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Mutations of intermediate effect are responsible for adaptation in evolving *Pseudomonas fluorescens* populations

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The fixation of a beneficial mutation represents the first step in adaptation, and the average effect of such mutations is therefore a fundamental property of evolving populations. It is nevertheless poorly characterized because the rarity of beneficial mutations makes it difficult to obtain reliable estimates of fitness. We obtained 68 genotypes each containing a single fixed beneficial mutation from experimental populations of *Pseudomonas fluorescens*, evolving in medium with serine as the sole carbon source and estimated the selective advantage of each by competition with the ancestor. The distribution of selection coefficients is modal and closely resembles the Weibull distribution. The average selection coefficient (2.1) and beneficial mutation rate (3.8×10^{-8}) are high relative to previous studies, possibly because the ancestral population grows poorly in serine-limited medium. Our experiment suggests that the initial stages of adaptation to stressful environments will involve the substitution of mutations with large effect on fitness.

Keywords: experimental evolution; fixation; beneficial mutation; fitness effects; adaptation; selection

1. INTRODUCTION

Evolution was originally conceived as an extremely gradual process, with natural selection acting on slight successive variations (Darwin 1859). Fisher (1930) developed a mathematical framework known as the geometric model of adaptation to describe the statistical basis of this gradualist interpretation. This model showed that although small mutations have a 50% chance of being beneficial, larger mutational effects possess a rapidly diminishing chance of being advantageous. Fisher's influence has led to a rich body of mathematical theory on phenotypic evolution, built on idea that small mutations are the genetic basis of adaptation. However, recent findings from the experimental study of adaptation suggest that morphological evolution often involves a modest number of genetic changes (Orr 2001). The development of quantitative trait locus analysis has made it possible to directly map the factors underlying a response to selection. This approach has provided considerable evidence that evolution often operates through a few large changes rather than many small changes (Bradshaw *et al.* 1998; Wang *et al.* 1999; Shapiro *et al.* 2004). Unfortunately, it is very difficult to determine whether these findings represent the exception or the norm. The problem is that the low frequency of beneficial mutations has prevented empirical work with statistical power. Recently, experimental evolution using microbes has provided a way to increase greatly the number of beneficial mutations likely to arise during an experiment (Bull *et al.* 1997; Imhof & Schlotterer 2001; Rozen *et al.* 2002). Microbial populations facilitate the study of mutations because microbes have short generation times and large population sizes, thereby enabling the collection of large numbers of mutants.

In this study, we conducted short-term experimental evolution with populations of the heterotrophic bacterium *Pseudomonas fluorescens* selected under stressful conditions. This enabled us to collect a library of genotypes, each containing a single fixed beneficial mutation. By competing these genotypes against the ancestor we obtained selection coefficients associated with each mutation. The objective of this study was to determine both the shape and scale of the distribution of these selection coefficients. In doing so, we seek a more comprehensive understanding of the genetic basis of adaptive evolution and the cause of phenotypic change through time.

2. MATERIAL AND METHODS

(a) *Ancestral strain*

We used clonal isolates of *Pseudomonas* strains, SBW25 and SBW25ΔpanB, to found 96 replicate lines. SBW25ΔpanB is an isogenic strain of SBW25 containing a complete deletion of the *panB* gene and when plated on indicator plates with a low concentration of pantothenate (2.4×10^{-6}%) grows noticeably smaller colonies than SBW25. The two strains were mixed in roughly equal proportions to form a common pool to start the experimental populations.

(b) *Selection experiment*

We grew populations on 96-well plates with each well containing an M9 salt solution (NH_4Cl 1 g l^{-1}, Na_2HPO_4 6 g l^{-1}, KH_2PO_4 3 g l^{-1}, NaCl 0.5 g l^{-1}) supplemented with a high concentration of pantothenate (2.4×10^{-3}%) and 0.3 g l^{-1} serine. *Pseudomonas fluorescens* has very poor growth on the carbon substrate serine, but will rapidly adapt to use the substrate within approximately 100 generations of selection (Barrett *et al.* 2005). Every 24 h we transferred selection lines by using a 96-pin replicator that transfers 0.06–0.07 µl of culture (approx. 7×10^3 cells) on each pin, about a 3000-fold dilution. After each transfer we scored the marker state of half of the lines using a ProtoCOL SR/HR counting system (Synoptics Ltd, Cambridge, UK). When the frequency of a marker state exceeded 95% we froze a sample from the line.

(c) *Competitive fitness assays*

For each line, we mixed evolved and ancestral genotypes to form a common pool that was inoculated into two replicate competition microcosms containing 200 µl of the original selection environment. Common pools were then diluted and spread on indicator plates to ascertain the initial ratio of the two competing genotypes. After incubation at 28 °C for 24 h, the culture was again diluted and spread on indicator plates. The intrinsic growth rate of a genotype was estimated as $r=\ln(N_f/N_0)$, where N_f is the final population size and N_0 is the initial population size. We then estimated selection coefficients as the proportional increase in growth rate of the evolved genotype compared to the ancestor, $S=(r_e-r_a)/r_a$, where r_e is the intrinsic growth rate of the evolved genotype and r_a is the intrinsic growth rate of the ancestral genotype.

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(d) *Statistical analysis*

Statistical analyses were performed using MATHEMATICA 5.0. For the purpose of describing the distribution of fitness effects from beneficial mutations, each genotype was treated as an independent observation. We used maximum likelihood to quantify the fit of alternative probability density functions to the observed distribution of selection coefficients and then used Akaike's Information Criterion (AIC) to compare the log likelihood of non-nested models. The model that is most consistent with the observations, while requiring the lower number of parameters, is the one with the lowest AIC.

3. RESULTS

We identified 70 genotypes with fixed mutations in 96 replicate lines. Two genotypes were discarded because they had negative selection coefficients and therefore they did not represent beneficial mutations. Hence, at least 68 beneficial mutations occurred over approximately 1.6×10^{10} cell divisions. The substitution rate for beneficial mutations per cell division is approximately $k \approx \mu\bar{p}$, where μ is the beneficial mutation rate and $\bar{p}$ is the average fixation probability calculated using a numerical branching process based on Rokyta *et al.*'s (2005) model but assuming population growth follows a Lotka–Volterra model of equal competitors and a common pool of resources (MATHEMATICA file available on request). Given an approximate substitution rate for beneficial mutations (k) of 4.2×10^{-9} per cell division, and an average probability of fixation ($\bar{p}$) of 0.11, we obtained an estimate of the beneficial mutation rate (μ) as 3.8×10^{-8} per cell division. The beneficial mutations had a large effect on fitness, as shown by the size of the competitive advantage that evolved genotypes held over the ancestor (mean $S=2.09$, $t=16.25$, d.f.$=67$, $p<0.0001$). The advantage varied substantially across genotypes ($F=2.71$, d.f.$=67$, $p<0.0001$, s.d.$=1.06$), with an observed range of 0.20–5.19.

The estimated selection coefficients of evolved genotypes are plotted as a histogram in figure 1. Among the alternative models compared with the empirical distribution, the Weibull model had the lowest AIC score (AIC$=199.379$, ΔAIC to next lowest model$=4.78$, see table 1). The Weibull distribution is a versatile distribution; it exhibits various shapes, depending on the coefficient of variation (CV). If the CV is greater than one, the distribution is L-shaped, whereas if the CV is less than one, it is bell-shaped. For the parameters of the Weibull distribution that maximized the likelihood of observing our data, the distribution is bell-shaped with a peak at intermediate values (mean$=2.09$, CV$=0.51$). In order to assess our confidence in the shape of the distribution, we calculated 95% confidence boundaries for the CV (0.43–0.61) of the model. For all values of the CV within these bounds, the Weibull distribution remains bell-shaped (figure 1).

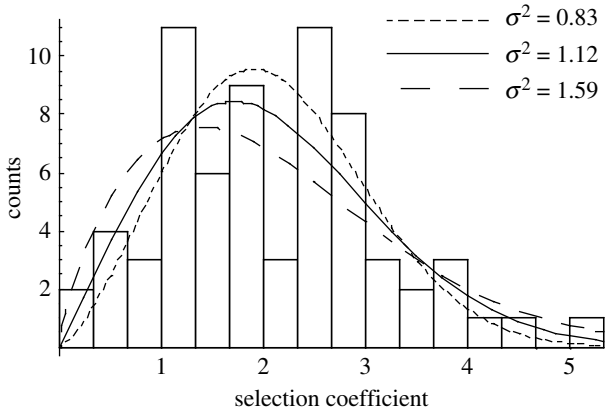

Figure 1. Distribution of fitness effects for fixed beneficial mutations. The solid line shows the Weibull distribution with parameters that maximize the likelihood of observing our data (mean$=2.09$, coefficient of variation$=0.51$). The dashed lines show the Weibull distribution at the 95% confidence boundaries for maximum likelihood of the coefficient of variation (short dashed line: mean$=2.03$, coefficient of variation$=0.43$; long dashed line: mean$=2.17$, coefficient of variation$=0.61$).

Table 1. Akaike's Information Criterion (AIC) for several probability density functions. (The model that is most consistent with the observations, while requiring the lower number of parameters, is the one with the lowest AIC.)

model	parameters	AIC	ΔAIC
Weibull	2	199.38	0
normal	2	204.16	4.78
logistic	2	206.16	6.79
log-normal	2	215.05	15.67
half-normal	1	216.54	17.16
gamma	2	235.76	36.38
exponential	1	238.46	39.08

4. DISCUSSION

The distribution of beneficial effects in experimental systems is poorly understood (Wilke 2004). Regardless of the specific probability distribution, however, extreme value theory predicts that the frequencies of fitness effects of beneficial mutations will be exponentially distributed (Gillespie 1984; Orr 2002). These beneficial mutations cannot be observed directly until they spread to an appreciable frequency in the population and become 'contending' mutations that will be fixed unless a superior competitor arises first. Since mutations of small effect will have very low probabilities of fixation, the distribution of mutations that actually become fixed, and thus contribute to adaptation, is predicted to shift from exponential to bell-shaped among fixed mutations (Orr 1998). High selective advantages will also make it more likely for beneficial mutations to escape extinction through clonal interference or population bottlenecks (Haldane 1927; Kimura 1983; Gerrish & Lenski 1998; Wahl *et al.* 2002; Campos & de Oliviera 2004; Wilke 2004). Thus, although mutations of larger effect may be less frequent, they will be selected more rapidly, and in consequence mutations of intermediate effect may be the most frequent among those that are actually fixed.

There have been very few empirical studies characterizing the statistical properties of beneficial mutations. Two studies have found support for exponentially distributed beneficial fitness effects (Imhof & Schlotterer 2001; Sanjuan *et al.* 2004), but they involved contending rather than fixed mutations. Rozen *et al.* (2002) reported that adaptation by

Escherichia coli to glucose-limited medium, in which the ancestor has high fitness, involved beneficial mutations whose frequencies were not distributed exponentially, but rather had a peaked distribution. Our results support this finding in a more extensive sample of mutations, and suggest that a bell-shaped distribution is appropriate for describing the properties of mutations that eventually become fixed. This pattern appears to be a robust feature of adaptation that transcends the specific features of the organism and the environment in question.

In previous studies investigating the fitness distribution of beneficial mutations (Lenski *et al.* 1991; Gerrish & Lenski 1998; Imhof & Schlotterer 2001; Rozen *et al.* 2002), estimates of selection coefficients were an order of magnitude lower than those reported here. The most likely cause of this discrepancy is that in these experiments *E. coli* was cultured in rich LB medium or glucose, environments in which the ancestor would have a nearly optimal phenotype. In contrast, the ancestral strain of *Pseudomonas* used in our experiment has very poor growth in carbon-limited serine environments (Barrett *et al.* 2005). Bull *et al.* (2000) found beneficial mutations of large effect ($S=0.8$–13.9) when they selected a bacteriophage at very high temperatures, but they did not collect enough mutants to determine the distribution of selection coefficients. Our experiment characterizes this distribution in a stressful environment, and shows that its shape remains peaked, although the average selection coefficient is much larger.

The rapid evolution of resistance to antibiotics, insecticides and herbicides by bacteria and other organisms has had serious consequences for human well-being. In all cases, the pest populations must adapt to novel and stressful conditions. This adaptation is ultimately the result of the substitution of spontaneous beneficial mutations, which is the fundamental unit of evolutionary change. Our experiment is one of a small handful of empirical studies that have investigated the distribution of selection coefficients of beneficial mutations, and the first study to determine this distribution in an environment where the ancestor has low fitness. We found that the first mutation fixed tended to have very high selective advantage, whereas the shape of the distribution resembled that found in a previous study conducted with a different organism in more favourable conditions. Experimental work seems to be leading towards the conclusion that the mutations responsible for the initial stages of adaptive evolution may have much larger effects than is usually supposed.

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