Phage to the rescue: adjunct phage treatment enhances the effectiveness of low antibiotic dose against *Staphylococcus aureus* biofilms *in vitro*

James Dickey and Véronique Perrot
Department of Biology, Emory University
Atlanta, GA.

J.D.'s current address: Medical College of Georgia, Augusta, GA., *jadickey@augusta.edu*

V.P: corresponding author, *vperrot@emory.edu*

Abstract

Phage therapy is drawing more interest as antibiotic resistance becomes an ever more serious threat to public health. Bacterial biofilms represent a major obstacle in the fight against bacterial infections as they are inherently refractory to many types of antibiotics. Treating biofilms with phage has shown promise in a handful of experimental and case studies. However, quantification of the effect of phage combined with antibiotics is needed to pave the way for larger clinical trials. Here we explore the effect of using phage in combination with a total of nine antibiotics, applied simultaneously or as a pretreatment before antibiotics are applied to in vitro biofilms of *Staphylococcus aureus*. Most antibiotics alone were ineffective at low dose, but the addition of phage to treatment regimens led to substantial improvements in efficacy. At high dose, antibiotics alone were effective, and in most cases the addition of phage to treatment regimens did not improve efficacy. Using phage with rifampin was also extremely effective at reducing treatment failures due to the ascent of resistance to rifampin used alone.

Introduction

Bacteriophages (phages), viruses that kill bacteria, have been used to treat bacterial infections since shortly after their discovery just over a century ago [1-3]. While research into and use of phage as antimicrobial treatment persisted in a handful of countries, most notably in the Republic of Georgia, the widespread use of antibiotics completely displaced phage therapy in the rest of the world. In recent years, the rise of antibiotic resistance and the spread of pathogen strains resistant to most or all antibiotics [4, 5] has spurred a renewed interest in the use of phages as therapeutic agents [6-10]. There is a growing body of evidence demonstrating the effectiveness of phage and phage products for treating natural [11] as well as experimental infections in animal models (e.g.,[12-19]. Phage have also been used through the FDA Expanded Access, aka compassionate use, for critically ill human patients infected with bacteria that fail to respond to antibiotics, sometimes with mixed success [20] but occasionally, and quite recently, with impressive outcomes [21-23].

The successful use of phage to treat infections in animals and for compassionate use therapy in human patients is encouraging, and it justifies developing protocols for phage therapy in humans. As is the case for new chemotherapeutic agents, properly controlled clinical trials demonstrating safety and efficacy in human patients are essential. However, we believe that

clinical trials that implement solely phage for the treatment of infections that are otherwise amenable to antibiotic therapy will face difficult and possibly insurmountable regulatory hurdles [24-26]. Alternatively, one could conduct non-inferiority clinical trials comparing treatments combining antibiotics with phage against treatment with antibiotics alone, a method analogous to those required for the introduction of a new antibiotic. While there is little or no reason to anticipate adverse reactions to phage in systemic treatment [27, 28], safety would have to be demonstrated in human volunteers, and regulatory permission for such an enterprise may be too difficult to obtain to explore seriously for systemic infections. To circumvent this hurdle, one could start non-inferiority trials for topical, rather than systemic, treatments of infections and target infections of the skin and mucosa, such as burn wounds, diabetic ulcers, and sinusitis.

The advantage of treating topical infections with the superficial administration of phage and antibiotics over antibiotics alone extends beyond simple regulatory circumstances. Even when they are genetically sensitive to antibiotics, bacteria infecting skin and surfaces of tissues commonly form a biofilm, a polysaccharide matrix that can allow bacteria to be phenotypically refractory to antibiotics [29-32]. There is evidence that phage may break open biofilms, making the bacteria within more susceptible to antibiotics [33, 34]. Furthermore, there is *in vitro* experimental evidence that combinations of phage and antibiotics are more effective than antibiotics alone for killing biofilm populations of *Pseudomonas aeruginosa*, [35, 36] and *Staphylococcus aureus* [37]. Finally, antibiotics and phage may not only be pharmacodynamically synergistic, but evolutionarily synergistic as well. When used in combination, phage may prevent the ascent of antibiotic resistant minority populations, and antibiotics may conversely prevent the ascent of phage resistant bacteria [35, 36], although resistance to the phage does not seem to appear readily in *S. aureus*.

In this study, we expand upon the earlier work by Chaudhry et al [36] with *P. aeruginosa* by examining the joint action of antibiotics and phage for treating biofilms of the Gram positive and common skin pathogen *Staphylococcus aureus*. In addition to using this very different bacterium, we examine a broader range of antibiotics, including but not limited to drugs that are traditionally considered bacteriostatic. We implement these antibiotics against *S. aureus* biofilms at high and low concentrations as a sole treatment, applied simultaneously with phage, and used in sequence following phage treatment. We also explore the effectiveness of phage treatment to suppress the ascent of resistance to rifampin, an antibiotic to which resistance arises quite rapidly, in *S. aureus* biofilms.

The results of this study provide additional support for the potential of the combined use of phage and antibiotics for the treatment of topical infections. They suggest that when applied with phage, low doses of antibiotics can be as effective as higher doses of antibiotic applied alone, and that phage can prevent treatment failure due to the ascent of antibiotic resistance.

Materials and Methods

Bacteria and phage strains

All experiments used the bacterium *S. aureus* Newman. A single phage isolated from the commercially available Eliava PYO phage cocktail was used. The PYO phage cocktail is routinely use to treat various skin and wound infections In the Republic of Georgia, and contains phages targeted to *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Proteus* and *E. coli* [6].

Unlike *E. coli* or *P. aeruginosa* and their lytic phages, *S. aureus* does not seem to readily evolve resistance to its phage. We do not observe the appearance of bacteria resistant to the PYO phage in standard culture: resistant colonies do not appear on phage-treated lawns, nor do cultures of *S. aureus* with phage become turbid overnight, as is commonly observed with *E. coli* and *P. aeruginosa* and their phages.

Phage imaging and sequencing

The phage was imaged by the Robert P. Apkarian Integrated Electron Microscopy Core at Emory University (Figure 1). The left panel shows an intact virion, and the right panel shows a virion with its tail contracted, indicating that the phage we used is likely a member of the family *Myoviridae*. Phage DNA was extracted using the Phage DNA Isolation Kit #46500 from Norgen Biotek Corp., and sequenced by the Molecular Evolution Core of the Parker H. Petit Institute for Bioengineering and Bioscience at the Georgia Institute of Technology. The sequence was deposited in NCBU under the BioProject accession number PRJNA477834. It clusters with Staphylococcus virus G1 (accession number AY954969) published in [38].

Figure 1: Transmission Electron Microscopy images of the PYO phage. The images were taken on a JEOL JEM-1400 120kV TEM.

Culture and sampling media

S. aureus Newman was grown in Muller Hinton II (MHII) broth (Difco). Bacterial densities were determined by serial dilution in saline and plating on Lysogeny Broth (LB) hard (1.6%) agar. Phage densities were estimated by mixing the serially diluted culture with a lawn of *S. aureus* Newman (0.1mL of a 1 in 10 dilution of a fresh overnight culture), adding 1.5 mL of soft (0.65%) LB agar, and pouring the mixture on the surface of 1% agar LB plates. When added to biofilms, phage was added at ~4e6 pfu/mL, at an MOI of ~0.1.

Antibiotics and MIC estimation

Antibiotics were purchased from Sigma (gentamycin (GEN), oxacillin (OXA), vancomycin (VAN), and tetracycline (TET)), AppliChem (ciprofloxacin (CIP), rifampin (RIF)), TCI (daptomycin (DAP)), MP Biochemicals (erythromycin (ERM), and Chem-Impex International (linezolid (LIN)). The minimum inhibitory concentration (MIC) for each drug was determined by the standard two-fold dilution protocol [39]. The MICs for each drug were, in µg/mL: CIP, 0.1875; DAP, 2; GEN, 0.75; OXA, 0.1875; VAN, 1.5; ERM, 0.375; LIN, 1.5; TET, 0.1875; RIF, 0.01. Each antibiotic was used at 2×MIC and 10×MIC. We chose these antibiotics to have a broad range of

115 pharmacodynamic properties. The first eight antibiotics listed above are grouped by their
116 pharmacodynamic characteristics: in classic assays of killing dynamics in liquid cultures at super
117 MIC concentrations, CIP, DAP and GEN kill rapidly (fast-acting bactericidal antibiotics); OXA and
118 VAN kill more slowly (slow-acting antibiotics); and LIN, ERM and TET prevent growth
119 (bacteriostatic antibiotics) ([40] and unpublished data).

120 *Biofilm establishment and preparation, treatment, and sampling*

121 To establish biofilms of *S. aureus*, 24-well tissue culture plates (TPP; well diameter: 15 mm,
122 surface of the biofilm: 177 mm^2) were inoculated with 2 mL MHII broth with ~ 10^6 cfu/mL of an
123 overnight culture of *S. aureus* Newman. Plates were incubated at 37˚C without shaking for 72 h
124 to establish a biofilm on the bottom of the wells. After 72 h incubation, each well was prepared
125 by removing the media using an aspirator and washed twice with 2 mL saline. After removal of
126 the last saline wash, 1 mL of broth containing antibiotic (antibiotic-only controls), phage
127 (phage-only controls and first phase of sequential treatments), or both (simultaneous
128 treatments) or neither (untreated controls), was added to each well and incubated without
129 shaking for 48 h. In the case of sequential treatment (phage first, antibiotic later), phage was
130 added, plates incubated without shaking at 37˚C for 24 h, at which time the appropriate
131 amount of antibiotic (2x or 10x MIC) was added to each well in saline, and incubated without
132 shaking at 37˚C for an additional 24 h for a total treatment time of 48 h.

133 To estimate the density of bacteria and phage after the 48 h incubation, the biofilms at the
134 bottom of the wells were disrupted with the flat end of a sterile wooden stick; the walls were
135 not disrupted. The bacteria and phage in the wells were further mixed by taking up and
136 returning 100 µL with the pipette. The densities of bacteria and phage in these suspended
137 cultures were then estimated by serial dilution and plating. When desired, the density of
138 bacteria resistant to RIF was estimated by plating on plates containing 10xMIC of RIF. When
139 most of the bacteria were able to form colonies on RIF-containing plates the population was
140 classified as resistant; otherwise it was classified as sensitive.

141 The initial, pre-treatment densities of bacterial in the biofilms were estimated as above by
142 adding 1 mL of saline, rather than broth, to the 72 h prepared biofilms. Since the sampling of a
143 biofilm requires its disruption, each estimate of densities was taken from a different well. In
144 addition, the density of phage added to the well was estimated by plating.

145 *Experimental design and sample size*

146 For all antibiotics except RIF, each combination of a given antibiotic at a given concentration,
147 used alone or with phage (either simultaneously or sequentially) was run three separate times
148 with three replicate wells each time. Because of various mishaps, the final number of data
149 points for each combination was between 6 and 9. For all antibiotics except RIF, the antibiotic-
150 free controls (no treatment and phage-only controls) were pooled, with a total of between 16
151 and 30 wells each. Thus, the results presented in Figures 2 and 3 all have the same antibiotic-
152 free controls. Experiments with RIF were done at a later time, and were run two separate times
153 with 10 replicates each time for each combination of RIF at 2x or 10xMIC, used alone or with

154 phage (either simultaneously or sequentially). Additionally they had their own untreated and
155 phage only controls (a total of 6 wells for each control).

156 *Measure of the efficacy of treatment and statistical analyses*

157 For our experiments, we define an effective treatment as one that leads to a decrease in the
158 density of bacteria recovered from a well relative to the density before treatment (initial
159 density). In our protocol, we do not remove the treatment media before disrupting the biofilm.
160 Instead, the biofilm is mixed with the planktonic population for sampling at the end of
161 treatment. A net decrease in bacterial density of the culture after time 0 indicates that the
162 treatment was effective at reducing the bacterial density in the biofilm. In addition, a decrease
163 in culture density would also indicate that treatment prevented the outgrowth of a planktonic
164 cells. As could be expected, there is a 1 to 2 log increase in bacterial density when only broth is
165 added to the biofilm (untreated controls; see Figures 2 and 3, and Tables 1 and 2). Thus, if there
166 is no significant change in density after 48 h, the treatment does not reduce the bacterial
167 density, but it does prevent growth of the biofilm or the outgrowth of a planktonic cells, or
168 some combination of both.

169 To determine the statistical significance of the change in bacterial or phage density after 48 h
170 treatment, we performed 2-tailed t-tests comparing the initial density and the density after
171 treatment. For each antibiotic, we wanted to determine if some treatments were more
172 effective than others. To do this, we ran an analysis of variance including only the treatments
173 that were effective (i.e., treatments that led to a significant decrease in bacterial density
174 compared with initial densities). Similarly, we wanted to determine if some treatments led to
175 greater phage growth, so we ran an analysis of variance including only the treatments that lead
176 to significant phage growth.

177 All statistical analyses were performed on log-transformed bacterial or phage densities. All
178 analyses were done using R in its RStudio incarnation [41]. In Figures 2, 3, and 4, the results are
179 represented by standard box-and-whisker plots, where in each box the black line shows the
180 median density of bacteria and phage, the box illustrates the range for 50% of the data (IQR),
181 and the whiskers show 99.3% of the data.

182

183 **Results**

184 **1 - Combination treatment with phage and antibiotics**

185 In this experiment, we measured the bacterial (and phage, where applicable) densities in
186 prepared biofilms of *S. aureus* before (initial density, shown as 0h on Figures 2 and 3) and after
187 48 hours of treatment with combinations of phage and antibiotics. As controls, we also
188 measured bacterial and phage densities after 48h of treatment with phage, antibiotics, or
189 neither (untreated control). As sampling is destructive, a set of wells were sacrificed to estimate
190 the initial density in biofilms at the time of treatment. The same initial density data is used for
191 all the 8 antibiotics in this experiment. In addition, we use the same data for the untreated and

192 phage-only controls for all 8 antibiotics. For the treatments with combination of phage and
193 antibiotic, we considered two regimens: simultaneous treatment (SIM), in which phage and
194 antibiotic were added together to the culture at time 0, and sequential treatment (SEQ), in
195 which the phage was added at time 0 and the antibiotics added 24 hours later. For the
196 sequential treatment, we estimated the density of phage and bacteria after 24 hours of
197 exposure to phage to ascertain the effect of the antibiotic on phage-treated cultures.

198 In Figure 2, we present the results for the cyclic peptide DAP. The results of the 8 antibiotics
199 tested here are presented in Figure 3 and summarized in Table 1. The format of Figure 3 is
200 identical to that described in the following for DAP.

201 **Figure 2: Combined action of daptomycin and phage**. Top panel: bacterial densities;
202 bottom panel: phage densities. Blue boxes: treatment with 2×MIC of the antibiotic; red
203 boxes: treatment with 10×MIC of the antibiotic. Densities after 48h of combined
204 antibiotic and phage treatments are shown in the shaded columns. In this figure and in
205 Figure 3, we use the same controls (the left side of the figure): (i) untreated, or treated
206 with phage only (same data for all 8 antibiotics), and (ii) treated only with the noted
207 antibiotic, daptomycin in this figure. The results for the simultaneous treatment (SIM)
208 with phage and 2× and 10×MIC daptomycin are shown in the middle shaded column.
209 The sequential treatment (SEQ), where the cultures were first treated with phage for
210 24 h then treated with the antibiotic for another 24 h, the bacterial and phage densities
211 at 48 h are shown on the right shaded column. The bacterial and phage densities after
212 24 h exposure to phage are shown between the two shaded columns. The dashed lines
213 across the panels indicate the initial densities of bacteria and phage. The short,
214 horizontal broken lines in the 3 columns to the right for the sequential treatment
215 denote the densities of bacteria (top panel) and phage (bottom panel) after 24 h
216 exposure to phage. The significance levels of the two-tailed t-tests comparing the
217 densities at 48 h with initial densities and with densities at the time antibiotic was
218 added in SEQ treatment are as follows: ***: $p<0.00001$, **: $p<0.001$, *: $p<0.05$; · :$p<0.1$
219 and NS: $p>0.1$.

220 In untreated controls, the viable cell density of *S. aureus* increased by 15 fold relative to the
221 initial density. In the phage-only controls, the cell density was reduced 33 fold, and the phage
222 density increased by 100 fold. At 2×MIC, DAP alone was not effective in reducing the viable cell
223 density of bacteria. DAP at 10×MIC and simultaneous and sequential treatments at both
224 antibiotic concentrations were effective in significantly reducing the viable cell density of *S.*
225 *aureus* in culture, and not significantly different from each other (second line of top panel of
226 Table 1). DAP added at both concentrations after 24 h of phage treatment (SEQ) was able to
227 further reduce the bacterial density: the blue and red boxes in the right shaded column are
228 significantly below the dashed line showing the bacterial density after 24 h of phage exposure.
229 This observation holds even when the antibiotic was added at 2×MIC, a concentration at which
230 DAP alone was unable to prevent the outgrowth of an intact biofilm.

231 There was a significant increase in phage density in all the combined therapy experiments with
232 DAP (second line of the bottom panel of Table 1). On the other hand, the increase in phage
233 density in simultaneous treatment with DAP was significantly less than in the absence of this
234 antibiotic. With sequential treatment, the density of phage increased to roughly that observed
235 in the absence of this antibiotic.

236 **Figure 3: Combined action of 8 different antibiotics and phage**. For each antibiotic: top
237 panel, bacterial densities; bottom panel, phage densities; densities after 48 h of
238 combined antibiotic and phage treatments are shown in the shaded columns; initial
239 densities and controls are shown in the left for comparison, and densities after 24 h of
240 phage treatment are shown for sequential treatment (SEQ). See Figure 2 for more
241 details.

242 **Table 1: Summary of the effects of different treatments on the densities of *S. aureus***
243 **and phage.** This table shows the changes in bacterial density (top panel) and phage density
244 (bottom panel) over 48 h compared with initial densities for 8 antibiotics and the phage and
245 antibiotic treatments on the right, and for the various controls on the left (no Tx=untreated; ø=
246 phage only; ab only=antibiotic only). Cells in green hues show a decrease compared with initial
247 densities; cells in red hues show an increase; the intensity of the colors indicates the magnitude
248 of the change in density (the more intense the color, the greater the change). The number in
249 each cell indicates the magnitude of the change, and the stars indicate the level of significance
250 of the t-test to determine if the change was significant, as follows: ***: $p<0.00001$; **: $p<0.001$;
251 *: $p<0.05$; · : $p<0.1$; NS: $p\geq0.1$.

252 In the top panel, green cells indicate that the treatment was effective. Considering only effective
253 treatments, the letters in the green cells indicate the groups of significance within each line: for
254 a given antibiotic, values with the same letter are not significantly different from each other, but
255 values with different letters are.

256 In the bottom panel, red cells indicate that the phage increased in density. Considering only
257 treatments that led to a significant increase in phage, letters in the red cells indicate the groups
258 of significance within each line: for a given antibiotic, values with the same letter are not
259 significantly different from each other, but values with different letters are.

260 In the following, we summarize the results of these experiments

261 <u>(1) Untreated and phage-only controls</u>

262 (i) In absence of phage or antibiotics, the density of bacteria increased by 15 fold.

263 (ii) Phage reduced the viable cell density in the culture by 33-fold, and the density of phage
264 increased by two orders of magnitude

265 <u>(2) Antibiotic-only controls</u>

266 (i) In the absence of phage and at 2×MIC, OXA was the only antibiotic that effectively killed *S.*
267 *aureus*; it reduced the viable cell density in the culture by nearly 50 fold. None of the seven

268 other antibiotics at 2×MIC significantly reduced the viable cell density of *S. aureus* in the
269 culture.

270 (ii) At 10×MIC, all eight antibiotics significantly reduced the density of *S. aureus* in the culture.
271 This included the officially bacteriostatic antibiotics (ERM, LIN and TET), which are expected to
272 prevent growth rather than kill. CIP and gentamicin were more effective than phage alone,
273 while VAN less effective than phage alone. The other five antibiotics were not significantly more
274 effective than phage alone.

275 <u>(3) Antibiotics and phage — Simultaneous treatment</u>

276 (i) In combination with 2×MIC of all 8 antibiotics, simultaneous treatment with phage reduced
277 the viable cell density of bacteria. Adding phage did not improve the performance of the
278 already effective OXA. For CIP and TET, the efficacy of the combination (CIP+Φ: 140; TET+Φ: 99,
279 Table 1) was significantly greater than that of phage acting alone (33). Since these antibiotics
280 alone at 2×MIC were unable to kill bacteria (CIP) or prevent outgrowth (TET), this is a case of
281 synergy, where the efficacy of combination of phage and antibiotic is significantly greater than
282 the sum of the individual efficacies. For the other six antibiotics, the efficacy of phage alone and
283 phage with 2×MIC of the antibiotic are not significantly different.

284 (ii) There was significant phage growth when antibiotics were added with phage at 2×MIC for all
285 antibiotics except GEN, although the phage increased less in the presence of most antibiotics
286 than in the phage-only control. Only OXA had no effect on phage growth.

287 (iii) Simultaneous treatment with phage and 10×MIC of all 8 antibiotics was effective at
288 reducing bacterial densities in the cultures, but not significantly more effective than the
289 antibiotic alone at 10×MIC for 7 out of the 8 antibiotics. For TET, adding phage actually reduced
290 the efficacy of the antibiotic. When comparing with phage alone, CIP and GEN at 10×MIC with
291 phage were more effective. There was antagonism between VAN and TET at 10×MIC and the
292 phage: the efficacy of the combination (VAN+Φ: 4.9; TET+Φ: 8.5; Table 1) was significantly
293 smaller than that of the most effective agent (phage acting alone: 33) were less effective.
294 Adding 10×MIC of the remaining four antibiotics to treatment with phage did not increase
295 treatment efficacy.

296 (iv) When added at 10×MIC simultaneously with phage, most antibiotics either prevented
297 phage growth (CIP, VAN, TET) or led to massive decreases in phage density (GEN, ERM, LIN).
298 Only DAP and OXA allowed some modest phage growth.

299 <u>(4) Antibiotics and phage — Sequential vs. simultaneous treatment.</u>

300 (i) For all antibiotics, treatments with phage first, then antibiotic at either concentration were
301 effective at reducing bacterial density. For VAN, ERM and TET, treatment with 10×MIC was
302 significantly less effective than treatment with 2×MIC, while for the other five antibiotics there
303 was no significant difference between the antibiotic concentrations.

304 (ii) The antibiotics that were not able to prevent bacterial growth of intact biofilms (antibiotic-
305 only controls) at 2×MIC, that is, all antibiotics but OXA, were effective at reducing the bacterial
306 density of cultures treated with phage for 24 h.

307 (iii) For CIP, DAP and LIN, simultaneous treatment with 2× or 10×MIC of the antibiotic was as
308 effective as sequential treatment at either antibiotic concentration.

309 (iv) GEN and OXA were more effective when added to phage treated cultures (sequential
310 treatment) than when added at the same time as phage (simultaneous treatment).

311 (v) VAN and TET showed a similar pattern: when used in the presence of phage in both
312 treatment regimens (SIM and SEQ), using the high antibiotic concentration led to significantly
313 less reduction in bacterial density. For TET, the most effective treatment was with 2×MIC with
314 both treatment regimens. For VAN, the most effective treatment was sequential treatment
315 with 2×MIC. Similarly, ERM was most effective when used in sequential treatment at 2×MIC.

316 (vi) When antibiotics were added in sequential treatment (after 24 h of phage exposure), the
317 phage had grown by two orders of magnitude. There was generally very little change in phage
318 density after antibiotics were added at either concentration, as can be seen in Figure 3, bottom
319 panel, right shaded column. The only exception was GEN, where there was a small but
320 significant decrease in phage density when the antibiotic was added at 2×MIC, and a larger
321 decrease when added at 10×MIC.

322 **2 - Phage preventing the ascent of antibiotic resistance**

323 We ran experiments similar as those described above with the additional goal of monitoring the
324 appearance of resistance to the antibiotic RIF. RIF is a potent killer of *S. aureus* at very low
325 concentrations (MIC=0.01 µg/mL), but it is rarely if ever used in the clinic because resistance to
326 the antibiotic emerges very quickly. In our hands, an overnight culture of *S. aureus* treated with
327 RIF at super-MIC concentrations is turbid by morning and the majority population resistant to
328 RIF, a phenomenon we never observed for any other antibiotics. We modified the design of the
329 experiment to include larger sample sizes (a total of 20 wells for each treatment group). We
330 never observed the appearance of resistance to the phage in all the experiments reported in
331 this paper, so we conclude that resistance to phage is not an issue at the time frame of a few
332 days in the biofilm cultures we used. The results are presented in Figure 4 and summarized in
333 Table 2.

334 **Figure 4. A.** Combined action of phage and RIF on SA biofilms. The legend is the same as
335 for Figures 2 and 3. 20 wells were used for antibiotic-only controls, and simultaneous
336 and sequential treatments, at each antibiotic concentration. The 2× and 10×MIC RIF-
337 only control data are highlighted in yellow: some wells became turbid over the 48h of
338 incubation, and some wells remained clear. The number of wells in each category is
339 indicated below the corresponding box. **B.** Resistance status of phage-free controls.
340 Bacterial densities are shown for each well, in solid orange dots for resistant wells and
341 open green triangles for sensitive wells. Note that some of the cultures dosed with

342 2×MIC of RIF reached high densities (>1e8 cfu/mL) even though they appear to be
343 sensitive to RIF.

344 **Table 2: Summary of the effects of different treatments on the densities of *S. aureus***
345 **and phage using RIF.** The top row of the table shows the changes in bacterial density
346 over 48 h compared with initial densities. For the RIF-only controls, the values for the
347 wells that did not become turbid are shown in the top row, and the values for the wells
348 that became turbid are shown on the second row. Otherwise, same legend as Table 1.

349 The work with RIF was done separately from that with the other 8 antibiotics, with its own set
350 of antibiotic-free controls. In this case, phage alone was able to prevent the net growth of
351 bacterial cultures, but unable to significantly reduce the bacteria below their initial densities.
352 Phage densities increased significantly, but slightly less than they did in the first experiment.

353 The RIF-only controls at both concentrations presented a pattern we didn't see in any of the
354 other 8 antibiotics: there were two, rather than one, qualitative outcomes. Some wells became
355 turbid, which is equivalent to treatment failure, with bacterial densities greater than 1e8
356 cfu/mL, and the other wells remained clear, with bacterial densities smaller than 1e8 cfu/mL.
357 Densities in the turbid wells were very close to the density in the untreated controls. For the
358 wells that remained clear (7 out of 19 wells for 2×MIC and 6 out of 18 wells for 10×MIC),
359 rifampin alone at 2×MIC did not significantly reduce the viable cell density of bacteria, as
360 observed with 7 of the other 8 antibiotics (OXA being the exception). RIF alone at 10×MIC was
361 effective at reducing bacterial density, albeit its efficacy was in the range of the weakly
362 bactericidal and bacteriostatic antibiotics (see Table 1).

363 We assessed the resistance status of the bacteria in the untreated wells, and wells treated with
364 RIF alone (Figure 4B). Not unexpectedly, the bacteria in the untreated wells were sensitive to
365 RIF, as were the bacteria in wells treated with RIF that remained clear. The dominant
366 populations of bacteria in the wells treated with 10xMIC RIF that became turbid were resistant,
367 as were most of the dominant populations in wells treated with 2×MIC RIF that became turbid.
368 In addition, two of such wells appeared sensitive, possibly because the bacteria were tested on
369 plates containing 10×MIC of the antibiotic. The dominant populations in these two wells may
370 have been resistant to 2×MIC but unable to form colonies in the presence of 10×MIC RIF.

371 When RIF was used simultaneously with phage, its effect was similar to that of RIF alone, but
372 without treatment failure. RIF at 2×MIC with phage did not lead to net growth or death, and RIF
373 at 10×MIC with phage had the same efficacy as RIF at 10×MIC alone when resistance did not
374 arise. RIF was the only antibiotic out of the 9 we tested where simultaneous treatment with
375 2×MIC of the antibiotic was not effective at reducing bacterial density.

376 When RIF was added to phage treated wells (sequential treatment), both concentrations
377 significantly reduced bacterial densities, with 10×MIC being significantly more effective than
378 2×MIC. As in simultaneous treatment, we did not observe any treatment failure in sequential
379 treatment. Overall, there were 17 treatment failures out of 37 wells treated with RIF only, and
380 none out of 80 wells treated with a combination of RIF and phage.

381

382 **Discussion**

383 Bacteria in biofilms are notoriously difficult to kill with antibiotics, and biofilms represent a
384 significant problem in treating many bacterial infections [29-31, 42, 43]. One of the most cited
385 factors contributing to this problem is the extracellular matrix that comprises the structure of
386 the biofilm [44, 45]. This polysaccharide matrix is thought to reduce the exposure of bacteria in
387 the biofilm to the antibiotic, resulting in bacteria that are exposed to a lower, and thus less
388 effective, dose of antibiotic.

389 Phages hold some promise in treating biofilm infections. As naturally occurring bacterial
390 viruses, phages may have evolved mechanisms to infect bacteria living in a biofilm [33, 46].
391 Indeed, direct effect of phage on biofilm structure has been demonstrated in *Klebsiella* [47], *P.*
392 *aeruginosa* [16, 48] and *S. aureus* [49]. Using phage alongside antibiotics may improve the
393 efficacy of conventional antibiotic treatment in topical infections, such as burns, skin ulcers, and
394 sinusitis. However, only a handful of experimental studies have quantified the interactions
395 between phage, antibiotics, and bacteria in biofilms. Chaudhry et al. [36] investigated how a
396 pair of phages improved the antimicrobial effects of five bactericidal antibiotics for the control
397 of biofilms of the Gram-negative bacterium *P. aeruginosa* in vitro. In general, they observed
398 that simultaneous use of phage and antibiotics improved the killing of bacteria for two
399 antibiotics (ceftazidime and colistin), while using antibiotics after phage pre-treatment
400 (sequential treatment) improved killing by another two antibiotics (GEN and tobramycin.
401 Kumaran et al. [37] studied the combined effect of five antibiotics, three bactericidal and two
402 bacteriostatic, and one phage on biofilms of *S. aureus*. In general, they find that the greatest
403 reduction in bacterial density in the biofilms is obtained when the biofilms are treated with
404 phage first for 24 h, then with antibiotics for 24 h (sequential treatment). However, Kumaran et
405 al. used very high concentrations of antibiotics, with two-fold increasing concentrations starting
406 at 8×MIC for VAN and LIN, 64×MIC for cefazoline and TET, and 256×MIC for dicloxacillin. This
407 large difference with the antibiotic concentrations we used (2 and 10×MIC) make direct
408 comparisons difficult.

409 This study initially served to extend the work of Chaudhry et al., as we sought to examine the
410 generality of the effectiveness of combined phage and antibiotic treatment against the
411 common skin pathogen and Gram positive *S. aureus*. As with the previous work, antibiotics
412 were applied at the same time as phage (simultaneous treatment) or after phage pre-treatment
413 (sequential treatment), but with a broader range of nine antibiotics that included some
414 bacteriostatic antibiotics. To allow us to fully gauge the effectiveness of our treatment regimens
415 in eliminating bacteria within a biofilm, we further modified Chaudhry et al.'s protocol by using
416 a different measure of efficacy. In our modified protocol, we estimated the bacterial density in
417 prepared biofilms before treatment, and after 48 h of exposure to antibiotic, phage, both, or
418 neither (untreated controls). Instead of comparing bacterial densities after treatment with
419 densities in untreated controls, we compared them with bacterial densities before treatment.
420 Our measure of treatment efficacy is thus the change in bacterial density during the course of

421 treatment. This more conservative estimate allows us to ascertain the ability of treatment
422 regimens to penetrate and kill bacteria within a biofilm. Samples taken after treatment
423 consisted of both biofilm and planktonic bacteria, whereas the samples taken at the time of
424 treatment consisted of only biofilm bacteria. Any treatment that resulted in lower bacterial
425 densities than were in biofilms before treatment must have been effective at penetrating and
426 killing bacteria within the biofilm. In addition, by estimating the bacterial density after 24 h of
427 phage treatment, we could estimate the efficacy of antibiotics on phage-treated biofilms (see
428 Figure 5 below).

429 Our results are consistent with that of Chaudhry et al, except that the improvement of killing
430 upon using phage applied only to low antibiotic doses: such doses would otherwise be
431 ineffective or marginally effective, but the inclusion of phage in these treatments led to
432 substantial killing of bacteria in *S. aureus* biofilms. This result held for simultaneous as well as
433 sequential phage treatments for seven out of the nine antibiotics we used, including the
434 bacteriostatic drugs. The exceptions are OXA, which was inherently effective at low dose, and
435 RIF, with which simultaneous treatment with 2×MIC was not effective at reducing bacterial
436 density. However, unlike the general findings of Chaudhry et al, increasing the dosage five-fold
437 led to no additional killing of bacteria when used with phage. An exception to this pattern is
438 GEN, where in our experiments, GEN was still qualitatively, albeit not always significantly, more
439 effective at high compared with low dose within each treatment protocol. In some cases,
440 increasing antibiotic concentration led to a decrease in the efficacy of treatment, such as with
441 VAN and TET in simultaneous treatments, and for VAN and ERM in sequential treatments. This
442 is in line with the observations of TOB against *P. aeruginosa* by Chaudhry et al.

443 However, Chaudhry et al. were only able to detect whether any treatment was an improvement
444 over untreated controls, and subsequently compare relative effectiveness between treatment
445 regimens. They could not detect actual reduction of bacteria within the biofilm itself. By
446 modifying the protocol, we are able to circumvent this limitation and quantify killing within the
447 biofilm, thus drawing conclusions about the efficacy of our regimens to penetrate and kill cells
448 within the biofilm.

449 Generally speaking, antibiotics at low dose (2×MIC) were unable to kill bacteria in biofilms while
450 the same antibiotics were able to kill at high dose (10×MIC). This observation interestingly held
451 for bacteriostatic as well as bactericidal antibiotics. Using antibiotics and phage at the same
452 time improved killing over antibiotics alone or phage alone in 2 out of 18 cases (9 antibiotics × 2
453 concentrations), that is, we can detect a synergistic effect between antibiotic and phage (the
454 efficacy of the combination of antibiotic and phage was greater than the sum of the efficacy of
455 each when acting separately) for CIP and TET at 2×MIC. There were two cases of antagonism,
456 VAN and TET at 10×MIC, where the efficacy of the combination was smaller than that of the
457 most effective agent acting alone. For all other cases (14 out of 18), the efficacy of the
458 combination was not significantly different from that of the most effective agent acting alone:
459 in some cases, the phage did most of the killing, in other cases it was the antibiotic. This would
460 indicate that at least in some situations, combined phage and antibiotic would be generally

more effective than antibiotic alone, not so much because of synergy between the two agents (only 2 in 18 cases) but because either agent alone is effective (12 out of 18 cases), and antagonism between the two is infrequent (2 out of 18 cases).

Figure 5: Change in bacterial density after adding antibiotics to intact or phage-treated biofilms. The change in bacterial density (log(final density) – log(initial density)) is shown for all 9 antibiotics used in this study at both antibiotic concentrations. The symbols indicate the difference in means, and the error bars indicate the 95% confidence interval obtained from t-tests. Values above the dashed line show an increase in bacterial density during the course of treatment; values below the dashed line a decrease. Antibiotics added to intact biofilms are shown in solid dots and solid lines (initial densities = bacterial densities in prepared biofilms, final densities = antibiotic-only controls); antibiotics added to biofilms exposed to phage for 24 h are shown in open triangles and dashed lines (initial densities = bacterial densities after 24 h of phage-only treatment, final densities = sequential treatment at 48 h).

The blue ovals highlight the 7 antibiotics used at 2×MIC that did not reduce the bacterial density when applied to intact biofilms (change in density at or above the dashed line) but significantly killed bacteria when added to phage-treated biofilms (change in density below the dashed line). The orange ovals highlight the 3 antibiotics where pretreatment with phage likely reduced the efficacy of 10×MIC antibiotic.

Our experimental design further allowed us to check if phage pre-treatment could alter the susceptibility of biofilm-bound bacteria to antibiotics. To answer this question, we estimated the efficacy of the antibiotics when added to intact biofilms (antibiotics-only controls), and when added to phage-treated biofilms (sequential treatment regimens). These efficacies are summarized in Figure 5. When used at 2×MIC, 7 out of 9 antibiotics showed a greatly increased efficacy against phage-treated biofilms compared with intact biofilms: for all but OXA and RIF, the antibiotic was able to kill effectively phage-treated biofilms, while the same concentration didn't kill intact biofilms (shown in blue ovals on Figure 5). OXA was able to kill intact biofilms, and RIF was not, but pre-treatment with phage did not change the efficacy of either antibiotic. When used at 10×MIC, there is little effect of phage pre-treatment on antibiotic efficacy. When there is little (CIP, LIN) or no (TET) overlap between confidence intervals, phage pre-treatment actually reduced the antibiotic efficacy (shown in orange ovals on Figure 5). In short, when it had a significant effect, phage pre-treatment improved the efficacy of low doses of antibiotics, but it decreased the efficacy of high doses of antibiotics. This result is particularly promising for antibiotics prone to toxic side effects, where keeping the dose low is desirable.

We also addressed the possibility that combining phage with antibiotics would reduce the appearance of antibiotic resistance. In the clinical setting, RIF is not used alone in treating infections as resistance commonly ascends during the course of treatment, leading to treatment failure. Indeed, we observed that about half of biofilms treated with RIF alone became turbid, the in vitro analog of treatment failure. To prevent treatment failure due to the ascent of resistance to RIF, RIF is used in combination with other antibiotics to treat infections

with *S. aureus* (see [50] for a review). Using phage and RIF, either at the same time or phage first, RIF second, did not increase the efficacy of the antibiotic, but it completely prevented treatment failures. This observation indicates that one could develop treatment regimens that incorporate phage with RIF in order to reduce the ascent of resistance, while simultaneously diminishing concern of antagonistic interactions between therapeutic agents.

As we described earlier, one of the more practical approaches to add phage to the arsenal of antimicrobials in the clinic is by joint topical application with antibiotics against surface infections in non-inferiority trials. Such surface infections occur in the form of biofilms, and our results provide evidence in support for the combined use of phage and antibiotics against *S. aureus* biofilms. Needless to say, these experiments were conducted in vitro on polystyrene plates, and further in vivo work is required to determine the efficacy of these treatments in real wounds.

Our results also offer evidence that when applied jointly with antibiotics, phage can suppress the ascent of antibiotic resistance that would otherwise have led to treatment failure. We submit that if joint application was successful against the resistance mutation-prone RIF, as it was our experiments, the benefits could hold for antibiotics with lower rates of resistance. Additionally, phage may provide other means to reduce the ascent of resistance. Combination treatment of phage and antibiotic, particularly when phage was used first and antibiotic second, restored efficacy to low dose antibiotics that would otherwise have been ineffective. By reducing the concentration of antibiotic, the force selecting for antibiotic resistance would be lower, thereby reducing the likelihood of selecting for highly resistant strains. Furthermore and for directly clinical reasons, it is often desirable to reduce the antibiotic dosage during the course of treatment, as different antibiotics are associated with toxic side effects or subject to contraindications. This study is particularly promising for such situations, as it demonstrates that when applied as an adjunct, phage can rescue the efficacy of low concentrations of antibiotics.

Acknowledgements and funding

This study was funded by NIH-NIGMS grant GM091875 to B.R Levin. The Robert P. Apkarian Integrated Electron Microscopy Core is subsidized by the Emory College of Arts and Sciences and the Emory University School of Medicine and is one of the Emory Integrated Core Facilities, and the JEOL JEM-1400 120kV TEM is supported by NIH grant S10 RR025679.

We thank A. Hanes for obtaining and donating the PYO phage cocktail, Robert Petit III for analyzing the sequencing data, Waqas Chaudhry for his expertise and consulting, Ingrid McCall for making the lab run smoothly, and Bruce Levin, kibbitzer-in-chief, for providing funding for and feedback on this study.

539 **References**

540 1. Twart, F., *An investigation on the nature of ultra-microscopic viruses.* Lancet, 1915. **186**(4814): p.
541 1241-1243.
542 2. d'Herelle, F., *Sur un microbe invisible antagoniste des bacilles dysentériques (On an invisible*
543 *microbe antagonistic to dysentery bacilli).* Comptes Rendus de l'Académie des Sciences, 1917.
544 **165**: p. 373-375.
545 3. d'Herelle, F., *The Bacteriophage and Its Belhavior*. 1926, Baltimore: Williams and Wilkins. 679.
546 4. O'Neill, J., *Tackling drug-resistant infections globally: final report and recommendation*, in
547 *Review on Antimicrobial Resistance*. 2016: London.
548 5. de Kraker, M.E., A.J. Stewardson, and S. Harbarth, *Will 10 Million People Die a Year due to*
549 *Antimicrobial Resistance by 2050?* PLoS Med, 2016. **13**(11): p. e1002184.
550 6. Abedon, S.T., et al., *Phage treatment of human infections.* Bacteriophage, 2011. **1**(2): p. 66-85.
551 7. Gorski, A., et al., *Phage Therapy: Combating Infections with Potential for Evolving from Merely a*
552 *Treatment for Complications to Targeting Diseases.* Front Microbiol, 2016. **7**: p. 1515.
553 8. Henein, A., *What are the limitations on the wider therapeutic use of phage?* Bacteriophage,
554 2013. **3**(2): p. e24872.
555 9. Shen, Y., et al., *A bacteriophage endolysin that eliminates intracellular streptococci.* Elife, 2016.
556 **5**.
557 10. Thandar, M., et al., *Novel Engineered Peptides of a Phage Lysin as Effective Antimicrobials*
558 *against Multidrug-Resistant Acinetobacter baumannii.* Antimicrob Agents Chemother, 2016.
559 **60**(5): p. 2671-9.
560 11. Hawkins, C., et al., *Topical treatment of Pseudomonas aeruginosa otitis of dogs with a*
561 *bacteriophage mixture: a before/after clinical trial.* Vet Microbiol, 2010. **146**(3-4): p. 309-13.
562 12. Smith, H.W. and M.B. Hugggins, *Successful treatment of experimental Escherichia coli infections*
563 *in mice using phage: its general superiority over antibiotics.* J. General Microbiology, 1982. **128**:
564 p. 307-318.
565 13. Bull, J.J., et al., *Dynamics of success and failure in phage and antibiotic therapy in experimental*
566 *infections.* BMC Microbiol, 2002. **2**: p. 35.
567 14. Biswas, B., et al., *Bacteriophage therapy rescues mice bacteremic from a clinical isolate of*
568 *vancomycin-resistant Enterococcus faecium.* Infect Immun, 2002. **70**(1): p. 204-10.
569 15. Ferreira, F.A., et al., *Comparison of in vitro and in vivo systems to study ica-independent*
570 *Staphylococcus aureus biofilms.* J Microbiol Methods, 2012. **88**(3): p. 393-8.
571 16. Alemayehu, D., et al., *Bacteriophages phiMR299-2 and phiNH-4 can eliminate Pseudomonas*
572 *aeruginosa in the murine lung and on cystic fibrosis lung airway cells.* MBio, 2012. **3**(2): p.
573 e00029-12.
574 17. Galtier, M., et al., *Bacteriophages to reduce gut carriage of antibiotic resistant uropathogens*
575 *with low impact on microbiota composition.* Environ Microbiol, 2016. **18**(7): p. 2237-45.
576 18. Roach, D.R., et al., *Synergy between the Host Immune System and Bacteriophage Is Essential for*
577 *Successful Phage Therapy against an Acute Respiratory Pathogen.* Cell Host Microbe, 2017.
578 **22**(1): p. 38-47.e4.
579 19. Waters, E.M., et al., *Phage therapy is highly effective against chronic lung infections with*
580 *Pseudomonas aeruginosa.* Thorax, 2017. **72**(7): p. 666-667.
581 20. Lang, G., et al., *Bacteriophage therapy of septic complications of orthopaedic surgery.* Rev Chir
582 Orthop Reparatrice Appar Mot, 1979. **65**(1): p. 33-7.
583 21. Schooley, R.T., et al., *Development and Use of Personalized Bacteriophage-Based Therapeutic*
584 *Cocktails To Treat a Patient with a Disseminated Resistant Acinetobacter baumannii Infection.*
585 Antimicrob Agents Chemother, 2017. **61**(10).

586 22. Jennes, S., et al., *Use of bacteriophages in the treatment of colistin-only-sensitive Pseudomonas*
587 *aeruginosa septicaemia in a patient with acute kidney injury-a case report.* Crit Care, 2017.
588 **21**(1): p. 129.
589 23. Chan, B.K., et al., *Phage selection restores antibiotic sensitivity in MDR Pseudomonas*
590 *aeruginosa.* Scientific Reports, 2016. **6**: p. 26717.
591 24. Rose, T., et al., *Experimental phage therapy of burn wound infection: difficult first steps.*
592 International Journal of Burns and Trauma, 2014. **4**(2): p. 66-73.
593 25. Pirnay, J.P., et al., *The phage therapy paradigm: pret-a-porter or sur-mesure?* Pharm Res, 2011.
594 **28**(4): p. 934-7.
595 26. Debarbieux, L., et al., *A bacteriophage journey at the European Medicines Agency.* FEMS
596 Microbiol Lett, 2016. **363**(2): p. fnv225.
597 27. Knouf, E.G., W.E. Ward, and et al., *Treatment of typhoid fever with type specific bacteriophage.* J
598 Am Med Assoc, 1946. **132**: p. 134-8.
599 28. Desranleau, J.M., *The treatment of typhoid fever by the use of Vi antityphoid bacteriophages; a*
600 *preliminary report.* Can J Public Health, 1948. **39**(8): p. 317-9.
601 29. Davies, D., *Understanding biofilm resistance to antibacterial agents.* Nat Rev Drug Discov, 2003.
602 **2**(2): p. 114-122.
603 30. Kirby, A.E., K. Garner, and B.R. Levin, *The relative contributions of physical structure and cell*
604 *density to the antibiotic susceptibility of bacteria in biofilms.* Antimicrob Agents Chemother,
605 2012. **56**(6): p. 2967-75.
606 31. Hall-Stoodley, L., J.W. Costerton, and P. Stoodley, *Bacterial biofilms: from the Natural*
607 *environment to infectious diseases.* Nat Rev Micro, 2004. **2**(2): p. 95-108.
608 32. Stewart, P.S. and M.J. Franklin, *Physiological heterogeneity in biofilms.* Nat Rev Microbiol, 2008.
609 **6**(3): p. 199-210.
610 33. Harper, D.R., et al., *Bacteriophages and Biofilms.* Antibiotics, 2014. **3**(3): p. 270-284.
611 34. Danis-Wlodarczyk, K., et al., *A proposed integrated approach for the preclinical evaluation of*
612 *phage therapy in Pseudomonas infections.* Sci Rep, 2016. **6**: p. 28115.
613 35. Torres-Barceló, C., et al., *A Window of Opportunity to Control the Bacterial Pathogen*
614 *Pseudomonas aeruginosa Combining Antibiotics and Phages.* PLOS ONE, 2014. **9**(9): p. e106628.
615 36. Chaudhry, W.N., et al., *Synergy and Order Effects of Antibiotics and Phages in Killing*
616 *Pseudomonas aeruginosa Biofilms.* PLoS One, 2017. **12**(1): p. e0168615.
617 37. Kumaran, D., et al., *Does Treatment Order Matter? Investigating the Ability of Bacteriophage to*
618 *Augment Antibiotic Activity against Staphylococcus aureus Biofilms.* Front Microbiol, 2018. **9**: p.
619 127.
620 38. Kwan, T., et al., *The complete genomes and proteomes of 27 Staphylococcus aureus*
621 *bacteriophages.* Proc Natl Acad Sci U S A, 2005. **102**(14): p. 5174-9.
622 39. EUCAST. *The European Comittee on Antimicrobail Susceptibility Testing -Clinical Breakpoints*.
623 2013; Available from: http://www.eucast.org/clinical_breakpoints/.
624 40. Johnson, P.J. and B.R. Levin, *Pharmacodynamics, population dynamics, and the evolution of*
625 *persistence in Staphylococcus aureus.* PLoS Genet, 2013. **9**(1): p. e1003123.
626 41. Team, R., *RStudio: Integrated Development Environment for R*. 2015, RStudio, Inc.: Boston, MA.
627 42. Bhattacharya, M., et al., *Prevention and treatment of Staphylococcus aureus biofilms.* Expert Rev
628 Anti Infect Ther, 2015. **13**(12): p. 1499-516.
629 43. Mah, T.F. and G.A. O'Toole, *Mechanisms of biofilm resistance to antimicrobial agents.* Trends
630 Microbiol, 2001. **9**(1): p. 34-9.
631 44. Colvin, K.M., et al., *The pel polysaccharide can serve a structural and protective role in the*
632 *biofilm matrix of Pseudomonas aeruginosa.* PLoS Pathog, 2011. **7**(1): p. e1001264.
633 45. Khan, W., et al., *Aminoglycoside resistance of Pseudomonas aeruginosa biofilms modulated by*
634 *extracellular polysaccharide.* Int Microbiol, 2010. **13**(4): p. 207-12.

635 46. Abedon, S.T., *Ecology of Anti-Biofilm Agents I: Antibiotics versus Bacteriophages.*
636 Pharmaceuticals, 2015. **8**(3): p. 525-558.
637 47. Verma, V., K. Harjai, and S. Chhibber, *Structural changes induced by a lytic bacteriophage make*
638 *ciprofloxacin effective against older biofilm of Klebsiella pneumoniae.* Biofouling, 2010. **26**(6): p.
639 729-37.
640 48. Kim, S., et al., *Pseudomonas aeruginosa bacteriophage PA1O requires type IV pili for infection*
641 *and shows broad bactericidal and biofilm removal activities.* Appl Environ Microbiol, 2012.
642 **78**(17): p. 6380-5.
643 49. Rahman, M., et al., *Characterization of induced Staphylococcus aureus bacteriophage SAP-26*
644 *and its anti-biofilm activity with rifampicin.* Biofouling, 2011. **27**(10): p. 1087-93.
645 50. Forrest, G.N. and K. Tamura, *Rifampin combination therapy for nonmycobacterial infections.* Clin
646 Microbiol Rev, 2010. **23**(1): p. 14-34.

647

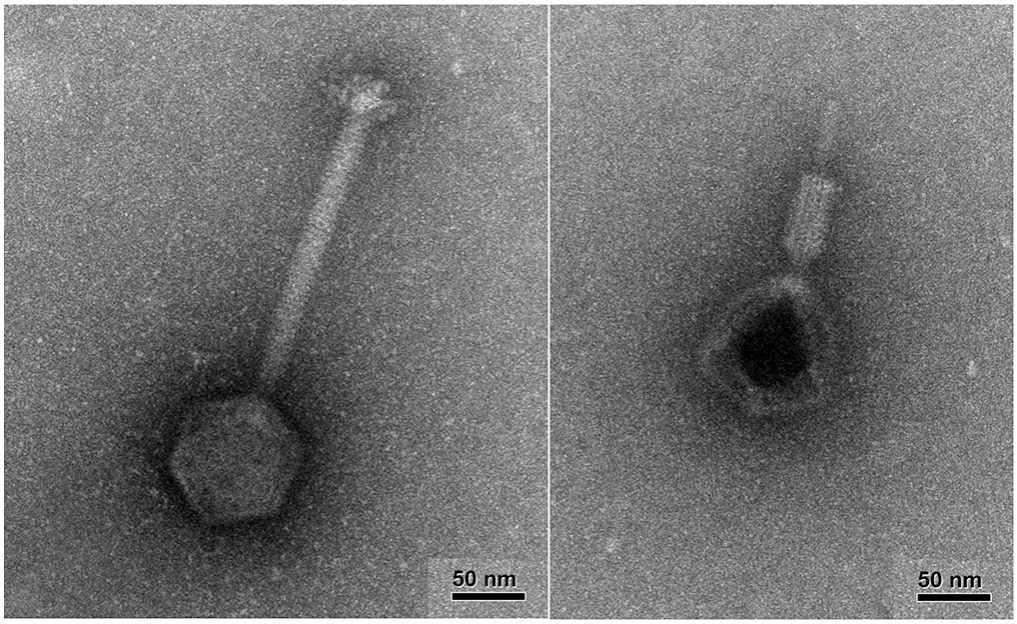

Figure 1: Transmission Electron Microscopy images of the PYO phage. The images were taken on a JEOL JEM-1400 120kV TEM.

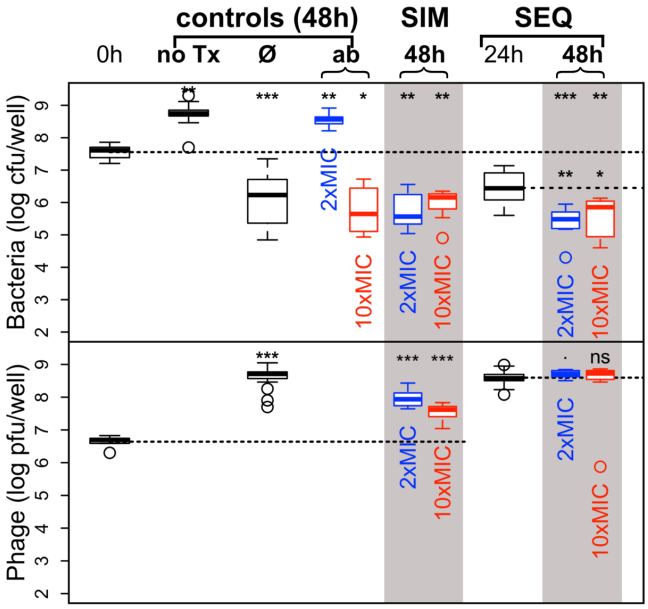

Figure 2: Combined action of daptomycin and phage. Top panel: bacterial densities; bottom panel: phage densities. Blue boxes: treatment with 2×MIC of the antibiotic; red boxes: treatment with 10×MIC of the antibiotic. Densities after 48h of combined antibiotic and phage treatments are shown in the shaded columns. In this figure and in Figure 3, we use the same controls (the left side of the figure): (i) untreated, or treated with phage only (same data for all 8 antibiotics), and (ii) treated only with the noted antibiotic, daptomycin in this figure. The results for the simultaneous treatment (SIM) with phage and 2× and 10×MIC daptomycin are shown in the middle shaded column. The sequential treatment (SEQ), where the cultures were first treated with phage for 24 h then treated with the antibiotic for another 24 h, the bacterial and phage densities at 48 h are shown on the right shaded column. The bacterial and phage densities after 24 h exposure to phage are shown between the two shaded columns. The dashed lines across the panels indicate the initial densities of bacteria and phage. The short, horizontal broken lines in the 3 columns to the right for the sequential treatment denote the densities of bacteria (top panel) and phage (bottom panel) after 24 h exposure to phage. The significance levels of the two-tailed t-tests comparing the densities at 48 h with initial densities and with densities at the time antibiotic was added in SEQ treatment are as follows: ***: $p<0.00001$, **: $p<0.001$, *: $p<0.05$; · :$p<0.1$ and NS: $p>0.1$.

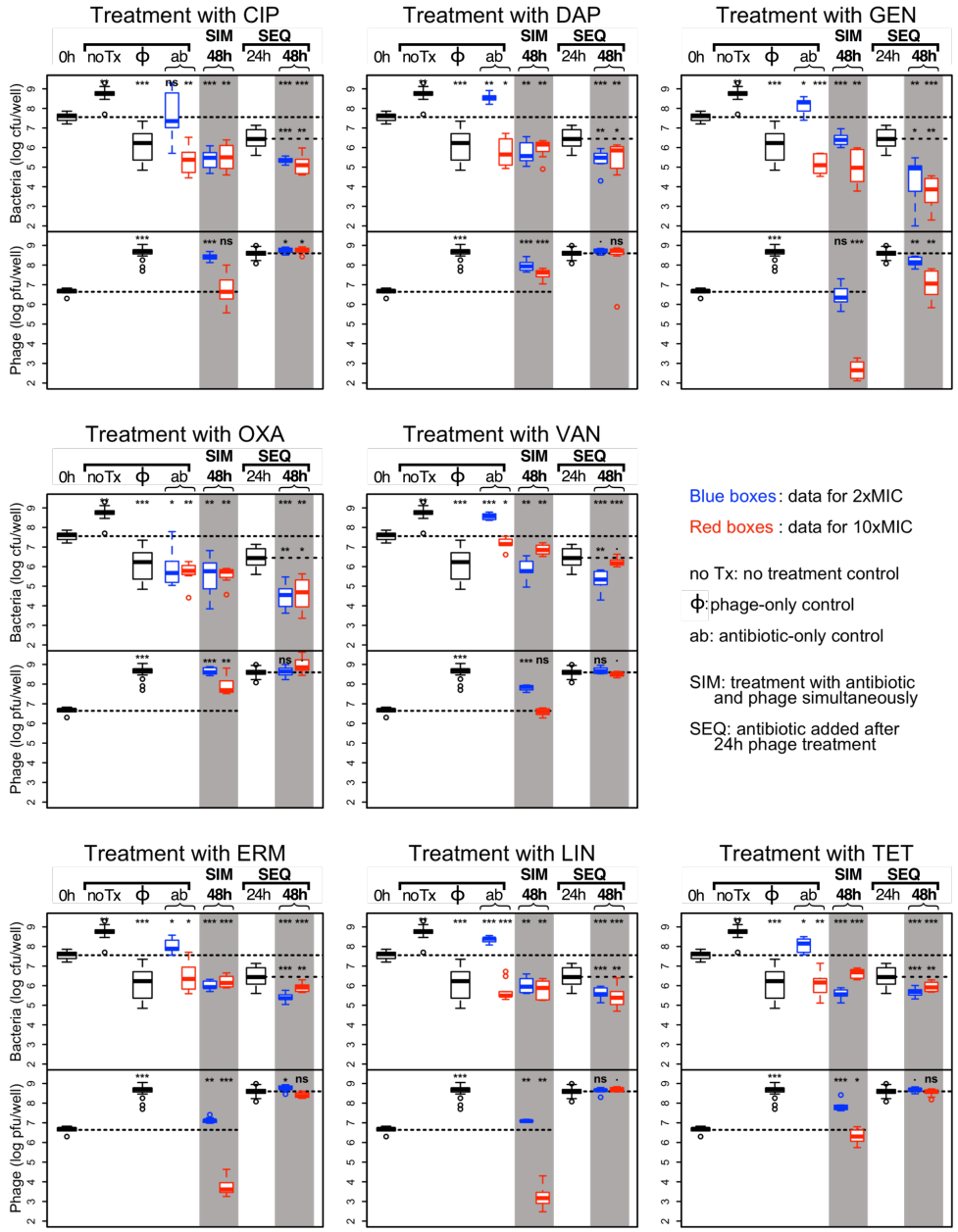

Figure 3: Combined action of 8 different antibiotics and phage. For each antibiotic: top panel, bacterial densities; bottom panel, phage densities; densities after 48 h of combined antibiotic and phage treatments are shown in the shaded columns; initial densities and controls are shown in the left for comparison, and densities after 24 h of phage treatment are shown for sequential treatment (SEQ). See Figure 2 for more details.

		controls							
		no Tx	ϕ	ab only		SIM		SEQ	
				2xMIC	10xMIC	2xMIC	10xMIC	2xMIC	10xMIC
Bacteria – Highly Cidal	CIP	15***	33*** b	1.3ns	160** a	140*** a	110** a	160*** a	270*** a
	DAP	15***	33*** a	10**	64* a	66** a	39** a	150*** a	100** a
	GEN	15***	33*** c	3.8*	260*** b	14*** c	360** b	1500** a	6700*** a
Bacteria – Weakly Cidal	OXA	15***	33*** b	46* b	73** b	100** b	100** b	1200*** a	900** a
	VAN	15***	33*** b	10***	2.4* c	48** b	4.9** c	180*** a	21*** b
Bacteria – Static	ERM	15***	33*** b	3*	13* b	36*** b	22*** b	140*** a	43*** b
	LIN	15***	33*** a	6.2***	67*** a	35** a	55** a	87*** a	140*** a
	TET	15***	33*** b	3.4*	26** b	99*** a	8.5*** c	78*** a	39*** b
Phage – Highly Cidal	CIP		100*** a			60*** b	1.2ns	130*** a	130*** a
	DAP		100*** a			21*** b,c	8*** c	120*** a	53* a,b
	GEN		100*** a			1.6ns	9500***	34*** b	2.4ns
Phage – Weakly Cidal	OXA		100*** a			100*** a	20** b	98*** a	210*** a
	VAN		100*** a			15*** b	1.1ns	110*** a	72*** a
Phage – Static	ERM		100*** a			3.1** b	740***	130*** a	5.4ns
	LIN		100*** a			2.8** b	2500**	98*** a	110*** a
	TET		100*** a			15*** b	2.1*	110*** a	79*** a

Table 1: Summary of the effects of different treatments on the densities of *S. aureus* and phage.

This table shows the changes in bacterial density (top panel) and phage density (bottom panel) over 48 h compared with initial densities for 8 antibiotics and the phage and antibiotic treatments on the right, and for the various controls on the left (no Tx=untreated; ø= phage only; ab only=antibiotic only). Cells in green hues show a decrease compared with initial densities; cells in red hues show an increase; the intensity of the colors indicates the magnitude of the change in density (the more intense the color, the greater the change). The number in each cell indicates the magnitude of the change, and the stars indicate the level of significance of the t-test to determine if the change was significant, as follows: ***: $p<0.00001$; **: $p<0.001$; *: $p<0.05$; · : $p<0.1$; NS: $p\geq0.1$.

In the top panel, green cells indicate that the treatment was effective. Considering only effective treatments, the letters in the green cells indicate the groups of significance within each line: for a given antibiotic, values with the same letter are not significantly different from each other, but values with different letters are.

In the bottom panel, red cells indicate that the phage increased in density. Considering only treatments that led to a significant increase in phage, letters in the red cells indicate the groups of significance within each line: for a given antibiotic, values with the same letter are not significantly different from each other, but values with different letters are.

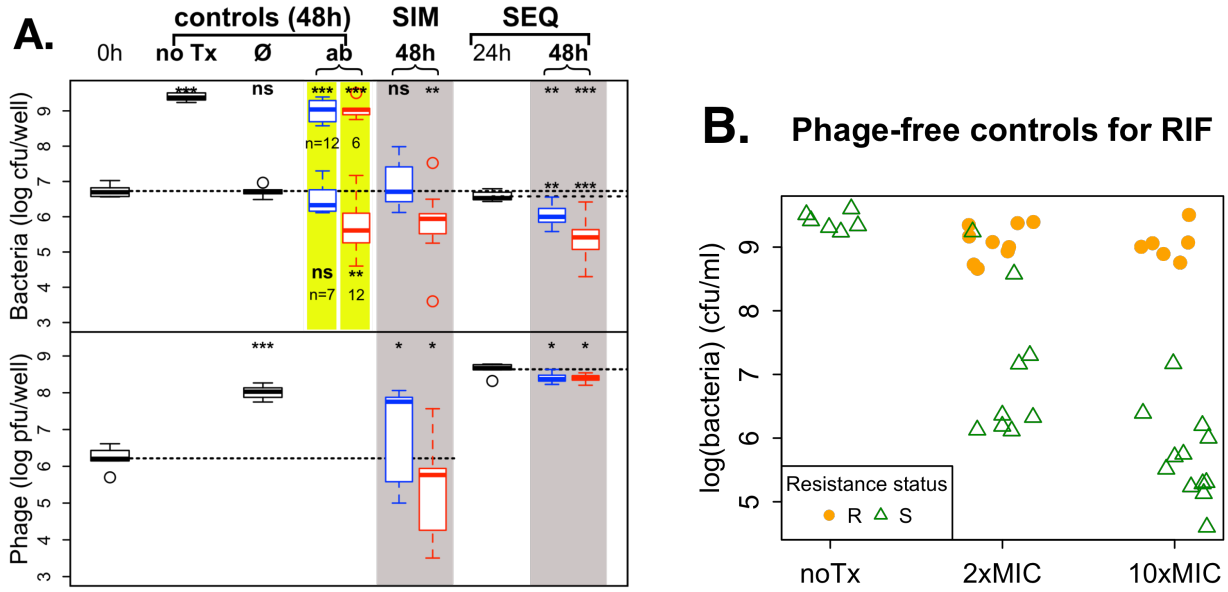

Figure 4. A. Combined action of phage and RIF on SA biofilms. The legend is the same as for Figures 2 and 3. 20 wells were used for antibiotic-only controls, and simultaneous and sequential treatments, at each antibiotic concentration. The 2× and 10×MIC RIF-only control data are highlighted in yellow: some wells became turbid over the 48h of incubation, and some wells remained clear. The number of wells in each category is indicated below the corresponding box. **B.** Resistance status of phage-free controls. Bacterial densities are shown for each well, in solid orange dots for resistant wells and open green triangles for sensitive wells. Note that some of the cultures dosed with 2×MIC of RIF reached high densities (>1e8 cfu/mL) even though they appear to be sensitive to RIF.

Table 2: Summary of the effects of different treatments on the densities of *S. aureus* and phage using RIF. The top row of the table shows the changes in bacterial density over 48 h compared with initial densities. For the RIF-only controls, the values for the wells that did not become turbid are shown in the top row, and the values for the wells that became turbid are shown on the second row. Otherwise, same legend as Table 1.

	controls				SIM		SEQ	
	no Tx	ϕ	ab only 2xMIC	ab only 10xMIC	2xMIC	10xMIC	2xMIC	10xMIC
Bacteria	470***	1ns	1.6ns	11** a,b	1.5ns	8.3** a,b	4.6** b	23*** a
			190***	210***				
Phage		62*** a			5.3* b	6.3*	150*** a	150*** a

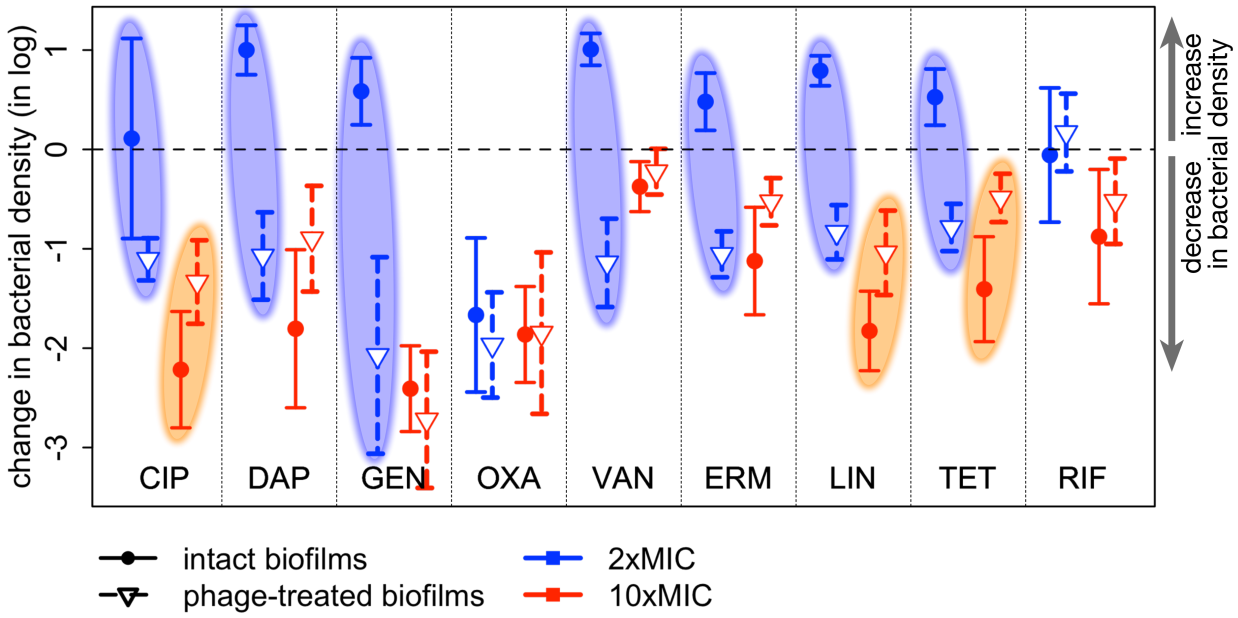

Figure 5: Change in bacterial density after adding antibiotics to intact or phage-treated biofilms. The change in bacterial density (log(final density) – log(initial density)) is shown for all 9 antibiotics used in this study at both antibiotic concentrations. The symbols indicate the difference in means, and the error bars indicate the 95% confidence interval obtained from t-tests. Values above the dashed line show an increase in bacterial density during the course of treatment; values below the dashed line a decrease. Antibiotics added to intact biofilms are shown in solid dots and solid lines (initial densities = bacterial densities in prepared biofilms, final densities = antibiotic-only controls); antibiotics added to biofilms exposed to phage for 24 h are shown in open triangles and dashed lines (initial densities = bacterial densities after 24 h of phage-only treatment, final densities = sequential treatment at 48 h).

The blue ovals highlight the 7 antibiotics used at 2×MIC that did not reduce the bacterial density when applied to intact biofilms (change in density at or above the dashed line) but significantly killed bacteria when added to phage-treated biofilms (change in density below the dashed line). The orange ovals highlight the 3 antibiotics where pretreatment with phage likely reduced the efficacy of 10×MIC antibiotic.