Escherichia coli mutators: selection criteria and migration effect

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In silico, it has been shown that mutator alleles that increase mutation rate can be selected for by generating adaptive mutations. *In vitro* and *in vivo*, competition between wild-type bacteria and isogenic mutator mutants is consistent with this view. However, *in vivo*, the gain of the mutator seems to be reduced when migration is allowed. *In vitro*, the advantage of mutators has been described as frequency-dependent, leading to mutator advantage only when they are sufficiently frequent. Using an *in vitro* system, it is demonstrated that (i) the selection of mutators is frequency-independent, yet depends on at least one mutator bacterium bearing an adaptive mutation (its presence depends on chance, mutation rates and population size of mutator bacteria); (ii) on average, the mutator gain is always equal to the ratio of the adaptive mutation frequency of the mutator versus wild-type; (iii) when migration into an empty niche is allowed, the mutator benefit is reduced if migration occurs after fixation of the adaptive mutation into the wild-type population. It is concluded that in all cases, mutator gain depends directly on the ratio of bacteria carrying a beneficial mutation in mutator versus wild-type lineages.

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INTRODUCTION

Numerous natural isolates of *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa* and *Neisseria meningitidis* species appear to be ‘mutators’ (LeClerc *et al.*, 1996; Matic *et al.*, 1997; Oliver *et al.*, 2000; Richardson & Stojiljkovic, 2001; Richardson *et al.*, 2002). When compared to wild-type strains, mutators have an increased mutation rate throughout their genome, due to mutations that disrupt some aspect of DNA replication or repair functions (Friedberg *et al.*, 1995). Most mutators from natural isolates are defective for the methyl-directed mismatch repair system (MMR). Among MMR-defective mutators, the majority are MutS-defective, a key component of the MMR (LeClerc *et al.*, 1996; Matic *et al.*, 1997; Oliver *et al.*, 2002; Richardson *et al.*, 2002). The MMR is the principal post-replicative pathway for DNA replication fidelity. In *E. coli*, the MMR uses *dam* DNA methylation (adenine methylation of GATC sequences) to discriminate between parental and neo-synthesized DNA strands. When replication errors occur, MutS recognizes base mismatches as well as insertions/deletions (up to 4 bases) and mediates the fixation of MutL. MutL recruits

MutH, which cleaves the newly synthesized, unmethylated strand at the nearest GATC sequence. Consequently, the UvrD (MutU) helicase unwinds the strands and the newly synthesized one is degraded by exonucleases in the direction of the mismatch. Finally, DNA polymerase III fills the resultant gap (Modrich & Lahue, 1996). Many other loci, such as *mutY* (MutY removes adenine from 8-oxo-G and A-G mismatches) or *xthA* (XthA is a 5′ abasic endonuclease), have been described in the laboratory as conferring a mutator phenotype when affected (Horst *et al.*, 1999).

The frequency of mutators in natural populations is higher than that generated by mutation and counterselection equilibrium. They may be selected for directly or indirectly (Boe *et al.*, 2000; Mao *et al.*, 1997; Ninio, 1991). It has been proposed that mutator loci could be indirectly selected by ‘hitch-hiking’, together with the advantageous mutations generated (Chao & Cox, 1983). The selection for advantageous mutations leads to the selection of conserved gene linkages and hence the selection of the mutator allele. This beneficial effect would counter-balance the negative impact of deleterious and lethal mutations also overproduced in mutator lineages. This scenario is consistent with *in silico*, *in vitro* and *in vivo* observations (Taddei *et al.*, 1997; Chao & Cox, 1983; Giraud *et al.*, 2001). For the *in vivo* model, the *mutS*⁺ genotype was restored in a *mutS*[−] strain that had previously evolved over 42 days in a mouse digestive tract (this strain was designated *r-mutS*⁺). In competition with the *mutS*⁺ ancestor strain, the *r-mutS*⁺ strain showed a

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Abbreviations: f(Spc^R), frequency of spontaneously occurring spectinomycin-resistant mutants; Nal^R, nalidixic-acid-resistant; Spc^R, spectinomycin-resistant.

similar gain to a *mutS*[−] strain in competition with the *mutS*⁺ ancestor strain (Giraud *et al.*, 2001). These results proved that the selection for mutators is indirect, due to other advantageous mutations and not to the mutator phenotype alone.

Due to their ability to adapt more rapidly than wild-type bacteria, and because their evolutionary clock is accelerated, mutators are frequently used as models by ecologists and evolutionists. Since mutators can be involved in pathologic processes (such as infections and cancers) where multiple adaptive steps seem to be required (Denamur *et al.*, 2002; Loeb, 1998; Oliver *et al.*, 2000; Richardson & Stojiljkovic, 2001), and acquire drug resistance more rapidly than wild-type (Giraud *et al.*, 2002; Oliver *et al.*, 2000), they are considered as risk factors. Thus, an understanding of the conditions that permit mutator selection could be useful in the treatment of infectious diseases and of tumours.

It is generally accepted that mutators are selected when they represent a given proportion of the total population; such a selection is termed frequency-dependent (Chao & Cox, 1983). This conclusion seems paradoxical as it would lead to mutator advantage only when they are already sufficiently frequent. It does not address the initial selective steps for reaching such a situation. A second paradox has been provided by *in vivo* results: in germ-free mice, competition experiments between the wild-type and *mutS*[−] strain led to mutator selection when both populations were inoculated in the same mouse. In contrast, when three mice were kept together – one mono-associated with mutators, one mono-associated with wild-type and the third one initially germ-free – the mutator advantage disappeared in the global population (Giraud *et al.*, 2001). Thus it seems that, for some reason that still remains unclear, allowing migration of strains between mice reduces the mutator advantage *in vivo*.

In this study, an *in vitro* model examining competition, under selective pressure, between mutators and wild-type *E. coli* was developed. *mutS*[−] and *xthA*-defective strains were chosen as mutators (*xthA*[−] is a weaker mutator than

mutS[−]). By varying the mutation rates, population sizes and relative frequency, we show that mutator selection depends solely on one parameter: the presence of at least one spontaneous pre-adapted bacterium within the mutator population. We further demonstrate that this presence is linked to the mutator population size, mutation rate and chance. These results suggest that a reduced mutator advantage associated with migration (Giraud *et al.*, 2001) is due to pre-migration fixation of an adapted clone in the wild-type population. This allows for separate expansion in the selective environment, prior to post-migration mutator/wild-type competition. In all cases, the mutator advantage depended directly on the ratio of bacteria carrying beneficial mutations in mutator versus wild-type populations.

METHODS

Bacterial strains. *Escherichia coli* strains used in this study are listed in Table 1. All were derived from the *E. coli* K12 MG1655, a fully sequenced strain (Blattner *et al.*, 1997). MGS and MGN were spontaneous mutants of MG1655 exhibiting resistance to streptomycin and nalidixic acid, respectively. To construct MGS21 the $\Delta(xth-pncA)90$ allele was obtained by P1 transduction (Miller, 1974) in MGS using a phage lysate developed in strain LG101 (Schaaper *et al.*, 1985). $\Delta(xth-pncA)90$ was co-transduced together with the *zdh-201::Tn10* marker. Transductants were selected on tetracycline-supplemented medium (tetracycline resistance is carried by the Tn10 transposon). The MGS21 mutator phenotype was confirmed by determining the frequency of spontaneously occurring spectinomycin-resistant mutants [*f*(*Spc*^R)].

Culture media and antibiotics. Liquid cultures were performed in 10 ml screw-cap tubes containing 10 ml Luria–Bertani (LB) medium with vigorous shaking, incubated at 37 °C. Colony forming units (c.f.u.) were determined on LB plates. When needed, the medium was supplemented with the following antibiotics: rifampicin (100 mg l^{−1}), tetracycline (15 mg l^{−1}), streptomycin (100 mg l^{−1}), nalidixic acid (Nal, 40 mg l^{−1}) or spectinomycin (Spc, 40 mg l^{−1}).

Determination of the frequency of spontaneously occurring *Spc*^R mutants. Liquid cultures were performed in 10 ml screw-cap tubes containing 10 ml LB medium with vigorous shaking, incubated at 37 °C overnight. The number of c.f.u. was determined on

Table 1. *E. coli* strains used in this study

Strains	Genotype*	Antibiotic marker†	Mutator phenotype	Source or reference
WTStr	<i>srl::Tn10 mutS</i> ⁺	Str ^R	Wild-type	Giraud <i>et al.</i> (2001)
WTNal	<i>srl::Tn10 mutS</i> ⁺	Nal ^R	Wild-type	Giraud <i>et al.</i> (2001)
MutStr	<i>srl::Tn10 mutS3</i>	Str ^R	Mutator	Giraud <i>et al.</i> (2001)
MutNal	<i>srl::Tn10 mutS3</i>	Nal ^R	Mutator	Giraud <i>et al.</i> (2001)
MGS		Str ^R	Wild-type	This work
MGN		Nal ^R	Wild-type	This work
MGS21	$\Delta(xth-pncA)90$ <i>zdh-201::Tn10</i>	Nal ^R	Mutator	This work

*Note that all strains are also *rph*-1, as for the MG1655 strain.

†Only resistances allowing differentiation of competitors are indicated here. Str^R, Streptomycin-resistant; Nal^R, nalidixic-acid-resistant.

LB plates and spectinomycin-supplemented LB plates. The $f(\text{Spc}^R)$ is defined as the ratio c.f.u. on spectinomycin plates/c.f.u. on LB plates. This was done several times for each strain.

Competition experiments. Liquid cultures supplemented with spectinomycin were inoculated with different dilutions of stationary-phase wild-type and mutator. Str^R and Nal^R markers (see Table 1) were used to distinguish between wild-type and mutator strains and markers were reversed to control for their effects. Spectinomycin represents a lethal selective pressure in these experiments. Appropriate dilutions of the overnight cultures were plated onto solid medium supplemented with streptomycin, nalidixic acid or spectinomycin to determine population sizes and Spc^R mutant initial frequencies in the inoculum. After 24 h of competition, appropriate dilutions were plated onto solid medium supplemented with streptomycin or nalidixic acid to determine wild-type and mutator population size.

Migration experiments. Wild-type and mutator overnight cultures were diluted separately in LB medium supplemented with spectinomycin. At different times, an equal volume of mutator and wild-type culture was mixed in a new culture tube (in the presence of spectinomycin, the ‘post-migration culture’) and incubated for 24 h at 37 °C. Mutator and wild-type population levels were measured as described above.

Quantitative mutator gain. This was calculated as the final mutator/wild-type ratio, divided by the initial mutator/wild-type ratio. The $\log_{10}$ of quantitative mutator gain defined a standard estimation of fitness. When this value is >0 , mutators have been selected for; when the value is 0 there is no benefit of the mutator clone; and when it is <0 , mutators are counterselected.

RESULTS

Is mutator selection frequency-dependent?

To determine if mutator selection could depend on their frequency within a population, a first set of competition experiments was performed. Wild-type bacteria (WTStr or WTNal; 2×10^5) with varying numbers of mutators (MutNal or MutStr) were inoculated in fresh medium supplemented with spectinomycin and incubated for 24 h. Both wild-type and mutator pre-cultures were initially sensitive to spectinomycin. Mean rates of generation of spontaneous Spc^R mutants [$f(\text{Spc}^R)$] were 6×10^{-6} and 3×10^{-4} for the wild-type strains and the mutator strains, respectively (independently of the markers). Results obtained after 24 h of culture showed that competition experiments performed with either WTStr and MutNal or WTNal and MutStr gave similar results (Fig. 1a). Hence, the antibiotic resistance markers (Nal^R and Str^R) did not affect competitiveness between the strains. When the initial inoculation ratio of mutator/wild-type was above 10^{-2} , the final mutator/wild-type ratio exceeded the initial ratio, indicating that mutators were selected. When the inoculation ratio was below 10^{-2} , mutators were counterselected. Noticeably, the mutator/wild-type ratio of 10^{-2} represented a critical threshold where mutators could be selected or counterselected. These results could be interpreted as frequency-dependent selection for the mutator lineage.

Fig. 1. Mutator selection is frequency-independent. (a) Competitions inoculated with 2×10^5 wild-type bacteria and different mutator size populations (from 1×10^2 to 4×10^5 , mutS^-). (b) Competitions inoculated with 2×10^7 wild-type bacteria and different mutator size populations (from 1×10^2 to 4×10^7 , mutS^-). $\circ$, Competitions between WTStr and MutNal; $\bullet$, competitions between WTNal and MutStr.

In a second set of competition experiments, the size of the wild-type population present in the inoculum was fixed at 2×10^7 . Again, mutators were selected for when present in the inoculum over a given ratio. Mutators were counterselected when they were under-represented in the inoculum. The use of different markers (Nal^R and Str^R) did not influence the outcome of the competition (Fig. 1b). This observation can again be seen as evidence for frequency-dependent selection of the mutator lineage.

However, the selection thresholds of both sets of experiments (Fig. 1a and b) are clearly different, 10^{-2} versus 10^{-4} . Thus, for a given initial wild-type population size, mutator selection appears to be frequency-dependent. When using different initial wild-type population sizes, the same absolute frequency did not lead to the selection of mutators. This paradoxical result led us to look for other explanations.

Mutator selection depends on the presence of a mutator bearing an adaptive mutation

In the two previous sets of experiments, the selective threshold corresponded to the same initial mutator population size (2.4×10^3 mean for MutStr and MutNal). This population size was similar to the inverse of the frequency of beneficial alleles in the mutators population [$1/f(\text{Spc}^R) = 3 \times 10^3$]. Below this population size, mutators were not selected for, whereas above this population size they were. This population size was considered to be a population

threshold, showing that the selection of the mutators was population-size-dependent.

Twenty-three competition experiments were performed with a mutator inoculum size comparable to the population threshold. In 16 out of the 23 experiments, mutators were selected, whereas in the other seven they were not. This could be due to the stochastic occurrence of adaptive mutations. To check whether mutator selection could be stochastic, a new set of competitions was initiated. The initial mutator population size was tenfold lower than the population threshold (5×10^2) and the wild-type population was 5×10^7 bacteria. As expected, the vast majority (109/117) of the trials led to the decline of mutators. However, in eight trials, mutator selection was observed (Fig. 2). Thus mutator selection depends on chance and the probability of mutators to be selected increases with mutator population size.

Finally, we studied the impact of the mutation rate on mutator selection. Competition experiments were realized between wild-type bacteria and *xth*⁻ mutators. *xth*⁻ mutants are known to generate mutations at a lower rate than *mutS*⁻

Fig. 2. Mutator gain at different initial mutator/wild-type ratios. Expected gain: ratios between $f(\text{Spc}^R)$ in initial mutators and wild-type populations (24 independent measurements). Competitions above mutator threshold (2.4×10^3): quantitative mutator gain for competition experiments inoculated with 10^7 wild-type bacteria (WTStr or WTNal) and 10^7 mutator bacteria (MutNal or MutStr) (14 independent competitions). Competitions below mutator threshold: quantitative mutator gain for competition experiments inoculated with 10^7 wild-type bacteria (WTStr or WTNal) and 10^2 mutator bacteria (MutNal or MutStr) (117 independent competitions). Each open circle corresponds to the ratio for one experiment and lines correspond to the mean of the ratios of all experiments. No marker effect was observed.

mutants. For this set of experiments, the $f(\text{Spc}^R)$ was 7.7×10^{-6} for the *xth*⁻ strain and 2.4×10^{-6} for the wild-type. As with the experiments using the *mutS*⁻ strains, a ratio threshold was observed when competitions were initiated with 10^6 (Fig. 3a) or 10^7 wild-type bacteria (Fig. 3b). In the majority of experiments, mutators were selected when their initial population size was over the ratio threshold and counterselected when under. These two ratios were different but correspond to a similar initial mutator population size, close to the inverse of the frequency of beneficial alleles in the mutator population [$1/f(\text{Spc}^R) = 1 \times 10^5$]. The dependence of mutator selection on population size was thus confirmed. The population threshold of *xth*⁻ mutators was higher than for *mutS*⁻ mutators, suggesting that strong mutators (in this case *mutS*⁻) would be selected in smaller populations than weak mutators (in this case *xth*⁻). This is expected if the presence of a beneficial variant was rate-limiting for mutator lineage selection.

On average, the mutator advantage is always equal to the ratio of mutator to wild-type adaptive mutation frequency

In the 8 of 117 '1:10⁵ competitions' inoculated with 5×10^2 mutators and 5×10^7 wild-type bacteria showing mutator selection (Fig. 2, third column), the quantitative mutator gain was higher than in '1:1 competitions' initiated with 4×10^7 mutators and 5×10^7 wild-type bacteria (Fig. 2, comparison between second and third columns). The mean gain of these eight competitions was 14-fold higher than that in the '1:1 competitions'. However, averaged over the 117

Fig. 3. Effect of mutation rates on mutator selection. (a) Competitions inoculated with 4×10^6 wild-type bacteria (MGN) and different mutator size populations (from 5×10^2 to 5×10^6 MGS21, *xth*⁻). (b) Competitions inoculated with 4×10^7 wild-type bacteria (MGN) and different mutator size populations (from 5×10^2 to 5×10^7 MGS21, *xth*⁻).

competitions, the quantitative mutator advantage was identical to that of the '1:1 competitions' experiments (56 ± 11 for '1:1 competitions' and 54 ± 27 for '1:10⁵ competitions'; Fig. 2, second and third columns). Independently of the initial mutator/wild-type ratio, the mean mutator quantitative advantages were equal to the ratio of the adaptive mutation frequency in mutators to wild-type (52 ± 6 ; Fig. 2, first column).

Mutators do not have to pay a migration cost to see their benefit reduced after migration

As we have shown that mutator selection, by second-order selection, is only dependent on the presence of a mutator bacterium bearing an adaptive mutation, it was of interest to test if the results showing a reduction of mutator advantage when migration was allowed between mouse guts (Giraud *et al.*, 2001) could also be explained by the presence or absence of adaptive bacteria in mutator and wild-type populations. To eliminate potential costs associated with migration between mouse guts, an *in vitro* migration model was developed where bacteria did not pay a migration cost because the initial and final niches were similar and migration was instantaneous. Mutators and wild-type were inoculated in fresh medium supplemented with spectinomycin. At different times, an equal volume of mutator and wild-type cultures was mixed together in a new culture tube (in presence of spectinomycin: the 'post-migration culture') and incubated for 24 h at 37 °C. In the initial cultures,

mutator and wild-type populations exhibited similar growth patterns and reached the same final population size. However, the mutator population grew faster than the wild-type, due to the presence of more numerous adapted variants at the onset of the experiments (Fig. 4a). In the 'post-migration cultures', on average the final mutator/wild-type ratio corresponded to the initial ratio of the adaptive mutation frequency in the mutator to that in the wild-type when migration occurred after up to 15 h of separate culture. With migrations occurring after 18, 21 and 24 h of separate culture, the final mutator/wild-type ratio was reduced to one (Fig. 4b). Without any migration cost, our results showed a reduction of the mutator advantage similar to previous results describing bacterial migration between mice (Giraud *et al.*, 2001); depending on adaptive and migration timing, mutator benefit could be diminished without invoking any migration cost.

DISCUSSION

This work investigated some apparent paradoxes of mutator selection and proposes new ways of solving them. Our data suggest that mutator selection is frequency-independent: competition studies initiated with the same mutator/wild-type ratio showed different outcomes, depending on the size of the inoculum. However, even when the size of the inoculum varied, a selective threshold was seen corresponding to the same initial mutator population size (2.4×10^3). This population size was close to the inverse of the frequency

Fig. 4. *In vitro* migration. (a) Separate cultures of mutators (MutStr or MutNal, ●) and wild-type (WTNal or WTStr, ○). Data points are the means of six replicates. (b) Mutator gain is measured as the ratio of mutator c.f.u./wild-type c.f.u. at the end of competitions over mutator c.f.u./wild-type c.f.u. at time 0, in the 'post-migration' culture. At 0 h, competitions were inoculated with 10^7 wild-type bacteria (WTStr or WTNal) and 10^7 mutator bacteria (MutNal or MutStr) both from overnight cultures without selection (same data as shown in Fig. 2). At 12, 15, 18, 21 and 24 h, competitions were inoculated with the same volume of wild-type (WTStr or WTNal) and mutator (MutNal or MutStr) cultures with selection [cultures shown in (a)]. At 21 and 24 h, this volume represented 10^7 wild-type bacteria and 10^7 mutator bacteria. Each open circle corresponds to the ratio for one experiment and lines correspond to the mean of the ratios of all experiments. No marker effect was observed.

of beneficial alleles in the mutator population [$1/f(\text{Spc}^R) = 3 \times 10^3$]. It is noticeable that at this population size the expected number of adapted mutants [product of $f(\text{Spc}^R)$ and of mutator population size] was close to 1. This means that, on average, there was one bacterium bearing a beneficial allele per mutator population. Over this threshold, the expected number of adapted mutants would be > 1 and mutators were indirectly selected. Below this threshold, there would be no beneficial variant in the mutator population and therefore mutators were not selected. Thus, mutator selection, against lethal selective pressure, solely depends on the pre-existence of at least one adapted bacterium in the initial population.

Consistent with this, it was observed that mutator selection depended on (i) population size; (ii) frequency of adapted mutants in the mutator population [in this case $f(\text{Spc}^R)$]; (iii) chance – in some cases, even when the expected number of adapted mutants in mutator population was < 1 (0.08), mutators increased in frequency in 8/117 competition studies. The stochastic and population-size effects on mutator selection had been previously predicted in theoretical studies (Tenaillon *et al.*, 1999) where the presence of one mutant (in our case adapted) in a given population depends on: (i) the size of the population; (ii) the frequency of generation of such mutants; and (iii) stochastic occurrence. From our results, it can be concluded that mutator selection by hitch-hiking solely depends on the presence of at least one adapted clone in the mutator population before replacement by the wild-type population. Note that the above results were obtained with an almost lethal selective pressure. In cases of a non-lethal selective pressure, lack of one adapted mutant in the initial mutator population can lead to mutator selection only if an adapted mutant can appear in the mutator population before its replacement by the wild-type population. This event should again depend on chance, mutation rate and total number of mutator genomes replicated before the fixation of the non-mutator lineage.

In a small subset of populations, chance can allow mutator selection when, on average, the number of adapted mutants is < 1 . In these cases, mutator selection was associated with a much larger gain (an increase of nearly 800-fold with an adapted mutant inoculum frequency of 0.08). However, averaging all competition, mutator gain was identical regardless of the initial expected number of adapted mutants in the mutator population. As mutator selection depends on the presence of adapted clones, mutator gain corresponds to the ratio of the adaptive mutation frequency in mutators versus wild-type.

Giraud *et al.* (2001) showed that competition between wild-type and *mutS*[−] strains (at a 1:1 ratio) in germ-free mice lead to mutator selection when both populations were inoculated in the same mouse. In contrast, when three mice were housed together, one colonized by mutators, one mono-associated with wild-type and the third initially germ-free, mutator advantage in the metapopulation (sum

of the three bacterial populations) disappeared (Giraud *et al.*, 2001). Genetic amnesia [i.e. the loss of temporary unused genetic functions in a given environment (Funchain *et al.*, 2000; Giraud *et al.*, 2001)] appeared after a longer period of time in mice and could not explain this reduction (Giraud *et al.*, 2001). The reduction of mutator advantage could be due to an intrinsic migration cost (i.e. a pleiotropic cost of *mutS*[−] allele during migration). In the absence of such hypothetical intrinsic migration costs, the reduction of the mutator advantage could also be due to the fact that wild-type and mutator populations were initially in different mice. Actually, separation could allow the adaptation of the wild-type population, as it has enough time to fix beneficial mutations. Our *in vitro* system allowed us to test the separation hypothesis in a context where mutators do not pay this hypothetical migration cost. In our experiments, mutators grew faster, presumably because the initial inoculum contained more adapted (*Spc*^R) mutants than the initial wild-type inoculum. In the 'post-migration cultures', mutators lost their advantage when the wild-type inoculum contained an equal number of pre-adapted mutants to the mutator inoculum. Thus, in the 'post-migration culture', the competition would be between pre-adapted mutators and pre-adapted wild-type bacteria. Under such circumstances, mutators were not favoured anymore. The mutators did not pay a migration cost, yet their advantage disappeared when competition happened after the fixation of beneficial alleles. The reduction of mutator gain was due to the fixation of adapted clones in the wild-type population, resulting from separate growth in the selective environment before competition. Under this scenario, and after wild-type adaptation, mutators are not favoured any more, probably because no more beneficial mutations can be obtained or perhaps the fitness gain associated with late-arising mutations is too low to be detected in a short time period.

From an evolutionary point of view, it is important to determine if mutators accelerate adaptive evolution in bacterial populations. In nature, *E. coli* population size may be extremely variable (Savage, 1977). If the population is fully adapted, at equilibrium, the mutator frequency is stable (Boe *et al.*, 2000; Ninio, 1991). Our results confirm that mutator selection occurs more often in large populations than in small ones. Thus, in large populations the probability that a mutator minority contains pre-adapted clones is higher than in small ones. In addition, the probability of mutator selection by hitch-hiking is also higher. It appears that mutators may be preferentially selected in large bacterial populations, where they moderately accelerate adaptive evolution, because wild-type populations are large enough to contain pre-adapted clones at the outset (Arjan *et al.*, 1999; Tenaillon *et al.*, 1999). In contrast, mutators greatly accelerate adaptive evolution in small bacterial populations where the probability for their selection is weaker (Arjan *et al.*, 1999; Tenaillon *et al.*, 1999). Thus, in this case it could be argued that the role of mutators as accelerators of adaptive evolution may be reduced.

However, we can modify this conclusion by considering sequential adaptation and chance. In infectious diseases, pathogens are submitted to a variety of stresses during the pathogenic process or subsequent sequential antibiotic treatments. Even if the initial population is large, the probability that the wild-type population could rapidly cross several selective barriers resulting in the repeated re-occurrence of narrow bottlenecks, is weaker than for mutators. Mutators can be selected and rapidly fixed during adaptation to the first stress. The large mutator population thus obtained can accelerate adaptation to the following stresses (Denamur *et al.*, 2002; Giraud *et al.*, 2002). In situations comparable to small populations, 8 out of 117 competition experiments (initiated with 10^2 mutators) showed mutator selection. Thus, by permitting mutator selection in small populations, chance allows mutators to accelerate adaptive evolution. So, sequential adaptation and chance could allow a mutator lineage to accelerate adaptive evolution of clonal populations, including the development of mutator tumours in humans (Loeb, 1998).

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