

Antagonistic coevolution between a bacterium and a bacteriophage

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Antagonistic coevolution between hosts and parasites is believed to play a pivotal role in host and parasite population dynamics, the evolutionary maintenance of sex and the evolution of parasite virulence. Furthermore, antagonistic coevolution is believed to be responsible for rapid differentiation of both hosts and parasites between geographically structured populations. Yet empirical evidence for host–parasite antagonistic coevolution, and its impact on between-population genetic divergence, is limited. Here we demonstrate a long-term arms race between the infectivity of a viral parasite (bacteriophage; phage) and the resistance of its bacterial host. Coevolution was largely driven by directional selection, with hosts becoming resistant to a wider range of parasite genotypes and parasites infective to a wider range of host genotypes. Coevolution followed divergent trajectories between replicate communities despite establishment with isogenic bacteria and phage, and resulted in bacteria adapted to their own, compared with other, phage populations.

Keywords: experimental evolution; arms race; microbes; local adaptation; directional selection

1. INTRODUCTION

Pathogenic parasites impose selection for resistant hosts, which in turn impose selection for infective parasites. This may result in rapid antagonistic coevolution: the reciprocal evolution of host resistance and parasite infectivity (Thompson 1994). Antagonistic coevolution is believed to play a critical role in host and parasite population dynamics (Thompson 1998), the evolution of parasite virulence (Bull 1994; Ebert & Hamilton 1996) and, under some conditions, explain the benefit of producing genetically variable offspring through sexual reproduction (Hamilton *et al.* 1990; West *et al.* 1999). Antagonistic coevolution is also believed to cause rapid between-population differentiation of both parasites and hosts, which may be critical to the maintenance of genetic variation, speciation events, and, in combination with migration, drive the coevolutionary process (Thompson 1999).

Antagonistic coevolution, and resultant between-population genetic differentiation, is thought to operate in numerous host–parasite systems, yet direct evidence of its operation is limited. Much work has focused on the interactions between agricultural plants and associated insect and fungal pathogens, where pathogens frequently evolve to overcome plant resistance (Thompson & Burdon 1992). However, in this context, host resistance ‘emerges’ only through the human introduction of novel, resistant cultivars. A number of studies of natural populations have reported genetic and phenotypic divergence between spatially distinct populations of co-occurring host and parasite species, often resulting in parasites better adapted to their own host population compared with other host populations (Parker 1985; Lively 1989; Ebert 1994; Berenbaum & Zangerl 1998; Burdon & Thrall 1999, 2000; Lively & Dybdahl 2000). Although these data

support a number of models of host–parasite coevolution, they do not provide a direct demonstration of it. The clearest example of antagonistic coevolution in natural populations is the interaction between snails and their trematode parasites (Lively 1989, 1999; Lively & Dybdahl 1998, 2000). Here, parasites showed increased infectivity through time to initially common snail genotypes, with a subsequent increase in the proportion of initially rare, resistant snail genotypes, as predicted by a time-lagged model of antagonistic coevolution driven by frequency-dependent selection (Lively & Dybdahl 1998). However, coevolution was not shown to be an ongoing process (only one coevolutionary cycle was observed) and it is possible that fluctuations in snail genotype frequency may have been caused by other selection pressures, either abiotic or from other parasites (Lythgoe 1998).

Many of the difficulties of studying coevolution in the field, such as the time-scale of coevolutionary change, measuring interactions across time and lack of control over the environment and genetics, can be overcome by using laboratory populations of microbes (Lenski & Levin 1985). Isogenic populations can be propagated in carefully controlled replicate environments, hence changes in host resistance and parasite infectivity (both within and between-communities) can be directly ascribed to mutation, followed by reciprocal selection between the interacting host and parasite populations, and not to other environmental or genetic variation. Furthermore, large population sizes and short generation times favour rapid coevolutionary change, and the ease of long-term storage (of host and parasite) allows interactions to be measured across both time and space.

The most frequently used microbial system for the study of antagonistic coevolution is bacteria and virulent phage. Virulent phage are viral parasites that bind to the bacterial cell surface, inject in their genetic material and use the host cellular machinery to replicate. Release of viral particles requires cell lysis, hence the interaction between host and parasite is entirely antagonistic. A num-

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ber of studies using laboratory populations of bacteria and phage have directly demonstrated reciprocal evolution of resistance and infectivity, but have been limited to a maximum of one cycle, after which bacteria evolve resistance that apparently cannot be overcome (Chao *et al.* 1977; Levin *et al.* 1977; Lenski 1984; Lenski & Levin 1985; Bohannan & Lenski 2000). Such asymmetry in this coevolutionary interaction is believed to result from the reduced evolutionary potential of the much smaller phage genomes and the requirement of phage to bind to specific bacterial receptors (Lenski 1984). Long-term antagonistic coevolution, with multiple cycles of resistance and infectivity and no obvious end-point, has been demonstrated in another microbial system: RNA viruses and spontaneously arising mutants that require the wild-type enzyme for successful replication (defective interfering particles) (Horodysli *et al.* 1983; DePolo *et al.* 1987). However, this system lacks the complexity of interspecific host–parasite interactions, and coevolutionary divergence was not addressed.

The work described here was initiated after preliminary studies, in which a bacteriophage of the bacterium *Pseudomonas fluorescens* SBW25 was found to persist after more than 300 (bacterial) generations of co-cultivation, suggested the possibility of an evolutionary arms race. After direct proof of persistent antagonistic coevolution was obtained, we examined between-population coevolutionary divergence in the absence of environmental or (initially) genetic variation. We also addressed whether coevolutionary divergence had resulted in local adaptation of either parasites or hosts: were parasites better at infecting hosts they coevolved with than hosts from other populations, and were hosts better at resisting parasites they coevolved with? Finally, we wanted to identify which of two, non-mutually exclusive, types of selection was predominantly responsible for driving coevolution. Selection may be directional, favouring hosts that are resistant to all encountered parasite genotypes and parasites that can infect all encountered host genotypes. Alternatively, selection may fluctuate, resulting in different, rather than greater, resistance ranges being alternately favoured through time (Thompson 1994). Unlike fluctuating selection, directional selection is unlikely to drive antagonistic coevolution indefinitely—resistance and infectivity ranges must ultimately be constrained either genetically or metabolically. It is assumed that coevolution is largely driven by fluctuating selection in natural populations (Vanderplank 1968; Parker 1994; Thompson 1994, 1999; Burdon & Thrall 1999; Lively 1999), whereas directional selection seems to operate in laboratory microbial populations (Chao *et al.* 1977; Levin *et al.* 1977; Horodysli *et al.* 1983; Lenski 1984; Lenski & Levin 1985; DePolo *et al.* 1987; Bohannan & Lenski 2000).

2. MATERIAL AND METHODS

(a) *Culturing techniques*

Cultures were propagated in static microcosms (25 ml glass universals containing 6 ml of standard King's medium B (KB)) in a 28 °C incubator. Twelve replicate microcosms were inoculated with 10^8 cells of *P. fluorescens* isolate SBW25 (Rainey & Travisano 1998) and 10^5 clonal particles of a naturally associated DNA phage, SBW25 Φ 2. Sixty microlitres of each culture

(on average 10^7 bacterial cells and 10^4 phage particles) was transferred to a fresh microcosm every 2 days for a total of 50 transfers (approximately 400 bacterial generations). Cultures were frozen at -80 °C in 20% glycerol at every second transfer. Bacterial colonies were isolated by plating diluted cultures onto standard KB agar plates. Phage populations were isolated by centrifuging cultures with 10% chloroform, which lysed and pelleted bacterial debris.

(b) *Measuring bacterial resistance and phage infectivity*

Coevolutionary arms races are characterized by the continual evolution of host defence and parasite counter defence. For simplicity, we consider only the binary traits of bacterial resistance or sensitivity and phage infectivity or non-infectivity. The resistance of a particular bacterial population to a particular phage population (or the infectivity of a phage population to a bacterial population) was determined by streaking 20 independent bacterial colonies across a perpendicular line of phage that had previously been streaked on a KB agar plate. A colony was defined as resistant if there was no inhibition of growth, otherwise it was defined as sensitive. Ancestral SBW25 was used on every plate as a control.

(c) *Measuring coevolutionary change*

Selection on phage infectivity was assumed to show a time-lag (Nee 1989; Lively & Dybdahl 1998), such that contemporary phage would be less able, than phage from the immediate future, to infect contemporary bacteria. Contemporary bacteria were therefore expected to be more resistant, than bacteria from the immediate past, to contemporary phage. To measure this, we determined, at every second transfer (*ca.* 15 bacterial generations), the resistance of bacterial populations to their contemporary phage and to the phage population from two transfers in the future. We simultaneously measured bacterial resistance to the ancestral phage, and the ability of phage populations to infect the ancestral bacteria.

(d) *Directional versus fluctuating selection*

Directional selection is expected to result in a positive relationship between time coevolving and the range of phage to which bacteria are resistant, and the range of bacteria that can be infected by phage. No such relationship is expected if coevolution was driven by predominantly fluctuating selection. We isolated bacteria and phage approximately every 10th transfer and measured, within each of the 12 replicates, the resistance of bacterial populations from all time-points, to phage populations from all time-points.

(e) *Parallel versus divergent coevolution*

We wanted to determine the magnitude of divergent and parallel coevolution between the 12 replicates. Bacteria and phage were isolated from each population at the final transfer and assayed for resistance or infectivity in all combinations, both within and between populations.

(f) *Statistical analyses*

To statistically demonstrate persistent time-lagged coevolution, we counted the number of times in each of the 12 replicates that future phage were able to infect more contemporary bacteria than contemporary phage, and contemporary bacteria were more resistant than past bacteria against contemporary phage. These were separately analysed using heterogeneity

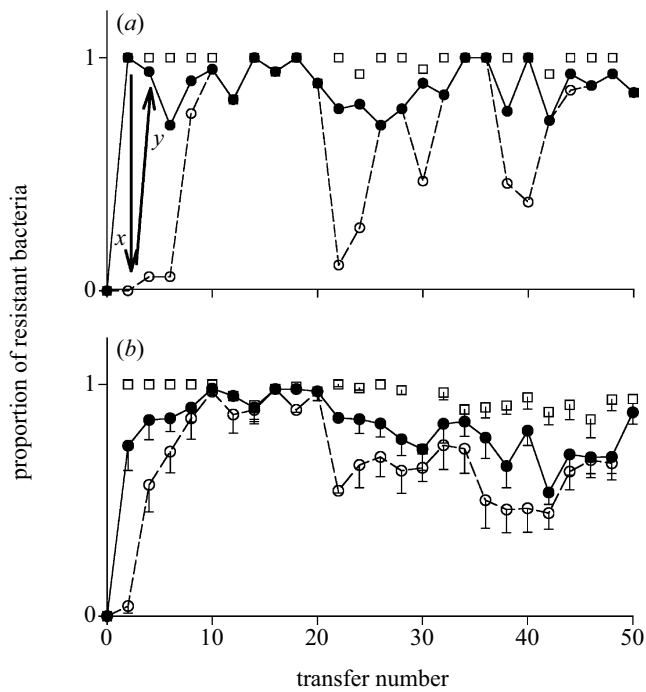


Figure 1. (a) Proportion of bacteria resistant to ancestral phage (open squares), contemporary phage (closed circles, solid lines) and phage from two transfers in the future (open circles, dashed lines) in the replicate that underwent the greatest rate of coevolutionary change. Time-lagged evolution of phage infectivity to resistant bacteria is demonstrated by the difference between the resistance of contemporary bacteria to contemporary and future phage populations (vertical arrow, *x*). Subsequent increases in bacterial resistance in response to phage evolution is demonstrated by the difference between the resistance of past and contemporary bacteria, to contemporary phage (diagonal arrow, *y*). (b) Mean (– s.e.m., *n* = 12) bacterial resistance of all populations; symbols as above.

G-tests (Sokal & Rohlf 1981), with the null hypotheses that there were no differences in the number of times that future phage were more infectious than contemporary phage, and the number of times that contemporary bacteria were more resistant than past bacteria.

To statistically distinguish between (predominantly) directional and fluctuating selection, within each replicate we calculated the mean resistance (proportion of resistant bacteria, *p*) of bacterial populations from each time-point to phage populations from all time-points. Mean infectivity ($1 - p$) of each phage population was determined in the same way. Mean resistance and infectivity were square-root arcsine transformed and regressed against time for each replicate. Sign tests were used to determine if these slopes were greater than zero in more than 50% of the replicates.

To determine the degree of parallel and divergent evolution, resistance or sensitivity of each bacterial colony was used as a binary response variable and phage and bacterial populations and their interaction fitted as factors in a generalized linear model using GLIM 4 (Crawley 1993). The total deviance explained by the main effects provided an estimate of the degree of parallel evolution, while the interaction provided an estimate of divergent evolution.

We also wanted to determine if divergent coevolution had resulted in either bacteria or phage becoming locally adapted.

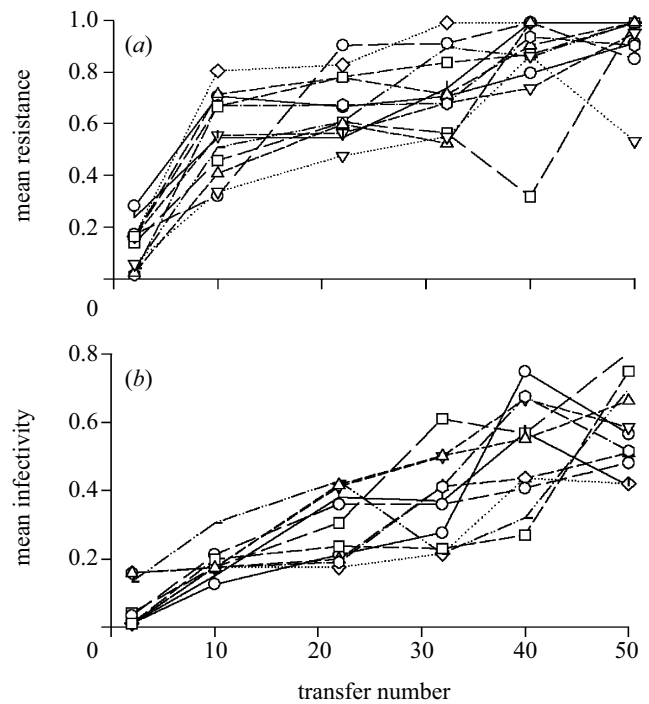


Figure 2. (a) The relationship between average bacterial resistance to phage isolated from multiple time-points (approximately every 10 transfers) and time spent coevolving (transfer number) within each replicate. Each of the 12 replicates is designated by a different symbol, connected through time by lines. (b) The relationship between average phage infectivity to bacteria isolated from multiple time-points (approximately every 10 transfers) and time spent coevolving (transfer number) within each replicate.

For local adaptation of bacteria, we determined the number of allopatric phage populations that were more infective (as determined from the proportion of sensitive bacterial colonies) than the sympatric phage population, and used a heterogeneity *G*-test to determine if more than 50% of allopatric phage were more infective than sympatric phage. An identical analysis was carried out to test for local phage adaptation, except that bacterial resistance was substituted for phage infectivity.

3. RESULTS

The data explicitly demonstrated time-lagged antagonistic coevolution between phage infectivity and bacterial resistance, with reciprocal increases in host resistance and phage infectivity observed throughout the experiment. Phage populations from two transfers in the future consistently showed greater infectivity to bacteria than contemporary phage (figure 1; $\chi^2_{12} = 62.5$, $p < 0.0001$). Likewise, contemporary bacteria were more resistant to phage than bacteria from two transfers in the past (figure 1; $\chi^2_{12} = 34.7$, $p < 0.001$). This pattern did not differ significantly between populations of either phage or bacteria (between-population heterogeneity: $p > 0.1$).

Two lines of evidence demonstrate that coevolution was largely driven by directional selection. First, bacteria resistant to their contemporary phage were always resistant to ancestral phage (figure 1), and phage populations from all time-points were able to infect ancestral bacteria. Second, we found that both mean bacterial resistance (figure 2a) and phage infectivity (figure 2b) increased with

Table 1. Proportion of bacteria from each final transfer population (B1–B12) resistant to each final transfer phage population (Φ 1– Φ 12). (Sympatric interactions are shown in bold.)

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
Φ 1	0.8	0.9	1	1	1	1	1	1	0.85	0.85	0.75	0.65
Φ 2	0.1	1	0.3	1	0.85	0.25	1	1	0.85	0.9	0.8	0.65
Φ 3	0.75	0.75	1	1	1	0.9	1	1	0.85	0.9	0.9	0.65
Φ 4	0.15	0.9	0.8	1	0.85	0.6	0.6	1	0.85	1	0.85	0.35
Φ 5	0.25	0.9	1	1	1	0.9	1	0.8	0.85	1	0.8	0.65
Φ 6	0.2	1	0.85	0.8	0.75	0.8	0.85	0.9	0.85	0.75	0.45	0.25
Φ 7	0.2	0.75	0.6	1	0.4	0.45	1	0.9	0.85	1	0.75	0.35
Φ 8	0	0.95	0.55	0.95	0.35	0.25	0.8	1	0.85	1	0.7	0.25
Φ 9	0	0.7	0.55	0.45	0.7	0.35	1	1	0.85	1	0.5	0.1
Φ 10	0	0.7	0.9	0.7	0.55	0.9	1	1	0.7	1	0.5	0.4
Φ 11	0	0.5	0.9	0.75	0.7	1	1	0.95	0.75	1	1	0.35
Φ 12	0	0.15	0	0.1	0.65	0.35	1	1	0.7	0.8	0.85	0.4

time in all 12 populations (sign test: $p < 0.001$, for both resistance and infectivity). Because we assayed phage populations as a whole, it is conceivable that each phage population was made up of numerous specialist phage genotypes, only capable of infecting certain bacterial genotypes. However, individual phage genotypes (obtained by growing diluted populations overnight on exponentially growing bacteria in soft KB agar, and resuspending individual plaque forming units) from the final transfer populations, had the same infectivity characteristics as the population from which they were isolated, demonstrating the evolution of generalist phage genotypes.

Analyses of the final transfer populations of bacteria and phage revealed considerable between-population divergence in the absence of environmental and, initially, genetic variation (table 1). The proportion of the non-residual deviance explained by the main effects provided an estimate of the degree of parallel evolution ($930/1489 = 62\%$), while the interaction provided an estimate of the magnitude of divergent evolution ($559/1489 = 38\%$). Divergence resulted in bacteria better able to resist phage from their own microcosm than other microcosms (table 1; $\chi^2_1 = 28.9$, $p < 0.0001$), suggesting that bacteria were locally adapted to phage, although there were two populations where this was not the case (heterogeneity: $\chi^2_{11} = 14.5$, $p = 0.051$). Phage were, not surprisingly, less able to infect their own than other bacterial populations (table 1; $\chi^2_1 = 20.95$, $p < 0.0001$), although this varied considerably between replicates (heterogeneity: $\chi^2_{11} = 26.3$, $p = 0.002$).

4. DISCUSSION

In this study, we present, to our knowledge, the first explicit demonstration of long-term antagonistic coevolution, characterized by multiple cycles of defence and counter defence, between a host and parasite species. Coevolution was largely driven by directional selection, with hosts becoming progressively more resistant to a wider range of parasite populations and parasites infective to a wider range of host populations. Furthermore, there was considerable between-population divergence in patterns of resistance and infectivity (*ca.* 40%) after approximately 400 bacterial generations, which resulted in

bacteria more resistant to their own, than other, phage populations.

Our study differs from previous work on bacteria and phage (Chao *et al.* 1977; Levin *et al.* 1977; Lenski 1984; Lenski & Levin 1985; Bohannan & Lenski 2000) in showing long-term coevolution. There are a number of possible explanations: this system has never been used before; previous studies have been less extensive; and, unlike other studies, evolution occurred in spatially structured (static) microcosms, which may provide spatial refuges for sensitive bacteria (Schrag & Mittler 1996), allowing the generation of infective phage mutants. Whatever the reason, this study demonstrates that phage are not fundamentally constrained in their ability to coevolve with bacteria (Lenski 1984). Antagonistic coevolution is therefore probably important in natural populations of bacteria and phage, contributing to their coexistence and between-population genetic differentiation. Furthermore, evidence of persistent bacteria–phage coevolution, particularly the evolution of generalist phage, has implications for the use of phage to treat pathogenic bacterial infections.

Knowledge of the mechanisms of phage infectivity and bacterial resistance in other systems (*Escherichia coli* and associated phage) allow us to speculate on the mechanistic bases of directional coevolution in this study. Phage infectivity requires irreversible binding to a specific bacterial receptor, which allows the injection of genetic material into the host cell (Goldberg *et al.* 1994). Bacterial resistance probably involves mutations that result in a change in structure, or even the entire loss, of the phage binding site (Hofnung *et al.* 1976). If these sites have a functional role in bacterial metabolism, resistance will be associated with a reduction in growth rate (Lenski 1988). Complete loss of the binding site is expected to result in resistance to all possible mutants of the phage, and be associated with the greatest cost (Schwartz 1980; Lenski 1984; Lenski & Levin 1985.) Completely resistant bacteria will therefore be largely outcompeted by bacteria with altered binding sites that are resistant to contemporary phage genotypes, allowing directional coevolution to continue. However, complete loss of the binding site might have been responsible for the small proportion of bacteria early on in the experiment (by transfer four) that were entirely

resistant to all subsequent phage populations (data not shown). Phage that are capable of infecting bacterial cells that have altered binding sites are believed to have acquired mutations resulting in reduced specificity of their binding (Schwartz 1980). (This lack of specificity is associated with a reduction in maximal population growth rate (Chao *et al.* 1977; Lenski & Levin 1985).) Subsequent bacterial resistance probably results from more radical changes in the structure of the binding site, preventing a wider range of phage genotypes successfully binding. Similarly, subsequent evolution of phage infectivity is likely to result from even less specificity for the binding site, hence the ability to infect a wider range of bacterial genotypes. Such mechanisms of resistance and infectivity would explain the long-term, predominantly directional, coevolution observed in this study.

Our finding that antagonistic coevolution was predominantly driven by directional selection is consistent with previous work using microbial laboratory systems (Chao *et al.* 1977; Levin *et al.* 1977; Horodysli *et al.* 1983; Lenski 1984; Lenski & Levin 1985; DePolo *et al.* 1987; Bohannan & Lenski 2000). By contrast, the extensive polymorphisms for resistance and infectivity found within most natural populations (Vanderplank 1968; Parker 1994; Burdon & Thrall 1999; Lively 1999), suggests that fluctuating, rather than directional, selection plays a more significant role in driving coevolution in the wild. Some models of coevolution, such as the 'matching alleles' model (Frank 1993), invoke implicit genetic constraints on coevolution driven by directional selection, as precise matching of parasite alleles to host alleles is required for parasite infectivity. (Selection fluctuates because a genotype's fitness will be a negative function of its frequency.) Although some data from natural populations are consistent with this model (Frank 1993, 1996; Lively 1999), there is good evidence that directional selection operates to produce generalist pathogens (capable of infecting a wide range of 'resistant' host genotypes) in many natural plant-pathogen systems (asymmetric 'gene-for-gene' coevolution (Flor 1956; Thompson & Burdon 1992; Parker 1994)). In these cases, theoretical results suggest selection fluctuates because of increasing metabolic costs associated with generalist strategies (Sasaki 2000).

This apparent discrepancy between the predominant type of selection driving coevolution in natural and laboratory populations may be in part an artefact of laboratory experiments. Preliminary data from this system suggest that increasing bacterial resistance and phage infectivity ranges are associated with declining growth rates, hence selection may have fluctuated if coevolution was allowed to continue for longer. Two other features of this and other laboratory microbial studies are likely to decrease the role of fluctuating selection in driving the coevolutionary process: resources were abundant, potentially decreasing the relative fitness costs of resistance and infectivity (Hochberg & Van Baalen 1998; Bohannan & Lenski 2000), and there was no migration between divergently coevolving populations (Gandon *et al.* 1996; Burdon & Thrall 1999; Lively 1999).

We found clear evidence of local adaptation of bacteria to phage in this study. It is commonly assumed that parasites, rather than hosts, will show evidence of local adaptation in the absence of migration: parasites generally have

larger population sizes and shorter generation times, hence evolve more rapidly (Ebert 1994; Kaltz & Shykoff 1998). That hosts were largely resistant to their contemporary phage and it was lack of evolution of phage, rather than bacteria, that impeded the coevolutionary process (figure 1; between transfers 10 and 20), suggests that bacteria were evolving more rapidly in this study. This can be explained by bacterial population sizes being on average at least three orders of magnitude greater than that of phage populations (data not shown). The result is consistent with the view that local adaptation is in part governed by the relative rates of evolution of the players involved.

To some extent it was surprising to find evidence of persistent antagonistic coevolution and coevolutionary divergence in these simple microbial populations. Replicate populations were initiated with a single identical genotype, so that all genetic variation on which selection acted to produce coevolution had to be generated *de novo* by mutation. Moreover, our experiments were carried out on an asexual species, which are thought to respond more slowly to selection than sexual species. In natural populations the rate of coevolutionary change could be increased by sexual reproduction (Hamilton *et al.* 1990; West *et al.* 1999), and by any factor that increases genetic diversity such as existing diversity (Thompson 1999) or migration (Gandon *et al.* 1996). Furthermore, existing between-population genetic differentiation is likely to enhance the divergence of coevolutionary trajectories (Thompson 1999). Antagonistic host-parasite coevolution is therefore likely to be widespread and play a major role in many evolutionary and ecological processes.

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