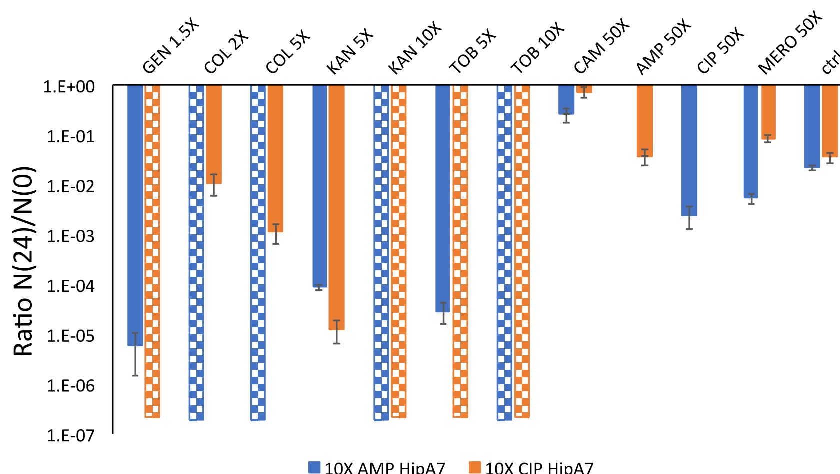

Figure 4—Susceptibility of HipA7 persisters to subsequent treatment with antibiotics. Ratio of the N(24)/N(0) of HipA7 *E. coli* generated by four hours of exposure to 10X MIC CIP or 10X MIC AMP N(0) followed by subsequent treatment with other antibiotics for 24 hours (N24). Hashed bars indicate that viable cell density measured at N(24) was below our detection limit of 10^2 CFU/mL. Mean and standard error of the N(24)/N(0) ratios 6 measurements from two independent experiments.

Low, but still super-MIC concentrations of the aminoglycosides and colistin kill the *E. coli* hipA7 persisters generated by exposure to both ciprofloxacin and ampicillin. Relative to the control, which was not subsequently treated with bactericidal antibiotics, the other drugs, chloramphenicol, ampicillin, ciprofloxacin and meropenem were ineffective at killing the hipA7 *E. coli* persisters. The ampicillin-generated hipA7 persisters were more susceptible to killing by colistin than the ciprofloxacin-generated persisters.

To explore the susceptibility of *S. aureus* persisters to killing by subsequent treatment with bactericidal antibiotics, using a protocol similar to that of Joers and colleagues [29] used to generate *E. coli* persisters. Overnight stationary phase MHII cultures of *S. aureus* Newman 1/10 in fresh MHII and 25X MIC of ampicillin was added immediately. After 24 hours, the viable cell density of these cultures, N(0) was approximately 10^{-3} that of the initial density. After 24 hours of exposure to the secondary antibiotics, the viable cell densities wee estimated. The results of this experiment are presented in Figure 5.

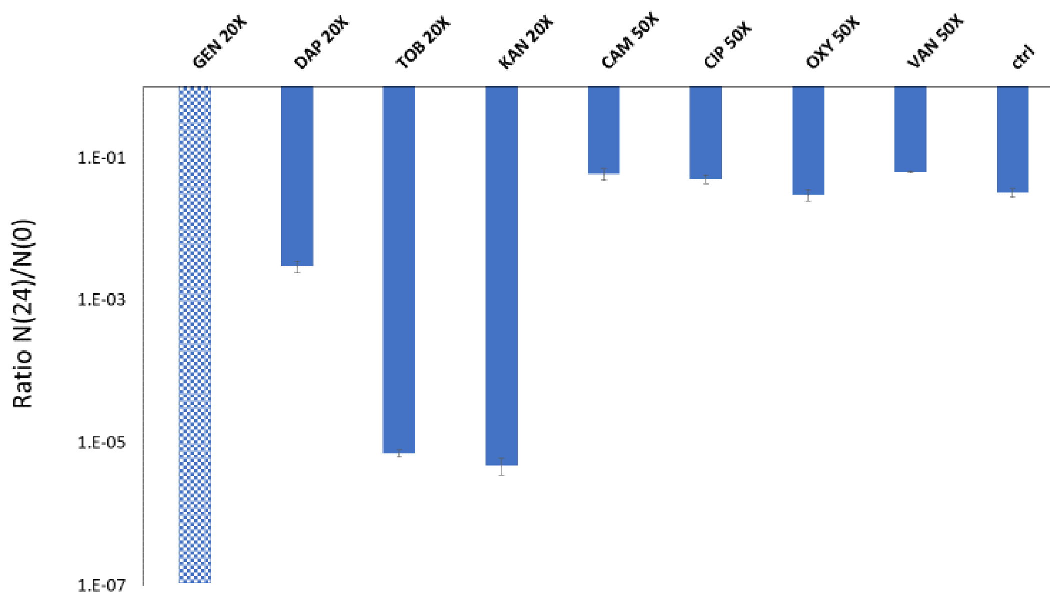

Figure 5—Susceptibility of *S. aureus* persisters generated by 24 hours of exposure to 25X MIC ampicillin to killing by bactericidal antibiotics, N(0). Ratio of the viable cell after 24 hours of treatment with bactericidal antibiotics N(24)/N(0). Hashed bars indicate that viable cell density measured at N(24) was below our detection limit of 10^2 CFU/mL. Mean and standard error of 6 ratios from two experiments.

At 20X MIC the aminoglycosides substantially reduce the viable cell density of these persisters cultures by 5 orders of magnitude for 20X MIC tobramycin and kanamycin and even more or 20X MIC gentamicin, where the viable cell density after 24 hours of exposure was below the detection limit. Daptomycin at 20X MIC was also effective in killing these persisters, albeit to an extent less than the aminoglycosides. Even at 50X MIC, the other antibiotics tested had no significant effect in reducing viable cell densities of these ampicillin-generated *S. aureus* persisters.

(iii) Bacteriostatic (BS) persisters
The bacteriostatic persisters (BS-persisters) were generated by exposing exponentially growing *E. coli* and *S. aureus* to the bacteriostatic drugs for 24 hours, at which time the second antibiotic was added, N0). The cultures were maintained for another 24 hours and the viable cell densities estimated N(24). As before, our measure of the efficacy of the second antibiotic for killing the BS-persisters is the N(24)/N(0) ratio. The results of these experiments are presented in Figure 6.

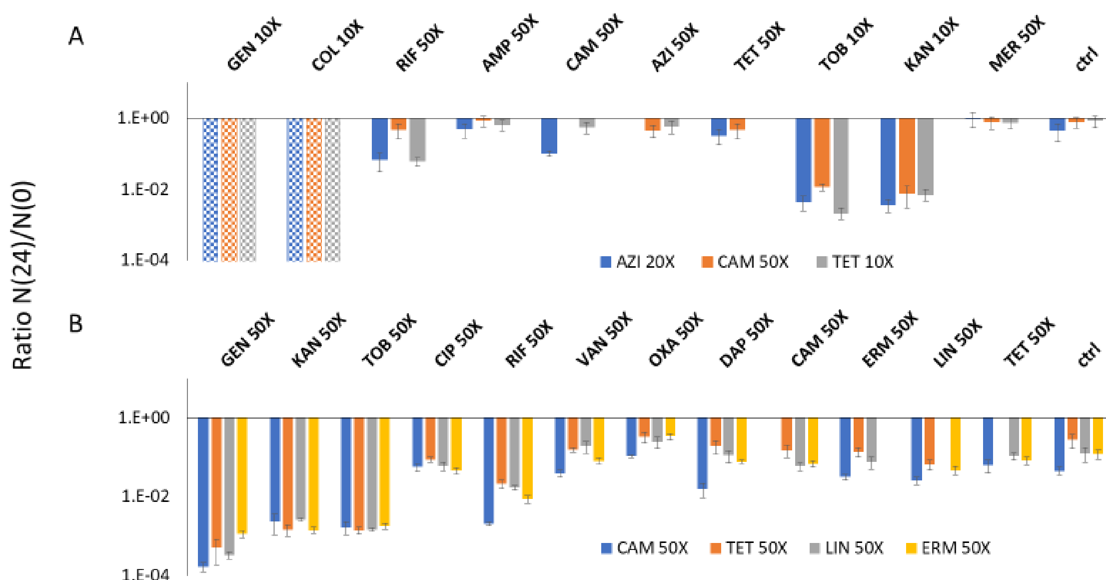

Figure 6—Effect of antibiotic treatment on bacteriostatic (BS-) persisters. Mean and standard error of the N(24)/N(0) ratios for (A) *E. coli* and (B) *S. aureus*. The concentrations of the antibiotics as multiples of MICs used to generate the BS persisters are noted in the figure legend. For *E. coli*, the concentration antibiotics used for subsequent treatment varied among the antibiotics and noted in the concentrations also noted. Hashed bars indicate that viable cell density measured at N(24) was below our detection limit of 10^2 CFU/mL. Mean and standard error of the N(24)/N(0) ratios of 6 measurements from two experiments. The densities of the cultures used to generate the persisters were between $6x10^6$ and $7x10^7$ CFU/mL for *E. coli* and $3x10^6$ and $1x10^8$ CFU/mL for *S. aureus*.

The *E. coli* BS persisters surviving exposure to azithromycin, chloramphenicol and tetracycline are readily killed by the aminoglycosides and colistin and marginally so by high concentrations of rifampin. The *S. aureus* persisters surviving exposure to chloramphenicol, tetracycline, erythromycin and linezolid are killed by the aminoglycosides and somewhat by high concentrations rifampin. The extent of the mortality of the *S. aureus* BS-persisters super-treated with aminoglycosides is

substantially less than the corresponding *E. coli* BS-persisters, Moreover, even in the absences of super-treatment, the *S. aureus* are killed at higher rate than the *E. coli* due to the exposure of these ribosome-targeting "bacteriostatic" antibiotics.

Discussion

The results of this study are inconsistent with what seems to be the common conviction [23-25] that stationary phase populations of bacteria are refractory to killing by antibiotics. Relatively ancient drugs belonging, in particular, the aminoglycosides kill stationary phase populations of *E. coli* and *S aureus.* Moreover, the peptide antimicrobials, colistin and daptomycin respectively, kill 48-hour *E. coli* and *S. aureus* at stationary phase. This observation is not new, it has been known for some time that the aminoglycosides and the cyclic peptides, daptomycin and colistin are capable of killing stationary phase bacteria [30-33].

Although there have been many studies of persistence, relatively little is known about the susceptibility of these non-replicating bacteria to antibiotics other than those employed to generate them. One exception to this is a study by Allison and colleagues, [34] that demonstrates that by adding metabolites, *E. coli* and *S. aureus* persisters in the form of biofilms become susceptible to killing by aminoglycosides. Our results with the ampicillin-generated planktonic *S. aureus* persisters are fully consistent with these results. In our experiment, however, the metabolites were added via the MHII nutrient broth into which the stationary culture was added.

We elected not to perform parallel experiments with "natural" *E. coli* persisters, because the densities of viable cells following exposure to ampicillin and ciprofloxacin were too low to properly explore the effects of subsequent treatment with bactericidal antibiotics. If, however, the Moyed mutant hipA7 persisters are similar to "natural" *E. coli* persisters, we would have to conclude that *E. coli* persisters are sensitive to killing by even low concentrations (1.5X MIC gentamicin) as well as other aminoglycosides and colistin. These results suggest that hipA7 persisters generated by exposure to ampicillin differ in the susceptibility to secondary treatment with bactericidal antibiotics. For example, ampicillin generated hipA7 persisters are far more sensitive to colistin than those generated by exposure to ciprofloxacin. We postulate that an explanation for this is that the outer membranes of *E. coli* surviving exposure to ampicillin are modified, as suggested by [35] and thereby more susceptible to the method of killing by this detergent-like peptide [36].

To our knowledge, this study is the first to explore the sensitivity of subsequent treatment by bactericidal antibiotics by bacteria not replicating because exposure to bacteriostatic antibiotics, as we call them, BS-persisters. As was the case for the hipA7 *E. coli* persisters, the aminoglycosides and the peptide colistin are highly effective at killing non-growing cells generated by exposing growing populations of *E. coli* to the ribosome-targeting bacteriostatic antibiotics azithromycin, chloramphenicol and tetracycline. While our results suggest that rifampin kill these BS persisters, relative to that observed for the aminoglycosides, even at high concentrations, the killing effect is

small. The other drugs tested were clearly ineffective. Similar results obtain for the *S. aureus* BS persisters, although even at 50X MIC the extent of killing by the aminoglycosides was small.

Relationship between BS- and "natural" persisters

From phenotypic perspective, BS-persisters and hipA7 *E. coli* and natural *S. aureus* persisters are similar: (i) they are not replicating, (ii) are metabolically inactive [37-40] and (iii) are in an environment where nutrients do not limit replication. We interpret the results of this study to suggest that BS- and natural persisters are also similar in their susceptibility to killing by bactericidal antibiotics and particularly the aminoglycosides and colistin.

To further explore the phenotypic similarity of BS- and hipA7 *E. coli* persisters, we tested their relative capacity to support the replication of bacteriophage and the extent to which they are killed by these viruses. The results of these experiments with three lytic phages, λ^{VIR}, T3 and T4, for hipA7 and BS-persisters are presented in Figures 7 and 8, respectively.

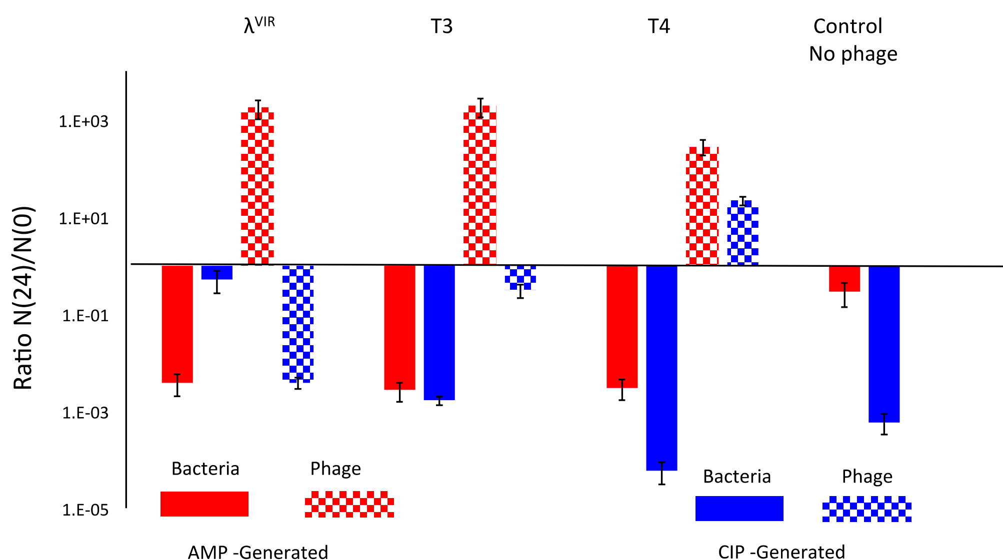

Figure 7. Effect of phage on *E. coli* hipA7 persisters. The ratio of the viable cell and free phage density after 24 hours of phage exposure, N(24) and the initial viable cell and free phage density, N(0) for hipA7 persisters generated with 10X MIC ampicillin and ciprofloxacin. Hashed bars indicate ratios for phage. Mean and standard error of 6-8 ratios from two experiments separate experiments.

Relative to the phage-free hipA7 controls on the right, ampicillin-generated hipA7 persisters are killed by all three phage and support the replication of those phage. There is little or no killing of the ciprofloxacin-generated hipA7 persisters by λ^{VIR} and T3 and some killing by T4 as well as the replication of those phage. The phage λ^{VIR} is the only phage that is clearly capable of killing BS persisters generated by exposure to all three

bacteriostatic antibiotics. While there is a suggestion that T4 may be replicating on the Tetracycline-generated BS- persisters, the extent of phage replication is, a best low For all the other situations exposure to these BS persisters resulted in a substantial decline in the density of phage. We postulate that the phage are adsorbing to the BS-persisters and injecting their DNA, but are incapable of replicating in these metabolically inactive cells and only λ^{VIR} is capable of killing these bacteria; a hypothesis that requires testing. What is clear, from these phage data, with respect to phage replication hip7A and BS-persisters are different.

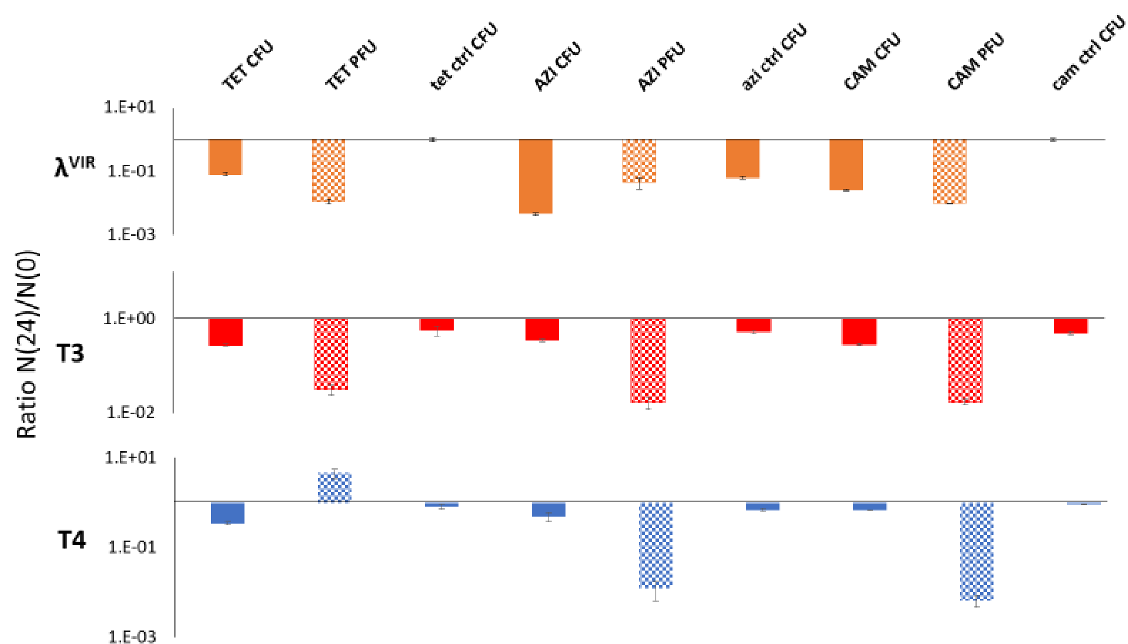

Figure 8 Effect of phages λ^{VIR}, T3 and T4 on *E. coli* bacteriostatic persisters. The ratio of the viable cell and free phage density after 24 hours of phage exposure, N(24) and the initial viable cell and free phage density, N(0) for bacteriostatic persisters generated with 10X MIC tetracycline, 20X MIC azithromycin and 50X MIC chloramphenicol. Phage free controls (ctrl) are included. Hashed bars indicate ratios for phage. Mean and standard error of 9 (bacteria) and 12 (phage) ratios from three independent experiments.

Potential clinical implications

Within the last decade, much emphasis has been placed on drug discovery as a means of combating persisters, particularly in the form of biofilms. A prime example of the sort is work by Kim Lewis and colleagues [41] which involves activation of a protease, ClpXP, and in turn inevitably forces the bacteria tested to proteolyze themselves. Despite growing efforts in the field, the results presented here suggest that existing antimicrobials ought to suffice. A main concern is that the antibiotics with this virtue are

among the more toxic drugs – aminoglycosides and the peptides, colistin and daptomycin [42-44]. It should be noted, however, relatively low, and possible non-toxic concentrations of the aminoglycosides and the peptide, colistin can kill *E. coli* BS- and hipA7 persisters. How important these planktonic persisters are clinically is, in at least our interpretation, not all that clear. At least theoretically for immune competent patients, most of the control of infections can be attributed to the innate immune system, and if persisters are as susceptible to phagocytosis and other elements of the innate immune system, they would play little or no little role in reducing the efficacy of antibiotic therapy [45]. Consistent with this yet-to-be tested hypothesis is observation that for immune competent patients, bacteriostatic drugs are as effective for treatment as highly bactericidal agents [46, 47].

Supplemental Information

Phage Used	Source
λ^{VIR}	Sylvain Moineau
T3	
T4	
T5	James Bull
T7	

Table S1 Coliphage used in this study.

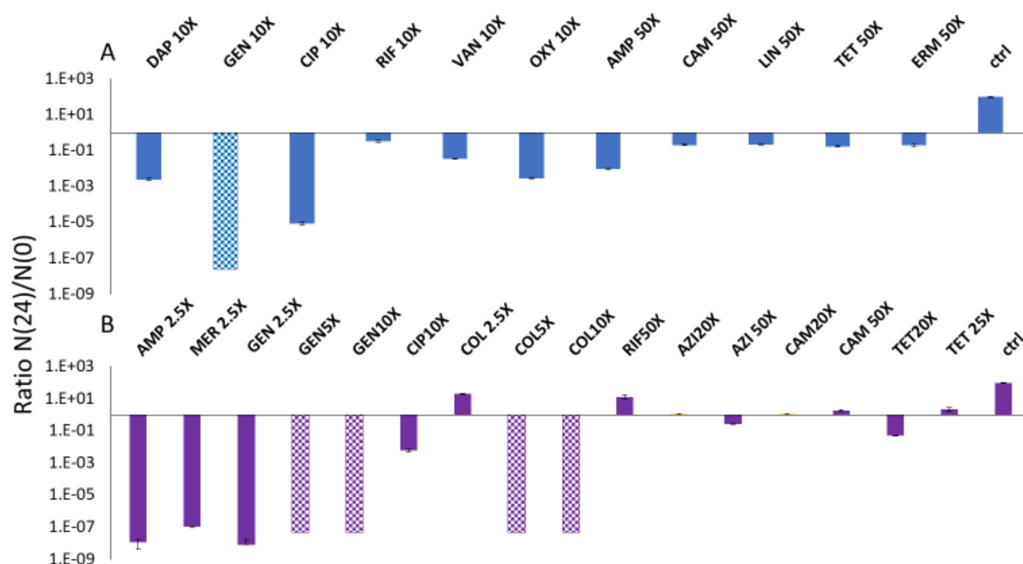

Figure S1 Effect of antibiotic treatment on exponentially growing populations. The ratio of the viable cell density after 24 hours of antibiotic exposure, N(24) and the initial viable cell density, N(0). (A) Growing populations of *S. aureus* treated with super-MIC concentrations of the bactericidal and bacteriostatic antibiotics. Hashed bars indicate that

the final viable cell density, N(24) was less than 10^2 CFU/mL for experiments with antibiotic concentrations exceeding 2.5X the MIC of the given antibiotic. (B) Growing populations of *E. coli* treated with bactericidal and bacteriostatic antibiotics. Mean and standard error for 6 ratios from two independent experiments.

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