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Antagonistic coevolution with parasites increases the cost of host deleterious mutations

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The fitness consequences of deleterious mutations are sometimes greater when individuals are parasitized, hence parasites may result in the more rapid purging of deleterious mutations from host populations. The significance of host deleterious mutations when hosts and parasites antagonistically coevolve (reciprocal evolution of host resistance and parasite infectivity) has not previously been experimentally investigated. We addressed this by coevolving the bacterium *Pseudomonas fluorescens* and a parasitic bacteriophage in laboratory microcosms, using bacteria with high and low mutation loads. Directional coevolution between bacterial resistance and phage infectivity occurred in all populations. Bacterial population fitness, as measured by competition experiments with ancestral genotypes in the absence of phage, declined with time spent coevolving. However, this decline was significantly more rapid in bacteria with high mutation loads, suggesting the cost of bacterial resistance to phage was greater in the presence of deleterious mutations (synergistic epistasis). As such, resistance to phage was more costly to evolve in the presence of a high mutation load. Consistent with these data, bacteria with high mutation loads underwent less rapid directional coevolution with their phage populations, and showed lower levels of resistance to their coevolving phage populations. These data suggest that coevolution with parasites increases the rate at which deleterious mutations are purged from host populations.

Keywords: Experimental evolution; *Pseudomonas Fluorescens*; bacteriophage; host–parasite coevolution; sex

1. INTRODUCTION

Infection by parasites is by definition costly to hosts, but may sometimes provide benefits. For example, parasites that have already infected their host may prevent the establishment of other, more pathogenic, parasites (Mueller *et al.* 2005). Here we focus on a potential longer term evolutionary benefit: parasites may result in more rapid purging of deleterious mutations from host populations. Two mechanisms have been proposed to explain this. First, parasitic infection (and stresses in general; Elena & de Visser 2003; Peck & Waxman 2000) may increase the relative costs of deleterious mutations, turning selectively minor mutations into effective lethals. Studies using organisms ranging from bacteria (Cooper *et al.* 2005) to beetles (Stevens *et al.* 1997) to sheep (Coltman *et al.* 1999) support this prediction, but studies on other organisms such as Daphnia (Haag *et al.* 2003; Salathe & Ebert 2003) found no such effect. Second, parasitic infection may result in synergistic epistasis between deleterious mutations (West *et al.* 1999). That is, the deleterious effects of multiple mutations are greater than that expected by the effect of the mutations in isolation. Despite a recent explicit experimental test (Cooper *et al.* 2005), there is currently no empirical support for this hypothesis.

The above mechanisms have been framed in terms of parasitism directly increasing the strength of selection against mutagenized hosts. However, parasites may also indirectly increase the strength of selection against deleterious mutations by imposing selection for hosts that are resistant to parasitism. Host resistance to parasites is sometimes costly in the absence of parasites (Kraaijeveld & Godfray 1997; Webster & Woolhouse 1999; Bohannan & Lenski 2000), and it is possible that the cost of resistance to parasites may either act synergistically with background deleterious mutations, or result in synergistic epistasis between background mutations. Under both scenarios, the difference in fitness between hosts with high and low mutation loads will be greater when hosts have also evolved resistance to parasites. Increasing costs of deleterious mutations resulting form parasite resistance is likely to affect the dynamics of host–parasite antagonistic coevolution (the reciprocal evolution of host defense and parasite counter-defense; Thompson 1994). Resistance evolution will occur less readily or not at all in populations with higher mutation loads, hence parasites will show greater levels of infectivity. Furthermore, if resistance evolution proceeds less rapidly in mutated host populations, there will be weaker selection for novel parasite infectivity traits, hence coevolution in general will proceed less rapidly or may stall completely.

We used experimental populations of the bacterium *Pseudomonas fluorescens* and a parasitic (lytic) bacteriophage (Buckling & Rainey 2002*a*) to address how antagonistic coevolution with parasites affects the cost of host deleterious mutations, and how this in turn affects the dynamics of coevolution. Phages invade bacterial cells,

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replicate and then lyse the cell to release the progeny, hence imposing strong selection for bacterial resistance. Bacterial resistance in turn imposes selection for infective phage, and a directional arms race ensues, whereby bacteria evolve resistance to an increasingly wide range of phage genotypes, and phage evolve infectivity to an increasingly wide range of bacterial genotypes (Buckling & Rainey 2002*a*). The short generation times and large population sizes allow coevolution to be observed over a matter of days, and the ability to store bacteria and phage in suspended animation allows direct assessment of relative fitness of ancestral and evolved types. We coevolved phage with replicate bacterial clones that had or had not undergone UV mutagenesis, and determined the relative fitness of bacterial genotypes, average levels of resistance to phage and the rate of coevolution through time.

2. MATERIAL AND METHODS

(a) *Generation of bacterial clones*

We derived three clones with low levels (L) of deleterious mutations, and three clones with higher levels (H) of deleterious mutations from *P. fluorescens* SBW25 (Rainey & Bailey 1996). The same number of clones were derived from an isogenic auxotrophic mutant of SBW25 (SBW25*panB*−; Rainey & Travisano 1998), which requires an exogenous source of pantothenic acid to grow, resulting in a total of 6 L and 6 H clones. Independent L clones were derived by propagating three SBW25 and three SBW25*panB*− populations in KB microcosms (30 ml glass universals with loose plastic lids, containing 6 ml of King's Medium B supplemented with 0.0024% pantothenic acid, negating any fitness cost of the *panB* knockout) in the absence of phage in an orbital shaker at 28 °C. Every 24 h, 60 µl of culture was transferred to fresh media, for a total of seven transfers, and then a single clone from each population was isolated after plating onto KB agar. Independent H clones were generated by exposure of diluted culture on a KB agar plate to UV light from a UVP TMW-20 50 Hz transilluminator (8 cm distance) for 10 s, and then isolating random surviving colonies. This treatment resulted in approximately 50% of colonies not growing (relative to non-UV treated controls), and it was assumed that surviving colonies would have acquired some deleterious mutations; this was confirmed from fitness assays (see below). Clones were independently stored in 20% glycerol/80% KB at −86 °C.

(b) *Culture conditions*

All 12 bacterial clones were grown overnight in KB microcosms in a 28 °C orbital shaker (200 rpm). Twelve tubes containing fresh media were each inoculated with 10^8 cells of one of the overnight cultures, along with 10^5 clonal particles of a naturally associated DNA phage SBW25ϕ2 (Buckling & Rainey 2002*a*,*b*), and grown at 28 °C in a static incubator for 48 h. Sixty microlitre of each culture was transferred to fresh KB microcosms, and the procedure repeated for a total of 32 transfers (approximately 240 bacterial generations). Cultures were frozen every second transfer.

(c) *Competition experiments*

The relative fitness of bacterial populations in the absence of phage was determined by competition experiments with the ancestral genotype with the opposite genetic marker (i.e. evolved SBW25 strains were competed against ancestral SBW25*panB*−, and vice versa). Prior to the competition assays, bacteria had to be isolated from phage by chemical treatment. A 5% Virkon (a commercially available disinfectant)/water solution was made, and added to KB to a concentration of 0.375% Virkon. 60 µl of culture was added to 6 ml of the Virkon/KB solution in glass universals, and left for 24 h, static at 28 °C. This procedure left the bacteria viable, and completely phage free. 60 µl of the Virkon cultures was added to a fresh static glass universal containing 6 ml KB, and grown for one day at 28 °C, to give a phage-free and Virkon-free stock (Morgan *et al.* 2005). The potential presence of phage in Virkon-treated cultures was tested for by plating out the cultures on to semi-solid agar seeded with *P. fluorescens* strain SBW25 and incubated at 28 °C for 24 h; plaques would indicate the presence of phage. In all cases the test proved negative.

Competition assays were carried out by first adding approximately 5×10^7 cells of overnight cultures of both evolved and ancestral strains at 50 : 50 ratio to KB microcosms. Accurate estimates of starting densities were determined by plating the mixture onto vitamin free KB agar supplemented with 4.8×10^{-6}% pantothenic acid; on this agar the pantothenate marker strain is readily distinguished by its greatly reduced size (Buckling *et al.* 2000). Cultures were then propagated for 24 h (to minimize evolution during the competition) in static tubes at 28 °C, and final densities determined in the same way. Relative fitness was calculated from the ratio of the estimated Malthusian parameters (m) of each competing type, $m=\ln(N_f/N_0)$, where N_0 is the starting density and N_f the final density (Lenski *et al.* 1991). Fitness of all twelve populations was assayed prior to coevolution with phage, and after 2, 16 and 32 transfers coevolving with phage. Each competition experiment was replicated at least 3 times, and the mean fitness for each population determined.

(d) *Measurement of bacterial resistance and phage infectivity*

Resistance of a bacterial population (or infectivity of a phage 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 (Buckling & Rainey 2002*a*). (Phage populations were isolated by centrifuging cultures with 10% chloroform, which lysed and pelleted bacterial debris.) A colony was defined as resistant if there was no inhibition of growth; otherwise it was defined as sensitive.

(e) *Measurement of coevolution*

Antagonistic coevolution in the *P. fluorescens*—SBW25Φ2 systems has been shown to be largely escalatory, with phage and bacteria showing increasingly wide infectivity and resistance ranges, respectively, through time (Buckling & Rainey 2002*a*). The rate of escalatory coevolution at any give time point can be determined from the change in the infectivity of phage populations to a bacterial population through time. Specifically, at every second transfer, we determined the resistance (proportion of resistant colonies) of bacterial populations to past (two transfers previous), contemporary and future (two transfers subsequent) sympatric phage populations. If coevolution was escalatory, we would expect, for multiple points, future phage to be better than contemporary phage, and for contemporary phage to be better than past phage at infecting contemporary bacteria, hence a negative slope of bacterial resistance against the time when the

phage population was isolated (past, contemporary and future). Thus the rate of coevolution can be determined from how much phage infectivity changed between past and future populations, which is given by the slope of infectivity against time (past, contemporary and future; Brockhurst *et al.* 2003).

3. RESULTS

At time zero, bacteria exposed to UV-mutagenesis (H bacteria) showed an approximately 5% reduction in fitness relative to the ancestral type (figure 1; 1-sample *t*-test: $t=9.71$, $p<0.001$), whereas bacteria propagated for 7 transfers (L bacteria) had the same level of fitness as the ancestor (figure 1; 1-sample *t*-test: $t=0.14$, $p=0.9$). The fitness of L and H bacteria decreased with increasing time spent coevolving with phage (slopes of fitness against time where negative in all lines; sign test: $p=0.0005$), but this rate of decrease in fitness was significantly greater in H than L bacteria (figure 1; Mann–Whitney test of slopes of fitness against time between H and L bacteria: $W=21$, $p=0.005$). Analysis of the data using Residual Maximum Likelihood (REML) Repeated Measures (using GenStat 7.2), with clone treated as a random factor, revealed the same patterns as the non-parametric analyses reported above ($p<0.001$ for the main effects of time and treatment, and the time by treatment interaction).

Figure 2 details the coevolutionary dynamics of bacteria and phages. Bacteria and phages were isolated at every second transfer, and the resistance of bacteria from each time point was determined against phages from two transfers in the past, contemporary phages, and phages from two transfers in the future. Resistance of bacteria from each time point to past, contemporary and future phage populations are plotted (from left to right, respectively) as three connected points, with the middle point (resistance to contemporary phage) indicating the time point from which the bacteria were isolated.

Bacteria were initially sensitive to the ancestral phage but bacterial resistance to ancestral phage rapidly evolved in all populations, with mean proportions of 0.94 and 0.69 resistant bacterial cells in L and H lines, respectively, by transfer 2 (first point on *t*2 lines in figure 2). Phage evolved to infect these bacteria, as shown by the greatly reduced proportion of bacteria at the second transfer resistant to their contemporary phage (middle point on *t*2 lines in figure 2; means = 0.22 and 0, for L and H lines, respectively). Bacteria then evolved resistance to these phages, as shown by the increased resistance of bacteria from transfer 4 against phage from transfer 2 (first point on *t*4 lines in figure 2; means = 0.8 and 0.26. for L and H lines, respectively). Multiple rounds of such resistance and infectivity coevolution are apparent from the consistently negative slopes of bacterial resistance plotted against past, contemporary and future phages. These results are consistent with our previous work showing an escalatory arms race between these organisms during early stages of their coevolutionary interaction (Buckling & Rainey 2002*a*; Brockhurst *et al.* 2003).

However, coevolutionary dynamics differed significantly between H and L lines in two major ways. First, average levels of bacterial resistance to their contemporary phage were lower in the H than the L lines (figure 2, middle points in lines; 2-sample *t*-test of resistance averaged through time: $t=2.41$, $p=0.036$), suggesting

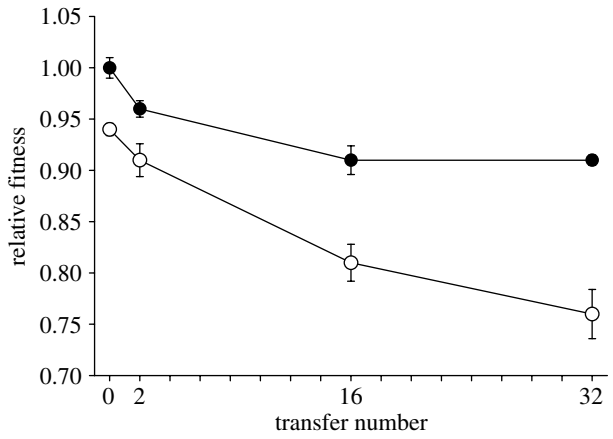

Figure 1. Fitness of bacteria with low (closed circles) and high (open circles) mutation loads during coevolution with bacteriophage. Points are mean (±s.e.) fitness when competing with ancestral bacteria in the absence of phage.

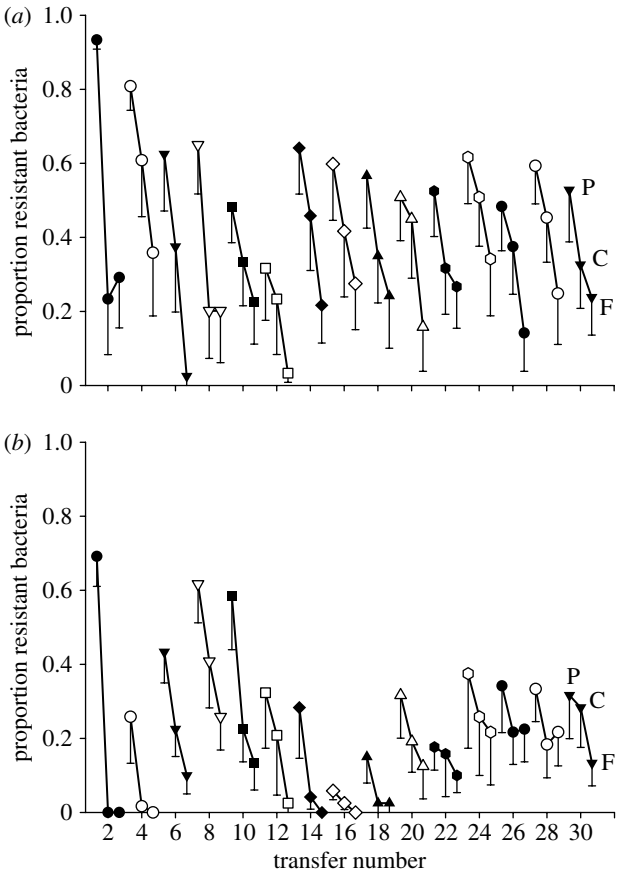

Figure 2. Rates of directional coevolution between bacteria and phage, when bacteria have low (*a*) and high (*b*) mutation loads. Lines connect the (mean − 1s.e.) proportion of resistant bacteria from a single time point (indicated by the middle point) to past (P), contemporary (C) and future (F) phage populations (from left to right). Bacterial resistance was measured every two transfers. The gradient of the proportion of resistant bacteria against time (past, contemporary and future) provide a measure of how rapidly bacteria and phage are coevolving.

that phage have a relative advantage when coevolving with bacteria with high compared to low mutational loads. Second, the rates of coevolution, as indicated by the slopes of bacterial resistance against past, contemporary and future phage, was greater in L than H lines (2-sample *t*-test of slopes averaged through time: $t=2.69$, $p=0.02$).

4. DISCUSSION

In this study we investigated the interaction between deleterious mutations in bacterial populations and antagonistic coevolutionary dynamics with parasitic bacteriophages. Our data provide support for three inter-related hypotheses. First, that fitness decreased more rapidly through coevolutionary time in bacterial populations with higher mutational loads suggests that the relative costs of evolving resistance to bacteriophages are greater in mutagenized populations (figure 1). Second, average levels of bacterial resistance to contemporary phage were lower in mutagenized bacterial populations, suggesting a relative advantage of phages coevolving with bacteria with high than low mutation loads (figure 2). Third, the rate of directional coevolution was lower when phages were coevolving with mutagenized bacterial populations (figure 2).

The interaction between background deleterious mutation and the cost of resistance to phage in this study is indicative of synergistic epistasis. However, because we were not able to control for the number of UV or resistance mutations or know their precise physiological effects we can only speculate as to why such an interaction may arise. Phage resistance is likely to have pleiotropic effects if phage bind to receptors that serve additional functions for the bacteria, such as nutrient uptake, as modification to prevent phage binding (Hofnung *et al.* 1976) may reduce their functional efficiency (Lenski 1988). If background deleterious mutations have arisen in, for example, other parts of the nutrient uptake pathway, this may result in a synergistic interaction between mutational effects. Alternatively, background deleterious mutation may reduce the ability of the bacterial genome to compensate for fitness costs associated with resistance to phage. It is also unclear from our study if resistance to phage results in synergistic epistasis between existing mutations, or synergistic epistasis occurs between existing mutations and parasite resistance. Knowledge of the precise number of mutations would be necessary to differentiate between these hypotheses (Elena & Lenski 1997; Cooper *et al.* 2005), and subsequent experiments will address this issue.

Our study is phenomenological insofar as we are unable at present to identify the biological mechanisms responsible for them. Nevertheless, our results are sufficiently clear to make certain deductions about their implications. Parasites can result in more rapid purging of deleterious mutations from populations, by increasing the relative cost of deleterious mutations. Such conclusions have been drawn from similar studies where the effect of parasites *per se*, rather than coevolution with parasites, has been addressed (Stevens *et al.* 1997; Coltman *et al.* 1999; Cooper *et al.* 2005). This study therefore extends the generality of this conclusion.

This study suggests that coevolution with parasites may increase the rate at which deleterious mutations are removed from host populations, but parasites can theoretically also increase the mutational load of their host populations. Parasite-imposed selection for resistant hosts can result in population bottlenecks, thereby decreasing effective population size, and increasing the rate at which deleterious mutations are accumulated (the stochastic loss of individuals with small mutational loads, Muller's ratchet, is accelerated; Howard & Lively 1994, 1998). It is unlikely, but possible, that such a process contributed to the decline in fitness (as measured in the absence of phages) through time of bacterial populations in this study. Previous studies using this system have demonstrated that phages can reduce bacterial within-population diversity (Buckling & Rainey 2002*b*; Brockhurst *et al.* 2004), but given the massive bacterial population sizes (approximately 10^9) and that colony morphological variation was detected in the majority of samples of only 100 individuals, it is unlikely that population bottlenecks would be sufficiently small. Note that this acceleration of Muller's ratchet resulting from coevolution with parasites has been invoked to explain the evolutionary advantage of sexual over asexual reproduction (see below), as sexual reproduction will allow the production of offspring with lower mutational loads than their parents (Howard & Lively 1994, 1998)

Finally, we cautiously extrapolate the results from this study to the evolutionary maintenance of sexual reproduction, as have several previous studies dealing with asexual organisms (Elena & Lenski 1997; Cooper *et al.* 2005). There are currently two major hypotheses to explain why sexual reproduction might have a selective advantage over asexual reproduction. First, sex may be beneficial when coevolving with parasites because it can result in genetically novel hosts to which parasites have yet to evolve means of successful infection ('parasite red queen hypothesis'; Hamilton 1980; Hamilton *et al.* 1990). Second, sex may allow mutations to be more effectively purged by increasing variance in the mutation load of offspring, assuming strong synergistic epistasis and relatively high genomic mutation rates ('the mutational deterministic hypothesis'; Kondrashov 1982, 1988). There is no empirical support for the mutational deterministic hypothesis, and only limited support for the red queen hypothesis (e.g. Jokela & Lively 1995; Gemmill *et al.* 1997), hence it has been proposed that hypotheses may themselves interact to increase the relative advantage of sex over asexual reproduction (Howard & Lively 1994, 1998; West *et al.* 1999). Of specific relevance to the current study is the suggestion that infection by parasites may increase the strength of synergistic epistasis to levels that make the mutational deterministic hypothesis theoretically possible (West *et al.* 1999). This study provides experimental evidence that parasites, by imposing selection on hosts for costly resistance to infection, can generate synergistic epistasis between deleterious mutations. However, convincing support for this hypothesis requires evidence of strong synergistic interactions between costly parasite resistance and deleterious mutations in sexual populations at mutation–selection balance.

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