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# Rate and effects of spontaneous mutations that affect fitness in mutator *Escherichia coli*

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Knowledge of the mutational parameters that affect the evolution of organisms is of key importance in understanding the evolution of several characteristics of many natural populations, including recombination and mutation rates. In this study, we estimated the rate and mean effect of spontaneous mutations that affect fitness in a mutator strain of *Escherichia coli* and review some of the estimation methods associated with mutation accumulation (MA) experiments. We performed an MA experiment where we followed the evolution of 50 independent mutator lines that were subjected to repeated bottlenecks of a single individual for approximately 1150 generations. From the decline in mean fitness and the increase in variance between lines, we estimated a minimum mutation rate to deleterious mutations of 0.005 ($\pm$0.001 with 95% confidence) and a maximum mean fitness effect per deleterious mutation of 0.03 ($\pm$0.01 with 95% confidence). We also show that any beneficial mutations that occur during the MA experiment have a small effect on the estimate of the rate and effect of deleterious mutations, unless their rate is extremely large. Extrapolating our results to the wild-type mutation rate, we find that our estimate of the mutational effects is slightly larger and the inferred deleterious mutation rate slightly lower than previous estimates obtained for non-mutator *E. coli*.

**Keywords:** mutation accumulation experiment; deleterious mutations; beneficial mutations; bottlenecks; mutator

## 1. INTRODUCTION

The genomic deleterious mutation rate ($U_d$) and the effects of deleterious mutations ($s_d$) on fitness are key parameters in many evolutionary theories, such as those trying to explain the evolution of recombination (Muller 1964; Maynard-Smith 1978; Kondrashov 1988; Barton & Charlesworth 1998; Barton 2010), ageing (Charlesworth & Hughes 1996; Hughes 2010), the advantage of outcrossing (Charlesworth & Charlesworth 1998), patterns of genetic diversity and molecular evolution in different genomic regions (Charlesworth *et al.* 2009), the degeneration of populations by Muller's ratchet (Combadao *et al.* 2007; Loewe & Hill 2010) or the frequency of mutator strains in microbial asexual populations (LeClerc *et al.* 1996; Matic *et al.* 1997; Sniegowski *et al.* 1997; Taddei *et al.* 1997; Tenaillon *et al.* 1999; Boe *et al.* 2000).

One of the classical ways to estimate mutation rates and mutational effects is a mutation accumulation (MA) experiment. This approach was pioneered in *Drosophila melanogaster* (Bateman 1959; Mukai 1964; Fernandez & Lopez-Fanjul 1996) and the same principle was later applied to several other organisms, namely *Saccharomyces cerevisiae* (Wloch *et al.* 2001; Zeyl & DeVisser 2001; Joseph & Hall 2004; Dickinson 2008), *Caenorhabditis elegans* (Keightley & Caballero 1997; Davies *et al.* 1999; Vassilieva & Lynch 1999; Estes *et al.* 2004), *Escherichia coli* (Kibota & Lynch 1996; Loewe *et al.* 2003) and some viruses (Elena & Moya 1999; Lazaro *et al.* 2003; Li & Roossinck 2004; de la Iglesia & Elena 2007). The principle relies on the assumption that, if a population is maintained with an extremely low effective population size, natural selection will be inefficient in purging deleterious mutations. So mutations will accumulate at the rate at which they appear. Given this, it is possible to get estimates of the rate of mutations and their mean effects on fitness related traits in an MA experiment from the rates of decline in the mean, $\Delta M$, and increase in variance, $\Delta V$. The Bateman–Mukai method gives an upper bound for $U_d$ as $\Delta M^2/\Delta V$ and a lower bound for the mean deleterious effect of a mutation, $s_d$, as $\Delta V/\Delta M$ (Mukai *et al.* 1972). More recently a maximum likelihood method was developed (Keightley 1994) to estimate the rate and the distribution of fitness effects in MA experiments. Assuming that mutation effects follow a continuous gamma distribution, it is possible to estimate its shape and scale parameters and, importantly, test if

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this fits the MA data better than a model that assumes a single value for $s_d$. Another method that also allows one to extract the shape of the distribution of mutation effects is the minimum distance method (Garcia-Dorado 1997), in which a distribution of fitness effects is assumed and a search is made for the mutation rate and distribution parameters that minimize the distance between the empirical distribution of the means observed in the MA lines and the theoretically expected distribution.

While MA experiments in many species involve a set-up in which the effective population size is kept extremely low, this is much more difficult in micro-organisms. For these, the population size fluctuates from one to several million individuals, allowing for natural selection to act during the exponential growth phase. Kibota & Lynch (1996) performed an MA experiment in *E. coli* involving 50 lines propagated during 300 bottlenecks in minimal medium. They then used the Bateman–Mukai method to estimate a value of $U_d$ of $1.7 \times 10^{-4}$ and a value of $s_d$ of 0.012 in this bacterium. Given that during colony growth selection can act against deleterious mutations, Kibota & Lynch (1996) performed a correction to adjust for this selection bias and obtained an estimate of $1.9 \times 10^{-4}$ for $U_d$. Recently, a framework has been developed that models precisely the type of scenario that occurs in a microbial MA experiment, where selection acts against deleterious mutations during the growth phase, and that allows for the estimation of $U_d$ and $s_d$ (Colato & Fontanari 2001; Gordo & Dionisio 2005). The model assumes an asexual population undergoing periodic bottlenecks of a single individual and then expands, just like the MA experiments in micro-organisms. Mutations occur following a Poisson distribution with mean $U_d$ and selection acts against new mutations, which are assumed to decrease fitness by an amount $s_d$. Multiplicative fitness is assumed and the distribution of mutations before the bottleneck can then be calculated, and from which the expected mean fitness of a clone at a given bottleneck is deduced. Under this model, it was shown that mean fitness decays exponentially with bottleneck number at a rate that depends on $U_d$ and $s_d$ (Gordo & Dionisio 2005). Although the model is deterministic, stochastic simulations in which genetic drift was incorporated showed that good estimates of $U_d$ and $s_d$ could be obtained.

All the MA experiments have shown that the number of mutations that decrease fitness is much larger than those that increase it. They also show that most deleterious mutations can impair fitness by only a few per cent or less, i.e. their effects may be small as theoretically expected (Haldane 1937; Drake *et al.* 1998; Orr 2000). However, this is not a general rule. In some organisms, such as *C. elegans* (Keightley & Caballero 1997; Vassilieva & Lynch 1999), and some viruses (Elena & Moya 1999), the average effect of deleterious mutations can be large.

The equilibrium frequency of micro-organisms with high mutation rate (mutators) and their dynamics, particularly in fluctuating environments (Taddei *et al.* 1997; Travis & Travis 2002), depend strongly on the values of $U_d$ and $s_d$. Mutators are expected to be maintained in natural populations at a low frequency because they generate more deleterious mutations and therefore suffer from a bigger mutational load. They may also occasionally increase in frequency by hitchhiking with beneficial mutations (LeClerc *et al.* 1996; Taddei *et al.* 1997; Le Chat *et al.* 2006). The mutation rates to both beneficial and deleterious mutations are the main factors determining the dynamics of mutators in a population and so it is of particular importance to estimate them. Notably, mutators are often associated with medically relevant phenotypes, such as virulence (Oliver *et al.* 2000) and antibiotic resistance (LeClerc *et al.* 1996; Chopra *et al.* 2003). Therefore, if we can predict the expected frequency of mutations and their effects in mutators, we can predict how these phenotypes may evolve.

Here, we performed an MA experiment in a mutator strain of *E. coli* that is deficient in one of the mismatch repair genes (*mutS*) to estimate the rate and fitness effects of spontaneous mutations. This strain has a higher mutation rate than the wild-type and, therefore, accumulates mutations more rapidly (Funchain *et al.* 2000).

We estimate a rate of 0.005 deleterious mutations per genome per generation, each causing a mean fitness decrease of 3 per cent. Similar to recent findings on other MA experiments in *S. cerevisiae* (Joseph & Hall 2004; Dickinson 2008) and *Arabidopsis thaliana* (Shaw *et al.* 2000), the dynamics of mean fitness in our mutator populations seem to indicate that spontaneously arising beneficial mutations may be occurring, although future experimental and theoretical work will be required to be able to determine their rate and effects.

## 2. MATERIAL AND METHODS

### (a) *Bacterial strains*

All strains used in this study were derived from *E. coli* K12 MG1655 through P1 transduction using as donor strains K12 MG1655 *srl*::Tn10 *mutS* $Str^R$ and K12 MG1655 *srl*::Tn10 $Str^R$, and as receptor strains the wild-type *E. coli* K12 MG1655 and *E. coli* K12 MG1655 Δ*ara*, all kindly provided by I. Matic. The ancestor bacterium for the MA experiment was the mutator *E. coli* K12 MG1655 *srl*::Tn10 *mutS*. The strain is resistant to tetracycline. The strain used as reference for competitive fitness measurements was *E. coli* K12 MG1655 *srl*::Tn10 *mutS* Δ*ara*. The deletion in the arabinose operon was used as a phenotypic marker to distinguish the two strains in competition.

### (b) *Media and antibiotics*

The MA lines were grown on plates containing Luria-Bertani (LB) supplemented with agar at 37°C. Every five bottlenecks, the medium was supplemented with tetracycline (20 $\mu g\,ml^{-1}$), to which the lines are resistant, to prevent contaminations. Tetrazolium agar (TA) medium containing 1 per cent peptone, 0.1 per cent yeast extract, 0.5 per cent sodium chloride, 1.7 per cent agar, 1 per cent arabinose and 0.005 per cent tetrazolium chloride was used to assess the frequency of each strain before and after the

competition (Lenski *et al.* 1991). The bacteria from the MA lines will give rise to white colonies and the competitors to red colonies in TA. The competitions were done in LB medium. For the dilutions, we used a solution of $MgSO_4$ at a concentration of 0.01 M. LB supplemented with agar and rifampicin (100 μg $ml^{-1}$) was used to measure the mutator strength.

### (c) *Estimation of mutator strength*

Liquid cultures of both the ancestor mutator and wild-type clones were grown in 2 ml tubes containing 1 ml LB medium at 37°C, for 24 h, with aeration. This was done 200 times independently. The mutation rate was estimated in both strains by plating 100 μl of the cultures on Petri dishes containing LB-agar supplemented with rifampicin and then counting the fraction of plates without any colonies ($p_0$) (Luria & Delbruck 1943). The mutator strength was calculated as the ratio between the natural logarithms of $p_0$ (an estimator of the mutation rate) for the mutator and wild-type. For the wild-type, $p_0$ was 0.975 and for the mutator, $p_0$ was 0.21, so the mutation rate of the mutator is about 62-fold higher than of the wild-type. This value is in agreement with previous studies in which differences as low as 30-fold and as high as 100-fold have been found (Giraud *et al.* 2001; de Visser & Rozen 2006).

### (d) *Mutation accumulation*

Fifty single clones of a population of mutator *E. coli* were randomly selected and spread on an agar plate by the streak-plate technique. Each of these 50 single colonies was the start of an independent line that went through 50 such bottlenecks (one every 24 h); the isolated colonies were always chosen at random. Every 10 bottlenecks, samples were frozen as follows: a part of the colony used to streak the next plate was grown on a 2 ml tube containing 1 ml LB medium and tetracycline at 37°C for 24 h, with agitation, and then stored in 15 per cent glycerol at −20°C. The number of generations elapsed between bottlenecks was measured by picking, diluting and plating one colony of three different randomly chosen lines. After 24 h of growth, the number of colony forming units (CFUs) was counted. The number of generations was estimated as follows: $G = \log_2(N_f/N_i)$, where $G$ is the number of generations, $N_f$ is the number of CFUs in the colony and $N_i$ is the number of individuals that started the colony, which is assumed to be unity.

### (e) *Fitness estimation*

Cultures of the MA lines were competed with the ancestral strain in an approximate proportion of 1 : 1. The initial ratio of each strain was measured by plating the mixture on TA agar plates. All competitions occurred in 50 ml screw-cap tubes containing 10 ml LB medium at 37°C, for 24 h, with agitation. After that, appropriate dilutions were plated onto TA medium to count CFUs.

Fitness was estimated as per the generation difference in growth rates between the evolved and reference strains (Hartl & Clark 1997)

$$W_a = 1 + G^{-1} \ln\left(\frac{N_{fa}/N_{fb}}{N_{ia}/N_{ib}}\right),$$

where $W_a$ is the fitness of the evolved strain $a$ against the ancestor strain $b$, $N_{fa}$ and $N_{fb}$ are the number of evolved and ancestor bacteria, respectively, after the competition, and $N_{ia}$ and $N_{ib}$ are the number of evolved and ancestor bacteria, respectively, before the competition.

The fitness of the ancestral clone was measured 36 times independently and each evolved clone was measured three times.

### (f) *Parameter estimation*

We followed the model by Gordo & Dionisio (2005) (adapted from Colato & Fontanari 2001), which assumes that the number of newly arising deleterious mutations is Poisson distributed with mean $U_d$ and each mutation causes a fitness decline of $s_d$. They showed that the distribution of mutations at a given generation ($G$) is Poisson with mean

$$\lambda(G) = \frac{U_d}{s_d}\left(1 - (1 - s_d)^G\right),$$

and that $U_d$ and $s_d$ can be estimated by

$$U_d = \frac{m_1}{(1 - (1 - s_d)^G)} \quad \text{and} \quad s_d = \frac{m_2}{m_1},$$

where $m_1$ is the slope of the natural logarithm of the mean fitness (of all 50 lines) with bottleneck number and $m_2$ is the slope of the natural logarithm of $F_i = \overline{W_i^2}/\overline{W_i}^2$ with bottleneck number $i$; $W$ is the mean fitness of each line at bottleneck $i$. In this model, it is assumed that no beneficial or compensatory mutations arise and that each deleterious mutation has a selection coefficient $s_d$. So, from the slopes $m_1$ and $m_2$ and their respective standard error ($\delta$), one can estimate $U_d$ and $s_d$ and an associated error which was calculated through error propagation as

$$\delta s_d = \left|\frac{\partial s_d}{\partial m_1}\delta m_1\right| + \left|\frac{\partial s_d}{\partial m_2}\delta m_2\right|$$

and

$$\delta U_d = \left|\frac{\partial U_d}{\partial s_d}\delta s_d\right| + \left|\frac{\partial U_d}{\partial m_1}\delta m_1\right|.$$

### (g) *Monte Carlo simulations*

Since variation in effects of deleterious mutations and the possible occurrence of beneficial mutations are ignored in the model described above, we investigated how violation of these assumptions would influence the mutational parameter estimates $U_d$ and $s_d$. We performed simulations where the MA process was modelled as follows: each population started with a single individual carrying no mutations; each generation the population doubled and each new individual inherited all the mutations from the parent plus a random number of new deleterious and beneficial mutations taken from Poisson distributions

with mean $U_d$ and $U_a$, respectively. To perform the selection step, we assumed multiplicative fitness $W_{da} = (1 - s_d)^d \times \prod_{i=0}^{a}(1 + s_{ai})$, where $d$ and $a$ are the number of accumulated deleterious and beneficial mutations, respectively; the effect of each beneficial mutation ($s_a$) was taken from an exponential distribution (Imhof & Schlotterer 2001; Perfeito *et al.* 2007). We studied how variation in the mean effect of beneficial mutations affects the estimates of the mutation rate and effects of deleterious mutations. We assume that back mutations are negligible and that new beneficial mutations that may be compensatory do not necessarily lead to a full restoration of fitness. This is generally observed in studies of mutations that compensate for the costs of antibiotic resistance mutations (Maisnier-Patin & Andersson 2004; Trindade *et al.* 2009). Bottlenecks were simulated by picking one random individual every $G$ generations to continue the evolution.

In the model we used to estimate $U_d$ and $s_d$, all deleterious mutations are assumed to have the same effect. To test how the estimates were affected by variation in $s_d$ values, we ran simulations assuming a gamma distribution, $\beta^\alpha s_d^{\alpha-1} e^{-\beta s_d}/\Gamma(\alpha)$, with different shape parameters ($\alpha$), which is a distribution commonly used (Keightley 1994) to fit data from MA experiments. One hundred simulations were done for each set of parameters.

## 3. RESULTS

### (a) *Trajectories of the mutation accumulation lines*

We followed the evolution of 50 independent lines of *E. coli* mutators that were subjected to 50 periodic bottlenecks (approx. 23 generations). Each line was derived from the same clone, whose fitness was estimated by direct head-to-head competition against a reference strain (points at bottleneck 0 in figure 1). Figure 1 shows the trajectories of each MA line throughout the experiment. The general trend is a decline in the mean fitness of the lines with increasing bottleneck number, as previously observed (Kibota & Lynch 1996; Funchain *et al.* 2000). As expected, the rate of decline in mean fitness for this mutator strain is much larger than was observed in wild-type *E. coli* (Kibota & Lynch 1996). Figure 2 shows the fitness distribution of mutants throughout the bottlenecks. The bell shape initially observed disappears with time. The mean of the distribution of fitnesses decreases and the variance increases as bottlenecks proceed, which is expected since the lines are accumulating mutations randomly. By bottleneck 50, all the lines except one had significantly lower fitness than the initial clone.

### (b) *Estimate of the rate and effect of mutations*

To estimate $U_d$ and $s_d$, we followed the model of Gordo & Dionisio (2005), which allows one to estimate these parameters from the decline in mean fitness ($W$) and from the increase in $F_i = \overline{W_i^2}/\overline{W_i}^2$ with bottleneck number $i$. This model is slightly different from that classically used in which the rate of change in mean($W$) and var($W$) is estimated (Bateman 1959; Mukai 1964; Kibota & Lynch 1996), since it was motivated by MA accumulation experiments in microbes and incorporates selection against deleterious mutations during the period of growth following each bottleneck. Figure 3*a* shows the evolution of the natural logarithm of mean fitness and respective variance among lines and figure 3*b* shows the natural logarithm of the $F$-values during the 50 bottlenecks, as well as the regression analysis. Despite the occasional apparent fitness increases observed during the experiment (as expected from measurement error), mean fitness decreased and variance increased, which is to be expected if deleterious mutations are being fixed at the bottleneck (Bateman 1959; Mukai 1964). We used the mean decrease in fitness and mean increase in $F$ to estimate $U_d$ and $s_d$ (Mukai 1964; Kibota & Lynch 1996; Colato & Fontanari 2001; Gordo & Dionisio 2005) and obtained an estimate of $U_d = 0.005$ deleterious mutations per genome per generation, with a 95% confidence interval of [0.004; 0.006] and $s_d = 0.03$ with a 95% confidence interval of [0.02; 0.04]. To compare these estimates with those previously obtained for wild-type strains, we divided $U_d$ by the strength of the mutator (estimated as described in §2), which is 62-fold. This gives us an estimate of $U_d$ for wild-type *E. coli* of $8 \times 10^{-5}$ deleterious mutations per genome per generation, slightly smaller from what was previously estimated using a wild-type strain—$2 \times 10^{-4}$ (Kibota & Lynch 1996). We should note though that not only the strain of *E. coli* which we are using is different from that of the previous study, but also the medium (rich medium in our MA experiment, minimal medium in Kibota & Lynch (1996) experiment) and the fitness assays differ (fitness assayed by head-to-head competition in this study and from growth curves in Kibota & Lynch (1996)).

Loewe *et al.* (2003) estimated a value of $U_d$ per day of 0.03 for wild-type *E. coli* in stationary phase. If we extrapolate our estimate of $U_d$ per generation of 0.005 to a day, we obtain a value of 0.115 mutations per day, which is much larger than the previous estimate. This indicates that the stable mutator phenotype of our strain is much stronger than the transient mutator phenotype observed by Loewe *et al.* (2003).

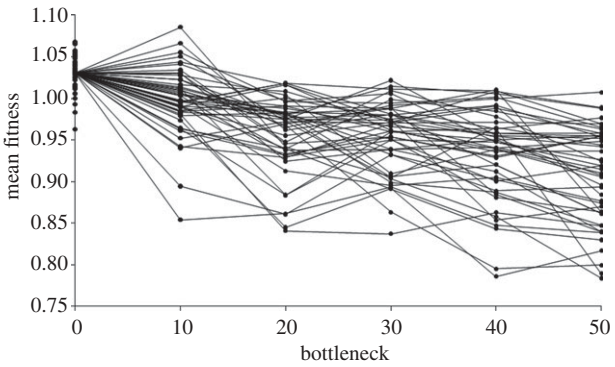


Figure 1. Fitness trajectories of all 50 lines throughout the bottlenecks. Each point is the mean of three competition experiments, except for bottleneck 0, where all 36 measurements of the original clone are reported.

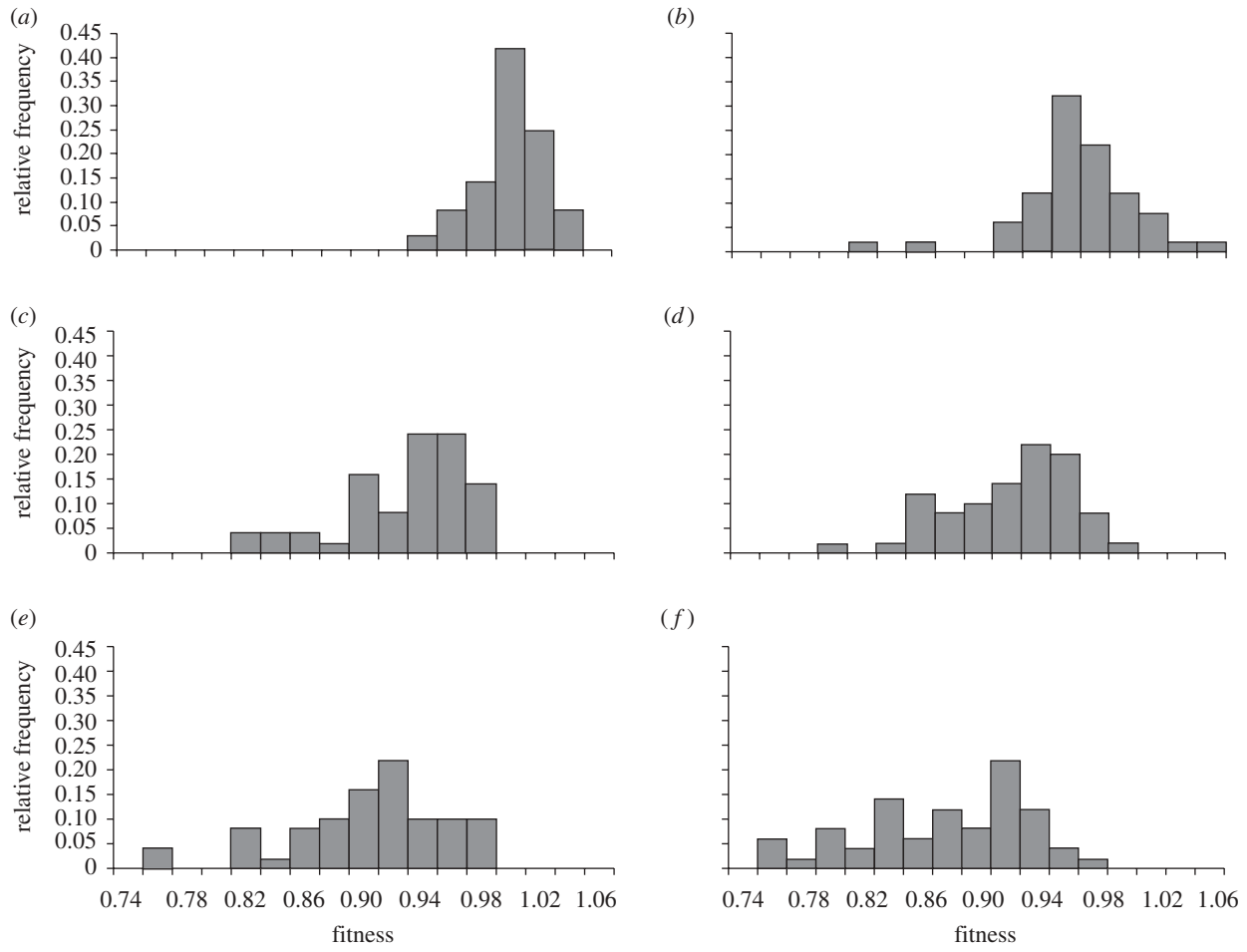


Figure 2. Distribution of fitnesses throughout the bottlenecks. In all cases, the fitness difference between the reference and the ancestral strains was normalized, such that the ancestor has mean fitness of unity. (*a*) shows the distribution of 36 independent fitness measurements of the ancestral strain and thus shows only experimental error; the others show the fitness distributions of the MA lines at the (*b*) 10th, (*c*) 20th, (*d*) 30th, (*e*) 40th and (*f*) 50th bottlenecks.

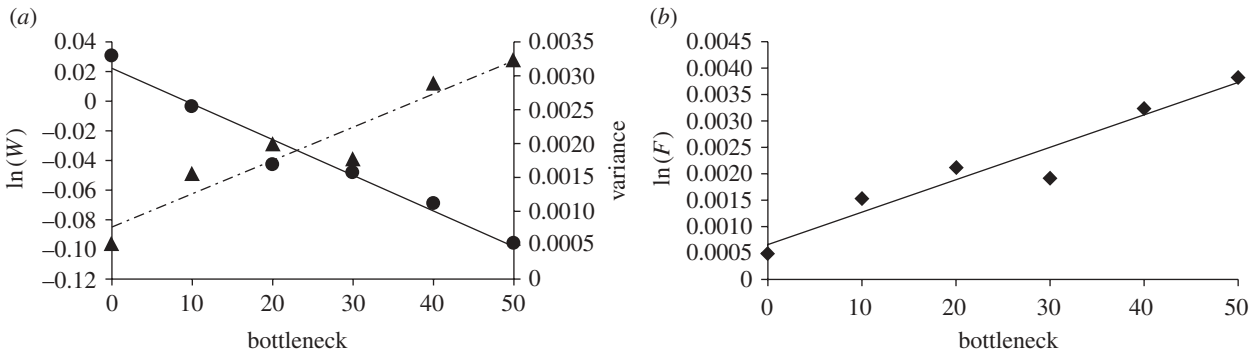


Figure 3. Changes in summary statistics of the 50 lines as a function of bottleneck number. In (*a*), the circles show the mean fitness ($W$) and triangles show the variance. In (*b*), diamonds show $F$ (see text for details). The corresponding slopes are the following: the slope of the $\ln(W)$ is $-0.0024$ (2 s.e. $= 0.0004$, $R^2 = 0.96$, $p < 0.01$), the slope of the variance is $4.9 \times 10^{-5}$ (2 s.e. $= 1.6 \times 10^{-5}$, $R^2 = 0.90$, $p < 0.01$) and the slope of the $\ln(F)$ is $6 \times 10^{-5}$ (2 s.e. $= 2 \times 10^{-5}$, $R^2 = 0.93$, $p < 0.01$).

### (c) *The impact of a distribution of deleterious effects on the estimates of* $U_d$ *and* $E[s_d]$

Our estimate of $U_d$ assumes that all deleterious mutations have the same effect, which is likely not to be the case. We used Monte Carlo simulations to investigate the impact of a distribution of selective coefficients on the estimates of $U_d$ and $s_d$. We simulated a haploid population undergoing bottlenecks of one individual every 23 generations (see §2) and assumed $U_d = 0.005$ mutations per genome per generation and a mean $E[s_d] = 0.03$, as estimated above. We then used a gamma distribution of $s_d$ with various shape parameters. In the simulation, we measured the decline in fitness through time and the increase in $F$. We then estimated $U_d$ and $s_d$ as described above for the experimental data. Table 1 summarizes these results. If the distribution is bell-shaped ($\alpha > 1$), then variation in $s_d$ has little impact on the estimates.

Table 1. Effect of having a distribution of deleterious mutations on the estimates of $U_d$ and $s_d$. (In all the simulations, the deleterious mutation parameters used were those determined experimentally—$U_d = 0.005$ and $E[s_d] = 0.03$. The numbers in brackets show twice the standard error.)

| $\alpha$ | estimated $s_d$ | estimated $U_d$ |
|---|---|---|
| constant $s$ | 0.028 [0.003] | 0.0052 [0.0003] |
| 0.01 | 0.04 [0.02] | 0.00013 [$2 \times 10^{-5}$] |
| 0.1 | 0.13 [0.02] | 0.00094 [$2 \times 10^{-5}$] |
| 0.5 | 0.07 [0.01] | 0.002 [$7 \times 10^{-5}$] |
| 1 | 0.039 [0.005] | 0.0033 [0.0003] |
| 5 | 0.028 [0.005] | 0.0054 [0.0006] |
| 7 | 0.028 [0.002] | 0.00469 [$9 \times 10^{-5}$] |
| 10 | 0.029 [0.006] | 0.0049 [0.0004] |
| 50 | 0.028 [0.006] | 0.0054 [0.0007] |
| 100 | 0.028 [0.003] | 0.0051 [0.0003] |

However, if $\alpha < 1$, then the estimates become biased for lower values of $U_d$ and higher values of $s_d$. For example, in simulations where the real $U_d = 0.005$ and $E[s_d] = 0.03$, if $\alpha = 5$, then $U_d$ is estimated to be 0.0054 ($\pm$0.0006) and $s_d$ 0.028 ($\pm$0.005); if $\alpha = 1$, then $U_d$ is estimated to be 0.0033 ($\pm$0.0003) and $s_d$ 0.039 ($\pm$0.005); and if $\alpha = 0.1$, then $U_d$ is estimated to be 0.00094 ($\pm$0.00001) and $s_d$ 0.13 ($\pm$0.02). This is the same type of bias that was observed for Bateman–Mukai estimates of deleterious mutation parameters from MA experiments (Keightley 1998). Importantly, if the distribution of deleterious effects has a shape parameter $\alpha \ll 1$, then we are massively underestimating $U_d$ (with $\alpha = 0.01$, $U_d$ is underestimated by more than an order of magnitude; see table 1) and grossly overestimating the mean deleterious effect.

### (d) *The impact of beneficial mutations on the estimates of* $U_d$ *and* $s_d$

Even though the vast majority of lines show an increasing fitness decline with increasing bottleneck number, the dynamics of fitness change between bottlenecks in some lines indicates that a considerable number of mutations could have occurred that caused increased fitness between bottlenecks. A significant fraction of beneficial mutations have been observed previously in MA experiments of *S. cerevisiae* (Joseph & Hall 2004; Dickinson 2008; Hall *et al.* 2008) and *A. thaliana* (Shaw *et al.* 2000), which points to a mutation rate to beneficial mutations in these organisms high enough to be detected even when natural selection is put at a minimum. We analysed the number of mutator lines that changed fitness when compared with the previous measurement, 10 bottlenecks before, to try to detect the presence of beneficial mutations (comparisons were done using *t*-test). When making no correction for multiple testing, we see that every 10 bottlenecks at least one line increases in fitness ($p < 0.05$), whereas when performing a Bonferroni correction, we do not detect any lines increasing in fitness ($p < 0.0002$). The Bonferroni method, however, may be unnecessarily stringent for many situations (Verhoeven *et al.* 2005; Narum 2006); so, we used a more recently developed method to correct for multiple comparisons—the Benjamini–Hochberg (B–H) method (Benjamini & Hochberg 1995), which adjusts for multiple comparisons by controlling false discovery rate instead of family-wise error rate. It is less conservative than the traditional Bonferroni method, yet it still provides adequate protection against Type I error in some scenarios (Narum 2006). If the B–H correction for multiple comparisons is used (at 5% significance level), then some lines do indeed show an increased fitness indicating the occurrence of beneficial mutations (table 2). In particular, in the interval between bottlenecks 20 and 30, we see that seven lines increased in fitness. If this is a real biological signal, rather than a statistical artefact, then by repeating the experiment we should observe a similar pattern. To test this, we propagated all 50 lines from bottleneck 20 under exactly the same conditions as before and measured their fitness change after a new round of 10 bottlenecks. Of these 50 new lines, two showed an increase in fitness (under the B–H correction), indicating a non-negligible frequency of occurrence of beneficial mutations. We note that the increases in fitness observed involved different lines from those of the previous experiment, and that it is difficult to rule out possible variation of the environment in the fitness assays, which could have caused lower estimates of fitness at bottleneck 20.

Table 2. Number of mutants that significantly changed their fitness each 10 bottlenecks (significant after applying the Benjamini–Hochberg correction at 5% significance level).

| bottleneck | increases in fitness | decreases in fitness |
|---|---|---|
| 10 | 1 | 21 |
| 20 | 1 | 15 |
| 30 | 4.5 from $(7 + 2)/2$[a] | 11 from $(11 + 11)/2$[a] |
| 40 | 0 | 8 |
| 50 | 1 | 8 |
| mean | 1.5 | 12.6 |
| total | 7.5 | 63 |

[a]An independent repeat of evolution from bottleneck 20–30 resulted in two lines with a significant increase in fitness and 11 with significant decrease in fitness.

It has been suggested that the number of compensatory mutations may increase during the accumulation of deleterious mutations (Lazaro *et al.* 2003; Gordo & Dionisio 2005; Silander *et al.* 2007). In fact for some viruses undergoing strong bottlenecks, stabilization in their fitness was observed after an initial fitness decline (Lazaro *et al.* 2003; Silander *et al.* 2007). In mutator *E. coli*, we did not observe such stabilization during the studied period.

The model we used to estimate the rate and mean effect of deleterious mutations assumes that the MA lines do not acquire any beneficial mutations. Since this assumption may not apply in these experiments, we used Monte Carlo simulations to investigate the impact of the presence of beneficial mutations on the estimates of $U_d$ and $s_d$. We simulated a haploid population undergoing bottlenecks of one individual every 23 generations (see §2). We assumed $U_d = 0.005$

Table 3. Effect of beneficial mutations on the estimates of deleterious mutations (in all the simulations, the deleterious mutation parameters used were those determined experimentally, $U_d = 0.005$ and $s_d = 0.03$. In some simulations, the model could not be applied (n.a.) because mean fitness increased. The numbers in brackets show twice the standard error).

| | $s_a = 0.01$ | | $s_a = 0.03$ | | $s_a = 0.05$ | |
|---|---|---|---|---|---|---|
| $U_a$ | estimated $s_d$ | estimated $U_d$ | estimated $s_d$ | estimated $U_d$ | estimated $s_d$ | estimated $U_d$ |
| 0 | 0.028 [0.003] | 0.0052 [0.0003] | 0.028 [0.003] | 0.0052 [0.0003] | 0.028 [0.003] | 0.0052 [0.0003] |
| 0.000001 | 0.023 [0.004] | 0.0057 [0.0006] | 0.035 [0.008] | 0.0041 [0.0006] | 0.03 [0.01] | 0.005 [0.001] |
| 0.00001 | 0.032 [0.008] | 0.0046 [0.0009] | 0.027 [0.003] | 0.0056 [0.0005] | 0.033 [0.007] | 0.0045 [0.0006] |
| 0.0001 | 0.031 [0.004] | 0.0049 [0.0003] | 0.036 [0.003] | 0.0038 [0.0001] | 0.11 [0.02] | 0.00218 [$6 \times 10^{-5}$] |
| 0.0004 | 0.027 [0.003] | 0.0055 [0.0003] | 0.042 [0.008] | 0.0036 [0.0004] | 0.6 [0.3] | 0.000753 [$6 \times 10^{-7}$] |
| 0.001 | 0.028 [0.005] | 0.0051 [0.0005] | 0.19 [0.06] | 0.000934 [$4 \times 10^{-6}$] | n.a. | n.a. |

mutations per genome per generation and $s_d = 0.03$, as estimated above, and tested several values of $U_a$ and $s_a$. From the mean fitness decrease and increase in $F$, we estimated $U_d$ and $s_d$ in the same way as for the experimental data. As table 3 shows, the beneficial mutations only affect the $U_d$ and $s_d$ estimates if their rate of appearance and mean effects are very high. If this is the case, then $U_d$ is underestimated and $s_d$ overestimated; given the current estimates of $s_a \sim 1\%$ (Imhof & Schlotterer 2001; Perfeito *et al.* 2007), it is possible that beneficial mutations may be slightly biasing our estimates of $U_d$ and $s_d$. So, both by the presence of beneficial mutations and by variation in $s_d$ values, the value of the genomic deleterious mutation rate may be massively underestimated and the mean effects of $s_d$ overestimated.

## 4. DISCUSSION

This work presents new estimates of the rate of fitness-affecting mutations in a mutator strain of *E. coli* using a classical MA experiment. As predicted, most of our lines decreased in fitness, supporting the notion that the vast majority of mutations are deleterious. As observed previously in other organisms, we also found that beneficial mutations may be fixing in our mutator MA lines, although future work should be done to be able to estimate the rate and effects of such mutations. We show here that the occurrence of beneficial mutations in MA lines leads to an underestimation of $U_d$ and an overestimation of $s_d$. This is the same type of bias that has been described previously if there is a distribution of effects of deleterious mutations (Keightley 1998).

Comparing our estimates with those of Kibota & Lynch (1996), we see slight differences: namely, the inferred mutation rate to deleterious mutations is lower ($8 \times 10^{-5}$ compared with $2 \times 10^{-4}$) and the estimated $s_d$ is higher (0.03 compared with 0.012). This could be due to the fact that in the previous study absolute growth rate was used to measure fitness, whereas we used competitive ability. It is reasonable to think that mildly deleterious mutations that affect growth are more deleterious when in direct competition than when bacteria are growing without competition. Also, there are differences in the environments in which the two studies were performed, which can lead to different estimates. Further, our results might be slightly biased by beneficial mutations, which, as we show here, are expected to bias $U_d$ downwards and $s_d$ upwards. Kibota & Lynch (1996) did not detect beneficial mutations, possibly because of their strain's lower overall mutation rate or because these mutations only increase growth rate while in competition. Finally, the difference in our estimates from those of Kibota & Lynch (1996) could also be due to the type of mutations that are increased by a deficiency in the mismatch repair genes. It is known that strains without a functional *mutS* gene have a higher rate of transitions (Cox *et al.* 1972) and these mutations might, on average, have a more deleterious effect than the mutations to which the wild-type strains are exposed.

Interestingly, when analysing mutations caused by random transposon insertions (involving the Tn10 element), Elena & Lenski (1997) estimated a mean $s_d$ of about 0.03, which is similar to our estimate. The mutator strain that we have studied also carries a Tn10 element and so it is possible that a fraction of the deleterious mutations that fixed in our lines could involve transpositions. Further studies involving whole-genome sequencing of several MA lines could allow not only the nature of the mutations accumulated to be identified but also their number, which would contribute to better estimates of the mutation rate, their effects and their type.

More recently, the rate and mean effect of deleterious mutations appearing during stationary phase in *E. coli* were estimated in another study (Loewe *et al.* 2003). These authors found an average effect of 3 per cent, as we do here. It is known that the concentration of the MutS protein (as well as other mismatch repair proteins) is lower in the stationary phase (Feng *et al.* 1996), so it seems that mutations caused by a lack of the mismatch repair system could be more deleterious than spontaneous mutations occurring in a wild-type background. The overall mutation rate in this mutator strain is expected to be 0.18 per genome per generation (based on the mutation rate for wild-type *E. coli* (Drake *et al.* 1998) and on the measured mutator strength), which is 36 times higher than the $U_d$ estimated in our experiments. This implies that the majority of mutations are effectively neutral in our experiments or that we do not have the power to measure many of them, which is in full agreement with previous indications from

other organisms that the distribution of $s_d$ is very leptokurtic (Loewe & Charlesworth 2006; Eyre-Walker & Keightley 2007). If this is the general case for micro-organisms, it is important to develop new tools that can allow estimation of the distribution of effects of deleterious mutations under selection. Furthermore, we should note that mutations with very small effect (about less than 0.1%) are extremely difficult to measure because they do not have detectable effects in typical fitness assays. Another potentially important issue that requires future experimental and theoretical work involves the study of epistasis between deleterious mutations. Elena & Lenski (1997) measured the degree of epistasis between pairs of slightly deleterious mutations caused by random transposon insertions in *E. coli* and found evidence for both positive and negative epistasis. Two more recent studies in bacteria found evidence for positive epistasis (Maisnier-Patin *et al.* 2005; Trindade *et al.* 2009). In an MA experiment in *Salmonella typhimurium*, the observed decline in mean fitness from accumulating an increasing number of mutations supported the existence of positive epistasis between deleterious alleles (Maisnier-Patin *et al.* 2005). Importantly, one of the reasons underlying this observation appears to be linked to the over-expression of chaperones that assist in protein folding and are therefore capable of reducing the impact of deleterious mutations as they accumulate. In a study in *E. coli* where pairwise epistasis between deleterious alleles conferring antibiotic resistance was measured, a high frequency of combinations exhibiting positive epistasis was also shown (Trindade *et al.* 2009). Incorporation of epistasis in modelling approaches aimed at analysing data from MA experiments may therefore be required.

We show here with experimental evolution that a clear massive amount of slightly deleterious mutations leads to a rapid fitness decline of micro-organisms with impairments in DNA repair, although sporadic adaptations may also occur. Previous studies have found evidence for the fixation of beneficial mutations in MA with other microbes (Hall *et al.* 2008). Many of the models trying to describe the evolution of microbial populations should therefore take into account a potentially large number of beneficial mutations. In particular, when MA procedures are used to estimate parameters, the occurrence of mutations that increase fitness may not be ignored. Furthermore, the fact that even under strong bottlenecks there might be a significant number of lineages of mutator bacteria that can increase fitness (which has not been observed in wild-type bacteria) may have important implications. If, in nature, bacteria undergo bottlenecks that are either purely demographic due to the process of infection of a new host by a few cells, or due to selection by the application of antibiotics, then there may be a higher number of mutator lineages that can survive at least temporarily, even though at the cost of accumulating deleterious mutations at a higher rate in the long term.

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