Parallel evolution of *Pseudomonas aeruginosa* phage resistance and virulence loss in response to phage treatment *in vivo* and *in vitro*

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Abstract

With rising antibiotic resistance, there has been increasing interest in treating pathogenic bacteria with bacteriophages (phage therapy). One limitation of phage therapy is the ease at which bacteria can evolve resistance. Negative effects of resistance may be mitigated when resistance results in reduced bacterial growth and virulence, or when phage coevolve to overcome resistance. Resistance evolution and its consequences are contingent on the bacteria-phage combination and their environmental context, making therapeutic outcomes hard to predict. One solution might be to conduct “*in vitro* evolutionary simulations” using bacteria-phage combinations from the therapeutic context. Overall, our aim was to investigate parallels between *in vitro* experiments and *in vivo* dynamics in a human participant. Evolutionary dynamics were similar, with high levels of resistance evolving quickly with limited evidence of phage evolution. Resistant bacteria – evolved *in vitro* and *in vivo* - had lower virulence. *In vivo*, this was linked to lower growth rates of resistant isolates, whereas *in vitro* phage resistant isolates evolved greater biofilm production. Population sequencing suggests resistance resulted from selection on *de novo* mutations rather than sorting of existing variants. These results highlight the speed at which phage resistance can evolve *in vivo*, and how *in vitro* experiments may give useful insights for clinical evolutionary outcomes.

Introduction

By 2050, as many as 10 million people may die annually from antimicrobial resistant infections (WHO, 2019). To combat this threat, phage therapy is being increasingly researched as a complement to antibiotics (Abedon, 2019; Kwiatek et al., 2020). Phage therapy uses viruses (phage) that infect pathogenic bacteria to clear infections (Kwiatek et al., 2020). Phage therapy has several advantages to antibiotics by being species specific (thus not harming beneficial microbiota) (Koskella and Meaden, 2013) and self-amplifying at infection sites (Górski et al., 2015). A major consideration with the use of therapeutic phages is the ease and speed (sometimes within hours) at which bacteria can evolve resistance, which can greatly limit the therapeutic potential of phages (Jariah and Hakim, 2019; Torres-Barceló, 2018; Torres-Barceló and Hochberg, 2016). However, phages may contribute to reducing the severity of infections even after resistance evolves. First, phage can sometimes evolve to overcome this resistance (i.e. bacteria-phage coevolution) and so they may continue lowering bacterial densities (Gandon et al., 2008; Hampton et al., 2020). Second, resistance can trade-off against bacterial growth rates and virulence; as the alteration or loss of bacterial surface structures, typically associated with phage resistance, can affect other fitness-related phenotypes such as nutrient uptake, biofilm formation and binding of host receptors (Mangalea and Duerkop, 2020). Predicting the evolutionary outcome of phage therapy is therefore of crucial importance (Abedon, 2017).

Bacteria-phage dynamics and its consequences for phage therapy are highly context-specific (Gandon et al., 2008; Hampton et al., 2020). Both human clinical and animal models studies report a range of outcomes with differing phage therapy success, and differences in the extent of resistance evolution and associated costs and virulence (Abedon, 2019; Brix et al., 2020; Kwiatek et al., 2020; Leitner et al., 2021). One solution to better understand the outcome

of *in vivo* therapy is to conduct controlled *in vitro* experiments with the relevant organisms. However, while most of our understanding of the ecology and evolution of bacteria-phage interactions comes from highly controlled *in vitro* environments (Gandon et al., 2008; Hampton et al., 2020), we do not know how well *in vitro* dynamics mirror *in vivo* dynamics quantitatively or even qualitatively.

There are good reasons to expect the outcomes may be very different. *In vivo* environments are expected to be more stressful for bacteria owing to limiting resources, presence of immune cells and competing microbiota (Everest, 2007). Consequently, these conditions may differ significantly from studies of bacteria-phage coevolution in nutrient rich microcosms which could drive differences in (co)evolutionary dynamics and associated trade-offs (Gandon et al., 2008; Hampton et al., 2020). In order to evolve phage resistance, bacteria must have sufficient mutation supply, which will primarily be determined by population size and gene flow (Gandon et al., 2008; Hampton et al., 2020; Morgan et al., 2010, 2005; Pal et al., 2007). Smaller population sizes *in vivo* may therefore limit resistance. Moreover, resistance costs and trade-offs may be greater as a consequence of the additional selection pressures facing bacteria, which may limit selection for resistance, making it easier for phage to overcome resistance, or reduce growth rates and virulence (Buckling et al., 2006; Scanlan et al., 2015).

Consequently, the main aim of this research was to determine how well *in vitro* bacteria – phage coevolutionary dynamics mirror dynamics in a clinical context. As a starting point, we followed bacteria-phage coevolutionary dynamics during a phage-mediated decolonisation trial in parallel with the dynamics between the same bacteria and phage *in vitro*. The nasal cavity can act as a source of infection, notably from highly drug-resistant *Pseudomonas aeruginosa* and *Staphylococcus aureus* when patients undergo hospitalisation and are treated with broad

spectrum antibiotics (Mainz et al., 2009; Wertheim et al., 2004). The trial was conducted to try and decolonise intensive care (ICU) patients with *P. aeruginosa* and *S. aureus*, using a phage cocktail prior to antibiotic treatment in prevention of ICU infections with these pathogens. Mucirocin (trade name Bactroban) and chlorhexidine are routinely used for nasal decolonisation in such contexts, but extensive use of these agents has led to resistance and new nasal antiseptics need to be considered (Edgeworth, 2011). We focused on a single patient, in which we could detect *P. aeruginosa* at the beginning of the trial and both *P. aeruginosa* and phage during treatment. Decolonisation of *P. aeruginosa* was ultimately successful in this patient. We used the pre-treatment *P. aeruginosa* isolates and evolved these populations *in vitro* in the absence and presence of treatment phages. We measured bacteria-phage (co)evolution and while we found extensive resistance evolution both *in vivo* and *in vitro*, we only found evidence of limited phage infectivity evolution *in vitro*. We determined the consequences of resistance, with respect to virulence, and the possible mechanisms linking this resistance-virulence trade-off using phenotypic (growth rate, biofilm assays) and metagenomic analyses for the *in vivo* and *in vitro* populations. As we only had access to samples from a single patient, we did not have a control group to understand the impact of phage application frequency *in vivo*. However, we were able to simulate this in our *in vitro* experiments, where we included two phage treatments: one where phages were added repeatedly (mimicking multiple phage doses during *in vivo* phage treatment) and one where phage were added once (single dose at the start of the experiment). By comparing these treatments with the *in vivo* treatment, we were able to gain further insight into how multiple phage doses may affect phage therapy.

Results

Resistance and infectivity (co)evolution

In vivo

Resistance to phage can evolve via *de novo* mutations or selection on pre-existing resistance and we therefore tested the resistance of ancestral bacterial isolates to ancestral phage and pooled phage isolates isolated *in vivo*. Of the 24 ancestral isolates isolated *in vivo* at day 0, 23 were susceptible to both the ancestral phage (14-1 and PNM) while one was resistant to 14-1 but susceptible to PNM. All ancestral bacteria were susceptible to phage isolated from the patient during and after decolonisation (Figure 1a). We considered whether bacteria and phage coevolved *in vivo* using time-shift assays to determine changes in resistance and infectivity through time. After two days, we saw evidence of resistance evolution, with more than half of bacterial isolates being resistant to ancestral phage. 1 of 2 isolates isolated at day 4 were phage resistant and no *P. aeruginosa* was detected from nasal swabs after this time point. Although both bacterial populations from days 2 and 4 showed observationally higher phage resistance (87.5% day 2, 100% day 4) against day 2 phage, this was non-significantly different to resistance shown to ancestral phage and phage from days 4 and 7 (Tukey HSD: $p > 0.05$; Supplementary File 1; Figure 1a). This non-significance is likely due to low statistical power from limited clonal numbers (only 8 isolates from day 2) and the consistency of resistance levels in bacterial populations from day 2 (apart from resistance to contemporary phage); overall, results showed equal levels of phage resistance over time *in vivo*. The absence of changes in phage infectivity through time did not warrant further investigation of the population dynamics of the two inoculated phages.

Figure 1. Time-shift assays of bacterial evolution and phage during phage therapy. The proportion of phage resistant bacterial isolates from (a) ancestral (pre-phage therapy, “Anc.”), and days 2 (T1) and 4 (T2) of *in vivo* (clinical) phage therapy (n = 8) tested against ancestral phage (“anc.”: 14-1 and PNM) and phage isolates from days 2 (T1), 4 (T2) and 7 (T3). The same ancestral bacterial isolates are evolved *in vitro* with phage added either (b) once, at the start of the experiment for comparison to (c) in which phage are added repeatedly, emulating clinical treatment. For (b) and (c) the proportion of bacterial isolates susceptible to phage at three time-points (T1 - T3: days 4, 8 and 12 of coevolution) is presented with small points as independent treatment replicates. Resistance of bacteria isolated at T3 to ancestral phage (“Anc.”: 14-1 and PNM) is indicated within the shaded region, note that these isolates are distinct from

those tested against phage from T1-T3. Filled points are the mean proportion of resistant isolates across replicates. Bars = $\pm$ SE.

In vitro

We used the same bacterial isolates and phages to determine how well the *in vivo* evolutionary dynamics paralleled *in vitro* experiments in the lab. To assess the impact of phage application schedule, we added phages once at the start of the experiment, repeatedly (as was the case *in vivo*) or not at all. The dynamics in the phage treatments largely mirrored the *in vivo* dynamics. Resistance to phage increased through time, reaching comparable levels to that observed *in vivo* (>50%). While phage resistance increased in both phage treatments (ANOVA comparing models with and without bacterial time: phage added: once $\chi^2_2 = 79.08$, $p < 0.001$; repeatedly $\chi^2_2 = 45.30$, $p < 0.001$; Figure 1b, c), there was a significantly higher proportion of bacteria resistant to contemporary phage present at the end of the experiment in the treatment where phages were added once (90.81%, $\pm$ SE = 0.0003) compared to when phages were added repeatedly (66.7%, $\pm$ SE = 7.64; Tukey HSD comparing phage added once and repeatedly treatments: estimate = -1.63, z-ratio = -3.75, $p = 0.0002$; Figure 1b, c). All isolates from the control condition were susceptible to ancestral phages.

The lower resistance of bacteria when phages were added repeatedly was driven by evolution of increased phage infectivity. When phages were added once, resistance to ancestral phage (14-1: 88.64%, $\pm$ SE = 2.77; PNM: 90.15%, $\pm$ SE = 2.60) did not differ relative to phage isolated at the end of experimental evolution (90.0%, $\pm$ SE = 4.27; Tukey HSD comparing endpoint phage resistance to contemporary phage to resistance to 14-1 ($p = 1.0$) and PNM ($p = 0.938$; Figure 1b). However, when phages were added repeatedly, resistance to ancestral phage (14-1: 94.41%, $\pm$ SE = 1.93; PNM: 96.5%, $\pm$ SE = 1.54) was significantly higher than to phage

isolated at the end of experiment (61.5%, $\pm$ SE = 12.5), indicative of increased phage infectivity (Tukey HSD comparing end-point phage resistance to contemporary phage to resistance to 14-1 (p < 0.001) and PNM (p < 0.001); Figure 1c). We saw no further evidence of phage evolution in either treatments, with phage resistance being independent to the time point from which phage were isolated during experimental evolution (ANOVA comparing models with and without phage time: phage added: once $\chi^2_2 = 0.41$; p = 0.814; repeatedly $\chi^2_2 = 0.68$; p = 0.713; Figure 1c). This suggests the increase in phage infectivity evolved before the first sampling time point (the second transfer, or 96 hours). The absence of any further changes in phages during experimental evolution did not warrant further investigation of the population dynamics of the two inoculated phages.

Phage resistance-virulence trade-offs

We next assessed whether phage application altered the virulence of *P. aeruginosa* populations *in vivo* and *in vitro* using a *G. mellonella* infection model. Here, virulence can be calculated as the inverse of *G. mellonella* survival over time: the more virulent the bacterial isolate, the shorter the time until death. *In vivo*, phage therapy reduced bacterial virulence by 95.5% on average (median hazard ratio = 0.045, 95%CI = 0.012 – 0.163). When inoculated with bacteria isolated before phage therapy, 479 (99.7%) of *G. mellonella* died within 46 hours, with death occurring at a mean time of 15 hours (Figure 2a). In contrast, after phage therapy, bacteria killed only 70% of *G. mellonella* within 50 hours, with mean time of death occurring at 20.5 hours (Figure 2a).

We also saw a reduction in virulence *in vitro* when populations had evolved with phage compared to without phage. Bacteria in the phage added once and phage added repeatedly treatments were 96.15% (median hazard ratio = 0.0385, 95%CI = 0.011 – 0.124) and 89.8%

(median hazard ratio = 0.102, 95%CI = 0.0294 – 0.355) less virulent on average compared to the no-phage control populations (Figure 2b). In the control populations, 96 (80%) of the *G. mellonella* died within 47 hours, with death occurring at a mean time of 19 hours. In contrast, only 32.5% and 41.2% of *G. mellonella* died in the phage added once and phage added repeatedly treatments respectively, with a mean time to death of 36.7 and 35.4 hours (Figure 2b).

This difference was driven by two impacts of phage application. First, there was a huge reduction in virulence of bacteria that were resistant to both phages. When resistant to both phage, virulence reduced by 95.69% compared to susceptible bacteria (median hazard ratio = 0.0431, 95%CI = 0.0139-0.122) and was 93.33% lower than bacteria resistant to only a single phage (median hazard ratio = 0.0663, 95%CI = 0.0179-0.247; Figure 2c). Meanwhile, there was no reduction in virulence for bacteria resistant to just a single phage (median hazard ratio = 0.653, 95%CI = 0.185 -2.09) when compared to phage susceptible bacteria. Second, even susceptible bacteria became less virulent after phage application, with a 91.14% median hazard ratio = 0.0886, 95%CI = 0.0246-0.344) reduction in the phage added once treatment and an 80.7% reduction when phage was added repeatedly (median hazard ratio = 0.193, 95%CI = 0.04-1.06; Figure 2c).

Figure 2. Survival curves of *G. mellonella* inoculated with *P. aeruginosa* isolates isolated (a) *in vivo* and (b, c) *in vitro*. (a) Virulence of bacterial isolates isolated from before (black) and after (grey) phage therapy. (b) Virulence of bacteria in control populations where no phage is present (yellow) is much greater than populations which were exposed to either a single (orange) or repeated (red) phage applications. (c) Virulence is significantly reduced in bacterial isolates resistant to both phages while bacteria resistant to one phage are non-significantly different in virulence to phage susceptible isolates. Lines represent the average prediction and shaded bands represent the 95% credible intervals of those predictions. Proportion and mean time to death of *G. mellonella* that died are presented as summary statistics for each treatment.

Growth rate

Evolving resistance against phage can trade-off with growth rate (Buckling et al., 2006; Koskella et al., 2011; Wright et al., 2018), and could be a plausible mechanism for virulence reduction. In the case of the *in vivo* isolates, the growth rate of pre-phage treatment isolates ($\bar{x} = 0.36$, 95%CI = 0.342 - 0.369) was significantly higher than that of post-treatment isolates ($\bar{x} = 0.25$, 95%CI = 0.195 - 0.302). Post-treatment isolates which evolved resistance (to one or both phage) had a significantly lower growth rate ($\bar{x} = 0.21$, 95%CI = 0.162 - 0.266) than the ancestral isolates ($\bar{x} = 0.36$, 95%CI = 0.342 - 0.369). However, the 95% CIs of the susceptible isolates' growth rate ($\bar{x} = 0.33$, 95%CI = 0.256 - 0.402) overlapped with the ancestral and resistant isolates, indicating non-significance, which was likely driven by a lack of statistical power ($n = 3$ susceptible isolates) (Figure 3a).

For *in vitro* isolates, growth rate was significantly affected by treatment ($\chi^2_2 = 20.14$, $p < 0.001$) with the control group having significantly higher growth rates ($\bar{x} = 0.43$, 95%CI = 0.373 - 0.477) compared to treatments in which phage were added once ($\bar{x} = 0.30$, 95%CI = 0.256 - 0.346; $p = 0.002$) or repeatedly ($\bar{x} = 0.276$, 95%CI = 0.235 - 0.318; $p < 0.001$; Figure 3b). No significant difference was observed in growth rates between each phage treatment (Tukey HSD: estimate = 0.025, t-ratio = 0.95, $p = 0.628$); and changes in growth were not associated with phage resistance (interaction between treatment and resistance: $\chi^2_2 = 0.37$, $p = 0.832$; fixed effect of resistance: $\chi^2_2 = 2.98$, $p = 0.226$; Figure 3b).

Figure 3. Phenotypic changes underpinning the resistance – virulence trade-off of bacterial populations. Populations were evolved (a, c) *in vivo*, and (b, d) *in vitro*. (a, b) Changes in bacterial growth were estimated for populations isolated (a) before and after phage treatment, and (b) between control and the phage treatment groups. Single points represent individual bacterial isolates of differing phage resistance levels: resistant to both phage (light blue), resistant to 14-1 or PNM (dark blue) or phage susceptible (red). Tops and bottoms of the bars represent the 75th and 25th percentiles of the data, the middle lines are the medians, and the whiskers extend from their respective hinge to the smallest or largest value no further than 1.5* interquartile range.

Biofilm production

Biofilm production is often altered in phage resistant isolates (Harrison et al., 2015; Kim et al., 2015; Scanlan and Buckling, 2012) and this can also affect virulence and growth rate (Li et al., 2008; Wang et al., 2011). Therefore, we compared the biofilm productivity between isolates evolved *in vitro* and *in vivo*. For *in vivo* isolates, there was no significant difference in biofilm production between isolates isolated pre- ($\bar{x} = 5.53$, 95%CI = 5.45 - 5.6) or post-phage therapy ($\bar{x} = 5.39$, 95%CI = 5.27 - 5.5), and between phage resistant ($\bar{x} = 5.38$, 95%CI = 5.27 - 5.50) and susceptible isolates ($\bar{x} = 5.39$, 95%CI = 5.1 - 5.69; Figure 3c).

For *in vitro* isolates, there was no significant difference in biofilm production between treatments (treatment x resistance interaction: $\chi^2_2 = 0.785$, $p = 0.675$; treatment: $\chi^2_2 = 4.52$, $p = 0.104$). However, phage resistance was associated with increased biofilm production ($\chi^2_2 = 19.12$, $p < 0.001$), with phage susceptible isolates ($\bar{x} = 5.35$, 95%CI = 5.16 - 5.54) having lower biofilm production than isolates resistant to one ($\bar{x} = 5.85$, 95%CI = 5.56 - 6.13; $p = 0.012$) or both phages ($\bar{x} = 5.93$, 95%CI = 5.73 - 6.13; $p = 0.004$; Figure 3d).

Genetic basis of evolutionary changes

Given the parallel phenotypic differences we observed under *in vivo* and *in vitro* conditions, we next explored whether the underlying genetic changes were the same for *in vitro* and *in vivo* evolved populations. Results were not affected whether we included only genetic variants occurring in genes of known function or all genetic variants (Figure 5-Figure supplement 1). As we were interested in attributing potential functions to the variants identified, we only present the results for genetic variants occurring in genes of known function. From the *in vitro* pooled

sequencing, we found significant changes in 26 different genes with known function (Figure 4), 16 of which changed in frequency compared to the ancestral population (selection on standing genetic variation) while 10 were novel mutations (*de novo* evolution).

Figure 4. Significant genetic changes identified *in vitro* and *in vivo*. The frequency of genes that differed significantly between treatments *in vitro* are shown. Genetic changes that were already present in the ancestral population (first column) are shown in blue, while *de novo* mutations are in orange. Only genes whose function was identified are included. Multiple rows of the same gene indicate different genomic variants of the same gene. Colour intensity shows how frequent the genetic change is in the

population. For *in vitro* treatments, each column is an independent replicate representing the results of the pooled sequencing. For the *in vivo* isolates (final column), all isolates were pooled to visualise the frequency of each genetic change *in vivo*.

In terms of genetic distance from the ancestral population, populations in the treatment where phage was added repeatedly diverged more than when phage was added once (Tukey HSD: $t = -4.32$, d.f. = 15, $p = 0.0017$), which diverged more from the ancestral populations than the control populations (Tukey HSD: $t = -8.81$, d.f. = 15, $p < 0.0001$; Figure 5a, b). Consistent with this, more novel SNPs/indels were found in the treatment populations where phages were added repeatedly (mean = 19.2) compared to where phages were added once (mean = 11.8, Tukey HSD: $t = -4.80$, d.f. = 15, $p = 0.0006$), which in turn had more novel SNPs/indels than control populations (mean = 2.5, Tukey HSD: $t = -6.105$, d.f. = 15, $p = 0.0001$; Figure 5b). This suggests novel SNP/indels were potentially more important determinants of phage resistance than selection acting on standing variation. Within-population diversity was highest in phage added repeatedly treatment (Tukey HSD phage added repeatedly vs. phage added once: $t = -5.96$, d.f. = 15, $p = 0.0001$; Tukey HSD phage added repeatedly vs. control: $t = -4.33$, d.f. = 15, $p = 0.0016$), but there was no difference between control populations and phage added once treatment (Tukey HSD: $t = -1.14$, d.f. = 15, $p = 0.2625$; Figure 5c).

Figure 5. Genomic changes for populations evolved *in vitro*. (a) The divergence of each treatment group as indicated by Euclidean distances of each gene from the ancestral isolates. Genetic changes were characterised as a change in frequency of an existing gene in the ancestor or selection on a mutation that was gained during experimental evolution. The presence of phage significantly affected (b) genotypic distance from the ancestor, (c) the number of new SNPs and (d) alpha diversity.

326

327 *In vitro* treatment significantly altered genetic composition (i.e. the centroids of phage
328 added once, phage added repeatedly and control populations were different, Figure 4,
329 PERMANOVA, $F_{3,15} = 16.05$, $R^2 = 0.76$, $p = 0.0001$). There was no difference in beta-diversity
330 (calculated from distance-to-centroids between groups) between treatments (homogeneity of
331 multi-variation dispersion ANOVA: $F_{3,15} = 1.65$, $p = 0.22$). Genetic changes associated with both
332 *in vitro* phage treatments, but not control, were flagellar hook-associated protein (*flgK*) and rod-
333 shape determining proteins (*mreC* and *mreD*) (Figure 4). Additionally, genetic changes in DNA
334 mismatch repair (*mutS*), elongation factor G (*fusA*), signal recognition particle (*ffs*), type 3
335 secretion system pilotin (*yscW*), and pilus assembly protein (*pilB*) were mainly found *in vitro* in
336 the phage added repeatedly treatment (Figure 4).

337 We examined whether these same mutations arose in the clonal *in vivo* isolates. Of the 50
338 genetic changes identified *in vitro*, the *in vivo* isolates had a mean of 11.8 changes (maximum =
339 23, minimum = 1). Changes in frequencies or mutations were found at 16/26 of the same genes
340 identified *in vitro* in at least one isolate (Figure 4). Crucially, some of the *de novo* mutations that
341 were observed at high frequency *in vitro* also had high frequency *in vivo* (*flgL* = 30%, *mutS* =
342 45%, *mreC* = 50%, *mreD* = 30%, *pilB* = 40%). In total, 31 of 50 genetic changes (62%) *in vitro*
343 were found *in vivo*. Of these, 77% were *de novo* mutations *in vitro*, indicating that *de novo*
344 mutations were also important for phage resistance *in vivo*.

345 We next assessed whether mutations arising in individual isolates *in vivo* could be linked
346 to phenotypic changes (resistance, virulence, biofilm productivity, growth rate). If strong
347 genotypic – phenotype links can be made, we would expect isolates to cluster according to their
348 trait value. However, we found no obvious genotype to phenotype links *in vivo* and due to the

small number of isolates, we were unable to conduct robust statistical analyses (Figure 5; Figure supplement 2).

Discussion

Parallel evolutionary dynamics were found *in vivo* and *in vitro*, showing that laboratory studies can be predictive of certain phenotypic outcomes of clinical phage therapy (phage-mediated decolonisation). Levels of phage resistance were comparable *in vivo* and *in vitro* with limited phage infectivity evolution occurring only *in vitro*. While bacteria went extinct *in vivo*, they persisted with phage *in vitro*, suggesting that *in vitro* experiments may be more important in predicting evolutionary than clinical outcomes. Additionally, phage resistance was associated with trade-offs with virulence and bacterial growth and similar mutations or genetic variants could explain these parallel evolutionary changes in both ecological contexts.

Parallel evolution is promising for future clinical work in which the trajectory of bacteria and phage (co)evolution may be predicted for individual patients. Given the difference in environments between that of a laboratory and human nasal cavity, it was surprising that evolution was so similar. The nasal cavity would have been more nutrient limited and spatially heterogenous and such conditions have been shown to change bacteria-phage interactions compared to evolution in high-nutrient laboratory growth medium (e.g. LB). For instance, bacteria-phage coevolution is altered in soil compared to growth medium because of increased costs of resistance in the former (Gomez and Buckling, 2011); and in plant leaves, bacteria and phage have failed to evolve while otherwise coevolving in the laboratory due to lower bacteria – phage contact rates (Hernandez and Koskella, 2019). Other media types can be used *in vitro* to replicate *in vivo* conditions more close (e.g. sputum media) than LB used here which may be

more appropriate for other infection types such as those infecting lungs of cystic fibrosis patients (Palmer et al., 2007, 2005).

Rapid phage resistance has been highlighted as a concern for phage therapy development (Torres-Barceló, 2018) and was found in this case study. Without ongoing reciprocal phage evolution, phage resistance is likely to lead to regrowth of bacteria during infections; this has been found in a few case studies but has been combatted by selecting a different phage (single or cocktail) (Aslam et al., 2020; Schooley et al., 2017; Wu et al., 2021; Zhvania et al., 2017). That many *de novo* mutations – as well as selection on existing variation - occurred during bacteria-phage coevolution *in vitro* and many were also found *in vivo* (77% of variants observed *in vivo* also evolved *de novo* during *in vitro* coevolution) further highlights the fact that stressful *in vivo* conditions do not prevent bacteria evolving resistance to phage. However, phage resistance was found to have growth rate costs which may further explain why some isolates remained sensitive to phage, and why decolonisation was ultimately successful. The natural nasal microbiota, and host immunity, may have further contributed to this success by outcompeting slower growing phage resistant isolates (Wang et al., 2019). Bacteria did not go extinct in our *in vitro* experiments which may be reflective of differences in environmental conditions such as nutrient availability and interspecies competition.

Resistance-virulence trade-offs have been found in previous *in vitro* animal models and clinical studies and is expected to improve treatment success in spite of phage resistance (Oechslin, 2018). Phage resistance however is not always costly and likely depends on the genetic background of the bacteria and phage, as well as the environmental context (Meaden et al., 2015; van Houte et al., 2016). The absence of clear links between resistance phenotypes and growth rate within our *in vitro* treatments, despite clear between-treatment effects, is presumably

because the cultures were initiated with genetically diverse clinical isolates, rather than a single ancestral clone. Ultimately, phage resistance was beneficial in controlling the pathogen in this case study and such benefits could have led to treatment success.

Population genomics of the isolates *in vivo* and *in vitro* revealed parallel genomic changes and the effects of phage selection at a molecular level. In line with previous studies, phage selection accelerated molecular evolution which resulted in populations being more diverged from their ancestral pool than control isolates (*in vitro*) (Betts et al., 2018; Morgan et al., 2010; Pal et al., 2007). This suggests that phage placed strong selection on bacteria and this further resulted in mutator strains (*mutS* genotype), especially where phage were added repeatedly *in vitro* and *in vivo*, that likely accelerated the rate of evolution further (Morgan et al., 2010). Between both *in vivo* and *in vitro* results, mutations selected by phage gave insight into potential mechanisms of resistance. In particular, mutations to *mreD* and *mreC* encoded for cell shape (Cabeen and Jacobs-Wagner, 2005) while missense mutations at *flgK* and *flgL* probably resulted in flagella loss (Macnab, 2003). The result of these mutations was likely reduced cell surface area which reduces the probability of phage absorption (Dennehy and Abedon, 2021). *pilB* mutations were also selected which is likely a surface mutation to prevent PNM (a pilus binding phage) from attaching (Ceyssens et al., 2011).

Strong genotype-phenotype links were difficult to distinguish as a high number of the same mutations or existing variants were selected upon both *in vivo* and *in vitro*, and no unique candidate genes were obvious to explain phenotypic differences (i.e. growth rate, biofilm production). Flagella and *mreC* mutations are associated with increased biofilm production (Whiteley et al., 2001) and as they were found in the *in vitro* isolates, this may suggest a generalistic phage defence strategy (Harper et al., 2014). However, as these same mutations were

found *in vivo*, without increases in biofilm production, strong genotype – phenotype links are unclear. Surface mutation is a common form of phage resistance (van Houte et al., 2016) but here it appears coupled with mechanisms that prevent absorption more generally; combined, these strategies could have reduced bacterial growth and/or virulence by impairing swarming motility (Haiko and Westerlund-Wikström, 2013). Motility (Alseth et al., 2019) and resource-use assays (using Biolog plates) may have provided some stronger genotype-phenotype correlations, but this was beyond the scope of this work. In contrast, other studies using the same phage but *P. aeruginosa* PA01 instead reported mutations predominantly at pilus and lipopolysaccharide receptors, showing that greater selection is placed on specific phage resistance strategies at phage binding sites (Wright et al., 2019, 2018). Our results highlight the importance of genetic background, and hence the use of clinical isolates over laboratory clones, in determining evolutionary trajectories (Betts et al., 2014; Friman et al., 2016; León and Bastías, 2015; Rossitto et al., 2018; van Houte et al., 2016).

How phages were applied *in vitro* had an impact on phage resistance. Although we observed a greater number of mutations where phages were added repeatedly (mimicking multiple phage doses), the proportion of resistant bacteria (resistance to contemporary phage) at the end of the experiment was significantly lower (61.5%) compared to where phages were just added at the start of the experiment (90%). That resistance of evolved bacteria to the ancestral phages (~90%) did not differ between treatments suggests that differences in resistance to evolved phages was caused by phage infectivity evolution in the repeated phage treatment. This infectivity evolution occurred before the first time point, with no subsequent changes, and was presumably the result of greater mutation supply rate in this repeated phage application treatment. It is interesting that although this “repeated” treatment mimics clinical methods more

441 closely, these same patterns were not observed *in vivo*. However, as bacteria went extinct *in vivo*,
442 it is likely phage generations were fewer which may have restricted phage evolution in the lower
443 resource environment (Lopez-Pascua and Buckling, 2008). Multiple (Manohar et al., 2018) and
444 higher (Barrow et al., 1998; Biswas et al., 2002; Debarbieux et al., 2010; Merril et al., 1996;
445 Sunagar et al., 2010; Vinodkumar et al., 2008; Wang et al., 2006; Wills et al., 2005) phage doses
446 have been shown previously to decrease morbidity and mortality in animal models. Our results
447 build upon this work to show that maintaining high phage titres may have a positive effect in
448 reducing resistant populations despite increasing the strength of selection for resistance.

449 Although our results suggest that results of laboratory studies may parallel phage
450 resistance – virulence trade-offs during *in vivo* phage therapy, there a number of important
451 caveats. Firstly, our *in vivo* results are based on that of a single patient which limits the
452 generalisability of our results. Nevertheless, that our replicated *in vitro* experiments agreed with
453 our *in vivo* findings, provides strength behind our conclusions. Secondly, our *in vitro* – *in vivo*
454 results were comparable with regards to the direction of change, but absolute effect sizes were
455 greater *in vitro*. For instance, we observed a higher proportion of phage resistant isolates *in vitro*
456 that were also far less virulent than isolates found *in vivo*. Therefore, it may be expected that
457 phenotypic changes will be exaggerated *in vitro*. These parallels were further dependent on the
458 different underlying mechanisms (biofilm formation or growth rate) mediating the resistance-
459 virulence trade-off having the same knock-on phenotypic outcomes. Had different environments
460 selected for differing phage resistance strategies, this may have resulted divergent phenotypic
461 outcomes for virulence (Alseth et al., 2019). Additionally, as this patient had a nasal
462 colonization, the role of the adaptive immune system would have been heavily reduced (Ooi et
463 al., 2008). The immune system has the potential to improve phage therapy success by

synergistically killing bacteria with phage which may diminish the potential for phage resistance by reducing bacterial growth (Leung and Weitz, 2017; Roach et al., 2017). Alternatively, immune cells may kill phage and therefore prevent phage acting on bacterial populations (Van Belleghem et al., 2018). Additionally, the nasal cavity has a diverse microbiome that may have contributed to *P. aeruginosa* decolonisation (Hu et al., 2018; Kumpitsch et al., 2019). The role of the microbiome will differ in infection type, with gut and respiratory infections potentially having a greater microbiome effect (Hu et al., 2018; Kumpitsch et al., 2019; Lloyd-Price et al., 2016) while septic infections may have a greater immune system effect (Van Belleghem et al., 2018). Therefore, it is important to compare laboratory vs *in vivo* phage therapy in a number of infection types. The genomic background of the bacteria may further affect how sensitive its resistance strategies are to the environment it is in as more costly resistance strategies are more likely to be selected *in vitro* than *in vivo* (van Houte et al., 2016). This encompasses not only genetic variation within species but also differences between species that may differ in infection sites, immune responses, virulence and sensitivity to phage (Abedon, 2019; Doffkay et al., 2015; van Houte et al., 2016). Further work using isolates from case studies and clinical trials will give an insight into the predictability of bacteria and phage evolution from laboratory to *in vivo* studies.

Materials and methods

Ethics statement

The decolonisation study protocol was approved by the Leading Ethics Committee of the “Université Catholique de Louvain” (Avis N°: B-403201111110). The study was performed in

accordance with the ethical standards as laid down in the Declaration of Helsinki and as revised in 2013. The patients gave informed consent and their anonymity was preserved.

Patient information, bacteria and phage strains and phage-bacteria evolution *in vivo*

Bacterial isolates were sampled from a patient as part of a clinical trial in Brussels, Belgium. The patient (85 years old, male) had been admitted to an intensive care unit in 2015 for treatment of burns (10% body area). Independent of his burns, a nasal colonisation of *P. aeruginosa* was observed. Here, the patient was treated every day with a three-phage cocktail (BFC-1) for five days, which consisted of two phages (14-1 and PNM) that could lytically infect *P. aeruginosa*. The phage cocktail included a third phage that specifically infects *S. aureus* bacterium, but this bacterium was not detected in this patient. The phage cocktail has previously been characterised to ensure no prophage elements are present or that the phage carry toxin-encoding genes that may increase bacterial resistance and/or virulence (Merabishvili et al., 2009). Furthermore, a purification process including the removal of endotoxins in phage production was used to ensure such waste products cannot harm patients (Merabishvili et al., 2009). As 14-1 infects via a lipopolysaccharide (LPS) (Betts et al., 2014; Kropinski et al., 1977) receptor and PNM uses type IV pili (Ceyssens et al., 2011), resistance to one phage is unlikely to result in cross-resistance and neither phage should compete for receptor sites (Wright et al., 2018). Swabs of both nasal cavities were taken on day 0 (commencement of phage therapy), 2, 4, 7 and 13 and frozen at -80 °C in lysogeny broth containing 20% glycerol. Individual *P. aeruginosa* isolates were obtained by plating swabs onto ceftrimide selection plates. Colonies were picked and inoculated into Luria broth (LB) and grown overnight, shaking at 180 r.p.m., at 37 °C to achieve high cell densities. Additionally, swabs were soaked in LB broth to amplify phage overnight in the same conditions. Phage was isolated from overnight cultures via chloroform extraction: 100 µL of chloroform was

added 900 μ L of culture, vortexed and then centrifuged (14000 r.p.m., 1 min); the supernatant was isolated and stored at 4 °C. Culture samples were frozen in 80% glycerol (final concentration 40%) at -80 °C. Spot assays on lawns of *P. aeruginosa* P573 (a phage susceptible control strain) confirmed the presence of phage at all time-points. Twelve individual plaques were picked at each time-point and amplified with an overnight culture of P573 to obtain clonal isolates. Individual isolates of *P. aeruginosa* were isolated from days 0 (n = 24), 2 (n = 8) and 4 (n = 2) for use in this study and swabs were negative on days 7 and 13.

Experimental *in vitro* phage-bacteria evolution

To examine whether findings *in vitro* are consistent with *in vivo* bacteria-phage (co)evolution, phage therapy was replicated in the laboratory. We used two treatments: (1) phage added “once”, at the start of the experiment, and (2) phage added at every second transfer (“repeated” treatment). The repeated phage treatment aimed to emulate the conditions of the clinical trial (while preventing the build up of too many ancestral phage in the “closed” *in vitro* system) and the “once” condition was used to assess whether repeated doses change the observed dynamics. The phages were identical to the two lytic phages (14-1 and PNM) used in the clinical trial. A control group in which bacteria evolved in the absence of phage was set up and there were 6 biological replicates in the control and each phage treatment.

Prior to experimental set-up, *in vivo* ancestral isolates (n = 24, same isolates as day 0) were individually amplified in LB media and cultured at room temperature for two days. Cell densities were estimated via optical density (wavelength 600 nm; OD₆₀₀) measures. Cultures were centrifuged (14000 r.p.m., 3 min), the supernatant removed, and resuspended in M9 buffer to equalise cell densities. These were added to a master-mix in which isolates were equally represented in equal ratio (total 10⁸ CFUs / mL; ~4 x 10⁶ CFUs / mL of each isolate). 100 μ L of

master-mix (10^7 CFUs; $\sim 4 \times 10^5$ CFUs per isolate) was inoculated into 5 mL LB media. 100 μ L of phage was added to treatment microcosms at a total multiplicity of infection (MOI) of 0.5. Microcosms were cultured, shaken (180 r.p.m.), at 37 °C. 1% transfers (50 μ L culture into 5 mL LB) took place every two days for a total of 12 days. At each transfer, culture samples were frozen in glycerol, and in the phage treatments, phages were extracted as described previously. Cultures were diluted and plated from frozen stocks onto LB agar and incubated for two days at 37 °C. From each biological treatment replicate and time-point (days 4, 8 and 12), 16 bacterial colonies were picked (96 total colonies) for time-shift assays to measure coevolutionary changes. An additional 24 isolates were isolated at day 12 from each treatment and control group replicate for bacterial virulence, growth rate and biofilm assays. Picked isolates were individually amplified in LB media overnight before being frozen as described previously.

Measuring resistance-infectivity (co)evolution

To assess whether bacteria and phage had coevolved *in vivo* and *in vitro*, resistance assays were conducted where bacteria were exposed to phage populations from the same/contemporary, past, and future time-points (time-shift assays) (Buckling and Rainey, 2002). If bacteria and phage coevolved, both bacteria and phage would show significant changes in resistance and infectivity, respectively, through time. Increases in resistance and infectivity would be indicative of arms race dynamics (ARD), while non-directional changes would be indicative of fluctuating selection dynamics (FSD) (Lopez Pascua et al., 2014). Infectivity/resistance was determined by spot assays in which 100 μ L of overnight bacterial cultures was added to 5-8 μ L soft LB agar overlay and 5 μ L of each ancestral phage, or evolved phage mixture, was spotted onto the overlay. For *in vivo* isolates, ancestral isolates (isolated pre-phage therapy) and bacterial isolates from days 2 and 4 were tested against the phage clonal isolates from days 2, 4 and 7. A bacterial isolate was

scored as susceptible (1 = susceptible, 0 = resistant) if at least one of twelve clonal phage isolates could infect. For *in vivo* analyses, each isolate was treated as an individual biological replicate. *In vitro* isolates from days 4, 8 and 12 were tested (16 colonies per time-point) against phage populations isolated from each time-point (Figure 6). Each treatment replicate from each time point were defined as biological replicates, while clonal isolates are biological replicates nested within each treatment replicate. In addition, we assessed the infectivity of both ancestral phage against the ancestral bacteria (n = 24), the *in vivo* isolates (n = 8), and the *in vitro* populations from day 12 (n = 24 per treatment replicate; Figure 6).

Figure 6. Selection process of colonies used for time-series assays measuring resistance-infectivity (co)evolution and phenotypic assays (end-point resistance, virulence, growth rate and biofilm) for isolates evolved *in vitro*. For time-series assays, 16 bacterial colonies were randomly selected from each treatment replicate (phage added once or repeatedly, n = 6 each) and time-point (T1-T3), alongside a phage extract that contains a pool of phage genotypes and species (14-

1 and PNM). Each colony was then tested for resistance to the pooled isolated across each time-point. For phenotypic assays, 24 colonies from T3 replicates of each treatment were screened for resistance to each phage. From these 24 colonies, bacterial colonies of each resistance type were randomly selected for further phenotypic assays. Created with icons from BioRender.com.

Virulence assays

Changes in bacterial virulence were measured using a *Galleria mellonella* infection model (Tsai et al., 2016). Although lacking an adaptive immune system, *G. mellonella* innate immune systems are comparable with that of vertebrates, making them suitable and well-used models of virulence in acute infections (Tsai et al., 2016). Note that we define virulence as increased host mortality resulting from parasite infection (May and Anderson, 1990). Virulence of all ancestral (n = 24) and *in vivo* isolates (n = 10) were measured, however two *in vivo* isolates (from day 2 of phage therapy) were excluded from analyses as time of death was not accurately determined. As few isolates remained phage susceptible (n = 3), these were pooled with phage resistant isolates to determine a change in virulence from pre- (ancestral, n = 24) to post-phage treatment (n = 8). For *in vitro* isolates, where possible, one resistant and one susceptible (to ancestral phage) isolate were selected from each treatment replicate to give a total of 12 resistant and 12 susceptible isolates, with 14 isolates from the “phage added once” and 10 isolates from the “phage added repeatedly” (Figure 6). As some treatment replicates contained no susceptible isolates, multiple susceptible isolates were chosen from the same replicate (Supplementary File 2). Seven *in vitro* isolates evolved resistance to one phage only (single resistance), therefore their virulence was measured to assess whether changes in virulence were mediated by resistance to one or both

phages. The virulence of *in vitro* isolates was contrasted to six isolates from the control group isolated from 6 independent replicates.

For each bacterial isolate (total of 56 biological replicates), 20 *G. mellonella* larvae (20 technical replicates) were infected with 10 μ L (~100 CFUs) of overnight culture (cells resuspended in M9 buffer) using a sterile syringe. Twenty *G. mellonella* larvae were injected with 10 μ L of M9 buffer as a technical control. Survival of each larvae was assessed every two hours for up to 58 hours.

Growth rate assays

We measured bacterial growth rates as a potential mechanism underlying virulence variation and cost of resistance for ancestral and all evolved isolates (*in vitro* and *in vivo*) tested in the virulence assay. Growth curves were measured in a 96 well plate with 10 μ L of culture (1.0×10^5 CFUs) from each isolate inoculated into individual wells containing 140 μ L LB broth. 26 wells remained as blank controls. The plate was incubated in a spectrophotometer (Biotek Instruments Ltd) at 37 °C in shaken conditions and OD₆₀₀ was measured every 20 mins for 24 hours to estimate changes in cellular density. Growth curves were independently replicated 3 times (technical replicates).

Biofilm assay

Biofilm production of isolates isolated for virulence and growth rate assays was measured using a resazurin assay, based upon a previously described protocol (Peeters et al., 2008). Overnight monocultures were diluted to 0.1 OD₆₀₀ (~ 10^9 CFU/mL) into M9 buffer. 100 μ L of each culture was inoculated into a sterile 96 well plate and incubated at 37 °C for 4 hrs for cellular adhesion. Then, the supernatant was removed, and wells were washed gently with 100 μ L of M9 buffer by pipetting up and down 3 times. 100 μ L of fresh LB media was added to each well including 25

controls (no bacteria) and plates were incubated for 16 hrs. Liquid was removed and wells were washed with 100 μ L M9 buffer. 100 μ L of fresh M9 was added followed by 10 μ L resazurin solution (final resazurin concentration: 100 μ M). Fluorescence (λ_{ex} : 560 nm and λ_{em} : 590 nm) was measured after 1 hr incubation. Biofilm assays were independently replicated 3 times (technical replicates).

Sequencing

We sequenced the genomes of the bacterial isolates used for the *in vitro* experiment to establish to what extent evolution was driven by *de novo* mutation or selection on existing genetic variation. This is especially pertinent as the experiment did not start from a clonal population, rather from 24 isolates (which included an isolate that was resistant to one phage). At the end of the experiment, we picked 24 isolates, extracted DNA from isolates individually and performed whole genome sequencing (WGS) on pools of the bacterial isolates that were isolated from each replicate (pool-seq). Total DNA extraction (19 pooled-isolate samples) was performed using the “Qiagen QIAmp DNA Mini Kit” following the manufacturer’s instructions. An Illumina HiSeq 2000 sequencer was used to generate 250 bp paired reads from a 500 bp insert library.

Reads were trimmed for the presence of Illumina adapter sequences and for quality scores below 10 using *TrimGalore* (v0.6.4_dev, powered by *Cutadapt*). *TrimGalore* also removed any paired end reads when one of the paired end reads was shorter than 20 bp. Trimmed reads were mapped to the *P. aeruginosa* UCBPP-14 reference genome (Locus: NC_008463) with *bwa-mem* (v0.7.17-r1188), and duplicates were marked using *samblaster* (v0.1.24). Subsequent bam files were split into mapped and unmapped reads, sorted and indexed using *samtools* (v0.1.19-44428cd). Across all files, ~93% of the reads mapped to the reference genome (minimum 92.5%), giving on average ~12,000,000 reads per replicate (minimum 8,807,569).

637 Variants were identified using *freebayes* (v1.3.2-dirty) with ploidy set to 24 (*-p 24*) and we
638 assumed pooled samples with a known number of genome copies (*--pooled-discrete*). Due to the
639 high ploidy of the pooled samples, we reduced the number of alleles that are considered at the
640 cost of sensitivity to lower frequency alleles at multiallelic loci by setting *--use-best-n-alleles 4*.

641 Next, we compiled the *vcf* files (standard output of SNP callers) into *R* using the package
642 *vcfR* and kept only the variants which were sequenced at a depth within two standard deviations
643 of the mean. Variants were further filtered to retain only those with quality scores >30. We used
644 the two replicate sequencing runs of the same samples to conservatively identify variants;
645 variants were only kept if they occurred in both sequencing runs and if there was less than 0.3
646 difference in frequency between runs. We then only kept variants that had different frequency
647 than the ancestor (> 1/24 difference in frequency). To identify significant differences in genetic
648 variants (SNPs and indels) between treatments, we first filtered unique that were present in at
649 least three replicates of any treatment. This identified 960 potential variants. We then performed
650 a Wilcoxon test on each variant, with frequency as the response variable and treatment (control,
651 phage added once, and phage added repeatedly) as the predictor. Significant genetic variants
652 were identified if the adjusted p value (*fdr* method) was < 0.05. Of these significant changes, 160
653 were existing genetic variants and 120 were novel mutations. We then checked where each
654 variant was in the genome and only retained variants that occurred in a gene. After these steps,
655 50 genetic variants were identified in genes of known function, and 236 in genes of unknown
656 function. We performed all downstream statistical analyses on (a) only genetic variants in genes
657 of known function and (b) all genetic variants.

658 For the *in vivo* isolates, DNA extraction and whole genome sequencing was conducted on
659 each isolate individually by MicrobesNG using in-house protocols (“MicrobesNG Sequencing

Service Methods v20210419,” n.d.). Whole genome sequencing was carried out on each isolate individually by MicrobesNG using Illumina sequencers (HiSeq/NovaSeq) to create 250 bp paired end reads. In-house processing by MicrobesNG trimmed the adapters and removed reads with a quality < 15. We then processed the reads as for the pooled sequencing, except ploidy was set to 1 when using *freebayes*. On average, 81% of reads mapped back to the reference genome, but there was more variation as compared to the *in vitro* experiment, with a minimum of 44% and a maximum of 94%. As we only had isolates from a single patient, there was limited statistical analysis we could do, so we primarily concentrated on whether the significant changes found *in vitro* were also present in the *in vivo* isolates.

Statistical analyses

All data was analysed using R (v. 4.0.3) in RStudio (Team, 2013) and all plots were made using the package “*ggplot2*” (Wickham, 2016). Model simplification was conducted using likelihood ratio tests and Tukey’s post-hoc multiple comparison tests were used to identify the most parsimonious model using the R package “*emmeans*” (Lenth, 2018). First, we established whether bacteria and phage had coevolved *in vivo* and in each *in vitro* treatment using general linear mixed effects models. Models were fitted using the package “*lme4*” (Bates et al., 2014). *In vivo* isolates from day 4 were excluded as their small sample size (n = 2) prevented model convergence. For *in vivo* isolates: infection (1 = susceptible, 0 = resistant) was analysed against phage time-point (fixed effect) with a binomial error structure. A random effect of “isolate” was included to account for the same bacterial isolate being used in infectivity assays across each phage time-point. For *in vitro* analyses, the proportion of isolates infected was analysed against fixed effects of phage and bacterial time-points with separate models for each phage treatment.

To see if there were any differences in phage resistance to ancestral phage or *in vitro* phage isolates at the end of the experiment, the proportion of isolates infected was analysed against fixed effects of phage treatment and phage type (ancestral or end – point phage). All *in vitro* models included a random effect of “replicate” (which treatment replicate isolates had been isolated from) with a binomial error structure.

Virulence of bacterial isolates was analysed based on survival of *Galleria* infected with different bacterial isolates. Survival curves were fitted using Bayesian regression models using the package “*rstanarm*” (Goodrich, 2020) and parameters were estimated using “*tidybayes*” (Kay and Mastny, 2020). For all models, we fit a proportional hazards model with an M-splines baseline hazard. Models were ran for 3000 iterations and 3 chains were used with uninformative priors. Model convergence was assessed using Rhat values (all values were 1) and manually checking chain mixing. For all models, log hazards were estimated for each treatment value and hazard ratios were calculated as the exponential of the difference between two log hazards. A hazard ratio below one indicates a decrease in virulence compared to the baseline treatment, with a value above one indicating an increase in virulence. Median hazard ratios with 95% credible intervals that do not cross 1 indicate a significant difference in virulence between two factors. Proportion of *Galleria* that died and mean time to death of *Galleria* that died were calculated as summary statistics for each treatment.

For virulence analyses of *in vivo* isolates, treatment (pre- or post-phage therapy) was the only predictor of survival, with a random effect of “isolate” added to account for multiple *Galleria* larvae being infected by the same isolate. For the *in vitro* isolates, survival was analysed against interacting fixed effects of resistance (resistant, single resistant, susceptible) and

treatment (no phage added, phage added once, phage added repeatedly). Here, an additional random effect of “replicate” was included to account for clonal non-independence.

Biofilm productivity of *in vitro* isolates was estimated using linear mixed effects models. Biofilm production (fluorescence 560/590nm) was log transformed to account for the data being abundance-based. Biofilm production was averaged over each technical replicate for each biological replicate (individual bacterial isolates). Biofilm production was used as the response variable and explained with interacting fixed effects of treatment and phage resistance, while treatment replicate was included as a random effect. Due to the low number of isolates, biofilm productivity of *in vivo* isolates from before and after phage treatment as well as phage susceptible and resistant isolates were estimated using a non-parametric bootstrap using the R package “boot” (Canty and Ripley, 2021). Non-overlapping 95% CIs were interpreted as being significantly different.

Growth rates were estimated using a rolling regression taking the steepest slope of the linear regression between $\ln OD_{600}$ and time in hours in a shifting window of every 7 time points (every 2.3 hrs). Growth rate was averaged over each technical replicate for each biological replicate (individual bacterial isolates). For *in vitro* isolates, growth rate was analysed against treatment and resistance in a linear mixed effects model with a random effect of treatment “replicate”. Growth rates of isolates pre- and post-phage treatment, and resistant and susceptible *in vivo* isolates were compared using 95% confidence intervals (CIs) that were estimated using a non-parametric bootstrap.

To explore genetic differences of *in vitro* isolates, we calculated several commonly used metrics using the frequency data of the 50 variants in known genes that differed significantly between our treatments: (1) the genetic distance from the ancestral population, calculated as the

sum of the difference of the proportion of each SNP/indel in each population from the ancestral proportion; (2) the number of unique *de novo* SNPs/indels in each population; (3) alpha diversity, calculated using a modified version of the Hardy-Weinberg equilibrium, such that $\alpha = \sum(1 - p_i^2 - q_i^2)$, where i is the position of each SNP/indel, p is the proportion of the SNP/indel and q is $1 - p$. This is equivalent to expected heterozygosity. Differences between these metrics were analysed using linear models. To test whether consistent genetic differences occur within treatments, we performed non-metric multidimensional scaling on the Euclidean distance matrix of SNPs/indels and their proportions in each population using “*metaMDS*” in the R package “*vegan*”. Nonmetric multidimensional scaling aims to collapse information from multiple dimensions (*i.e.*, from different populations and multiple SNPs/indels per population) into just a few, allowing differences between samples to be visualized and interpreted. Permutational ANOVA tests were run using “*vegan::adonis*”, with Euclidean distance as the response term and treatment as the predictor variable with 9999 iterations. Changes in beta-diversity were examined using “*vegan::betadisper*” based on the same response and predictor variables as in the PERMANOVA.

For the *in vivo* sequencing, we looked for the presence of variants found to be significant *in vitro* in the *in vivo* isolates. To see if any of the genetic changes could explain differences in phenotypes of the isolates, we used non-metric multidimensional scaling as described previously. Phenotypic traits of the isolates (virulence, growth rate, resistance profile, and biofilm production) were then mapped onto the resulting NMDS plot to see whether observed genetic changes correlated with any changes in phenotypes (Figure 5-Figure supplement 2).

Data cleaning

Before growth rate analyses, data points from the first 2.5 hrs were removed as a sharp increase in OD was observed before lag phase independent of exponential growth (Figure 3; Figure supplement 1).

In the *in vitro* dataset, one replicate in the control and one in the susceptible treatments were found to be outliers that significantly impacted data analysis. In the susceptible treatment, the outlier (711) had a value 2.9x greater than the group mean (246.9) and this was 2.7x greater than the next nearest value (266.1). Similarly, in the control group, the outlier (708.6) had a value 2.26x greater than the group mean (313.5) and this was 2.5x greater than the next nearest value (278). We subsequently determined the impact of these values individually and when together on model output by sequentially removing these from the dataset (Supplementary File 3 and 4). Resistance was significant regardless of outlier removal (Supplementary File 3) as were comparisons between resistant and susceptible groups (Supplementary File 4). Although treatment became significant when the susceptible outlier was removed, all Tukey-HSD comparisons were non-significant. The only change detected in Tukey HSD comparisons was that between the single resistant and susceptible isolates which was found when the susceptible and both outliers were removed (Supplementary File 4).

References

- Abedon, S.T., 2019. Use of phage therapy to treat long-standing, persistent, or chronic bacterial infections. *Adv. Drug Deliv. Rev.* 145, 18–39. <https://doi.org/10.1016/j.addr.2018.06.018>
- Abedon, S.T., 2017. Information Phage Therapy Research Should Report. *Pharmaceuticals* 10, 43. <https://doi.org/10.3390/ph10020043>
- Alseth, E.O., Pursey, E., Luján, A.M., McLeod, I., Rollie, C., Westra, E.R., 2019. Bacterial biodiversity drives the evolution of CRISPR-based phage resistance. *Nature* 574, 549–552. <https://doi.org/10.1038/s41586-019-1662-9>
- Aslam, S., Lampley, E., Wooten, D., Karris, M., Benson, C., Strathdee, S., Schooley, R.T., 2020. Lessons Learned From the First 10 Consecutive Cases of Intravenous Bacteriophage Therapy to Treat Multidrug-Resistant Bacterial Infections at a Single

Center in the United States. *Open Forum Infect. Dis.* 7, ofaa389.
<https://doi.org/10.1093/ofid/ofaa389>

Barrow, P., Lovell, M., Berchieri, A., 1998. Use of lytic bacteriophage for control of experimental *Escherichia coli* septicemia and meningitis in chickens and calves. *Clin. Diagn. Lab. Immunol.* 5, 294–298.

Bates, D., Maechler, M., Bolker, B., Walker, S., 2014. lme4: Linear mixed-effects models using Eigen and S4. R Package Version.

Betts, A., Gray, C., Zelek, M., MacLean, R.C., King, K.C., 2018. High parasite diversity accelerates host adaptation and diversification. *Science* 360, 907–911.
<https://doi.org/10.1126/science.aam9974>

Betts, A., Kaltz, O., Hochberg, M.E., 2014. Contrasted coevolutionary dynamics between a bacterial pathogen and its bacteriophages. *Proc. Natl. Acad. Sci. U.S.A.* 111, 11109–11114. <https://doi.org/10.1073/pnas.1406763111>

Biswas, B., Adhya, S., Washart, P., Paul, B., Trostel, A.N., Powell, B., Carlton, R., Merrill, C.R., 2002. Bacteriophage Therapy Rescues Mice Bacteremic from a Clinical Isolate of Vancomycin-Resistant *Enterococcus faecium*. *Infect. Immun.* 70, 204–210.
<https://doi.org/10.1128/IAI.70.1.204-210.2002>

Brix, A., Cafora, M., Aureli, M., Pistocchi, A., 2020. Animal Models to Translate Phage Therapy to Human Medicine. *Int J Mol Sci* 21, 3715. <https://doi.org/10.3390/ijms21103715>

Buckling, A., Rainey, P.B., 2002. Antagonistic coevolution between a bacterium and a bacteriophage. *Proc. Royal Soc. B* 269, 931–936.
<https://doi.org/10.1098/rspb.2001.1945>

Buckling, A., Wei, Y., Massey, R.C., Brockhurst, M.A., Hochberg, M.E., 2006. Antagonistic coevolution with parasites increases the cost of host deleterious mutations. *Proc. Royal Soc. B* 273, 45–49. <https://doi.org/10.1098/rspb.2005.3279>

Cabeen, M.T., Jacobs-Wagner, C., 2005. Bacterial cell shape. *Nat Rev Microbiol* 3, 601–610.
<https://doi.org/10.1038/nrmicro1205>

Canty, A., Ripley, B., 2021. boot: Bootstrap Functions.

Ceyssens, P.-J., Glonti, T., Kropinski, Andrew M., Lavigne, R., Chanishvili, N., Kulakov, L., Lashkhi, N., Tediashvili, M., Merabishvili, M., 2011. Phenotypic and genotypic variations within a single bacteriophage species. *Virol J* 8, 134. <https://doi.org/10.1186/1743-422X-8-134>

Debarbieux, L., Leduc, D., Maura, D., Morello, E., Criscuolo, A., Grossi, O., Balloy, V., Touqui, L., 2010. Bacteriophages Can Treat and Prevent *Pseudomonas aeruginosa* Lung Infections. *J Infect Dis* 201, 1096–1104. <https://doi.org/10.1086/651135>

Dennehy, J.J., Abedon, S.T., 2021. Adsorption: Phage Acquisition of Bacteria, in: Harper, D.R., Abedon, S.T., Burrowes, B.H., McConville, M.L. (Eds.), *Bacteriophages*. Springer International Publishing, Cham, pp. 93–117. https://doi.org/10.1007/978-3-319-41986-2_2

Doffkay, Z., Dömötör, D., Kovács, T., Rákhely, G., 2015. Bacteriophage therapy against plant, animal and human pathogens. *Acta Biol. Szeged.* 59, 291–302.

Edgeworth, J.D., 2011. Has decolonization played a central role in the decline in UK methicillin-resistant *Staphylococcus aureus* transmission? A focus on evidence from intensive care. *Journal of Antimicrobial Chemotherapy* 66, ii41–ii47. <https://doi.org/10.1093/jac/dkq325>

Everest, P., 2007. Stress and bacteria: microbial endocrinology. *Gut* 56, 1037–1038.
<https://doi.org/10.1136/gut.2006.117150>

Friman, V.-P., Soanes-Brown, D., Sierocinski, P., Molin, S., Johansen, H.K., Merabishvili, M., Pirnay, J.-P., De Vos, D., Buckling, A., 2016. Pre-adapting parasitic phages to a pathogen leads to increased pathogen clearance and lowered resistance evolution with *Pseudomonas aeruginosa* cystic fibrosis bacterial isolates. *J. Evol. Biol.* 29, 188–198.
<https://doi.org/10.1111/jeb.12774>

- Gandon, S., Buckling, A., Decaestecker, E., Day, T., 2008. Host-parasite coevolution and patterns of adaptation across time and space. *J. Evol. Biol.* 21, 1861–1866. <https://doi.org/10.1111/j.1420-9101.2008.01598.x>
- Gomez, P., Buckling, A., 2011. Bacteria-Phage Antagonistic Coevolution in Soil. *Science* 332, 106–109. <https://doi.org/10.1126/science.1198767>
- Goodrich, B., 2020. rstanarm: Bayesian Applied Regression Modeling via Stan.
- Górski, A., Dąbrowska, K., Hodyra-Stefaniak, K., Borysowski, J., Międzybrodzki, R., Weber-Dąbrowska, B., 2015. Phages targeting infected tissues: novel approach to phage therapy. *Future Microbiol.* 10, 199–204. <https://doi.org/10.2217/fmb.14.126>
- Haiko, J., Westerlund-Wikström, B., 2013. The Role of the Bacterial Flagellum in Adhesion and Virulence. *Biology* 2, 1242–1267. <https://doi.org/10.3390/biology2041242>
- Hampton, H.G., Watson, B.N.J., Fineran, P.C., 2020. The arms race between bacteria and their phage foes. *Nature* 577, 327–336. <https://doi.org/10.1038/s41586-019-1894-8>
- Harper, D., Parracho, H., Walker, J., Sharp, R., Hughes, G., Werthén, M., Lehman, S., Morales, S., 2014. Bacteriophages and Biofilms. *Antibiotics* 3, 270–284. <https://doi.org/10.3390/antibiotics3030270>
- Harrison, E., Truman, J., Wright, R., Spiers, A.J., Paterson, S., Brockhurst, M.A., 2015. Plasmid carriage can limit bacteria–phage coevolution. *Biol. Lett.* 11, 20150361. <https://doi.org/10.1098/rsbl.2015.0361>
- Hernandez, C.A., Koskella, B., 2019. Phage resistance evolution *in vitro* is not reflective of *in vivo* outcome in a plant-bacteria-phage system*. *Evolution* 73, 2461–2475. <https://doi.org/10.1111/evo.13833>
- Hu, Y.O.O., Hugerth, L.W., Bengtsson, C., Alisjahbana, A., Seifert, M., Kamal, A., Sjöling, Å., Midtvedt, T., Norin, E., Du, J., Engstrand, L., 2018. Bacteriophages Synergize with the Gut Microbial Community To Combat *Salmonella*. *mSystems* 3, e00119-18, <https://doi.org/10.1128/mSystems.00119-18>
- Jariah, R.O.A., Hakim, M.S., 2019. Interaction of phages, bacteria, and the human immune system: Evolutionary changes in phage therapy. *Rev Med Virol* 29. <https://doi.org/10.1002/rmv.2055>
- Kay, M., Mastny, T., 2020. tidybayes: Tidy Data and “Geoms” for Bayesian Models.
- Kim, M.S., Kim, Y.D., Hong, S.S., Park, K., Ko, K.S., Myung, H., 2015. Phage-Encoded Colanic Acid-Degrading Enzyme Permits Lytic Phage Infection of a Capsule-Forming Resistant Mutant *Escherichia coli* Strain. *Appl. Environ. Microbiol.* 81, 900–909. <https://doi.org/10.1128/AEM.02606-14>
- Koskella, B., Meaden, S., 2013. Understanding Bacteriophage Specificity in Natural Microbial Communities. *Viruses* 5, 806–823. <https://doi.org/10.3390/v5030806>
- Koskella, B., Taylor, T.B., Bates, J., Buckling, A., 2011. Using experimental evolution to explore natural patterns between bacterial motility and resistance to bacteriophages. *ISME J* 5, 1809–1817. <https://doi.org/10.1038/ismej.2011.47>
- Kropinski, A.M., Chan, L., Jarrell, K., Milazzo, F.H., 1977. The nature of *Pseudomonas aeruginosa* strain PAO bacteriophage receptors. *Can. J. Microbiol.* 23, 653–658. <https://doi.org/10.1139/m77-098>
- Kumpitsch, C., Koskinen, K., Schöpf, V., Moissl-Eichinger, C., 2019. The microbiome of the upper respiratory tract in health and disease. *BMC Biol* 17, 87. <https://doi.org/10.1186/s12915-019-0703-z>
- Kwiatek, M., Parasion, S., Nakonieczna, A., 2020. Therapeutic bacteriophages as a rescue treatment for drug-resistant infections – an *in vivo* studies overview. *J Appl Microbiol* 128, 985–1002. <https://doi.org/10.1111/jam.14535>
- Leitner, L., Ujmajuridze, A., Chanishvili, N., Goderdzishvili, M., Chkonia, I., Riggava, S., Chkhotua, A., Changashvili, G., McCallin, S., Schneider, M.P., Liechti, M.D., Mehnert, U., Bachmann, L.M., Sybesma, W., Kessler, T.M., 2021. Intravesical bacteriophages for

treating urinary tract infections in patients undergoing transurethral resection of the prostate: a randomised, placebo-controlled, double-blind clinical trial. *The Lancet Infectious Diseases* 21, 427–436. [https://doi.org/10.1016/S1473-3099\(20\)30330-3](https://doi.org/10.1016/S1473-3099(20)30330-3)

Lenth, R., 2018. Emmeans: Estimated marginal means, aka least-squares means. R Package Version.

León, M., Bastías, R., 2015. Virulence reduction in bacteriophage resistant bacteria. *Front. Microbiol.* 06. <https://doi.org/10.3389/fmicb.2015.00343>

Leung, C.Y. (Joey), Weitz, J.S., 2017. Modeling the synergistic elimination of bacteria by phage and the innate immune system. *J. Theor. Biol.* 429, 241–252. <https://doi.org/10.1016/j.jtbi.2017.06.037>

Li, L., Zhou, R., Li, T., Kang, M., Wan, Y., Xu, Z., Chen, H., 2008. Enhanced biofilm formation and reduced virulence of *Actinobacillus pleuropneumoniae* luxS mutant. *Microb. Pathog.* 45, 192–200. <https://doi.org/10.1016/j.micpath.2008.05.008>

Lloyd-Price, J., Abu-Ali, G., Huttenhower, C., 2016. The healthy human microbiome. *Genome Med* 8, 51. <https://doi.org/10.1186/s13073-016-0307-y>

Lopez Pascua, L., Hall, A.R., Best, A., Morgan, A.D., Boots, M., Buckling, A., 2014. Higher resources decrease fluctuating selection during host-parasite coevolution. *Ecol Lett* 17, 1380–1388. <https://doi.org/10.1111/ele.12337>

Lopez-Pascua, L., Buckling, A., 2008. Increasing productivity accelerates host–parasite coevolution. *J Evol Biol* 21, 853–860. <https://doi.org/10.1111/j.1420-9101.2008.01501.x>

Macnab, R.M., 2003. How Bacteria Assemble Flagella. *Annu. Rev. Microbiol.* 57, 77–100. <https://doi.org/10.1146/annurev.micro.57.030502.090832>

Mainz, J.G., Naehrlich, L., Schien, M., Kading, M., Schiller, I., Mayr, S., Schneider, G., Wiedemann, B., Wiehlmann, L., Cramer, N., Pfister, W., Kahl, B.C., Beck, J.F., Tummeler, B., 2009. Concordant genotype of upper and lower airways *P aeruginosa* and *S aureus* isolates in cystic fibrosis. *Thorax* 64, 535–540. <https://doi.org/10.1136/thx.2008.104711>

Mangalea, M.R., Duerkop, B.A., 2020. Fitness trade-offs resulting from bacteriophage resistance potentiate synergistic antibacterial strategies. *Infect Immun* IAI.00926-19, iai;IAI.00926-19v1. <https://doi.org/10.1128/IAI.00926-19>

Manohar, P., Nachimuthu, R., Lopes, B.S., 2018. The therapeutic potential of bacteriophages targeting gram-negative bacteria using *Galleria mellonella* infection model. *BMC Microbiol* 18. <https://doi.org/10.1186/s12866-018-1234-4>

May, R.M., Anderson, R.M., 1990. Parasite—host coevolution. *Parasitology* 100, S89–S101. <https://doi.org/10.1017/S0031182000073042>

Meaden, S., Paszkiewicz, K., Koskella, B., 2015. The cost of phage resistance in a plant pathogenic bacterium is context-dependent. *Evolution* 69, 1321–1328. <https://doi.org/10.1111/evo.12652>

Merabishvili, M., Pirnay, J.-P., Verbeken, G., Chanishvili, N., Tediashvili, M., Lashkhi, N., Glonti, T., Krylov, V., Mast, J., Van Parys, L., Lavigne, R., Volckaert, G., Mattheus, W., Verween, G., De Corte, P., Rose, T., Jennes, S., Zizi, M., De Vos, D., Vaneechoutte, M., 2009. Quality-Controlled Small-Scale Production of a Well-Defined Bacteriophage Cocktail for Use in Human Clinical Trials. *PLoS ONE* 4, e4944. <https://doi.org/10.1371/journal.pone.0004944>

Merril, C.R., Biswas, B., Carlton, R., Jensen, N.C., Creed, G.J., Zullo, S., Adhya, S., 1996. Long-circulating bacteriophage as antibacterial agents. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3188–3192. <https://doi.org/10.1073/pnas.93.8.3188>

MicrobesNG Sequencing Service Methods v20210419, n.d.

Morgan, A.D., Bonsall, M.B., Buckling, A., 2010. Impact of bacterial mutation rate on coevolutionary dynamics between bacteria and phages. *Evolution* 64, 2980–7. <https://doi.org/10.1111/j.1558-5646.2010.01037.x>

- Morgan, A.D., Gandon, S., Buckling, A., 2005. The effect of migration on local adaptation in a coevolving host–parasite system. *Nature* 437, 253–256. <https://doi.org/10.1038/nature03913>
- Oechslin, F., 2018. Resistance Development to Bacteriophages Occurring during Bacteriophage Therapy. *Viruses* 10, 351. <https://doi.org/10.3390/v10070351>
- Ooi, E.H., Wormald, P.-J., Tan, L.W., 2008. Innate Immunity in the Paranasal Sinuses: A Review of Nasal Host Defenses. *Am. J. Rhinol.* 22, 13–19. <https://doi.org/10.2500/ajr.2008.22.3127>
- Pal, C., Maciá, M.D., Oliver, A., Schachar, I., Buckling, A., 2007. Coevolution with viruses drives the evolution of bacterial mutation rates. *Nature* 450, 1079–1081. <https://doi.org/10.1038/nature06350>
- Palmer, K.L., Aye, L.M., Whiteley, M., 2007. Nutritional Cues Control *Pseudomonas aeruginosa* Multicellular Behavior in Cystic Fibrosis Sputum. *J Bacteriol* 189, 8079–8087. <https://doi.org/10.1128/JB.01138-07>
- Palmer, K.L., Mashburn, L.M., Singh, P.K., Whiteley, M., 2005. Cystic Fibrosis Sputum Supports Growth and Cues Key Aspects of *Pseudomonas aeruginosa* Physiology. *J Bacteriol* 187, 5267–5277. <https://doi.org/10.1128/JB.187.15.5267-5277.2005>
- Peeters, E., Nelis, H.J., Coenye, T., 2008. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *J. Microbiol. Methods* 72, 157–165. <https://doi.org/10.1016/j.mimet.2007.11.010>
- Roach, D.R., Leung, C.Y., Henry, M., Morello, E., Singh, D., Di Santo, J.P., Weitz, J.S., Debarbieux, L., 2017. Synergy between the Host Immune System and Bacteriophage Is Essential for Successful Phage Therapy against an Acute Respiratory Pathogen. *Cell Host & Microbe* 22, 38–47.e4. <https://doi.org/10.1016/j.chom.2017.06.018>
- Rossitto, M., Fiscarelli, E.V., Rosati, P., 2018. Challenges and Promises for Planning Future Clinical Research Into Bacteriophage Therapy Against *Pseudomonas aeruginosa* in Cystic Fibrosis. An Argumentative Review. *Front. Microbiol.* 9, 775. <https://doi.org/10.3389/fmicb.2018.00775>
- Scanlan, P.D., Buckling, A., 2012. Co-evolution with lytic phage selects for the mucoid phenotype of *Pseudomonas fluorescens* SBW25. *ISME J* 6, 1148–1158. <https://doi.org/10.1038/ismej.2011.174>
- Scanlan, P.D., Buckling, A., Hall, A.R., 2015. Experimental evolution and bacterial resistance: (co)evolutionary costs and trade-offs as opportunities in phage therapy research. *Bacteriophage* 5, e1050153. <https://doi.org/10.1080/21597081.2015.1050153>
- Schooley, R.T., Biswas, B., Gill, J.J., Hernandez-Morales, A., Lancaster, J., Lessor, L., Barr, J.J., Reed, S.L., Rohwer, F., Benler, S., Segall, A.M., Taplitz, R., Smith, D.M., Kerr, K., Kumaraswamy, M., Nizet, V., Lin, L., McCauley, M.D., Strathdee, S.A., Benson, C.A., Pope, R.K., Leroux, B.M., Picel, A.C., Mateczun, A.J., Cilwa, K.E., Regeimbal, J.M., Estrella, L.A., Wolfe, D.M., Henry, M.S., Quinones, J., Salka, S., Bishop-Lilly, K.A., Young, R., Hamilton, T., 2017. Development and Use of Personalized Bacteriophage-Based Therapeutic Cocktails To Treat a Patient with a Disseminated Resistant *Acinetobacter baumannii* Infection. *Antimicrob Agents Chemother* 61, e00954-17, e00954-17. <https://doi.org/10.1128/AAC.00954-17>
- Sunagar, R., Patil, S.A., Chandrakanth, R.K., 2010. Bacteriophage therapy for *Staphylococcus aureus* bacteremia in streptozotocin-induced diabetic mice. *Res. Microbiol.* 161, 854–860. <https://doi.org/10.1016/j.resmic.2010.09.011>
- Team, R.C., 2013. R: a language and environment for statistical computing.
- Torres-Barceló, C., 2018. Phage Therapy Faces Evolutionary Challenges. *Viruses* 10, 323. <https://doi.org/10.3390/v10060323>
- Torres-Barceló, C., Hochberg, M.E., 2016. Evolutionary Rationale for Phages as Complements of Antibiotics. *Trends Microbiol.* 24, 249–256. <https://doi.org/10.1016/j.tim.2015.12.011>

- Tsai, C.J.-Y., Loh, J.M.S., Proft, T., 2016. *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence* 7, 214–229. <https://doi.org/10.1080/21505594.2015.1135289>
- Van Bellegheem, J., Dąbrowska, K., Vaneechoutte, M., Barr, J., Bollyky, P., 2018. Interactions between Bacteriophage, Bacteria, and the Mammalian Immune System. *Viruses* 11, 10. <https://doi.org/10.3390/v11010010>
- van Houte, S., Buckling, A., Westra, E.R., 2016. Evolutionary Ecology of Prokaryotic Immune Mechanisms. *Microbiol. Mol. Biol. Rev.* 80, 745–763. <https://doi.org/10.1128/MMBR.00011-16>
- Vinodkumar, C., Kalsurmath, S., Neelagund, Y., 2008. Utility of lytic bacteriophage in the treatment of multidrug-resistant *Pseudomonas aeruginosa* septicemia in mice. *Indian J Pathol Microbiol* 51, 360. <https://doi.org/10.4103/0377-4929.42511>
- Wang, J., Hu, B., Xu, M., Yan, Q., Liu, S., Zhu, X., Sun, Z., Tao, D., Ding, L., Reed, E., Gong, J., Li, Q.Q., Hu, J., 2006. Therapeutic effectiveness of bacteriophages in the rescue of mice with extended spectrum beta-lactamase-producing *Escherichia coli* bacteremia. *Int. J. Mol. Med.* 17, 347–355.
- Wang, X., Wei, Z., Yang, K., Wang, J., Jousset, A., Xu, Y., Shen, Q., Friman, V.-P., 2019. Phage combination therapies for bacterial wilt disease in tomato. *Nat Biotechnol* 37, 1513–1520. <https://doi.org/10.1038/s41587-019-0328-3>
- Wang, Y., Zhang, W., Wu, Z., Lu, C., 2011. Reduced virulence is an important characteristic of biofilm infection of *Streptococcus suis*: Biofilms of *Streptococcus suis* cause reduced virulence. *FEMS Microbiol. Lett.* 316, 36–43. <https://doi.org/10.1111/j.1574-6968.2010.02189.x>
- Wertheim, H.F., Vos, M.C., Ott, A., van Belkum, A., Voss, A., Kluytmans, J.A., van Keulen, P.H., Vandenbroucke-Grauls, C.M., Meester, M.H., Verbrugh, H.A., 2004. Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. *The Lancet* 364, 703–705. [https://doi.org/10.1016/S0140-6736\(04\)16897-9](https://doi.org/10.1016/S0140-6736(04)16897-9)
- Whiteley, M., Banger, M.G., Bumgarner, R.E., Parsek, M.R., Teitzel, G.M., Lory, S., Greenberg, E.P., 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* 413, 860–864. <https://doi.org/10.1038/35101627>
- WHO, 2019. No time to wait: securing the future from drug-resistant infections. Report to the secretary-general of the United Nations. https://www.who.int/antimicrobial-resistance/interagency-coordination-group/IACG_final_report_EN.pdf?ua=1.
- Wickham, H., 2016. *ggplot2: elegant graphics for data analysis*. Springer, New York.
- Wills, Q.F., Kerrigan, C., Soothill, J.S., 2005. Experimental Bacteriophage Protection against *Staphylococcus aureus* Abscesses in a Rabbit Model. *Antimicrob. Agents Chemother.* 49, 1220–1221. <https://doi.org/10.1128/AAC.49.3.1220-1221.2005>
- Wright, R.C.T., Friman, V.-P., Smith, M.C.M., Brockhurst, M.A., 2019. Resistance Evolution against Phage Combinations Depends on the Timing and Order of Exposure. *mBio* 10, e01652-19. <https://doi.org/10.1128/mBio.01652-19>
- Wright, R.C.T., Friman, V.-P., Smith, M.C.M., Brockhurst, M.A., 2018. Cross-resistance is modular in bacteria–phage interactions. *PLoS Biol* 16, e2006057. <https://doi.org/10.1371/journal.pbio.2006057>
- Wu, N., Dai, J., Guo, M., Li, Jianhui, Zhou, X., Li, F., Gao, Y., Qu, H., Lu, H., Jin, J., Li, T., Shi, L., Wu, Q., Tan, R., Zhu, M., Yang, L., Ling, Y., Xing, S., Zhang, J., Yao, B., Le, S., Gu, J., Qin, J., Li, Jie, Cheng, M., Tan, D., Li, L., Zhang, Y., Zhu, Z., Cai, J., Song, Z., Guo, X., Chen, L.-K., Zhu, T., 2021. Pre-optimized phage therapy on secondary *Acinetobacter baumannii* infection in four critical COVID-19 patients. *Emerg. Microbes Infect.* 10, 612–618. <https://doi.org/10.1080/22221751.2021.1902754>

1032 Zhvania, P., Hoyle, N.S., Nadareishvili, L., Nizharadze, D., Kutateladze, M., 2017. Phage
1033 Therapy in a 16-Year-Old Boy with Netherton Syndrome. Front. Med. 4, 94.
1034 <https://doi.org/10.3389/fmed.2017.00094>
1035

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Supplementary material
Parallel resistance evolution to phages during clinical phage therapy and in vitro

Supplementary figures

(a) Replicate 1

1045 (a) Replicate 2

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(c) Replicate 3

Figure 3 - Figure supplement 1. Growth curves of bacterial clones as measured by change in log-transformed optical density (OD₆₀₀) over time (hours). Measures from the first 2.5 hours were excluded (blue line) as a rise in OD was observed independent of bacterial logistic growth which may skew the rolling regression process. Growth curves were independently replicated a total of 3 times (a - c) owing to cellular clumping which may have skewed OD measures. (“anc” = ancestral clones; “blank” = uninoculated wells; “control” = control clones; mix add” = clones from repeated phage treatment; “mix” = clones from treatment where phage were added once; “resist” = phage resistant clones; “susc” =

susceptible clones; “sing_res” = *in vitro* clones resistant to one phage; “TP1” = *in vivo* clones from day 2; “TP2” = *in vivo* clones from day 4).

Figure 5 - Figure supplement 1. Genomic changes for populations evolved *in vitro*, including genetic variants occurring in genes of known and unknown function. (a) The divergence of each treatment group as indicated by Euclidean distances of each gene from the ancestral isolates. Genetic changes were characterised as a change in frequency of an existing gene in the ancestor or selection on a mutation that was gained during experimental evolution.

1066 The presence of phage significantly affected (b) genotypic distance from the ancestor, (c) the
1067 number of new SNPs and (d) alpha diversity.

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Figure 5 - Figure supplement 2. Associations between genetic similarity and phenotypic traits for *in vivo* clones. Individual points represent individual clones isolated at day 2 (n = 8) and day 4 (n = 2) of phage treatment. Genes that diverged in frequency *in vitro* from the ancestor are indicated in grey while *in vivo* clones are overlayed according to their individual genetic make-up. Across quantitative (a – c) and qualitative (d) phenotypic traits, no clustering in genotypes and clones of particular phenotypes is observed.

Supplementary Tables

Supplementary File 1. Pairwise comparisons of resistance of *in vivo* clones from day 2 against phage from contemporary (day 2) and two future time-points (days 4 and 7). P-values adjusted using the Tukey method of comparing a family of three estimates. “SE” = standard error.

Supplementary File 2. Susceptible colonies picked from *in vitro* phage treatments for phenotypic assays and sequencing.

Supplementary File 3. The effect of outlier removal on the significance of model terms (treatment and phage resistance). Outlier removed states in which treatment each outlier was identified and subsequently removed. Significant p-values in bold.

Supplementary File 4. The effect of outlier removal on Tukey HSD contrasts comparing biofilm productivity between phage resistance groups. Significant p-values highlighted in bold.