Terminal investment induced by a bacteriophage in a rhizosphere bacterium [version 1; referees: 2 approved with reservations]

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Abstract
Despite knowledge about microbial responses to abiotic stress, few studies have investigated stress responses to antagonistic species, such as competitors, predators and pathogens. While it is often assumed that interacting populations of bacteria and phage will coevolve resistance and exploitation strategies, an alternative is that individual bacteria tolerate or evade phage predation through inducible responses to phage presence. Using the microbial model Pseudomonas fluorescens SBW25 and its lytic DNA phage SBW25Φ2, we demonstrate the existence of an inducible response in the form of a transient increase in population growth rate, and found that the response was induced by phage binding. This response was accompanied by a decrease in bacterial cell size, which we propose to be an associated cost. We discuss these results in the context of bacterial ecology and phage-bacteria co-evolution.
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Introduction
Pathogens are ubiquitous in natural communities and the antagonistic interactions they establish with their hosts are recognized as one of the main drivers of evolutionary diversification. Hosts can reduce the impact of pathogens through three non-mutually exclusive processes: (i) avoidance of either infected individuals, habitats where the pathogen is prevalent, or of the pathogen itself, (ii) resistance to the actual infection process or post-infection immune defences, and (iii) tolerance. Research on these responses has generally focused on animal and plant models, but there is growing appreciation that microbes, particularly bacteria, can exhibit similar responses. For instance, bacteria can be selected for heightened levels of genetic resistance towards infection by pathogens. On the other hand, although bacteria are known to display plastic responses to various types of environmental stresses and to competition, it is unknown whether they can do so when faced with natural enemies such as bacteriophages.

Plastic responses are an adaptive phenotypic change following an environmental stimulus, occurring without a concurrent change in the genotype. They may involve behavioural, physiological or phenological changes, and be triggered by direct or indirect contact with the stimulus or through communication with neighbouring organisms. Phenotypic plasticity is considered to be a genetic adaptation to variable environments, but given the diversity of associated mechanisms and behaviours, it is not known to what extent different stimuli translate into different responses.

Individual-level interactions between bacteria and phage may be conducive to induced responses. The first step of bacteriophage infection is the binding of phage proteins to bacterial surface proteins, which then triggers conformational changes to both proteins. Surface proteins used by the bacterium for signal transduction are known to be targets of bacteriophage adsorption and as such could trigger a response when bacteriophage binding is detected. Such a response would allow a bacterium to react to the pathogen and to eventually either evade or reduce the effects of the infection. Lytic phages are prime candidates for organisms against which bacteria may have evolved a stress response, because they typically interact with their host over short timescales, and death is inevitable once the phage has injected its DNA into a sensitive bacterial cell.

In addition, bacteriophages are widely distributed in the environment and interact with their hosts over relatively small spatial scales and throughout most of the year. This could select for the expression of induced structural, physiological or behavioural responses to different enemies. Also, bacteria employ signalling pathways and have a known ability to communicate within populations. Such pathways could induce and synchronise inducible responses before predators and pathogens are encountered, or at least before they have spread through the population, or before the point beyond which cell death is certain. All of these factors suggest that plastic stress responses to phage should be a common feature of bacterial cells and that such responses would have important repercussions for ecological and evolutionary interactions between phage and bacterial populations. Although molecular responses of bacteria to bacteriophages have been characterized, the behavioral, ecological, and selective consequences of such responses are not known.

Here we demonstrate that when confronted with phage, bacteria express transient increases in division rate at a cost to individual biomass accumulation. Specifically, we employ the rhizosphere bacterium Pseudomonas fluorescens SBW25 to investigate how its population growth rate is affected by exposure to inactivated populations of its lytic bacteriophage SBW25Φ. We find that bacteria exposed to inactivated phage increase their fission rate nearly two-fold at 24 hours post-exposure. This is followed by a continual decrease in fission rate relative to the control. We also show that bacteria exposed to inactivated phage were smaller in size compared to controls. All of these effects were enhanced as the density of inactivated phage was increased. The results are consistent with a competitive strategy that increases allocation to reproduction under stressful conditions (i.e., “terminal investment”). Terminal investment is well characterised for other host-parasite associations, but to our knowledge has not previously been observed in bacteria and phage.

Results
Bacteria exposed to UV-inactivated phage display a statistically significant higher growth rate over the first 24 hours post-exposure than non-phage controls (Kruskal-Wallis, df=3, P = 0.006; Figure 1). After this period, the estimated doubling time of exposed bacteria increased (i.e., their populations grew slower), and did so for the next 48 hours. This decrease in growth rate compared to controls is suggestive of a cost to the higher fission rate observed over the first 48 hours. It is also possible that differences seen at earlier time points are due to differences in bacterial fitness or growth rates, as spontaneous mutants are known to occur in both controls and phage-exposed populations. This was measured for four consecutive replicates, and the bars are standard errors.

![Figure 1. Maximum doubling time (in hours) of biomass produced by bacteria exposed to different concentrations of UV-inactivated phage.](image-url)
24 hours (Figure 1). During the fourth day post-exposure, control and treatment bacteria showed no significant differences in doubling time (KW, df=3, P > 0.05). That exposed bacteria returned to their ancestral growth rate suggests that the response over the first 24 hours was due to phenotypic plasticity and not selection on faster growing genotypes. There was a marginally significant effect on population growth for bacteria exposed to different phage concentrations (KW, df=2, P < 0.02), suggesting that the encounter rate between bacteria and phage is important in determining the population-level strength of the fission response.

We hypothesized that faster doubling times would come at a cost to cell size, since cells would have less time to metabolize and convert absorbed nutrients into cell structure. Twenty-four hours post-exposure, we found that phage-treated bacteria were two to three times smaller (as measured by mean cellular width) than the control (KW, df=3, P < 0.0001; Figure 2). This difference in size gradually decreased over the following 3 days, but in contrast to growth rate (Figure 1), bacteria did not attain their ancestral cell size by the end of the experiment (Figure 2). Analyses of the distribution of several flow cytometry profiles showed that a difference in cell shape is unlikely to explain this result (see data associated to this article). Finally, observations using a transmission electron microscope showed that the cells remained rod-shaped for all treatments.

**Discussion**

Our experiments reveal a previously unexplored behavioural response to bacteriophage predation: phage induce bacteria to reproduce earlier in their cell cycle. We hypothesize that this response increases the survival chances of progeny under natural conditions and demonstrate that this behaviour comes at a fitness cost of reduced size of daughter cells. Our experiments with UV-inactivated phage further demonstrate that this response is specifically due to phage binding.

These results support and extend both theoretical\(^1\)\(^2\) and empirical\(^3\)^1\(^4\) predictions that victims may lessen the fitness impact of their natural enemies through early reproduction, to cases where phenotypic responses are plastic and temporary. Increased allocation to reproduction in stressful environments—termed “fecundity compensation” or “terminal investment”\(^5\)\(^6\) – although never studied in bacteria-phage associations to our knowledge – has been extensively studied for other host-parasite (or organism - stressor) interactions. Terminal investment is characterized by increased reproductive rate or the earlier onset of reproduction, if the prospect of future reproduction is low\(^7\). Examples of such responses include faster host maturation\(^8\), increased oviposition rate\(^9\), and the modification of traits involved in the onset of reproduction\(^10\)^\(^11\).

Phenotypically plastic responses are important in that they allow individuals to cope with environmental change during their lifetimes\(^12\). As such, plasticity is expected to be favoured in variable environments when the costs of induction and phenotypic change compensate for probabilistic (expected) fitness loss\(^13\). Although it is difficult to generalize about constitutive costs of resistance across biological systems\(^14\)^\(^15\), limited evidence suggests that genetically evolved, constitutive resistance in bacteria to their lytic phage could have costs of as much as 5–10% to relative fitness\(^16\).

We employed inactivated bacteriophages to evaluate how phage contact with the bacterial outer membrane mediates bacterial responses. Bacteria could be selected to exhibit an escape response in several, non-mutually exclusive ways. First, non-virulent phage may signal the presence of virulent phage in the local environment (i.e., the bacterium does not perish following initial phage contact). Senescent (inactive) phage are present in natural environments\(^17\), and many phages bind to outer membrane proteins without being infective (e.g. the bacterium is resistant;\(^18\)). Moreover, it is possible that phage could detach if they sense the host to be unsuitable\(^19\). Second, when phage infect the bacterium there may be a ‘race’ between the time it takes a bacterial cell to divide (and potentially survive) and the point of no recovery associated with the maturation of phage progeny and bacterial cell lysis. Third, the response...
Supplement Figure 2 demonstrated that this method was sufficient to kill all phage (see wavelength) at 5 cm distance for 4 hours. Extensive pilot studies to UV light (Model 4.LC, Vilber Lourmat, Deutschland, 254 nm) for 8 minutes at 13000 rpm, and inoculating the pellet into fresh KB medium. Phages were then isolated by centrifuging the remaining supernatant (see above) and placing in fresh KB medium. 1% of each population was transferred every 24 hours into new KB medium. Each of the 4 treatments was replicated 6 times and arranged arbitrarily in a rack for incubation.

Methods

Bacteria cultures

Ancestral Pseudomonas fluorescens SBW25\(^\text{\textsuperscript{39}}\) were inoculated into 30 ml microcosms containing 6 mL of King’s B medium (KB), and allowed to grow under alternating rotational agitation (200 rpm for 1 minute every 30 minutes). Every 48 h following plating on solid agar, 10 CFU of the smooth morphotype were transferred into fresh KB medium. After 10 transfers, the culture was composed of smooth morphotypes only. We continued this selection procedure for another 10 transfers and then arbitrarily isolated a single CFU, which was used for all experiments described below. Experiments were conducted at 28°C in KB medium under constant rotational agitation (200 rpm).

Phage cultures

We grew an arbitrarily selected clone of the ancestral phage SBW25\(\text{\textsuperscript{26}}\) on an exponentially growing culture of fixed smooth P. fluorescens SBW25 in 3 mL of KB for 48 hours. This resulted in a culture containing approximately 10\(^4\) phage per ml. The sample was then centrifuged for 3 minutes at 8000 rpm in a 1.5 ml Eppendorf tube, and the pellet discarded. Centrifugation was repeated three times to ensure all bacteria were removed (see Supplement Figure 1). Phages were then isolated by centrifuging the remaining supernatant for 8 minutes at 13000 rpm, and inoculating the pellet into fresh KB medium. The sample was thoroughly vortexed and exposed to UV light (Model 4.LC, Vilber Lourmat, Deutschland, 254 nm wavelength) at 5 cm distance for 4 hours. Extensive pilot studies demonstrated that this method was sufficient to kill all phage (see Supplement Figure 2).

Preliminary tests

We conducted a series of preliminary tests to verify how UV-inactivated phage affected bacterial hosts. First, observations under a transmission electron microscope showed that UV-inactivated phage were still intact and able to bind to their bacterial hosts. Second, we checked that bound UV-inactivated phage did not introduce phage DNA into the bacteria. This was done by inoculating 1 ml of UV-inactivated phage into 6 overnight bacterial cultures. Inactivated phage were allowed 4h to attach to the bacterial outer membrane. We separated phage and bacterial fractions by filtration using a 0.2 µm filter. We then conducted a full DNA extraction (WholeBlood NucleoSpin DNA extraction kit, Macherey-Nagel) of the filter. PCR was done using TPF1f (GATGTGAGAAAGCGATACACGG) and TPF1r (GAGAGAAGCGGGAGAGTGAA) sequences developed for this study, which selectively amplify a 550 bp fragment of the phage DNA and a 1200 bp fragment of the bacterial DNA (see Supplement Figure 1 for detailed protocols). We did not find any evidence that UV-inactivated phage was present in samples putatively containing bacteria only, thus confirming that the DNA of inactivated phage was not incorporated in the bacterial cell.

Experiments using UV-inactivated phage

We conducted an experiment to understand how UV-inactivated phage affected bacterial behaviour. Fixed smooth SBW25 bacteria were first cultivated in 6 ml KB in 30 mL universal glass vials. 20 µL of exponentially growing bacteria (c 10\(^4\) bacterial cells) were transferred into fresh KB medium with either no phage or UV-inactivated phage at ratios of 1:10, 1:2, and 1:1 (corresponding to approximately 10\(^4\), 5x10\(^4\), and 10\(^5\) phage per ml), and then allowed to interact for 4 hours under alternating shaking (200 rpm for 1 minute every 30 minutes). Bacteria were then separated from bound phage by centrifuging (see above) and placed in fresh KB medium. 1% of each population was transferred every 24 hours into new KB medium. Each of the 4 treatments was replicated 6 times and arranged arbitrarily in a rack for incubation.

Measures

Biomass doubling time (used as a proxy for population fitness) was measured in a Fluorostar Optima spectrophotometer (28°C, constant agitation, 250 measures at 650 nm over 24 hours) each day, using the following formula:

\[
D_t = \frac{\Delta \ln(N)}{\ln(N_e) - \ln(N_0)}
\]

where \(N_e\) and \(N_0\) are the total biomasses (measured as optical density, OD) before and after the exponential growth phase, and \(\Delta t\) is the duration of the exponential phase. Exponential phase was determined by conducting a series of windowed linear regressions over the full growth curve, and retaining the part of the curve with the largest slope (computer code given in suppl. materials part 3).

Individual cell size was measured by flow-cytometry using a FACS CantoII (BD BioSciences, San Jose, California, USA), and data (forward scatter) were analysed using the flowCore package\(^\text{\textsuperscript{13}}\).
in R 2.12.0\cite{r}. Each measure was performed on a sample of 2\times10^6 cells without dyes.

We also estimated the sensitivity of the different treatments to live phage by measuring changes in bacterial populations. At each 24-hour transfer, 1% of the bacterial population was placed in 2 mL of fresh KB, and 20 \mu L of amplified phage (ca 10^9 viral particles) were added (a control without phage was conducted simultaneously). Bacteria CFUs were counted on solid agar after 48 hours of incubation to estimate population size.

Due to non-normality of the data as assessed by a Shapiro test, we used a Kruskal-Wallis test to determine the significance of the between-treatments effects.

**Author contributions**

TP, TB and MEH designed the research, TP and EM conducted the microbiology experiments, TP and CGB conducted the molecular biology experiments, TP, TB and MEH analyzed the results and wrote the paper, all authors contributed to revisions.

**Competing interests**

No competing interests were disclosed.

**Grant information**

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### Supplementary material

1. **Molecular biology protocol and results**

   **PCR cycle** – 6 minutes at 95°C, then 30 cycles of 1 minute at 94°C, 1 minute at 55°C, 2 minutes at 72°C, then 10 minutes at 72°C.

   **PCR buffer** – 5 \mu L of buffer, 4 \mu L of primers at 10 pM/mL (for both TPV1f and TPV1r), 2 \mu L of dNTP at 5 pM/mL, 1.5 \mu L of MgCl2 at 25 mM, 5.35 \mu L of H2O, 3 \mu L of sample DNA, 0.15 \mu L of TaqPol - conducted with a GoTaq FlexiDNA Polymerase M8301 kit from Promega.

![Supplementary Figure 1. Sample gel obtained on 9 total DNA extractions (A-C: bacteria and page, D-F: phage only, following extraction as explained in text, G-I: bacteria following exposure to inactivated phage, whose DNA was extracted after removal of inactivated phages). The primers TPV1f and TPV1r yield a 1200 bp amplicon in the bacteria, and a 500 bp amplicon in phages. Our separation method for bacteria and phage was complete, since only DNA of the intended organism was found in any given sample.](image)

2. **Preliminary experiments and phage**

   We verified the efficiency of the phage inactivation protocol by incubating the bacterial strain used for the main experiment with either live phage or phage exposed to UV for 2hrs or 4hrs. We measured the Malthusian fitness of 6 host populations near carrying capacity at low temperature (4°C, growth restrictive) over the course of 24hrs (the difference with the experiment presented in the main text is that inactivated phage were not removed over the course of this pilot study).

![Supplementary Figure 2. Bacterial mortality as a function of phage inactivation. Because bacteria do not grow at 4°C, we can directly measure phage-induced mortality. After 4 hours of exposure to UV, we observed that phages do not introduce significant mortality in the bacterial population. Kruskal-Wallis test (df = 2, p = 0.02) reveals differences between treatments, with 0h and 2h being significantly different (t-test, df = 7, p < 10^-3), 0h/4h being significantly different (t-test, df = 8, p < 10^-5), and 2h and 4h being similar (t-test, p = 0.16) - all p-values were Bonferroni-corrected to account for multiple testing. Similarly, 2h and 4h are not significantly different from 0 (p-values of -5 -5 0.14 and 0.89 respectively, after correction).](image)
3. Determination of the maximal growth rate (R code)
givegrowth = function (y, x = c(1:length(y)), bw = 12) 
## y: optical density
## x: times of the measures
## bw: number of points to include in regression

list.of.coeff <- NULL
for (i in 1:(length(x) - bw)) {
  part.x <- x[i:(i + bw)]
  part.y <- y[i:(i + bw)]
  cur.lm <- lm(part.y ~ part.x)
  list.of.coeff[i] <- return(as.numeric(coeff[2]))
}

References


36. Lafferty KD: The marine snail, Cerithidea californica, matures at smaller sizes

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Current Referee Status:  

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1. Is there a subpopulation of the UV inactivated phage that are destroyed in the process of creating them, such that addition to bacterial cultures might constitute addition of DNA that can be taken up through transformation? If so, is it possible that this transformed DNA is being used as a nutrient by the bacteria? This might explain the slight increase in growth rate of phage-exposed bacteria in the experiments. There is precedent in other bacterial systems, but I do not know whether this provides an alternative explanation in the current study. The authors should take this possibility into account.

2. It is unclear what constitutes the controls performed in this study. One choice of control would be to obtain the UV-inactivated phage, and then remove these particles via centrifugation. The particle-free supernatant would then be added to controls, so that all components (except phage presence) would be otherwise identical across treatments and controls. However, it is unclear whether this was the approach used, and therefore I am worried that the chosen control is insufficient for drawing proper conclusions in the work.

Overall, the work seems very preliminary, and the data presented are not strongly supportive of the conclusions drawn.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.
responses of bacteria to phage in the environment.

This includes possible changes in bacterial growth rate and cell size, as both have been shown to affect the rate of adsorption of phages by host cells (e.g. Hadas et al. 1997). In this paper, Poisot and coauthors investigate the inducible response of bacteria to phages by using UV-treated phages that are capable of binding to, but not infecting their host cells. They find that bacteria encountering UV-treated phages have a faster doubling time and smaller cell size than the control bacterial populations but that this response is short-lived and does not confer resistance to the phage.

This is a very intriguing result that confirms work from studies using live phages (e.g. Gómez, P. and A. Buckling. 2011) and suggests that binding of phages, regardless of subsequent infection success, might play a key role in shaping bacterial population dynamics. The approach taken is a very nice way to look for inducible responses to phage and the results are, for the most part, very clear. I do, however, wonder about the independence of the results for doubling time (measured as optical density) and cell size. Surely the optical density measure is affected by the cell size? The authors state that “Analyses of the distribution of several flow cytometry profiles showed that a difference in cell shape is unlikely to explain this result (see data associated to this article)” Since this is an absolutely central result of the finding, I would find it very helpful if the authors actually presented and discussed this evidence. In fact, it was not clear to me which data were in support of this. Otherwise, I do not think that the results should be used to primarily suggest a phage-induced response of increased growth rate, with a cost of decreased cell size. Instead, perhaps it is a response of decreased cell size with a subsequent small change in doubling time? I imagine the authors have the analyses to rule out the latter possibility. Further to this, when the authors do look directly at colony forming units, rather than optical density, in their analyses of bacterial resistance to live phages they do not find a difference among the treatments. In this case, when bacteria were exposed to live phages, bacterial populations that had been exposed to inactivated phages grew to the same densities over 24 hours as those that had not been exposed to inactivated phages. I find this hard to interpret as it could suggest that a) the previous results were primarily indicative of a change in cell size, rather than growth rate, or even that b) the bacteria from the inactivated phage treatments do have a higher growth rate but were more susceptible to phages and thus had the same CFU. It would be helpful if the authors could discuss this result in more detail.

I have a few additional points of clarification that I think would help readers fully understand the results.

First, I wonder whether the authors could clarify their thoughts on the mechanism underlying the change to smaller cell size and/or increased doubling time of bacteria encountering inactivated phages. For example, could it be that small cell size is a response to altered numbers or activity of receptors on the bacterial cell surface? It seems that phage binding to receptors could alter their function and thus those bacterial cells with bound inactivated phages could be smaller due to decreased uptake of resources.

Second, I think the finding that bacteria treated with inactivated phages show changes in growth rate and/or cell size but do not differ in terms of their resistance to live phages is quite interesting! I would be keen to know what the infection rates of the control and treated populations were, as this would help with interpretation of the result. It seems surprising that there is no change in resistance, as previous evidence suggests a strong correlation between cell size and growth rate with adsorption rate. Might this result give insight to the mechanism underlying the changes observed? The authors mention that smaller surface area would mean a lower encounter rate with phages, but this doesn’t seem to be the case when the cells are exposed to live phages.

As a very minor point, I wonder whether the authors meant to say that bacteria were separated from
unbound, rather than bound, phages in their methods section, as it is unclear how centrifugation would separate bacteria from bound phages.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

**Competing Interests:** No competing interests were disclosed.