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Test of synergistic interactions among deleterious mutations in bacteria

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Identifying the forces responsible for the origin and maintenance of sexuality remains one of the greatest unsolved problems in biology[1–6]. The mutational deterministic hypothesis postulates that sex is an adaptation that allows deleterious mutations to be purged from the genome; it requires synergistic interactions, which means that two mutations would be more harmful together than expected from their separate effects[4,5]. We generated 225 genotypes of *Escherichia coli* carrying one, two or three successive mutations and measured their fitness relative to an unmutated competitor. The relationship between mutation number and average fitness is nearly log-linear. We also constructed 27 recombinant genotypes having pairs of mutations whose separate and combined effects on fitness were determined. Several pairs exhibit significant interactions for fitness, but they are antagonistic as often as they are synergistic. These results do not support the mutational deterministic hypothesis for the evolution of sex.

Within a population of organisms, an asexually reproducing genotype should have twice the fitness of its sexual counterparts, all else being equal[1,2]. Therefore, it is difficult to understand why sexual reproduction is so widespread in nature[3]. This problem remains unsolved not for want of theories, but rather for lack of compelling data. Among some 20 hypotheses[5], two postulate that sex is an adaptation for purging deleterious mutations from the genome[4–6]. One of these two, Muller's ratchet[7,8], depends on random genetic drift and thus provides an advantage for sex only in small populations. In contrast, the mutational deterministic hypothesis[4,5] is effective in large populations but requires synergistic epistasis, such that multiple mutations are typically more harmful together than would be expected from their separate effects (Fig. 1). In mathematical form, let

$$\ln W_k = -\alpha k - \beta k^2,$$

where W_k is the average fitness of genotypes with k mutations, $\alpha > 0$ for deleterious mutations, and β defines the interaction between mutations. The intercept is fixed at zero because fitness values are expressed relative to an unmutated progenitor. If mutations interact synergistically ($\beta > 0$), then the equilibrium load of deleterious mutations is lower in a sexual than in an asexual population[9,10], providing an advantage for sex. (This requirement for synergistic epistasis has recently been extended to bacterial transformation, a form of non-mendelian sex[11].)

There are few data to indicate whether deleterious mutations typically interact in a synergistic manner, and previous attempts to address this issue have been hampered by methodological limitations. For example, some studies have examined interactions between whole chromosomes isolated from nature[12,13], whereas others allowed spontaneous mutations to accumulate by population bottlenecks[14,15] or have used mutagens to achieve the same effect[13,16]; these approaches all depend on indirect estimates of mutation number in experimental genomes. Other studies have examined interactions among defined sets of mutations[17], which may not be representative of the whole genome. There is the added problem that genomes with several mutations may be more difficult to obtain, and hence to measure, than genomes with only one mutation. Also, most studies have relied on incomplete fitness estimates in organisms with complex life-cycles, such as fruitflies. Moreover, dominance and inbreeding effects may obscure interactions in diploid organisms[18]. Finally, a recent experiment with

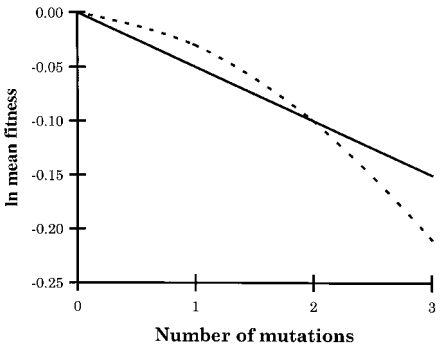

Figure 1 Hypothetical effects of increasing numbers of deleterious mutations on fitness. Each curve is based on the general model given in the text. The solid line illustrates multiplicative effects ($\alpha = 0.05$; $\beta = 0$); each additional mutation causes a comparable reduction in fitness. The dotted curve shows synergistic epistasis ($\alpha = 0.01$; $\beta = 0.02$); each additional mutation causes a disproportionate fitness reduction. The mutational deterministic hypothesis for the evolution of sex requires synergistic interactions among deleterious mutations.

yeast suggests that sex provides an advantage by the elimination of deleterious mutations, but it cannot distinguish between the effects of Muller's ratchet and synergistic epistasis[19]. Our approach avoids these methodological problems.

The most direct approach for determining the relationship between mutation number and fitness is to construct genotypes with different numbers of random mutations and measure their relative fitness. The bacterium *E. coli* provides an excellent system for this approach. The particular strain we used was sampled from a population that was propagated in a defined environment for 10,000 generations, during which time its rate of evolutionary adaptation slowed dramatically[20]. Hence, this strain was near a selective optimum in that environment. Three mini-Tn*10* transposons, each having a different antibiotic-resistance marker, were used to generate insertion mutations[21]. These transposons had been modified so that the site of insertion was effectively random[21]. We identified mutants under permissive conditions, on a nutritionally rich medium; we then measured their fitness under stringent conditions, in the same minimal medium in which their progenitor evolved. Hence, the sample is unbiased with respect to recovery of mutations, and fitness is expressed in the same environment as the organism's recent selective history. We constructed 225 genotypes, including 75 each with one, two or three insertion mutations. Each numerical class was balanced with respect to the three resistance markers. For every genotype, we performed at least three competition assays, which typically ran for 40 generations, to measure fitness relative to an unmutated genotype. Relative fitness is simply defined as the ratio of the net growth rates of the mutated and unmutated genotypes during competition for limited resources.

Figure 2 shows the relationship between mutation number and average fitness. A log-linear regression explains more than 99% of the variation in sample means ($F_{1,2} = 2{,}864.6$, $P = 0.0003$, $r^2 = 0.9993$). Inclusion of a quadratic term, as postulated by the mutational deterministic hypothesis, does not significantly improve the model's fit to the data ($F_{1,1} = 1.4353$, $P = 0.4428$). To maximize statistical power, one would like to perform these regressions using each mutant as an independent observation. In practice, this approach introduces technical difficulties because: (1) several mutants have zero fitness, so that the logarithm is undefined; and (2) the variance in fitness increases with mutation number, which violates an assumption of least-squares regression. To circumvent these problems and achieve statistical power commensurate with a sample of 225 genotypes, we used a robust method called Tukey's jack-knife[22] to obtain 225 quasi-independent estimates of the linear coefficient, α, and the quadratic coefficient, β. The jack-knifed estimate of α is 0.0273 (standard error = 0.0059, $t_{224} = 4.6510$, $P < 0.0001$). The corresponding estimate of β is 0.0037, which does not differ significantly from zero (standard error = 0.0070, $t_{224} = 0.5247$, $P = 0.6003$). Thus, there is no clear tendency for deleterious mutations to interact synergistically.

This finding is a negative result, and the objection can be raised that our experiment lacked sufficient power to reject the null hypothesis. Statistical power reflects several factors, including sample size and the ratio of signal to noise. Computation of variance components[22] indicates that the genetic variance in fitness among the 225 mutants ($s_G^2 = 0.0204$, $F_{224,460} = 132.7$, $P < 0.0001$) was 40-fold greater than the error variance due to imprecision in measuring fitness ($s^2 = 0.0005$). Thus, the negative result cannot be due to noisy measurements. More problematic for resolving the shape of the fitness function is the existence of substantial fitness variation among genotypes within each numerical class, which is a fact of life (but one ignored in most theoretical treatments of this subject). The most useful summary of statistical power is given by the confidence limits for any parameter of interest. Using standard errors from the jack-knife analysis, the true value of β is between −0.0101 and 0.0174 with 95% confidence. (We can also use the jack-knife analysis to obtain the 95% confidence interval for the ratio β/α, which runs from −0.2100 to 0.4774.) The question is then: how large must β be for synergistic epistasis to be important with respect to the hypothetical advantage of sex? There is no easy answer to this question; it depends on other variables, including especially the genomic deleterious mutation rate[4,5] (which can vary greatly even among genotypes of the same species[23]). For example, if β is positive and not too much smaller than α (such that $\beta/\alpha = 0.4$), then the increase in mean fitness due to sex is only a few per cent when the genomic deleterious mutation rate is 0.1, but the advantage of sex is about twofold when the rate is 2.0 (ref. 24). The genomic deleterious mutation rate in *E. coli* is $<10^{-3}$ for a genotype with functional DNA repair mechanisms[25], and so the benefit of sex is very slight even for the highest value of β consistent with our experiment. However, our primary objective is not to evaluate whether sex would be advantageous for *E. coli*. Rather, we seek to determine if there is a general tendency for genetic architectures to exhibit synergistic epistasis among deleterious mutations, which does not appear to be true. But given the statistical uncertainty inherent in this negative result, we conservatively summarize the regression analyses by reiterating that precise measurements of the fitness of 225 genotypes, each having from one to three mutations, were insufficient to demonstrate significant synergistic epistasis. Any comparable study that seeks to prove synergistic epistasis must

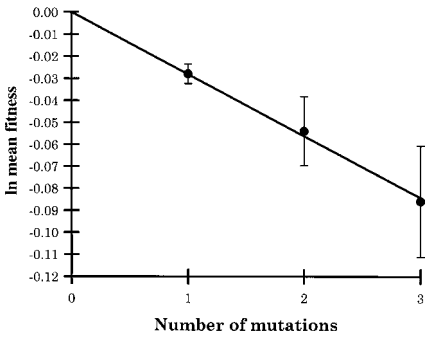

Figure 2 Observed effect of increasing the number of deleterious mutations on fitness in the bacterium, *E. coli*. Each point represents the average fitness value for 75 different genotypes carrying one, two or three insertion mutations. Error bars indicate standard errors, using the jack-knife method[22]. The solid line shows the best fit of a log-linear (multiplicative) model to the data. A model with synergistic epistasis, which includes an extra quadratic term, does not yield a significant improvement in the fit to the data.

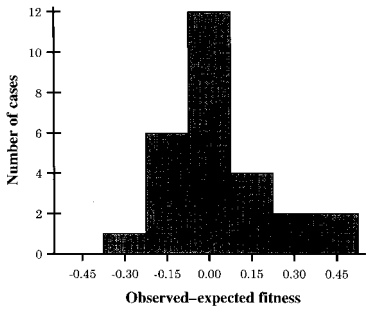

Figure 3 Distribution of observed minus expected fitness values for 27 different double mutants. The expected fitness values are calculated from fitness values of the corresponding single mutants assuming no interaction (multiplicative effects). Synergistic interactions are neither stronger nor more common than antagonistic interactions.

(1) examine many more genotypes, (2) generate a much wider range of numbers of mutations per genome, or (3) show that some other organism has a much greater tendency for synergistic epistasis than does *E. coli*.

A roughly log-linear fitness function has two possible explanations. One is that the effects of mutations on fitness are almost always independent. Alternatively, deleterious mutations may often interact, but synergistic and antagonistic interactions may be more or less equally common, so that on average there is no clear deviation from log-linearity. Otto and Feldman[26] recently showed theoretically that "variability among loci in the sign and strength of epistasis … decreases the parameter space over which increased recombination may evolve" and noted that "no data currently exist on the variance in epistatic interactions among random mutations." Therefore, we devised a second experiment to test these alternative explanations by examining the variability in the sign and strength of interactions among deleterious mutations.

In our first experiment, genotypes with multiple mutations were produced by successive rounds of mutagenesis. By contrast, in this second experiment, we constructed genotypes with multiple mutations by recombining mutations from genotypes that had single mutations. Thus, we could measure the effect of each particular mutation alone and in combination with other mutations. We chose three mutations, which had a broad range of fitness effects, from each of the three sets of genotypes with single mutations. We used P1 transduction (see Methods) to construct all 27 combinations of two mutations from different sets. The fitness values for each double mutant and its corresponding single mutants were measured simultaneously, with 10-fold replication each. The paired fitness values for two single mutants were used to generate the expected fitness values for the double mutants, assuming no interactions (multiplicative effects).

Figure 3 shows the distribution of observed minus expected fitness values for the 27 double mutants. A skewness test[22] indicates that the distribution is effectively symmetrical ($t_{26} = 1.5336$, $P = 0.1372$); synergistic interactions are neither stronger nor more common than antagonistic interactions. For each double mutant, we ran a paired *t*-test of the difference between observed and expected values. Seven cases exhibit synergistic epistasis, whereas seven others indicate antagonistic interactions (all $P < 0.05$). Because we performed 27 tests for interactions, and not all were independent (using the same nine mutations), we then applied the Bonferroni method[27] to adjust significance levels for the multiplicity of tests. Even with this conservative approach, three synergistic and four antagonistic interactions are significant. Therefore, the mutational deterministic hypothesis seems to fail not because interactions between deleterious mutations are very rare, but rather because synergistic and antagonistic interactions are both common.

Note added in proof. If fitness is measured as a relative rate, as in our study, then an additive null model may be more appropriate theoretically for testing the mutational deterministic hypothesis (S. P. Otto, personal communication), whereas the multiplicative model is more plausible biologically. In fact, the statistical results for both experiments reported here are nearly identical using additive and multiplicative models, with the additive model yielding even less support for the mutational deterministic hypothesis. □

Methods

Transposon mutagenesis. Insertion mutations were generated using mini-Tn*10* constructs and methods described in ref. 21. Briefly, transposase genes are not carried by the mini-Tn*10* but are encoded by the delivery vector (a defective λ phage), so that secondary transpositions cannot occur. Also, the transposase genes have been engineered to yield insertions that are effectively random with respect to target sequence[21]. Southern blots confirmed that insertions were well dispersed through the genome; there were few, if any, identical insertions among 60 mutants screened[28]. The three mini-Tn*10* constructs used here encode resistance to chloramphenicol, kanamycin or tetracycline. The site of an insertion mutation, and not the associated resistance marker, is primarily responsible for its fitness effect[28]. Mutants were selected on TA agar[29] supplemented with antibiotic at the appropriate concentration[21]. Plates were incubated for 72 h, after which no additional colonies were observed; mutants were chosen at random with respect to their time of appearance[28].

P1 transduction. Recombinant genotypes that had two particular mutations were constructed by transduction using P1*vir* and the method described in ref. 30. Recombinants were selected on TA agar containing the relevant antibiotics. Each mutation's presence was confirmed by a Southern blot (using the resistance genes as probes), in which single and double mutants were run on the same gel. As a control to test for any effect of the transduction procedure on fitness, we performed four separate transductions to generate each of three double mutants; we measured the fitness of each construct with fivefold replication. A nested ANOVA confirms that the three double mutants have different fitness values ($F_{2,9} = 307.1$, $P < 0.0001$) whereas independent constructions of the same double mutant have the same fitness ($F_{9,48} = 0.6704$, $P = 0.7312$).

Culture conditions and fitness assays. The fitness of each mutant genotype was measured relative to a common competitor at 37 °C in DM broth[29], in which ammonium and glucose provide nitrogen and carbon, respectively. To distinguish competitors in the fitness assays, we used a common competitor[28] that was Ara^+, whereas all the mutants were Ara^-; the common competitor and mutants produce white and red colonies, respectively, on TA agar[29]. We ran 195 control assays using the unmutated Ara^- progenitor and the Ara^+ common competitor; the fitness for each mutant was then standardized relative to the average value of these controls[28]. Before each fitness assay, the two competitors were separately grown in DM to acclimatize them to the experimental conditions. They were then each diluted 200-fold into the same fresh DM broth and a sample was immediately plated on TA agar to estimate their initial densities. Every day, for six days, the mixed population was diluted 100-fold into fresh DM; the population then grew 100-fold, or ~6.6 (= $\log_2 100$) cell generations, after which the glucose was depleted. After six days, a sample was plated on TA agar to obtain each competitor's final density. Some mutants had such low fitness that their final frequencies were below 5%; for those mutants, we reran the competitions for three days (or, in a few cases, one day) to ensure sufficient numbers in the final samples to obtain accurate fitness estimates. For each competitor, we calculated its growth rate as $m = \ln(100^t \cdot N_t/N_0)/t$, where N_0 and N_t are initial and final densities, respectively, and t is the number of days of competition. (All densities are expressed before dilution; the factor of 100^t reflects the 100-fold dilution and re-growth each day.) The relative fitness of two genotypes is defined as the ratio of their growth rates during competition for the same pool of nutrients[29]. This fitness measure thus reflects differences in reproduction and survival during every phase (lag, growth and stationary) of the serial batch regime[29].

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Antisaccade performance predicted by neuronal activity in the supplementary eye field

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The voluntary control of gaze implies the ability to make saccadic eye movements specified by abstract instructions, as well as the ability to repress unwanted orientating to sudden stimuli. Both of these abilities are challenged in the antisaccade task, because it requires subjects to look at an unmarked location opposite to a flashed stimulus, without glancing at it[1,2]. Performance on this task depends on the frontal/prefrontal cortex and related structures[3–8], but the neuronal operations underlying antisaccades are not understood. It is not known, for example, how excited visual neurons that normally trigger a saccade to a target (a prosaccade) can activate oculomotor neurons directing gaze in the opposite direction. Visual neurons might, perhaps, alter their receptive fields depending on whether they receive a pro- or antisaccade instruction. If the receptive field is not altered, the antisaccade goal must be computed and imposed from the top down to the appropriate oculomotor neurons. Here we show, using recordings from the supplementary eye field (a frontal cortex oculomotor centre) in monkeys, that visual and movement neurons retain the same spatial selectivity across randomly mixed pro- and antisaccade trials. However, these neurons consistently fire more before antisaccades than prosaccades with the same trajectories, suggesting a mechanism through which voluntary antisaccade commands can override reflexive glances.

In an antisaccade, a single visual stimulus acts both as a landmark for and a distractor from the goal of the required movement. The need to use the target location conflicts with the interdiction to glance at it. If a powerful reflex attracts gaze to the target, or at least draws attention to it[9], how can it be repressed? The answer depends on how the antisaccade is programmed by the brain. We know that each cerebral hemisphere contains: visual neurons that respond to stimuli appearing in one visual hemifield, at specific locations called receptive fields; movement neurons activated shortly before saccades directed towards subregions of the same hemifield, called movement fields; and visual-and-movement neurons that have both a receptive field and a movement field, usually in close spatial correspondence when prosaccades are generated. We began by considering the hypothesis that the instruction to make an antisaccade causes receptive fields to shift from one hemifield to the other so that both the visual neurons and the appropriate movement neurons they would activate after the shift would be in the same hemisphere. The initial reflex, no longer sustained by visual activity in the opposite hemisphere, would be short-lived or nonexistent. We know that receptive-field shifts are possible as they have been observed in other experiments[10]. Alternatively if visual receptive fields do not move, the antisaccade goal must be calculated and imposed on the neurons that have the appropriate movement field. Under this hypothesis, the flashed stimulus would give rise to concurrent streams of neuronal signals; one, if dominant, would trigger a reflexive prosaccade, and the other would initiate the correct antisaccade. The conflict of neuronal signals thus created would have to be resolved at a 'winner-take-all' junction in the brain, where the strengths (for example, the intensity of firing) of different saccadic commands are weighed and the strongest one is translated into behaviour. The 'winner-take-all' principle has been found to account for perceptual decisions[11]. It should be noted, however, that if the rule 'one neuron, one vote' applies, the odds of winning are very poor for antisaccade commands, given the ubiquity of neurons that discharge more strongly before saccades to visual targets than before self-initiated saccades.

We searched for evidence supporting the first hypothesis (the shift in receptive field), in the two oculomotor centres found indispensable for performing antisaccades: none were found in either the frontal eye field (FEF)[12] or the supplementary eye field (SEF). However, we observed that neuronal discharges in the SEF were consistently greater before antisaccades than before prosaccades with the same trajectories. This bias could be the means by

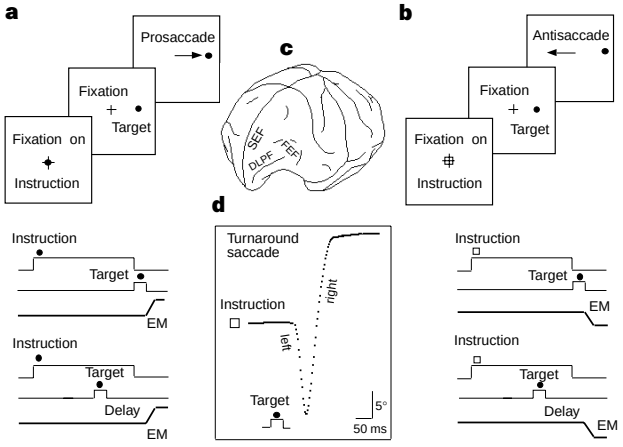

Figure 1 Spatial display and sequence of stimuli and saccades. **a**, Prosaccade trial; **b**, antisaccade trial. Top, the spatial display of stimuli and saccades; bottom, the traces indicate the sequence of events. Each trial began with a fixation point stimulus encoding, by its shape, the instruction to make a prosaccade (small dot) or an antisaccade (small square). The disappearance of the fixation point was the trigger signal for the eye movement (Em). An eccentric visual stimulus (always a dot) was flashed for 50–100 ms when the fixation point disappeared (trigger signal for the saccade) or at variable times during the fixation of the instruction cue. **c**, Location of the frontal eye field (FEF), supplementary eye field (SEF), and dorsolateral prefrontal cortex (DLPF). **d**, Sample record of an occasional 'turnaround saccade' occurring on an antisaccade trial. The first saccade was directed in error to the visual stimulus, then, without a pause, the correct antisaccade followed.