

Distribution of fitness effects among beneficial mutations before selection in experimental populations of bacteria

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The extent to which a population diverges from its ancestor through adaptive evolution depends on variation supplied by novel beneficial mutations. Extending earlier work^{1,2}, recent theory makes two predictions that seem to be robust to biological details: the distribution of fitness effects among beneficial mutations before selection should be (i) exponential and (ii) invariant, meaning it is always exponential regardless of the fitness rank of the wild-type allele^{3,4}. Here we test these predictions by assaying the fitness of 665 independently derived single-step mutations in the bacterium *Pseudomonas fluorescens* across a range of environments. We show that the distribution of fitness effects among beneficial mutations is indistinguishable from an exponential despite marked variation in the fitness rank of the wild type across environments. These results suggest that the initial step in adaptive evolution—the production of novel beneficial mutants from which selection sorts—is very general, being characterized by an approximately exponential distribution with many mutations of small effect and few of large effect. We also document substantial variation in the pleiotropic costs of antibiotic resistance, a result that may have implications for strategies aimed at eliminating resistant pathogens in animal and human populations.

The magnitude of the fitness increase associated with the first step of an adaptive walk depends on the characteristics of the distribution of fitness values from which new beneficial mutations are drawn. One plausible suggestion for the form of this distribution is that novel beneficial mutations be viewed as draws from the right tail of a probability distribution of fitness values². Provided that mutation rates per site are low enough to ignore double mutants⁵ and that the fitness of the wild-type sequence from which novel mutants are derived is not too low (formally, the wild-type sequence represents the leftmost draw from the right tail of the distribution of fitness values), two nonintuitive predictions about the distribution of fitness values among beneficial mutants can be made^{3,4}. First, the fitness effect among beneficial mutations, defined as the difference in fitness between a genotype carrying a beneficial mutation and the wild type, is exponentially distributed regardless of the underlying distribution of

fitness values. Second, the distribution of fitness effects is invariant, meaning that it will always be exponential regardless of the fitness of the wild-type. These predictions seem to be robust to biological details such as the strength of selection, mutation rate heterogeneity among sites and the manner of reproduction; thus, the theory may represent a

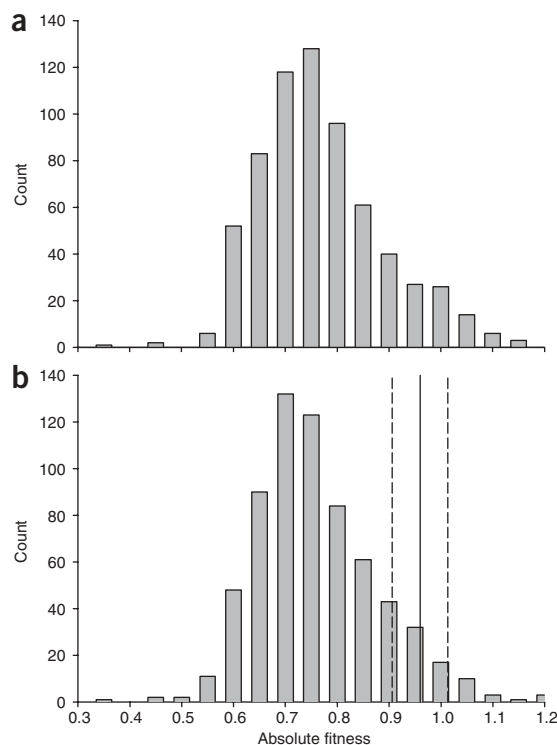


Figure 1 Frequency distributions of absolute fitness in selective (a) and permissive (b) environments. The wild type did not grow in the selective environment. Wild-type fitness in the permissive environment is marked by the solid vertical line; dashed lines denote the 95% confidence limits.

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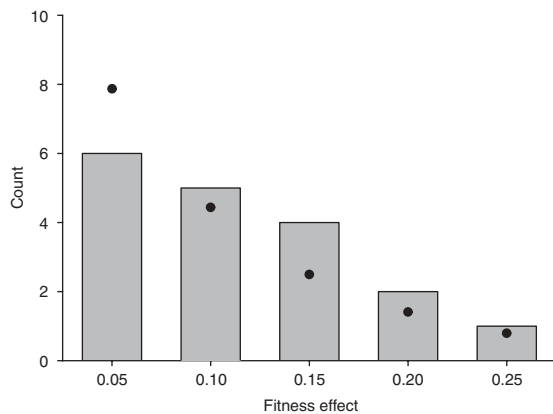


Figure 2 Distribution of fitness effects among 18 beneficial mutants assayed in the permissive environment. Bars indicate the observed distribution of fitness effects. The dots indicate expected values given an exponential distribution with mean $\beta = 0.087$ that was used to fit the data (see **Table 1**).

general explanation for what natural selection ‘sees’ when it sorts among new mutations during adaptation.

To test these predictions empirically, we estimated the distribution of fitness effects among novel, independently derived, single-step mutants of the bacterium *P. fluorescens* before the biasing effects of selection, drift and clonal competition. We obtained a collection of single-mutation strains using a conventional fluctuation-style assay⁶ for resistance to the quinolone antibiotic nalidixic acid and then assayed their fitness under a range of permissive conditions (that is, without antibiotics). This method allows us to collect novel genotypes derived from independent mutational events occurring naturally during population expansion over a limited number of generations without regard to their pleiotropic fitness effects in the assay environments. Few, if any, of the genotypes isolated in this way are expected to carry second-site mutations. Although we expect the fitness effect of most mutations to be deleterious relative to the wild type under permissive conditions,

previous work has shown beneficial mutations do occasionally arise in both clinical⁷ and laboratory⁸ settings; their rarity necessitates surveying large numbers of mutants. Resistance to nalidixic acid is typically conferred through chromosomal alterations caused by modifications of DNA gyrase or topoisomerase genes that prevent quinolone binding (and thus DNA replication) or changes to the quantity or characteristics of porins that reduce the concentration of quinolones in the cell^{9–11}. At least 18 nonsynonymous substitutions within one DNA gyrase gene, *gyrA*, are known to confer resistance⁸, and two of these have been shown to have pleiotropic effects on fitness in the absence of antibiotic¹¹. Thus, although we do not know the specific identity of the mutations conferring resistance in our experiment, we can be confident that a range of mutational targets with detectable fitness effects are available to selection, as hypothesized by theory.

We isolated 665 nalidixic acid-resistant mutants and assayed their fitness in the presence and absence of antibiotic (**Fig. 1**). By design, all mutants included in the assay were beneficial in the selective environment, in the sense that they are resistant to nalidixic acid. Their distribution of absolute fitness was roughly normal, with a slight right-skew (**Fig. 1a**), and analysis of variance demonstrated statistically significant genetic variation in fitness ($F_{664, 641} = 7.55$, $P < 0.0001$). In the permissive environment, the distribution of absolute fitness was superficially similar, there was again statistically significant genetic variance in fitness ($F_{664, 659} = 6.04$, $P < 0.0001$) and the majority of mutants were deleterious relative to the wild type (**Fig. 1b**). The assay uncovered 28 mutants with fitness greater than the wild type under permissive conditions, irrespective of formal tests of significance, corresponding to a beneficial mutation rate of 7.6×10^{-11} per gene per cell division (see Methods). Although we do not know the molecular changes responsible for conferring high fitness in the permissive environment, an analysis of variance of absolute fitness under selective and permissive conditions showed significant genetic variance ($F_{27,54} = 11.10$, $P < 0.0001$) and genotype-by-environment interaction variance ($F_{27,54} = 5.71$, $P < 0.0001$) among the 28 mutants, suggesting that the differences in fitness observed among these genotypes were underlain by different mutations. Moreover, we are confident that our mutants were a single mutational step from the wild type: we estimated the genome-wide probability of obtaining a

Table 1 Maximum likelihood analysis of fitness effects

Environment	n	σ	LogL	Gamma model			Exponential model		LRT (P value)
				ML α	ML β	ML $\alpha\beta$	LogL	ML β	
Experiment 1									
LB	18	0.0185	25.45	1.95 [0.69–3.48]	0.045 [0.02–0.15]	0.089	24.31	0.087 [0.06–0.14]	2.28 $P = 0.27$
Experiment 2									
LB	20	0.067	27.99	48.5 [0.71–109.4]	0.0018 [0.00083–0.114]	0.086	24.27	0.070 [0.056–0.13]	7.44 $P = 0.26$
Glucose	8	0.024	18.02	0.76 [0.53–171]	0.030 [0.00016–0.051]	0.023	17.95	0.024 [0.013–0.052]	0.14 $P = 0.94$
Mannitol	14	0.021	27.44	2.25 [0.628–35.12]	0.021 [0.0010–0.081]	0.047	26.63	0.044 [0.029–0.079]	1.61 $P = 0.50$
Sorbitol	25	0.039	50.38	51.9 [0.83–112]	0.0008 [0.00043–0.043]	0.039	47.02	0.031 [0.029–0.059]	6.70 $P = 0.49$

n , the number of favorable mutants analyzed in each experiment or environment ($n + 1$ gives the ranking of the wild type). σ , standard error of fitness effect estimates. LogL, log-likelihood of the data under each model. The exponential model is nested in the gamma model (α is set to 1). ML, maximum likelihood estimates. 95% confidence intervals (based on 1,000 bootstrap samples) are indicated in brackets. Note that the gamma distribution fitted to the data has mean $\alpha\beta$, and the exponential distribution has mean β . These estimates of the mean effect of beneficial mutations are therefore directly comparable. LRT, likelihood ratio test statistic comparing the fit of the gamma model with the fit of a reduced model assuming an exponential distribution (which assumes $\alpha = 1$, leaving only β free to vary). P -values were not based on asymptotic theory but rather were estimated using 1,000 bootstrap samples (**Supplementary Note**).

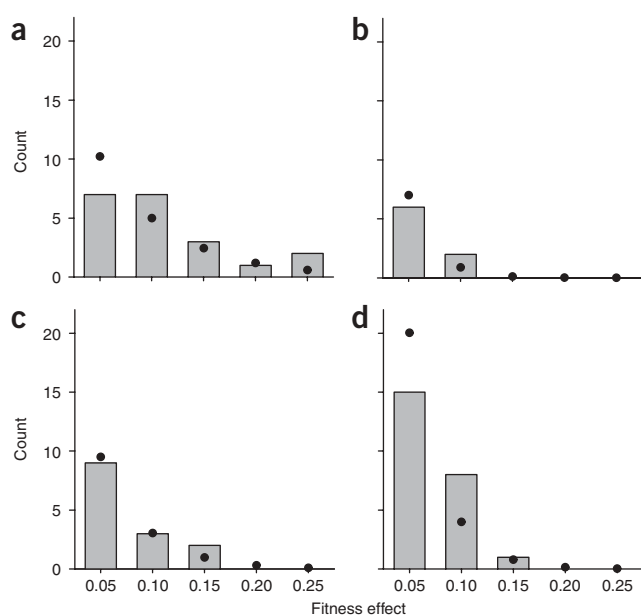


Figure 3 Observed (bars) and expected (dots) distribution of fitness effects among beneficial mutants assayed in (a) LB (fitness rank of wild type, 21); (b) glucose (fitness rank of wild type, 9); (c) mannitol (fitness rank of wild type, 15) and (d) sorbitol (fitness rank of wild-type, 26). Expected values were calculated for each environment using an exponential distribution with means obtained from the likelihood analysis (see **Table 1**).

double mutant to be 2.04×10^{-14} per genome per generation (see Methods). When corrected for the number of populations sampled in our experiment ($N = 2,016$), the chance that our mutant collection contains a double mutant is approximately 1 in 1,000,000.

To test the prediction that the distribution of fitness effects among beneficial mutants is exponentially distributed and invariant^{3,4}, we conducted two experiments. In the first (experiment 1) we obtained more accurate estimates of fitness in the permissive environment by identifying the 40 fittest mutants in permissive conditions from the previous experiment and reassaying their fitness eight times, together with the wild type. We then considered the fitness effects among beneficial mutants and asked whether the exponential distribution provided a good fit for the data using a maximum likelihood framework (see Methods). This assay identified 18 mutants fitter than the wild-type, all of which had a fitness greater than or indistinguishable from the wild type in the previous experiment. We detected statistically significant genetic variance in fitness among these mutants ($F_{17,125} = 2.68$, $P = 0.0009$). The likelihood of the data under a model using a full gamma distribution for specifying fitness effects is not improved relative to a reduced model assuming an exponential distribution, suggesting the exponential provides a satisfactory description of the data (**Fig. 2**, **Table 1**). The selection coefficient associated with this collection of mutants (calculated as $s_j = [W_j - W_{wt}] / W_{wt}$ where W_j is the absolute fitness of the j th type and W_{wt} is the absolute fitness of the wild type) was 0.086 ± 0.059 (mean \pm s.d.), which is comparable in size with selection coefficients estimated during the early stages of adaptation in laboratory populations of bacteria^{12,13}.

The second experiment (experiment 2) tested the prediction that the distribution of beneficial effects is invariant, meaning that the distribution of fitness effects should always be exponential, independent of the fitness rank of the wild type. We assayed the fitness of the

95 highest-ranking mutants in the permissive environment from the initial assay of 665 mutants in four qualitatively different environments. Despite marked variation in the rank of wild-type fitness across environments (from $i = 9$ to 26), the distribution of fitness effects was exponential in all environments (**Fig. 3** and **Table 1**). Furthermore, the mean fitness effect in our experiment was statistically indistinguishable across environments (**Fig. 4a**; analysis of covariance (ANCOVA) between fitness rank of wild type and fitness effects: $F_{1,65} = 0.30$, $P = 0.5832$; data were transformed by natural log before analysis; likelihood ratio test = 5.69, $P = 0.13$). This result is notable because theory predicts that the mean fitness effect should depend on the size of the fitness gap between the fittest and the second most fit mutation³, a value that is likely to differ across environments. Our results suggest, by contrast, that the average fitness effect among beneficial mutants is conserved across the four environments used here. Furthermore, the size of the fitness gaps (the difference in fitness among mutants of neighboring rank when genotypes are ranked from fittest to least fit) in **Figure 4b** increases with absolute fitness (and thus with fitness rank), as would be expected if beneficial mutations represented draws from an exponential distribution^{3,4}.

Our results are likely to be robust to three sources of bias in our experimental protocol. First, if a limited number of genetic routes to nalidixic acid resistance exist, we may have sampled the same mutation multiple times. As a consequence, we may have overestimated the

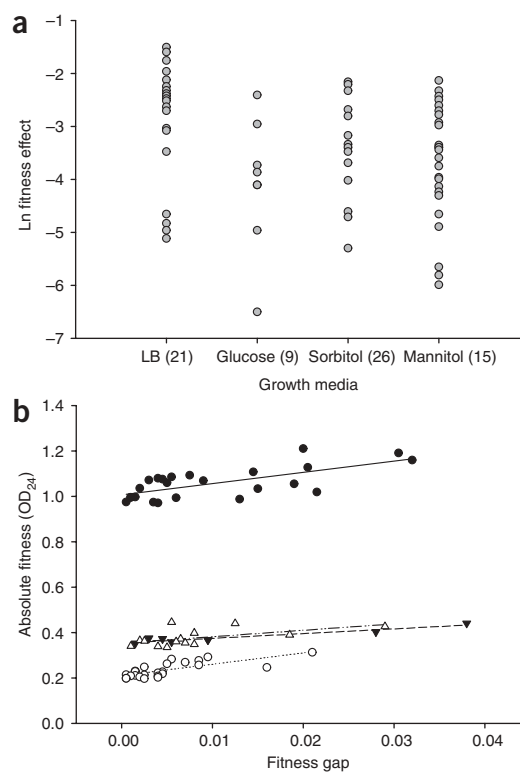


Figure 4 Mean fitness effects among beneficial mutants across environments in experiment 2. (a) Fitness effects of beneficial mutants across four environments. Numbers in parentheses give the fitness rank of the wild type. (b) Covariance of absolute fitness of beneficial mutants and the fitness gap with the mutant of next-lowest rank across four environments (filled circles: LB, fitness rank of wild-type: 21; open circles: sorbitol, fitness rank of wild-type: 26; filled triangles: glucose, fitness rank of wild-type: 9; open triangles: mannitol, fitness rank of wild-type: 15).

number of genetically unique beneficial mutants in our collection and thus may have underestimated the true fitness rank of the wild-type and inadvertently inflated our 'genetic' degrees of freedom. Our estimates of the number of genetically unique beneficial mutants must therefore be treated with caution, although it seems unlikely that such an overestimation should affect the distribution of fitness effects, as doing so simply increases the sample size of independently arising mutations. Future work will address this issue by sequencing the strains in our collection. Second, in order to be confident that we have identified single-step mutations, we restricted attention to mutations with a specific phenotype—nalidixic acid resistance—and their pleiotropic effects in different environments rather than the full spectrum of mutational effects in any specific environment. The mutations in our collection thus represent a fraction of all possible mutations in a given environment. There is no compelling reason to think that these mutations should not be a random sample of all possible one-step mutations, and so the theory being tested should apply with equal force to this subsample. Third, despite our extensive assay, we have collected only a few dozen beneficial mutants. This, together with experimental error in assaying fitness, potentially limits our statistical power to reject the exponential distribution. Monte Carlo simulations suggest that, even with a modest number of mutants (20–30) and the range of precision in fitness estimates (σ) we report here, our analysis has power to reject the exponential distribution in favor of a range of alternative gamma distributions (see **Supplementary Note** online). It is worth noting, however, that the range of α values we report (0.69–3.48; **Table 1**) for the gamma distribution means that alternative distributions that are L-shaped or slightly bell-shaped cannot be formally rejected.

The unit process of adaptive evolution involves the substitution of a single beneficial mutation by natural selection. Our results shed light on the characteristics of the first part of this process, the origin of *de novo* genetic variation in fitness through mutation, and complement recent work on the distribution of fitness effects among mutations fixed by selection^{14–17}. In line with previous work¹⁸, we have shown that the distribution of fitness effects among single-step beneficial mutants can be characterized adequately by an exponential distribution. Furthermore, our results confirm the nonintuitive prediction that the distribution of fitness effects is invariant: it is always exponential regardless of the environment in which variation is ultimately sorted by selection. Taken together, these results are consistent with recent theory^{3,4} and imply that the spectrum of beneficial mutational effects exposed to natural selection may be very general, being characterized by many mutations of small effect and few of large effect. It is notable that despite the complexity of the genotype-phenotype map, even for relatively simple characters such as the antibiotic resistance studied here, a simple statistical model can capture most, if not all, of the evolutionarily relevant information required to predict the first step in the response to selection.

Our results also bear on the more applied problem of antibiotic resistance. It is often suggested that an appropriate means of eliminating resistant genotypes is to stop using antibiotics, the assumption being that resistance incurs a substantial cost in the absence of antibiotic. This strategy has been questioned on the grounds that resistant strains may evolve high fitness in the absence of antibiotic selection through compensatory mutations that do not compromise resistance^{19,20}. Our results suggest a further reason to doubt the effectiveness of this strategy: the pleiotropic costs of resistance are highly variable (**Fig. 1b** and ref. 21) and may lead to the generation, in a single mutational step, of genotypes that are both resistant and have high fitness in permissive environments. Post-hoc tests of significance

between the beneficial mutants and the wild type from experiment 1 showed six resistant genotypes that were significantly fitter than the wild type in the permissive environment (Dunnett's one-tailed *t*-test: critical value = 2.648, $MS_{\text{error}} = 0.0105$, d.f. = 132, $P < 0.05$). Such 'universally superior' genotypes may therefore arise approximately once in every 100 resistant mutants. Although the probability of obtaining such a mutant in a single mutational step may seem vanishingly small, the large population sizes and short generation times characteristic of most microbial pathogens makes it likely that they will arise occasionally in natural populations. Strategies designed to eliminate or control antibiotic resistance would do well to take this variation into account.

METHODS

Mutant isolation. Approximately 500 cells of the wild-type *P. fluorescens* strain SBW25 were inoculated into each well of a 96-well microwell plate containing 195 μl of Luria Bertrani (LB) medium, which was then incubated overnight at 28 °C (which was the temperature for all incubations) in an orbital shaker (150 rpm). Final population sizes under these conditions were approximately 2×10^8 cells. After overnight growth, the cultures were spread on LB agar plates containing 100 $\mu\text{g ml}^{-1}$ nalidixic acid and were incubated overnight. From those plates containing viable colonies, a single colony was used to inoculate 6 ml of LB medium plus nalidixic acid; after overnight growth, this culture was frozen for further analysis. A total of 2,016 populations were assayed in this way, resulting in 673 resistant mutants. Following the procedure of Luria and Delbruck⁶, we obtained a mutation rate of 2.47×10^{-9} mutations per cell division for this genetic target. This estimate, based on the fraction of populations yielding no mutants, is likely to be robust to various assumptions made in the Luria-Delbruck procedure²². Given 28 beneficial mutations identified in the preliminary assay and assuming a single target for antibiotic resistance, this mutation rate corresponds to a beneficial mutation rate of 7.6×10^{-11} mutations per cell division. To calculate the genome-wide probability of a double mutant, we assumed that a single gene confers resistance. We assumed a Poisson distribution of mutations to calculate the probability of a double mutant and then multiplied by 6,700, the approximate number of genes in *P. fluorescens*.

Distribution of fitness effects in selective and permissive media. We assayed the fitness of all resistant mutants and the wild type as the change in cell density of each genotype grown in pure culture after 24 h growth in permissive (LB) and selective (LB plus nalidixic acid) media. Genotypes were first grown overnight from frozen stocks, and 5 μl of the overnight culture was inoculated into 195 μl of media. Cell density was measured as optical density at 630 nm on an ELX800 Microplate Reader. Space constraints necessitated using only 665 mutants from the total of 673 collected and conducting the analysis in three blocks. The wild-type was included in each of the three blocks, and two replicates of each genotype were run. Analysis of variance did not show any statistically significant variation among blocks for the wild type, the only genotype to be assayed at all time points ($F_{2,3} = 0.32$, $P = 0.7479$), so raw fitness data were used in all further analyses. In experiment 1, the fitness of the 40 fittest mutants in permissive medium (LB, the same medium used in antibiotic selection but lacking nalidixic acid) was assayed using the same procedure as above with all genotypes assayed on the same microwell plate. The experiment was divided into two blocks that differed in the location of genotypes within a microwell plate. In total, there were four replicate plates per block, giving a total of eight replicate estimates of fitness for each genotype. Analysis of variance did not show any significant block effects ($F_{1,279} = 0.002$, $P = 0.6879$). Although the fitness rank of the wild type in this experiment ($i = 19$) is substantially higher than in the previous assay ($i = 29$), the 95% confidence limits for the two fitness estimates overlap ($i = 19$: 0.9505–1.0977; $i = 29$: 0.9055–1.0135).

To test the prediction that the fitness effects are exponentially distributed, we used a maximum likelihood (ML) framework. We modeled the fitness effects as draws from a gamma distribution, characterized by a shape parameter α and a scale parameter β , which encompasses a variety of shapes, and includes the exponential as a special case ($\alpha = 1$). With our parameterization, fitness effects

have mean $\alpha\beta$ and variance $\alpha\beta^2$. We assume a set of n fitness effects measures, $D = (x_1, x_2, \dots, x_n)$, where x_i is the estimate of the mean fitness difference between the i^{th} beneficial genotype (irrespective of its actual ranking in fitness) and the wild type. The likelihood of such a data set is

$$L = \prod_{i=1}^n \int_{-\infty}^{+\infty} I_{(x_i + \varepsilon > 0)} f_{\Gamma}(x_i + \varepsilon) \Phi(\varepsilon) d\varepsilon$$

where I is the indicator function, f_{Γ} is the probability density function of a gamma distribution with parameters α and β , and Φ is the probability density function of a Gaussian distribution with mean 0 and variance σ^2 (to account for the experimental error around estimates of fitness effects). L is maximized, yielding ML estimates of the parameters α and β . Twice the difference between the logarithm of the full likelihood above and a reduced likelihood, assuming an exponential distribution (by setting α to 1), provides a test of the hypothesis that fitness effects are exponentially distributed. Instead of relying on asymptotic properties of likelihood ratio tests, P -values for the likelihood ratio test under the hypothesis of exponentially distributed fitness effects as well as confidence intervals for α and β were obtained by parametric bootstrapping (Supplementary Note). All likelihood calculations and bootstrap simulations were carried out using Mathematica 5.0 (ref. 23). Likelihoods were maximized numerically using the function NMaximize.

Invariance of fitness effects across novel environments (experiment 2). To test the prediction that the distribution of fitness effects among beneficial mutants is invariant, we chose the 95 fittest mutants in the permissive medium from the preliminary assay plus the wild type and assayed their fitness in four environments. The environments were LB, as before, and three others constructed of M9 salts, with either glucose, sorbitol or mannitol as the sole carbon source at a concentration of 0.4%.

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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