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High Deleterious Genomic Mutation Rate in Stationary Phase of *Escherichia coli*

Laurence Loewe,*† Volker Textor, Siegfried Scherer

In natural habitats, bacteria spend most of their time in some form of growth arrest. Little is known about deleterious mutations in such stages, and consequently there is limited understanding of what evolutionary events occur. In a deleterious mutation accumulation experiment in prolonged stationary phase of *Escherichia coli*, about 0.03 slightly deleterious mutations were observed per genome per day. This is over an order of magnitude higher than extrapolations from fast-growing cells, but in line with inferences from observations in adaptive stationary phase mutation experiments. These findings may affect understanding of bacterial evolution and the emergence of bacterial pathogenicity.

Mutation rates and selection coefficients are fundamental for understanding evolutionary processes. Terumi Mukai was the first to determine both parameters for deleterious mutations in a population of fruit flies (*1*). Similar studies have led to considerable insights into spontaneous deleterious mutations (*2–4*). While most experiments targeted the fruit fly *Drosophila melanogaster* or the worm *Caenorhabditis elegans*, few have studied microorganisms. Aside from more general work on RNA viruses (*5*), deleterious mutation parameters have been investigated in yeast (*6*, *7*), in *Salmonella typhimurium* (*8*), and in *Escherichia coli* (*9–11*). All of these studies have estimated mutation rates per generation during logarithmic growth but have not investigated stationary phase. A recent study on stress-induced mutations in aging colonies of 787 *E. coli* strains showed that the average frequency of mutations to resistance against the antibiotic rifampicin was sevenfold higher in 7-day-old colonies as compared to 1-day-old colonies (*12*), but the rate of deleterious mutations has not been measured.

In natural habitats, bacterial populations spend most of their time under nutritional constraints (*13*, *14*), and rates of growth may be very slow, because essential nutrients are consumed much faster than they become available. At least in one *E. coli* assay system (the Lac frameshift reversion assay), to evade death by starvation under such conditions, a subpopulation can turn into transient mutators to increase the probability of an adaptive mutation arising that allows growth to resume on an alternative nutrient (*15–17*). In another assay system, cells with a growth advantage in stationary phase (GASP) repeatedly take over the population (*14*, *18*). The mutational processes used in these systems differ mechanistically from those occurring during the logarithmic growth phase (*14*, *19*), as recombination and DNA polymerases induced by SOS (a DNA damage response) appear to play a key role (*15*, *19*, *20*). Cells in stationary phase might divide one to two times per day (*14*, *21*) or even less than once per 3 days (*22*); however, to accumulate mutations they need neither to divide (*23*) nor to replicate their DNA globally (*24*). Given the importance of stationary phase in the naturally occurring life cycle of bacteria, the deleterious mutation rate during that phase could be crucial for understanding bacterial evolution. Here, we report on a Bateman-Mukai (BM) analysis of a stationary phase mutation accumulation experiment that estimates this rate (*25*).

Because it is difficult to estimate the number of generations per day in the stationary phase, we have abandoned the generation-centric view of mutation rates in favor of a clock-like view and have measured mutation rates per genome per day. Figure S1 depicts the design of a 101-day stationary phase mutation accumulation experiment with 98 replicate lines. Fitness was measured in terms of maximal growth rate *m*, and the resulting distributions of growth rate showed a decrease in mean and an increase in variance over time (Fig. 1), leading to a typical BM plot (Fig. 2). Depending on the distributions selected for estimating mutational parameters as well as the method used [BM or maximum-likelihood (ML)], a number of different values were obtained [supporting online material (SOM) Text]. The most reliable values are deleterious mutation rates of $U_{BM} = 0.027$ and $U_{ML} = 0.045 \pm 0.004$ per genome per day with deleterious selection coefficients of $s_{BM} = 3.4\%$ and $s_{ML} = 2.3 \pm 0.2\%$, assuming that all mutations have the same effect [ML values with 95% confidence intervals (*26*)]. If mutational effects are distributed exponentially, then $U_{ML} = 0.091 \pm 0.01$ per genome per day and $s_{ML} = 1.1 \pm 0.1\%$. Different shape parameters of a Gamma distribution gave equal or increasing likelihoods for more leptocurtic distributions leading to corresponding higher U and lower s estimates. Therefore, inferences on the distribution of mutational effects are not possible here (*26*). It was important to allow long-term stationary phase lines to acclimate to logarithmic growth for at least one or two additional serial transfers, because temporal changes of gene expression can lead to much slower growth rates. In summary, the values to remember are $U \approx 0.03$ mutations per genome per day with a selection coefficient of $s \approx 3\%$.

The selection coefficients found here are in general agreement with $s_{max} \approx 1.2\%$ from the *E. coli* log-phase mutation accumulation experiment of Kibota and Lynch (*9*) and similar to the 2.7% reported for artificial random transposon insertions by Elena and Lenski (*27*). To understand the mutation rate found here is more challenging. Any purely replication-based model fails: Assuming two generations per day in stationary phase (*21*) and $U_{deleterious}$ of 0.0002 mutations per genome per generation (*9*) results in one to two orders of

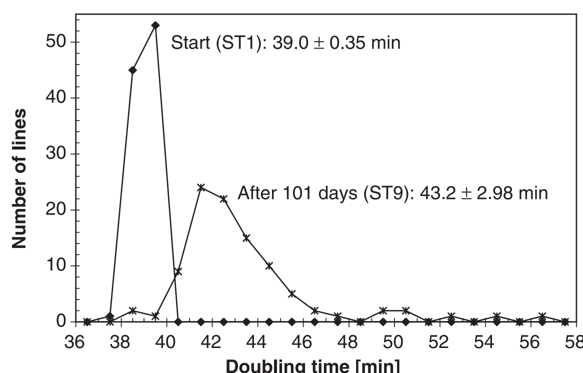

Fig. 1. The distribution of doubling times in fresh medium before serial transfer 1 (ST1, serial transfer 1) and after (ST9) prolonged periods in the stationary phase. Each distribution (mean ± standard deviation) contains one measurement of each line that experienced the corresponding serial transfer. The dilution of the inoculum was 2401-fold in all measurements. The long tail in ST9 shifts the arithmetic mean toward higher doubling times.

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magnitude less than the deleterious mutation rate found here. Assuming either 25 generations per day in stationary phase or that all mutations are slightly deleterious [U_{total} of 0.0025 (*3*)] yields a rate that is still less than one-fifth of what we observe. To explain our values, we would have to assume constant log-phase growth and a mutator phenotype (*11*, *28*), which is not a very plausible scenario for a nonmutator strain in stationary phase. The mutation rates of three randomly chosen lines did not show differences compared with the founder strain (SOM Text).

During stationary phase, at least in one assay system, a subpopulation of cells can turn into transient mutators (*17*, *29*), producing mutations that confer a growth-promoting or adaptive phenotype. Fixation of an adaptive mutation fixes all other (potentially deleterious) mutations linked to it. This hitchhiking effect suggests a large impact of transient mutators on apparent population-wide substitution rates.

Bull *et al.* (*30*), using the Torkelson *et al.* data from *E. coli* (*17*), estimated a mutation rate that is equivalent to 0.00035 mutations per day per mutational target after a 4-day starvation course at 37°C. If one assumes that the genome of *E. coli* contains about 85 (≅ 0.03/0.00035) mutational targets with similar properties that affect growth rate, then an overall mutation rate of ~0.03 per genome per day results. This mutation rate was observed in a small fraction of a starving stationary phase culture of normal cells of *E. coli* but not in long-term mutators. The general phenomenon of transient mutability induced by starvation has recently been shown to occur in most (but not all) natural isolates of *E. coli* (*12*). Cells that survive the longest in such experiments may do so because their transiently high mutation rates repeatedly generate selected phenotypes. If all other cells die, the effective population size in the stationary phase might be much smaller than one would assume initially. Resulting hitchhiking events may significantly accelerate evolution in the stationary phase. Thus, it appears that mutagenesis in response to starvation might generate many more mutations than DNA replication errors that occur during cell division.

This suggests a correlation between deleterious mutation rate estimates and the time bacteria spend in the stationary phase in mutation accumulation experiments. Support comes from a comparison of our study with the two replication-based deleterious mutation accumulation experiments available that used the same strain, i.e., REL606 (*9*, *10*). It is striking (Fig. 3) that a BM analysis of the data reported by Cooper and Lenski (6.7 generations per day at 37°C in liquid glucose minimal medium) led to a higher deleterious mutation rate than the one found by Kibota and Lynch (25 generations per day at 37°C on modified Davis minimal agar plates).

Mutation rates in stationary phase are likely to be influenced by (i) temperature-dependent biochemical reactions, (ii) the genetic background of the strain (*12*), and (iii) the complex population genetics of stationary phase that determines the fixation probability of mutants in the population. Our data indicate that the time bacteria spend in stationary phase must be very important for their evolution and, given the ubiquity of stationary phase or near-stationary phase conditions in nature, our results suggest we should reshape our views about bacterial evolution (*31*). Although such high mutation rates can be deadly for bacteria caught in stationary phase, they probably play an important role in escaping local extinction, accelerating adaptation (*32*), and evolving pathogenicity (*33*).

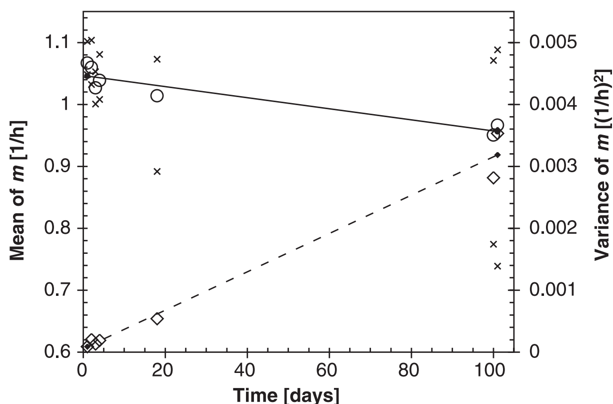

Fig. 2. BM analysis of the stationary phase mutation accumulation experiment. This plot corresponds to a deleterious mutation rate U_{BM} of 0.026 per genome per day with a deleterious selection coefficient s_{BM} of 3.5%, assuming mutational effects are equal. All growth rates (*m*) of this plot were observed at a 2401-fold dilution. The solid line and circles denote means (M); the dashed line and diamonds denote variances (V). The highest and lowest growth rates are marked (×). From the slope of the regression lines, $U_{BM} = (\Delta M)^2/\Delta V$ and $s_{BM} = \Delta V/\Delta M$ were calculated, where Δ is the difference for 1 day (*9*).

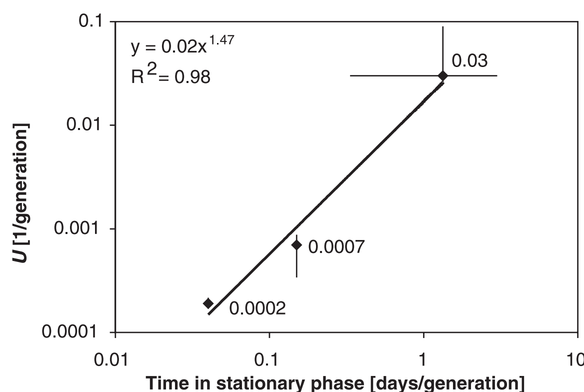

Fig. 3. Mutation rates per generation depend on time spent in stationary phase. All estimates have been derived from descendants of the same strain of *Escherichia coli* growing at 37°C. The lowest mutation rate was observed over 7500 generations in 300 days (*9*), the highest rate in Fig. 2. The link between these extremes comes from 10,000 generations of mutation accumulation in genes not required for metabolism in Lenski's long-term serial transfer evolution experiment [data for Bateman-Mukai analysis could be estimated from figure 4 in (*10*)]. U denotes BM estimates of deleterious genomic mutation rates. Error bars, estimated range of values.

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Supporting Online Material
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Supporting Online Material

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1. Materials and methods

1.1 Bacterial strains

An asexual non-mutator strain of *Escherichia coli* B (REL606) was used that had been described earlier (*1, 2*). It was grown at 37°C in LB (Luria Broth; 50 g Tryptone + 25 g NaCl + 25 g Yeast extract in 5 l distilled water well mixed, adjusted to pH 7.4 and distributed to many small flasks before autoclaving).

1.2 Mutation accumulation

The experimental design of the stationary phase mutation accumulation experiment can be found in Figure S1. The first 4 serial transfers were designed to allow regeneration from freezing and to measure the precise initial condition of the 99 lines that were derived from a frozen culture of the same strain that had also been used in Lenksi's evolution experiments (*2*) and in Kibota and Lynch's bacterial mutation accumulation experiment (*1*).

Each well of the low dilution plate of ST1 contained 240 µl LB and was directly inoculated with 5µl from one 1 ml sample that was thawed in ca. 30 min from storage at -70°C to room temperature. This sample had been frozen to -70°C (in 2M glycerol) from the stationary phase of the strain obtained after growing it for two consecutive nights in 100 ml LB. Each well of the high dilution plate contained 240 µl LB (as all the plates in this study) and was inoculated with 5 µl from the corresponding well on the low dilution plate (after mixing; this is how all high dilution plates in this study were produced). ST1-4 comprised 24 h observation for each ST after adding 5µl of the stationary phase culture from the corresponding well of the previous transfers low dilution plate to 240µl LB.

The long-term plates after ST4 were wrapped in Parafilm™ to prevent loss of liquid. They were incubated at 37°C in the dark without shaking (except pipette mixing during rare transfers like the 1:1 dilutions for producing plates in the big, long arrows; ST5-6 used 5µl of stationary phase culture for corresponding fitness measurements). Mutation accumulation and growth measurements were performed in HoneyComb2 plates (Labsystems, Helsinki, Finland).

To assess heritability of the evolved phenotypes, the high dilution plate of ST6 was stored at -70°C after growth to stationary phase (no glycerol added) until further analysis in ST7-9. After thawing (<1h) ST7 was inoculated like all other transfers. One line was lost in this process. The data from ST7 could not be used, because cells needed the whole transfer to recover to their normal speed and thus showed extremely slow growth curves (mean doubling time 1.6 ± 0.9 hours for the high dilution plate).

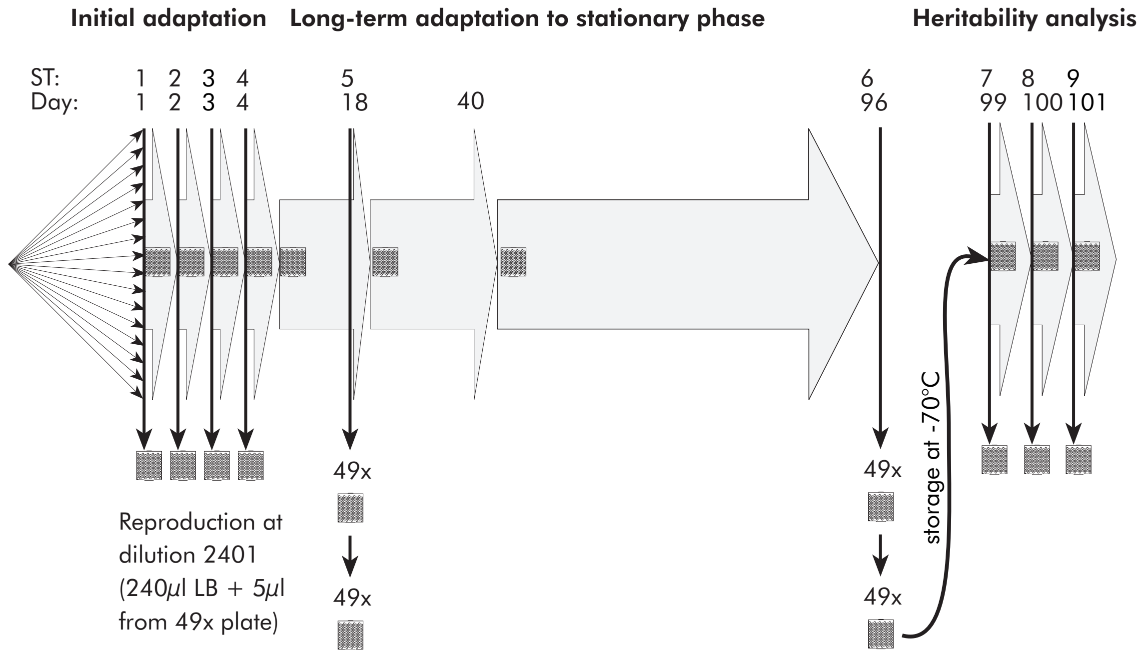

Figure S1. *Experimental design of the stationary phase mutation accumulation experiment.* ST denotes the Serial Transfer that was conducted after the given days in stationary phase.

1.3 Measurement of maximal growth rates

Cell lines from ST1-6 were analyzed immediately after sampling for growth at 37±0.1°C under continuous shaking in the microtiter-like HoneyComb2 plate in the Bioscreen C system (Labsystems, Helsinki, Finland) using the same batch of growth medium for all plates. ST7-9 were analysed in the same way, except that they experienced one freeze-thaw event between ST6 and ST7. Optical density (OD) was measured at 600 nm every 5 minutes for 24 hours. Minimal, middle and maximal values for the steepest slope of a regression line through at least 3 measured log-OD values were computed and checked by eye. The mean of these three values was used to compute the

maximal growth rate per hour = Malthusian parameter m = $\ln(OD_{t+1h}/OD_t) = \ln(2)/\text{doublingtime}$ [see p5 in (*3*)], assuming a linear relationship between optical density and population size. The Malthusian parameter is the best way to measure fitness for multiplicative fitness models. A total of 1800 growth curves representing more than 500 000 OD values were analysed.

1.4 Estimation of mutational parameters

For haploids with equal mutation effects, $U_{BM} = (\Delta M)^2/\Delta V$ and $s_{BM} = \Delta V/\Delta M$, where U_{BM} is the deleterious mutation rate per genome over the time interval Δt, s_{BM} is the selection coefficient, ΔM is the decrease of mean of m, ΔV is the increase of variance of m and everything is scaled to Δt = one day (see p341-343 in (*4*) and refs (*1, 5*)). Actual observations were used for the initial distribution of fitness (no artificial variance of 0, no scaling of mean). Maximum likelihood analysis was conducted as described (*6*).

1.5 Measurement of heritable mutation rates

To determine whether lines had become heritable mutators after long-term stationary phase mutation accumulation, we measured the mutation rate for reversion from the Ara^- genotype to Ara^+ as described (*7*) for the founding strain. Mutation rates were determined for the founding strain of the experiment and for the randomly chosen lines 20, 40 and 69 originating from ST7 (stored with 2M glycerol at -70°C). A Luria-Delbrück fluctuation assay format was applied (*8, 9*). After thawing and 5 overnight transfers in Davis minimal medium with 2.5% glucose [see (*7, 10*)], six 41 ml cultures were started for each line. After overnight growth, the cell number of each culture was determined and all cells of each culture were plated on Davis minimal agar medium containing 4% arabinose as the sole carbon source [see (*7, 10*)]. The Ara^+ mutants per culture were counted. Mutation rates were estimated by using the maximum likelihood method (*11*) as described by ref (*9*). 95% confidence intervals were obtained from formulae 23-25 in ref (*9*). Final mutation rates were computed assuming that the probability of mutation is distributed evenly over the division cycle (i.e. divide by 1.44 times total number of cells in culture, see (*9*)) and final lower and upper bounds used the largest and smallest estimates for the number of cells in the culture. Two mutation rate measurement replicates A and B were conducted.

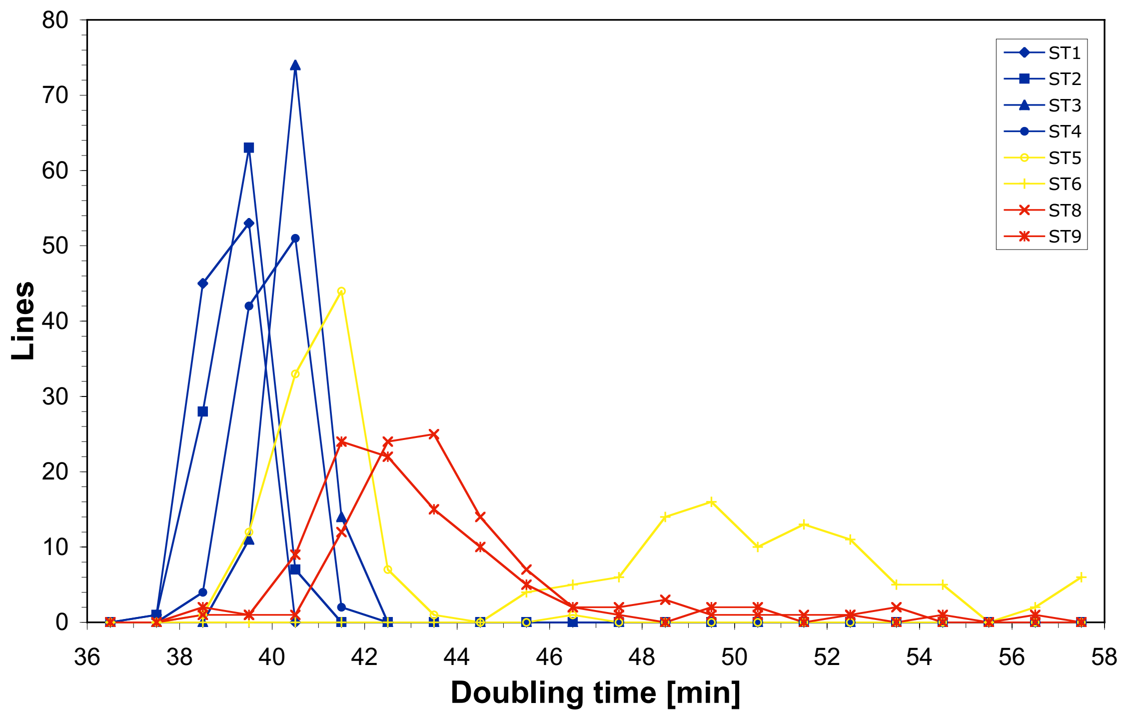

Figure S2. *Overview over growth rate distributions at high dilutions.* Please note that the variability within groups (ST1-4, blue, initial variability; ST8-9, red, final variability) can be significant. ST5-6 (yellow) have been measured *directly* from long-term stationary phase without opportunity to recover. Each distribution contains one measurement of each line that experienced the corresponding serial transfer (ST). The dilution of the inoculum was 2401 fold in all measurements. Descriptive statistics of these distributions are given in Table S1. Please note the long tails in ST5-9; the last point in ST6 includes all longer doubling times as well.

2. Overview over growth rates at high dilutions

To allow the reader to get a better feeling for the variability that comes with the experimental system, Figure S2 and Table S1 give an overview over all interesting growth rate distributions as measured from high-dilution plates (2401 fold).

Table S1. *Overview over growth rate distributions at high dilutions.*
See Figure S2 for more details. All values give the doubling time in minutes.

SerialTransfer	Mean	StDev	Min	Max
1	38.99	0.35	37.75	39.68
2	39.25	0.52	37.68	40.29
3	40.52	0.45	39.50	41.56
4	40.05	0.53	38.48	41.26
5	41.04	0.98	38.76	46.64
6	51.17	4.43	45.12	70.49
8	43.91	2.68	38.83	53.71
9	43.22	2.98	38.22	56.29

3. Heritability of growth rate changes

Heritability of the slow growth acquired after 100 days of mutation accumulation in stationary phase was demonstrated in ST8 and ST9. These two consecutive serial transfers showed only marginal differences that do not seem to be significant in the light of the accuracy of the method (see fluctuations in ST1-4). However, ST8-9 showed faster growth than had been observed in ST6. Thus part of the slow growth in ST6 might stem from temporary patterns of gene expression that vary among lines. However, it is not very likely that patterns of gene expression still influence growth after freezing-thawing *and* 2 additional serial transfers with fresh medium. Furthermore, the characteristic shape of the distributions in ST1-4 is markedly different from that in ST8-9 (note the very long tail). Finally, particularly slow lines remain slow, if compared across ST6, ST8 and ST9, with no significant changes between ST8 and ST9, which is also clear evidence for heritability. Thus, we used the data from ST8-9 to compute the final mutation rates.

4. Influence of different viable cell counts in the inoculi on growth rates

If fresh medium is inoculated with such a number of cells that the first cells enter stationary phase (due to overcrowding) before the last cells leave the lag-phase, then the apparent maximal growth rate will be smaller than the real one. We cannot see another mechanism for inoculum concentration to influence maximal growth rate, since we do not use the absolute OD values from either lag phase or stationary phase, but only the slope of a straight line fitted to LogOD. From our comparisons of low dilution plates (49x) and high dilution plates (49x49) we know that a 49 fold difference in the number of inoculated cells does not necessarily influence the maximal growth speed we observe, although it does so sometimes by a few percent. While we found considerable heterogeneity (up to 10 fold and more) in cell density between lines after one month in stationary phase, such variability is not responsible for the observed effect on growth rate, for the following reasons:

(i) Bateman-Mukai computations of mutation rates using fitness values of the low-dilution lines are close to the values obtained when using the high-dilution lines (after excluding those transfers, where condensed water blurred growth rate measurements).

(ii) Variability in cell density after long-term stationary phase does not apply to ST8 and ST9, because these were sampled from fresh overnight cultures that do not show such a large variability in cell density in stationary phase.

(iii) If mere pipetting errors were responsible for a decrease of mean and an increase of variance of growth rate, then one would expect *much* more variability from one transfer to the next in the 2 series of consecutive over-night transfers (ST1-4 and ST8-9).

(iv) Mere random pipetting errors cannot explain, why the slowest lines in ST9 are immediate descendants of the slowest lines in ST8 and ST6 (comparison not shown).

To summarize, the inferred mutation rates do not appear to be generated by the variability in the number of viable cells in inocula.

5. Mutational parameter estimates

Table S2 and Table S3 provide an overview over the various estimates of the mutational parameters that can be deducted by using different combinations of serial transfers or dilutions. As values that use ST6 as final point are most probably influenced by temporary patterns of gene expression, these cannot be taken at face value. Quality of measurements was generally higher with the high dilution plates.

Table S2. *Maximum-Likelihood estimates of mutational parameters.*
U = mutation rate per genome per generation assuming a Gamma distribution of mutational effects with the shape-parameter β, s = deleterious selection coefficient in percent assuming the same distribution, d = dilution of inoculum, ST = serial transfers used, ± give 95% confidence intervals. Shape-parameter of inf and 1 correspond to mutations with equal effects and to mutations with exponentially distributed effects, respectively.

ST	**d**	**β**	**U**	**s**
1-2+8-9	49x49	inf	0.045±0.004	2.3±0.2
1-2+8-9	49x49	1	0.091±0.01	1.1±0.1
1+9	49x49	inf	0.037±0.004	2.6±0.1
1+9	49x49	1	0.067±0.008	1.4±0.2
1+8	49x49	inf	0.045±0.004	2.5±0.1
1+8	49x49	1	0.111±0.01	1.0±0.1
1+6	49x49	inf	0.198±0.01	1.3±0.1
1+6	49x49	1	0.410±0.02	0.63±0.03
1+6	49x	inf	0.129±0.008	2.4±0.1
1+6	49x	1	0.272±0.016	1.13±0.06

Table S3. *Bateman-Mukai estimates of mutational parameters.*

U_{min} = lower bound for the mutation rate per genome per generation, s_{max} = upper bound for the deleterious selection coefficient in percent, d = dilution of inoculum, ST = serial transfers used to compute the slope of the regression lines.

ST	d	U_{min}	s_{max}
1-4+8-9	49x49	0.027	3.4
1-5+8-9	49x49	0.026	3.5
1+8-9	49x49	0.038	2.9
1+8-9	49x	0.089	1.6
1+6	49x	0.12	2.5
1-6	49x49	0.16	1.5

6. Influence of long-term stationary phase on heritable mutation rates

It is important for the conclusions presented in this paper that mutability was transient. The main study that reported on the evolution of heritable mutators in this strain of *E. coli* (*7*) found 3 mutators to evolve among 12 lines after 2500, 3000 and 8500 generations, corresponding to 375, 450 and 1275 daily serial transfers. We doubt that so many mutators could have arisen in the 100 days of our experiment without the help of transient mutators. But even with the help of transient mutators, we still expect the majority of lines to be non-mutators, as it appears unlikely that mutation repair has been damaged in every line. The mutation rates measured in the founding strain and in the randomly chosen lines 20, 40 and 69 (Table S4) indeed demonstrate that these three lines do not show elevated mutation rates. Thus it seems unlikely that our results are due to heritable mutators.

Table S4: *Mutation rates for the reversion from the Ara$^-$ genotype to Ara$^+$.*
Measurements A and B are two independent replicates. Multiply all numbers by 10^{-10}. The numbers in brackets give lower and upper 95% confidence limits.

Strain	**Measurement A**	**Measurement B**
Founder	2.5 (0.9 - 10.2)	2.0 (0.9 - 4.5)
Line 20	2.0 (0.8 - 4.9)	1.1 (0.4 - 3.7)
Line 40	1.9 (0.9 - 4.4)	1.1 (0.2 - 4.6)
Line 69	2.7 (1.3 - 4.2)	2.6 (1.1 - 5.4)

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TECHNICAL COMMENT ABSTRACTS

COMMENT ON "High Deleterious Genomic Mutation Rate in Stationary Phase of *Escherichia coli*"

J. Arjan G. M. de Visser and Daniel E. Rozen

Loewe et al. (Reports, 28 Nov. 2003, p. 1558) presented an estimate of the deleterious mutation rate in stationary phase of *E. coli* that is likely erroneous. Rather than measuring deleterious mutations, the authors have probably measured negative pleiotropic effects of positively selected mutations that provide a growth advantage in stationary phase.

Full text at www.sciencemag.org/cgi/content/full/304/5670/518c

RESPONSE TO COMMENT ON "High Deleterious Genomic Mutation Rate in Stationary Phase of *Escherichia coli*"

Laurence Loewe

de Visser and Rozen greatly underestimate the effects of so-called GASP mutations. Since the deleterious mutations we reported are dwarfed by growth arrest, linked GASP mutations can easily hitchhike them to fixation. Thus, antagonistic pleiotropy is unnecessary to explain our data, although it cannot be ruled out. Circumstantial evidence supports our original claim and may suggest "cooperative evolution" in bacteria.

Full text at www.sciencemag.org/cgi/content/full/304/5670/518d

Comment on "High Deleterious Genomic Mutation Rate in Stationary Phase of *Escherichia coli*"

Loewe *et al*. (*1*) presented an estimate of the deleterious mutation rate for *Escherichia coli* in stationary phase that is two orders of magnitude higher than an earlier estimate using the same strain of *E. coli* (*2*). From this striking discrepancy in mutation rate estimates, they concluded that cells held in stationary phase become transient mutators and rapidly accumulate deleterious mutations. However, we believe that problems with experimental design and data interpretation render these conclusions erroneous.

The conventional method to study deleterious mutations is to conduct a mutation accumulation experiment, in which replicate populations started from a single ancestor are serially passaged through bottlenecks (*3*). In such small populations, where natural selection is largely absent, deleterious mutations can accumulate relatively freely by genetic drift. As a result of mutation accumulation, the mean fitness of replicate populations is expected to decrease over time while the variance across populations increases. Estimates of deleterious mutation rate and mean effect of each mutation can be inferred from these data using standard Bateman-Mukai and maximum-likelihood analyses (*3*).

Loewe *et al*. (*1*) conducted such an experiment, but rather than passaging cells through bottlenecks, the cells were maintained in stationary phase at high densities [on the order of 10^7 cells after an initial decline from about 10^9 cells (*4*)] for about 100 days. Maximum growth rates were then determined in fresh medium and the anticipated response was observed: Mean growth rate declined while the variance across populations increased. These changes in fitness were then used to derive estimates of rate and mean effect of deleterious mutations. Loewe *et al.* compared their high estimate of deleterious mutation rate (translating per-generation into per-day estimates by assuming a certain number of cell generations per day) with estimates from studies in which stationary phase conditions were less prominent, and concluded that the mutation rate must have been transiently increased during stationary phase conditions (*1*).

We believe that an alternative explanation is more likely. The probability for mutation fixation by genetic drift within the ~100 days of the experiment is extremely small for populations of 10^7 cells. Instead, the long-term maintenance of large populations of *E. coli* in stationary phase, as in (*1*), allows for the frequent and rapid selection of spontaneous mutants with growth advantages during stationary phase [so-called GASP mutants (*4*)]. Furthermore, GASP mutants are known to have reduced fitness during rapid growth conditions due to antagonistic pleiotropy (AP) (*5*). It is thus likely that Loewe *et al.*, rather than measuring the effects of accumulated deleterious mutations, were instead measuring the negative pleiotropic effects under rapid-growth conditions of mutations that reached fixation by natural selection under conditions of restricted growth. This would also cause a mean decline in maximum growth rate, as well as an increase in across-population variance if these nonselected pleiotropic effects are diverse. To distinguish between these possibilities, Loewe *et al.* would need to assay fitness of their populations under the same conditions in which the mutations arose (i.e., during stationary phase). Instead, their assays were conducted during rapid growth conditions, which would not measure the effects of mutations accumulated by genetic drift—however unlikely those would be. These two problems—large populations experiencing strong natural selection, and fitness assays performed under conditions different from those that prevailed in stationary phase—make the inferences drawn in (*1*) doubtful.

In a subsequent analysis, Loewe *et al*. interpreted the loss of niche breath observed in a long-term evolution experiment with *E. coli* (*6*) as resulting from mutation accumulation, while the conclusion of this study was instead that antagonistic pleiotropy caused most of the observed loss. It may be true that *E. coli* held in stationary phase have elevated mutation rates (*7*), and it remains an important and worthwhile problem to determine the extent of these rate increases and their associated effects on fitness. However, it will be a challenge to develop protocols that allow the free accumulation in stationary phase of deleterious mutations by genetic drift, while simultaneously avoiding selection of GASP mutants.

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Response to Comment on "High Deleterious Genomic Mutation Rate in Stationary Phase of *Escherichia coli*"

It did not escape our attention that fixation times for mutations would be excessive in our experiment, if effective population size (N_e) was similar to census population size. Therefore, we pointed to fixation of adaptive mutations (e.g., growth advantage in stationary phase, or GASP mutations) that reduce N_e drastically (*1–5*): One cell that can grow in stationary phase, while all others cannot, will sweep through the population, fixing all its slightly deleterious mutations (SDMs). GASP experiments suggest several sweeps in 100 days (*6, 7*), leading to repeated bottlenecks. The resulting tiny N_e in our experiment justifies the mutation accumulation (MA) design if we assume that a reasonable number of sites can accumulate SDMs mild enough not to interfere with GASP-sweeps, and if the sweep-causing advantageous mutations themselves have no negative side effects on exponential growth. de Visser and Rozen (*8*) claim that there is enough evidence for antagonistic pleiotropy (AP) to doubt the second assumption. However, their postulated high variability of nonselected pleiotropic effects seems rather unlikely with repeated quasi-deterministic sequences of GASP mutations assayed in constant environments.

AP always implies that the same mutations that promote growth in stationary phase, slow growth during log phase. Without detailed genetic analysis, these effects of AP are hard to separate (*9, 10*) from other SDMs on the same nonrecombining genome (predicted by MA). Thus, circumstantial evidence must decide whether MA or AP contributed more to our observations.

de Visser and Rozen cite Vasi and Lenski (*11*), who reported growth patterns similar to ours, but provided no data to conclusively distinguish AP from MA. Cooper and Lenski (*9*) attempted to differentiate between AP and MA, but did not carry out a formal genetic analysis of the mutants. They based their test largely on a presumptive log-linear decline in performance under MA versus a log-curvilinear decline under AP. This does not have a strong theoretical basis, especially if N_e changes or mutants inactivating crucial pathways are occasionally fixed under AP (*9*). Moreover, a detailed analysis suggests that the statistical power of their data is insufficient to detect deviations from log-linearity. A subsequent genetic analysis (*12*) of the most eroded catabolic function found in (*9*) is suggestive of AP, but not conclusive. Unfortunately, the genes involved showed extraordinarily high mutation rates (*12*) and only a few-fold higher increase (*1*) is necessary to explain the observed parallel evolution by MA.

de Visser and Rozen (*8*) suggested measuring growth rates in stationary phase (*13*), but this would simply provide evidence for the presence of GASP mutations. Furthermore, it is problematic to derive static assay conditions from an arbitrary snapshot of stationary phase, which is highly dynamic (*6, 14–17*). However, two other approaches might help quantify the contributions of MA and AP. First, comparisons of DNA sequences could reveal increased mutation rates predicted by MA, as in the studies which found adaptive mutations in stationary phase to be accompanied by transiently high mutation rates in many unselected genes (*18–23*). These studies inspired the interpretation of our experiment. A prominent role for transposable elements (TE) in MA is suggested by the similarity of mutational effects found in Bateman-Mukai analyses of data in (*1, 9, 24*) to the effects of TE mutagenesis (*25*). Confirmation comes from similar catabolic decay rates (*9*) and TE mutation rates (*26*) between mutators and non-mutators. Point mutations appear less important for Lenski's lines (*27*). Second, functional characterization of GASP mutations at the molecular level, combined with high-precision growth curves when placed in an isogenic background, can quantify their effect on fitness during exponential growth. The extensive double checks needed to exclude mutations generated by the molecular methods used (*12, 28*) appear within reach for some well-studied GASP mutations [e.g., (*12–14*)].

Should GASP mutations regularly have deleterious side effects (AP), our findings present bacteria with an evolutionary dilemma: To either avoid extinction by acquiring GASP mutations, or avoid collecting excess GASP mutations because removing side-effects would require improbable back-mutations. GASP mutations without negative side effects will survive better over the long term, once growth resumes. Thus, frequencies of stationary phase mutators (that survive by adapting) and nonmutators (that preserve quality genes) may indeed be associated with a complex evolutionary fitness tradeoff (*11*) that depends heavily on horizontal gene transfer (*29, 30*). The mutators may not "cheat" (*13*), but rather "cooperate" with nonmutators to generate frequent adaptive mutations without degrading adapted genes in the species. Such "cooperative evolution" may be pivotal for the success of bacteria—AP would prevent it.

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