The ISME Journal (2013) 7, 2242–2244

www.nature.com/ismej

SHORT COMMUNICATION

Coevolution with phages does not influence the evolution of bacterial mutation rates in soil

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Coevolution with phages drive the evolution of high bacterial mutation rates *in vitro*, but the relevance of this finding to natural populations is unclear. Here, we investigated how coevolution affects mutation rate evolution in soil, in the presence and absence of the rest of the natural microbial community. Although mutation rate on average increased threefold, neither coevolving phages nor the rest of natural community significantly affected mutation rates. Our results suggest that features of the soil over and above directly interacting organisms constrain the evolution of strong mutators, helping to explain their relatively low frequency compared with some laboratory and clinical settings.
The ISME Journal (2013) **7,** 2242–2244; doi:10.1038/ismej.2013.105; published online 4 July 2013
Subject Category: Microbial population and community ecology
Keywords: bacteria mutator; coevolution; mutation rate; phage; *Pseudomonas fluorescens*; soil

Mutator bacteria readily evolve in experimental populations (Sniegowski *et al.*, 1997; Giraud *et al.*, 2001; Pal *et al.*, 2007), and are found at relatively high frequencies in both natural populations (LeClerc *et al.*, 1996; Matic *et al.*, 1997) and in clinical opportunistic pathogen populations (Oliver *et al.*, 2000). Although mutators may increase adaptability to novel environmental conditions (Sniegowski *et al.*, 1997; Giraud *et al.*, 2001; Pal *et al.*, 2007), they are also prone to the accumulation of deleterious mutations (McDonald *et al.*, 2012). The long-term maintenance of high mutation rates requires rapidly changing selection pressures (Tenaillon *et al.*, 1999; Denamur and Matic, 2006; Desai and Fisher, 2011), in addition to the possible slow transition rate by point mutation from mutators to non-mutators (Denamur *et al.*, 2000). One of the most likely causes of rapidly changing selection pressures is coevolution with lytic bacteriophages (Pal *et al.*, 2007). Lytic bacteriophages are ubiquitous, and require bacterial cell lysis following 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 (Bohannan and Lenski, 2000; Buckling and Rainey, 2002); hence, creating conditions where mutator alleles may increase in frequency by hitch-hiking with the beneficial resistance mutations they generate.

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Received 5 February 2013; revised 2 May 2013; accepted 24 May 2013; published online 4 July 2013

Recent *in vitro* work has shown that coevolution between the bacterium *Pseudomonas fluorescens* SBW25 (Rainey and Bailey, 1996) and a lytic dsDNA phage (Buckling and Rainey, 2002) rapidly results in the evolution of elevated mutations rates, and competition between wild-type and isogenic mutator bacteria confirm the selective advantage in the presence but not the absence of coevolving phages (Pal *et al.*, 2007). However, the relevance of this finding to natural populations is unclear. Although this bacteria and phage readily coevolve in their natural environment, soil (Gómez and Buckling, 2011), phage-imposed selection for resistance is lower in soil than *in vitro* despite long-term persistence of the phage population (Buckling and Rainey, 2002; Gómez and Buckling, 2011). Moreover, selection for mutation rates imposed by a single phage may be unimportant when experiencing the myriad selection pressures resulting from the community as a whole. Here, we determine the simultaneous roles of a tightly coevolving phage and interactions with the rest of the natural microbial and viral community (NMC) in driving the evolution of mutation rates of *P. fluorescens* in soil. We inoculated 32 sterile soil microcosms; $10 \times 10\,cm^2$ petri dishes containing 100 g of twice autoclaved (unsieved) compost (John Innes no. 2), with *P. fluorescens* SBW25, half of which were then inoculated with a soil wash of the NMC, and half with phage SBW25, in a fully factorial design, as described previously in Gómez and Buckling (2011).

After 48 days evolution in soil, we determined the mutation rates of *P. fluorescens* SBW25 using simple fluctuation tests (Luria and Delbruck, 1943). Twenty bacteria colonies of *P. fluorescens* SBW25 were taken randomly from each of the 32 populations,

and we grew all the colonies from each population together King's media B (KB) overnight. Four new cultures of each of the ancestral bacteria and the 32 evolved bacterial cultures were established with 100 cells from a previous culture, and grown for 24 h at 28 °C in a shaken (200 r.p.m.) incubator, before plating onto both normal KB agar and KB agar supplemented with rifampicin (100 μg ml^{-1}). Bacterial mutation rates were estimated by fluctuation tests for antibiotic resistance (Luria and Delbruck, 1943), having previously established that no colonies from the evolved populations in the soil microcosms were resistant to rifampicin. Mutation rates were calculated and corrected for sampling error using the Ma–Sandri–Sarkar maximum likelihood estimator (MSS-MLE) method (Rosche and Foster, 2000), implemented in the FALCOR web package (Hall *et al*., 2009).

P. fluorescens showed an average threefold increase in mutation rates (Figure 1; one sample *t*-test for all populations against ancestral: $t_{31} = 6.04$, $P < 0.01$), perhaps because the specific conditions represents a slightly novel and stressful environment for *P. fluorescens*. However, populations evolved in the presence of phages did not evolve higher mutation rates than populations evolved in the absence ($F_{1,28} = 4.1$; $P = 0.06$); if anything, the presence of phage resulted in lower mutation rates. The absence of a clear difference in mutation rates between treatments cannot be explained by the absence of phage-imposed selection in the phage treatments: phage persisted throughout the experiment (Gómez and Buckling, 2011), and some bacteria exposed to phages evolved phage resistance to ancestral and sympatric phages, whereas resistance was undetected in the phage-free treatments (Supplementary Figure S1). Moreover, the presence of the NMC or the interaction between phages and the NMC had no impact on mean mutation rates (Figure 1; $P > 0.2$ in both cases).

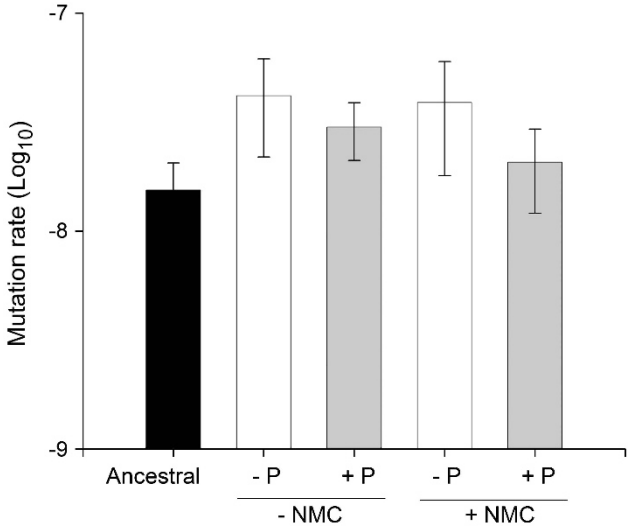

Figure 1 Mean (± s.e.m.) mutation rates of the ancestral *P. fluorescens* SBW25 bacteria (black bar) and evolved bacteria populations isolated after 48 days evolving with (+P; grey bar) and without phages (−P; white bar) and in the absence (−NMC) and presence (+NMC) of the natural microbial community. Mutation rate was determined by fluctuation test to rifampicin resistance and calculated by The Ma–Sandri–Sarkar maximum likelihood estimator (MSS-MLE) method. For evolved bacteria, each bar represents the average of eight independent populations from four replicates per population.

The likelihood of mutators successfully invading populations is increased by population size (which increases the chance of mutators generating beneficial mutations and thus hitch-hiking to high frequencies), time and the strength of selection (Tenaillon *et al*., 1999). We suggest that population size cannot explain the absence of phage-imposed increases in mutation rate because *P. fluorescens* population densities were extremely large (~10^7 g^{-1} soil; Gómez and Buckling, 2011), and only 10–100-fold less than that observed *in vitro* (Buckling and Hodgson, 2007). Although we do not know the generation time of bacteria in soil, it is inevitably longer than in a media, hence the absence of a large change in mutation rate resulting from phage-imposed selection may have resulted from a time constraint of the experiment: strong mutators may not have had a chance to reach high frequencies. To investigate this possibility, we competed a *lacZ*-marked of *P. fluorescens* SBW25 (wild-type) against a *mutL*-knockout mutant of *P. fluorescens* SBW25 (a 100-fold mutator) (Pal *et al*., 2007) in the presence and the absence of phages for 10 days in soil—a period long enough for resistance evolution to occur and hence for any relative advantage of mutators in the absence versus the presence of phages to be uncovered. Mutators suffered a general competitive cost in soil ($t_{11} = 3.69$, $P < 0.01$ in the absence of any genetic marker; Pal *et al*., 2007), possibly due to pleiotropic effects of the knockout, and that was not influenced by phages (Figure 2; $F_{1,10} = 3.453$; $P = 0.09$). We can therefore conclude that phage simply do not impose sufficiently strong selection on bacteria in soil for mutator alleles to hitch-hike to detectable frequencies.

Why do phages select for large increases *in vitro* but not *in vivo*, having ruled out the impact of the rest of natural microbial community? Numerical simulations (Pal *et al*., 2007) suggest a major factor affecting the evolution of mutators when coevolving with phages is the cost associated with phage resistance: when costs are high, there is weaker selection both for resistance and the mutator alleles that generate resistance mutations. Crucially, costs of resistance are much greater in soil than *in vitro* (Gómez and Buckling, 2011). Moreover, the coevolutionary dynamics between bacteria and virus are qualitatively different in soil compared with *in vitro*: coevolution in soil is characterised by fluctuations in resistance and infectivity through space and time (Gómez and Buckling, 2011), whereas coevolution *in vitro* follows and arms race dynamic (Gandon *et al*., 2008), resulting in bacteria and phages evolving increasingly wide resistance and infectivity ranges through time, respectively (Buckling and Rainey, 2002).

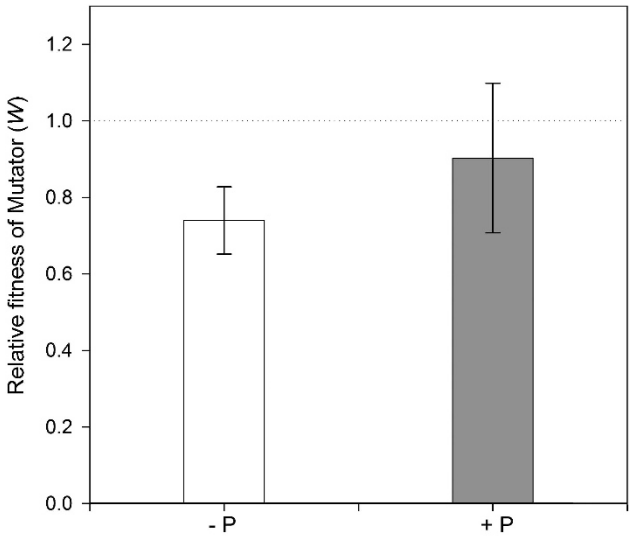

Figure 2 Relative fitness of the *P. fluorescens* SBW25 mutator (*mutL)* bacteria to the wild-type bacteria in the presence (+P; grey bar) and the absence (−P; white bar) of phages after 10 days of competition in soil. Bars represent mean (±s.e.m.) relative fitness ($n=6$). Note that if relative fitness = 1, mutator and wild-type bacteria are equally fit.

In summary, direct evolutionary interactions with viruses and the other members of soil microbial communities appear to have relatively minor roles in driving the evolution of bacterial mutators in soil. Other features of the soil environment appear to select for relatively low mutation rates, as confirmed by the fitness cost of the 100-fold mutator in the competition experiments. We cautiously generalise the results from this experimental system on the basis that spatial patterns of phage adaption to bacteria in natural soil communities are consistent with rapid bacteria–virus coevolution being the norm (Vos *et al.*, 2009). These results may therefore help to explain why bacteria with high mutation rates (10–1000-fold higher than the wild-type) are found at relatively low frequencies (<2%) in soil, but can be found at much higher frequencies in the lab and in clinical infections.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

We thank two anonymous reviews for comments on the manuscript. The work was supported by European Research Council. PG was supported by a Marie Curie Intra-European Fellowship (PIEF-GA-2010-272945) within the European Union Seventh Framework Programme.

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Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)