High deleterious genomic mutation rate in stationary phase of *Escherichia coli*

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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).

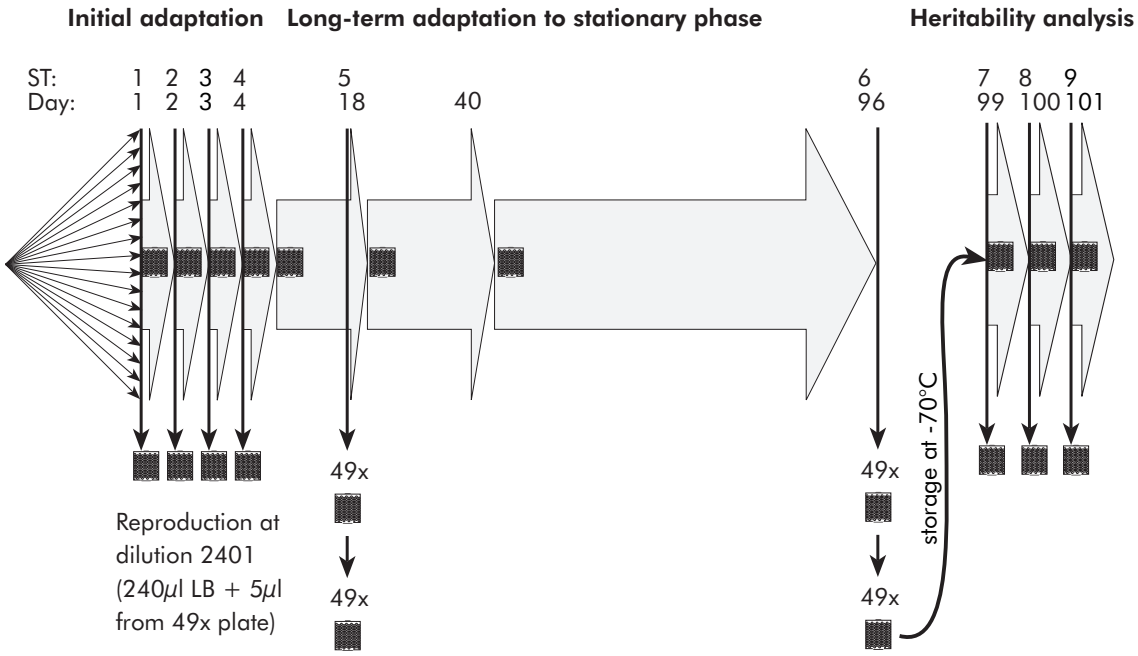

Figure S1. *Experimental design of the stationary phase mutation accumulation experiment.* ST denotes the Serial Transfer that was conducted after the given days in stationary phase.

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)/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.

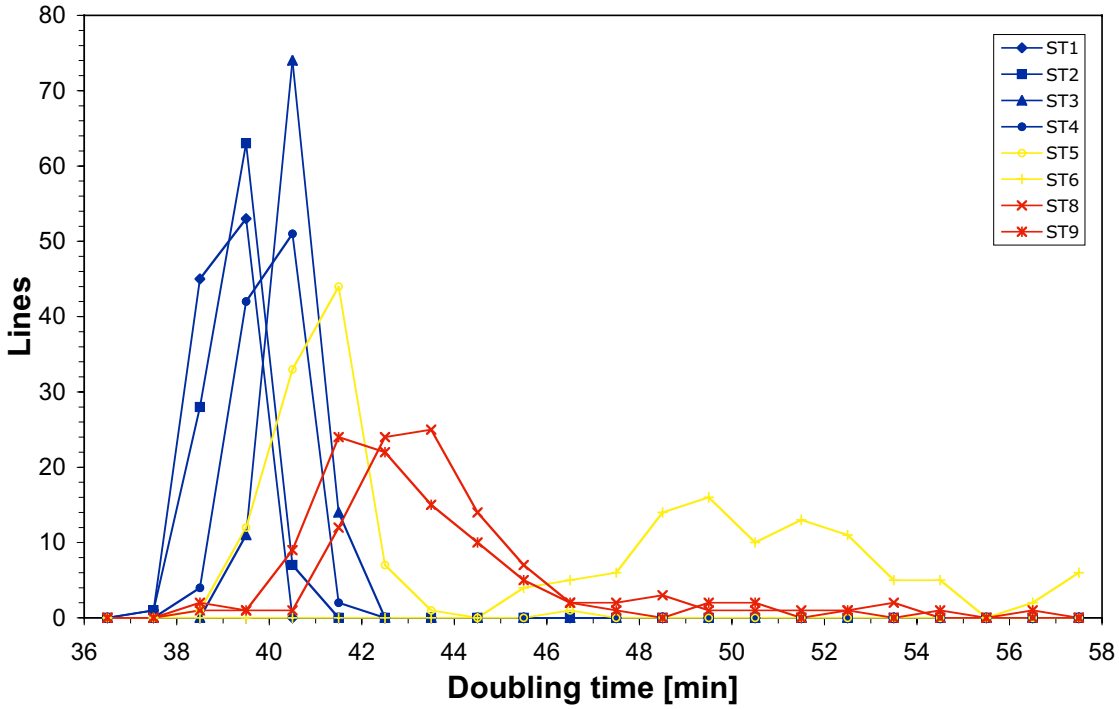

Figure S2. *Overview over growth rate distributions at high dilutions.* Please note that the variability within groups (ST1-4, blue, initial variability; ST8-9, red, final variability) can be significant. ST5-6 (yellow) have been measured *directly* from long-term stationary phase without opportunity 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)

7. References

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