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Joint antibiotic and phage therapy: addressing the limitations of a seemingly ideal phage for treating *Staphylococcus aureus* infections

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Abstract

In response to increasing frequencies of antibiotic-resistant pathogens, there has been a resurrection of interest in the use of bacteriophage to treat bacterial infections: phage therapy. Here we explore the potential of a seemingly ideal phage, PYO^{Sa}, for combination phage and antibiotic treatment of *Staphylococcus aureus* infections. (i) This K-like phage has a broad host range; all 83 clinical isolates of *S.aureus* tested were susceptible to PYO^{Sa}. (ii) Because the mode of action of PYO^{Sa} *S. aureus* is unlikely to generate surface mutants resistant to PYO^{Sa}; none were observed in the 13 clinical isolates tested. (iii) PYO^{Sa} kills *S. aureus* at high rates. On the downside, the results of our experiments and tests of the joint action of PYO^{Sa} and antibiotics raise issues that must be addressed before PYO^{Sa} is employed clinically. Despite the maintenance of the phage, due to the ascent of potentially pathogenic small colony variants, PYO^{Sa} does not clear populations of *S. aureus*; following an initial demise the bacterial populations return to densities similar to that of phage-free controls. Bacteriostatic antibiotics prevent PYO^{Sa} from replicating on *S. aureus and bactericidal* antibiotics are antagonistic to the action of PYO^{Sa}. Using a combination of mathematical modeling and in vitro experiments, we postulate and present evidence for a mechanism to account for the demise–resurrection dynamics of PYO^{Sa} and *S. aureus*. Critically for phage therapy, our experimental results suggest that treatment with PYO^{Sa} followed by bactericidal antibiotics can clear populations of *S. aureus* more effectively than the antibiotics alone

Keywords: Phage therapy; population dynamics; *Staphylococcus aureus*

Significance Statement

The increasing frequency of multiply antibiotic-resistant pathogens has fostered a quest for alternative means to treat bacterial infections. Prominent in this quest is a therapy that predates antibiotics: bacteriophage. This study explores the potential of a phage, PYO^{Sa}, for treating *Staphylococcus aureus* infections in combination with antibiotics. On first consideration, this phage, isolated from a commercial therapeutic cocktail, seems ideal for this purpose. The results of this population dynamic study identify a limitation and potential liability of using PYO^{Sa} for therapy. Due to the production of potentially pathogenic small colony variants, PYO^{Sa} alone cannot eliminate *S. aureus* populations. We demonstrate that by following the administration of PYO^{Sa} with bactericidal antibiotics, this limitation and potential liability can be addressed.

Introduction

Driven by well-warranted concerns about the increasing frequencies of infections with antibiotic-resistant pathogens, there has been a resurrection of interest in, research on, and clinical trials with a therapy that predates antibiotics by more than 15 years: bacteriophage [1-4]. One direction phage therapy research has taken is to engineer lytic (virulent) phages with properties that are anticipated to maximize their efficacy for treating bacterial infections in mammals [5-7]. Primary among these properties are (i) a broad host range for the target bacterial species, (ii) mechanisms that prevent the generation of envelope or other kinds of resistance in the target bacteria [8], (iii) the capacity to thwart the innate and adaptive immune systems of bacteria, respectively restriction-modification and CRISPR-Cas [7, 9, 10], (iv) the ability to survive, kill, and replicate on pathogenic bacteria colonizing or infecting mammalian hosts [11, 12] and (v) little or no negative effects on the treated host [8].

To these five desired properties for therapeutic bacteriophage, we add a sixth: synergy with antibiotics. Phage-only treatment may be reasonable for compassionate therapy where the bacteria responsible for the infection are resistant to all available antibiotics [13-15]. But from a practical perspective, for phage to become a widely employed mechanism for treating bacterial infections, they would have to be effective in combination with antibiotics. It would be unethical and unacceptable to clinicians and regulatory agencies to use phage independently for infections that can be effectively treated with existing antibiotics.

Although not specifically engineered for these properties, there is a *Staphylococcal* phage isolated from a therapeutic phage collection from the Eliava Institute in Tbilisi; we call PYO^{Sa} that on first consideration appears to have all six of the properties required to be an effective agent for therapy. (i) PYO^{Sa} is likely to have a broad host range for *S. aureus.* The receptor of this K-like Myoviridae is N-acetylglucosamine in the wall-teichoic acid backbone of *Staphylococcus aureus* and is shared among most [16], if not all *S. aureus;* thereby suggesting PYO^{Sa} should be able to adsorb to and potentially replicate on and kill a vast number of clinical isolates of *S. aureus*. (ii) *S. aureus* may not be able to generate classical, surface modification mutants resistant to PYO^{Sa}. Since the structure of the receptor of PYO^{Sa} is critical to the viability, replication, and virulence of these bacteria, the modifications in this receptor [17] may not be consistent with the viability or pathogenicity of *S. aureus* [18]. (iii) The replication of PYO^{Sa} is unlikely to be prevented by restriction-modification (RM) or CRISPR-Cas. Despite a genome size of 127 KB, the PYO ^{Sa} phage has no GATC restriction sites for the *S. aureus* restriction enzyme Sau3A1 and only one restriction site, GGNCC, for the Sau961 restriction endonuclease [19, 20]. There is no evidence for a functional CRISPR-Cas system in *S. aureus* or, to our knowledge, other mechanisms by which *S. aureus* may prevent the replication of this phage [21]. (iv) There is evidence that PYO^{Sa}–like phages can replicate in mammals. Early treatment with a phage with a different name but the same properties as PYO^{Sa}, $Statu^{v}$, prevented mortality in otherwise lethal peritoneal infections of *S. aureus* in mice [22].

A PYO-like phage has also been successfully used therapeutically in humans [23]. (v) No deleterious effects of a PYO-like phage were observed in recent placebo-controlled trials with volunteers asymptotically colonized by *S. aureus* [19]. (vi) Finally, there is evidence to suggest synergy with antibiotics. *In vitro,* PYO^{Sa} increased the efficacy of low concentrations of antibiotics for the treatment of biofilm populations of *S. aureus* [24].

With *in vitro* parameter estimation, population and evolutionary dynamic studies, and experiments with PYO^{Sa} and *Staphylococcus aureus* Newman in combination with three different bacteriostatic and six different bactericidal antibiotics, we explore just how well PYO^{Sa} fits the above criteria list for combination antibiotic and phage therapy. Our results suggest that PYO^{Sa} scores well on most of these tests but does not get an "A". As a consequence of selection for potentially pathogenic small colony variants, SCVs, by itself PYO^{Sa} does not clear *S. aureus* infections; after an initial demise when confronted with this phage, although the phage continue to be present, the densities of the bacteria return to levels similar to that in the absence of this virus. The ribosome targeting bacteriostatic antibiotics tested to prevent the replication of PYO^{Sa} and, when employed simultaneously with bactericidal antibiotics, the efficacy of PYO^{Sa} for killing *S. aureus* is less than that when the phage is used alone. More positively and most importantly, from a therapeutic perspective, we demonstrate that in experimental populations if treatment with PYO^{Sa} is followed by the administration of bactericidal antibiotics*, S. aureus* cultures with this phage are cleared more effectively than with these antibiotics alone.

Results

(i) <u>PYO^{Sa} has a broad host range for *Staphylococcus aureus*:</u> We use two assays to determine the host range of PYO^{Sa}: (i) the production of zones of inhibition in soft agar lawns and (ii) changes in the optical density of exponentially growing liquid cultures of *S. aureus* mixed with this phage. By both criteria *Staphylococcus aureus* Newman and 12 clinical isolates of methicillin-sensitive *S. aureus* from the Network on Antimicrobial Resistance (NARSA) collection [25] were all sensitive to PYO^{Sa} and appeared to be unable to generate classically resistant mutants. Additional evidence for a broad host range of PYO^{Sa} comes from a survey of 71 clinical isolates of *S. aureus,* including 54 MRSA strains and phylogenetically similar species performed by LabCorp™ (Supplemental Table 1).

(ii) <u>*S. aureus* appear to be unable to generate classical, surface modification, mutants resistant to PYO^{Sa}:</u> Evidence for this comes from experiments with *S. aureus* Newman and 12 clinical isolates from the NARSA collection [26]. Single colonies of each strain were grown in the presence of $\sim 10^6$ phage particles per ml, and after 24 hours of exposure to the phage in liquid culture, the optical densities of exponentially growing *S. aureus* were no greater than that of the media without the bacteria, and by plating, we were unable to detect colonies from these cultures.

(iii) Small colony variants and the maintenance of the bacteria and phage:
With the bacterial growth and phage infection parameters estimated for *S. aureus* Newman and PYO^{Sa}, a complete clearance of the bacteria in these experiments after less than 24 hours of exposure to PYO is anticipated from a simple mass action model of the population dynamics of bacteria and phage [27]. To determine whether this is the case empirically, we divided a culture with ~ 10^4 *S. aureus* Newman into 28 tubes. We let these cultures replicate for 2 hours, for an average density of 3 x 10^7 and then added ~ 6 x 10^8 PYO^{Sa}. At 24 hours, all 28 cultures were clear, and no bacterial colonies were found on the LB plate samplings of these cultures. However, by day seven, 26 of these 28 independent cultures were turbid or somewhat turbid. These turbid cultures all had phage at densities in excess of $5x10^6$. Upon plating these 7-day cultures, colonies were detected with two distinct phenotypes: colonies similar to those produced by the ancestral *S. aureus* Newman and much smaller colonies, small colony variants (SCVs) (Figure 1A).

To elucidate why PYO^{Sa} does not kill all of the *S. aureus* Newman in these liquid cultures, we prepared three independent 10 ml cultures with ~ 10^8 *S. aureus* Newman and ~10^7 PYO^{Sa} and serially passaged these cultures for five days, transferring 1/100 of these cultures to fresh media each day. The results of these serial transfer experiments are presented in Figure 1B. As anticipated from the experiment with the 28 tube cultures, at 24 hours, all the flasks were clear. However, we expect the densities of bacteria in these cultures estimated by plating, ~10^3 per ml, is an underestimate of the real density due to killing by the phage on the plates. Additional support for the hypothesis that the densities of *S. aureus* in these liquid cultures is substantially greater than the CFU estimate is that the density of the PYO^{Sa} phage did not decline at each transfer, and therefore, the phage must have been replicating. The latter can be seen with a simple model [28] with the phage infection parameters estimated for PYO^{Sa} and *S. aureus* Newman. To maintain a population in a culture diluted by 100-fold the phage density must increase by at least 100-fold at each transfer and for this to occur, the product of the adsorption rated constant, δ, the burst size β, and the density of sensitive bacteria, N, has to exceed 100. With the estimated values, δ=4.8x10^{-7} and β=80, the density of bacteria in these cultures would have to be at least 2.6 X 10^6 for the phage not to be diluted out.

Most intriguingly, in all three independent serial transfer populations with PYO^{Sa}, the CFU estimates of the density of *S. aureus* Newman started to increase after the 2nd transfer. By the 4th transfer, the density of cells in these cultures was similar to that of the phage-free controls, and the phage continued to be maintained, presumably because of the maintenance of a susceptible population. The bacteria on sampling plates included those with colonies like those of wild type *S. aureus* Newman but also SCVs. By the spot test criterion, the cultures derived from the wild type colonies taken at the end of the 5th transfer were susceptible to the PYO^{Sa} used to start these experiments as well as the co-existing PYO^{Sa}. On the other hand, by the spot test criterion the SCVs isolated from these cultures were resistant to PYO^{Sa}. The SCVs tested grow at lower rates than the wild type colony *S. aureus* Newman from whence

they are derived, respectively 2.02±0.28 and 0.75±0.27 per cell/per hour. See the Supplemental Material II for a consideration of the between-experiment variation in these serial transfer population dynamics.

As can be seen in Figure 1C, the liquid serial transfer cultures generated from SCVs are unable to support the phage, and SCVs maintain lower densities than the normal colony, phage-free controls. How then are the phage maintained in Figure 1B? One possibility is that the population is polymorphic and includes sensitive cells capable of supporting the phage as well as SCVs. Consistent with this hypothesis are the results of the experiment presented in Figure 1D. When wild type S. aureus Newman are mixed in equal frequency with SCVs, the phage are maintained and the total population density is similar to that in phage-free controls. Another possibility is that by either a genetic or phenotypic mechanism, the normal colony *S. aureus* isolated after the density of bacteria recovered in the serial transfer experiment in Figure 1B evolved to be better able to survive encounters with PYO than the ancestral *S. aureus?* To test this hypothesis, we repeated the serial transfer PYO^{Sa}-*S. aureus* Newman experiment with bacteria isolated from the final transfers of the three independent experiments in Figure 1E. The results of these experiments are consistent with this reduced susceptibility hypothesis. The initial decline in the bacterial density with the presumptive "evolved" *S. aureus* is less than that of the ancestral *S. aureus* Newman (Figure 1B). Moreover, within a single transfer, the bacterial density returned to that of the phage-free controls and the phage declined, albeit at a rate less than that anticipated if they were not replicating at all, in two of the three cultures. See the Supplemental Material II for a consideration of the between experiment variation in the population dynamics observed in Figures 1B and 1E.

Based on the preceding results and interpretations, we postulate that the bacteria are of three states, wild type, W, evolved, E, and small colony variants, S. In accordance with this hypothesis, cultures derived from the wild type, *S. aureus* Newman, the evolved, E, and SCV, S populations would respond differently when exposed to PYO^{Sa}. To test this hypothesis, we co-cultured high densities of each state of the bacteria with PYO^{Sa} and determined the relative change in densities of both bacteria and phage, respectively $10^8$and 10^7 cells and particles per ml, after 24 hours (Figure 1F). Each of the three states exhibited a unique dynamic. The density of the wild type *S aureus* Newman exposed to the phage declined to an extent greater than that of the evolved, and the density of the SCVs exposed to the phage increased. While the density of the phage increased with a wild type host, for both the evolved and SCV isolates, the phage density declined.

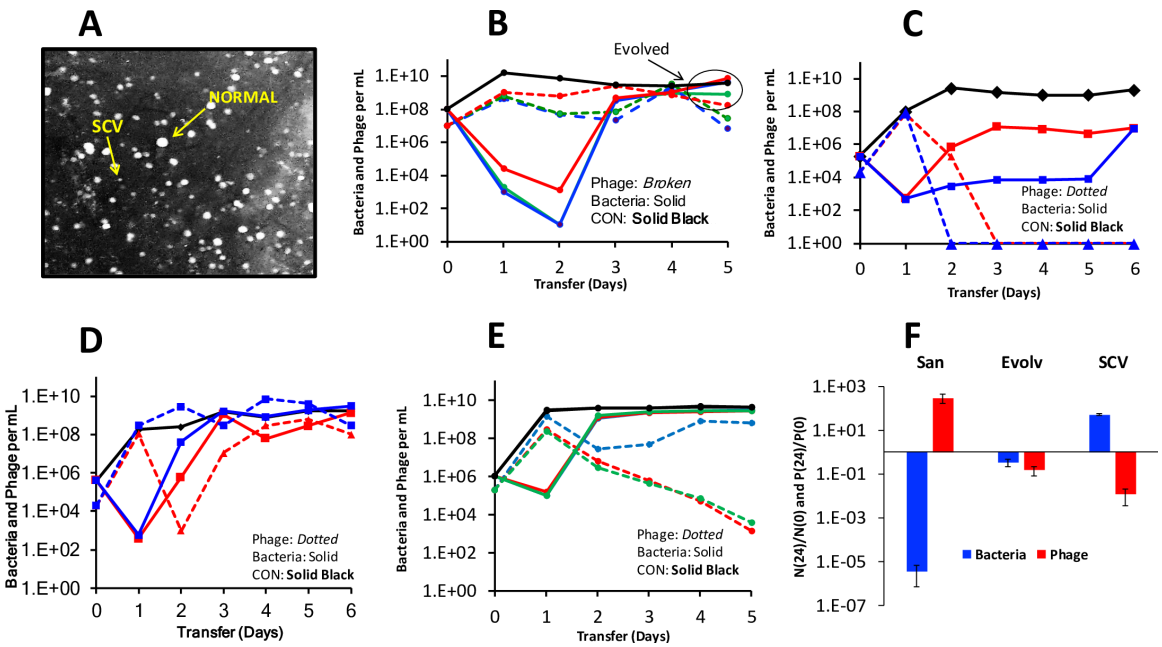

Figure 1 – Small colony variants and the population dynamics of PYO^{Sa} and *S. aureus* Newman in serial transfer culture. **A-** Normal and small colony variants visualized. **B-** Changes in densities of *S. aureus* Newman and PYO^{Sa} in three independent 1/100 serial transfer experiments. **C-** Changes in densities of *S. aureus* Newman and PYO^{Sa} in serial transfer cultures initiated two small colony variants isolated from the 5th transfer of the cultures with PYO and *S. aureus* Newman in Figure 1B.. **D-** Changes in densities of *S. aureus* Newman and PYO^{Sa} in serial transfer cultures initiated with PYO^{Sa} and equal densities of cultures derived from SCV colonies and the ancestral *S. aureus* Newman. **E-** Changes in densities of *S. aureus* Newman and PYO^{Sa} in serial transfer cultures initiated with the evolved normal colony variants isolated from the 5th transfer of the cultures with PYO and *S. aureus* Newman in Figure 1B. **F-** Changes in the ratio of bacteria and phage at time 0 and 24 hours. Wild type *S. aureus* Newman (WT), Evolved (EVOL), and small colony variants (SCV). With blue columns representing changes in bacteria and red the changes in phage. Three independent replicas.

iii) <u>A hypothesis</u>: One hypothesis for the dynamic presented in Figure 1B that is consistent with all the observations is that evolved *S. aureus* Newman, E, continually generate SCVs, S, in the presence of phage. At a relatively high rate, these SCVs generate sensitive bacteria, E. In this interpretation, the phage are maintained by a mechanism first suggested by Max Delbruck [29], the continuous reversion from the "resistant" SCVs to the susceptible evolved variants is what Chaudhry and collaborators [30] call 'leaky resistance'. To illustrate how this mechanism can account for the observed dynamics, we use the heuristic model depicted in Figure 2A, for the equations and more details, see Supplemental Information Section III. In this model, there are three populations of bacteria, wild type, W, evolved bacteria, E, and small colony variants, S, and one population of phage V, where W, E, S and V are densities as well as the designations of these populations. The phage adsorb to these bacteria with rate constants, δ_W, δ_E, and δ_S, and have burst sizes, β_W, β_E and β_S, for the

W, EVOL, and SCV, respectively. At rates μ_{EW}, μ_{WE},, μES and μSE per cell per hour, the bacteria change states, respectively, E→W, W→E, E→S, and S→E.

A numerical solution to this model, a simulated serial transfer experiment, is depicted in Figure 2B for a population of 10^7 sensitive cells (S) per ml mixed with 10^6 phage (V) per ml and ten evolved (E) and SCVs (SCV) cells per ml. As observed in Figure 1B, during the first transfer the total density of the bacterial population declines while that of the phage increases so that by the end of the second transfer, the bacterial population recovers but is now dominated by SCVs. The phage continue to be maintained as do minority populations of evolved bacteria and sensitive cells. The density of the minority populations of bacteria would be too low to be seen in the experiments, like that depicted in Figure 1B. Contrary to the prediction of this model, however, is that in the experiments, the dominant population of bacteria is not composed solely of SCVs, is mixed with both SCVs and evolved at higher densities.

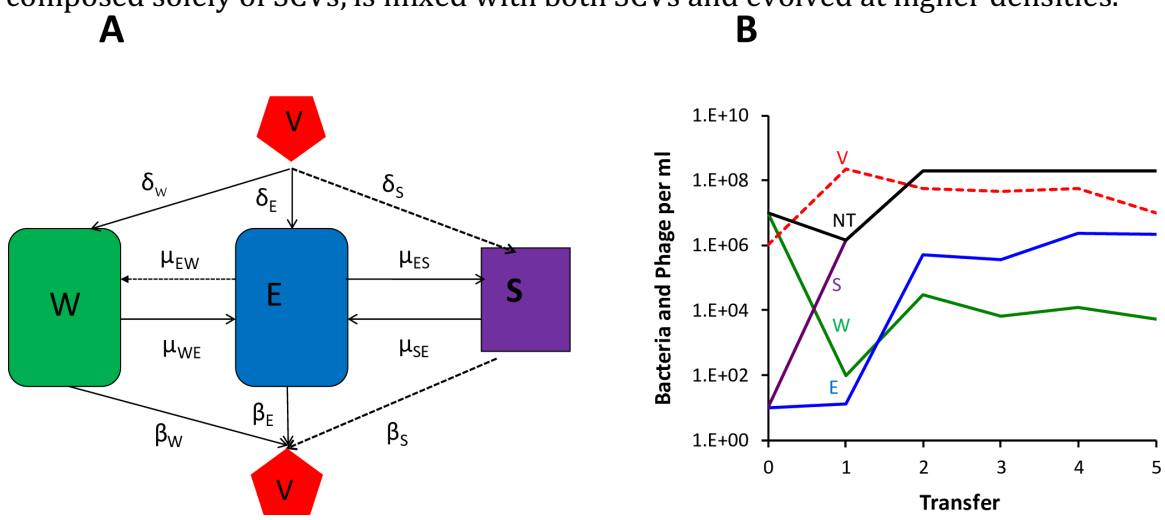

Figure 2 – A population dynamic mechanism to account for observed changes in the densities of bacteria and phage in Figure 1B. **A-** A diagram of the leaky resistance model. See the text for a brief description of the model and the Supplemental Material section III for a more detailed distribution of the model, the equations, and the method used for the simulation. **B-** Simulated changes in the densities of bacteria and phage in a serial transfer population initiated with high densities of wild type cells, W, and phage, V, and minority populations of evolved cells, E and, small colony variants, S. See the supplemental material section III figure S2A for the values of the parameters used for these simulations.

In addition to the experimental results presented in Figure 1B, other observations are consistent with the predictions of this model, as considered in the Supplemental Material III. (i) In the absence of phage, serial transfer cultures of SCVs produce increasing numbers of normal phenotype *S. aureus*: the SCVs are at a marked selective disadvantage. (ii) Cultures initiated with SCVs as the only bacteria do not decline when exposed to the phage but are unable to maintain the phage. (iii) Population initiated with the evolved bacteria and phage do not decline when mixed with the phage, and in some cases, the phage are lost. (iv) Mixtures of sensitive bacteria and

SCVs do not decline when exposed to the phage but maintain the phage. (v) When liquid cultures of the presumptive evolved cells derived from experiments like that depicted in Figure 1B are plated, small colony variant are present at substantial densities. This is not the case when the ancestral *S. aureus* Newman are plated.

(iv) PYO^{Sa} does not replicate on stationary phase *S. aureus.* In infected hosts, many of the bacteria will not be replicating, and thereby, phage would be particularly effective for treatment if they, like some antibiotics [31], could kill non-replicating *S. aureus.* To determine whether PYOSa can kill and replicate on non-growing bacteria, three independent 48-hour stationary phase cultures were mixed with PYO^{Sa} for average initial densities of ~4E9 *S. aureus* and 10^6 PYO^{Sa}. The bacteria and phage were incubated with shaking for 24 hours, and the viable cell and phage densities were estimated and compared to the initial densities. There was no evidence for the stationary phase bacteria being killed, the mean and standard error of the N(24)/N(0) ratio was 0.96 ± 0.03. Moreover and critically, there was a significant decline in the density of phage, N(24)/N(0) = 0.043 ± 0.006. Thus, not only does PYO^{Sa} not kill stationary phase *S. aureus*, these non-replicating bacteria act as a sink and will reduce the density of PYO^{Sa} from treated hosts.

The results of experiments to determine whether the decline in the density of phage can be attributed to the medium, such as due to a high pH, were negative. The density of PYO^{Sa} did not decline in sterile filtrates of 48-hour stationary phase cultures. There is, however, the suggestion that the bacterial must be viable to lead to the reduction in the viable density. When the *S. aureus* in the 48-hour stationary phase culture are killed with chloroform, the density of PYO^{Sa} does not decline.

(v) The joint action of PYO^{Sa} and antibiotics. To determine whether the action of antibiotics and PYO^{Sa} would be synergistic or antagonistic in killing *S. aureus* Newman and the effect of these drugs on replication of PYO^{Sa}, we followed the change in the densities of bacteria and phage over 24 hours of exposure to both antibiotics and phage together and phage alone. For these experiments, we mixed growing cultures of *S. aureus* Newman and PYO^{Sa} in MHII at a density of $4X10^6$ with $4X10^5$, respectively and super MIC concentrations of the antibiotics. The densities of bacteria and phage were estimated just before the antibiotics were added and 24 hours later, respectively N(0) and N(24). In these experiments, the antibiotics and phage were introduced into the growing cultures of *S. aureus* Newman simultaneously, AB+PYO, the antibiotics and then the phage 30 minutes later, AB→PYO, the phage first and the antibiotics 30 minutes later, PYO→AB. As controls, we also treated parallel cultures of *S. aureus* Newman with only the antibiotics, AB, and only the phage. The results presented in Figure 3 and Figure 4 are the means standard errors in the N(24)/N(0) ratios for the bacteria and phage for three independent experiments with each antibiotic and phage combination.

In Figure 3 we present the results of these experiments for three bacteriostatic antibiotics, tetracycline (TET), azithromycin (AZT), and Linezolid (LZD). The greatest decline in the density of bacteria and increase in the density of phage obtains in the

experiment where phage are used alone. There is clearly a negative synergy between these bacteriostatic antibiotics and the phage. Whether administered simultaneously or sequentially, at the concentrations used, these antibiotics prevent PYO from killing *S. aureus* Newman, and when administered simultaneously with azithromycin and linezolid, the phage density declines.

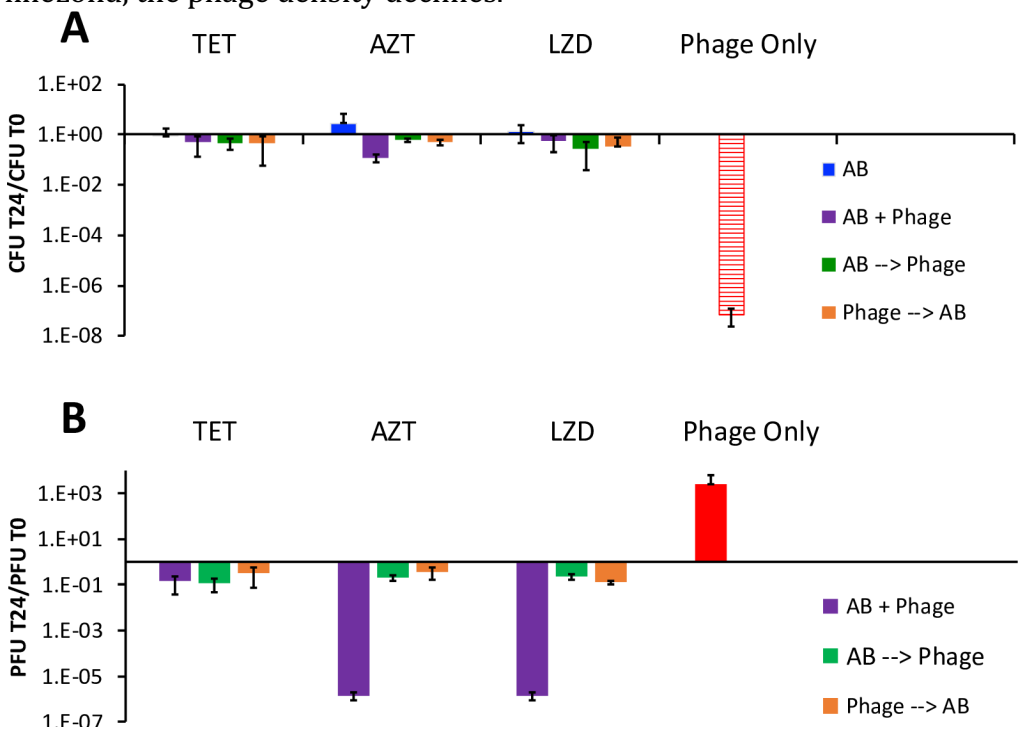

Figure 3. Joint action of bacteriostatic antibiotics and PYO^{Sa}. The concentrations of these antibiotics are as multiples of the MICs, respectively TET 16.7, AZI 2.3, LZD - 9.1. **A-** The ratio of the change in density of S. *aureus* after 24 hours of exposure to antibiotics, antibiotics and phage or phage alone. Hash red, the density of *S. aureus* recovered was below the detection limit, ~10^2 cells per ml. **B-** The ratio of the change in the density of PYO^{Sa} after 24 hours of confronting *S. aureus* Newman in combination with antibiotics or alone.

In Figure 4, we present the results of experiments with bactericidal antibiotics, rifampin (RIF), oxacillin (OXN), ciprofloxacin (CIP), vancomycin (VAN), daptomycin (DAP) and Kanamycin (KAN). As observed in Figure 3 for the parallel experiments with bacteriostatic antibiotics, the most significant decline in the viable density of *S. aureus,* the N(24)/N(0) ratio, was for the phage only treatment. While these data suggest simultaneous or sequential administration of PYO^{Sa} may increase the rate at which oxacillin, ciprofloxacin, and kanamycin, kill *S. aureus* at the concentrations employed. At these antibiotic concentrations, the contribution of the phage to killing the bacteria is modest at best. Moreover, as was observed for the parallel experiment with the static drugs (Figure 3) the phage kill more *S. aureus* in the absence of antibiotics than with these drugs, an antagonistic interaction once again. The failure of rifampin to reduce the viable density of *S. aureus* can be attributed to rifampin resistance emerging. It should be noted; however, in one of the three treatments

where PYO^{Sa} was used before adding rifampin, it prevented the ascent of resistance. Most interestingly, while treatment with rifampin, oxacillin, and ciprofloxacin allowed for PYO^{Sa} to replicate, that was not the case for vancomycin, daptomycin, and kanamycin, which appeared to suppress replication of PYO^{Sa}.

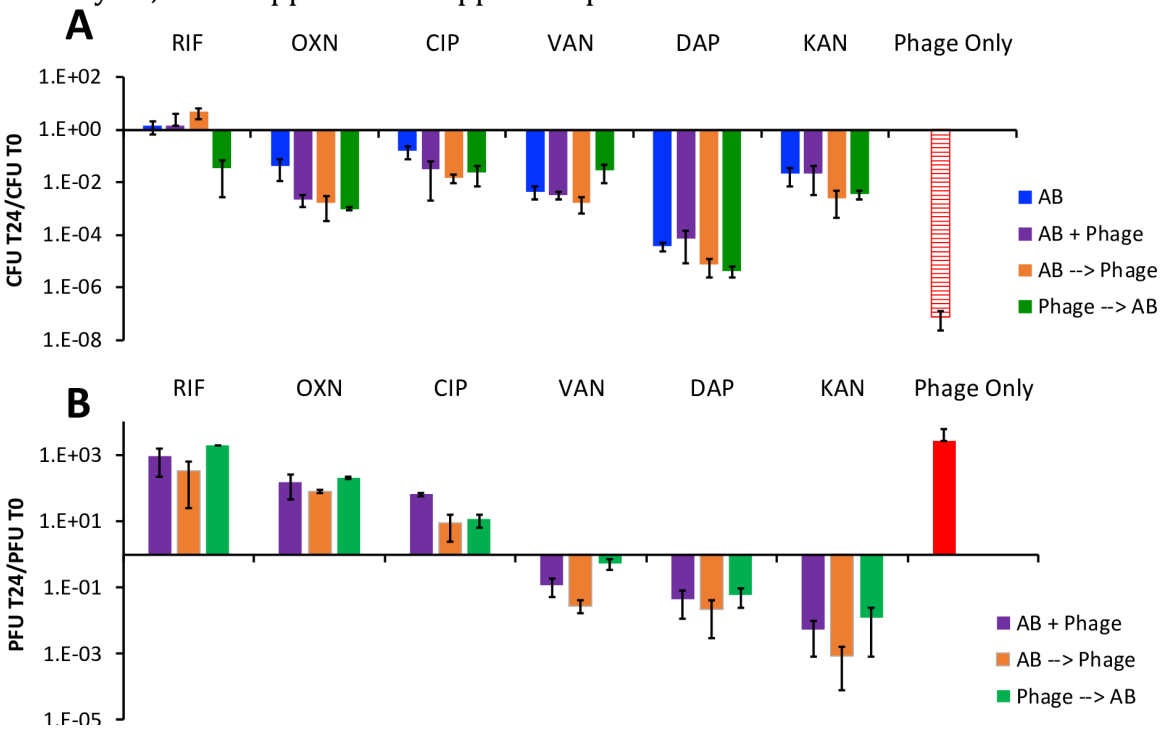

Figure 4. The joint action of bactericidal antibiotics and PYO^{Sa}. Multiples of MICs of the different antibiotics RIF-3.2, OXN - 1.6, CIP 2.29, VAN4.57, DAP 1.60, KAN 2.0. **A-** The ratio of the change in density of *S. aureus* after 24 hours of exposure to antibiotics, antibiotics, and phage or phage alone. Hash red, the density of *S. aureus* recovered was below the detection limit, ~10^2 per ml. **B-** The ratio of the change in the density of PYO^{Sa} after 24 hours of confronting *S. aureus* Newman in combination with antibiotics or alone.

(vi) <u>Sequential treatment, phage followed by antibiotics:</u> The serial transfer results presented in Figure 1B indicate that as a consequence of the emergence and ascent of SCVs, by itself, the PYO^{Sa} will not be able to control an *S. aureus* population for an extended time. The bacterial population recovers from its initial demise due to the generation of SCVs that do not support the replication of PYO^{Sa}. Although the phage continue to be present, the density of the bacteria returns to levels similar to that of the phage-free control (Figure 1B). What will obtain if antibiotics are administered to these populations? To address this question, we performed the serial transfer experiments with the addition of bactericidal antibiotics following the profound reduction in the density of *S. aureus* observed during the first 24 hours, as observed in Figure 1B. The results of these experiments are presented in Figure 5.

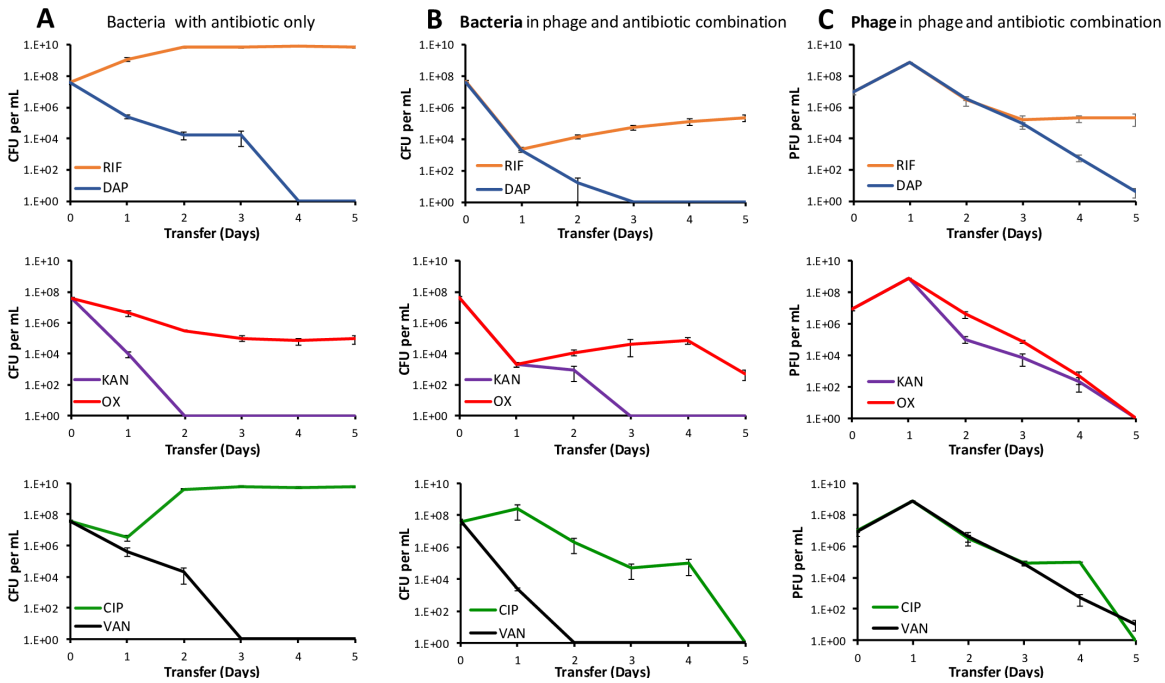

Figure 5. Changes in the densities of bacteria and phage in serial transfer cultures treated with antibiotics alone and treatment with antibiotics and phage. **Column A-** Bacteria treated with just the antibiotic. **Column B-** The bacteria in antibiotics and phage cultures. **Column C -** The phage in antibiotics and phage cultures. Mean and standard errors of 3 independent experiments**. Row 1** - treatment with rifampin and daptomycin, 0.2 and 64 μg/ml, respectively. **Row 2 –** treatment with kanamycin and oxacillin, 46 and 3 μg/ml respectively, **Row 3** – treatment with ciprofloxacin and vancomycin, 0.5 and 8 μg/ml respectively.

Three of the antibiotics alone eliminated the *S. aureus* population, daptomycin, kanamycin and vancomycin (Figures 5 A, D and G). By the second transfer, the density of *S. aureus* in the rifampin treated cultures was similar to that of the antibiotic-free controls (Figure 5A). The reason for the failure of this antibiotic to clear these cultures was the emergence of mutants resistant to this drug. All six colonies tested from the 5th transfer were resistant to rifampin. At the same time, the viable cells recovered from the phage-antibiotic cultures were as sensitive to rifampin as was the antibiotic-free controls. Neither oxacillin nor ciprofloxacin alone cleared the cultures. In the case of oxacillin, the viable cell density declined but continued to persist at a density of approximately 10^5 cells per ml. The bacteria recovered from these cultures were sensitive to oxacillin. We postulate that this leveling off can be attributed to persistence [32]; see the supplemental material IV. In the case of ciprofloxacin, the initial exposure led to a substantial decline in the viable cell density of *S. aureus*, following the second transfer the bacterial population recovered and was sustained at densities similar to that in the antibiotic-free controls. Based on the MIC data, the colonies of *S. aureus* recovered at the end of this experiment were susceptible to ciprofloxacin. We postulate that these resurrection dynamics can be attributed to heteroresistance [33], see the supplemental material V.

(vii) Mechanism for the high rates of transition between states: Arguably, from a clinical perspective, the molecular and genetic mechanisms responsible for the generation of the SCV's that prevent the clearance of *S. aureus* population by PYO^{Sa} is of only modest interest. On the other hand, from at least an academic perspective, elucidating the mechanisms responsible for the high rates of transition between wild type colony *S. aureus* and the SCVs responsible for the failure of PYO^{Sa} to eliminate the bacteria in serial culture (Figure 1B) is of substantial interest. The results reported in a recent article by Guérillot and colleagues [34] may well be key to understanding the mechanism for the high rate of production of SCVs and their instability. They show that exposure to rifampin induces chromosomal inversions mediated by a restriction endonuclease, which in turn activates a prophage that generates phenotypic and genetic diversity in the form of SCVs. We postulate that infections with PYO^{Sa} have a similar effect. Moreover, in their study, Guérillot and colleagues observed three states of *S. aureus* exposed to rifampin, wild type (WT), SCVs, and a third they call Normal Colony Variants, (NCVs) that have a high rate of transition to and from SCVs. We postulate that these NCVs are similar to what we call "Evolved", E, state considered in Figure 1 and Figure 2.

To explore the validity of this genomic instability hypothesis, we sequenced genomic DNA isolated from purified colonies of four SCV and three 'evolved, E, mutants recovered from cultures exposed to PYO^{Sa} in an experiment of the type shown in Figure 1B. The only mutations found in the evolved and SCV cultures were a point mutation in *femA* (G368D), and in one evolved mutant a frameshift mutation in *nusB* (S93fs). We did not find any evidence for additional acquired point mutations in the evolved or SCV genomic sequences. There was no evidence of deletions, duplications or insertions in any of the genomes. The absence of additional mutations in any of the evolved or SCV mutants suggests that these phenotypes might be the result of one or more structural rearrangements. Interestingly, Guérillot and colleagues [34] have shown that exposure of *S. aureus* to rifampin promotes an inversion in the chromosome, resulting in an SCV phenotype. By analogy, we suggest that exposure to the PYO^{Sa} phage could promote structural rearrangements in the *S. aureus* chromosome, resulting in the evolved and SCV phenotypes (the rearrangements could be sequential inversions).

Discussion and conclusions

On first consideration, PYO^{Sa} seems to be an ideal phage for treating *Staphylococcus aureus* infections. The results of this study provide evidence in support of three virtues of PYO^{Sa} as a therapeutic phage. 1) PYO^{Sa} is likely to kill virtually all methicillin-resistant as well as methicillin-sensitive *S. aureus. (2) S. aureus* are unable to generate classical surface resistant mutants to PYO^{Sa}, thus, cocktails of multiple phages would not be needed to ensure coverage or prevent resistance. (3) *S. aureus* Newman, PYO^{Sa} has a high adsorption rate and burst size, and, when first confronting growing populations of *S. aureus*, the bacteria are killed, and the phage replicate at a high rate.

On the downside, our experiments raise caveats about the use of PYO^{Sa} for treating *S. aureus* infections alone and suggest a possible liability as well. Not only is PYO^{Sa} unable to clear cultures of *S. aureus* Newman, it selects for potentially pathogenic small colony variants, SCVs [35-38] that are refractory to this phage. Through a "leaky resistance" mechanism [39], the phage continue to be maintained, and the bacterial population continues to persist at densities not much less than they do in the absence of PYO^{Sa}. Although not observed for the SCVs tested here, at least some SCVs are more resistant to antibiotics than the bacteria from whence they are derived [40].

The observation that exposing *S. aureus* to rifampin induces a chromosomal inversion with an associated SCV phenotype . shows the potential for genomic instability to contribute to selected phenotypic variants. Genomic instability, including duplications, amplifications, deletions, and inversions, is an under-appreciated source of selected mutant phenotypes, including resistance to antibiotics and survival of other stressful environments [41] Chromosomal rearrangements occur spontaneously, but their rates can be greatly induced by environmental selective pressures, for example, by exposure to agents that cause DNA damage. Chromosomal rearrangements can be maintained by selection, and, with the exception of deletions, they are reversible. Our observations of the evolution of *S. aureus* after exposure to PYO^{Sa}, to produce SCV and evolved variants that had not acquired point mutations (Table S2), and SCV variants that generate evolved at a high rate (Figure 1B) are supportive of genomic rearrangements as a plausible underlying cause of these phenotypes. Details of the genetic mechanisms will be of interest for a full understanding of the bacterial response to the phage and will need to be elucidated in the future.

For phage therapy to be a practical and acceptable enterprise, these viruses would have to be used in combination with antibiotics. Our results indicate that when administered simultaneously, or nearly simultaneously with antibiotics, PYO^{Sa} does worse in killing *S. aureus* than it does alone PYO^{Sa}. The ribosome-targeting bacteriostatic antibiotics, tetracycline, azithromycin, and linezolid, suppress the ability of PYO^{Sa} to kill *S. aureus* Newman. This observation is consistent with the failure of PYO^{Sa} to replicate on stationary phase populations of *S. aureus*. It is also consistent with the Numbers Game hypothesis for the action of bacteriostatic antibiotics [42]. Presumably, the number of free ribosomes is too low to support the protein synthesis needed for replication of the phage.

When mixed with bactericidal antibiotics, PYO^{Sa} is less effective in killing *S. aureus* than it is in the absence of these drugs. We postulate that this can be attributed to pharmaco and population dynamics of the joint action of antibiotics and phage; the antibiotics reduce the densities of the bacteria substantially and thereby lower the capacity of the phage to replicate (Supplemental Material IV).

<u>Sequential treatment with PYO^{Sa} and antibiotics:</u> Our experiments also suggest a way to deal with the major caveat and potential liability of treatment with PYO^{Sa} alone, the

recovery of the bacterial population due to the ascent of small colony variants. The administration of bactericidal antibiotics following the initial decline in the density of bacteria due to PYO^{Sa} prevents this recovery and eliminates the SCVs. The latter is not the case when the antibiotics are used alone. One interpretation of this is that sequential treatment with phage and bactericidal antibiotics may well be more effective than treatment with antibiotics alone as well as phage alone.

Conclusion and Recommendation: We interpret the results of this *in silico* and *in vitro* to suggest that PYO^{Sa} will be effective for treatment of *S. aureus* infections, but only if the administration of bactericidal antibiotics follows that of phage. The next step will, of course, be to test this sequential phage and antibiotic treatment hypothesis with *S. aureus* infections in experimental animals.

We suggest that the *in vitro* methods used to explore the potential efficacy of PYO^{Sa} would also be useful for evaluating other phages being developed for treating bacterial infections. Existing data suggested that PYO^{Sa} met all of the criteria desired for a phage to be effective for therapy; yet, the *in vitro* experiments performed here uncovered a limitation and potential liability of using this phage for therapy that would not have anticipated and they also subsequently revealed a way to deal with said limitation and liability.

Materials and Methods:

Strains and growth media

Unless otherwise noted, all experiments were performed from derivatives of the parent strain *S. aureus* Newman (ATCC 25904). The parent *S. aureus* Newman was obtained from Bill Schafer of Emory University. The small colony variant and evolved strains were obtained from PYO^{Sa} challenged *S. aureus* Newman by experiments performed in our lab. The investigation for classical resistance was performed in the following MSSA strains obtained from Abraham Moller in the Reid Lab at Emory University: NRS52, NRS102, NRS180, NRS110, NRS252, NRS253, NRS266, NRS109, NRS148, and NRS205.

Bacterial cultures were grown at 37°C in Mueller-Hinter II Broth [275710, BD™]. PYO^{Sa} lysates were prepared from single plaques at 37°C in MHII broth alongside wild-type *S. aureus* Newman by plate lysis. Specifically, individual phage plaques were picked with a sterile stick, resuspended in 4ml of phage soft agar with .1ml of overnight bacterial culture and plated on top of phage plates. The plates were then incubated at 37°C overnight. The soft agar was scraped with a sterile iron scoop, resuspended in 10ml MHII with ~.5ml of chloroform to kill the surviving bacteria. The lysates were then centrifuged to remove the agar, sterilized by filtration (0.2 μm) and stored at 4°C.

Sampling bacterial and phage densities

Bacteria and phage densities were estimated by serial dilutions in 0.85% NaCl solution followed by plating. The total density of bacteria was estimated on LB (1.6%) agar plates. To estimate the densities of free phage, chloroform was added to suspensions before serial dilutions. These suspensions were mixed with 0.1 mL of overnight MHII grown cultures of wild-type *S. aureus* Newman (about 5×10^8cells per mL) in 4 mL of LB soft (0.65%) agar and poured onto semi-hard (1%) LB agar plates.

Parameter estimations

The parameters critical for the interaction of the PYO^{Sa} phage and *S. aureus* Newman used in this study were estimated in independent experiments M9M. The maximum growth rate of different clones of *S. aureus* Newman was measured by Bioscreen, as described in [43]. Phage burst sizes (β) were estimated with one-step growth experiments similar to [44]. Adsorption of PYO^{Sa} to *S. aureus* was estimated as described in [44].

Serial transfer experiments

All serial transfer experiments were carried out in 10ml MHII cultures grown at 37°C with vigorous shaking. The cultures were initiated by 1:100 dilution from 10-mL overnight cultures grown from single colonies. Phage was added to these cultures to reach the initial density of approximately 10^6 PFU/mL. At the end of each transfer, 100 µL of each culture was transferred into flasks with fresh medium (1:100 dilution). Simultaneously, 100 µL samples were taken for estimating the densities of colony-forming units (CFU) and plaque-forming units (PFU), by serial dilution and plating on solid agar.

Antibiotics and their Sources

Tetracycline, Oxacillin, Vancomycin, Kanamycin, Streptomycin- SIGMA. Azithromycin- TOCRIS. Daptomycin- MP Biochemicals. Rifampin- APPLICHEM. Linezolid- CHEM IMPEX INT'L

Whole-genome sequencing

For sequencing individual clones of ancestral, SCV and evolved *S. aureus*, genomic DNA was prepared using the MasterPure Gram-Positive kit, following the manufacturer's instructions (Epicentre, Illumina Inc., Madison, Wisconsin). Final DNA was resuspended in EB buffer. Genomic DNA concentrations were measured in a Qubit 2.0 Fluorometer (Invitrogen via ThermoFisher Scientific). DNA was diluted to 0.2 ng/µL in water (Sigma-Aldrich, Sweden), and the samples were prepared for whole genome sequencing according to Nextera® XT DNA Library Preparation

Guide (Illumina Inc., Madison, Wisconsin). After the PCR clean up-step, samples were validated for DNA fragment size distribution using the Agilent High Sensitivity D1000 ScreenTape System (Agilent Technologies, Santa Clara, California). Sequencing was performed using a MiSeq™ desktop sequencer, according to the manufacturer's instructions (Illumina Inc., Madison, Wisconsin). The sequencing data were aligned and analyzed in CLC Genomics Workbench version 11.0 (CLCbio, Qiagen, Denmark).

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SUPPLEMENTAL MATERIAL

Combination antibiotic and phage therapy: dealing with the limitations of a seemingly ideal phage for treating *Staphylococcus aureus* infections

I. Host range

In our report, we summarized the evidence for PYO^{Sa}'s host range included all 83 clinical isolates of *Staphylococcus aureus* tested. Much of those data are from LabCorp. The methods used are summarized in the following and the results obtained are summarized in Supplemental Table S1.

Methods

The methods LabCorp employed to test the sensitivity of these strains to PYO were somewhat different than those we employed and presented in our report.

(i) The *S. aureus* strains tested were streaked for single colonies on TSB agar.

(ii) These single colonies were inoculated in 3 ml LB broth and grown overnight with shaking at 37°C.

(iii) The overnight cultures were diluted by 1:30 in 3ml LB broth and grown with shaking at 37°C to an OD 600nm of between 0.3-1.0.

(iv) 300ul of these cultures were added to 3ml LB soft (0.3% or 0.5%) agar supplemented with 3ul 1M $CaCl_2$ and 3ul 1M $MgCl_2$ which were spread onto LB agar plates to form a lawn.

(v) 5µl of the PYO lysates were spotted onto the lawns and incubated overnight.

(vi) Lysates were prepared from original spot plates and then re-spotted on the original spot plate host bacteria following the above conditions.

The Key to scoring the results of this assay follows:

Key	
-	No lysis
-/+	Weak lysis
+	Partial lysis (cloudy)
++	Complete lysis

Table S1 Assay for the susceptibility of *S. aureus* to phage PYO^{Sa}. Data provided by LabCorp

Species	Strain	Spot	Re-spot
Staphylococcus aureus	27660	++	++
Staphylococcus aureus	RN6390	+	-/+
Staphylococcus aureus	502A	++	++
Staphylococcus aureus	6538	++	++
Staphylococcus aureus	25923	++	++
Staphylococcus aureus subsp. aureus	12600	++	++
Staphylococcus aureus	RN4220	++	++
Staphylococcus aureus subsp. aureus	BAA-1721	++	++
Staphylococcus aureus subsp. aureus	14775	++	++
Staphylococcus aureus	AR0461	++	++
Methicillin-resistant S. aureus	017-243	++	++
Methicillin-resistant S. aureus	041-332	+	-/+
Methicillin-resistant S. aureus	045-188	++	++
Methicillin-resistant S. aureus	038-401	+	++
Methicillin-resistant S. aureus	049-841	++	++
Methicillin-resistant S. aureus	USA300	+	-
Methicillin-resistant S. aureus	002-639	+	+
Methicillin-resistant S. aureus	049-60	++	++
Methicillin-resistant S. aureus	026-485	++	++
Methicillin-resistant S. aureus	011-719	++	++
Methicillin-resistant S. aureus	045-696	++	++
Methicillin-resistant S. aureus	BAA-1707	+	-/+
Methicillin-resistant S. aureus	BAA-1717	+	+
Methicillin-resistant S. aureus	BAA-1720	++	++
Methicillin-resistant S. aureus	BAA-1747	+	-
Methicillin-resistant S. aureus	BAA-1754	++	+
Methicillin-resistant S. aureus	BAA-1761	++	++
Methicillin-resistant S. aureus	BAA-1763	++	++
Species	**Strain**	**Spot**	**Re-spot**
Methicillin-resistant S. aureus	BAA-1764	++	-/+

Methicillin-resistant S. aureus	BAA-1766	++	+
Methicillin-resistant S. aureus	BAA-1768	++	++
Methicillin-resistant S. aureus	BAA-41	++	++
Methicillin-resistant S. aureus	BAA-42	++	-/+
Multidrug-resistant resistant S. aureus	BAA-44	++	+
Methicillin-resistant S. aureus	BAA-1683	++	+
Methicillin-resistant S. aureus	BAA-2094	++	++
Methicillin-resistant S. aureus	BAA-2313	++	++
Methicillin-resistant S. aureus	33592	++	+
Methicillin-resistant S. epidermis	700583	-/+	-
Methicillin-resistant S. haemolyticus	700564	-/+	-
Staphylococcus aureus subsp. aureus	BAA-1718	++	++
Methicillin-resistant S. aureus	AR0462	++	-
Methicillin-resistant S. aureus	AR0463	++	++
Methicillin-resistant S. aureus	AR0464	-/+	-/+
Methicillin-resistant S. aureus	AR0465	++	+
Methicillin-resistant S. aureus	AR0466	+	-
Methicillin-resistant S. aureus	AR0467	++	+
Methicillin-resistant S. aureus	AR0468	++	+
Methicillin-resistant S. aureus	AR0469	++	+
Methicillin-resistant S. aureus	AR0470	++	+
Methicillin-resistant S. aureus	AR0471	++	-
Methicillin-resistant S. aureus	AR0472	++	++
Methicillin-resistant S. aureus	AR0473	++	++
Methicillin-resistant S. aureus	AR0474	++	+
Methicillin-resistant S. aureus	AR0475	+	-
Methicillin-resistant S. aureus	AR0476	++	++
Methicillin-resistant S. aureus	AR0477	++	++
Methicillin-resistant S. aureus	AR0478	++	++
Methicillin-resistant S. aureus	AR0479	++	++
Species	**Strain**	**Spot**	**Re-spot**
Methicillin-resistant S. aureus	AR0480	+	+

Methicillin-resistant S. aureus	AR0481	++	++
Methicillin-resistant S. aureus	AR0482	++	++
Methicillin-resistant S. aureus	AR0483	++	++
Methicillin-resistant S. aureus	AR0484	++	++
Methicillin-resistant S. aureus	AR0485	++	++
Methicillin-resistant S. aureus	AR0486	++	++
Methicillin-resistant S. aureus	AR0487	+	-/+
Methicillin-resistant S. aureus	AR0488	+	-/+
Methicillin-resistant S. aureus	AR0489	+	-
Methicillin-resistant S. aureus	AR0490	++	++
Methicillin-resistant S. aureus	AR0491	-/+	-
Methicillin-resistant S. aureus	AR0492	+	-

II. Variation in the demise – resurrection population dynamics presented in Figure 1B.

To explore the generality of the PYO^{Sa} – *S. aureus* Newman serial transfer results presented in Figure 1B and the experiments with PYO^{Sa} and *S. aureus* in the absence of transfer, we performed the Figure 1B experiments with ten 2 ml and six 10ml cultures. Both had effectively the same initial densities of phage and bacteria. The dynamics of only one of the ten 2 ml samples serially transferred were similar to the demise–resurrection dynamics observed from Figure 1B. While all six 10 ml serial transfer cultures were turbid by the 5th transfer, some did not become turbid until the 4th transfer. We interpret this observation to be consistent with the hypothesis that the resurrection of these phage-exposed bacteria is a stochastic process in *S. aureus* and thus is more likely to occur in the 10ml populations because the total number of bacteria is 5-fold greater than that in 2 ml cultures. As noted in our report, this result is what would be anticipated if the generation of SCVs is the product of genomic instability [34].

Also consistent with this stochastic genome instability hypothesis is that the time before the recovery of the *S. aureus* population due to the ascent of SCVs is variable. In Figure 1B, all three serial transfer populations started the recovery to high density by the second transfer. This was not the case for a repeat of this experiment. The recovery of one of the three populations started at the end of the first transfer (Figure S1A).

In Figure 1D, where the serial transfer cultures were initiated with *S. aureus* isolated at the end of serial transfer experiments with the PYO^{Sa}, the "Evolved" bacteria, the phage were maintained in one of three cultures and were lost in two. In the repeat of this experiment with three independently "Evolved" strains, the phage were lost in all three (Figure S1B).

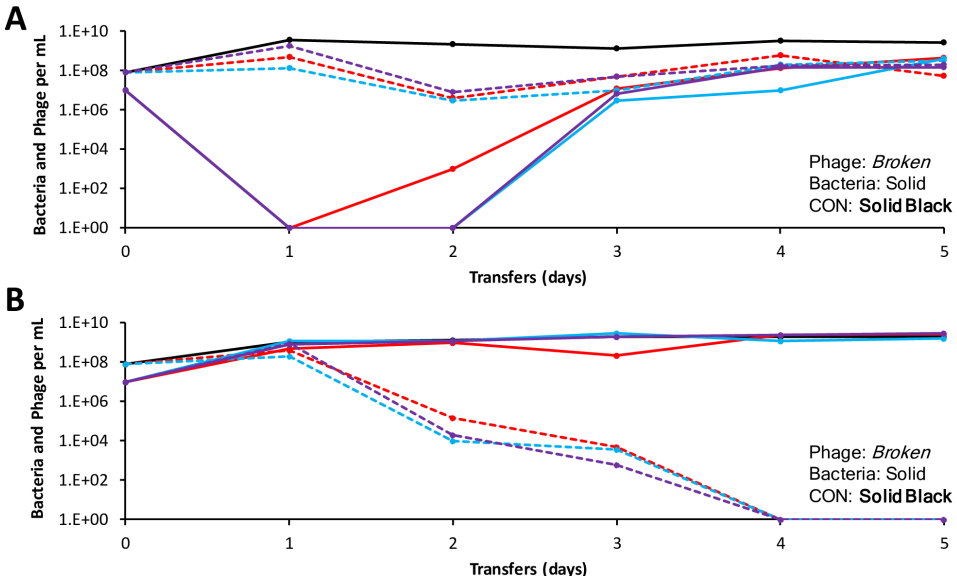

Figure S1 Changes in the densities of bacteria and phage in (1/100) 10 ml serial transfer cultures of *S. aureus* Newman and PYO^{Sa} and a phage-free control, CON. **A)** Three independent cultures initiated with *S. aureus* Newman that had not previously been exposed to PYO^{Sa}. **B)** Three independent cultures initiated with *S. aureus* Newman that obtained from the 5th transfer of independent serial passage experiments with *S. aureus* Newman mixed with PYO^{Sa}.

Whole genome sequence analysis of mutants selected in the presence of PYO^{Sa}
Genomic DNA isolated from purified colonies of three 'evolved, E, and four SCV recovered from cultures exposed to PYO^{Sa} in an experiment of the type shown in Figure 1B. The data show that with the exception of *femA* G368D and *nusB* S93fs no point mutations were selected during the evolution of these two phenotypes in the presence of the PYO^{Sa} phage (Table S2).

Table S2 Whole genome sequencing data for wild-type *S. aureus* Newman, and of Evolved, and SCV mutants after culturing in the presence of PYO^{Sa}.

Strain/Mutant	Genotype	Mutations
S. aureus Newman	Wild-type	-
Evolved culture 1	*femA* G386D	*nusB* S93fs
Evolved culture 3	-	-
Evolved culture 5	*femA* G386D	-
SCV culture 2	*femA* G386D	-
SCV culture 3	*femA* G386D	-
SCV culture 4	*femA* G386D	-
SCV culture 5	*femA* G386D	-

III. A hypothesis to account for the population and evolution dynamics of the *S. aureus* and PYO^{Sa}.

In our article, we postulate that the population dynamics observed for *S. aureus* Newman and PYO^{Sa} in serial transfer culture, particularly the rapid decline in the bacterial population followed by its resurrection and maintenance at high densities along with the phage (Figure 1B), can be attributed to ascent of PYO^{Sa} sensitive evolved bacteria that continually produce phage resistant small colony variants at high rate. The following is the mathematical - computer simulation model upon which we base this hypothesis.

<u>A heuristic model of the population dynamics of phage and bacteria with transitions between the states of the bacteria</u>**:** There is one population of lytic phage, V and three populations of bacteria, a wild type, evolved, and small colony variant, respectively W, E, S as designations and densities, particles and cells per ml. The bacteria grow at maximum rates, v_W, v_E and v_S per cell per hour with their net growth rates equal being equal to the product of the maximum growth rates and a hyperbolic function of concentration of the limiting resource, r , µg/ml. a strain , $v_i \bullet \psi_i(r) = v_i \bullet \frac{r}{(r+k_i)}$ [45].

The subscript i represent W, E or S. The phage adsorb to the bacteria with rate constants δ_W, δ_E, and δ_S per hour per ml. We assume, however, that while the phage adsorb to the small colony variants, SCVs, S, these phage are lost and do not kill these bacteria. Upon adsorption, W and E produce β phage particles per cell. [27]. We assume that there is a transition between states, where μ_{WE} and μ_{EW} per cell per hour are the transitions for W→E and E→W, μ_{ES} and μ_{SE} are the transitions between E→S and S→E. Finally, we assume that resources are consumed at a rate proportional to the net growth rates of the bacteria, and a conversion efficiency parameter, *e* µg/ml, which is the amount of resource needed to produce a new cell. [46].

With these definitions and assumption, the rates of change in the densities of the different bacteria, phage, and the concentration of the resource in batch culture are given by a set of five coupled differential equations:

$$\frac{dr}{dt} = -\psi(r) \bullet e \bullet (W \bullet v_W + E \bullet v_E) - e \bullet \psi_S(r) \bullet S \bullet v_S$$

$$\frac{dW}{dt} = \psi(r) \bullet v_W \bullet W - \delta_W \bullet W \bullet V \bullet \psi(r) + \mu_{EW} \bullet E \bullet \psi(r) - \mu_{WE} \bullet W \bullet \psi(r)$$

$$\frac{dE}{dt} = \psi(r) \bullet v_E \bullet E - \delta_E \bullet E \bullet V \bullet \psi(r) + \mu_{WE} \bullet W \bullet \psi(r) - \mu_{EW} \bullet E \bullet \psi(r) - \mu_{ES} \bullet E \bullet \psi(r) + \mu_{SE} \bullet S \bullet \psi_S(r)$$

$$\frac{dS}{dt} = \psi_S(r) \bullet v_S \bullet S + \mu_{ES} \bullet E \bullet \psi(r) - \mu_{SE} \bullet S \bullet \psi_S(r)$$

$$\frac{dV}{dt} = V \bullet \beta \bullet (\delta_W \bullet W + \delta_E \bullet E) \bullet \psi(r) - \delta_S \bullet V \bullet S \bullet \psi_S \bullet (r)$$

$$where\ \psi(r) = \frac{r}{(r+k)} \quad and\ \psi_S(r) = \frac{r}{(r+k_S)}$$

Numerical Solutions - simulations

To solve this set of coupled differential equations and those for the models that follow, we use Berkeley Madonna. The population growth and phage infection parameters employed for these numerical solutions, simulations, are of the range estimated for PYO^{Sa} and *S. aureus* Newman in MHII medium. To simulate a serial transfer mode of population maintenance, every 24 hours, there is a 100-fold reduction in densities of the bacteria and phage and the resource concentration is restored to its maximum level, 1000µg per ml. For copies of the program and instructions for it use, write to blevin@emory.edu. In their study, Guerillot and colleagues [34] presented evidence that when *S. aureus* are exposed to rifampin, unstable chromosome rearrangements generate SCVs. Here we are assuming that infections with PYO^{Sa} also generate unstable chromosome rearrangements in *S. aureus* and produces the Evolved population which continually generates SCVs in the presence of the phage. As a consequence of their presumed genomic instabilty, these SCVs return at a predictable rate to the susceptible, evolved state. In this interpretation, in the absence of PYO^{Sa}, the transition rate between E-->S, μ_{ES} is markedly lower than it is in the absence of these phage.

Simulation Results: In Figure S2, we follow the changes in the densities of the bacteria and phage in simulated serial populations. As observed in Figure 2B, the model predicts that upon the first encounter with the phage, V, the density of susceptible cells, W, and the total cell density of the bacteria, NT, will declines whilst that of the phage increases. In subsequent transfers, the population of bacteria recovers and becomes dominated by SCVs. As a consequence of the transitions between the different states of bacteria, the phage and all three bacterial populations are maintained with SCVs dominating the bacterial community. If we make the SCVs less efficient in the use of the resource, $e_C = 5x10^{-6}$ rather than $5x10^{-7}$, the total density of SCV population is lower (Figure S2A). When cultures of just SCVs are started without the phage, they are ultimately diluted out due to their lower growth rate and low production rate in the absence of the phage and are overtaken by the evolved population being generated through reversion from the SCVs (Figure S2B). When the evolved cells and phage are mixed, as observed in Figure 1D, the population recovers to full density more rapidly than when sensitive cells and phage are

mixed (Figure S2C). However, in these simulations, the phage continue to be maintained, which was the case for only one of the two parallel experiments in the main body of the paper and in none of the parallel experiments presented in supplemental Figure S1B. This model can also account for why the mixture of the ancestral *S. aureus* Newman and SCVs can maintain the phage (Figure 1E) with little effect on the density of bacteria (Figure 2SD).

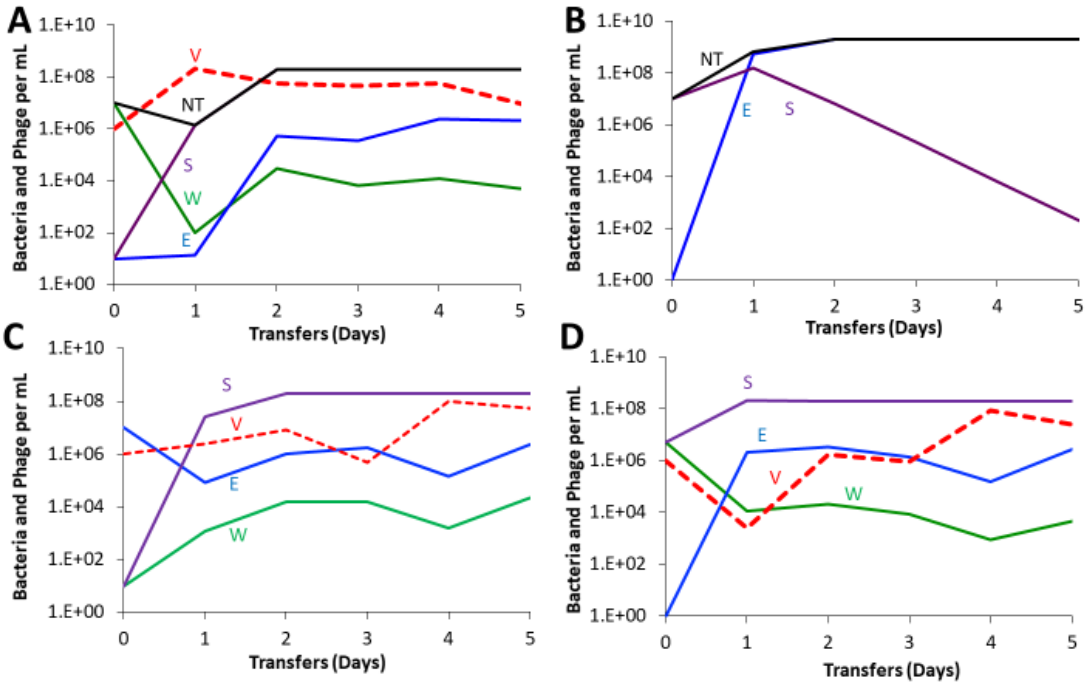

Figure S2 Simulated serial transfer populations. Changes in the densities of bacteria and phage in serial transfer culture. Standard parameters, $v_W = v_E = 2.0$, $v_S = 0.5$, $\delta_W = \delta_E = \delta_S = 4.8\times10^{-7}$, $\beta_W = \beta_E = 80$, $\beta_S = 0$, $e_S = 5\times10^{-7}$, C=1000, k=1, $\mu_{SE} = 10^{-5}$. NT is the total density of bacteria. **A)** Serial transfer population initiated with 10^7 wild-type, W, 10^6 phage, V, 10 S and 10 E. $\mu_{WE}=10^{-5}$ $\mu_{EW}=\mu_{ES}=\mu_{SE}=10^{-3}$. **B)** No phage, $\mu_{WE}=10^{-5}$, $\mu_{EW}=0$, $\mu_{ES} = 0$, $\mu_{SE} = 10^{-3}$, and populations initiated with equal densities of wild-type, W, and SCVs, S. **C)** Population initiated with Evolved cells, and phage $\mu_{WE}=10^{-5}$, $\mu_{WE}=\mu_{ES}=\mu_{SE}=10^{-3}$. **D)** Populations initiated with a mixture of wild-type cells, SCVs and phage, $u_{WE}=10^{-5}$, $\mu_{WE}=\mu_{ES}=\mu_{SE}=10^{-3}$.

IV – The joint action of bactericidal antibiotics and phage.

In our article, we postulate that the reason bactericidal antibiotics in combination with phage do worse than phage alone (Figure 4) can be attributed to the antibiotics reducing the density of bacteria and thereby the capacity of the phage to replicate. We illustrate this with the following model of the joint action of antibiotics and a bactericidal antibiotic.

Model of the joint action of antibiotics and phage:

There are two populations of bacteria enumerated in cells per ml. The populations are respectively W (wild-type, which are sensitive to antibiotics) and P (persisters which are sensitive to the phage but phenotypically resistant to antibiotics). There is a single antibiotic of concentration A μg/ml, a lytic phage of density V particles per ml, and a limiting resource r μg/ml. The sensitive bacteria grow at a maximum rate, v_N, with the net growth/death rate ψ(A,r) being proportional to the concentration of the antibiotic and the limiting resource voila,

$$\psi(A,r) = \left[v_{WMAX} - \left[(v_{WMAX} - v_{WMIN}) \bullet \left(\frac{\left(\frac{A}{MIC}\right)^{\kappa}}{\left(\left(\frac{A}{MIC}\right)^{\kappa} - \left(\frac{v_{WMIN}}{v_{WMAX}}\right) \right)} \right) \right] \right] \bullet \frac{r}{(r+k)}$$

Where v_{WMAX} (>0) is the maximum growth rate, v_{WMIN} (<0) is the minimum growth rate/maximum kill rate, MIC the minimum inhibitory concentration of the antibiotic, and a shape parameter, κ, such that the greater the value of κ the more acute the function.

The persisters, P, are non-replicating bacteria that are resistant to the antibiotic. The phage adsorb to the persisters but do not replicate on them. The rate constant of adsorption of the phage to sensitive cells is δ_W and that to the persisters δ_P. Infections of phage to sensitive cells produce β phage particles per cell. With a rate y per cell per hour, sensitive cells produce persisters, W-->P, and with a rate x per cell per hour, persisters produce sensitive cells, P -->W. With these definitions and assumptions, the rates of change in the densities of phage and the concentration of the resources are given by

$$\frac{dr}{dt} = -\psi(A,r) \bullet W \bullet e - v_{MAXW} \bullet \psi(r) \bullet W \bullet e$$

$$\frac{dW}{dt} = \psi(A,r) \bullet W - \delta_W \bullet W \bullet V \bullet \psi(r) - y \bullet W + x \bullet P$$

$$\frac{dP}{dt} = y \bullet W - x \bullet P - \delta_P \bullet P \bullet V \bullet \psi(r)$$

$$\frac{dV}{dt} = \delta_W \bullet V \bullet W \bullet \beta \bullet \psi(r) - \delta_P \bullet P \bullet V \bullet \psi(r)$$

where $\psi(r) = \frac{r}{(r+k)}$ *and e μg is the amount of the* lim*iting resource need to produce a new cell*

Numerical solutions- simulations:

To solve these equations and simulate the dynamics, we use Berkeley Madonna. Copies of the programs are available from blevin@emory.edu.

Results

In Figure S3, we illustrate the rate of change in the total density of bacteria with a bactericidal antibiotic alone, with phage alone, and with the phage and antibiotic together. The rate of decline in the density of bacteria is lowest in the simulations where antibiotics are used alone. The highest rate of decline in the density of bacteria is obtained in the simulation, where phage are used alone. When phage are used in combination with bactericidal antibiotics, they do not increase in density to the same extent that they do in the absence of the antibiotics. The leveling off in the density of bacteria is a consequence of persistence [47] [48, 49].

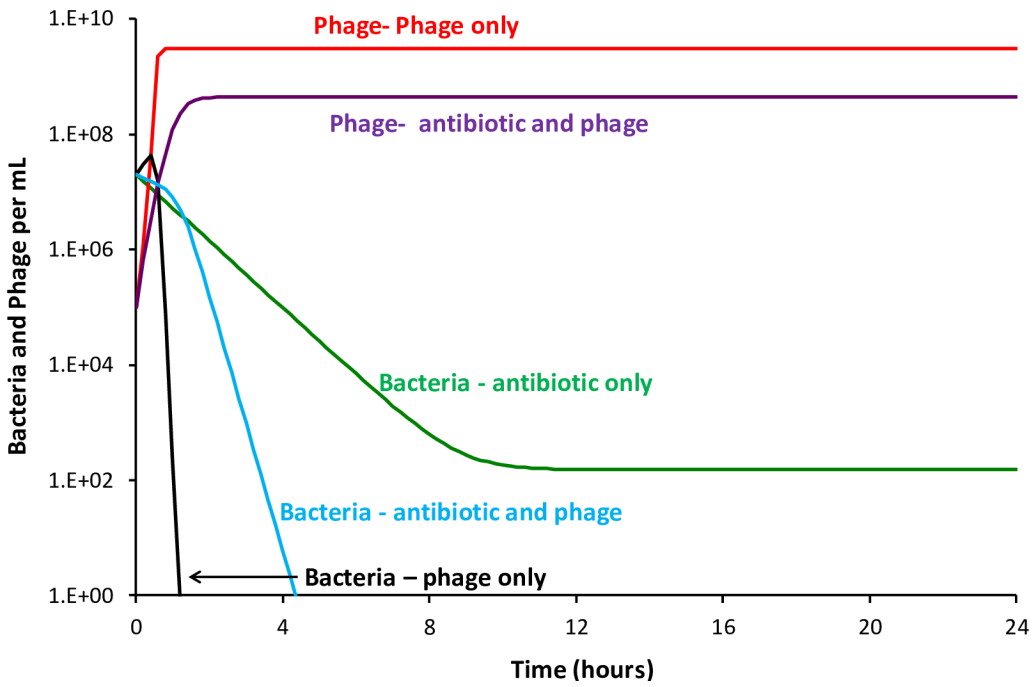

Figure S3 Simulation results: changes in the densities of bacteria and phage with different treatments. Parameter values. v_{SMAX}=2.0, v_{SMIN} -2.0, κ=1.0, MIC=1.0, e=5x10^{-7}, k=1.0, x=y=10^{-5}, δ_P=10^{-8}, δ_S=10^{-8}, β=50, A =2 μg/ml (no decay), r(0)=1000.

V. Persistence and heteroresistance

In the absence of phage in serial transfer culture, the super MIC concentration of oxacillin does not eliminate the bacteria but rather maintains the density at levels markedly lower than the antibiotic-free controls (Figure 5 A center). We postulate that this can be attributed to persistence in serial transfer culture. In the absence of phage, ciprofloxacin initially reduces the density of *S. aureus* but in subsequent transfers, the density of these bacteria return to levels within the range anticipated for antibiotic-free controls (Figure 5A bottom). We postulated that these pharmacodynamics can be attributed to heteroresistance [33]. In the following, we present the evidence for persistence for oxacillin-treated cultures and heteroresistance for ciprofloxacin-treated cultures. Using a mathematical - computer simulation model, we will illustrate how these postulated mechanisms can account for the observed pharmacodynamics of these drugs with *S. aureus* Newman.

A model for antibiotics in serial transfer culture with persistence and heteroresistance

There are three populations of bacteria, wild type, heteroresistant, and persisters with designations and densities, W, H, and P cells per ml; a single antibiotic of concentration, A μg/ml; and a limiting resource of concentration r μg/ml. As in our model of the joint action of antibiotics and phage (II), we assume that the rate of growth, of bacteria of each of these states, $\psi_i(A,r)$ is a product of a Hill function for the antibiotic [47], and a Monod function for the resource [45].

$$\psi_i(A,r) = \left[v_{iMAX} - \left[(v_{iMAX} - v_{iMIN}) \bullet \left(\frac{\left(\frac{A}{MIC_i}\right)^{\kappa}}{\left(\left(\frac{A}{MIC_i}\right)^{\kappa} - \left(\frac{v_{iMIN}}{v_{iMAX}}\right) \right)} \right) \right] \right] \bullet \frac{r}{(r+k)}$$

The subscript i represents the bacterial population, W, H, or P. For strain i, v_{iMAX} (>0) is the maximum growth rate, v_{iMIN} (<0) the minimum growth rate/maximum kill rate, and MIC_i the minimum inhibitory concentration of the antibiotic. Bacteria of the same state have the same Hill coefficient and Monod constant, respectively κ and k.

In these simulations, we assume the persisters can replicate, $v_{PMAX}>0$, and are resistant to the antibiotics, $v_{PMIN}=0$. As in [33], the MIC for the antibiotic of the heteroresistant cells is greater than the sensitive, $MIC_H > MIC_S$. There is a transition from W to P and from P to W respectively at rates, x and y per cell per hour, and a transition from W to H and H to W at rates, x_H, and y_H per cell per hour. Finally, we assume that the concentration of the antibiotic can decline at a rate d per hour. With these definitions and assumptions, the rates of change in the densities of the bacterial population and concentration of the antibiotic and resource are given by:

$$\frac{dr}{dt} = -\psi(r) \bullet e \bullet (W \bullet v_{MAXW} + H \bullet v_{MAXH})$$

$$\frac{dW}{dt} = \psi_W(A,r) \bullet W - x \bullet W + y \bullet P - x_H \bullet W + y_H \bullet H$$

$$\frac{dP}{dt} = v_{MAXP} \bullet \psi(r) \bullet P + x \bullet W - y \bullet P$$

$$\frac{dH}{dt} = \psi_H(A,r) \bullet H + x_H \bullet W - y_H \bullet H$$

$$\frac{dA}{dt} = -d \bullet A$$

$$where\ \psi(r) = \frac{r}{(r+k)}$$

Numerical solutions: To solve these equations, we simulate the changes in the densities of the bacterial populations and changes in the concentration of the antibiotics with

Berkeley Madonna. As in our experiments, in these simulations every 24 hours the density of the bacteria is reduced by a factor of 100, and new resources and antibiotics are added.

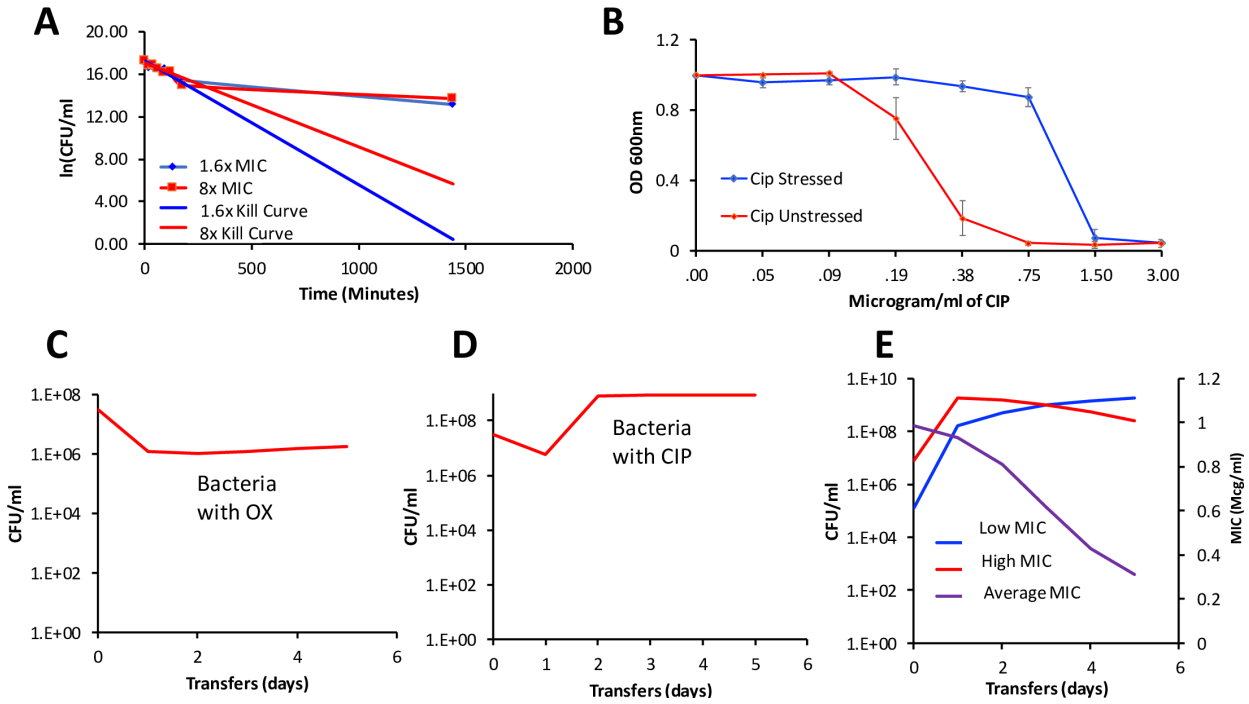

Figure S4 Antibiotic *S. aureus* pharmacodynamics **A)** Natural log of the change in density of growing cultures of *S. aureus* Newman exposed to 1.6X and 8X MIC, low and high for 24 hours (1440 minutes). The lines with the data points are experimental changes in density estimated by plating; while, the lines without data points are the results of a linear regression of the changes in density estimated during the first 180 minutes of exposure. **B)** Changes in the optical density of experimental cultures of *S. aureus* Newman exposed to 5µg/ml ciprofloxacin, mean and standard error of the ODs for three replicas. In orange are cells that were treated with ciprofloxacin and then transferred without the presence of this drug for seven days before the MIC determination was performed. In blue are cells that were treated with ciprofloxacin for one week before the MIC was performed. **C, D, E)** Simulation Results Common parameters: v_{MAXS}=1.5, v_{MINS}=-0.6 ,v_{MAXSR} = 1.2 , v_{MINSR}=-3 , ks=1 ,kh=1 ,k=1 ,e=5.0E-7 **C)** Changes in the densities of bacteria in serial transfer culture with persistence with parameters in the range estimated for *S. aureus* in oxacillin. Specific parameters: vp=0.2, MIC_S= 1.8, x_P=1.0e-4, y_P=1E-3, A_{MAX}=3. Exposure to oxacillin does not start until the second transfer (compare to Figure 5A center). **D) and E)** – Heteroresistance for ciprofloxacin MIC_S= 0.218, MIC_H=1, x= 1E-6, y=1e-3, x_P=0, y_P=0, A_{MAX}=0.5 Changes in the densities of bacteria in serial transfer culture with heteroresistance with parameters in the range estimated for *S. aureus* in ciprofloxacin. Exposure to ciprofloxacin doesn't start until the second transfer (compare to Figure 5A bottom). **E)** Unique Parameters for E: MIC_S= 0.218, MIC_H=1, x=1E-6, 1E-3 x_P=0, y_P=0. Simulation of the changes in the densities bacteria and average MIC of the antibiotic Mixture of the low MIC sensitive and the high MIC heteroresistant populations in serial transfer culture in the absence of the antibiotics.

In Figure S4A, we present the results of time-kill experiments with *S. aureus* Newman exposed to oxacillin. The observation that the rate of decline in the viable density of sensitive cells during the first few hours of exposure considerably exceeds the rate of decline later in the experiment is what would be anticipated for persistence. In Figure S4B, we present the evidence for heteroresistance; higher concentrations of ciprofloxacin are needed to kill cultures initiated with bacteria exposed to ciprofloxacin than to kill bacteria cultured in the absence of this drug. If we allow for persistence with parameters in the range estimated for oxacillin the predicted changes in the density of *S. aureus* Newman in serial transfer culture with oxacillin are similar to those observed (compare Figures S4A and S4C). If we allow for heteroresistance with the parameter in the range estimated for *S. aureus* Newman in serial transfer culture with ciprofloxacin the results are similar to those observed (compare figures S4B and S4D). One property of heteroresistance is that when the bacteria are removed from the drug, the resistant population will decline in frequency, and the average MIC will decline to levels similar to that of the original sensitive population [33], see Figure S4E.

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