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Persistence of costly novel genes in the absence of positive selection

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Abstract

Many genetic changes that ultimately lead to adaptive evolution come with a short-term cost expressed in terms of reduced survival and reproduction. In the absence of genetic drift, it is unclear how such costly mutations may persist. Here we experimentally demonstrate that parasites can promote the persistence of costly genetic variants. We employed a genetically engineered strain (GMMO) of the bacterium *Pseudomonas fluorescens* as a model of the acquisition of a new gene either through a major mutation or through horizontal transfer, and examined its persistence in different evolving communities comprising an ancestral strain and a lytic bacteriophage. Whereas competition resulted in the elimination of the GMMO, inclusion of the phage promoted GMMO persistence. We provide evidence for why this effect is due to the differential susceptibility of GMMO and ancestral bacteria to phage.

Introduction

Microbes have a great capacity to colonize new environments and to respond efficiently to new selective pressures. This capacity to evolve is considered to be due to the modification of existing genes through mutation (Lenski *et al.*, 1991; Taddei *et al.*, 1997; Desai *et al.*, 2007), and to the acquisition of new genes by horizontal transfer (HGT) (Guttman & Dykhuizen, 1994; Lan & Reeves, 1996; Lawrence, 1997; Ochman *et al.*, 2000; Gogarten & Townsend, 2005). Whereas advantageous mutations of large effect are more efficient in moving the population from its phenotypic mean to a new adaptive peak, they should be uncommon because large mutations are more likely to cause deleterious effects to the organism (Fisher, 1930; Burch & Chao, 1999). However data on morphological (Orr & Coyne, 1992; Spiers *et al.*, 2002) and metabolic (Barret *et al.*, 2006) evolution as well as the evolution to antibiotic (Lenski *et al.*, 1991; Woodford & Ellington, 2006) and insecticide (Roush & McKenzie, 1987) resistance, suggest that adaptation often occurs by the acquisition of costly mutations of intermediate or large effect. These require the persistence of (at least initially) costly mutations in the environment to the arrival of the selective conditions favouring the novel genotype, or the appearance of compensatory mutations. But, how can individuals with costly mutations persist in a nonselective environment without being out-competed by fitter congeners in the absence of drift? For example, it has been estimated that, on average, 6% of all bacteria genomes are transferred sequences (Lawrence & Ochman, 1998; Kurland, 2000; Ochman *et al.*, 2000). An organism's genome is constituted of an ensemble of coadapted genes with a kinetically optimized system (Ehrenberg & Kurland, 1984; Kurland, 1992). However, this rampant accumulation of horizontally transferred genes in microbial genomes is paradoxical, because the arrival of new genetic material into the genome lowers the efficiency of the functional components, thus decreasing the fitness of the cell. A stochastic model of the evolution of HGT taking into account inactivating mutation rates, selection coefficients, invasion and infection rates, and population size led Berg & Kurland (2002) to conclude that only genes subjected to strong positive selection can be fixed

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in the population. But, because the conditions of positive selection on HGT are exceedingly rare, its role in prokaryote evolution remains unresolved (Kurland *et al.*, 2003).

Ecology and evolution can contribute to the persistence of novel genes through differential effects on reproduction and survival, involving for example interactions with resources, competitors, mutualists and natural enemies (Fussmann *et al.*, 2007). Moreover, theory suggests that combinations of two or more of these interactions in simple communities could lead to unexpected outcomes (Abrams & Matsuda, 1996; Holt & Polis, 1997; Abrams & Chen, 2002a,b; Fussmann *et al.*, 2007). Could the presence of species expected to reduce the persistence of costly genes actually promote it due to interactions with other species in simple communities? Natural enemies such as predators, parasites and pathogens, may exacerbate the effects of competition through indirect links in simple food webs (e.g. Holt, 1977; Holt *et al.*, 1994). For instance, they can provide density dependent regulation on the stronger competitor, allowing coexistence of competing organisms even in the absence of strong directional selection (Thingstad, 2000).

Bacteriophages are ubiquitous bacterial parasites, widely distributed and abundant in natural environments with densities estimated in sea water to 10^7 viron particles per milliliter. Their importance in maintaining prokaryotic diversity and species coexistence has been recognized especially in marine environments (Fuhrman & Schwalbach, 2003); but is has also been tested empirically under laboratory conditions of bacterial model systems (Buckling & Rainey, 2002; Brockhurst *et al.*, 2006). Therefore, if phages can alter the balance of competitive interactions, we then hypothesize that bacteria carrying costly novel genes—be they horizontally transferred genes or mutations—could theoretically persist in the presence of species-specific bacteriophages attacking preferentially the nonmodified bacteria. This study aims at experimentally testing this hypothesis.

Our simple community consisted of the rhizophere bacterium, *P. fluorescens* SBW25 [herein referred to as the wild type (WT)], a genetically modified variant, GM-BCA *P. fluorescens* 23.10R (the GMMO) (Timms-Wilson *et al.*, 2000), and the lytic phage associated with the ancestor, SBW25-φ2. The GMMO was a spontaneous SBW25 rifampin mutant modified by the insertion of a gene cluster (*phzABCDEFG*) for phenazine-1-carboxylique acid (PCA) biosynthesis (which is effective in the biological control of certain fungal pathogens), and a kanamicin resistance gene, *nptI*, inserted via a disarmed Tn5 vector, (Tomashow & Weller, 1988; Mavrodi *et al.*, 1998; Ellis *et al.*, 1999, 2000; Timms-Wilson *et al.*, 2000, 2004). We employed the GMMO as a model organism emerging from within the WT population by the acquisition of a novel gene through HGT. Thus, the GMMO and WT are different genotypes, differing only in the modified functions (phenazine production and antibiotic resistance to rifampin and kanamycin) and possible pleiotropic or epistatic effects of the inserted genes. As detailed below, whereas competition resulted in the elimination of the GMMO, inclusion of the phage promoted GMMO persistence through its differential impact on the WT.

Materials and methods

Experimental regime

Cultures were propagated in 30 mL glass microcosms containing 6 mL of standard King's B (KB) medium. A single clone of *P. fluorescens* SBW25 was isolated from the isogenic ancestor (provided by Dr. P.B. Rainey) and grown for 24 h at 28 °C under constant orbital shaking at 0.45 *g* units. Six populations were then created by transferring 60 μL from this culture and were allowed to diversify for 24 h under the same conditions. These populations constituted the six replicates of WT treatments in the experiment. The same procedure was employed to generate the six replicate populations of the genetically modified microorganism GM-BCA *P. fluorescens* 23.10R (GMMO) (provided by Dr. T. M. Timms-Wilson).

Thirty six experimental populations consisting of six treatments and six replicate microcosms per treatment were initiated by inoculating $c10^7$ bacterial cells into microcosms. Eighteen of these microcosms were inoculated with $c10^5$ particles of a single culture of the lytic bacteriophage SBW25φ2. The six treatments were as follows: (1 and 2) the WT strain with or without the phage; (3 and 4) the GMMO strain with or without the phage; and, (5 and 6) approximately equal numbers of each strain evolved in competition in the presence or absence of the phage. The microcosms were incubated at 28 °C in an orbital shaker at 200 rpm for 1 min every 30 min (referred to below as 'standard conditions'). Sixty microlitres of each culture was transferred every two days into microcosms containing 6 mL of fresh KB media, for a total of 16 transfers. A total of 150 μL samples of each population were kept in 80% glycerol at −80 °C. At the end of the experiment, 33 μL of the frozen cultures were used to assay bacterial densities by counting colonies grown on KB-agar plates. The presence of phage at the end of the experiment was determined by vortexing 900 μL of bacterial culture in 100 μL of chloroform. Ten microlitres from the supernatant containing phage was plated on soft agar with the top layer containing exponentially growing *P. fluorescens*. Phage presence was confirmed in all microcosms of the phage treatments.

Growth curves

We determined the growth rate of the ancestral strain of *P. fluorescens* SBW25, the genetically modified GM-BCA *P. fluorescens* 23.10R (GMMO), and populations

of *P. fluorescens* SBW25 that were propagated for 32 days with transfer to new KB media every day (fast growers) or every two days (slow growers). Six clones of each strain were grown on fresh KB for 48 h under standard conditions and their OD was determined with a spectrophotometer at 660 nm. The growth rate (r) was calculated as the $\log(OD_{660}$ 24 h$/OD_{660}$ initial).

Phage selectivity

A test was performed to assess if the initial invasion of phage in treatments containing the WT and the GMMO depended on their relative densities. Six WT populations and one population of the GMMO were created from a single colony of the ancestral strain and allowed to diversify for 24 h under standard conditions. Six replicate microcosms containing 100%, 75%, 50%, 25% or 0% of the WT strain in competition with the GMMO were established both with and without phage ($c10^5$ phage particles). Bacterial densities were estimated by colony counting at the start of the experiment, and then again after 5 h and 48 h of incubation under standard conditions.

Resistance to phage

Twelve bacterial lines with different maximal growth rates (fast growing: r = 0.58, 0.63, 0.58, 0.66, 0.62 and 0.59; slow growing: r = 0.73, 0.32, 0.38, 0.42, 0.29 and 0.09) were confronted with 12 phage populations that had coevolved with the WT or the GMMO strain for 32 days under standard conditions (144 combinations). Resistance of the SBW25 bacterial lines (fast or slow growers) was determined by streaking 12 independent colonies of each bacterial line across a line of phage population inoculated onto a KB agar plate. A colony was scored as sensitive if growth was inhibited; otherwise it was defined as resistant (Buckling & Rainey, 2002). A bacterial population was considered susceptible if more than 70% of individual colonies were sensitive.

Statistical analyses

A one-way ANOVA was used to compare the growth rates of the WT and GMMO strains. The analyses of population dynamics was done in separate two-way ANOVAs for each strain, with competition and phage as factors. For this, log transformed mean population densities at the end of the experiment were compared across treatments. The analysis of phage selectivity was done after 5 h and 48 h in separate analyses by means of a two-way ANOVA for each strain, with presence or absence of phage and initial bacterial frequency as factors. All ANOVAS were performed using GLM in JMP 5.1(1989). A nominal logistic fit was performed to analyse the effect of growth rate and phage type on resistance to phage between fast and slow growing SBW25 lines.

Results

When we grew the ancestral strains of the WT and GMMO in King's B media (see Materials and methods), we found that the former multiplied significantly faster than the latter (r_{WT} = 0.58; r_{GMMO} = 0.38; $F_{1,10}$ = 13.07, $P < 0.0047$), and reached a higher final density ($ODmax_{WT}$ = 1.448; $ODmax_{GMMO}$ = 1.122; F_1 = 14.08, $P < 0.0038$) (Fig. 1). Thus, at least one cost of the inserted genes in the GMMO is to lower reproduction rate in nutrient-rich media. We next set out to determine how different species' interactions in the simple community affected GMMO persistence.

We founded six replicate populations of each strain and propagated them in shaken microcosms for 16 transfers (c 120 bacterial generations). The six experimental treatments included each bacterium alone in the presence (2) or absence (2) of the phage and the two bacterial types in the presence (1) or absence (1) of the phage. The behaviour of each strain was considered separately. The three factors tested in the experiment and the interactions among them, explained 59% and 94% of the variance observed in average bacterial population densities of the WT and the GMMO respectively. The same general pattern was observed when the population density of each microcosm at the end of the experiment

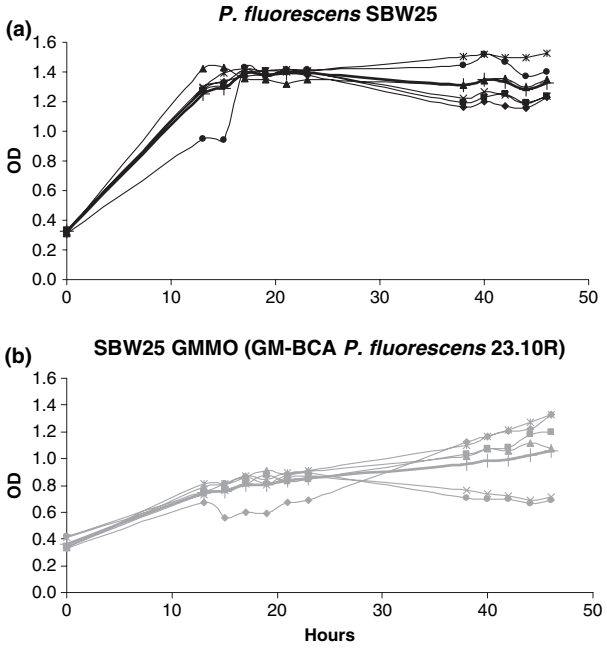

Fig. 1 Growth curves of the six strains of the (a) ancestral SBW25 [wild type (WT)] and (b) the GM-BCA *Pseudomonas fluorescens* 23.10R [genetically engineered strains (GMMO)] obtained by measuring the OD_{660} at different moments of the incubation period in KB media under standard conditions. Thick lines represent the average value of the six replicates.

was considered, suggesting that the observed pattern was consistent during the experiment (Fig. 2). Whereas each bacterial strain maintained similar population densities of $c.10^9$ cfu mL^{-1} when growing alone (Fig. 2a), they differed significantly in their response to competition (Fig. 2b,d) and in predation by the bacteriophage (Fig. 2c,d). In fact, we observed that the WT populations were significantly reduced in the presence of the phage ($F_{1,20} = 27.88$, $P < 0.0001$) but were not significantly affected by competition with the GMMO ($F_{1,20} = 0.1534$, P = 0.699). When in the presence of the phage only, the WT lost about 36% of its population to phage predation, as compared with 75% for the GMMO ($F_{1,20} = 7.4$, $P < 0.021$) (Fig. 2c).

But in contrast to the WT, the GMMO populations where highly affected by competition ($F_{1,20} = 321.95$, $P < 0.0001$), and the presence of phage was significantly important in the presence of competitors ($F_{1,20} = 52.3177$, $P < 0.0001$). Thus, while the GMMO decreased progressively in density to its extinction by the seventh transfer (*c.* 50 generations) (Fig. 2b), when the two strains were grown together, in the presence of phage, the GMMO sometimes persisted throughout the entire experiment (Fig. 2d; Fig. S1). Contrary to expectations, we observed that phage increased the persistence of the GMMO when in competition with the WT. One-tailed Fisher exact tests supported the statistical significance of this result for two out of three time points (T7, $P = 0.001$; T10, $P = 0.03$; T16, $P = 0.09$).

We then confronted the two bacteria types at different initial frequencies to the phage, and measured each of their densities after 5 h and 48 h of incubation in fresh KB media. After 5 h, there were no significant changes in bacterial density in the absence of phage, indicating that bacteria were in the lag phase ($F_{3,98} = 2.35$; $P = 0.07$; Fig. 3a) and due to their low densities, competition was not occurring. The phage was able to reduce bacterial density significantly in this phase, with a larger overall impact on the WT strain compared with the GMMO ($F_{1,84} = 79.65$, $P < 0.0001$) (Fig. 3b). Moreover, initial strain frequency did not produce significant differences in the WT populations in the presence of phage after 5 h ($F_{3,44} = 2.72$, $P = 0.06$). By contrast, there was a significant effect of strain frequency on GMMO population density in the presence of phage ($F_{3,40} = 16.04$, $P < 0.0001$), and this difference is mostly explained by the treatment when the GMMO was alone (100% Fig. 3b). Irrespective of the relative frequencies of the two strains, after 48 h the WT populations attained higher average densities in the absence than in the presence of phage (cf. Fig. 3c,d) ($F_{1,40} = 9.04$, $P = 0.0048$). The GMMO, by contrast achieved similar average densities (c 10^8 cfu mL^{-1}) in the presence and in the absence of phage ($F_{1,40} = 0.06$, $P < 0.801$), suggesting that the WT population is more affected by phage predation than the GMMO.

We then tested the hypothesis that the differences in growth rates between the two strains are at the basis of

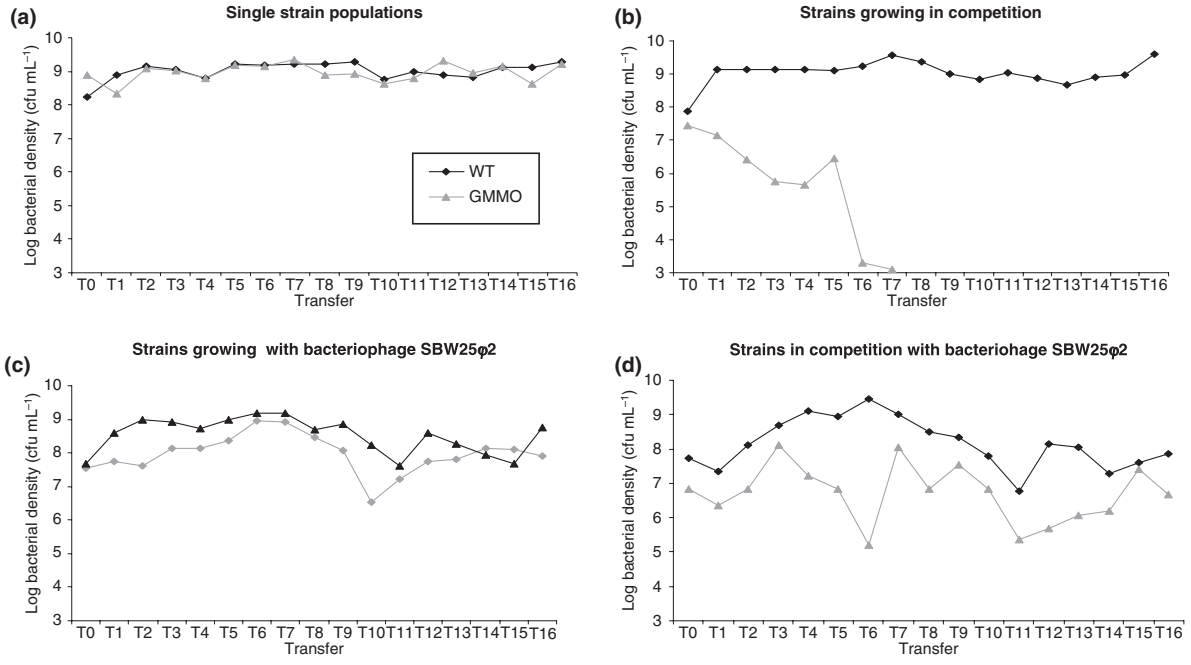

Fig. 2 Bacterial population dynamics of the *Pseudomonas fluorescens* SBW25 [wild type (WT-black line)] and the GM-BCA *P. fluorescens* 23.10R [genetically engineered strains (GMMO-grey line)] through time, under different evolving treatments. Data are in log scale and lines represent mean population densities ± SE of six replicates.

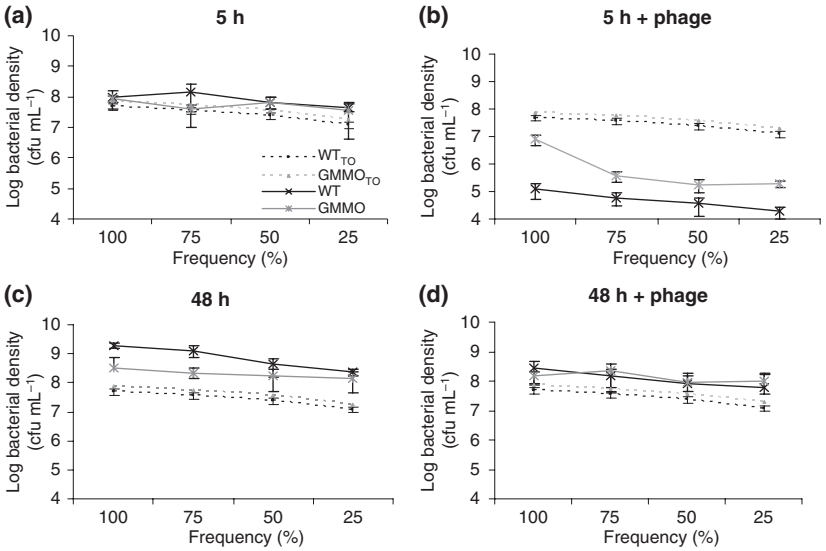

Fig. 3 Bacterial density of wild type (WT) and genetically engineered strain (GMMO) populations growing alone (100%) or at different strain frequencies (75%, 50% or 25%) in competing microcosms. Note that, for example, the cultures containing 75% of the WT, were grown with 25% of the GMMO, and vice versa. The initial densities of each of the strains (T0, dotted lines) are presented in all figures as comparison with the densities achieved after either 5 or 48 h of incubation in the absence (a, c) or presence (b, d) of the phage. Data are on a log scale and lines represent mean population densities ± SE of six replicates.

Table 1 Number of populations of fast or slow growing SBW25 bacteria lines that were susceptible to predation by phage and had coevolved with the wild type (WT) or the genetically engineered strains (GMMO) strain for 32 days under standard conditions.

Bacteria/phage	Fast growing r = 0.61	Slow growing r = 0.37	Total
WT phage	6	1	7
GMMO Phage	16	3	19
Total	22	4	26

A total of 144 phage-bacteria population combinations were tested and 26 were susceptible to phage predation.

their differential susceptibility to phage. We estimated the resistance to phage of twelve SBW25 WT populations with different growth rates (six fast growers, mean r = 0.61; and six slow growers, mean r = 0.37). Twelve phage populations were isolated after *c* 70 generations (10 transfers) of coevolution with the WT or GMMO strains and confronted with 12 clones from each of the 12 bacterial populations. We found a highly significant effect of growth rate on bacterial susceptibility to phage ($\chi^2_1 = 9.36$, $P < 0.0022$). As expected, slow growing bacteria were more resistant to phage predation ($F_{1,84} = 79.65$, $P < 0.0001$) (Table 1).

Discussion

In our system, the acquired phenazine gene is not under strong positive directional selection (see Fig. S2), and evolutionary models would predict that the GMMO will always be out-competed by the WT (Berg & Kurland, 2002; Novozhilov *et al.*, 2005). So how can the GMMO persist in the presence of phage and the WT when it pays a cost to harbouring the inserted gene (Fig. 1), is outcompeted by the WT (Fig. 2b), and is more affected by the phage than the WT (Fig. 2c)?

In systems with complex interspecific interactions involving pairs of prey species attacked by the same predator, some ecological models predict coexistence when one species is superior at resource competition (greater ability to deplete resource levels) and the other at apparent competition (higher resistance to natural enemies) (Holt & Pickering, 1985; Holt *et al.*, 1994)). The 'killing the winner' hypothesis predicts the coexistence of two competing species if a virus acts as a balancing factor on species with different growth rates, by preferentially attacking the most abundant one (Thingstad, 2000). Given both the higher growth rate of the WT (Fig. 1) and its dominance at competition (Fig. 2b), we therefore predict that its coexistence with the GMMO necessitates that it be differentially impacted by the phage. Our results strongly suggest that at least initially, the WT is significantly more susceptible to bacteriophage predation (Fig. 3b). One possible explanation for these differences is that given the faster growth rate of the WT, it may be the only of the two strains in a physiological state that favours phage infection (Adams, 1959). Our results support the predictions of the 'killing the winner hypothesis' in that the bacteriophage preferentially attacks the competitor with the higher growth rate. However, contrary to our system, this hypothesis is based on a model of host-specific viruses and does not consider evolutionary dynamics in the bacteria–virus interaction. In fact, although short-term ecological dynamics of the bacteria–phage interaction favour the GMMO strain over the WT (Fig. 3b,d), the long-term dynamics of the coevolutionary race between the bacteria and the phage favour the WT strain, as indicated by its higher average population densities when growing in the absence of the GMMO, but in the presence of the phage (Fig. 2c). This can simply be explained by the lower intrinsic growth rate of the GMMO with respect to the WT.

Kawecki (1998) developed a model of coexistence that includes host-parasite-coevolution, in which a parasite capable of host choice can become specialized on its preferred host (see also Kawecki & Holt, 2002; Nuismer & Thompson, 2006). Recent experimental study (Heineman *et al.*, 2008) indicates that bacteriophages are indeed capable of evolving preferences for more suitable hosts, possibly through modifications to tail fibres (Montag *et al.*, 1987), which are key in determining phage adsorption to specific surface proteins on bacteria. A recent study on coevolutionary interactions between wild type *P. fluorescens* SBW25 and its bacteriophage φ2 indicates that phage adapts to its host by producing both specialists and generalists (Poullain *et al.*, 2008). Crucially, we suggest that some degree of specialization is likely to occur at the strain level, because inter-population differences between the WT and the GMMO as suitable hosts are ostensibly greater than within-strain variation. In fact, our results showed a significant effect of phage type (coevolving with WT or GMMO) on bacterial susceptibility ($\chi^2_1 = 8.06$, $P < 0.0045$) (Table 1). Nevertheless, additional experimental work is necessary to confirm this prediction.

In our experimental system, the differences in growth rates of the two competing strains might have induced disruptive selection on the phage population, generating partially decoupled coevolutionary dynamics between the WT and the early-infective phages on the one hand, and the GMMO and the later-infective phages on the other. Such specialization could change the outcome of competition in agreement with the 'kill the winner hypothesis', allowing the persistence of novel genes in the population. Thus our results show that phage permits the persistence of the GMMO in the absence of direct selection for the encoded phenazine gene, despite the GMMO being both more affected by phage as compared to the WT (Fig. 2c) and an inferior competitor (Fig. 2b). The long-term persistence in more realistic environments remains to be addressed.

Conclusions

Very little is known about how novel genotypes—be they point mutations of major effect or horizontally transferred genes—persist in nonselective environments (Kurland *et al.*, 2003). Such persistence could be important to establishment and spread because the selective conditions favouring the novel genotype may be rare or temporally variable (Novozhilov *et al.*, 2005). Our findings point to specialization of natural enemies on ancestors and on novel genotypes in promoting persistence. Both ecological and evolutionary forces may promote such specialization, particularly when there are differences in host susceptibility to infection (Kawecki, 1998) due for example, to the differences in host growth rate, host quality (Heineman *et al.*, 2008), spatial heterogeneity (Kawecki & Ebert, 2004), or temporal variability in host relative frequencies (Wolinska *et al.*, 2006). We suggest that this unexpected effect of natural enemies may be of fundamental importance in the dynamics of biodiversity, and could have applications in the management of pathogens, antibiotic resistance and genetically modified microorganisms.

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Author contributions

P.E.-P., A.B. and M.E.H. conceived and designed the experiments. P.E.-P., N.F. and C.G.-B. performed the experiments. P.E.-P. analysed the data. M.E.H. contributed reagents/materials/analysis tools. P.E.-P. and M.E.H. wrote the paper.

Competing interests

The authors have declared that no competing interests exist.

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Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1 Bacterial population dynamics of the wild type (WT) and genetically engineered strains (GMMO) for each of the six replicate microcosms of the competition treatment in the presence of the phage.

Figure S2 Testing for effects of phenazine on bacterial growth and phage resistance.

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