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Discussion

Experimental coevolution with bacteria and phage The *Pseudomonas fluorescens*—Φ2 model system

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Abstract

Parasites are ubiquitous in biological systems and antagonistic coevolution between hosts and parasites is thought be a major ecological and evolutionary force. Recent experiments using laboratory populations of bacteria and their parasitic viruses, phage, have provided the first direct empirical evidence of antagonistic coevolution in action. In this article we describe this model system and synthesise recent findings that address the causes and consequences of antagonistic coevolution.

Keywords: Experimental evolution; Host; Parasite; Resistance; Infectivity; Arms race; Microbes; Local adaptation; Diversity; Metapopulation; Migration

1. Introduction

Antagonistic host–parasite coevolution, the reciprocal evolution of host defence and parasite counter-defence, is a pervasive evolutionary force. It is implicated in a wide range of biological processes including: host–parasite population dynamics (Thompson, 1998), the evolution of parasite virulence (Bull, 1994), the evolutionary maintenance of sex (Barton and Charlesworth, 1998; Hamilton et al., 1990; West et al., 1999) and genetic divergence between populations (Brockhurst et al., 2005, 2004; Buckling and Rainey, 2002b; Thompson, 1999). However, in spite of its widespread theoretical importance, direct empirical evidence of coevolution in action is scarce, although indirect evidence consistent with coevolution is abundant in a range of natural host–parasite systems (Dybdahl and Lively, 1998; Little and Ebert, 1999; Niemi et al., 2006; Thrall et al., 2001). The main reasons for this lack of direct evidence are to do with the difficulties associated with conducting evolutionary experiments in natural populations.

In recent years, laboratory populations of microbes have emerged as powerful model systems for testing evolutionary theory (Elena and Lenski, 2003). The advantages of using laboratory populations of microbes are several-fold (Lenski and Levin, 1985). Isogenic populations can be propagated in carefully controlled replicate environments; hence, changes can be directly ascribed to mutation and selection, and not to environmental or standing genetic variation. In addition, short generation times (~7 bacterial generations per day) and large population sizes (10^9 bacterial cells per millilitre) favour rapid evolutionary change and allow large-scale replication. Finally, the ease of long-term storage allows ancestral genotypes and evolutionary intermediates to be retained in suspended animation allowing evolutionary change to be assayed through time as well as space (Lenski et al., 1991).

Bacteria and their specific obligate killing viral parasites, lytic phage, provide an ideal testing ground for coevolutionary theory (Bohannan and Lenski, 2000; Buckling and Rainey, 2002a). Lytic phage typically replicate by binding to specific bacterial cell surface structures, then injecting their genetic material into the bacterial cell and utilizing the cellular machinery to produce multiple phage progeny, which are released via lysis of the bacterial cell. Bacteria have been shown to readily evolve resistance to phage via mutational changes of the cell surface structures to which the phage bind (Bohannan and Lenski, 2000; Lenski, 1988). However, coevolution in most bacteria-phage systems appears to be limited to one or two cycles of reciprocal evolution of resistance and infectivity [e.g., *Escherichia coli* and various T-phage (Bohannan and Lenski, 2000); *Pseudomonas syringae* and Φ6 (Lythgoe and Chao,

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2003); *Pseudomonas aeruginosa* and PP7 (Brockhurst et al., 2005, 2006a)]. Crucially, however, more persistent cycles of antagonistic coevolution have been observed in several bacteria-phage systems (Buckling and Rainey, 2002a; Mizoguchi et al., 2003), the best studied being the interaction between *Pseudomonas fluorescens* SBW25 and the lytic phage Φ2 (Brockhurst et al., 2003; Buckling and Rainey, 2002a; Buckling et al., 2006), which is the focus of this paper. This has allowed, for the first time, experiments that test coevolutionary theory to be conducted over coevolutionary timescales. The aim of this review is to briefly outline the *P. fluorescens*-phage experimental system and synthesise published results using this system that address the causes and consequences of antagonistic coevolution.

2. Methodology

2.1. Selection experiments

Populations of *P. fluorescens* and phage Φ2 are typically propagated in heterogeneous microcosms (Buckling and Rainey, 2002a; Rainey and Travisano, 1998) (statically incubated glass bottles containing growth media) by batch culture, where a fixed proportion of each population is inoculated into a fresh microcosm at regular intervals, termed transfers. Each transfer allows for approximately seven bacterial generations. Simple manipulations of these culture conditions allow for the testing of the effects of a wide variety of environmental and genetic variables upon coevolutionary dynamics (see Table 1).

2.2. Measurement of coevolution

Bacterial resistance and phage infectivity is measured using a simple assay whereby bacterial colonies are streaked across a line of phage that has been previously applied to an agar plate. Bacteria are scored as resistant if no inhibition of growth is observed after crossing the line of phage. To determine if antagonistic coevolution has occurred, we measure how the infectivity of phage populations and resistance of bacterial populations change through time. Specifically, to determine the rate and magnitude of phage infectivity evolution, at certain transfers we determine the resistance (proportion resistant colonies) of bacterial populations to past (two transfers previous), contemporary and future (two transfers subsequent) sympatric phage populations. Selection on phage infectivity is assumed to show a time-lag (Nee, 1989; Dybdahl and Lively, 1998), such that contemporary phage would be less able, than phage from the immediate future, to infect contemporary bacteria; and phage from the immediate past would be less able to infect contemporary bacteria than contemporary phage. Phage infectivity evolution can then be detected from a negative slope of bacterial resistance to phage through time (past, contemporary and future). The rate of phage infectivity evolution can be calculated from the magnitude of this slope (Fig. 1a; see also, Brockhurst et al., 2003). Similarly, we can determine the rate of bacterial resistance evolution from the

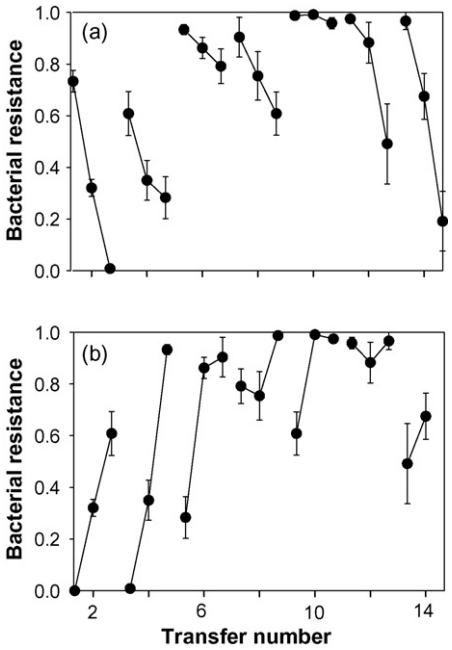

Fig. 1. Rates of phage infectivity (a) and bacterial resistance (b) evolution in coevolving populations of *P. fluorescens* and phage. (a) Lines represent bacterial resistance to past, contemporary and future sympatric phage populations. (b) Lines represent resistance of past, contemporary and future bacterial populations to a given sympatric phage population. The slope of each line provides a measure of the rate of evolutionary change over a four-transfer period. Adapted from Brockhurst et al. (2003).

Table 1
Experimental manipulations, their effects and the broader relevance of such treatments in published articles utilizing the *P. fluorescens*—phage system

Manipulation	Effect	Relevance	Ref.
Periodic shaking of microcosms	Increased within-population dispersal	Coevolution dynamics	Brockhurst et al. (2003)
Constant shaking of microcosms	Destroyed environmental spatial heterogeneity	Evolution of diversity	Brockhurst et al. (2004)
Alter time period between transfer to fresh microcosm	Varied the disturbance regime	Evolution of diversity	Morgan and Buckling (2004)
Mixing proportions of culture from different microcosms prior to transfer	Increased between-population migration	Coevolution dynamics; local adaptation	Brockhurst et al. (in press), Morgan et al. (2007, 2005)
UV mutagenesis of ancestral bacterial clones	Increased mutational load in founding host genotypes	Coevolution dynamics; evolution of sex	Buckling et al. (2006)
Holding bacteria or phage populations constant for varying numbers of transfers	Altered effective relative generation time of host or parasite	Coevolution dynamics; local adaptation	Morgan and Buckling (2006)

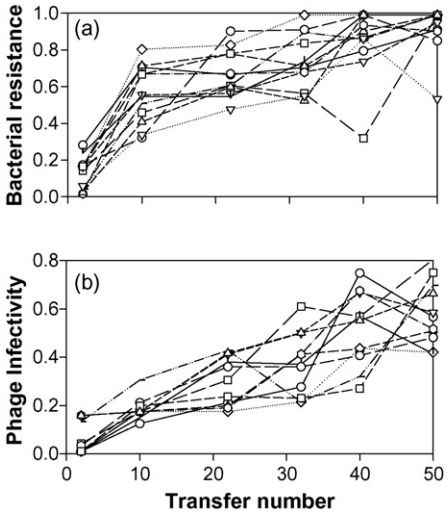

Fig. 2. (a) The relationship between average bacterial resistance to phage isolated from multiple time-points and time spent coevolving within each replicate. (b) The relationship between average phage infectivity to bacteria isolated from multiple time-points and time spent coevolving within each replicate. Adapted from Buckling and Rainey (2002a).

positive slope of resistance to phage populations of past, contemporary and future sympatric bacterial populations (Fig. 1b). Taken together these data provide evidence for persistent reciprocal coevolutionary change.

3. Key results

3.1. Basic coevolutionary dynamics

Long-term selection experiments have shown that dynamic antagonistic coevolution between *P. fluorescens* and phage Φ2 is persistent over evolutionary timescales [50 transfers or approximately 400 bacterial generations (Buckling and Rainey, 2002a)], leading to multiple rounds of reciprocal selection for resistance and infectivity, respectively (Fig. 1a and b). During this time, selection is predominantly directional, favouring the evolution of generalists with wider resistance and infectivity ranges through time, in bacteria and phage populations, respectively (Fig. 2a and b; see also Buckling and Rainey, 2002a). While coevolutionary selection is predominantly directional, analysis of interactions between individual bacterial and phage clones across replicate populations reveals that it is broadly divergent between populations, leading to bacteria being better able to resist sympatric phage (i.e., from same population) compared to allopatric phage (i.e., from different populations) (Buckling and Rainey, 2002a). The observed coevolutionary dynamics are broadly consistent with an asymmetrical multilocus gene-for-gene host–parasite interaction that allows for the evolution of generalists (Buckling and Rainey, 2002a; Morgan et al., 2005; Sasaki, 2000). In accordance with this, there are fitness costs associated with resistance to phage infection in that resistant bacterial genotypes display reduced competitive ability relative to susceptible bacteria in the absence of phage (Brockhurst et al., 2004). Furthermore, the cost of resistance increases with time spent coevolving and hence resistance range (Buckling et al., 2006). Whether there are costs associated with phage infectivity range expansion is the subject of current investigation.

3.2. Factors affecting coevolutionary dynamics

Trade-offs associated with resistance and infectivity are expected to impose an eventual limit to the coevolutionary escalation because subsequent reciprocal adaptations are too costly (Sasaki, 2000). As such, the genetic backgrounds of antagonists are expected to impact upon coeovlutionary dynamics. Indeed, bacteria with high mutational loads and consequently reduced fitness, induced via UV mutagenesis, are less able to coevolve with phage, displaying lower levels of evolved resistance and lower rates of coevolution than unmutagenised bacteria (Buckling et al., 2006). This was likely to be caused by synergisitic epistatic interactions between deleterious mutations and costly resistance mutations. Indeed, bacterial fitness decreased far more rapidly in mutagenised compared to unmutagenised bacterial populations over coevolutionary time, suggesting that when the relative cost of resistance evolution is increased, the rate and extent of coevolution is reduced (Buckling et al., 2006).

Population structure is also thought to have profound consequences for coevolutionary dynamics (Thompson, 2005). Populations of *P. fluorescens* and phage Φ2 are inherently structured because they are cultured in non-shaken tubes (Rainey and Travisano, 1998). Periodic population mixing events (vigorous shaking of the tubes for 1 min in every 30 min) increase within-population dispersal, and lead to higher phage transmission rates (Brockhurst et al., 2003). This increases the likelihood of encountering an infectious phage genotype, increasing selection for bacterial resistance, that in turn increases selection for phage infectivity; a process that leads to the doubling of the rate of coevolution in high compared to low mixing populations (Brockhurst et al., 2003). An additional effect of population mixing is the evolution of more broadly resistant bacteria and more broadly infective phage in high mixing populations such that dispersal appears to increase selection for generalist genotypes. This is presumably as a result of the greater coevolutionary rate and the increased chance of encountering divergent phage and bacteria, respectively, from elsewhere in the microcosm (Brockhurst et al., 2003).

At a larger spatial scale, divergence between microcosms in directly selected traits [resistance and infectivity (Buckling and Rainey, 2002a; Morgan et al., 2005)], suggests that different populations follow different coevolutionary trajectories. Whether parasite or host is ahead in the arms-race will depend on which partner has the greater evolutionary potential (Gandon and Michalakis, 2002); in the *P. fluorescens*-phage system, bacteria typically have the upper hand (Morgan et al., 2005). This asymmetry is probably because of the much greater population and genome sizes of bacteria compared to phage in this and other systems (Lenski and Levin, 1985; Morgan et al., 2005). Migration between microcosms is likely to alter the

evolutionary potential of antagonists by providing genetic variation. In support of this, simultaneous migration of bacteria and phage between populations confers greater evolutionary benefit to phage than bacteria, because phage display lower evolutionary potential in the absence of migration (Morgan et al., 2007). Similarly, immigration of phage-susceptible bacteria into coevolving populations increases phage evolutionary potential through increased transmission opportunities, increasing phage infectivity evolution and thereby accelerating coevolution (Brockhurst et al., in press).

3.3. Local adaptation

Divergent coevolutionary trajectories of populations can lead to parasite (or host) local adaptation: the higher performance of local versus foreign parasites on local hosts (Kawecki and Ebert, 2004). On average, the species with the most genetic variance in the fitness related traits important for the coevolutionary interaction (resistance and infectivity) is most likely to be locally adapted. Migration can increase within-population genetic variation and hence the rate of adaptation, thus relative rates of migration are thought to be a crucial determinant of which partner is ahead in the coevolutionary arms race; all other variables being equal, the species that migrates the most is therefore likely to have an evolutionary advantage (Gandon, 2002; Gandon et al., 1996; Gandon and Michalakis, 2002; Lively, 1999; Morgan et al., 2005). However, if migration rates are too high, genetic variation may be purged through homogenisation of populations, potentially retarding the rate of adaptation (Lenormand, 2002). However, when bacteria migrate more than phage, this has no effect on patterns of local adaptation: bacteria remain locally adapted and phage locally maladapted. This is consistent with the idea that bacteria already have the evolutionary advantage, and increasing within-popualtion genetic variation further provides no additional benefit. However, when phage migrate more than bacteria, phage gain an evolutionary advantage and are on average locally adapted while bacteria are locally maladapted (Morgan et al., 2005).

Local adaptation can theoretically also be affected by the relative generation time of host and parasites (Gandon and Michalakis, 2002). On the one hand, shorter relative generation times should increase the probability of that species being locally adapted, because this allows more rapid adaptation to genetic changes in the other species. On the other, rapid response to selection can purge genetic diversity, reducing the likelihood of local adaptation. Relative generation times can be indirectly manipulated regularly removing one of the coevolving partners and replacing it with a population from an earlier time point. Thus, one partner undergoes more generations than the other. At early stages of coevolution, bacteria and phage that undergo relatively more generations have an evolutionary advantage, but local adaptation is not affected (Morgan and Buckling, 2006). This is because populations were not sufficiently diverged to allow local adaptation to occur. At later stages, manipulations of relative generation time have no effect on either average levels of resistance or infectivity, or altered the level of local adaptation relative to the control populations (in which bacteria were locally adapted), probably because traits other than resistance and infectivity were under strong selection (Morgan and Buckling, 2006). Taken together, these data suggest that the relative generation times of hosts and parasites may not be an important determinant of local adaptation in this system.

3.4. Consequences for host diversity

When propagated in spatially heterogeneous environments in the absence of phage, isogenic *P. fluorescens* populations rapidly diversify, generating numerous niche specialist types that are readily distinguished by their (heritable) colony morphologies on agar plates (Rainey and Travisano, 1998). These can be grouped into three distinct classes based on colony morphology and niche occupation. Smooth (SM) morphotypes, resembling the ancestral morphotype, inhabit the liquid phase; wrinkly spreader (WS) morphotypes form a biofilm at the air-broth interface; fuzzy-spreader (FS) morphotypes colonise the harsher, less aerobic bottom of the vials. Competition for resources is responsible for the origin and maintenance of diversity, as demonstrated by the operation of negative frequency dependent selection; a fitness advantage when rare because there is less intense competition within their niche (Ayala and Campbell, 1974). In contrast, little diversification occurs in spatially homogenous environments (shaken microcosms) (Rainey and Travisano, 1998).

In the presence of phage, diversification dynamics are markedly different. In heterogeneous environments, where diversity is otherwise high, phage reduce diversity. This arises because phage cause a reduction in bacterial density, weakening negative frequency dependent selection (Brockhurst et al., 2004; Buckling and Rainey, 2002b). By contrast, in homogeneous environments, where diversity is otherwise low, phage increase diversity as a result of fitness trade-offs between bacterial resistance and competitive ability (Brockhurst et al., 2004), allowing coexistence of phage-sensitive and phage-resistant genotypes. A similar interaction between the abiotic environment and phage was reported when the impact of disturbance (non-specific mass mortality events) frequency and phage on diversity was investigated (Morgan and Buckling, 2004). In the absence of phage, diversity of spatial niche specialists peaked at intermediate disturbance frequency: the fastest growing and most competitive niche specialists tended to dominate at high and low disturbance frequencies, respectively (Buckling et al., 2000; Morgan and Buckling, 2004). By contrast, there was no relationship between diversity and disturbance frequency in the presence of phages, probably for the same reasons as above: phage reduced diversity when diversity was otherwise high, because of reductions in bacterial density, but increased diversity when it was otherwise low, because of coexistence of sensitive and resistant morphs (Brockhurst et al., 2004; Buckling and Rainey, 2002b; Morgan and Buckling, 2004).

Under both homogeneous and heterogeneous environmental conditions, phage cause an increase in between-population diversity because different populations are dominated by different morphotypes (Brockhurst et al., 2004; Buckling and

Rainey, 2002b). Thus, in addition to causing the divergence of directly selected traits between populations [resistance and infectivity (Buckling and Rainey, 2002a)], coevolution also causes the divergence of other ecologically important traits (Brockhurst et al., 2004; Buckling and Rainey, 2002b).

4. Broader relevance

The coevolutionary interaction between *P. fluorescens* and phage Φ2 exhibits much of the complex dynamics anticipated from field and theoretical studies, and as such is becoming a key model system for the study of antagonistic host–parasite coevolution. The theoretical ideas investigated empirically with this system are of broad relevance to our understanding of host parasite interactions in general, yet have proved difficult to test in natural populations. However, the generality of the coevolutionary patterns observed in this system may be somewhat limited to host–parasite systems that undergo a degree of directional selection. Such systems include certain plant–pathogen interactions (Burdon and Thrall, 1999; Thrall and Burdon, 2003) that broadly comply with a mutilocus gene-for-gene model of coevolutionary interaction, which allows for the evolution of generalist host and parasite types (Damgaard, 1999; Sasaki, 2000; Thrall and Burdon, 2002). By contrast, in many host–parasite systems, infection relies upon highly specific matching of host and parasite genotypes and selection is predominantly fluctuating [i.e., matching alleles interactions (Agrawal and Lively, 2002, 2003)]. Furthermore, lytic phage are obligate killing and therefore do not form chronic associations with their hosts, as do most classic parasites. This therefore limits the types of questions which can be asked with bacteria-phage systems, it is, for example, difficult to define ''virulence'' for obligate killing phage (although see, Messenger et al., 1999). The pit-falls of extrapolation from microbial systems to all living things are clear, and discussed elsewhere (for example, Levin and Bergstrom, 2000), yet microbial systems provide an essential stepping-stone between theory and the natural world.

Understanding of coevolutionary interactions between bacteria and phage are clearly of relevance to the evolutionary ecology of bacteria. Indeed, phage are believed to play a crucial role in the maintenance of bacterial diversity in the environment (Brockhurst et al., 2006b; Weinbauer and Rassoulzadegan, 2004), where diverse bacterial consortia perform a wide range of ecosystem services (Arrigo, 2005; Bell et al., 2005; Fitter et al., 2005). Furthermore, bacterial pathogens are highly likely to encounter lytic phages, yet the evolutionary impact has been little explored [but see recent studies of the pathogens *P. aeruginosa* and *E. coli* O157:H7 and associated phage (Brockhurst et al., 2005; Mizoguchi et al., 2003)]. Due to the inexorable rise of antibiotic resistance, interest in using viral parasites of bacteria (phage) to treat infections has increased and phage therapy is beginning to be seen as a viable alternative by the medical profession (Levin and Bull, 2004; Summers, 2001). The potential advantage of using phage to treat bacterial infection is that they self-replicate within the bacterial cells they are killing. Furthermore, our work suggests that phage may also be able to coevolve in vivo, allowing them to ''keep up'' with bacterial resistance evolution. Therefore, unlike antibiotics, further doses of phage may not be required to maintain an effective antibacterial concentration in the patient. As noted in Section 1, studies of other bacteria-phage interactions have not reported extensive coevolutionary interactions, raising the possibility that our findings are somewhat the exception to the rule. This, however, seems unlikely, as too few interactions have been studied in detail. Furthermore, most other studies have used lab-adapted strains of bacteria and phage, which may display reduced evolutionary potential (Buckling et al., 2003; Colegrave and Buckling, 2005). Indeed, both systems in which extensive coevolution has been observed utilised environmentally isolated bacteria suggesting that such coevolutionary interactions between bacteria and phage may well be widespread in the environment (Buckling and Rainey, 2002a; Mizoguchi et al., 2003).

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