LETTERS

Coevolution with viruses drives the evolution of bacterial mutation rates

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Bacteria with greatly elevated mutation rates (mutators) are frequently found in natural[1–3] and laboratory[4,5] populations, and are often associated with clinical infections[6,7]. Although mutators may increase adaptability to novel environmental conditions, they are also prone to the accumulation of deleterious mutations. The long-term maintenance of high bacterial mutation rates is therefore likely to be driven by rapidly changing selection pressures[8–14], in addition to the possible slow transition rate by point mutation from mutators to non-mutators[15]. One of the most likely causes of rapidly changing selection pressures is antagonistic coevolution with parasites[16,17]. Here we show whether coevolution with viral parasites could drive the evolution of bacterial mutation rates in laboratory populations of the bacterium *Pseudomonas fluorescens*[18]. After fewer than 200 bacterial generations, 25% of the populations coevolving with phages had evolved 10- to 100-fold increases in mutation rates owing to mutations in mismatch-repair genes; no populations evolving in the absence of phages showed any significant change in mutation rate. Furthermore, mutator populations had a higher probability of driving their phage populations extinct, strongly suggesting that mutators have an advantage against phages in the coevolutionary arms race. Given their ubiquity, bacteriophages may play an important role in the evolution of bacterial mutation rates.

Antagonistic coevolution with parasites (the reciprocal evolution of host defence and parasite counter-defence mechanisms) has long been recognized as a potentially important force in the evolutionary maintenance of sexual reproduction[16]. Sex often results in offspring that are genetically distinct from their parents, and hence may be more resistant to parasites that are adapted to parental genotypes[16]. Increased mutation rates in bacterial populations may confer similar indirect benefits in the absence of sex. Lytic bacteriophages are ubiquitous and require bacterial cell lysis after infection and replication to transmit to new hosts; hence there is very strong reciprocal selection for bacterial resistance and phage infectivity. This interaction can lead to ongoing antagonistic coevolution between bacteria and phages[18,19], which creates conditions where mutator alleles may increase in frequency by hitch-hiking with the beneficial resistance mutations they generate.

We performed simple computer simulations to address the conditions under which coevolution with bacteriophages could result in the evolution of elevated mutation rates in bacteria, and the consequences of mutators to the fitness of the coevolving phage populations (see Supplementary Information). Despite an inherent cost to being a mutator (an increased chance of accumulating deleterious mutations at loci under stabilizing selection), mutators were able to increase in frequency under a wide range of conditions. An example of the dynamics of a mutator allele and one particular host genotype is shown in Fig. 1a. Furthermore, increasing the mutation supply rate of the bacterial population (the product of population size and mutation rate) caused a decrease in bacteriophage fitness (Fig. 1b) as a result of an increased resistance of bacteria to their contemporary phage population. The success of mutators decreased with increased costs associated with resistance to phages, increased temporal fluctuations in population sizes of bacteria and phages, and when the specificity of interaction between bacteria and phages[20,21] allowed generalists with wide resistance and infectivity ranges, respectively, to evolve.

To experimentally address whether coevolution with bacteriophages drives the evolution of mutation rates, we evolved 36 populations of the common plant-colonizing bacterium *P. fluorescens*[22] in laboratory microcosms in the presence of a naturally associated lytic DNA phage[18], and 36 populations in the absence of phages. Cultures were propagated by batch culture in King's Media B (KB), diluting 1% of each culture in fresh media on a daily basis, for a total of 24

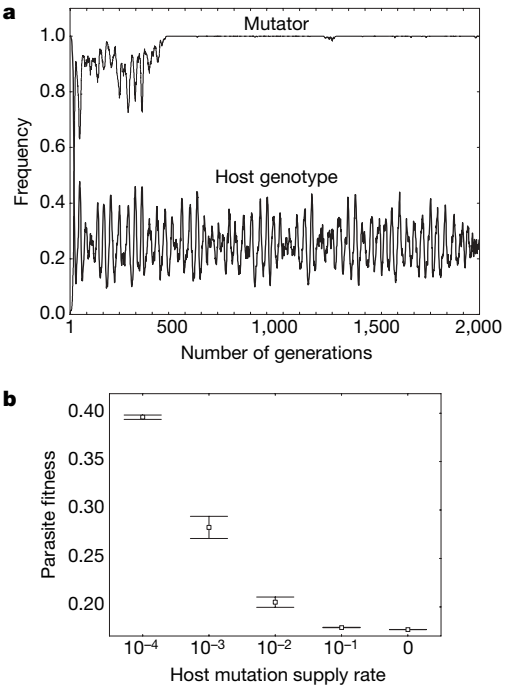

Figure 1 | Simulation results (see Supplementary Information). a, Change in frequency of the mutator through time under a Matching Alleles model ($a = 1$). Graph also shows the dynamics of one (out of four possible) host-resistance genotypes, which fluctuates through time as a result of coevolution with phages. For details on parameter set used, see Supplementary Information. **b**, Mean ± two standard errors of the mean fitness (measured over the final 500 generations of a 2,000 generation simulation) of phage populations as a function of the mutation supply rate (product of mutation rate and population size) of the host population.

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transfers (approximately 170 bacterial generations), and frozen every six transfers. Previous studies have shown that SBW25 and SBW25ϕ2 undergo antagonistic coevolution in KB[18,20,23], and we confirmed this result in the present study (see Supplementary Information).

We estimated the mutation rates of each population using fluctuation tests[4,24]. After 24 transfers, we found that mutation rates had increased 10- to 100-fold in 9 out of 36 populations coevolving with phages (Fig. 2). No significant increases in mutation rates were observed in any of the populations evolving in the absence of phages. We subsequently estimated mutation rates in all populations coevolving with phages at transfers 6, 12 and 18, and found a steady increase in the frequency of populations with elevated mutation rates through time (Fig. 3). We continued evolving the nine mutator populations identified at transfer 24 for a further 24 transfers and found that mutation rates remained at significantly higher levels than the ancestor in all cases.

Bacterial mutation rates can increase through phenotypic stress responses[25] as well as through genetic mutation. To confirm a genetic basis to the elevated mutation rates in our populations, we sought to identify the genes in which the mutations occurred. We isolated four clones from each of the nine mutator populations from transfer 24; of these, mutator clones were identified in seven populations (one out of four clones were mutators in three populations; three out of four in one population; and four out of four in three populations). In the other two populations, mutators must have been at relatively low frequencies. We chose a single random mutator clone from each of these seven populations for further analysis. Previous studies suggest that most mutators result from mutations in the methyl-directed mismatch repair (MMR) system[1,2,4–6], so we attempted to systematically complement MMR alleles with wild-type alleles from the closely related bacteria *P. aeruginosa*[26,27] (see Supplementary Information). In six out seven cases, wild-type mutation rates were restored by this complementation process: five with the *mutL* wild-type *P. aeruginosa* allele, and one with the *mutS* allele (see Supplementary Table 3). It is unclear which mutations were responsible for the elevated mutation rate in the seventh clone.

We have shown that mutators are more likely to evolve when bacteria coevolve with phages than when they evolve in isolation, but it is less clear why. In our simulations, mutators are indirectly favoured because they hitch-hike with alleles that confer resistance to coevolving phage populations. An increase in the frequency of mutators should therefore reduce phage fitness. Strong support for this hypothesis is that phage populations showed a greater tendency to be driven extinct when associated with mutator bacteria, compared with extinction rates of phages with non-mutators (Fig. 3; randomization test: $P = 0.015$; see Supplementary Information). Coevolution with phages could also reduce bacterial population density, which could result in the evolution of mutation rates through both an increased probability of genetic drift and reduced mutation supply rate (the product of mutation rate and population size)[28]. To address this possibility, we measured population densities of bacteria coevolving with phages across time points (transfer 6, 12, 18). We found no differences between populations with or without elevated mutation rates, strongly suggesting that population size was not an important factor contributing to patterns of mutation rate evolution (Mann–Whitney *U*-test, $P > 0.4$ for all three time points).

To confirm the benefits of elevated mutation rates of bacteria when coevolving with phages, we constructed a *mutS* knockout of *P. fluorescens* SBW25 (see Supplementary Information), which conferred an approximately 100-fold higher mutation rate. When the wild-type and *mutS* mutant were competed (see Supplementary Information), we generally found a massive selective advantage in the presence, but not the absence, of phages (Fig. 4). However, this advantage was positively frequency dependent, such that the mutators could always invade when initiated at frequencies of 10^{-2} and 10^{-4}, but that invasion success was limited when initiated at frequencies of 10^{-6}. This last result is consistent with the results of our first experiment, where mutators only increased in frequency in a quarter of populations over the course of 170 generations. Such positive frequency-dependent selection of mutators is consistent with previous theoretical[10–12] and experimental studies[5,29]. (see also simulations in the Supplementary Information). It presumably results from the wild-type population having a higher probability of evolving beneficial mutations when the mutator population is at a very low frequency. In a separate experiment, we found that populations of the *mutS* knockout were much more likely to drive their coevolving populations of phages extinct than were populations of the wild type (phage were at undetectable levels in 3 out of 24 versus 10 out of 24 replicate populations in the presence of the mutator and wild type, respectively; Fisher's exact test, $P = 0.02$). Thus these experiments initiated with mutator and wild-type genotypes confirm results from our *de novo* evolution experiments (1) that mutators are likely to have a selective advantage when coevolving with phages, and (2) that the mutators provide an advantage relative to phage in the coevolutionary arms race, presumably because of the more rapid generation of resistance mutations.

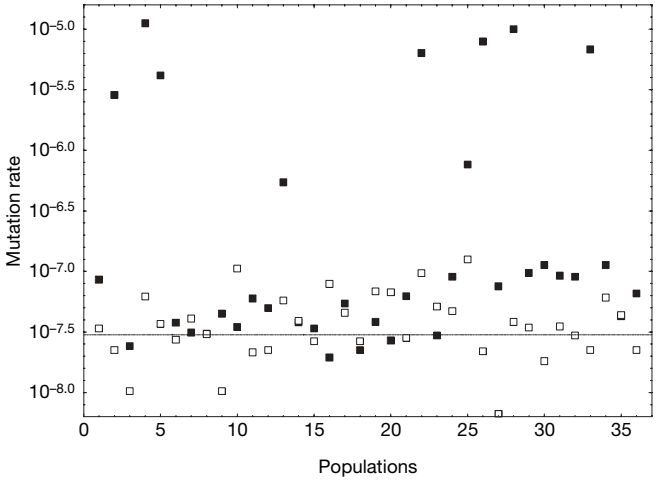

Figure 2 | Relative mutation rates. The estimated mutation rate (for rifampicin resistance) of bacteria in populations evolving with (closed symbols) and without (open symbols) phages; the line indicates the ancestor. Relative estimates using streptomycin gave the same qualitative results. Nine out of 36 populations coevolving with phages had evolved significantly higher mutation rates than the ancestor (Mann–Whitney *U*-test, $n = 6$ for evolved population and ancestral populations, $P < 0.01$ for all cases), whereas no control populations were mutators ($P > 0.1$ for all cases). The number of mutator populations was higher in the presence versus the absence of phages (Fisher's exact test: $P = 0.001$).

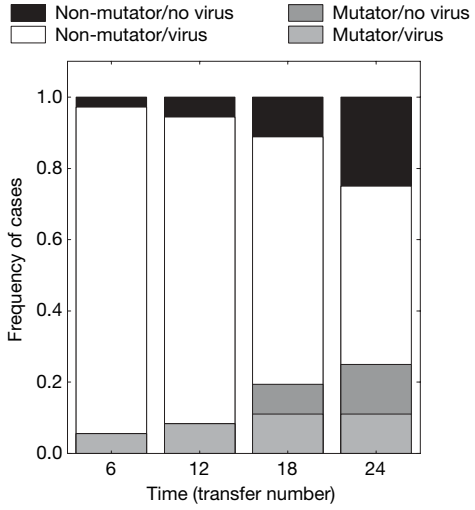

Figure 3 | The frequency of coevolving populations of bacteria evolving elevated mutation rates and driving phages extinct, through time. By transfer 24, 6 out of 9 mutator populations had driven their phages extinct, while 9 out of 27 non-mutator populations had driven their phages extinct.

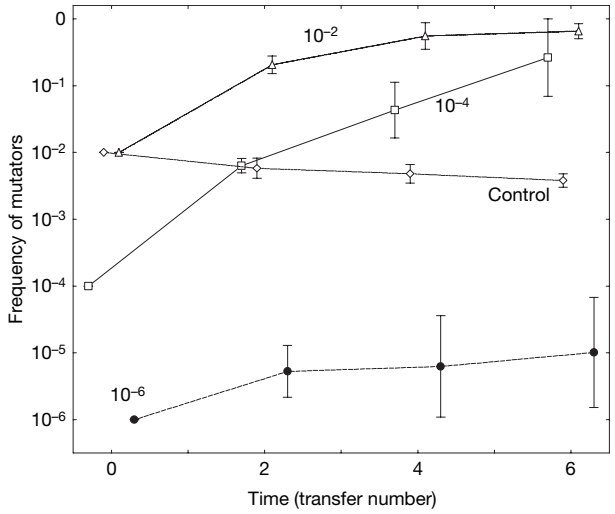

Figure 4 | Competition experiments between wild-type and isogenic mutator. Mean ± 95% confidence intervals through time. There was a significant increase in the frequency of mutators through time when initiated at frequencies of 0.01 (triangles; $F_{3,20} = 113.9$, $P < 0.0001$) and 10^{-4} (squares; $F_{3,20} = 46.1$, $P < 0.0001$), but not at 10^{-6} (filled circles; $F_{3,20} = 1.5$, $P > 0.2$ in the presence of phages. There was a significant decrease in mutator frequency in the absence of phages when mutators were initiated at 0.01 (open diamonds; $F_{3,20} = 7.1$, $P < 0.001$).

Here we have shown that antagonistic coevolution with phages can drive the evolution of elevated mutation rates in bacterial populations. The most probable explanation for this result is that mutator alleles hitch-hike with the beneficial phage resistance mutation they generated. The ubiquity of bacteriophages suggests that they may play a pivotal role in explaining why mutators persist at relatively high frequencies in many natural bacterial populations. As such, targeting phage populations may weaken selection for mutator bacteria in clinical infections. More generally, the study provides the first direct experimental evidence that a mechanism that increases genetic variation can be individually advantageous when coevolving with parasites.

METHODS SUMMARY

Study organisms and culture conditions. We coevolved the common plant-colonizing bacterium, *Pseudomonas fluorescens* SBW25 (ref. 22) and a naturally associated DNA phage, SBW25Φ2 (ref. 18). Note that we do not yet know if bacterial mutation rates affect phage mutation rates, either physiologically or by imposing selection. Seventy-two microcosms (25 ml glass universal bottle microcosms containing 6 ml King's medium B (KB)) were inoculated with 10^8 cells of *P. fluorescens* SBW25 (ref. 22). Half of the 72 microcosms were inoculated with 10^5 clonal particles of DNA phage SBW25Φ2 (ref. 18). Under these conditions, phages fail to completely lyse bacterial populations, because of the rapid emergence of bacteria resistant to the ancestral phage. Populations were propagated in a shaken incubator (200 r.p.m.; 0.9*g*) at 28 °C. Sixty microlitres of each culture was transferred to fresh medium every 24 h, for 24 transfers (approximately 170 generations). After every sixth transfer, populations were frozen in 1:4 v:v glycerol:KB solution at −86 °C.

Measurement of mutation frequency. We used modified fluctuation tests to estimate bacterial mutation rates[4,24]. Six microcosms per population were inoculated with 100–1000 bacterial cells and were allowed to grow for 24 h in a shaken (0.9*g*) 28 °C incubator. We regularly checked for the presence of pre-existing mutants of the trait investigated (antibiotic resistance) in the starting populations. Final cell density was determined by plating dilutions on non-selective solid medium (KB). The number of mutants was estimated by plating 60 μl of each culture on solid selective medium (KB plates mixed with rifampicin (100 mg ml^{-1}) or streptomycin (50 mg ml^{-1})). Jones median estimator was used to calculate mutation rate from the average and median frequency of mutant colonies[30]. Importantly, presence of phage in bacterial cells had no direct effect on mutation rates: increased mutation rates remained after isolating bacterial populations from phage using Virkon[20]. Note that Virkon treatment caused up to 40% bacterial mortality.

Received 9 July; accepted 4 October 2007.
Published online 2 December 2007.

1. LeClerc, J. E., Li, B. G., Payne, W. L. & Cebula, T. A. High mutation frequencies among *Escherichia coli* and *Salmonella* pathogens. *Science* **274,** 1208–1211 (1996).
2. Matic, I. *et al.* Highly variable mutation rates in commensal and pathogenic *Escherichia coli*. *Science* **277,** 1833–1834 (1997).
3. Trong, H. N. G., Prunier, A. L. & Leclercq, R. Hypermutable and fluoroquinolone-resistant clinical isolates of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **49,** 2098–2101 (2005).
4. Sniegowski, P. D., Gerrish, P. J. & Lenski, R. E. Evolution of high mutation rates in experimental populations of *E. coli*. *Nature* **387,** 703–705 (1997).
5. Giraud, A. *et al.* Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut. *Science* **291,** 2606–2608 (2001).
6. Oliver, A., Canton, R., Campo, P., Baquero, F. & Blazquez, J. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* **288,** 1251–1253 (2000).
7. Denamur, E. *et al.* High frequency of mutator strains among human uropathogenic *Escherichia coli* isolates. *J. Bacteriol.* **184,** 605–609 (2002).
8. Leigh, E. G. Natural selection and mutability. *Am. Nat.* **104,** 301–305 (1970).
9. Ishii, K., Matsuda, H., Iwasa, Y. & Sasaki, A. Evolutionarily stable mutation-rate in a periodically changing environment. *Genetics* **121,** 163–174 (1989).
10. Taddei, F. *et al.* Role of mutator alleles in adaptive evolution. *Nature* **387,** 700–702 (1997).
11. Tenaillon, O., Toupance, B., Le Nagard, H., Taddei, F. & Godelle, B. Mutators, population size, adaptive landscape and the adaptation of asexual populations of bacteria. *Genetics* **152,** 485–493 (1999).
12. Tanaka, M. M., Bergstrom, C. T. & Levin, B. R. The evolution of mutator genes in bacterial populations: the roles of environmental change and timing. *Genetics* **164,** 843–854 (2003).
13. Palmer, M. E. & Lipsitch, M. The influence of hitchhiking and deleterious mutation upon asexual mutation rates. *Genetics* **173,** 461–472 (2006).
14. Andre, J. B. & Godelle, B. The evolution of mutation rate in finite asexual populations. *Genetics* **172,** 611–626 (2006).
15. Denamur, E. *et al.* Evolutionary implications of the frequent horizontal transfer of mismatch repair genes. *Cell* **103,** 711–721 (2000).
16. Hamilton, W. D., Axelrod, R. & Tanese, R. Sexual reproduction as an adaptation to resist parasites (a review). *Proc. Natl Acad. Sci. USA* **87,** 3566–3573 (1990).
17. West, S. A., Lively, C. M. & Read, A. F. A pluralist approach to sex and recombination. *J. Evol. Biol.* **12,** 1003–1012 (1999).
18. Buckling, A. & Rainey, P. B. Antagonistic coevolution between a bacterium and a bacteriophage. *Proc. R. Soc. Lond. B* **269,** 931–936 (2002).
19. Mizoguchi, K. *et al.* Coevolution of bacteriophage PP01 and *Escherichia coli* O157: H7 in continuous culture. *Appl. Environ. Microbiol.* **69,** 170–176 (2003).
20. Morgan, A. D., Gandon, S. & Buckling, A. The effect of migration on local adaptation in a coevolving host–parasite system. *Nature* **437,** 253–256 (2005).
21. Agrawal, A. & Lively, C. M. Infection genetics: gene-for-gene versus matching-alleles models and all points in between. *Evol. Ecol. Res.* **4,** 79–90 (2002).
22. Rainey, P. B. & Bailey, M. J. Physical and genetic map of the *Pseudomonas fluorescens* SBW25 chromosome. *Mol. Microbiol.* **19,** 521–533 (1996).
23. Morgan, A. D. & Buckling, A. Relative number of generations of hosts and parasites does not influence parasite local adaptation in coevolving populations of bacteria and phages. *J. Evol. Biol.* **19,** 1956–1963 (2006).
24. Luria, S. & Delbruck, M. Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* **28,** 491–511 (1943).
25. Bjedov, I. *et al.* Stress-induced mutagenesis in bacteria. *Science* **300,** 1404–1409 (2003).
26. Oliver, A., Levin, B. R., Juan, C., Baquero, F. & Blazquez, J. Hypermutation and the preexistence of antibiotic-resistant *Pseudomonas aeruginosa* mutants: implications for susceptibility testing and treatment of chronic infections. *Antimicrob. Agents Chemother.* **48,** 4226–4233 (2004).
27. Oliver, A., Baquero, F. & Blazquez, J. The mismatch repair system (mutS, mutL and uvrD genes) in *Pseudomonas aeruginosa*: molecular characterization of naturally occurring mutants. *Mol. Microbiol.* **43,** 1641–1650 (2002).
28. de Visser, J., Zeyl, C. W., Gerrish, P. J., Blanchard, J. L. & Lenski, R. E. Diminishing returns from mutation supply rate in asexual populations. *Science* **283,** 404–406 (1999).
29. Chao, L. & Cox, E. C. Competition between high and low mutating strains of *Escherichia coli*. *Evolution Int. J. Org. Evolution* **37,** 125–134 (1983).
30. Rosche, W. A. & Foster, P. L. Determining mutation rates in bacterial populations. *Methods* **20,** 4–17 (2000).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank A. Spiers for providing sequence data. This work was funded by NERC UK (A.B. and C.P.); the Royal Society (A.B.); EMBO and Hungarian Research Grant (C.P.); Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Spanish Network for the Research in Infectious Diseases (A.O. and M.D.M.).

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