Research Article

Virus-Host Coevolution as a Tool for Controlling Bacterial Resistance to Phage Therapy

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Abstract

Bacterial resistance to antibiotics is a global public health concern. New treatments are needed to combat resistant strains, among which phage therapy is a promising option. Probably the main advantages of phage therapy are its high specificity as well as rapid viral adaptability, which in principle allows using phage evolution to overcome resistance. Here, we have performed serial coevolution passages between *Escherichia coli* and its phage T7 to investigate the ability of coevolved phages to reduce the emergence of resistances. We find that the initial bacterial population is less likely to undergo resistance when challenged with experimentally coevolved phages than when challenged with the wild-type phage. Hence, our findings suggest that coevolved phage preparations could be used to increase the efficacy of phage therapy.

Keywords: Bacterial resistance; Phage therapy; Phage-bacteria

1. Introduction

The emergence of multi-drug-resistant bacteria calls for novel therapeutic strategies. In this context, bacteriophages (phages), whose therapeutic use was long-ago suggested, are being now reconsidered. Phages are natural parasites of bacteria and the most abundant entity in the biosphere, making them an attractive tool for fighting against bacterial pathogens [1-4]. One important difference between antibiotics and phages is

that many antibiotics cover a broad spectrum of bacteria, whereas phage are typically species- or genus-specific. In acute infections, specificity can be problematic because treatment often precedes diagnosis, although phage cocktails can be used to broaden host range and improve effectiveness. Yet, specificity is clearly advantageous for treating long-term infections, since side-effects such as damage to the physiological microbiota are avoided [5]. On the other hand, long-term treatments are particularly prone to the emergence of resistances, although resistance also emerges among acute disease-causing bacteria at the host population level [6]. Bacteria and their phages coevolve in nature, and understanding phage-bacteria evolutionary dynamics should help us design better phage therapy interventions [7].

The emergence of bacterial resistance against phages is in many cases rapid and frequent, but unlike antibiotics, phages can evolve resistance-breaking by spontaneous mutation and natural selection [4, 8, 9]. In addition, phage-resistant bacteria are generally costly, producing a drop in virulence in many cases. Generally, phages mutate faster than their hosts [10, 11], which provides them a clear evolutionary advantage. However, bacteria have evolved elaborate and flexible mechanisms to block infection. These mechanisms include point mutations in specific proteins like phage receptors, restriction-modification systems, and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas adaptive immunity [8, 12]. In turn, phages have evolved specific genes to inactivate the CRISPR-Cas system, called anti-CRISPRs (Acrs), as expected under an evolutionary arms race [13-15]. Antagonistic phage-bacteria coevolution can have multiple population-level implications [16, 17], including increased genetic diversity in both phage and bacteria [18], directional increases in host resistance and parasite infectivity [19], and negative frequency-dependent selection causing fluctuations in allele frequency [20, 21].

Promoting phage-bacteria coevolution in the laboratory may thus help us obtain phages capable of infecting a wider range of variants in the bacterial population and to overcome the emergence of resistances during treatment. Previous studies examined phage-bacteria coevolution mainly in chemostats and reported a variety of outcomes, including phage and host persistence, as well as full host resistance leading to phage extinction [16]. Here, we provide basic proof of concept for a coevolution approach to phage therapy using *Escherichia coli* and its phage T7 as model system. Previous work using this phage-bacteria system showed that the evolution of both resistance and resistance breaking are common [22]. *E. coli* is the most abundant commensal bacterium in the mammalian intestine and some strains are well-known pathogens [23]. Pathogenic strains have been reported worldwide, and their appearance is associated to the emergence of resistance. We chose bacteriophage T7, a double-stranded DNA phage, because it shows a rapid and highly lytic infection cycle, which is an interesting feature for phage therapy but also a convenient feature for basic studies. + viruses are the most abundant type of phage and, despite their lower mutation rates compared to RNA phages, they can also adapt efficiently under controlled laboratory conditions [24]. In addition, relatively large double-stranded DNA viruses are interesting from the therapeutic point of view because they can more easily incorporate transgenes aimed at improving treatment efficacy. We find that, after only ten serial transfers of coevolution with their host, the resulting coevolved phages improve their lytic activity and are superior to the founder phage in terms of preventing the growth of phage-resistant mutants.

2. Materials and Methods

2.1 Bacterial strain, bacteriophage and culture conditions

Escherichia coli C IJ1862 strain and bacteriophage T7 were kindly provided by Prof. James J. Bull (University of Texas). General biology of the phage can be found elsewhere [25]. *E. coli* C IJ1862 was cultured in Lysogeny Broth (LB) medium at 37°C in an orbital shaker (250 rpm). A stock of IJ1862 was obtained by growing the bacterium to stationary phase and storage at -70°C in glycerol 20% (v/v). Serial dilutions from this stock were performed to isolate three independent colonies, which were used to initiate coevolution experiments. Isolated bacterial colonies were picked randomly and resuspended in 50 µL LB. Serial dilutions were prepared, plated onto LB plates and incubated until single colonies were observed to determine bacterial density of these colony-derived populations. In parallel, 100 µL of IJ1862 were mixed with 100 µL of serial dilutions of phage T7 and poured onto dishes containing LB medium semi-solidified with soft agar (0.7%) and supplemented with 5 mM of $CaCl_2$ to obtain isolated plaques. After 6 h of incubation at 37°C, three independent plaques were picked randomly, resuspended in 50 µL of LB, and stored at -70°C. These three plaques were used to initiate the coevolution experiments.

2.2 Coevolution passages

Three independent coevolution lines (C1-C3) were initiated, each derived from a single plaque-derived virus and a single colony-derived bacterial culture as detailed above. For each coevolution line, 10^5 plaque forming units (PFU) of phage were used to inoculate 10^8 colony forming units (CFU) of IJ1862 cells at their exponential growth phase in liquid LB medium supplemented with 5 mM $CaCl_2$. Infected cultures were incubated under agitation (650 rpm) at 37°C in a Thermomixer 24-tube shaker (Eppendorf). After 24 h, the culture was diluted 1:100 in fresh LB medium to initiate the next passage. Ten serial passages of coevolution were performed. After each passage, the viral titer was determined by plaque assay. For this, bacteria were first cleared by centrifugation (16, 000 × *g*, 1 min) and the supernatant was poured onto dishes containing the bacterium in soft agar. In addition, after each passage, optical density (OD_{600}) was used to measure bacterial density (CFU/mL). A calibration curve between OD_{600} and bacterial density was made by performing serial dilutions, plating and colony counting. When bacterial density was below the sensitivity limit of OD_{600} measurements, colony counting was performed.

2.3 Determination of bacterial lysis

Coevolved and founder phages were assayed for their ability to lyse cells from the non-coevolved bacterial stock. To accomplish this aim, 10^8 CFU from the founder bacterial culture were inoculated with 10^5 PFU of each phage line (founder and coevolved phages) in liquid LB medium supplemented with 5 mM $CaCl_2$ and incubated at 37°C and 600 rpm in a Varioskan LUX multimode microplate reader (Thermo Scientific) to determine bacterial density. Six independent technical replicates were done per line, and OD_{600} readings were obtained every 4 min for the first 2 h and every 15 min subsequently, for a total time of 100 h.

2.4 Determination of phage-resistant colonies and resistance breaking

We inoculated 10^6 CFU of the non-coevolved bacterial stock with 10^7 PFU of founder or coevolved phages and poured it onto LB plates solidified with soft agar (supplemented with 5 mM $CaCl_2$). At this multiplicity of infection, we expected all bacteria to be lysed except resistant mutants, which should form isolated colonies capable of growing in presence of the phage. Plates were incubated at 37°C until candidate resistant

colonies were observed. Each assay was done in triplicate. Candidate colonies were picked and stored at -70°C in LB with glycerol 20% (v/v) for further testing. To test for resistance, candidate colonies were spotted onto LB with soft agar containing 10^7 PFU of the relevant phage and incubated at 37°C to determine whether cells grew or were lysed.

3. Results

3.1 Phage-host coevolution

Coevolution was initiated using three pairs of T7 plaque-derived and *E. coli* colony-derived populations. For this, each colony was expanded in liquid culture and inoculated with phage derived from a single plaque, resulting in three coevolution lines (C1-C3). Infected cultures were maintained in agitation for 24 h and diluted 1:100 to initiate the next passage, up to ten serial passages during which the phage and the host could in principle coevolve. In all the coevolution lines, the bacterium and the phage coexisted (Figure 1). In line C1, a six-fold drop in bacterial density occurred during the first passage, leading to a low-density plateau. The first infection produced a high phage titer (10^{10} PFU/mL) as determined by the plaque assay, but phage titer gradually decreased until reaching a plateau at passage 5 of around 10^6 PFU/mL. This suggests that the phage exhausted the host population initially, resulting in low densities of both bacteria and phage, and that fully resistant bacteria did not evolve or had low fitness. In contrast, in line C2, the phage titer remained high throughout the 10 passages, whereas the bacterial density dropped initially, but then rebounded drastically in passage 3, suggesting the emergence of resistant bacteria in the population. Finally, line C3 showed a qualitatively similar pattern to C2, albeit bacterial density rebounded later, suggesting the emergence of resistant variants around passage 6.

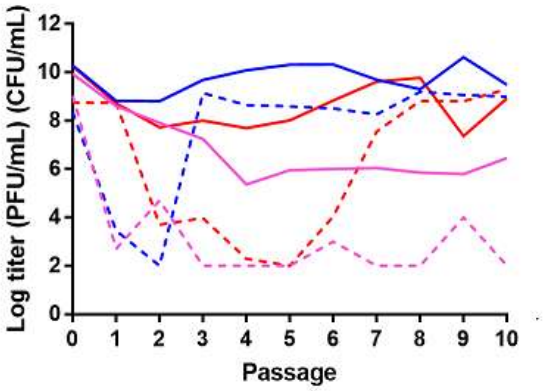

Figure 1: Viral titers and bacterial densities along coevolution passages. Viral titers (PFU/mL) are shown in solid lines, whereas dotted lines represent bacterial densities (CFU/mL). Pink: line C1; blue: line C2; red: line C3.

3.2 Dynamics of bacterial regrowth following challenge with phage

We compared the ability of the founder and coevolved phages to clear the initial bacterial population and, in particular, to prevent regrowth after initial lysis. For this, we challenged 10^8 CFU of the non-coevolved bacteria with 10^5 PFU of phages (founder and coevolved lines) in liquid medium and measured optical density in real time over 100 h of culture (Figure 2). Six replicate cultures were carried out for each condition. Bacterial growth was halted approximately 45 min after phage inoculation and lysis occurred within the first 90 min in all cases. After lysis, bacterial density remained stably low for at least 5 h post inoculation (hpi). Cultures that were challenged with coevolved phages (C1-C3) showed lower log-density than those infected

with the founder phage, indicating more efficient lysis. Phages from line C1 showed the highest antibacterial efficacy, followed by C3 and C2 (t-tests: founder versus C1, P=0.0160; founder versus C2, P=0.0314; founder versus C3, P=0.0420). In most cases, this was followed by a slow and gradual phase of bacterial regrowth, which was first evident in cultures treated with founder phages and was maximally delayed for those treated with C1 phages, whereas C2 and C3 showed intermediate rebound times. In C3-treated cultures we observed a sharp increase in log-density around 20 hours post inoculation (hpi) in one replicate, clearly showing the emergence of a high-fitness, phage-resistant bacterial mutant. Removing this outlier, we found significantly lower log-densities at 50 hpi in cultures treated with each of the three coevolved phages compared to those treated with founder virus (t-tests: founder versus C1, P=0.0012; founder versus C2, P=0.0097; founder versus C3, P=0.0180). At the final time point (100 hpi), we found lower log-densities in cultures treated with C1 coevolved phages compared to those treated with the founder phage, but no differences were found for lines C2 and C3.

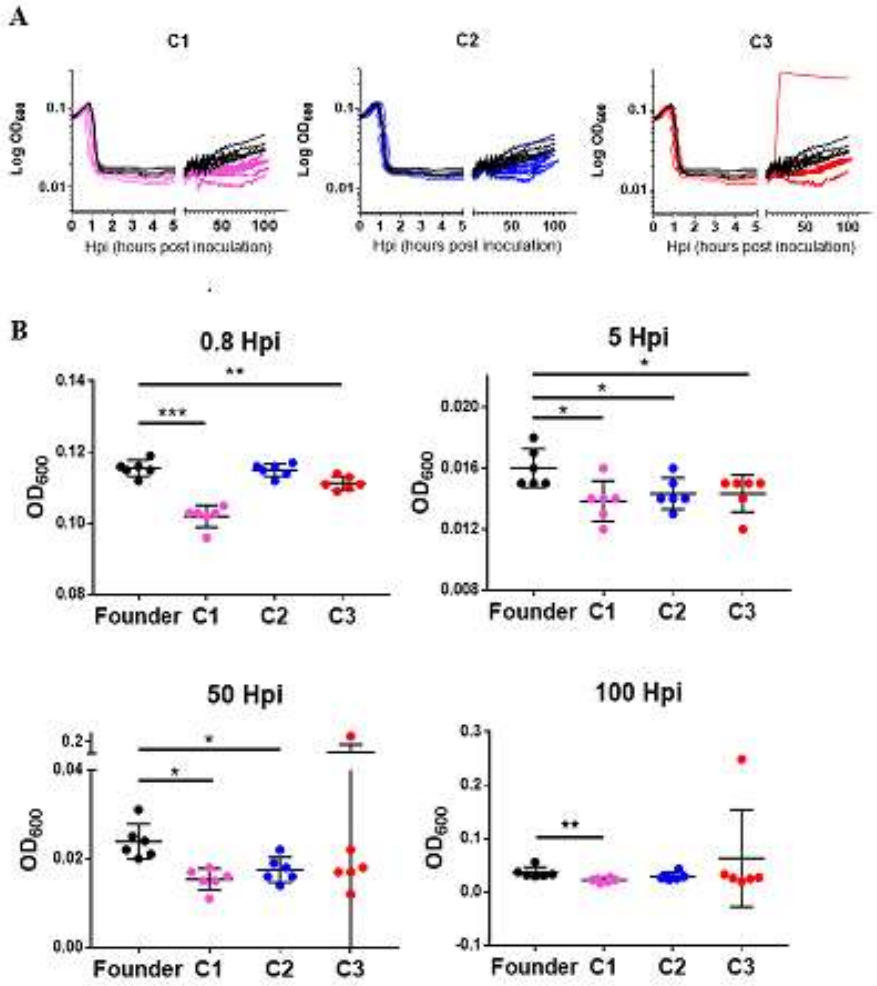

Figure 2: Dynamics of bacterial regrowth following challenge with phage. **(**A) Optical density of cultures of the non-coevolved bacteria infected with founder or coevolved phages. Six experimental replicates were done for each condition. Cultures treated with the founder phage (black), C1 (pink), C2 (blue), or C3 (red) are shown; (B) Optical densities at specific time points (0, 5, 50 and 100 hours post inoculation).

3.3 Analysis of phage-resistant colonies and resistance breaking

The above assays provided an overview of the increased ability of the coevolved phages to lyse bacteria and prevent regrowth, but did not provide information about the number of phage-resistant bacterial mutants emerged. For instance, a single high-fitness resistant mutant may repopulate the entire culture, as suggested by the outlier found in one replicate treated with C3 phages. To achieve this goal, we inoculated 10^6 CFU of the non-coevolved bacteria with 10^7 PFU of the founder or coevolved phage in dishes overlaid with soft agar, which allowed us to visualize resistant colonies. In dishes inoculated with the founder phage, we found between 20 and 100 resistant colonies (resistance rate ranging from 2×10^{-6} to 10^{-5}), whereas for C1-C3 phages the number of resistant colonies was between zero and five (resistance rate ranging from 0 to 5×10^{-7}). Whilst most colonies were remarkably small even after 3 days of incubation, a few colonies showed sizes comparable to those observed in the absence of phage. This indicates that levels of resistance and/or the fitness costs of resistance were amply variable, as also suggested by the optical density measurements. We picked 26 colonies randomly from dishes infected with the founder phage and 15 (all) from dishes infected with C1, C2, or C3 and evaluated their resistance to each phage by spotting them onto phage-containing dishes in soft agar (Figure 3). All 15 colonies derived from coevolved-infected cultures were resistant to all phages (founder, C1, C2, and C3), whereas only 9/26 colonies derived from founder-infected cultures were resistant to all phages, revealing a significant association between the origin of the colony (pre-infected with founder versus coevolved phage) and its subsequent resistance status (Fisher exact test: $P<0.001$). This suggests, first, that C1-C3 populations contained phages capable of infecting *E. coli* variants that were resistant to the non-coevolved phage. On the other hand, these results also suggest that some *E. coli* resistance mechanisms were efficient against all T7 phage variants.

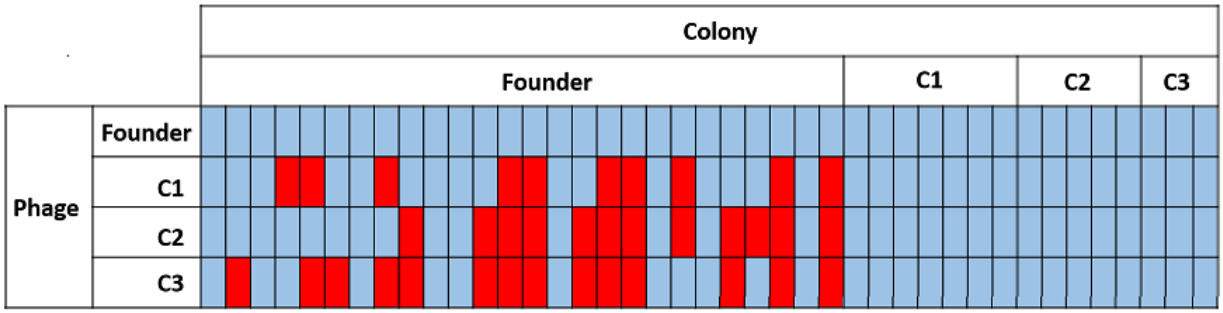

Figure 3: Analysis of phage-resistant colonies and resistance breaking. Resistance was tested by spotting each colony on each phage. Each column corresponds to an isolated colony from the indicated line, and each row corresponds to a phage line. Blue: bacterial growth. Red: lysis.

4. Discussion

Bacteria and viruses are excellent systems for studying basic evolutionary processes such as mutation, selection, and genetic drift in real time under controlled conditions, which in turn are important for understanding the emerge of resistances [26, 27]. Although phage therapy is an interesting alternative to classical antibiotics, it also faces the problem of bacterial resistance. Yet, as opposed to antibiotics, evolvability is an inherent property of phages. Here, we sought to explore the ability of phage to engage in a

short-term antagonistic coevolution process with its host under controlled laboratory conditions, and we tested whether the resulting coevolved phages could be a better choice than the wild-type phage in terms of lysis strength and/or delaying resistance. We found that in 3/3 coevolution lines the phage did not go extinct, suggesting that coevolution is a robust and, hence, easy-to-implement process. Providing proof of concept for the utility of coevolved phages, they delayed the timing of bacterial regrowth and produced fewer resistance colonies than an equivalent dose of wild-type non-coevolved phage.

In future work, it would be useful to sequence the coevolved populations of phages and bacteria to gain information about the mechanisms underlying this antagonistic coevolution. Sequencing should allow us to identify specific mutations responsible for resistance and resistance breaking, and to determine their abundance in natural populations and clinical isolates. For future applications, it may be required to identify resistance-breaking mutations in the phage genome and prepare phage cocktails of known composition that include these mutants, instead of using raw coevolved preparations. CRISPR-Cas loci are important drivers of phage-host antagonistic coevolution [28, 29]. The *E. coli* strain used here belongs to phylogenic group C, which contains two pairs of CRISPR loci, each associated with a different type of Cas genes [30]. Yet, there are other possible molecular determinants of antagonistic coevolution, such as loss of the receptor and use of alternative receptors by the phage [8, 31].

Finally, given their wide abundance and diversity, multiple candidate phages might be available to fight against a given bacterium, but we currently lack a general understanding on which type of phage should perform best. On one hand, RNA phages may be more evolvable than DNA phages because they mutate faster, but large DNA phages might display a larger number of alternative infection mechanisms owing to their higher genome complexity and, thus, may counteract resistance better. Comparative studies of the coevolutionary process of a given bacterial species with different phages may help illuminate this point.

Acknowledgments

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