Phage fitness may help predict phage therapy efficacy

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Abbreviations: MST, mean survival time; PB, pilus-binding; NPB, non-pilus binding.

We isolated 6 phages from 2 environmental water sources and assessed their ability to treat Pseudomonas aeruginosa infection of Drosophila melanogaster. We found all 6 phages were able to significantly increase mean survival time (MST) of infected D. melanogaster. Although phage traits, such as adsorption rate, burst size, and lysis time, varied significantly among these phages, none of the traits correlated significantly with MST. Phage growth rate determined in vitro, however, was found to be significantly correlated with MST. Overall, our study shows that infected D. melanogaster can be used as a model system to test the therapeutic efficacy of phages. In addition, a more comprehensive characteristic, like the in vitro growth rate, seems to be a better indicator in predicting therapeutic success than constituent traits like the adsorption rate, burst size, or lysis time.

Introduction

Unlike many other discoveries for which their practical applications are not immediately obvious, the antibacterial potential of bacteriophage (phage) was recognized at once and vigorously pursued by one of its discoverers.1-3 However, in the early days of phage therapy, inconsistent treatments and poor results, due to lack of basic understanding of phage biology, contributed to the decline of using phage as a therapeutic or prophylactic agent against bacterial infections.3 The demise of phage therapy, at least in Western Europe and North America, was later hastened by the discovery and subsequent widespread use of antibiotics. Ironically, the current renewed interest in phage therapy is mainly driven by the emergence of antibiotic-resistant bacterial strains and the dwindling supply of available antibiotics in the development pipeline.4 Expanded use of phages as a low-cost alternative to manage bacterial problems in non-human cases, such as animals, agriculture,5,6 and food industry,7 also contribute to the recent resurgent interest in phage applications.

The sheer number of phages in the environment8 presents a challenge to finding the most effective phage in treating bacterial pathogens. The most common approach to assessing the therapeutic efficacy of a specific phage is to conduct in vivo studies.5,9-19 However, since results from such studies can directly inform us whether a specific phage is effective against the bacterial infection, often it is not immediately clear why one phage is more efficacious than the others in treating the infection, not to mention the effort and resource that are needed for screening a large number of potential candidates. A possible alternative to direct in vivo screening is to identify phage traits that can be used as a proxy for in vivo efficacy. Theoretical studies of phage therapy, which routinely incorporate basic phage traits, such as adsorption rate, lysis time, and burst size, into mathematical models to investigate population dynamics of phage and targeted bacteria20-23 can be an excellent starting point for identifying important phage traits that can effectively depress or eliminate the infecting bacteria population.

Based on others20-23 and our24-26 previous studies, we hypothesize that any phage trait that contributes to a faster phage growth (replication) will also result in a faster or more clearing of the infecting bacteria population; consequently increasing the efficacy of phage treatment. All else being equal, a higher adsorption rate24 or burst size should result in a higher phage growth rate, while an optimal lysis time would maximize the growth rate as well.24-26 That is, we would expect to observe a correlation between various phage traits (including the phage in vitro or ex situ growth rate) and therapeutic efficacy.

To test our hypothesis, we adopted a model system13 involving the phage treatment of a systemic, lethal Pseudomonas aeruginosa infection within Drosophila melanogaster. P. aeruginosa is a ubiquitous Gram-negative bacterium associated with many human medical conditions, such as nosocomial27 and burn...
wound infections, as well as being a major cause of morbidity and mortality in cystic fibrosis patients. D. melanogaster has previously been used as a model system to study P. aeruginosa infections, including one study evaluating the efficacy of phage therapy in treating infection. In this previous study, Heo et al. evaluated the efficacy of 2 Caudovirales phage strains, MPK1 and MPK6, in treating P. aeruginosa infections in mice and in D. melanogaster. While only 2 phages were tested, there was general agreement that these phages were capable of treating infections in both the animal and insect systems. In this current study, we take advantage of the convenience of the Drosophila system to evaluate the feasibility of using phage traits to predict the efficacy of phage therapy.

**Results**

*Pseudomonas aeruginosa* phages from the environment

We isolated 6 phages from 2 water sources: a wastewater treatment plant in Menands, New York (phages HWPB-1, HWPB-2, HWNPB-2, and HWNPB-3) and river water from the Passaic River in New Jersey (phages HWNPB-1 and HWPB-3). Three phages require the presence of the type IV pili, a known virulence factor, for attachment and infection (i.e., the HWPB phages) while the remaining 3 (the HWNPB phages) do not. Partial genomic sequencing revealed that our phage collection encompasses all 3 commonly found *P. aeruginosa* phage families. HWPB-1 and HWPB-3 showed high sequence similarity to Siphoviridae phages M6, YuA and MP1412. HWPB-2 showed high sequence similarity to ϕKMV and related members. All 3 non-pilus-binding (NPB) phages showed high sequence similarity to Myoviridae PB-1 and related members. Since these phages utilize the lipopolysaccharide for cell entry, it is possible that our non-pilus-binding phages would also use the same receptor.

**Phage traits and growth rate**

We determined several phage traits, such as adsorption rate, lysis time, and burst size for each of the isolated phages. Table 1 and Figure 1 show these measurements. Overall, the 6 phages have significantly different adsorption rates (Kruskal-Wallis $\chi^2 = 15.0117, p = 0.0103$). There is no significant difference within the pilus-binding (PB) (Krusksal-Wallis $\chi^2 = 5.6000, p = 0.0608$) or non-pilus-binding (NPB) (Krusksal-Wallis $\chi^2 = 5.9556, p = 0.0509$) phages. However, there are significant differences between PB and NPB phages (Kruskal-Wallis: $\chi^2 = 10.9649, p = 0.0009$). The PB phages have an average adsorption rate of 6.31 (± 4.37, standard error) $\times 10^{-5}$ cell$^{-1}$ mL$^{-1}$ h$^{-1}$, while the NPB phages have a higher average adsorption rate of 3.98 (± 1.94) $\times 10^{-6}$ cell$^{-1}$ mL$^{-1}$ h$^{-1}$.

Analysis of one-step growth curves (Fig. 2) further showed significant differences in lysis times (Krusksal-Wallis $\chi^2 = 15.5372, p = 0.0083$) and burst sizes (Krusksal-Wallis $\chi^2 = 12.1345, p = 0.0330$). Lysis times ranged from 47 to 100 min (Table 1) whereas burst sizes ranged from 37 to 589 phages per infected cell. There is significant difference between the PB and NPB phages for the lysis time (Kruskal-Wallis test: $\chi^2 = 5.2999, p = 0.0213$) but not for the burst size (Kruskal-Wallis test: $\chi^2 = 1.4211, p = 0.2332$). Overall, there is a significant difference in growth rate among the phages (Kruskal-Wallis test: $\chi^2 = 16.5789, p = 0.0054$), with each phage’s growth rate being significantly different from the others, even after Bonferroni corrections to account for multiple comparisons (Table 1). However, there is not a significant difference between the PB and NPB phages (Kruskal-Wallis test: $\chi^2 = 1.4211, p = 0.2332$). The phage growth rate ranged from 0.33 to 4.84 h$^{-1}$ (Table 1).

**Therapeutic efficacy of phage treatment**

To test the therapeutic efficacy of our phages, adult female D. melanogaster were injected with $\sim10^3$ *P. aeruginosa* and 6 hours later either injected with $\sim2.40 \times 10^4$–$1.20 \times 10^7$ phages for treatment or sterile LB as a control. If left untreated, an inoculum of this size is fatal within 24–30 h at 25°C (Fig. 3). At the time of phage treatment, bacterial numbers had increased to $\sim10^6$ bacteria, thus giving a multiplicity of infection (MOI) of $\sim10$ at the time of phage treatment. The survival curves for the phage treatments are shown in Figure 3.

Overall, there are significant differences among the survival curves (Log-rank test: $\chi^2 = 370, P < 0.0001$). Fitting of Cox proportional hazard model showed that phage treatment significantly

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**Table 1.** Phage traits, in vitro growth rate, and mean survival time

| Measurement | Adsorption rate $\times 10^5$ | Lysis time | Burst size | Growth rate | Mean ST | Hazard ratio |
|-------------|-------------------------------|------------|------------|-------------|---------|-------------|
| LB          | n/a                           | n/a        | n/a        | n/a         | 22.8 ± 0.48 | 1.00        |
| HWPB-1      | 8.31 ± 3.07                   | 100 ± 0    | 392.8 ± 220.1 | 1.53 ± 0.03 | 32.8 ± 0.82 | 0.12 (0.08 – 0.17) |
| HWPB-2      | 8.66 ± 1.33                   | 50 ± 0     | 260.0 ± 101.9 | 4.84 ± 0.03 | 45.7 ± 1.19 | 0.04 (0.03 – 0.05) |
| HWPB-3      | 1.96 ± 0.68                   | 93 ± 7     | 36.8 ± 15.1  | 0.72 ± 0.06 | 31.5 ± 0.80 | 0.14 (0.10 – 0.20) |
| HWNPB-1     | 45.70 ± 4.84                  | 70 ± 6     | 589.4 ± 12.6 | 0.33 ± 0.02 | 27.8 ± 0.64 | 0.27 (0.18 – 0.38) |
| HWNPB-2     | 54.90 ± 7.85                  | 47 ± 3     | 246.1 ± 55.3 | 2.41 ± 0.06 | 33.2 ± 0.79 | 0.11 (0.08 – 0.16) |
| HWNPB-3     | 18.70 ± 7.74                  | 47 ± 3     | 116.5 ± 42.5 | 1.21 ± 0.13 | 32.0 ± 0.84 | 0.13 (0.09 – 0.19) |

*The units for each phage trait and measurement are: adsorption rate, cell$^{-1}$ mL$^{-1}$ h$^{-1}$; lysis time, min; burst size, phage/cell; growth rate (h$^{-1}$); mean survival time (using the Kaplan-Meier estimator), h.  
1Showing mean ± standard error.  
2Showing mean (95% confidence interval).
increased the survival of the infected flies (Table 1). The Kaplan-Meier estimate of mean survival time of untreated flies was 22.8 ± 0.48 h, whereas those for the phage-treated flies ranged from 27.8 ± 0.64 h for the least efficacious phage treatment to 45.7 ± 1.19 h for the most (Table 1). From these survival analyses, 3 distinct clusters of phages with varying degrees of therapeutic efficacy are discernible: HWPB-2 > HWPB-1 ≈ HWPB-3 ≈ HWNPB-2 ≈ HWNPB-3 > HWNPB-1.

Correlations between phage traits and therapeutic efficacy
The main reason for the current study was to see whether any phage trait determined in vitro would correlate significantly with its therapeutic efficacy. None of the individual phage traits: adsorption rate (r = −0.38, p = 0.4585), lysis time (r = −0.36, p = 0.4803), or burst size (r = −0.22, p = 0.6753), were significantly correlated with the mean survival time (MST). However, phage growth rate determined in vitro was significantly correlated with MST (r = 0.97, p = 0.0015) (Fig. 4). This positive correlation remains significant even after the most effective phage (HWPB-2) is removed from the analysis (Pearson’s r = 0.83, p = 0.0807; Kendall’s τ = 1, p = 0.0167). In general, the faster the phage is able to grow in vitro, the better the phage is able to combat bacterial infection.

Discussion
Initial attempts at using phage to treat bacterial infections were mired mainly by lack of basic understanding of phage
After almost a century, researchers are able to compile simple rules and propose best practices for a successful application of phage therapy. One of the future challenges for phage therapy will be to efficiently identify the most efficacious phages among many potential candidates. As a first step, it would be valuable to know if phage characteristics can be used to predict therapeutic efficacy in vivo.

In this study, each phage in our collection, to various degrees, is able to significantly increase the mean survival time (MST) of P. aeruginosa-infected flies. However, the amount of MST increase does not correlate with individual phage traits. Conversely, we did observe a positive correlation between phage in vitro growth rate and therapeutic efficacy. One possibility for the lack of correlation between individual phage traits and therapeutic efficacy is that the trait values estimated in vitro do not reflect what they would have been if measured in vivo. While in vivo and in vitro measurements are likely to be different, we do expect that their rank-orders will remain the same. That is, a phage with a high in vitro adsorption rate would be expected to have a high in vivo adsorption rate as well. Therefore, if a significant correlation existed we should be able to detect it (e.g., with nonparametric correlation tests). The more likely reason for the lack of correlation is that individual phage traits are poor surrogates for phage in vitro growth rate; even though the phage in vitro growth rate is a function of individual phage traits. For example, a phage with a high adsorption rate but a long lysis time may not grow as fast as a phage with a slightly lower adsorption rate but a much shorter lysis time. Nevertheless, our study showed that therapeutic efficacy is positively correlated with phage in vitro growth rate, a much more immediate proxy for bacterial clearance rate. This finding also corroborates a previous study by Henry et al.
who used 9 phages to treat *P. aeruginosa* infection in a mouse pulmonary system. While no statistical analysis was conducted to investigate whether phage in vitro characteristics (both efficiency of plating and lysis kinetics in their study) were related to bacteria in vivo growth in phage-treated mice (determined via a bioluminescent bacterial strain) and probability of survival, it was apparent from this study that a faster in vitro lysis of the targeted bacteria by phage corresponded closely to a more reduced bacterial load in the infected mice, which in turn resulted in a better chance of surviving *P. aeruginosa* infection. A different study by Bull et al.\(^{18}\) however, showed that phage growth rate, while positively correlated with the therapeutic efficacy, was not the most important determinant of it. In this study, mice were infected with *E. coli* O18:K1:H7 strain that expresses the K1 capsule on its surface and treated with 2 types of phages: K1-dependent and K1-independent. It was found that K1-dependent phages, as a whole, are much more efficient at rescuing infected mice when compared to the K1-independent phages (by 6 orders of magnitude, as judged by the minimum treatment dose). Even though the K1-dependent phages were found to have moderately higher in vivo\(^{18}\) and in vitro (at least in serum)\(^{19}\) growth rates, the primary factor in determining the efficacy is likely the presence of the endosialidases in the K1-dependent phages. Apparently, phage growth rate alone may not be enough to be used as a proxy for therapeutic efficacy.

In summary, our current study demonstrated that phage in vitro growth rate can be used as a good starting point for evaluating myriad of potential phages for prophylactic or therapeutic purpose. However, examination of additional factors may also be necessary to determine differences in treatment effectiveness.

### Materials and Methods

**Fly, bacteria, and bacteriophages**

The *Drosophila melanogaster* individuals used in the experiments were 4–6 day-old females from a laboratory population established in 2008 with flies caught at the Indian Ladder Farm in Voorheesville, New York. This population is maintained as a large outbred population kept at 25°C with a 12:12 light-dark cycle. Adult females used in infection assays were collected from low larval density vials established by placing 10 males and 10 females in a vial for 24 h.

The *Pseudomonas aeruginosa* strains MPAO1 and PW8621 were obtained from the University of Washington Genome Center.\(^{45}\) MPAO1 is the laboratory wild-type strain PAO1 and PW8621 a pilA transposant mutant of MPAO1, thus lacking the type IV pili. The *P. aeruginosa* clinical isolates PA14\(^{46}\) and MPAO1 were used for phage isolation.

Phages capable of infecting *P. aeruginosa* MPAO1 were isolated from a wastewater treatment plant in Menands, New York and water from the Passaic River in New Jersey. For isolation, 100 μL of the water sample was incubated with 25 mL log-phase MPAO1 in LB (Luria-Bertani) broth at 30°C waterbath shaker for 24 h. A portion of the culture was then centrifuged and the supernatant passed through a 0.2 μm filter. Approximately 100 μL of the filtrate was plated on MPAO1 lawn. A single plaque was randomly picked and used to infect 25 mL MPAO1 culture as described above. After centrifugation and filtration of the culture, the phage lysate was stored at 4°C as stock.

Phages HWPB-1, HWPB-2, and HWNPB-2 were isolated from sewer water sampled from the wastewater treatment plant, whereas HWPB-3 and HWNPB-1 were isolated from the river water sample. All phages used MPAO1 as the enrichment host, except for HWNPB-3, which was isolated from the wastewater sample but enriched with PA14. Except for HWNPB-3, which is able to plaque on both MPAO1 and PA14 lawns, the other 5 phages can only plaque on MPAO1.

PW8621 was used to determine whether type IV pilus is required for infection. Phages unable to form plaques on PW8621 are likely to utilize the type IV pilus for adsorption and are classified as HWPB (Heather Wilson Pilus-Binding) phages. Otherwise, they are classified as HWNPB (Heather Wilson Non-Pilus-Binding) phages.

**Phage purification, DNA isolation and sequencing**

Approximately 10\(^{10}\) phage particles were collected, purified, and DNA isolated as described in Lee and Clark.\(^{47}\) The purified DNA was digested by various restriction enzymes for 16 h, following manufacturers’ instructions and cloned into either pSMRT (Lucigen) or pUC19. Recombinant plasmids were confirmed by PCR, then purified and used as templates for DNA sequencing by the DNA Analysis Facility on Science Hill at Yale University. The BLAST tool\(^{48}\) from the National Center for Biotechnology Information (NCBI) was used to identify homologous sequences. Overall, at least 4 Kb of sequences for each phage were compared to GeneBank.

**Adsorption rate determination**

Methods described in Shao and Wang\(^{24}\) were adapted to determine phage adsorption rate. Final concentrations of ~10\(^{4}\) pfu/mL of phage were mixed with ~1.1 × 10\(^{9}\) cfu/mL log-phase MPAO1 in 5 mL at 30°C. Samples (0.5 mL) were withdrawn every 5 min for 20 min and filtered through a 0.2 μm AcroPrep 96 filter plate (Pall, Ann Arbor MI). The filtrate was plated on MPAO1 lawn to determine the free phage concentration. The adsorption rate was estimated by fitting the data to the model of ln(Pf/P0) = −rt, where P0 and P are the phage concentrations at times 0 and t min, respectively, r the adsorption rate constant, and B is the initial cell concentration, which was determined with a spiral plater (Autoplate 4000, Advanced Instruments Inc.) and Qcount colony counter (Advanced Instruments Inc.). The bacterial cell concentration was assumed to be constant throughout the assay as there was only a negligible increase in bacterial cell count throughout the 20-min assay (data not shown). The mean adsorption rate is based on 3 replicates for each phage.

**One-step growth curves**

Methods described by Wang\(^{26}\) were adapted for use to determine the lysis time and burst size of each phage. In brief, final concentrations of approximately 2.3–3.5 × 10\(^{8}\) pfu/mL of phage were mixed with ~1.10 × 10\(^{9}\) cfu/mL log-phase MPAO1 for 20 min at 25°C, then diluted 10,000-fold in LB broth to
untreated flies following experimental infection with MPAO1. To establish bacterial infection, 50 female flies per replicates. The growth rate of each phage was estimated with 3 independent replicates.

**Phage growth rate**

Phage growth rate was determined using method adapted from Wang. In brief, final concentrations of ~4 × 10^4 pfu/mL phages were mixed with ~1 × 10^8 cfu/mL log-phase MPAO1 in 3 mL LB broth. The mixture was incubated at 37°C and agitated in a tissue culture roller drum (New Brunswick Scientific) at setting 7. Growth rate was calculated as \( w = \ln(P_1/P_0)/3 \), where \( P_0 \) and \( P_1 \) are free phage concentrations at times 0 and 3 h, respectively. The growth rate of each phage was estimated with 3 independent replicates.

**Therapeutic efficacy of phage in treating *P. aeruginosa* infected *D. melanogaster***

The therapeutic efficacy of the phages was determined by comparing the survival of phage-treated *D. melanogaster* to untreated flies following experimental infection with *P. aeruginosa* MPAO1. To establish bacterial infection, 50 female flies per treatment group were injected by piercing the thorax with a 0.1 mm diameter minuten pin (Fine Science Tools) that had been dipped in an LB culture of MPAO1 diluted to OD_{600} = 0.097 ± 0.011, thus resulting in an initial inoculum of ~10^3 cfu/fly. Six hours post bacterial infection, flies were injected using a Nanoject injector (Drummond Scientific) with 50.6 nl of either sterile LB or filtered phage lysate with a concentration of ~10^4 pfu/fly. After phage treatment, flies were checked for survival every 6 h for 72 h. Three replicates of the experiment were performed for each phage.

**Statistical analysis**

All statistical analyses were conducted with R. The outliers package was used for outlier detection in determining the lysis time. The survival package was used for Kaplan-Meier estimation of the mean and median survival times and Cox proportional hazard model for estimating treatment efficacy. Flies surviving past the 72-h end point were right-censored. For each treatment, all flies were pooled together for survival analyses and estimates.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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