Adjunct phage treatment enhances the effectiveness of low antibiotic concentration against *Staphylococcus aureus* biofilms *in vitro*

James Dickey[1#] and Véronique Perrot[1*]

[1] Department of Biology, Emory University, Atlanta, GA.

[#] Current address: Medical College of Georgia, Augusta, GA., *jadickey@augusta.edu*

* Corresponding author

E-mail address: *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 concentration (2×MIC), but the addition of phage to treatment regimens led to substantial improvements in efficacy. At high concentration (10×MIC), 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 very effective at reducing the outgrowth of resistant strains during the course of treatment.

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 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).

44 The successful use of phage to treat infections in animals and for compassionate use therapy in
45 human patients is encouraging, and it justifies developing protocols for phage therapy in
46 humans. As is the case for new chemotherapeutic agents, properly controlled clinical trials
47 demonstrating safety and efficacy in human patients are essential. However, clinical trials that
48 implement solely phage for the treatment of infections that are otherwise amenable to
49 antibiotic therapy will face difficult regulatory hurdles (24-26). Alternatively, one could conduct
50 clinical trials to compare treatments combining antibiotics with phage with treatment with
51 antibiotics alone. While there is little or no reason to anticipate adverse reactions to phage in
52 systemic treatment (27, 28), one could start with trials for topical, rather than systemic,
53 treatments of infections and target infections of the skin and mucosa, such as burn wounds,
54 diabetic ulcers, and sinusitis.

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

67 In this study, we expand upon the earlier work by Chaudhry et al (36) with *P. aeruginosa* by
68 examining the joint action of antibiotics and phage for treating in vitro biofilms of the Gram
69 positive and common skin pathogen *Staphylococcus aureus*. In addition to using this very
70 different bacterium, we examine a broader range of antibiotics, including but not limited to
71 drugs that are traditionally considered bacteriostatic. We implement these antibiotics against *S.*
72 *aureus* biofilms at high and low concentrations as a sole treatment, applied simultaneously with
73 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 concentrations of antibiotics can be as effective as higher concentrations 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 given to the lab by William M. Schafer. 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). The single phage isolated from the cocktail is hereafter referred to as PYO phage.

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. Left: intact virion. Right: virion with its tail contracted.

101 *Culture and sampling media*

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

108 *Antibiotics and MIC estimation*

109 Antibiotics were purchased from Sigma (gentamicin (GEN), oxacillin (OXA), vancomycin (VAN),
110 and tetracycline (TET)), AppliChem (ciprofloxacin (CIP), rifampin (RIF)), TCI (daptomycin (DAP)),
111 MP Biochemicals (erythromycin (ERM), and Chem-Impex International (linezolid (LIN)). The
112 minimum inhibitory concentration (MIC) for each drug was determined by the standard two-
113 fold dilution protocol (39). In the expriments, each antibiotic was used at 2×MIC and 10×MIC.
114 We chose these antibiotics to have a broad range of pharmacodynamic properties. The first
115 eight antibiotics listed above are grouped by their pharmacodynamic characteristics: in classic
116 assays of killing dynamics in liquid cultures at super MIC concentrations, CIP, DAP and GEN kill
117 rapidly (fast-acting bactericidal antibiotics); OXA and VAN kill more slowly (slow-acting
118 antibiotics); and LIN, ERM and TET prevent growth (bacteriostatic antibiotics) (40).

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

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

130 appropriate amount of antibiotic (2x or 10x MIC) was added to each well in saline, and
131 incubated without shaking at 37˚C for an additional 24 h for a total treatment time of 48 h.

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

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

144 *Experimental design and sample size*

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

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

156 For our experiments, we define an effective treatment as one that leads to a decrease in the
157 density of bacteria recovered from a well relative to the density before treatment (initial

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

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

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

182

183 **Results**

184 **1 – Bacterial strain characterization**

185 For *S. aureus* Newman, the MICs for each drug were, in µg/mL: CIP, 0.1875; DAP, 2; GEN, 0.75;
186 OXA, 0.1875; VAN, 1.5; ERM, 0.375; LIN, 1.5; TET, 0.1875; RIF, 0.01. Unlike *E. coli* or *P.*
187 *aeruginosa* and their lytic phages, *S. aureus* does not seem to readily evolve resistance to its
188 phage. We do not observe the appearance of bacteria resistant to the PYO phage in standard
189 culture: resistant colonies do not appear on phage-treated lawns, nor do cultures of *S. aureus*
190 with phage become turbid overnight, as is commonly observed with *E. coli* and *P. aeruginosa*
191 and their phages.

192 **2 - Combination treatment with phage and antibiotics**

193 In this experiment, we measured the bacterial (and phage, where applicable) densities in
194 prepared biofilms of *S. aureus* before (initial density, shown as 0h on Figure 2) and after 48
195 hours of treatment with combinations of phage and antibiotics. As controls, we also measured
196 bacterial and phage densities after 48h of treatment with phage, antibiotics, or neither
197 (untreated control). As sampling is destructive, a set of wells were sacrificed to estimate the
198 initial density in biofilms at the time of treatment. The same initial density data is used for all
199 the 8 antibiotics in this experiment. In addition, we use the same data for the untreated and
200 phage-only controls for all 8 antibiotics.

201 The results of all the antibiotics except RIF are presented in Figure 2 and summarized in Table 1.

202 **Figure 2: Combined action of 8 antibiotics and phage**. For each plot, top panel:
203 bacterial densities; bottom panel: phage densities. Blue boxes: treatment with 2×MIC of
204 the antibiotic; red boxes: treatment with 10×MIC of the antibiotic. Densities after 48h of
205 combined antibiotic and phage treatments are shown in the shaded columns. For each
206 plot we use the same controls (the left side of the plot): (i) untreated, or treated with
207 phage only (same data for all 8 antibiotics), and (ii) treated only with the noted
208 antibiotic for that plot. For each plot, the results for the simultaneous treatment (SIM)
209 with phage and 2× and 10×MIC of the antibiotic are shown in the middle shaded
210 column. The sequential treatment (SEQ), where the cultures were first treated with
211 phage for 24 h then treated with the antibiotic for another 24 h, the bacterial and phage
212 densities at 48 h are shown on the right shaded column. The bacterial and phage
213 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: $p>0.1$.

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

		controls				SIM		SEQ	
		no Tx	☐	ab only 2xMIC	ab only 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
	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
	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
	Weakly Cidal								
	OXA		100*** a			100*** a	20** b	98*** a	210*** a
	VAN		100*** a			15*** b	1.1ns	110*** a	72*** a
	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

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: $p\geq0.1$.

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

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

242

243 For the convenience of the reader we describe in some details one representative plot in Figure
244 2; we arbitrarily chose DAP for this purpose. In untreated controls, the viable cell density of *S.*
245 *aureus* increased by 15 fold relative to the initial density. In the phage-only controls, the cell
246 density was reduced 33 fold, and the phage density increased by 100 fold. At 2×MIC, DAP alone
247 was not effective in reducing the viable cell density of bacteria. DAP at 10×MIC and
248 simultaneous and sequential treatments at both antibiotic concentrations were effective in
249 significantly reducing the viable cell density of *S. aureus* in culture, and not significantly
250 different from each other (second line of top panel of Table 1). DAP added at both
251 concentrations after 24 h of phage treatment (SEQ) was able to further reduce the bacterial
252 density: the blue and red boxes in the right shaded column are significantly below the dashed
253 line showing the bacterial density after 24 h of phage exposure (middle plot of the top row of
254 Figure 2). This observation holds even when the antibiotic was added at 2×MIC, a concentration
255 at which DAP alone was unable to prevent the outgrowth of an intact biofilm.

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

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

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

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

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

266 (2) Antibiotic-only controls

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

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

276 (3) Antibiotics and phage — Simultaneous treatment

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

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

288 (iii) Simultaneous treatment with phage and 10×MIC of all 8 antibiotics was effective at
289 reducing bacterial densities in the cultures, but not significantly more effective than the

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

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

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

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

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

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

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

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

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

323 **2 - Phage preventing the ascent of antibiotic resistance**

324 We ran experiments similar as those described above with the additional goal of monitoring the
325 appearance of resistance to the antibiotic RIF. RIF is a potent killer of *S. aureus* at very low
326 concentrations (MIC=0.01 µg/mL), but it is rarely if ever used in the clinic because resistance to
327 the antibiotic emerges very quickly. In our hands, an overnight culture of *S. aureus* treated with
328 RIF at super-MIC concentrations is turbid by morning and the majority population resistant to
329 RIF, a phenomenon we never observed for any other antibiotics. We modified the design of the
330 experiment to include larger sample sizes (a total of 20 wells for each treatment group). We
331 never observed the appearance of resistance to the phage in all the experiments reported in
332 this paper, so we conclude that resistance to phage is not an issue at the time frame of a few
333 days in the biofilm cultures we used. The results are presented in Figure 3 and summarized in
334 Table 2.

335 **Figure 3. Treatment of *S. aureus* biofilm with RIF and phage**. **A.** Combined action of
336 phage and RIF on *S. aureus* biofilms. The legend is the same as for Figures 2 and 3. 20
337 wells were used for antibiotic-only controls, and simultaneous and sequential
338 treatments, at each antibiotic concentration. The 2× and 10×MIC RIF-only control data
339 are highlighted in yellow: some wells became turbid over the 48h of incubation
340 (bacterial density ~ 1e9; data is the same as the points at the top of figure 4B), and some
341 wells remained clear (bacterial density ~ 1e5 - 1e7; data is the same as the points at the
342 bottom of figure 4B). The number of wells in each category is indicated below the
343 corresponding box. **B.** Resistance status of phage-free controls. Bacterial densities are
344 shown for each well, in solid orange dots for resistant wells and open green triangles for
345 sensitive wells. Note that some of the cultures dosed with 2×MIC of RIF reached high

346 densities (>1e8 cfu/mL) even though they appear to be sensitive to RIF when tested on
347 plates containing 10×MIC RIF.

348 **Table 2: Summary of the effects of different treatments on the densities of *S. aureus***
349 **and phage using RIF.**

	controls							
			ab only		SIM		SEQ	
	no Tx	ϕ	2xMIC	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

350

351 The top row of the table shows the changes in bacterial density over 48 h compared
352 with initial densities. For the RIF-only controls, the values for the wells that did not
353 become turbid are shown in the top row, and the values for the wells that became
354 turbid are shown on the second row. Otherwise, same legend as Table 1.

355 The work with RIF was done separately from that with the other 8 antibiotics, with its own set
356 of antibiotic-free controls. We notice that the initial bacterial density of the prepared biofilms
357 was about 6 times lower than for the first 8 antibiotics despite our using the same experimental
358 protocol. The phage density was about the same, so the MOI was slightly higher for the RIF
359 treatment. In this case, phage alone was able to prevent the net growth of bacterial cultures,
360 but unable to significantly reduce the bacteria below their initial densities. Phage densities
361 increased significantly, but slightly less than they did in the first experiment.

362 The RIF-only controls at both concentrations presented a pattern we didn't see in any of the
363 other 8 antibiotics: there were two, rather than one, qualitative outcomes. Some wells became
364 turbid, which is equivalent to treatment failure, with bacterial densities greater than 1e8
365 cfu/mL, and the other wells remained clear, with bacterial densities smaller than 1e8 cfu/mL.
366 Densities in the turbid wells were very close to the density in the untreated controls. For the
367 wells that remained clear (7 out of 19 wells for 2×MIC and 6 out of 18 wells for 10×MIC),

368 rifampin alone at 2×MIC did not significantly reduce the viable cell density of bacteria, as
369 observed with 7 of the other 8 antibiotics (OXA being the exception). RIF alone at 10×MIC was
370 effective at reducing bacterial density, albeit its efficacy was in the range of the weakly
371 bactericidal and bacteriostatic antibiotics (see Table 1).

372 We assessed the resistance status of the bacteria in the untreated wells, and wells treated with
373 RIF alone (Figure 4B). Not unexpectedly, the bacteria in the untreated wells were sensitive to
374 RIF, as were the bacteria in wells treated with RIF that remained clear. The dominant
375 populations of bacteria in the wells treated with 10×MIC RIF that became turbid were resistant,
376 as were most of the dominant populations in wells treated with 2×MIC RIF that became turbid.
377 In addition, two of such wells appeared sensitive, possibly because the bacteria were tested on
378 plates containing 10×MIC of the antibiotic. The dominant populations in these two wells may
379 have been resistant to 2×MIC but unable to form colonies in the presence of 10×MIC RIF.

380 When RIF was used simultaneously with phage, its effect was similar to that of RIF alone, but
381 without treatment failure. RIF at 2×MIC with phage did not lead to net growth or death, and RIF
382 at 10×MIC with phage had the same efficacy as RIF at 10×MIC alone when resistance did not
383 arise. RIF was the only antibiotic out of the 9 we tested where simultaneous treatment with
384 2×MIC of the antibiotic was not effective at reducing bacterial density.

385 When RIF was added to phage treated wells (sequential treatment), both concentrations
386 significantly reduced bacterial densities, with 10×MIC being significantly more effective than
387 2×MIC. As in simultaneous treatment, we did not observe any treatment failure in sequential
388 treatment. Overall, there were 17 treatment failures out of 37 wells treated with RIF only, and
389 none out of 80 wells treated with a combination of RIF and phage.

390

391 **Discussion**

392 Bacteria in biofilms are notoriously difficult to kill with antibiotics, and biofilms represent a
393 significant problem in treating many bacterial infections (29-31, 42, 43). One of the most cited
394 factors contributing to this problem is the extracellular matrix that comprises the structure of
395 the biofilm (44, 45). This polysaccharide matrix is thought to reduce the exposure of bacteria in

396 the biofilm to the antibiotic, resulting in bacteria that are exposed to a lower, and thus less
397 effective, concentration of antibiotic.

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

418 This study initially served to extend the work of Chaudhry et al., as we sought to examine the
419 generality of the effectiveness of combined phage and antibiotic treatment against the
420 common skin pathogen and Gram positive *S. aureus*. As with the previous work, antibiotics
421 were applied at the same time as phage (simultaneous treatment) or after phage pre-treatment
422 (sequential treatment), but with a broader range of nine antibiotics that included some
423 bacteriostatic antibiotics. To allow us to fully gauge the effectiveness of our treatment regimens
424 in eliminating bacteria within a biofilm, we further modified Chaudhry et al.'s protocol by using
425 a different measure of efficacy. In our modified protocol, we estimated the bacterial density in

426 prepared biofilms before treatment, and after 48 h of exposure to antibiotic, phage, both, or
427 neither (untreated controls). Instead of comparing bacterial densities after treatment with
428 densities in untreated controls, we compared them with bacterial densities before treatment.
429 Our measure of treatment efficacy is thus the change in bacterial density during the course of
430 treatment. This more conservative estimate allows us to ascertain the ability of treatment
431 regimens to penetrate and kill bacteria within a biofilm. Samples taken after treatment
432 consisted of both biofilm and planktonic bacteria, whereas the samples taken at the time of
433 treatment consisted of only biofilm bacteria. Any treatment that resulted in lower bacterial
434 densities than were in biofilms before treatment must have been effective at penetrating and
435 killing bacteria within the biofilm. In addition, by estimating the bacterial density after 24 h of
436 phage treatment, we could estimate the efficacy of antibiotics on phage-treated biofilms (see
437 Figure 4 below).

438 **Figure 4: Change in bacterial density after adding antibiotics to intact or phage-treated**
439 **biofilms**. The change in bacterial density (log(final density) – log(initial density)) is
440 shown for all 9 antibiotics used in this study at both antibiotic concentrations. The
441 symbols indicate the difference in means, and the error bars indicate the 95%
442 confidence interval obtained from t-tests. Values above the dashed line show an
443 increase in bacterial density during the course of treatment; values below the dashed
444 line a decrease. Antibiotics added to intact biofilms are shown in solid dots and solid
445 lines (initial densities = bacterial densities in prepared biofilms, final densities =
446 antibiotic-only controls); antibiotics added to biofilms exposed to phage for 24 h are
447 shown in open triangles and dashed lines (initial densities = bacterial densities after 24 h
448 of phage-only treatment, final densities = sequential treatment at 48 h).

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

454 Our results are consistent with that of Chaudhry et al, except that we observed better killing
455 with antibiotic and phage combinations only for low antibiotic concentrations. Such
456 concentrations would otherwise be ineffective or marginally effective, but the inclusion of
457 phage in these treatments led to substantial killing of bacteria in *S. aureus* biofilms. However,
458 unlike the general findings of Chaudhry et al, increasing the concentration five-fold led to no
459 additional killing of bacteria when used with phage. An exception to this pattern is GEN, where
460 in our experiments, GEN was still qualitatively, albeit not always significantly, more effective at
461 high compared with low concentration within each treatment protocol. In some cases,
462 increasing antibiotic concentration led to a decrease in the efficacy of treatment, such as with
463 VAN and TET in simultaneous treatments, and for VAN and ERM in sequential treatments. This
464 is in line with the observations of TOB against *P. aeruginosa* by Chaudhry et al.

465 Generally speaking, antibiotics at low concentration (2×MIC) were unable to kill bacteria in
466 biofilms while the same antibiotics were able to kill at high concentration (10×MIC).
467 Interestingly, this observation held for bacteriostatic as well as bactericidal antibiotics. Using
468 antibiotics and phage at the same time (SIM treatments) improved killing over antibiotics alone
469 or phage alone in 2 out of 18 cases (9 antibiotics × 2 concentrations). For these two cases (CIP
470 and TET at 2×MIC), the antibiotic alone did not reduce bacterial densities, so we can
471 conservatively put their efficacy at 0, if not as a negative number. Since the efficacy of the
472 combination was significantly greater than that of phage alone, there is synergy between
473 antibiotic and phage. There were two cases of antagonism, VAN and TET at 10×MIC, where the
474 efficacy of the combination was smaller than that of the most effective agent acting alone. For
475 all other cases (14 out of 18), the efficacy of the combination was not significantly different
476 from that of the most effective agent acting alone: in some cases, the phage appeared to do
477 most of the killing, in other cases it was the antibiotic. This would indicate that at least in some
478 situations, combined phage and antibiotic would be generally more effective than antibiotic
479 alone, not so much because of synergy between the two agents (only 2 in 18 cases) but because
480 either agent alone is effective (12 out of 18 cases), and antagonism between the two is
481 infrequent (2 out of 18 cases).

482 Our experimental design further allowed us to check if phage pre-treatment could alter the
483 susceptibility of biofilm-bound bacteria to antibiotics. To answer this question, we estimated

484 the efficacy of the antibiotics when added to intact biofilms (antibiotics-only controls), and
485 when added to phage-treated biofilms (sequential treatment regimens). These efficacies are
486 summarized in Figure 4. When used at 2×MIC, 7 out of 9 antibiotics showed a greatly increased
487 efficacy against phage-treated biofilms compared with intact biofilms: for all but OXA and RIF,
488 the antibiotic was able to kill effectively phage-treated biofilms, while the same concentration
489 didn't kill intact biofilms (shown in blue ovals on Figure 4). OXA was able to kill intact biofilms,
490 and RIF was not, but pre-treatment with phage did not change the efficacy of either antibiotic.
491 When used at 10×MIC, there is little effect of phage pre-treatment on antibiotic efficacy. When
492 there is little (CIP, LIN) or no (TET) overlap between confidence intervals, phage pre-treatment
493 actually reduced the antibiotic efficacy (shown in orange ovals on Figure 4). In short, when it
494 had a significant effect, phage pre-treatment improved the efficacy of low concentrations of
495 antibiotics, but it decreased the efficacy of high concentrations of antibiotics. This result is
496 particularly promising for antibiotics prone to toxic side effects, where keeping the
497 concentration low is desirable.

498 We also addressed the possibility that combining phage with antibiotics would reduce the
499 appearance of antibiotic resistance. In clinical settings, RIF is not used alone in treating
500 infections as resistance commonly ascends during the course of treatment, leading to
501 treatment failure. Indeed, we observed that about half of biofilms treated with RIF alone
502 became turbid, the in vitro analog of treatment failure. To prevent treatment failure due to the
503 ascent of resistance to RIF, RIF is used in combination with other antibiotics to treat infections
504 with *S. aureus* (see (50) for a review). Using phage and RIF, either at the same time or phage
505 first, RIF second, did not increase the efficacy of the antibiotic, but it completely prevented the
506 ascent of RIF resistant bacteria. This observation indicates that one could develop treatment
507 regimens that incorporate phage with RIF in order to reduce the ascent of resistance, while
508 simultaneously diminishing concern of antagonistic interactions between therapeutic agents.

509

510 Our results provide evidence supporting the combined use of phage and antibiotics against *S.*
511 *aureus* biofilms. We observed that the combination of phage and antibiotics prevented the
512 ascent of antibiotic resistant bacteria, and, especially when phage was used first and antibiotic
513 second, it restored efficacy to low concentration antibiotics that would otherwise have been

514 ineffective. These results are promising for the topical use of combinations of antibiotics and
515 phage to treat surface infections, as such surface infections occur in the form of biofilms.

516 However, more experiments are needed. Future work should examine how general these
517 results are for different strains of *S. aureus*, particularly as different clinical isolates are known
518 to differ in their biofilm-forming abilities. Furthermore, the work could also be expanded to
519 examine the effectiveness of other phages, which may have different pharmacodynamic
520 properties from the single phage tested here. And while promising, these experiments must
521 eventually be conducted in vivo, such as in mouse cutaneous wounds, as the complex dynamics
522 of drug and phage bioavailability, host immune system, bacterial dynamics and kinetics can
523 result in vastly different results from these in vitro experiments. Effectiveness of combined
524 therapy would have to be tested in living model systems in order to pave the way for human
525 clinical trials. Nevertheless, our results are a first step toward a better understanding of the
526 dynamics of treating bacteria with phage and antibiotics. .

527

528

529

530 **Acknowledgements**

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

535 **References**

536

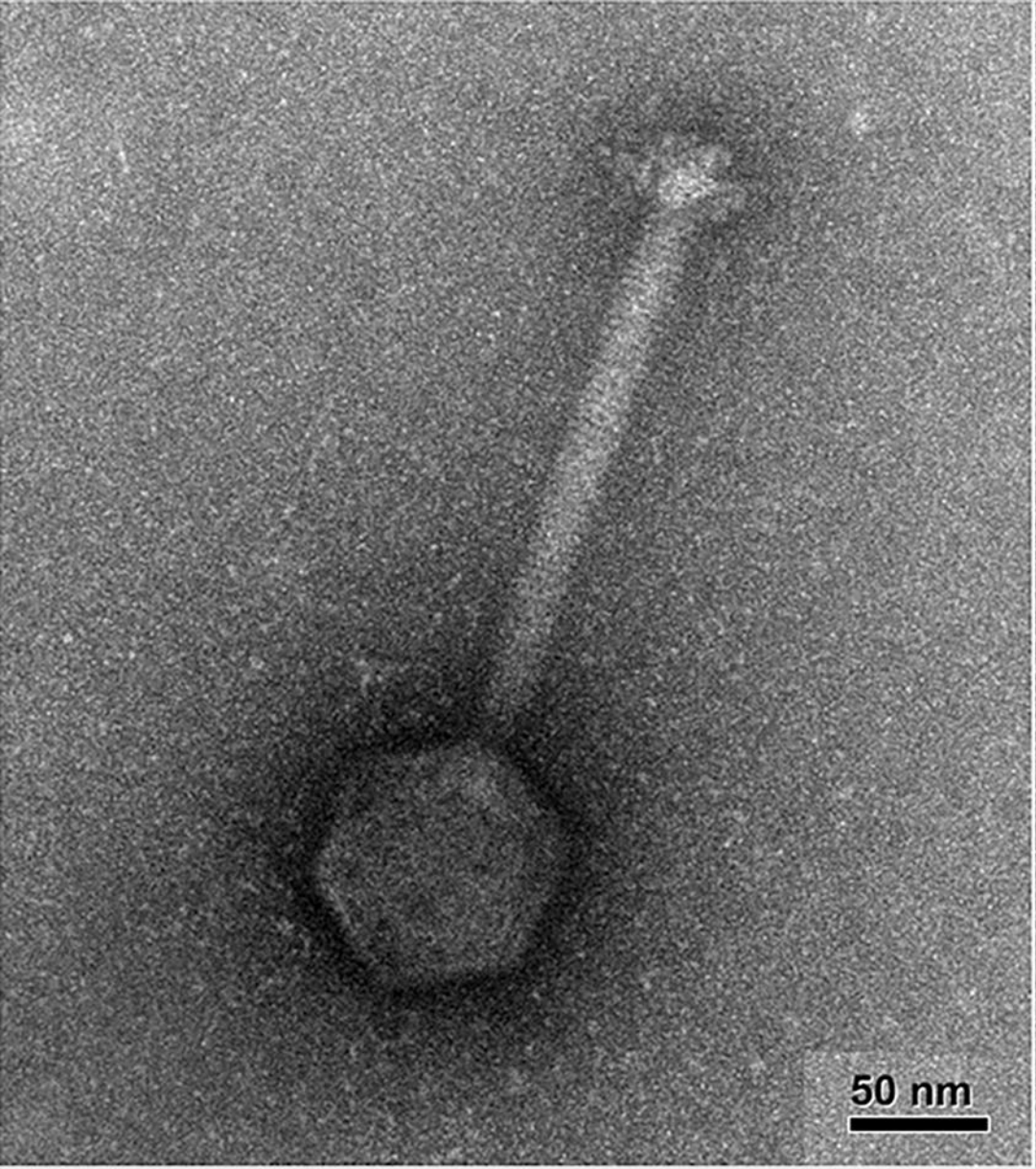
537

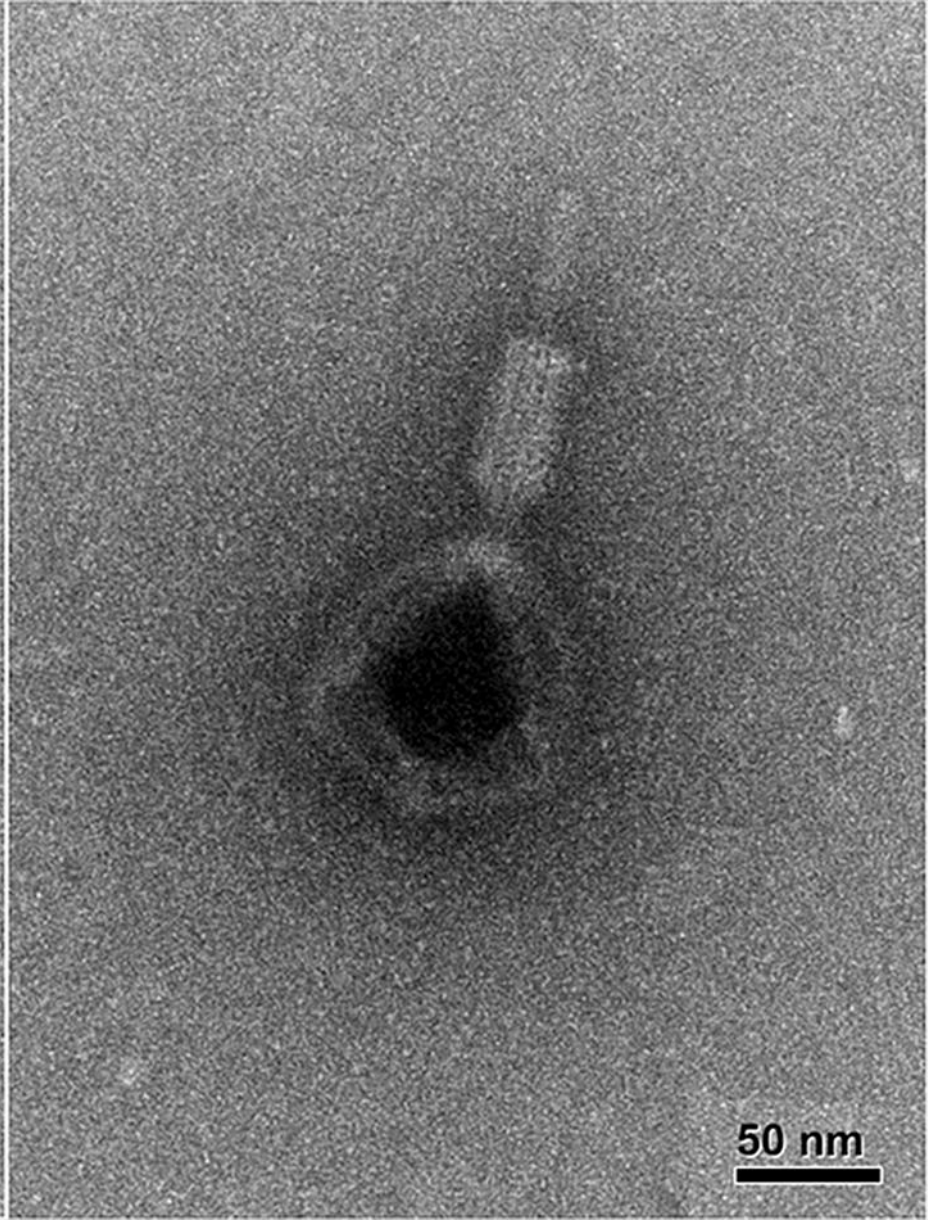
538 1. Twart F. An investigation on the nature of ultra-microscopic viruses. Lancet.
539 1915;186(4814):1241-3.
540 2. d'Herelle F. Sur un microbe invisible antagoniste des bacilles dysentériques (On an invisible
541 microbe antagonistic to dysentery bacilli). Comptes Rendus de l'Académie des Sciences. 1917;165:373-5.
542 3. d'Herelle F. The Bacteriophage and Its Belhavior. Baltimore: Williams and Wilkins; 1926. 679 p.
543 4. O'Neill J. Tackling drug-resistant infections globally: final report and recommendation. London;
544 2016.
545 5. de Kraker ME, Stewardson AJ, Harbarth S. Will 10 Million People Die a Year due to Antimicrobial
546 Resistance by 2050? PLoS Med. 2016;13(11):e1002184.
547 6. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. Phage treatment of human infections.
548 Bacteriophage. 2011;1(2):66-85.
549 7. Gorski A, Miedzybrodzki R, Weber-Dabrowska B, Fortuna W, Letkiewicz S, Rogoz P, et al. Phage
550 Therapy: Combating Infections with Potential for Evolving from Merely a Treatment for Complications to
551 Targeting Diseases. Frontiers in microbiology. 2016;7:1515.
552 8. Henein A. What are the limitations on the wider therapeutic use of phage? Bacteriophage.
553 2013;3(2):e24872.
554 9. Shen Y, Barros M, Vennemann T, Gallagher DT, Yin Y, Linden SB, et al. A bacteriophage endolysin
555 that eliminates intracellular streptococci. eLife. 2016;5.
556 10. Thandar M, Lood R, Winer BY, Deutsch DR, Euler CW, Fischetti VA. Novel Engineered Peptides of
557 a Phage Lysin as Effective Antimicrobials against Multidrug-Resistant Acinetobacter baumannii.
558 Antimicrobial agents and chemotherapy. 2016;60(5):2671-9.
559 11. Hawkins C, Harper D, Burch D, Anggard E, Soothill J. Topical treatment of Pseudomonas
560 aeruginosa otitis of dogs with a bacteriophage mixture: a before/after clinical trial. Veterinary
561 microbiology. 2010;146(3-4):309-13.
562 12. Smith HW, Hugggins MB. Successful treatment of experimental Escherichia coli infections in
563 mice using phage: its general superiority over antibiotics. J General Microbiology. 1982;128:307-18.
564 13. Bull JJ, Levin BR, DeRouin T, Walker N, Bloch CA. Dynamics of success and failure in phage and
565 antibiotic therapy in experimental infections. BMC microbiology. 2002;2:35.
566 14. Biswas B, Adhya S, Washart P, Paul B, Trostel AN, Powell B, et al. Bacteriophage therapy rescues
567 mice bacteremic from a clinical isolate of vancomycin-resistant Enterococcus faecium. Infect Immun.
568 2002;70(1):204-10.
569 15. Ferreira FA, Souza RR, Bonelli RR, Americo MA, Fracalanzza SE, Figueiredo AM. Comparison of in
570 vitro and in vivo systems to study ica-independent Staphylococcus aureus biofilms. Journal of
571 microbiological methods. 2012;88(3):393-8.
572 16. Alemayehu D, Casey PG, McAuliffe O, Guinane CM, Martin JG, Shanahan F, et al. Bacteriophages
573 phiMR299-2 and phiNH-4 can eliminate Pseudomonas aeruginosa in the murine lung and on cystic
574 fibrosis lung airway cells. mBio. 2012;3(2):e00029-12.
575 17. Galtier M, De Sordi L, Maura D, Arachchi H, Volant S, Dillies MA, et al. Bacteriophages to reduce
576 gut carriage of antibiotic resistant uropathogens with low impact on microbiota composition.
577 Environmental microbiology. 2016;18(7):2237-45.
578 18. Roach DR, Leung CY, Henry M, Morello E, Singh D, Di Santo JP, et al. Synergy between the Host
579 Immune System and Bacteriophage Is Essential for Successful Phage Therapy against an Acute
580 Respiratory Pathogen. Cell host & microbe. 2017;22(1):38-47.e4.

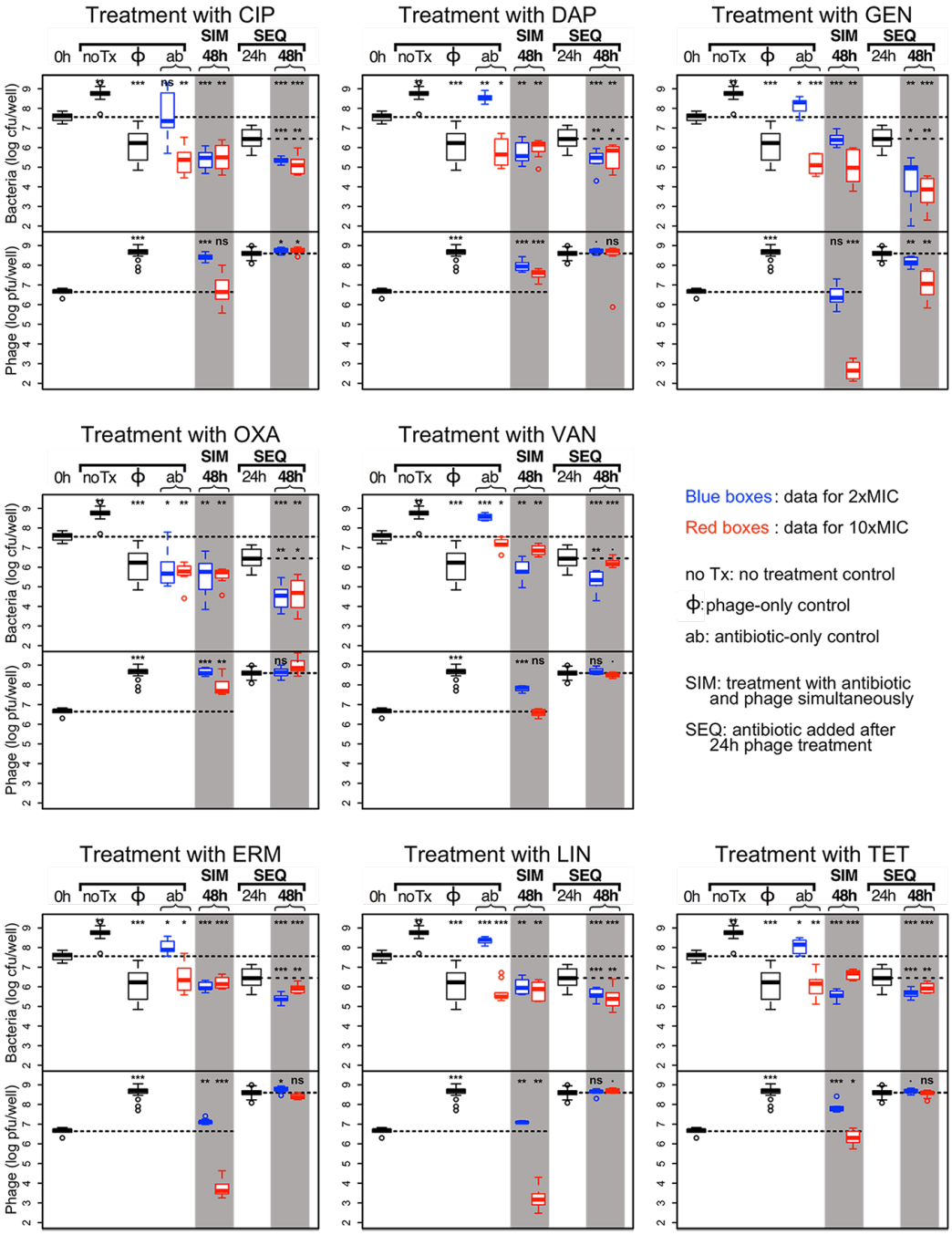
581 19. Waters EM, Neill DR, Kaman B, Sahota JS, Clokie MRJ, Winstanley C, et al. Phage therapy is
582 highly effective against chronic lung infections with Pseudomonas aeruginosa. Thorax. 2017;72(7):666-7.
583 20. Lang G, Kehr P, Mathevon H, Clavert JM, Sejourne P, Pointu J. Bacteriophage therapy of septic
584 complications of orthopaedic surgery. Revue de chirurgie orthopedique et reparatrice de l'appareil
585 moteur. 1979;65(1):33-7.
586 21. Schooley RT, Biswas B, Gill JJ, Hernandez-Morales A, Lancaster J, Lessor L, et al. Development
587 and Use of Personalized Bacteriophage-Based Therapeutic Cocktails To Treat a Patient with a
588 Disseminated Resistant Acinetobacter baumannii Infection. Antimicrobial agents and chemotherapy.
589 2017;61(10).
590 22. Jennes S, Merabishvili M, Soentjens P, Pang KW, Rose T, Keersebilck E, et al. Use of
591 bacteriophages in the treatment of colistin-only-sensitive Pseudomonas aeruginosa septicaemia in a
592 patient with acute kidney injury-a case report. Critical care (London, England). 2017;21(1):129.
593 23. Chan BK, Sistrom M, Wertz JE, Kortright KE, Narayan D, Turner PE. Phage selection restores
594 antibiotic sensitivity in MDR Pseudomonas aeruginosa. Scientific reports. 2016;6:26717.
595 24. Rose T, Verbeken G, Vos DD, Merabishvili M, Vaneechoutte M, Lavigne R, et al. Experimental
596 phage therapy of burn wound infection: difficult first steps. International journal of burns and trauma.
597 2014;4(2):66-73.
598 25. Pirnay JP, De Vos D, Verbeken G, Merabishvili M, Chanishvili N, Vaneechoutte M, et al. The
599 phage therapy paradigm: pret-a-porter or sur-mesure? Pharmaceutical research. 2011;28(4):934-7.
600 26. Debarbieux L, Pirnay JP, Verbeken G, De Vos D, Merabishvili M, Huys I, et al. A bacteriophage
601 journey at the European Medicines Agency. FEMS microbiology letters. 2016;363(2):fnv225.
602 27. Knouf EG, Ward WE, et al. Treatment of typhoid fever with type specific bacteriophage. J Am
603 Med Assoc. 1946;132:134-8.
604 28. Desranleau JM. The treatment of typhoid fever by the use of Vi antityphoid bacteriophages; a
605 preliminary report. Can J Public Health. 1948;39(8):317-9.
606 29. Davies D. Understanding biofilm resistance to antibacterial agents. Nat Rev Drug Discov.
607 2003;2(2):114-22.
608 30. Kirby AE, Garner K, Levin BR. The relative contributions of physical structure and cell density to
609 the antibiotic susceptibility of bacteria in biofilms. Antimicrobial agents and chemotherapy.
610 2012;56(6):2967-75.
611 31. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the Natural environment to
612 infectious diseases. Nat Rev Micro. 2004;2(2):95-108.
613 32. Stewart PS, Franklin MJ. Physiological heterogeneity in biofilms. Nature reviews Microbiology.
614 2008;6(3):199-210.
615 33. Harper DR, Parracho HMRT, Walker J, Sharp R, Hughes G, Werthén M, et al. Bacteriophages and
616 Biofilms. Antibiotics. 2014;3(3):270-84.
617 34. Danis-Wlodarczyk K, Vandenheuvel D, Jang HB, Briers Y, Olszak T, Arabski M, et al. A proposed
618 integrated approach for the preclinical evaluation of phage therapy in Pseudomonas infections.
619 Scientific reports. 2016;6:28115.
620 35. Torres-Barceló C, Arias-Sánchez FI, Vasse M, Ramsayer J, Kaltz O, Hochberg ME. A Window of
621 Opportunity to Control the Bacterial Pathogen Pseudomonas aeruginosa Combining Antibiotics and
622 Phages. PloS one. 2014;9(9):e106628.
623 36. Chaudhry WN, Concepcion-Acevedo J, Park T, Andleeb S, Bull JJ, Levin BR. Synergy and Order
624 Effects of Antibiotics and Phages in Killing Pseudomonas aeruginosa Biofilms. PloS one.
625 2017;12(1):e0168615.
626 37. Kumaran D, Taha M, Yi Q, Ramirez-Arcos S, Diallo JS, Carli A, et al. Does Treatment Order
627 Matter? Investigating the Ability of Bacteriophage to Augment Antibiotic Activity against Staphylococcus
628 aureus Biofilms. Frontiers in microbiology. 2018;9:127.

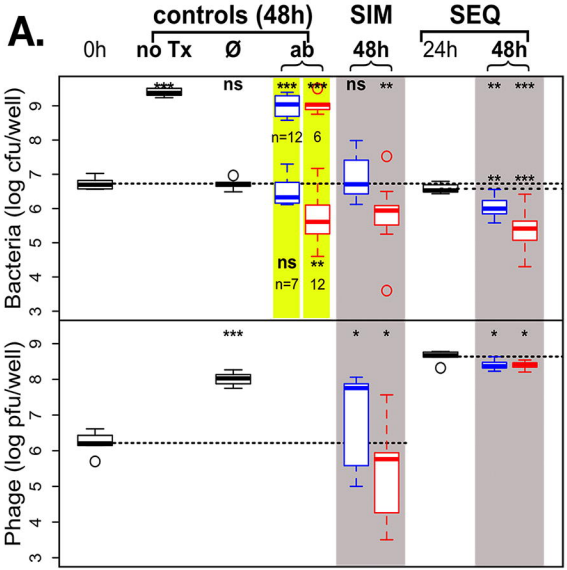
629 38. Kwan T, Liu J, DuBow M, Gros P, Pelletier J. The complete genomes and proteomes of 27
630 Staphylococcus aureus bacteriophages. Proceedings of the National Academy of Sciences of the United
631 States of America. 2005;102(14):5174-9.
632 39. Jorgensen JH, Ferraro MJ. Antimicrobial susceptibility testing: a review of general principles and
633 contemporary practices. Clinical infectious diseases : an official publication of the Infectious Diseases
634 Society of America. 2009;49(11):1749-55.
635 40. Johnson PJ, Levin BR. Pharmacodynamics, population dynamics, and the evolution of
636 persistence in Staphylococcus aureus. PLoS Genet. 2013;9(1):e1003123.
637 41. Team R. RStudio: Integrated Development Environment for R. Boston, MA: RStudio, Inc.; 2015.
638 42. Bhattacharya M, Wozniak DJ, Stoodley P, Hall-Stoodley L. Prevention and treatment of
639 Staphylococcus aureus biofilms. Expert review of anti-infective therapy. 2015;13(12):1499-516.
640 43. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends
641 Microbiol. 2001;9(1):34-9.
642 44. Colvin KM, Gordon VD, Murakami K, Borlee BR, Wozniak DJ, Wong GC, et al. The pel
643 polysaccharide can serve a structural and protective role in the biofilm matrix of Pseudomonas
644 aeruginosa. PLoS pathogens. 2011;7(1):e1001264.
645 45. Khan W, Bernier SP, Kuchma SL, Hammond JH, Hasan F, O'Toole GA. Aminoglycoside resistance
646 of Pseudomonas aeruginosa biofilms modulated by extracellular polysaccharide. International
647 microbiology : the official journal of the Spanish Society for Microbiology. 2010;13(4):207-12.
648 46. Abedon ST. Ecology of Anti-Biofilm Agents I: Antibiotics versus Bacteriophages. Pharmaceuticals.
649 2015;8(3):525-58.
650 47. Verma V, Harjai K, Chhibber S. Structural changes induced by a lytic bacteriophage make
651 ciprofloxacin effective against older biofilm of Klebsiella pneumoniae. Biofouling. 2010;26(6):729-37.
652 48. Kim S, Rahman M, Seol SY, Yoon SS, Kim J. Pseudomonas aeruginosa bacteriophage PA1O
653 requires type IV pili for infection and shows broad bactericidal and biofilm removal activities. Applied
654 and environmental microbiology. 2012;78(17):6380-5.
655 49. Rahman M, Kim S, Kim SM, Seol SY, Kim J. Characterization of induced Staphylococcus aureus
656 bacteriophage SAP-26 and its anti-biofilm activity with rifampicin. Biofouling. 2011;27(10):1087-93.
657 50. Forrest GN, Tamura K. Rifampin combination therapy for nonmycobacterial infections. Clin
658 Microbiol Rev. 2010;23(1):14-34.

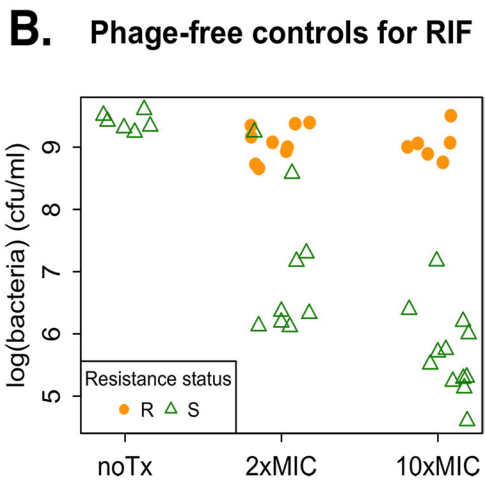
659

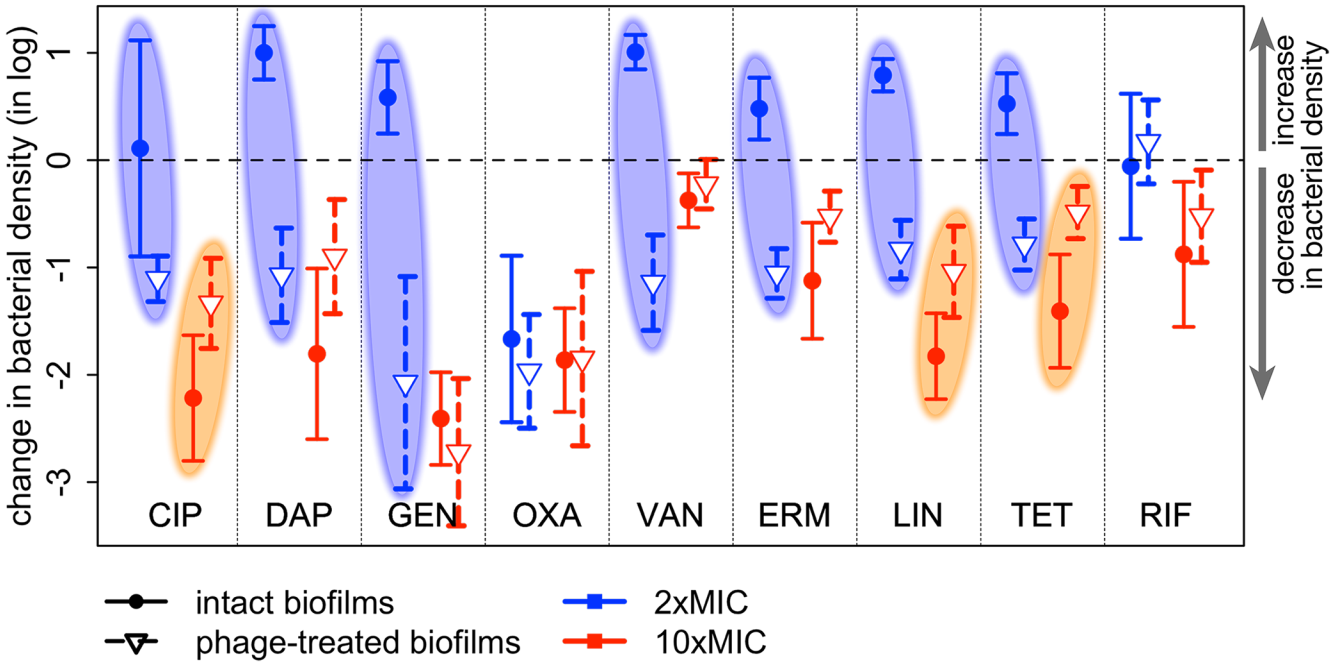

50 nm


50 nm


Treatment with CIP
Treatment with DAP
Treatment with GEN
Treatment with OXA
Treatment with VAN
Treatment with ERM
Treatment with LIN
Treatment with TET
0h
noTx
ϕ
ab
SIM
SEQ
48h
24h
48h
Bacteria (log cfu/well)
Phage (log pfu/well)
Blue boxes: data for 2xMIC
Red boxes : data for 10xMIC
no Tx: no treatment control
ϕ:phage-only control
ab: antibiotic-only control
SIM: treatment with antibiotic and phage simultaneously
SEQ: antibiotic added after 24h phage treatment


A.
controls (48h)
SIM
SEQ
0h
no Tx
Ø
ab
48h
24h
48h
Bacteria (log cfu/well)
Phage (log pfu/well)
ns
***
**
*
n=12
6
n=7
12


B.
Phage-free controls for RIF
log(bacteria) (cfu/ml)
Resistance status
R
S
noTx
2xMIC
10xMIC


change in bacterial density (in log)
1
0
-1
-2
-3
CIP
DAP
GEN
OXA
VAN
ERM
LIN
TET
RIF
increase in bacterial density
decrease in bacterial density
intact biofilms
phage-treated biofilms
2xMIC
10xMIC