Bacteriophage 5:2, e1050153; April/May/June 2015; © 2015 Taylor & Francis Group, LLC

Experimental evolution and bacterial resistance: (co)evolutionary costs and trade-offs as opportunities in phage therapy research

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Antagonistic coevolution between bacteria and phages (reciprocal selection for resistance and infectivity) has been demonstrated in a wide range of natural ecosystems, as well as experimental populations of microbes, yet exploiting knowledge of coevolution for the prophylactic and therapeutic use of phages is under-explored. In this addendum to our recent paper we discuss how real-time coevolution studies using experimental populations of bacteria and phages can provide novel insight into the changes in bacterial phenotypes that result from resistance evolution against coevolving phages, and how this may ultimately improve our understanding of phage therapy and ability to design effective treatments.

Keywords: Antagonistic coevolution, bacterial resistance, experimental evolution, *Pseudomonas fluorescens*, pleiotropic costs, phage therapy, trade-offs

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Submitted: 03/24/2015

Revised: 05/06/2015

Accepted: 05/07/2015

http://dx.doi.org/10.1080/21597081.2015.1050153

Addendum to: Scanlan PD, Hall AR, Blackshields G, Friman VP, Davis MR, Jr., Goldberg JB, Buckling A. Coevolution with bacteriophages drives genome-wide host evolution and constrains the acquisition of abiotic-beneficial mutations. Mol Biol Evol 2015.

The rise of antibiotic resistance in clinically relevant populations of bacteria has led to resurgent interest in the use of bacteriophages (phages) to control and prevent bacterial infections.[1] However, as with antibiotics, many studies of both natural and experimental populations have shown that bacteria can rapidly evolve resistance to phages, which clearly poses a prospective problem to the widespread use of phage therapy.[2] Although the most obvious consequence of resistance to phages is the potential failure to control infection, the evolution of resistance to phages can also entail a number of correlated responses, including changes in other phenotypic traits that are relevant to both the outcomes of infection by bacterial pathogens and prospects for managing them by phage therapy.

One possible solution to help predict and manage the potentially adverse consequences of resistance evolution is to apply an evolutionary ecology framework to phage therapy research.[2,3] This type of research framework can directly aid development of treatment strategies, such as phage cocktails, that minimise the likelihood of resistance evolution.[3-5] However, experimental evolution also allows for investigation of the wider effects of resistance evolution, such as costs in terms of growth rate or competitive ability for the bacterial host, and any other phenotypic effects relevant to pathogenesis or treatment,[6] and for these effects to be studied in different bacteria-phage combinations and experimental environments. This may be important for assessing the biotic and abiotic factors that determine the success or failure of phage therapy treatments and, given the abundance of phages in nature, the evolution of bacterial pathogens in general.

One aspect of phage therapy research that has received relatively little attention relates to the capacity for bacteria and phages to undergo antagonistic coevolution.[2] Antagonistic coevolution entails reciprocal selection for host resistance and parasite infectivity, such that both host and parasite phenotypes change over evolutionary time. Coevolution between bacteria and phages has been demonstrated in a wide range of natural ecosystems including soil, arboreal and marine environments,[7-9] as well as experimental populations of microbes.[10-12] Nonetheless, the consequences of coevolution for phage therapy, and potential to exploit coevolution to improve treatment, remain

largely unexplored. In this addendum to our recent paper,[13] we address how knowledge gained from experimental coevolution studies can potentially facilitate phage therapy research.

Several different bacteria-phage combinations have been used to study antagonistic coevolution in real-time, including pathogenic bacteria and their phages, such as *Escherchia coli* O157:H7 with phage PP01[11] and *Pseudomonas aeruginosa* with various lytic phages.[12] The *Pseudomonas fluorescens*-phi2 model system is among the most widely used experimental systems for studying long-term bacteria-virus coevolution.[14] This coevolutionary interaction has been studied both in nature (soil environments) and *in vitro*,[7,15] allowing the same process to be observed in controlled, simplified conditions and in the natural habitat of these species. Most research with this system has focused on theoretical and fundamental aspects of host-parasite coevolution,[14,16] but much of the knowledge gained has additional applied aspects which are also relevant to clinical microbiology and phage therapy research. For example, coevolution between *P. fluorescens* and phi2 can select for bacterial phenotypes that are also observed in clinically relevant populations of bacteria. This includes bacteria with elevated mutations rates, altered LPS profiles and mucoid phenotypes.[13,17]

One important mechanism by which the evolution of bacterial resistance may alter the outcomes of infection or treatment is through indirect effects of phage-resistance mutations on bacterial traits that contribute to virulence. Decreased virulence associated with phage-resistance evolution has been shown for a number of species including human pathogens such as *Escherichia coli,*[6,18] *Serratia marcescens*[19] and *Staphylococcus aureus,*[20] and also the fish pathogen *Flavobacterium columnare.*[21] Such effects can arise if phage resistance mutations pleiotropically alter the expression of bacterial virulence factors, or simply reduce bacterial growth rate or competitive ability.[22] Potentially, phage therapy strategies that account for these effects can attain better treatment outcomes.[23] For example, if phage combinations can be identified where the corresponding resistance mechanisms incur large fitness costs across all potential target bacteria, then even if resistance evolves it may be less likely to spread, and may result in less virulent infections when it does. The potential for rapid adaptation of phages to their hosts may also be exploited by pre-adapting phages to bacteria *in vitro*, with recent studies showing that preadaptation to a specific host can both increase infectivity[5] and reduce the likelihood of subsequent resistance evolution.[24]

What are the additional implications of coevolution (evolution of resistance *and* infectivity) over and above the evolution of resistance *or* infectivity, during a clinical bacterial infection? First, coevolution could lower the mean density of the infecting bacterial population if phages adapt to overcome evolved host resistance, although the relevance of this for clinical infections is unclear at present. Second, some costs associated with bacterial resistance may be specific to coevolution. As mentioned above, pleiotropic costs associated with resistance mutations in terms of growth rate and competitive ability have been known for some time.[25] However, one advantage of investigating such effects during long-term coevolution, as opposed to focusing on the immediate effects of resistance alleles on bacterial phenotypes, is the potential to identify effects that only emerge over extended time-scales or during reciprocal adaptation and counter-adaptation of both host and parasite. For example, in the *P. fluorescens*-phi2 system the costs associated with host resistance are greatest for bacteria that have sequentially acquired resistance to a wide range of phage phenotypes, as occurs during long-term coevolution but not necessarily during a single round of resistance evolution.[16] We recently observed a novel type of cost associated with resistance evolution in this system that we detected in an experiment lasting hundreds of generations: coevolution can constrain bacterial adaptation to other components of the environment, probably resulting from negative epistasis between phage-resistance mutations and mutations associated with growth in the abiotic environment.[13]

This finding emerged from a comparative genetic and phenotypic analysis of *P. fluorescens* SBW25 that had been grown for up to 400 bacterial generations in two treatment groups: with phages ('coevolution') and without phages ('evolution').[13,16] In each treatment we grew 6 replicate populations in microcosms containing simple nutrient media (Kings B media). Each population was transferred every second day and sampled at various time-points over the course of the experiment. At the end of the experiment, coevolved host populations had lower competitive fitness in the absence of phages compared with evolved populations. In other words, parasitised populations failed to adapt to the abiotic environment, and whole-genome sequencing showed that bacteria evolved with and without phages acquired different specific sets of mutations. Additional experiments showed that mutations acquired during adaptation to the abiotic environment were no longer beneficial after a subsequent period of coevolution with phages, implying strong negative epistasis between parasite resistance and growth-promoting mutations.

Coevolution with phages also altered two other bacterial traits that, in other species, are relevant to the outcomes of infection and treatment. First, coevolving bacteria acquired higher genomic mutation rates and fixed a greater number of mutations. This resulted in greater among-population divergence compared to populations evolved in the absence of phages. Crucially, elevated mutation rates have been observed in clinical isolates of key pathogenic species,[26] are associated with increased levels of antibiotic resistance,[27] and potentially alter bacterial capacity for adaptation to the within-host environment.[28] Although this suggests that coevolution with phages may contribute to variation of bacterial mutation rates in some scenarios, experiments with *P. fluorescens* and phi2 in soil microcosms show that phage-driven selection for lineages with mutator alleles is highly sensitive to ecological variation.[29] Second, coevolved bacteria also displayed altered LPS profiles, consistent with other data showing that phage predation selects for changes to this clinically relevant trait.[30]

As outlined above, a range of different costs of coevolution with phages have been previously demonstrated, mostly

resulting from the immediate effects of resistance mechanisms on the bacterial phenotype. Our experiment suggests that bacteria may also pay costs that are only manifest over evolutionary timescales: ongoing coevolution with phages constrained their ability to adapt to the abiotic environment. This finding has clear implications for phage therapy: over long time scales, such as those associated with chronic infections (as opposed to acute infections that occur over much shorter time scales), coevolution with phages might constrain bacterial adaptation to the host environment (for example human, animal or plant host).

A key challenge for understanding the relevance of such effects will be improving our general understanding of how adaptation to the host environment contributes to the likelihood of establishing a chronic infection and response to treatment. This is likely to vary among different types of infection. Nevertheless, in chronic infections of the respiratory system such as cystic fibrosis and chronic obstructive pulmonary disease, bacteria including *Pseudomonas aeruginosa, Burkholderia cepacia and Staphylococcus aureus* are known to evolve numerous adaptations to the host environment that facilitate the establishment of chronic infections under adverse conditions (hypoxia, host immune response and osmolarity).[31] In this context phage therapy could potentially impede pathogen adaptation to the host environment, although whether the effects observed in our system apply to other bacteria-phage combinations will also depend strongly on the genetic architecture of resistance and adaptation.

In conclusion, long-term experimental coevolution allows identification of effects that may alter the outcomes of infection and phage therapy. This includes pleiotropic effects of resistance alleles on traits such as growth rate or virulence, and effects that are only observed over (co)evolutionary time-scales such as changes in bacterial mutation rate or adaptation to the abiotic environment. Whether our recent findings in experimental microcosms[13] translate to natural and clinically relevant scenarios remains to be seen, but the current development of appropriate model systems that use suitable phages and clinically and agriculturally relevant bacteria[12,22,23] is expanding the possibilities for exploiting knowledge of resistance evolution and coevolutionary costs to populations that are directly relevant to phage therapy. This may ultimately help to explain variation in effectiveness among phage therapy treatments and to design new strategies that minimise the likelihood of resistance evolution and/or account for any 'side effects' of resistance evolution such as changes in bacterial growth rate, adaptation, mutation rate, LPS profile or virulence.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

We thank the editor and anonymous reviewers.

Funding

PDS is funded by a Marie Curie Intra-European Research Fellowship. AH is funded by an Ambizione grant from the Swiss National Science Foundation. AB is funded by the Royal Society, NERC, BBSRC, Leverhulme Trust and AXA research fund.

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