Characterizing the tolerance of phage therapy candidate OMKO1 to environmental stress

Michael Blazanin[1,⊗], Wai Tin Lam[1,⊗], Benjamin K. Chan[1], and Paul E. Turner[1,2,*]

[1]Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT 06511, USA.

[2]Program in Microbiology, Yale School of Medicine, New Haven, CT 06520, USA.

[⊗]These authors contributed equally to the work, and are listed in alphabetical order.

*Correspondence to: Paul E. Turner, Department of Ecology and Evolutionary Biology, Yale University, 165 Prospect St, New Haven, CT 06520, USA; paul.turner@yale.edu

Keywords: bacteriophage, thermotolerance, virus

Short title: Tolerance of OMKO1 to environmental stress

Abstract

Antibiotic resistant bacterial pathogens are increasingly prevalent, driving the need for alternative approaches to chemical antibiotics when treating infections. One such approach is bacteriophage therapy: the use of bacteria-specific viruses that lyse (kill) their host cells. However, while the effect of environmental conditions (e.g. elevated temperature) on antibiotic efficacy is well-studied, the generalized effects of environmental stressors on the potency of phage therapy candidates are seldom studied. Therapeutic phage OMKO1 infects and kills the opportunistic human pathogen *Pseudomonas aeruginosa*, while selecting for the evolution of antibiotic re-sensitivity in the target bacterial population. Here, we used phage OMKO1 as a model to test how a therapeutic virus degrades in different environments, and whether exposure to an environmental stressor affects subsequent growth ability on host bacteria. We observed that OMKO1 particles were highly tolerant to different saline concentrations, but rapidly deactivated at elevated temperatures and under high concentrations of urea. We also found that exposure to elevated temperature reduced the growth ability of surviving OMKO1 particles, suggesting a temperature-induced phenotypic shift. Our findings demonstrate that OMKO1 is highly tolerant to a range of conditions that could be experienced in and outside the human body, while also showing the need for careful characterization of therapeutic phages to ensure that environmental exposure does not compromise their expected potency, dosing, and pharmacokinetics.

30 **Introduction**

31 Widespread use of antibiotics – particularly in human therapy and animal agriculture – has selected for
32 the evolution of multi-drug resistant bacterial pathogens, commonly associated with poorer prognosis
33 and higher morbidity in human infections [1, 2]. One alternative or complementary approach to treating
34 bacterial infections with chemical antibiotics is bacteriophage therapy [3], where bacteria-specific
35 viruses with lytic replication cycles are used to kill (lyse) target bacterial cells. With any therapeutic
36 treatment, understanding the effect of environmental conditions on efficacy is paramount. The effects
37 of conditions like high temperature on the stability and application of chemical antibiotics is classically
38 shown [4-7]. In contrast, despite increasing development of phage therapy approaches, the effects of
39 various environmental conditions on phage stability and subsequent reproduction on host cells often
40 remains untested. Here, we use a therapeutic phage for a common human pathogen as a model of
41 phage stability under a range of environmental conditions.

42 The need for novel approaches to treat bacterial pathogens varies greatly between infections: some
43 strains of bacterial pathogens remain treatable with conventional antibiotics, while others show
44 resistance across multiple drug classes, sometimes to all currently-approved antibiotics [8]. One of these
45 multi-drug resistant bacterial pathogens is *Pseudomonas aeruginosa*, which has been identified by the
46 World Health Organization as a high-priority threat to human health [9]. *P. aeruginosa* is a widespread
47 gram-negative opportunistic pathogen that is commonly found in both natural habitats (e.g., soil, fresh
48 water) and artificial environments (e.g., sewage, households, hospitals, and contaminated medical
49 equipment) [10-13]. For these reasons, *P. aeruginosa* is frequently encountered by humans and causes
50 urinary-tract and respiratory infections in immunocompromised individuals, as well as fouling surgically-
51 implanted materials and devices [14, 15] . Individuals with cystic fibrosis (CF), non-CF bronchiectasis, and
52 chronic-obstructive pulmonary disease (COPD) are especially vulnerable to lung infections caused by *P.*
53 *aeruginosa*. Treatment of *P. aeruginosa* with chemical antibiotics is often ineffective because the
54 bacteria readily form biofilms that limit the penetrance of antibiotic molecules and due to multi-drug
55 efflux (Mex) systems: protein complexes that actively remove various types of antibiotics from the cell
56 [16-18].

57 As an alternative or complementary approach to treating *P. aeruginosa*, phage therapy has many
58 attractive advantages, such as the ability for the phage 'drug' to self-amplify within the infection site.
59 However, phage therapy also has some disadvantages, most notably the rapid evolution of bacterial
60 resistance to lytic phage infection [19]. We previously described a naturally-occurring phage that
61 leverages this inevitability of phage-resistance evolution as a strength [20]. The dsDNA phage OMKO1
62 (virus family Myoviridae) attacks *P. aeruginosa*, while affecting the ability for the target bacteria to
63 maintain resistance to various antibiotics. When bacterial strains evolve resistance to phage OMKO1,
64 the mutants can show drug re-sensitivity, suggesting compromised ability for mechanisms such as
65 MexAB and MexXY efflux pumps to remove antibiotics from the cell. Thus, phage OMKO1 can be doubly
66 effective – the virus kills phage-susceptible bacterial cells, and also can drive evolution of phage
67 resistance-associated loss of antibiotic resistance. This 'evolutionary tradeoff' is a mechanistic example
68 of how phages can synergistically interact with chemical antibiotics, to beneficially extend the usefulness
69 of currently-approved drugs. For example, phage OMKO1 and ceftazidime were used successfully in

70 emergency treatment of a 76-year-old patient, to resolve a chronic multi-drug resistant *P. aeruginosa*
71 mediastinal and aortic graft infection [21]. Phage OMKO1 is also currently being tested to resolve or
72 reduce *P. aeruginosa* infections in the lungs of CF, non-CF bronchiectasis and COPD patients when
73 administered via aerosol-delivery (nebulizer) treatment.

74 While phage OMKO1 already has been used successfully for patient treatment, its stability across
75 environmental conditions remains uncharacterized. Phages for therapeutic use can be manufactured in
76 large volumes then stored, and treatment can be administered through various routes, including
77 intravenous, oral suspension, nebulizer, and direct injection to the site of infection. However, it is well-
78 known that virus particles can degrade over time (i.e., become inactivated for cellular infection), and
79 that the rate of degradation can depend on the degree of environmental stress, including elevated
80 temperature, and exposure to high concentrations of salt and urea [22-30]. Because degradation alters
81 the concentration of active virus particles, it can create inaccuracies for treatment dosing and
82 administration. In addition, depending on the sensitivity of phage particles to conditions present in the
83 human body, environmentally-induced degradation could also play a role in the pharmacokinetics
84 during treatment. Thus, it is vital to test how exposure to environmental stressors affects the titer and
85 activity of phage-therapy candidates. Here, we use phage OMKO1 as a general model to test the adverse
86 biological effects of three environmental stressors (salt, urea, and heat) – within and beyond the
87 conditions experienced in the human body – on the stability and subsequent infectivity of a virus
88 successfully used in phage therapy.

89

90 **Materials and Methods**

91 Strains

92 *P. aeruginosa* strain PA01 was provided by B. Kazmierczak (Yale School of Medicine) and cultured on
93 1.5% agar plates and liquid media made from Lysogeny Broth (LB: 10 g/L tryptone, 10 g/L NaCl, 5 g/L
94 yeast extract). Bacteria were incubated at 37°C, and overnight batch cultures were grown with shaking
95 (100 rpm) in 10mL broth, by inoculating with a single randomly-chosen colony. Bacterial stocks were
96 stored in 40% glycerol at -80°C.

97 Phage OMKO1 was originally isolated from an aquatic sample (Dodge Pond, East Lyme, CT) enriched on
98 PAO1 (Chan et al., 2016). High-titer stocks (lysates) of the phage were obtained by mixing 10μl of the
99 original stock with 10ml of PAO1 culture in exponential phase. After 12 hours to allow phage population
100 growth, the mixture was centrifuged and filtered (0.22μm) to remove bacteria to obtain a cell free
101 lysate. Phage lysates were stored at 4°C. Five biological replicate OMKO1 stocks (S3, S8, S11, S16, R3)
102 used in some experiments were generated by adding 10μl of the original stock into 5 independent 10ml
103 PAO1 cultures in exponential phase. For simplicity, these were labeled stocks A, B, C, D, and E,
104 respectively.

105 Measuring Bacterial and Phage Densities

106 The density of a bacterial suspension was estimated using measurements of optical density at 600nm
107 (OD600), based on a pre-generated standard curve for conversions between OD600 and colony forming
108 units (CFU) per mL in a bacterial culture.

109 Estimates of phage titers were obtained via classic plaque-assay methods [31]. Lysate samples were
110 serially diluted in LB, and 100 µL of phage dilution were mixed with 100 µL overnight PAO1 culture and
111 added to 4 mL 'soft' (0.75%) agar LB held at 55°C. After gentle vortexing, the mixture was immediately
112 spread onto a 1.5% agar LB plate. When solidified, plates were inverted and incubated overnight at
113 37°C. Plates with countable plaques were used to calculate the number of plaque-forming-units (PFUs)
114 per mL in the lysate.

115 Analysis

116 All analysis was carried out in R (3.6.0) using dplyr (0.8.2), figures were made using ggplot2 (3.2.0) and
117 ggsignif (0.6.0) [32-35].

118 Urea & salt stress survival
119 To measure effects of urea (carbamide) and salt (NaCl) on phage stability, 10 µL of phage stock A (see
120 Strains) was added to 1 mL of either urea or salt solution at a defined concentration, and vortexed to
121 mix thoroughly. Only a single replicate (n=1) was exposed to each concentration. After a specified time,
122 50 µL were removed and immediately diluted 200-fold to terminate the stress condition. Then, the
123 stressed samples were titered in triplicate to calculate survival following stress (except the controls,
124 0.17M [i.e., LB medium] for saline and 0M for urea, which were titered singly). We then fit a generalized
125 linear model and carried out an ANCOVA. For urea, the model was of $\log_{10}$(percent survival) as a
126 function of urea concentration (as a factor) and the interaction between concentration and duration; for
127 salt, of $\log_{10}$(percent survival) as a function of saline concentration (as a factor) and the interaction
128 between concentration and duration. Values below the limit of detection and treatments that had fewer
129 than 3 points above the limit of detection were excluded from statistical analyses and plots.

130 Heat stress survival
131 To measure effects of heat on phage stability, for each temperature-duration treatment 100 µL of a
132 single OMKO1 phage stock (for Fig 3A, only Stock A) stored in LB media was split evenly into two
133 replicate 250 µL Eppendorf tubes and placed in a pre-heated MyCycler PCR block (BioRad Laboratories,
134 Inc., Hercules, CA) with an established temperature gradient (30-80°C, at 5°C intervals). Tubes were
135 removed from the heat block after specified times, and immediately cooled on ice to halt the stressful
136 condition. The two replicate tubes were mixed together, and then titered in triplicate to calculate
137 percent survival. We then fit a generalized linear model and carried out an ANCOVA. For Fig 3A, the
138 model was of $\log_{10}$(percent survival) as a function of temperature (as a factor) and the interaction
139 between temperature and duration; for Fig 3B, of $\log_{10}$(percent survival) as a function of phage stock
140 and the interaction of phage stock and duration. Values below the limit of detection and treatments that
141 had fewer than 3 points above the limit of detection were excluded from statistical analyses and plots.

142 Measuring phage fitness by bacterial growth curves
143 To measure phage fitness post heat stress, phages were subjected to heat stress as described above
144 then titered. These samples were then normalized by concentration to combine a defined number of
145 post-stress PFUs with bacteria in a 200 µL volume of LB in replicate wells (n = 3 or 6) of a 96-well plate
146 (Corning Inc., Corning, NY). For most populations, 200 PFUs were inoculated in each well. However,
147 because of low phage survival rates at longer heat shock times, some wells received fewer PFUs (Fig S2).

148 Across all wells, however, bacteria and phage were inoculated at a constant multiplicity of infection
149 (MOI; ratio of phage particles to bacterial cells) of 10^{-5}. Plates were incubated overnight at 37°C and an
150 automated spectrophotometer (INFINITE F500 microplate reader, TECAN Schweiz AG, Männedorf,
151 Switzerland) was used to monitor changes in the bacterial density (OD600), as described above. Growth
152 curves were smoothed and the first local maximum optical density of the bacterial population was used
153 as a reverse proxy for the fitness of phage [36]; see R script at https://github.com/mikeblazanin/tin-
154 omko1. We then fit a linear model and carried out an ANOVA for peak bacterial density as a function of
155 heat shock treatment (as a factor), phage stock, and treatment-stock interaction. Treatments which
156 were not initially inoculated with 200 PFUs were excluded from statistical analyses. Post-hoc pairwise
157 comparisons between all levels of the heat shock treatment were performed with Tukey's Honest
158 Significant Differences.

159

160 **Results**

161 To estimate the tolerance of phage OMKO1 to salt stress, we exposed samples of phage OMKO1 to
162 seven different NaCl concentrations ranging from 0.5M to 5M saline (n=1 per treatment), including
163 0.17M saline (i.e., LB medium) as a control. In each treatment environment, subsamples were obtained
164 at 5, 30, 60 and 90 minutes to create 28 test measures (7 salt environments x 4 time points). We then
165 measured the number of phage particles (PFU) that remained viable ('survived') for infection ability on
166 *P. aeruginosa* bacteria in each subsample. These measures were compared to the number of phage
167 particles measured at 5 minutes in the control environment to estimate changes in percent survival over
168 time. Results (Fig. 1) showed that saline concentration did not significantly alter the rate of particle
169 decay over time (slope of the lines in Fig 1; ANCOVA: $F(8, 16) = 0.93$, $p = 0.52$). We concluded that phage
170 OMKO1 viability was unaffected by salt exposure under the conditions tested.

171

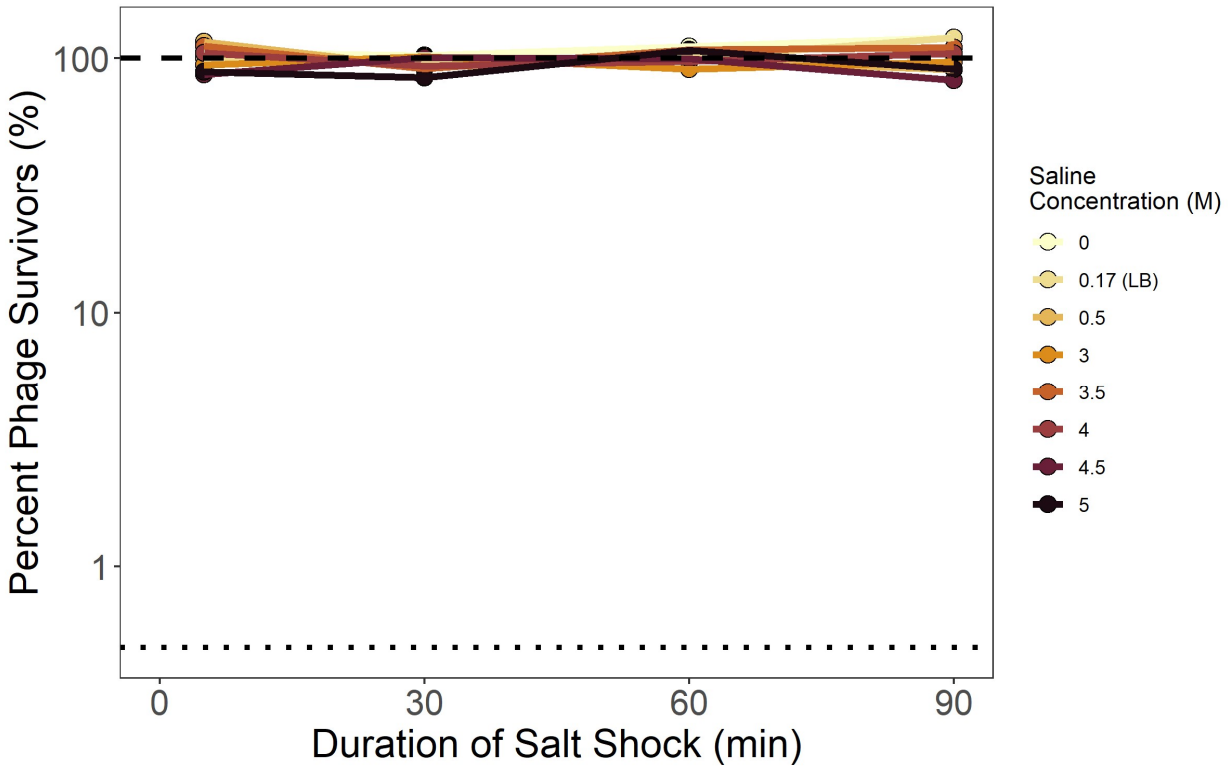


Figure 1. Phage OMKO1 stability is unaffected by saline concentration

To measure phage particle survival of saline stress, phage OMKO1 was exposed to a range of salt concentrations then titered over time. Mean percent survival was calculated and is plotted relative to the titer of the 5-minutes control (0.17M – LB media). The dashed line denotes 100% survival, while the dotted line denotes the limit of detection.

To estimate the tolerance of phage OMKO1 to urea stress, we exposed samples of phage OMKO1 to eleven different urea concentrations ranging from 1M to 10M urea (n=1 per treatment), including 0M urea as a control. In each treatment environment, subsamples were obtained at 5, 30, 60 and 90 minutes, to create 44 test measures (11 urea environments x 4 time points). We then measured the number (PFU) of surviving viable phage particles over time. These measures were compared to the particle number measured at 5 minutes in the 0M control to estimate changes in percent survival over time. Results (Fig 2) showed that urea concentration had a significant effect on the rate of decay of phage particles (slope of the lines in Fig 2; ANCOVA: $F(6, 12) = 128.49$, $p < 0.001$). Specifically, we observed that particle survival was unaffected in 0M urea, whereas phage samples showed significantly negative rates of decay in the five lowest urea concentrations (1M through 5M urea), and that the remaining highest concentrations of urea (6M through 10M urea) caused such rapid particle decay that most measures in these environments were unobtainable (i.e., fell below the limit of detection) (Table 1). We concluded that urea concentration was an environmental factor capable of affecting phage OMKO1 survival, depending on the level and time duration of urea exposure.

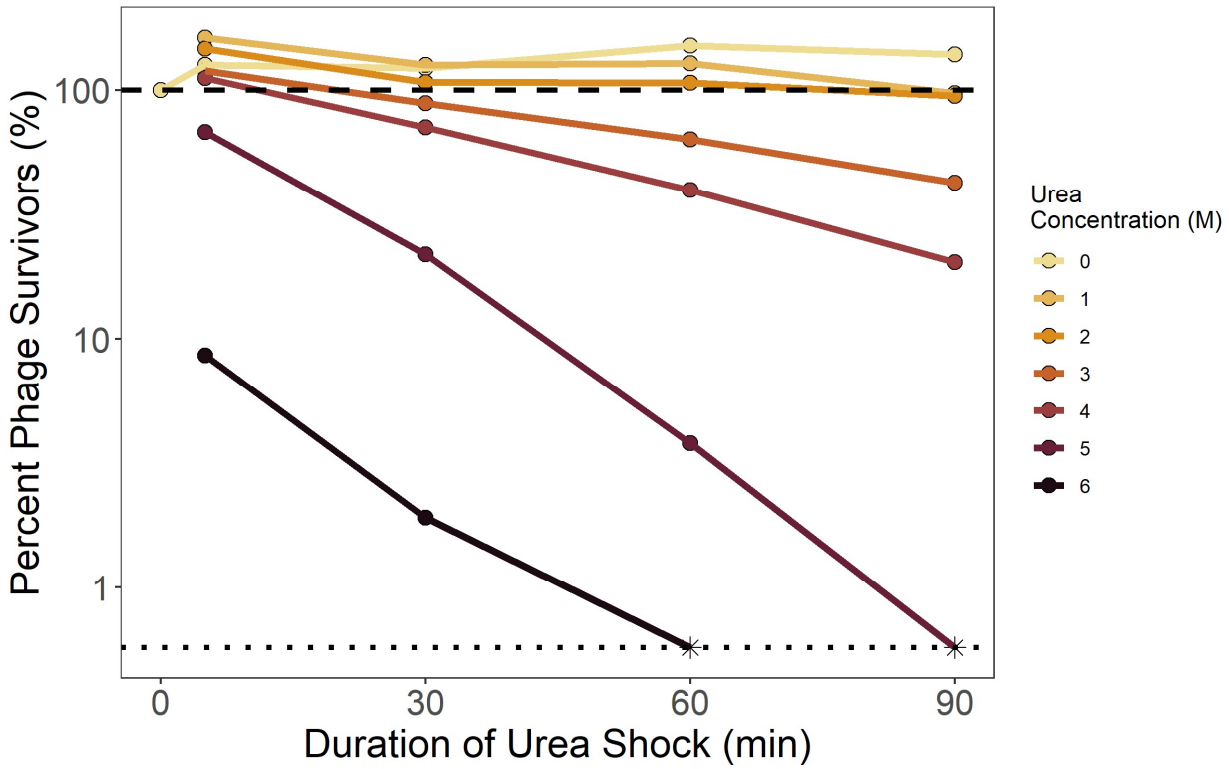


Figure 2. Degradation of phage OMKO1 under different urea concentrations.

To measure phage particle survival of urea stress, phage OMKO1 was exposed to a range of urea concentrations then titered over time. Mean percent survival was calculated and is plotted relative to the titer of the 0-minute control (0M). The dashed line denotes 100% survival, the dotted line denotes the limit of detection, and asterisks denote observations that fell below that limit. Concentrations 7M through 10M were tested but caused such rapid particle decay that all measures fell below the limit of detection.

Table 1. Multiple regression shows significant phage OMKO1 decay at 1M and higher urea concentrations. Parameter estimates of the rate of decay over time (slope) depending on urea concentration were evaluated to detect increased decay (decreased slopes) against the null hypothesis of 0 slope using one-tailed t-tests (df = 12) and a Bonferroni correction (6 tests).

Urea Concentration (M)	Estimated Coefficient	t-value	Bonferroni-adjusted p-value
0	0.0014	2.57	1
1	-0.0023	-3.64	0.010
2	-0.0020	-3.08	0.028
3	-0.0052	-8.26	<0.001
4	-0.0087	-13.70	<0.001
5	-0.0228	-22.04	<0.001
6-10	NA	NA	NA

To estimate the tolerance of phage OMKO1 to heat stress, we exposed samples of phage OMKO1 to six different temperatures ranging from 55°C to 80°C for 5, 30, 60, or 90 minutes, (n = 1 per temperature-duration combination) creating 24 test combinations (6 temperature environments x 4 time points). We then measured the percent of surviving viable phage particles, relative to the number estimated at 5 minutes at 55°C, as described above. Results (Fig 3A) showed a significant effect of temperature shock on phage OMKO1 decay rate (ANCOVA, $F(4, 8) = 125.01$, $p = <0.001$). At the two lowest temperatures (55°C and 60°C), there was no significant evidence of OMKO1 decay over time (Table 2). However, at 65°C there was a significant moderate signal of decay, which became more pronounced at 70°C. 75°C and 80°C caused such rapid particle decay that most measures in these environments were unobtainable (i.e., fell below the limit of detection) (Table 1). Abnormal plaque morphology was also observed in heat-treated phages (data not shown), suggesting possible phenotypic alteration of phage OMKO1 following heat treatment, explored further below. We concluded that heat shock was an environmental factor capable of affecting phage OMKO1 survival, depending on the level and time duration of temperature exposure.

Table 2. Multiple regression shows significant phage OMKO1 decay at 65°C and higher temperatures. Parameter estimates of the rate of decay over time (slope) depending on temperature were evaluated to detect increased decay (decreased slopes) against the null hypothesis of 0 slope using one-tailed t-tests (df = 8) and a Bonferroni correction (4 tests).

Temperature (°C)	Estimated Coefficient	t-value	Bonferroni-adjusted p-value
55	0.0008	0.98	1
60	0.0005	0.58	1
65	-0.0028	-3.35	0.020
70	-0.0182	-22.08	<0.001
75-80	NA	NA	NA

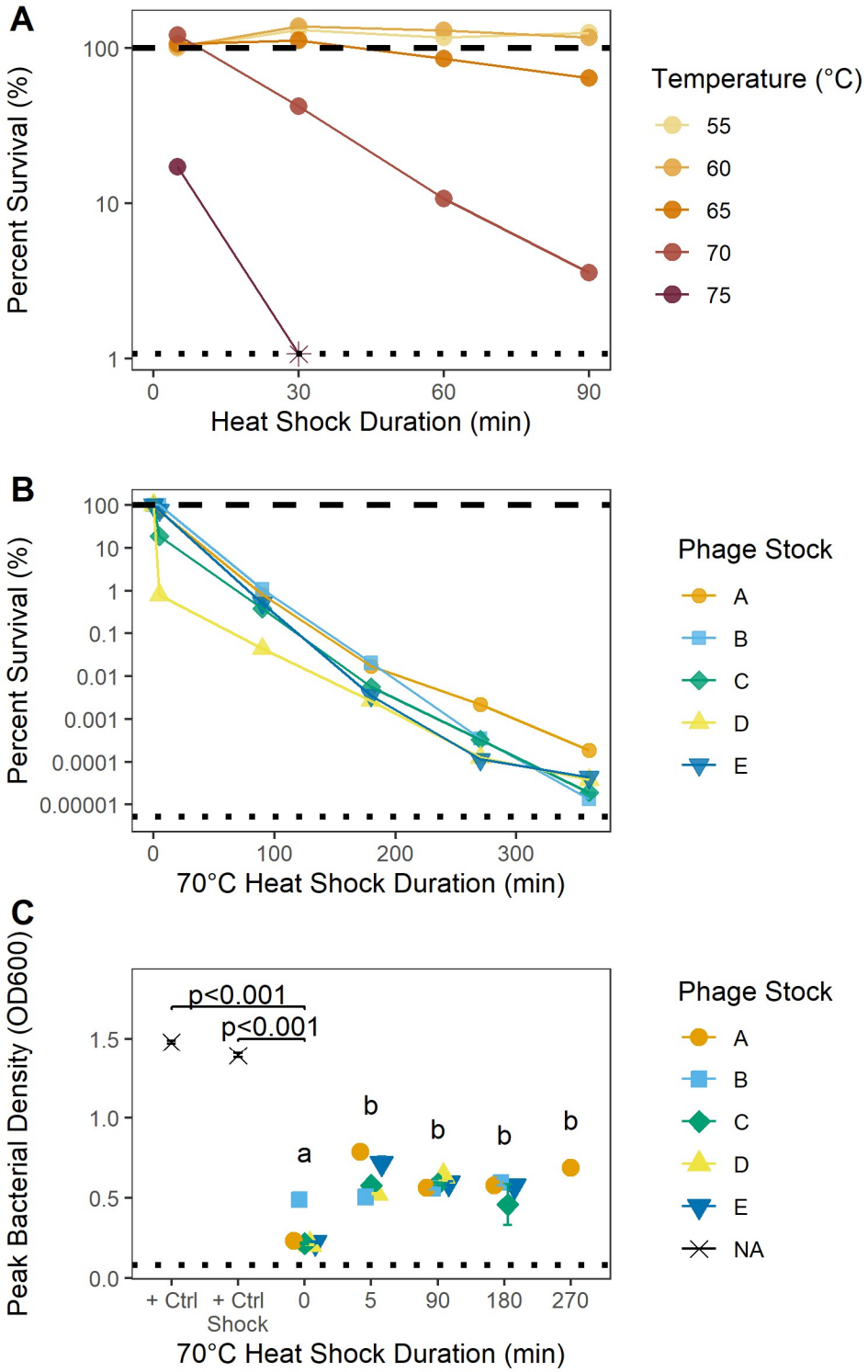
Given the observed substantial decay of phage OMKO1 induced by elevated temperatures (Fig. 3A), we next sought to examine whether heat-induced decay continues exponentially over longer periods of time, as well as the degree of variation in heat-induced decay among different stocks of the phage. We generated five independent phage stocks (A through E) and exposed each to a 5-minute control and four prolonged periods (90, 180, 270 and 360 minutes) of 70°C heat stress (n=1 per stock-duration combination). We then measured the percentage of surviving viable phage particles. Results (Fig 3B) showed significant decay over time (ANCOVA effect of duration, $F(1, 20) = 519.1$, $p < 0.001$) and some weak variation among biological replicates in decay dynamics (ANCOVA effect of stock, $F(4, 20) = 2.54$, $p = 0.07$; ANCOVA interaction between stock and duration, $F(4, 20) = 0.95$, $p = 0.45$). Additionally, as expected, we found very similar fit estimates for the comparable decay rate of phage OMKO1 between the two experiments [Fig 3A slope of $\log_{10}$(percent) at 70°C = -0.0167, Fig 3B slope of $\log_{10}$(percent) of stock A = -0.0161]. We concluded that prolonged exposure of phage OMKO1 to a deleterious heat-shock

238 environment caused reduced percent survival over time, and that biological replicates of the phage
239 behaved similarly in the assayed conditions.

240 Finally, we sought to understand whether the effects of temperature might extend beyond the simple
241 viability of phage particles (as assessed by their ability to form plaques). In particular, we tested how the
242 duration of high temperature exposure affected the growth ability of surviving phage particles on
243 susceptible host bacteria. To do so, we took the phage particles that had been heat-shocked at 70°C for
244 different durations and used dilutions in LB to normalize their concentrations according to how many
245 plaques they formed. Because of low titers, some populations after 270 minutes of heat shock and all
246 populations after 300 minutes of heat shock were unable to be standardized to the necessary
247 concentration (see Fig S1 for data). Then, we inoculated these standardized suspensions with *P.*
248 *aeruginosa* host bacteria in assay wells of a 96-well plate. We grew these cultures for >12 hours,
249 measuring the density (OD600) of bacteria every five minutes using an automated spectrophotometer
250 (see Methods). As expected, the observations revealed that bacterial density increased initially, peaked
251 at a maximum value, and then declined over time as the phage in the mixture lysed the bacterial
252 population (data not shown). As a proxy for phage fitness (virus growth ability on the bacterial
253 population), we computationally determined the peak bacterial density in each well. Here, the peak
254 density is thus negatively related to the fitness of the test phage population, because more fit phage
255 would suppress bacterial density sooner and to a greater degree, producing lower peak densities. For
256 comparison, we included two controls where bacteria were cultured in the absence of phage for >12
257 hours as described above, in normal LB medium and in LB medium that was heat-shocked for 360
258 minutes.

259 As shown in Fig. 3C, we observed a significant effect of phage OMKO1 heat-shock exposure on
260 subsequent fitness of the viruses, as measured by peak density of the bacterial population (ANOVA, $F(6,$
261 $49) = 1478.5$, $p < 0.001$); in addition, we observed significant variation among the phage stocks in both
262 peak density in the absence of heat shock, and how peak bacterial density responded to heat-shocked
263 viruses (ANOVA effect of stock, $F(4, 49) = 13.73$, $p = < 0.001$; ANOVA interaction between stock and
264 treatment, $F(11, 49) = 27.95$, $p < 0.001$). As expected, the presence of phage OMKO1 reduced peak
265 bacterial density relative to the uninfected bacterial controls, indicating that phage negatively affected
266 bacterial growth (Table 3). Additionally, any phage exposure to heat shock allowed the peak bacterial
267 density to measurably increase, demonstrating that heat shock generally decreased virus fitness (Tukey
268 Honest Significant Differences among all heat stress treatments showed significant differences between
269 0 minutes and all other treatments at $p < 0.01$, see Fig 3C). However, increasing durations of heat shock
270 exposure did not lead to further measurable increases in peak bacterial density, indicating that phage
271 fitness did not further decline with heat-shock duration (Tukey HSD finds no significant differences
272 among 5 minutes through 270 minutes treatments, $p \geq 0.69$). Surprisingly, this remained true even after
273 extremely long heat shocks, where the survival rate was very low (e.g. after 180 minutes survival was
274 ~0.01%; Fig. 3B). Note that this increase in peak bacterial density could not be explained by the effect of
275 heat-shocked medium alone, as heat shocked media reduces peak bacterial density (+ Ctrl Shock vs +
276 Ctrl, two-sample t-test of unequal variance, $t = -4.18$, $df = 8.86$, $p = 0.002$). These results indicated that,
277 in addition to the negative effects of heat shock on phage survival (Figs 3A and B), any exposure to heat

278 stress reduced the subsequent phenotypic ability (fitness) of phage OMKO1, shown by decreased impact
279 of viruses on the growth of susceptible bacteria.



280

281 **Figure 3. Inactivation & fitness suppression of OMKO1 by thermal stress**

282 **A.** To measure phage particle survival of heat stress at different temperatures, OMKO1 was
283 exposed to one of a range of temperatures for 5, 30, 60, or 90 minutes, then titered. Percent
284 survival was calculated and is plotted relative to the titer of the 0-minute 55°C treatment. The
285 dashed line denotes 100% survival, while asterisks denote the first observations that fell below
286 the limit of detection (dotted line). 80°C was tested but caused such rapid particle decay that all
287 measures fell below the limit of detection. **B.** To measure phage particle survival of heat stress
288 over longer periods of time, and to measure variation in heat stress tolerance between different
289 stocks of OMKO1, five stocks were exposed to 70°C for 5, 90, 180, 270, or 360 minutes, then
290 titered. Percent survival was calculated and is plotted relative to the source stock. The dashed
291 line denotes 100% survival, while the dotted line denotes the highest limit of detection across
292 the five stocks. **C.** To determine whether phage growth was altered by exposure to heat stress
293 among surviving phage particles, heat shocked phage particles (from Fig 3B) were inoculated
294 with bacteria and grown overnight with repeated measurements of bacterial density. The height
295 of the peak bacterial density was computationally determined and is plotted relative to the
296 negative control wells with no bacteria (dotted line). Thus, this peak height is a reverse proxy for
297 phage fitness. Error bars denote 95% confidence intervals among replicate wells (non-visible
298 error bars are smaller than the points). Positive control bacteria were grown in the absence of
299 phage in LB media ("+ Ctrl") or LB media that had been heat shocked for 360 mins at 70°C ("+
300 Ctrl Shock"). T-tests showed significant reduction in peak bacterial density between positive
301 controls and unshocked phage (see Table 3), and Tukey Honest Significant Differences were
302 used among all heat shock treatments (see main text).

303

304 **Table 3. Phage OMKO1 suppression of peak bacterial density is robust to presence/absence of**
305 **heat-shocked media in controls.** Two-sample one-tailed unequal variance t-tests between both
306 positive controls (+ Ctrl is bacteria alone, + Ctrl Shock is bacteria alone in media shocked at 70°C
307 for 360 mins) and each of the 0 minutes heat shock populations show Bonferroni-corrected (10
308 tests) significant reductions in peak bacterial growth.

Contrast	Estimated Difference	df	t-value	Bonferroni-adjusted p-value
+ Ctrl – 0 mins Phage A	-1.24	5.35	-106.6	<0.001
+ Ctrl – 0 mins Phage B	-0.99	6.38	-65.2	<0.001
+ Ctrl – 0 mins Phage C	-1.26	5.77	-105.6	<0.001
+ Ctrl – 0 mins Phage D	-1.27	5.24	-109.2	<0.001
+ Ctrl – 0 mins Phage E	-1.25	6.29	-101.8	<0.001
+ Ctrl Shock – 0 mins Phage A	-1.16	5.17	-68.9	<0.001
+ Ctrl Shock – 0 mins Phage B	-0.90	6.98	-46.6	<0.001
+ Ctrl Shock – 0 mins Phage C	-1.18	5.38	-69.1	<0.001
+ Ctrl Shock – 0 mins Phage D	-1.18	5.11	-70.5	<0.001
+ Ctrl Shock – 0 mins Phage E	-1.17	5.68	-67.7	<0.001

309

310 **Discussion**

311 Due to increasing interest in phage therapy to complement or replace traditional chemical antibiotics,
312 we sought to understand the stability and response of phage particles to environmental stressors. Here,
313 we examined how the therapeutic phage OMKO1 responded to possible degradation caused by salt,
314 urea, and heat stressors as a model of the environmental robustness of a phage therapy candidate. We
315 observed no measurable adverse effect of salinity on phage OMKO1 survival (Fig 1), but strong effects of
316 both increased urea concentrations (Fig 2) and elevated temperatures (Fig 3A, B) on virus survival. In
317 addition, we found that heat stress generally induced additional reductions in virus fitness, as heat-
318 shock surviving phage particles had reduced abilities to suppress a susceptible bacterial population
319 when compared to non-stressed viruses (Fig 3C).

320 High temperatures induced both increased decay of phage particles and decreased phage lysis of their
321 bacterial hosts. Phage OMKO1 particles decay approximately exponentially (Fig 3B, linear on log-linear
322 axes), with higher temperatures increasing the rate of degradation, although we have limited resolution
323 to detect a bi-phasic decay, as observed elsewhere [25]. Intriguingly, however, OMKO1 populations
324 experience a drastic reduction in fitness after even a brief exposure to heat stress, while increased
325 durations of heat stress have little additional effect (Fig 3C). Along with the observed abnormal plaque
326 morphology after heat shock, these results indicate a strong adverse phenotypic response to
327 environmental temperature conditions. We propose two possible explanations for this observation. The
328 first is that the population of OMKO1 particles is heterogenous in resistance to heat shock, and that
329 stability trades off with fitness. While this sort of stability-function tradeoff is thought to be common for
330 proteins [37, 38], this explanation is incompatible with the observation of simultaneous high survival
331 and large decreases in fitness for two of the replicate populations (A and E, see Fig 3B, C). The second
332 explanation is that high temperatures alter viral particle conformations to a lower-fitness state. Thus,
333 any exposure to heat shock alters the state and phenotype of all surviving viral particles, consistent with
334 the observed changes in fitness following heat shock (Fig 3C). While this bistability is environmentally
335 induced, phage particle bistability elsewhere has been previously reported to promote the evolution of
336 novel host use [39] .

337 Our observation of deactivation of phage OMKO1 by elevated urea concentrations was consistent with
338 previously-published findings on other phages. For example, in some of the earliest work published on
339 this topic tested the survival of particles of coliphages (viruses that specifically infect *Escherichia coli*)
340 over time in a 4.61M urea solution, observing decay anywhere from 0 to 7 orders of magnitude within
341 30 minutes [27]. Similarly, phage T4 experienced 90% decay after one minute of exposure to 2.5M urea
342 [28]. By comparison, phage OMKO1 decayed more slowly (~80% after 30 minutes at 5M, Fig 2),
343 suggesting that this virus has relatively greater tolerance for elevated urea concentrations compared
344 with the limited evidence from other published studies on phage survival.

345 In contrast to our findings with urea, our failure to find an effect of saline stress diverged from
346 previously-published results on other phages. Phages T2, T4, T6 and ps_1 have been reported to be
347 deactivated when they are rapidly osmotically shocked from 4M to 0M NaCl, and several phages decay
348 in 0M NaCl, all conditions where we observed no effect [26, 29, 30]. Experimental differences may have
349 contributed to these divergent results, including differences in the rate of dilution and thus severity of

osmotic shock (in the former case) or in the shorter length of time our experiments lasted (in the latter case). Additionally, our findings likely reflect some degree of underlying differences in susceptibility to osmotic shock between different phages, where OMKO1 is more tolerant of osmotic shock and saline stress than other reported phages.

Our results are also relevant to the understanding of how environmental stressors might affect use of phage OMKO1 in human therapy. All of our experiments were designed to span (and exceed) the range of environmental conditions that phage particles could experience during storage or within therapy applications in the human body. For example, within the human body the concentrations of salt range from roughly 30mM in urine to 135-145 mM in blood [40], all conditions where we saw no increase in phage OMKO1 decay rate (Fig 1); urea concentrations range from 2.6-6.5mM in human blood up to 325mM in human urine [41], again conditions where we observed no increase in phage decay rate (Fig 2); and human body temperatures can reach a maximum of around 40°C while phage storage conditions could reach perhaps 50°C, again conditions where we expect no effect on phage OMKO1 decay rate based on our findings (Fig 3A). These indications of the general stability of phage OMKO1 despite possible environmental stressors are promising for its further potential uses in clinical applications.

Future studies on the stability of phage OMKO1 and other therapeutic phages should expand the types of examined stressors, consider interactions between multiple stressors, and begin to more deeply elucidate the nature of the observed phage particle bistability. Our findings were limited by the scope and nature of our experimental designs. Additionally, we carried out 14 statistical tests, so we would expect nearly one of those tests to have been significant by chance (we observed 11 to be significant at $p<0.05$). Future work can expand on the scope of this study by considering the effects of pH and electromagnetic radiation, as well as interactions among any environmental stressors or between these stressors and storage conditions [e.g. freezing, lyophilizing (freeze drying)]. Additionally, biophysical and imaging approaches could be utilized to understand the mechanistic details behind phage particle stability and conformation, like those underlying the observed changes in phage fitness after any duration of heat stress exposure. While these experiments would deepen our understanding of phage biology and stability, our current work has revealed the limits of the stability of phage OMKO1 and highlighted intriguing nonlinear responses of phages to environmental stress, confirming the need for careful characterization and storage of therapeutic phages for widespread clinical use.

Acknowledgements

We thank Alita Burmeister, Caroline Turner, and Michael Wiser for helpful feedback on earlier versions of the manuscript, and the support for Wai Tin Lam from the Chinese University of Hong Kong.

Data Availability

All data and analysis are available at https://github.com/mikeblazanin/tin-omko1.

387 **References**

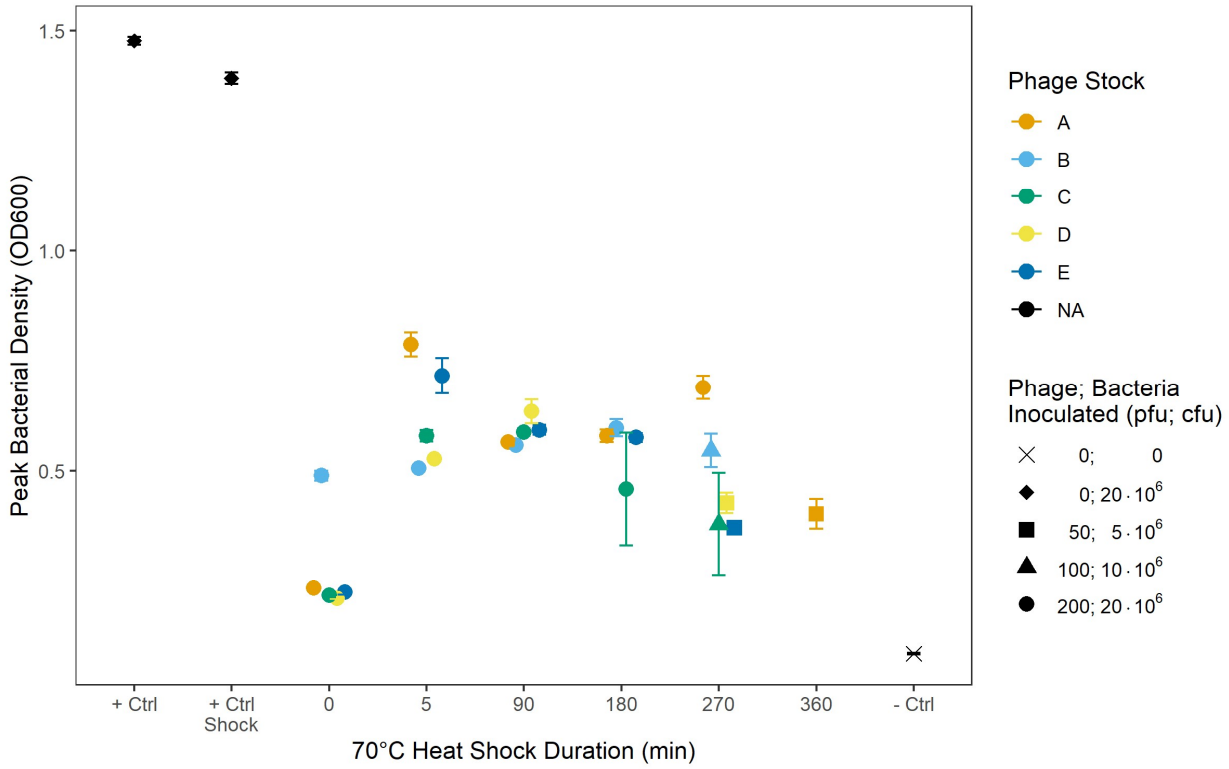
388 1. de Kraker ME, Davey PG, Grundmann H. Mortality and hospital stay associated with resistant
389 Staphylococcus aureus and Escherichia coli bacteremia: estimating the burden of antibiotic resistance in
390 Europe. PLoS medicine. 2011;8(10):e1001104. Epub 2011/10/25. doi: 10.1371/journal.pmed.1001104.
391 PubMed PMID: 22022233; PubMed Central PMCID: PMCPMC3191157.
392 2. Michael CA, Dominey-Howes D, Labbate M. The antimicrobial resistance crisis: causes,
393 consequences, and management. Frontiers in public health. 2014;2:145. Epub 2014/10/04. doi:
394 10.3389/fpubh.2014.00145. PubMed PMID: 25279369; PubMed Central PMCID: PMCPMC4165128.
395 3. Kortright KE, Chan BK, Koff JL, Turner PE. Phage Therapy: A Renewed Approach to Combat
396 Antibiotic-Resistant Bacteria. Cell host & microbe. 2019;25(2):219-32. Epub 2019/02/15. doi:
397 10.1016/j.chom.2019.01.014. PubMed PMID: 30763536.
398 4. Landerkin GB, Katznelson H. Stability of antibiotics in honey and sugar syrup as affected by
399 temperature. Applied microbiology. 1957;5(3):152-4. Epub 1957/05/01. PubMed PMID: 13435725;
400 PubMed Central PMCID: PMCPMC1057279.
401 5. Wick WE. Influence of antibiotic stability on the results of in vitro testing procedures. Journal of
402 bacteriology. 1964;87(5):1162-70. Epub 1964/05/01. PubMed PMID: 5874538; PubMed Central PMCID:
403 PMCPMC277162.
404 6. Samara E, Moriarty TF, Decosterd LA, Richards RG, Gautier E, Wahl P. Antibiotic stability over six
405 weeks in aqueous solution at body temperature with and without heat treatment that mimics the curing
406 of bone cement. Bone & joint research. 2017;6(5):296-306. Epub 2017/05/19. doi: 10.1302/2046-
407 3758.65.bjr-2017-0276.r1. PubMed PMID: 28515059; PubMed Central PMCID: PMCPMC5457644.
408 7. Svahn O, Bjorklund E. Describing sorption of pharmaceuticals to lake and river sediments, and
409 sewage sludge from UNESCO Biosphere Reserve Kristianstads Vattenrike by chromatographic
410 asymmetry factors and recovery measurements. Journal of chromatography A. 2015;1415:73-82. Epub
411 2015/09/13. doi: 10.1016/j.chroma.2015.08.061. PubMed PMID: 26362805.
412 8. Theuretzbacher U. Global antibacterial resistance: The never-ending story. Journal of global
413 antimicrobial resistance. 2013;1(2):63-9. Epub 2013/06/01. doi: 10.1016/j.jgar.2013.03.010. PubMed
414 PMID: 27873580.
415 9. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery,
416 research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and
417 tuberculosis. The Lancet Infectious Diseases. 2018;18(3):318-27. doi: https://doi.org/10.1016/S1473-
418 3099(17)30753-3.
419 10. Fazeli H, Akbari R, Moghim S, Narimani T, Arabestani MR, Ghoddousi AR. Pseudomonas
420 aeruginosa infections in patients, hospital means, and personnel's specimens. J Res Med Sci.
421 2012;17(4):332-7. Epub 2012/12/26. PubMed PMID: 23267393; PubMed Central PMCID:
422 PMCPMC3526125.
423 11. Mena KD, Gerba CP. Risk assessment of Pseudomonas aeruginosa in water. Reviews of
424 environmental contamination and toxicology. 2009;201:71-115. Epub 2009/06/02. doi: 10.1007/978-1-
425 4419-0032-6_3. PubMed PMID: 19484589.

426 12. Remold SK, Brown CK, Farris JE, Hundley TC, Perpich JA, Purdy ME. Differential habitat use and
427 niche partitioning by Pseudomonas species in human homes. Microbial ecology. 2011;62(3):505-17.
428 Epub 2011/04/20. doi: 10.1007/s00248-011-9844-5. PubMed PMID: 21503776.
429 13. Slekovec C, Plantin J, Cholley P, Thouverez M, Talon D, Bertrand X, et al. Tracking down
430 antibiotic-resistant Pseudomonas aeruginosa isolates in a wastewater network. PloS one.
431 2012;7(12):e49300-e. doi: 10.1371/journal.pone.0049300. PubMed PMID: 23284623.
432 14. Garau J, Gomez L. Pseudomonas aeruginosa pneumonia. Current opinion in infectious diseases.
433 2003;16(2):135-43. Epub 2003/05/08. doi: 10.1097/00001432-200304000-00010. PubMed PMID:
434 12734446.
435 15. Mittal R, Aggarwal S, Sharma S, Chhibber S, Harjai K. Urinary tract infections caused by
436 Pseudomonas aeruginosa: A minireview. Journal of Infection and Public Health. 2009;2(3):101-11. doi:
437 https://doi.org/10.1016/j.jiph.2009.08.003.
438 16. Aeschlimann JR. The role of multidrug efflux pumps in the antibiotic resistance of Pseudomonas
439 aeruginosa and other gram-negative bacteria. Insights from the Society of Infectious Diseases
440 Pharmacists. Pharmacotherapy. 2003;23(7):916-24. Epub 2003/07/30. doi:
441 10.1592/phco.23.7.916.32722. PubMed PMID: 12885104.
442 17. Mah TF, Pitts B, Pellock B, Walker GC, Stewart PS, O'Toole GA. A genetic basis for Pseudomonas
443 aeruginosa biofilm antibiotic resistance. Nature. 2003;426(6964):306-10. Epub 2003/11/25. doi:
444 10.1038/nature02122. PubMed PMID: 14628055.
445 18. Walters MC, 3rd, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. Contributions of antibiotic
446 penetration, oxygen limitation, and low metabolic activity to tolerance of Pseudomonas aeruginosa
447 biofilms to ciprofloxacin and tobramycin. Antimicrobial agents and chemotherapy. 2003;47(1):317-23.
448 Epub 2002/12/25. PubMed PMID: 12499208; PubMed Central PMCID: PMCPMC148957.
449 19. Seed KD. Battling Phages: How Bacteria Defend against Viral Attack. PLoS pathogens.
450 2015;11(6):e1004847. Epub 2015/06/13. doi: 10.1371/journal.ppat.1004847. PubMed PMID: 26066799;
451 PubMed Central PMCID: PMCPMC4465916.
452 20. Chan BK, Sistrom M, Wertz JE, Kortright KE, Narayan D, Turner PE. Phage selection restores
453 antibiotic sensitivity in MDR Pseudomonas aeruginosa. Sci Rep. 2016;6:26717. Epub 2016/05/27. doi:
454 10.1038/srep26717. PubMed PMID: 27225966; PubMed Central PMCID: PMCPMC4880932.
455 21. Chan BK, Turner PE, Kim S, Mojibian HR, Elefteriades JA, Narayan D. Phage treatment of an
456 aortic graft infected with Pseudomonas aeruginosa. Evol Med Public Health. 2018;2018(1):60-6. Epub
457 2018/03/29. doi: 10.1093/emph/eoy005. PubMed PMID: 29588855; PubMed Central PMCID:
458 PMCPMC5842392.
459 22. Pollard EC, Solosko W. The thermal inactivation of T4 and lambda bacteriophage. Biophysical
460 journal. 1971;11(1):66-74. doi: 10.1016/S0006-3495(71)86195-7. PubMed PMID: 5539000.
461 23. Goldhill DH, Turner PE. The evolution of life history trade-offs in viruses. Current opinion in
462 virology. 2014;8:79-84. Epub 2014/08/05. doi: 10.1016/j.coviro.2014.07.005. PubMed PMID: 25087040.
463 24. Jonczyk E, Klak M, Miedzybrodzki R, Gorski A. The influence of external factors on
464 bacteriophages--review. Folia microbiologica. 2011;56(3):191-200. Epub 2011/06/01. doi:
465 10.1007/s12223-011-0039-8. PubMed PMID: 21625877; PubMed Central PMCID: PMCPMC3131515.

466 25. Bleichrodt JF, Blok J, Berends-Van Abkoude ER. Thermal inactivation of bacteriophage phi X174
467 and two of its mutants. Virology. 1968;36(3):343-55. Epub 1968/11/01. doi: 10.1016/0042-
468 6822(68)90160-8. PubMed PMID: 5722182.
469 26. Anderson TF. The reactions of bacterial viruses with their host cells. The Botanical Review.
470 1949;15(7):464-505.
471 27. Burnet FM. The Classification of Dysentery-Coli Bacteriophages. III. A Correlation of the
472 Serological Classification with Certain Biochemical Tests. Journal of Pathology and Bacteriology.
473 1933;37:179-84.
474 28. Sato GH. Activation of bacteriophage by urea. Science (New York, NY). 1956;123(3203):891-2.
475 Epub 1956/05/18. doi: 10.1126/science.123.3203.891. PubMed PMID: 13324109.
476 29. Seaman PF, Day MJ. Isolation and characterization of a bacteriophage with an unusually large
477 genome from the Great Salt Plains National Wildlife Refuge, Oklahoma, USA. FEMS microbiology
478 ecology. 2007;60(1):1-13. Epub 2007/01/26. doi: 10.1111/j.1574-6941.2006.00277.x. PubMed PMID:
479 17250749.
480 30. Whitman PA, Marshall RT. Characterization of two psychrophilic Pseudomonas bacteriophages
481 isolated from ground beef. Applied microbiology. 1971;22(3):463-8. Epub 1971/09/01. PubMed PMID:
482 4107517; PubMed Central PMCID: PMCPMC376334.
483 31. Dulbecco R, Vogt M. Some problems of animal virology as studied by the plaque technique. Cold
484 Spring Harbor symposia on quantitative biology. 1953;18:273-9. Epub 1953/01/01. doi:
485 10.1101/sqb.1953.018.01.039. PubMed PMID: 13168995.
486 32. Team RC. R: A Language and Environment for Statistical Computing [Manual]. Vienna, Austria: R
487 Foundation for Statistical Computing; 2019. Available from: https://www.r-project.org/.
488 33. Wickham H, François R, Henry L, Müller K. dplyr: A Grammar of Data Manipulation [Manual].
489 2019. Available from: https://cran.r-project.org/package=dplyr.
490 34. Wickham H. ggplot2: Elegant Graphics for Data Analysis: Springer-Verlag New York; 2016.
491 35. Ahlmann-Eltze C. ggsignif: Significance Brackets for 'ggplot2' [Manual]. 2019. Available from:
492 https://cran.r-project.org/package=ggsignif.
493 36. Turner PE, Draghi JA, Wilpiszeski R. High-throughput analysis of growth differences among
494 phage strains. Journal of microbiological methods. 2012;88(1):117-21. Epub 2011/11/22. doi:
495 10.1016/j.mimet.2011.10.020. PubMed PMID: 22101310.
496 37. Beadle BM, Shoichet BK. Structural bases of stability-function tradeoffs in enzymes. Journal of
497 molecular biology. 2002;321(2):285-96. Epub 2002/07/30. doi: 10.1016/s0022-2836(02)00599-5.
498 PubMed PMID: 12144785.
499 38. Studer RA, Christin PA, Williams MA, Orengo CA. Stability-activity tradeoffs constrain the
500 adaptive evolution of RubisCO. Proceedings of the National Academy of Sciences of the United States of
501 America. 2014;111(6):2223-8. Epub 2014/01/29. doi: 10.1073/pnas.1310811111. PubMed PMID:
502 24469821; PubMed Central PMCID: PMCPMC3926066.
503 39. Petrie KL, Palmer ND, Johnson DT, Medina SJ, Yan SJ, Li V, et al. Destabilizing mutations encode
504 nongenetic variation that drives evolutionary innovation. Science (New York, NY). 2018;359(6383):1542-
505 5. Epub 2018/03/31. doi: 10.1126/science.aar1954. PubMed PMID: 29599247.
506 40. Reynolds RM, Padfield PL, Seckl JR. Disorders of sodium balance. BMJ (Clinical research ed).
507 2006;332(7543):702-5. doi: 10.1136/bmj.332.7543.702. PubMed PMID: 16565125.

508 41. Liu L, Mo H, Wei S, Raftery D. Quantitative analysis of urea in human urine and serum by 1H
509 nuclear magnetic resonance. The Analyst. 2012;137(3):595-600. Epub 12/16. doi: 10.1039/c2an15780b.
510 PubMed PMID: 22179722.

511



512

513 **Figure S1. Fitness suppression of OMKO1 by thermal stress across all tested conditions.** To
514 determine whether phage growth was altered by exposure to heat stress among surviving phage
515 particles, heat shocked phage particles (from Fig 3B) were inoculated with bacteria and grown
516 overnight with repeated measurements of bacterial density. The height of the peak bacterial
517 density was computationally determined and is plotted. Thus, this peak height is a reverse proxy
518 for phage fitness. Error bars denote 95% confidence intervals among replicate wells. Positive
519 control bacteria were grown in the absence of phage in LB media ("+ Ctrl") or LB media that had
520 been heat shocked for 360 mins at 70°C ("+ Ctrl Shock"), while negative control wells contained
521 only media. Note that, due to low survival after long durations of heat shock, some growth
522 curves were initially inoculated with reduced numbers of bacteria and phage (holding MOI
523 constant), confounding observed differences in peak bacterial density.