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Genomic buffering mitigates the effects of deleterious mutations in bacteria

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The relationship between the number of randomly accumulated mutations in a genome and fitness is a key parameter in evolutionary biology[1–5]. Mutations may interact such that their combined effect on fitness is additive (no epistasis), reinforced (synergistic epistasis) or mitigated (antagonistic epistasis). We measured the decrease in fitness caused by increasing mutation number in the bacterium *Salmonella typhimurium* using a regulated, error-prone DNA polymerase (polymerase IV, DinB). As mutations accumulated, fitness costs increased at a diminishing rate. This suggests that random mutations interact such that their combined effect on fitness is mitigated and that the genome is buffered against the fitness reduction caused by accumulated mutations. Levels of the heat shock chaperones DnaK and GroEL increased in lineages that had accumulated many mutations, and experimental overproduction of GroEL further increased the fitness of lineages containing deleterious mutations. These findings suggest that overexpression of chaperones contributes to antagonistic epistasis.

Synergistic and antagonistic epistasis has previously been reported in DNA-based organisms[6–12]. But these results have been difficult to generalize, because in some cases only a limited number of specific mutations were studied and in other cases the numbers and types of mutation were unknown. We allowed random mutations of an experimentally determined number and type to accumulate in the genome of *S. typhimurium*. Bacteria with different mutation rates were serially passaged by repeated streaking every 48 h on agar plates to generate new colonies initiated by a single cell. The repeated one-cell bottleneck increased genetic drift and allowed all types of mutation to come to fixation with roughly the same probability. We varied mutation rates over a range of several hundred-fold by means of an introduced plasmid encoding the DinB polymerase[13] expressed under control of the arabinose-inducible promoter P_{BAD}. By increasing the level of inducer in the growth medium, the amount of DinB synthesized in the cells could be increased and maintained at a set

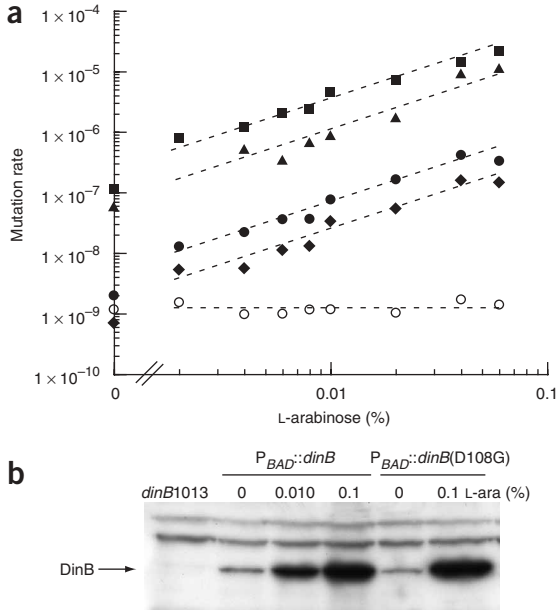

Figure 1 Mutation rates as a function of DinB inducer (L-arabinose) concentration. (**a**) Rifampicin (closed circles) and nalidixic acid (diamond) resistance is caused by base-pair substitutions; chlorate resistance (squares), by loss-of-function mutations; and Lac$^-$→Lac$^+$ reversion (triangles), by –1 frameshift mutations. The mutation rate to rifampicin resistance for an inactive DinB variant (open circles) containing a mutation in the conserved catalytic site (D108G) is also indicated. (**b**) Amount of DinB protein produced at different L-arabinose concentrations as determined by western blotting. Cell lysates were prepared from strains DA7974 (Δ*dinB*1013::*cam*), DA7699 (P_{BAD}::*dinB*) and DA9217 (P_{BAD}::*dinB*(D108G)).

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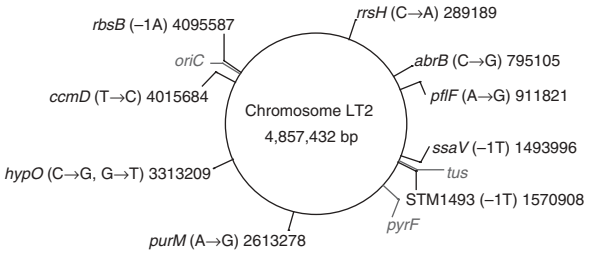

Figure 2 Chromosomal locations of mutations. Sequence changes were identified in the evolved lineages of *S. typhimurium*. Indicated are gene (type of mutation) and nucleotide position. Genomic reference points *oriC*, *tus* and *pyrF* are indicated.

level. When overproduced, DinB generates a variety of mutation types with a preference for transversions and frameshifts[13]. After propagating the bacteria for many generations with different mutation rates, we reset the mutation rate to normal by removing the DinB inducer and then measured fitness.

We confirmed the relationship between DinB level and mutation by measuring the mutation rate of several known targets in bacteria carrying the P_{BAD}::*dinB* plasmid and grown in Luria Bertani broth with increasing levels of the inducer. We physically estimated the level of DinB protein for each inducer level. As inducer concentration rose, the level of DinB protein increased and the mutation rates for all tested targets reached a maximum of 230 times higher than the baseline mutation rate (**Fig. 1**).

We tested mutant DinB variants with mutations in the conserved catalytic site (323GAT→GGT, D108G) or DNA binding site (Δ140–141AG) to show that mutagenesis resulted from DinB polymerase activity. When overexpressed, these mutant DinB proteins caused no increase in mutation rates (**Fig. 1a**), indicating that the increase in mutation rate was due to errors introduced by DinB polymerase. These tests also showed the correlation between mutation rate and DinB levels and calibrated the system for tests of fitness costs.

Accumulation of random mutations before fitness testing was achieved by serial passage of bacteria on solid Luria Bertani medium containing amounts of DinB inducer chosen on the basis of our mutation rate measurements (**Fig. 1a**). For each inducer concentration, we passaged eight independent parallel lineages. Control experiments showed that the mutation rates on solid and liquid Luria Bertani media were similar and that a given concentration of inducer caused a similar increase in mutation rates. This allowed us to compare experiments done in liquid culture, which measured the increase in mutation rates (**Fig. 1a**), with those done on solid medium, where accumulation of random mutations occurred.

We assessed the number and distribution of accumulated mutations by sequencing 209 kbp of random DNA from the genomes of two lineages passaged with elevated DinB levels (**Supplementary Fig. 1** online). We found ten mutations at unique sites distributed throughout the chromosome (**Fig. 2** and **Supplementary Table 1** online), suggesting that the mutations occurred at random. We estimated the total number of mutations accumulated in the genome by extrapolating from the changes in the sequenced DNA to the number of mutations per genome. From this number, we estimated the number of mutations accumulated per genome for each lineage on the basis of the mutation rate at each particular level of DinB and the number of generations of growth at that mutation rate (**Supplementary Note** online).

We determined fitness of the serially passaged lineages as the generation time during exponential growth in Luria Bertani broth in the absence of DinB overproduction. Without inducer, the *dinB*-carrying plasmid caused no change in the mutation rate or growth rate (data not shown). Fitness was expressed relative to that of the nonevolved parental control strain (set to 1). **Figure 3a** shows the relationship between number of mutations accumulated per genome and mean fitness of the evolved lineages at 37 °C. The average number of mutations accumulated per genome varied from 0 (nonevolved parental lineage) to 233 (lineages evolved for 1,000 generations with the highest mutation rate). Six lineages were excluded from the analysis because their mutation rates changed during the experiment (**Supplementary Table 2** online).

As expected, mean fitness decreased as more mutations accumulated (**Fig. 3a** and **Supplementary Fig. 2** online). To determine whether the rate of fitness decline reflected epistasis, we analyzed

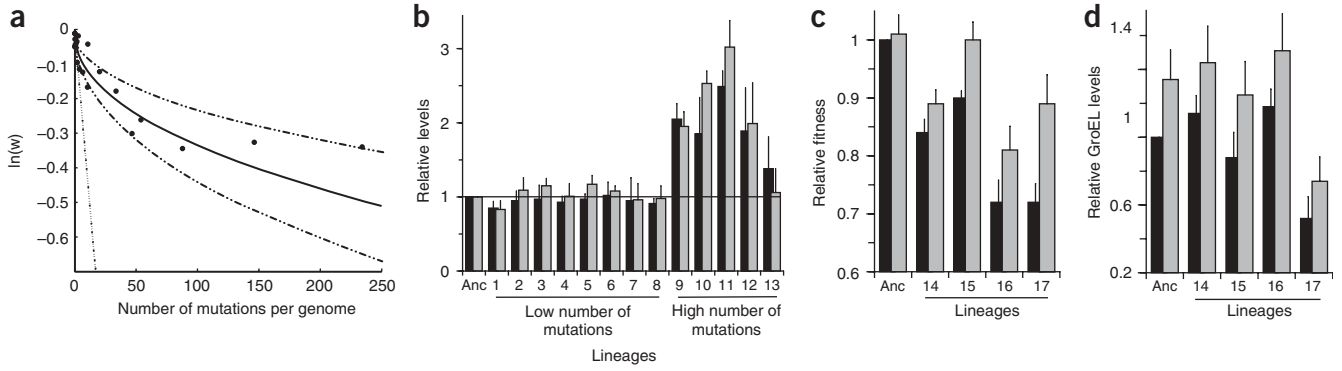

Figure 3 Effects of mutation accumulation on fitness and chaperone levels. (**a**) Decrease in mean fitness as a function of number of mutations accumulated. Each data point is the average fitness of five to eight lineages. Outer lines indicate the 95% confidence interval. The straight stippled line shows the expected fitness loss when applying a nonepistatic model with $\alpha = 0.041$. (**b**) Steady-state levels of chaperones GroEL (black) and DnaK (gray) as determined by western blots. Anc is the ancestral nonevolved strain. Low number of mutations indicates lineages that were serially passaged for 1,000 generations without arabinose (1–8) and high number of mutations lineages that were passaged for 1,000 generations with 0.1% arabinose (9–13). (**c**) Relative fitness and (**d**) steady-state levels of GroEL in strains carrying the plasmids pBB541 and pBB528 for expression of *groELS* under control of an IPTG-inducible promoter. The plasmid-containing ancestral nonevolved strain (Anc) and four lineages evolved without arabinose (14 and 15), 0.040% (16) and 0.1% L-arabinose (17) were grown in Luria Bertani broth at 37 °C without (black) and with (gray) IPTG. All levels were related to that obtained in the ancestral strain (1.0). Standard errors are indicated.

the data set (**Supplementary Table 3** online) using the regression model[14] $\ln(w) = -\alpha n^{\beta}$, where w describes fitness, α describes the extent of fitness decay, n is the number of accumulated mutations and β is the extent of epistasis. Antagonistic epistasis is indicated if $0 < \beta < 1$. There is no epistasis if $\beta = 1$, and epistasis is synergistic if $\beta > 1$. We fitted the parameters in the model by generalized least squares regression[15,16]; the parameter estimates for the fitted model were $\alpha = 0.041$ (standard error = 0.0054, $P < 10^{-10}$) and $\beta = 0.46$ (standard error = 0.038, $P < 10^{-10}$ for $\beta - 1$). Thus, antagonistic epistasis is indicated, because the parameter β lies between 0 and 1.

The finding that fitness declines with strong antagonistic epistasis was unexpected in view of previous experiments in which epistasis was either not observed[7–10,12] or was synergistic[6,11]. But antagonistic epistasis was predicted from *in silico* experiments, studies of the effect of mutations on RNA folding[14,17] and analyses of RNA viruses[18–20]. There are also several potential experimental biases that could make epistasis seem to be antagonistic[21]. One possibility is that we introduced a bias in colony picking. Because colonies were always picked at random, however, this is unlikely. A second explanation is that lineages with severe fitness reduction were not recovered during streaking on agar plates, and as a result, the actual fitness decline was underestimated. But control experiments showed that bacteria with a generation time of 2.5 h, compared with 20 min for the parental strain, could be recovered as colonies after 48 h of growth. Because all evolved lineages showed generation times shorter than 1 h, it is unlikely that the experiment missed a substantial number of slow-growing lineages. Other processes that could cause an underestimation of the fitness loss include the appearance of compensatory mutations during colony growth and the creation of synthetic lethals that reduce the number of mutations that are allowed in surviving clones. Our calculations suggest that neither compensatory mutations nor synthetic lethals are likely to cause the observed antagonistic epistasis, even though they can not be completely rejected (**Supplementary Note**).

The above potential biases should be more pronounced at high mutation accumulation and low fitness. But when we excluded lineages with the lowest fitness and the most extensive mutation accumulation ($n > 6$) from the data set, epistasis was still antagonistic and the β value was similar to that for the complete data set ($\beta = 0.46$). Another potential explanation for the antagonistic epistasis is that the accumulated mutations were occurring repeatedly in the same targets. Our sequencing data refutes such an interpretation, as the identified mutations occurred in different genes located across the whole chromosome (**Fig. 2**). Epistasis was previously reported to be absent in *Escherichia coli*[22]. Compared with our experiment, the mutation accumulation was low in their experiment; it is conceivable that an antagonistic effect would appear with more extensive mutation accumulation.

Another explanation for the antagonistic epistasis might be a physiological buffering mechanism. In several other organisms, the phenotypic penetrance of deleterious mutations may be reduced by the action of chaperones[23–25]. Thus, as mutations accumulate in the serially passaged lineages, an increased level of misfolded proteins could cause an upregulation of the levels of chaperones, which would buffer the phenotypic penetrance of later deleterious mutations. To examine this possibility, we measured the steady-state levels of two main chaperons, GroEL and DnaK, during logarithmic growth of cells from the five lineages that had accumulated the most mutations. The level of one or both of these chaperones was substantially elevated in all five lineages (**Fig. 3b**). In contrast, eight lineages that had been passaged without any induced mutagenesis had normal levels of chaperones, indicating that chaperone upregulation was a consequence of mutation accumulation and not of serial passage. These findings suggest that overproduction of chaperones contributes to antagonistic epistasis and imply that bacteria might be able to sense their level of mutagenic damage by responding to the level of misfolded protein. To substantiate this conclusion, we determined whether experimental overproduction of chaperones could increase fitness. We introduced a plasmid carrying the *groELS* genes under control of an IPTG-inducible promoter[26] into four lineages. When GroEL levels were increased moderately (by a factor of $\sim$1.5), fitness improved substantially (**Fig. 3c,d**). In contrast, similar GroELS overproduction did not increase fitness of the ancestral strain. This finding supports the idea that chaperone overproduction can buffer the negative fitness effects of deleterious mutations[24] but does not exclude the possibility that the genomic architecture of the organism also contributes to the antagonistic epistasis[18–20].

The antagonistic epistasis reported here has several broader implications. First, it argues against the mutational deterministic model for evolution of sex and recombination (at least in bacterial systems), which depends on synergistic epistasis[5]. Second, the strong antagonistic epistasis observed here suggests that a system for protein refolding might have the effect of abating the fitness cost of accumulated deleterious mutations, even though the rate of mutation accumulation (Muller's ratchet) might increase[27,28]. Finally, these findings also suggest that models that propose a regulated increase in mutation rate in response to starvation (hypermutable state)[29,30] might be prohibitively expensive with regard to the resulting fitness reduction.

METHODS

Strains and plasmids used (**Supplementary Table 4** online); determination of DinB, DnaK and GroEL levels; mutation rate determinations; overproduction of GroEL; and DNA sequencing are described in **Supplementary Methods** online.

Mutation accumulation experiment. We grew eight lineages of the parental nonevolved strain DA7699 (wild-type chromosome, P_{BAD}::*dinB*) on Luria Bertani agar plates containing 0, 0.004 %, 0.01%, 0.04% or 0.1% of L-arabinose and ampicillin to maintain the plasmid. At 48-h intervals, we randomly picked single colonies without regard to size or appearance, restreaked them onto fresh agar plates and grew them at 37 °C. Each serial passage (corresponding to 25 generations of growth) was repeated up to 40 times.

Fitness assay. After serial passage 8, 15, 25 and 40, we determined the growth rate in Luria Bertani broth at 37 °C by measuring the absorbance at 540 nm. Absorbance was proportional to viable count in the measured range. Relative fitness was defined as the ratio of the generation time of the parental strain and the serially passaged strains[9,14].

Statistical analyses. The parameters in the nonlinear $\ln(w) = -\alpha n^{\beta}$ model were estimated by generalized least squares regression[15,16]. The generalized model allows for heteroscedastic errors. The statistical analysis was done using R version 1.9.0.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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