doi:10.1111/j.1558-5646.2012.01722.x

ANTIBIOTIC RESISTANCE AND STRESS IN THE LIGHT OF FISHER'S MODEL

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Received March 1, 2012
Accepted June 4, 2012

The role of mutations in evolution depends upon the distribution of their effects on fitness. This distribution is likely to depend on the environment. Indeed genotype-by-environment interactions are key for the process of local adaptation and ecological specialization. An important trait in bacterial evolution is antibiotic resistance, which presents a clear case of change in the direction of selection between environments with and without antibiotics. Here, we study the distribution of fitness effects of mutations, conferring antibiotic resistance to *Escherichia coli*, in benign and stressful environments without drugs. We interpret the distributions in the light of a fitness landscape model that assumes a single fitness peak. We find that mutation effects (*s*) are well described by a shifted gamma distribution, with a shift parameter that reflects the distance to the fitness peak and varies across environments. Consistent with the theoretical predictions of Fisher's geometrical model, with a Gaussian relationship between phenotype and fitness, we find that the main effect of stress is to increase the variance in *s*. Our findings are in agreement with the results of a recent meta-analysis, which suggest that a simple fitness landscape model may capture the variation of mutation effects across species and environments.

KEY WORDS: **Distribution of fitness effects, fitness landscape, genotype-by-environment, mutation, selection.**

Most nonneutral mutations are deleterious. The distribution of their fitness effects (DFE) (Eyre-Walker and Keightley 2007) is paramount to most evolutionary theories (Charlesworth and Charlesworth 1998) and possibly depends on the genetic background (Barrick et al. 2010; Sousa et al. 2012) and environmental conditions (Kishony and Leibler 2003). In the context of antibiotic resistance, the dependence on the environment is clear—a given mutation conferring resistance to a bacterium is beneficial in the presence of the drug, but generally costly in its absence (Andersson and Hughes 2010; MacLean et al. 2010). In addition, the cost of resistance may vary according to the drug-free environment (MacLean and Buckling 2009; Hall et al. 2011). Importantly, the effects of resistance to commonly used antibiotics were shown to be variable in vivo (Andersson and Hughes 2010), with some resistances entailing no cost (Rodriguez-Rojas et al. 2010). Nevertheless, the form of DFE of mutations in any given environment is poorly known, specially the effects of common mutations such as SNPs. Two recent studies addressed this issue. One involves a nalidixic acid resistance in *Pseudomonas fluorescens*, where 18 mutations, beneficial in a benign environment, were tested in almost 100 environments differing in the carbon substrates (Bataillon et al. 2011). Strong genotype-by-environment (GxE) interactions were found and many resistance mutations remained beneficial in different environments, implying that a cost of adaptation due to an environmental change was not substantial. Another where 20 mutants of a RNA virus were tested in different plant hosts and different DFE were found across environments (Lalic et al. 2011).

It is commonly thought that selection is stronger in stressful environments, so that stress leads to increased effect of deleterious mutations (Jimenez et al. 1994; Kondrashov and Houle 1994; Shabalina et al. 1997; Korona 1999; Vassilieva et al. 2000; Szafraniec et al. 2001; Jasnos et al. 2008). However that has not always been observed (Kishony and Leibler 2003; Jasnos et al. 2008; Paulander et al. 2009; Hall et al. 2011; Maclellan et al. 2012), and therefore a gradient of stress does not necessarily

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amount to a correspondent gradient in selection pressure (Nichols et al. 2011; see review of Agrawal and Whitlock 2010). In relation to antibiotic resistance, it is of public health importance to determine if stress has a predictable influence on its costs.

The influence of the environment on the DFE can be theoretically predicted in the light of fitness landscape models (Martin and Lenormand 2006a; Lourenço et al. 2011). Indeed, Fisher, when he proposed his model (Fisher geometrical model, FGM), suggested that a change in the environment could be theoretically thought of as a change in the distance to the optimum of a given population or a change in the position of the optimum itself (Fisher 1930). Generally, an environmental change can lead to a change in the landscape beyond the position of the peak. These different landscape displacements are responsible for generating different patterns of adaptation to stress (for a review see Agrawal and Whitlock 2010). Martin and Lenormand (2006b) predicted that the average selection against new mutations should remain fairly constant, but variance should increase, if a stress would simply lead to change in the location of optimum. Most of the other types of changes will have an impact on the average selection, either increasing or decreasing its strength.

Here we study the DFE of mutations, which confer resistance to four types of antibiotics, commonly used in the clinic. We determined the fitness effects of such mutations in different environments: the plenty and the famine and also at high temperature characteristic of human infections. This choice allows us to study the effect on DFE, of common stresses that bacteria face. We define stress as a context in which the absolute fitness of bacteria is reduced relative to a benign condition (an environment with optimal temperature and multiple resources which allow for rapid growth).

We ask the following questions: Does stress increase the deleterious effects of mutations? And/or does it increase the variance? Given the target genes and the specific environments studied, is it possible to anticipate the effects of the mutations? If so, can deleterious mutations become beneficial under stress? Can a single peak fitness landscape model, such as FGM, provide an adequate description of DFE in different environments?

As expected, we observed strong GxE interactions in the sample of 25 resistances studied (19 resistance mutations accrued from Trindade et al. (2009) study and an extra group of six mutations conferring resistance to D-cycloserine; see Table 1 for a more detailed description). The fitness effects of antibiotic resistance mutations generally differ across the environments and, importantly, very costly resistance mutations in a benign environment can become beneficial in a stressful environment. This implies that polymorphism for such resistance mutations is to be expected if in nature environment fluctuates between benign and stressful conditions. Although it is hard to anticipate the outcome of stress on the effect of mutations, we were able to predict that, there was a high chance for some of the rifampicin-resistant mutants (Rif^r) to be beneficial in the stressful environments and that streptomycin-resistant mutants (Str^r) enjoyed an increased probability of being deleterious at high temperature. We finally tested if the theoretical predictions (Martin and Lenormand 2006a), in the context of FGM, which assume that mutations affect pleiotropically a number of quantitative traits under stabilizing selection, could reasonably describe the DFE, as it has been proposed for other types of mutations in *Escherichia coli* (Martin et al. 2007). As predicted by this theory, we found that a shifted gamma distribution aptly describes the distribution of the studied mutation effects, and that stress mainly increases the variance of mutations' effects on fitness, with less impact on their average effect.

Material and Methods

BACTERIAL STRAINS AND ENVIRONMENTAL CONDITIONS

The strains used were *E. coli* K12 MG1655 wild-type (ara^+) and Δara. Rifampicin-resistant, streptomycin-resistant, and nalidixic acid resistant clones were the same used in Trindade et al. (2009). D-cycloserine-resistant mutants were derived from the ancestral strain *E. coli* K12 MG1655 by plating the clones at 37°C on plates containing M9 minimal medium (MM) supplemented with agar and the respective antibiotic. We used MM instead of Luria-Bertani (LB) because D-cycloserine activity is known to be inhibited in the presence of alanine (Bondi et al. 1957; Hoeprich 1965), which is one of the composites of LB medium. MM supplemented with D-cycloserine at the final concentration of 0.04 mM was used in the selection process. All the D-cycloserine spontaneous resistant clones were tested for mutator phenotype by plating 100 μl of an overnight culture of both ancestral and each of the resistant clones in LB supplemented with rifampicin at the end-concentration of 100 μg/mL. The five clones presenting evidence of high mutation rate were not considered for further analysis. To study different stressful conditions, three different environments were chosen: LB at 37° and M9 MM, both at 37° (MM), and at 40° (MM40). All the three environments were antibiotic free.

DETECTION OF MUTATIONS

The known target gene for resistance to D-cyloserine is *cycA* (Fehér et al. 2006). Mutations responsible for resistance were identified by amplifying and sequencing the target gene. The primers used to amplify the *cycA* gene were: 5′-TTTCC AGCCAC ATGATGAAA-3′ and 5′-GTTTATGGGGTCTGGCAAAA-3′. The first primer as well as 5′-AGCGCGAAGACGTAGAACAT-3′, 5′-GTCGGCCTGGTCAT GTGGC-3′, 5′-TACCAATAAAC AGCCAGCCC-3′, 5′-CGTGGTGGCGATCACGG CTT-3′ and

Table 1. Genetic basis of the resistance mutations.

Gene	Nucleotide change	Amino acid change[1]	Antibiotic resistance	Reference
gyrA	GAC to GGC	D 87 G	Nalidixic acid	Trindade et al. (2009)
gyrA	TCG to TTG	S 83 L	Nalidixic acid	Trindade et al. (2009)
gyrA	GAC to TAC	D 87 Y	Nalidixic acid	Trindade et al. (2009)
rpoB	GAC to GTC	D 516 V	Rifampicin	Trindade et al. (2009)
rpoB	CAC to AAC	H 526 N	Rifampicin	Trindade et al. (2009)
rpoB	CAC to CTC	H 526 L	Rifampicin	Trindade et al. (2009)
rpoB	CAC to TAC	H 526 Y	Rifampicin	Trindade et al. (2009)
rpoB	ATC to TTC	I 572 F	Rifampicin	Trindade et al. (2009)
rpoB	GAC to TAC	D 516 Y	Rifampicin	Trindade et al. (2009)
rpoB	GAC to AAC	D 516 N	Rifampicin	Trindade et al. (2009)
rpoB	CGT to CAT	R 529 H	Rifampicin	Trindade et al. (2009)
rpoB	TCT to TTT	S 512 F	Rifampicin	Trindade et al. (2009)
rpoB	TCC to TTC	S 531 F	Rifampicin	Trindade et al. (2009)
rpoB	CAC to GAC	H 526 D	Rifampicin	Trindade et al. (2009)
rpsL	AAA to AGA	K 43 R	Streptomycin	Trindade et al. (2009)
rpsL	AAA to ACA	K 43 T	Streptomycin	Trindade et al. (2009)
rpsL	AAA to GAA	K 88 E	Streptomycin	Trindade et al. (2009)
rpsL	AAA to AAC	K 43 N	Streptomycin	Trindade et al. (2009)
rpsL	AAA to AGA	K 88 R	Streptomycin	Trindade et al. (2009)
cycA	TTG to TAG	L 432 Stop	D-cycloserine	This study
cycA	:: ACG	*cycA* 1048::T	D-cycloserine	This study
cycA	TCG to TAG	S 275 Stop	D-cycloserine	This study
cycA	GTT to TTT	V 284 F	D-cycloserine	This study
cycA	GAA to AAA	E 237 K	D-cycloserine	This study
cycA	AGC to CGC	S 313 R	D-cycloserine	This study

[1]***Escherichia coli*** **strain K12 MG1655.**

5′-CGAGGCGACCCAGTCGGAGA-3′ were used for sequencing directly from the PCR product.

GROWTH CURVES AND FITNESS ASSAYS

The ranking of the stress provided by each studied environment was generated based on the comparison of the three main phases of growth, namely, duration of the lag phase (L), division time at the exponential phase ($1/r$), and total carrying capacity measured at the stationary phase. Four growth curves of the ancestral population of *E. coli* were made in each studied environment and in the same conditions as the competitions. Overnight culture concentrations were adjusted such that the initial optical density (OD) was the same in every environment. To measure fitness effects of the resistance mutations in the three different environmental conditions, competition assays were done. The resistant mutants were competed against a reference strain, *E. coli* K12 MG1655 Δara, in the three different antibiotic free environments, in an approximate proportion of 1:1. To do so, we grew both resistant and reference strains in LB or MM at 37° or MM at 40° (all liquid media) for 24 h with aeration. Accurate values of each strain initial ratio were estimated by plating a dilution of the mixture in TA agar plates. Competitions were performed in 50-mL screw-cap tubes containing 10 mL of each medium by a period of 24 h at the corresponding temperature with aeration. By the end of this 24-h competition period, appropriate dilutions were platted onto TA agar plates to obtain the final ratios of resistant and reference strains. The fitness effect of each mutant strain—that is, the selection coefficient—was estimated as the per generation difference in Malthusian parameters for the resistant strain and the marker strain:

$$s = \frac{1}{t} \times \ln\left(\frac{R_f}{R_i}\right)$$

where t corresponds to the number of generations and R_f and R_i to the final and initial ratios between resistant and reference strains, respectively. The selection coefficient was discounted by the cost of the Δara marker, if it was the case.

DETERMINATION OF DISTANCE TO FITNESS PEAK

To study the rate of fitness increase and a potential fitness plateau, we performed experimental evolution in each of the three studied environments. Three replicate populations were studied, all founded from the same ancestral clone. They were propagated

daily by passage of 100 μl of a 10,000 diluted culture to 10 mL of the corresponding medium. Every five passages, a sample of each population was frozen with 15% glycerol at –80°C. Competitions were then made from the frozen samples at different time points (four competitions per time point) and the relative fitness estimated.

STATISTICAL ANALYSIS

All statistical tests were done using R and Mathematica (Wolfram Research 2010). To query about the presence of different fitness effects operating for the same genotype among different environments, we made pairwise comparisons between the fitness of each of the 25 resistance mutations in different environmental conditions using a Wilcoxon rank-sum test with a Bonferroni correction for multiple testing. To test for increase in the effects of mutations with stress, a Friedman test was performed. To test for increased variance of effects with stress, a Pitman test was performed. Finally, we fitted a linear mixed model by restricted maximum likelihood using the lme4 package in R, considering genotype, environment, and GxE as random factors. We further tested if the distribution of fitness effects of the mutations causing antibiotic resistance were compatible with the predictions of Fisher's geometrical model. Fisher model predicts that a shifted gamma distribution is a good approximation for the DFE. We estimated the parameters of this distribution using maximum likelihood and tested if it provided a good fit to the data using a Kolmogorov–Smirnov (KS) test. To determine the effect of measurement error of the fitness effects of mutations, we produced 100 new datasets where a normally distributed error was added top the data. We then estimated the parameters of the shifted gamma distribution (α_{mle}, β_{mle}, s_{0mle}) for each of these datasets, by maximum likelihood. The results of each parameter are given as supplemental information (Fig. S2). The maximum likelihood analysis was done using Mathematica 8.0 (Wolfram Research 2010).

Results

RANKING ENVIRONMENTS ACCORDING TO STRESS

We first ranked the environments by assaying how well the wild-type sensitive *E. coli* grows in each of the three target environments: rich medium (LB), MM with a single carbon source (MM) and MM at high temperature (MM40) (Fig. 1). There are three main phases during growth in each of the environments: lag phage, with duration L, exponential phase with constant growth rate r, and stationary phase, characterized by a carrying capacity K. The number of cells used to seed a growing culture can potentially influence all growth phases. For this reason, this number was carefully controlled and maintained constant across environments. All phases of growth are important when mutants compete with the wild type, and each of these fitness components can be altered in a novel stressful environment.

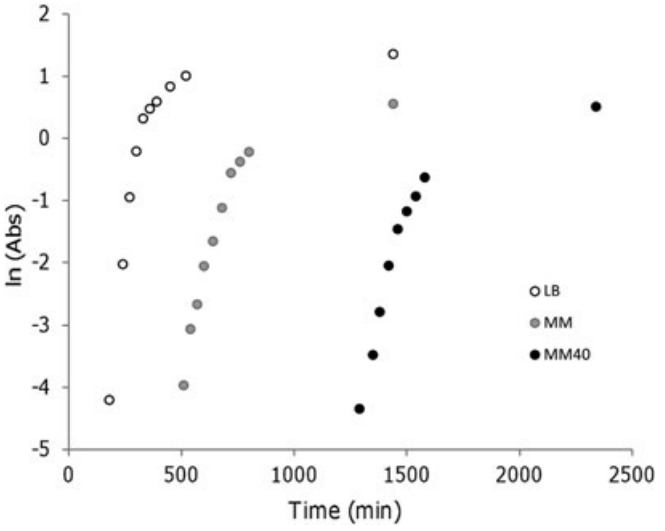

Figure 1. Growth of *Escherichia coli* in the three environments for ranking of stress. The growth curves of the wild-type sensitive bacteria in LB, MM, and MM40 allow the determination of lag phase duration, *L*, division time, 1/*r*, and carrying capacity, *K*.

We define an environment as benign if there are plenty of resources and conditions for a rapid growth and high carrying capacity. This is the case of the LB medium, where the wild-type *E. coli* has a lag phase (L) of 91 min (2SE = 14), a division time ($1/r$) at exponential phase of 33.6 min (2SE = 0.7), and a total carrying capacity after 24 h (K, OD_{600nm}) of 3.97 (2SE = 0.09). An environment is defined as stressful if any of the fitness components is reduced in comparison to the benign environment (LB). Comparing the three major fitness components of the growth curve between MM and LB, we observe that the lag phase is increased by 357% (L_{MM} = 324 min [2SE = 29]), $1/r$ is increased by 192% ($1/r_{MM}$ = 65 min [2SE = 5]), and K is decreased by 44% (K_{MM} = 1.76 [2SE = 0.05]). This indicates that MM is detrimental for all of the fitness components. The reduction in fitness is even higher when we add a thermal stress (MM40). This particular kind of stress (or the combined effect of scarcity of resources and increased temperature) affected mainly the lag phase. For this environment, L has increased 1395% (L_{MM40} = 1266 min [2SE = 88]), $1/r$ 198% ($1/r_{MM40}$ = 67 min [2SE = 9]), and K was reduced by 45% (K_{MM40} = 1.79 [2SE = 0.09]). Given these values, we considered that the environments are ordered by increased stress as follows: LB, MM, and MM40.

GxE INTERACTIONS

To query about how the effects of mutations vary with increasing stress, we studied a sample of 25 clones each carrying a single mutation and measured their fitness effects through direct

competition with the ancestral in each of the three environments (see section Growth Curves and Fitness Assays for detailed description of the competition assay). All the mutations confer resistance to a single antibiotic (streptomycin, rifampicin, nalidixic acid or D-cycloserine) and the vast majority of them are single-nucleotide changes (see Table 1 for the genetic basis of the resistance mutations).

Figure 2 shows the competitive fitness effects obtained across environments for all the mutations studied. Of the 25 mutations, only nine cause similar fitness effects across all three environments (Wilcoxon test $P > 0.025$, considering Bonferroni correction for $n = 2$ comparisons). When we fit a linear mixed model we find a significant contribution of genotype (G) ($P < 0.05$), environment (E) ($P < 0.05$), and GxE ($P < 0.05$) in explaining the fitness effects. The proportion of variance attributed to GxE interactions was 37%.

In all cases, mutations were mostly deleterious in the absence of antibiotics, as expected. In LB, no single beneficial mutation was detected, whereas in MM, four mutations conferring resistance to rifampicin were beneficial (*D516V*, *D516N*, *H526N,* and *H526Y* in *rpoB*). The mutation *D516V* remained beneficial at higher temperature, and an additional mutation became advantageous (*S512F*). Almost half of the mutations had the biggest cost in the most stressful environment, when compared to the other two environments. A similar situation, where increased temperature corresponded to an increase in the selection pressure was also observed in yeast (Szafraniec et al. 2001; Jasnos et al. 2008). There is a significant correlation of the mutation effects across environments: Pearson's correlation coefficient (r) was 0.75 ($P <$ 0.0001, 95% CI [0.502–0.883]) between LB and MM, 0.85 ($P <$ 0.0001, 95% CI [0.678–0.931]) between LB and MM40, and 0.72 ($P < 0.0001$, 95% CI [0.455–0.869]) between MM and MM40 (Fig. S1).

The mean fitness effect of mutations was –0.077 in LB, –0.055 in MM, and –0.18 in MM40. The mean effects differed according to the environment (Friedman test, df = 2, $P < 0.01$). A post-hoc test shows that the difference is due to the selection coefficients measured in MM40, because there was no significant difference between the mean effect of mutations in LB and MM. Given these results, we conclude that stress does not always lead to an increment in the mean effect of mutations in *E. coli*. However, increasing the temperature does seem to show a trend to increase the mean effect of resistance mutations.

The variance in mutational effects was: 0.005 in LB, 0.0125 in MM, and 0.07 in MM40. Although the mean fitness effect does not necessarily increase with stress, the variance is higher in the two stressful environments. Furthermore, these data suggest that the variance increases with increasing stress (Pitman's T test, $P < 0.005$ for MM vs. LB, $P < 0.0005$ for MM40 vs. LB, and $P < 0.0005$ for MM40 vs. MM).

DISTRIBUTION OF MUTATION EFFECTS AND FGM

An increase of variance with stress is expected under certain fitness landscape models (Agrawal and Whitlock 2010). One model that has been very successful at explaining DFE in bacteria is FGM. In the Fisherian framework, if stress is interpreted as an increased distance from the optimum, then an increase in the variance of mutational effects of random mutations is expected to occur. This is precisely what we have found here, even though we note that antibiotic resistance mutations are a nonrandom subset of all mutations. FGM is also a model from which the DFE of mutations can naturally emerge. If we assume a Gaussian relation of phenotype to fitness in a single peak landscape then the DFE can be predicted. Recent theoretical analysis of FGM (Martin and Lenormand 2006a; Gros et al. 2009; Lourenço et al. 2011) has provided expressions for the DFE of all mutations in a given environment. The parameters of such distribution ($f(s)$) relate to organism complexity (measured by the number of traits that contribute to fitness) and its distance to the fitness peak (s_0). More specifically, Martin and Lenormand (2006a) showed that away from the optimum the DFE is well approximated by a shifted gamma distribution, with shape parameter β, scale parameter α, and shift parameter s_0:

$$f(s) = \frac{e^{-(s_0 - s)}/\alpha(s_0 - s)^{\beta-1}\alpha^{\beta}}{\Gamma(\beta)}$$

We asked if a shifted gamma distribution could reasonably explain the DFE measured in each of the three environments. We estimated the three parameters of these distributions by maximum likelihood and tested if they provided a reasonable fit by a KS test. In the case of LB, the maximum likelihood values of the parameters were $\alpha = 0.06$ (95% CI [0.013–0.1]), $\beta = 1.5$ (95% CI [0.32–2.6]), $s_0 = -0.0076$ (95% CI [0.002–0.018]), for MM were $\alpha = 0.054$ (95% CI [0–0.12]), $\beta = 4.35$ (95% CI [0–12.2]), $s_0 = -0.18$ (95% CI [0.012–0.35]) and finally for MM40 were $\alpha = 0.204$ (95% CI [0.04–0.36]), $\beta = 1.81$ (95% CI [0.2–3.4]), $s_0 = -0.17$ (95% CI [0.1–0.24]). The good fit of the data, provided by the distributions generated with these estimated parameters, for all the three studied environments (see Fig. 3), was evaluated by KS test (LB: KS test $P = 0.66$; MM: KS test $P = 0.56$: MM40 KS test $P = 0.83$). Based on the parameters obtained under this model, the effective number of traits (n_e) can be estimated as $n_e = \beta(2 + 4\varepsilon)/(1+\varepsilon)^2$, where $\varepsilon = 2s_0/|E(s)|$, which leads to a value around three in the different environments ($n_e = 2.98$ in LB, $n_e = 3.58$ in MM, and $n_e = 2.84$ in MM40).

EXPERIMENTAL EVOLUTION TOWARDS A FITNESS PEAK

The parameters of the shifted gamma distribution obtained in the previous analysis, interpreted in the light of FGM, imply that s_0 reflects the level of maladaptation in a given environment.

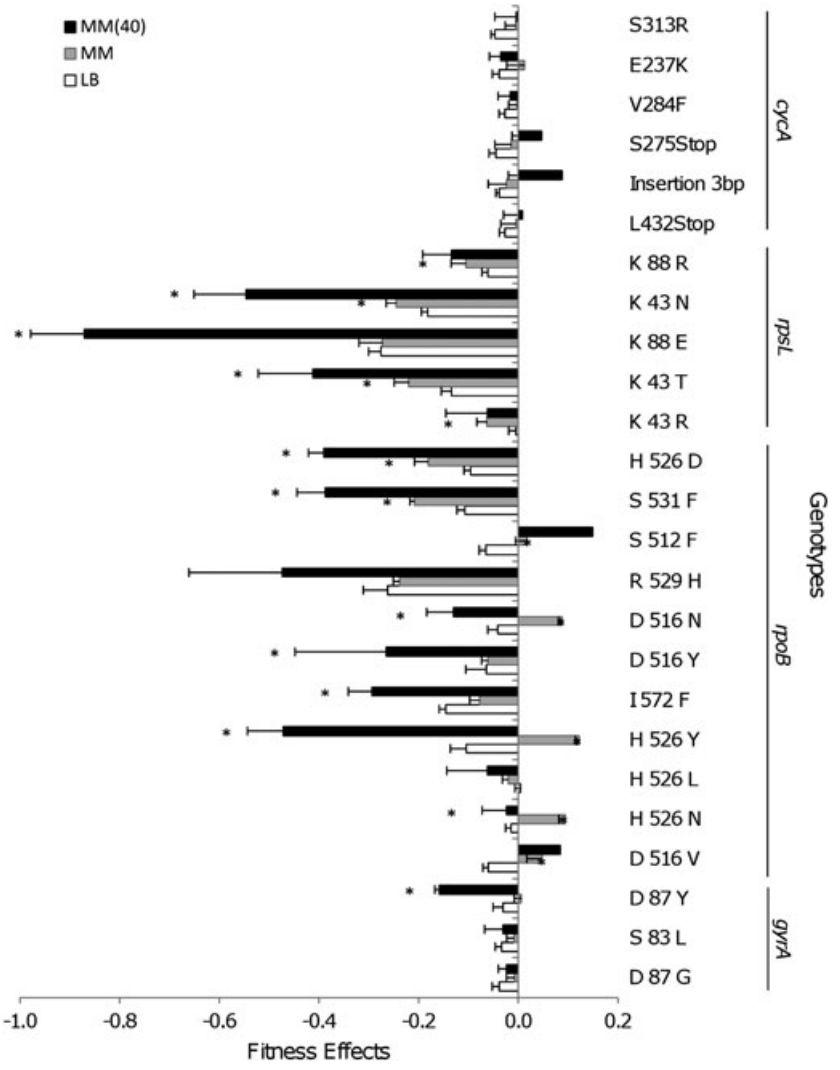

Figure 2. The effects of each resistance mutation in the three environments. The first three alleles correspond to mutations in *gyrA* related with resistance to nalidixic acid. The next 11 alleles correspond to mutations in *rpoB*, conferring resistance to rifampicin. The following five alleles correspond to mutations in *rpsL* that provide resistance to streptomycin and finally, the last six genetic changes correspond to mutations in *cycA* related with resistance to D-cycloserine. Asterisks (*) indicate statistically significant differences on the mean fitness effect of each mutation between two consecutive environments.

This theoretical framework suggests that if the wild-type sensitive *E. coli* is allowed to adapt in each particular environment, then it would reach a fitness peak at a distance close to s_0. To test for this prediction, we performed experimental evolution of the wild-type in each environment (three replicate populations). We followed the dynamics of mean population fitness change for a period of several hundreds of generations and estimated fitness through direct competition with the ancestral carrying a genetic marker, along time. This procedure continued until no significant fitness increase could be detected after the following 100 generations (see Fig. 4). If FGM can describe reasonably well the initial process of adaptation, then it is expected that the greatest fitness increase is observed in the initial stages of the adaptive walk, with the later stages being characterized by beneficial mutations of very small fitness effects. Figure 4 shows the dynamics of fitness increase in the first hundreds of generations. In LB, very little adaptation could be detected over about 500 generations of evolution. In MM, a clear rapid adaptation occurred during the first 100 generations of evolution with smaller increments afterwards. Finally, in MM40 the rate of fitness increase was slower initially, but from 500 to 700 no significant increase could be detected. If we consider the maximum fitness value achieved (in the adaptation to each environment), over the hundreds of generations studied, as a proxy for the distance to the peak, then the observed increments in fitness (s_0 [LB] = 0.04, s_0 [MM] = 0.17, and s_0 [MM40] = 0.15) are very similar to those predicted by maximum likelihood estimated value of the shifted gamma, s_0 (s_0 [LB] = 0.0076, s_0 [MM] = 0.18, and s_0 [MM40] = 0.17).

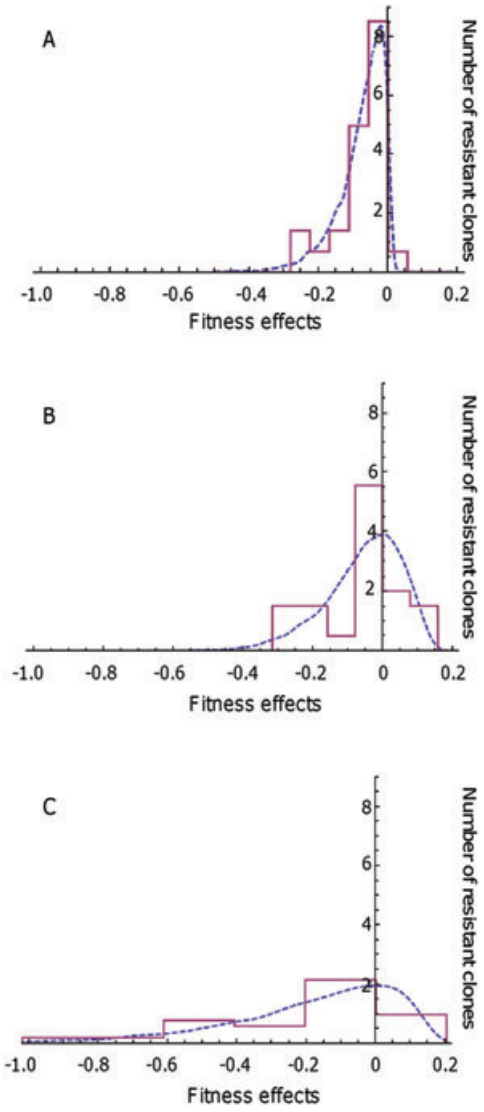

Figure 3. Distribution of measured fitness effects of mutations and the maximum likelihood estimate of the shifted gamma as predicted by FGM (dotted line). (A) In LB, (B) In MM, (C) In MM40.

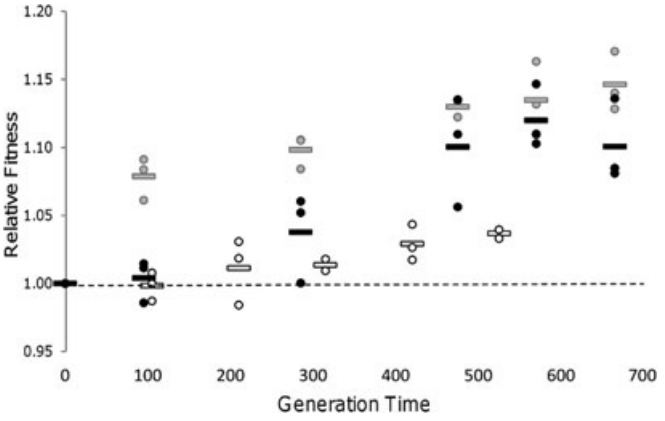

Figure 4. Experimental evolution toward adaptation in LB (A), MM (B), and MM40 (C). LB is represented in white, MM in gray, and MM40 in black. The mean fitness effect for each environment between populations is represented as a horizontal bar of the corresponding color.

This means that the DFE of the resistance mutations in these genes had substantial information to allow prediction of the position of nearest fitness peak.

Discussion

Robustness to mutation and environmental change is important for understanding evolutionary dynamics and maintenance of genetic variation in populations. A genotype presents mutational robustness if a large fraction of random mutations will have very small fitness effects. It presents environmental robustness if it is highly fit in many different environments. We have measured the distribution of fitness effects of mutations, all of which cause antibiotic resistance, in three different environments. These can be ranked according to the level of stress they cause in the growth dynamics of *E. coli*. The results obtained support the claim that stress generally leads to an increase in the variance of mutation effects to a larger extent than in the mean, which is in agreement with the recent meta-analysis of Martin and Lenormand (2006b). The results presented here also add to the findings of pervasive GxE interactions in *E. coli*, in particular those related with insertions, caused by single random transpositions of mini-Tn10 transposons (Remold and Lenski 2001). For random transposition, the variance in fitness among lines increased in stressful conditions, whereas the average deleterious effect remained unchanged. Interestingly, in that study, a few insertions were found to confer beneficial fitness effects in the more stressful environment. Here, we find that two of the mutations causing a disruption of the protein (one stop codon and one small insertion) were beneficial.

One of the biggest challenges yet to be accomplished is to elucidate the mechanism by which different classes of stresses affects the mean effect of selection (Agrawal and Whitlock 2010). A large sample of random mutations at the genomic level constitutes a powerful approach to address this issue. In this study, we consider a subset of mutations that are nonrandom in relation to a given stress, so one can attempt to make predictions on its effect. Although this was not the main goal of this work, it is important to consider what is known about the selective pressure imposed by the stresses studied here in relation to the mutation target genes (*rpoB* which codes for the β-subunit of the RNA polymerase [RNAP]; *rpsL* which codes for 30S ribosomal subunit; *gyrA* which codes for the subunit A of the DNA gyrase and *cycA*, coding for D-serine, D-alanine, and glycine permease) and relate that knowledge with the mutation effects that were observed.

Mutations in RNAP are the predominant driving force of adaptation to the scarcity of nutrients (Herring et al. 2006;

Applebee et al. 2008). In particular, RNAP has been identified as a target to be reprogrammed with specific kinetic properties that should increase the amount of transcription of key limiting genes for biomass production in MM (Conrad et al. 2010). Given this, it is not surprising that we found four mutations, deleterious in LB, to become beneficial in MM. Furthermore, one of these mutations remained advantageous when the temperature was raised and an additional deleterious mutation in LB was also beneficial in MM40. These mutations were located in the *rpoB* gene, which encodes a subunit of the RNAP. Interestingly, adaptive mutations in subunits of the RNAP have recently been recurrently found in laboratory experiments where *E. coli* was adapted to grow in MM at high temperature (Tenaillon et al. 2012). In fact, in the work reported by Tenaillon et al. (2012), where whole genome sequencing of clones from populations of *E. coli* adapted to high temperature was done, *rpoB* was one of the main target genes carrying adaptive mutations. One of the mutations studied here (*I572F*) was also found in that study. Interestingly, this mutation is deleterious in all the environments we tested, including high temperature. This may indicate that *I572F* is highly epistatic, its effect may depend on the genetic background where it arises, and thus it may be beneficial in combination with some other(s) mutation(s). In fact, Trindade et al. (2009) demonstrated that *I572F* presents high levels of positive epistasis with different alleles of *rpsL* and *gyrA* genes. We note that *rpoB* is currently used as a phylogenetic marker in Eubacteria (Case et al. 2007). Whereas such markers are expected to be conserved at the species level, being an obvious target for adaptation raises concerns about its level of conservation and thus about its performance as a species-specific marker.

Escherichia coli's growth rate is higher in rich medium than in poor medium (see Fig. 1). It is known that its protein synthetic ability is increased in the latter situation, not because the elongation rate is much higher but because the number of working ribosomes is bigger (Poulsen et al. 1993; Farewell and Neidhardt 1998). If the elongation rate is fairly constant in these two situations, mutations that affect this function (as the ones in *rpsL* [Kurland et al. 1996]) should have a similar fitness cost in both environments. On the other hand, it has been shown that the rate of elongation increases proportionally to the temperature between 37°C and 44°C, concomitantly with an increase in rate of protein degradation (Farewell and Neidhardt 1998). If a raise in elongation rate is needed to face the problem of protein degradation, then it can be expected that genetic defects that impair this function might have a bigger impact in this environment. In accordance with this last prediction, in our study, we observe that mutations in *rpsL* become more deleterious when the temperature was raised.

The dependence of DNA topology on growth temperature has been extensively established (Tse-Dinh et al. 1997). About 10% of the mutations that confer nalidixic acid resistance and map to the locus *gyrA* confer thermotolerance (Friedman et al. 1991). Moreover, mutations in this gene have been previously implicated in adaptation to high temperature (48°C) (Droffner and Yamamoto 1991), so one could expect that some *gyrA* mutations could be beneficial at high temperature. None of the *gyrA* mutations studied here showed advantageous effects at 40°C, however that does not preclude an advantage at higher temperatures.

Although *rpoB*, *rpsL,* and *gyrA* are essential genes, the other target studied here, *cycA*, is not. Given this, we could expect the mutation effects on this gene to be weaker. This was in fact the case, as the vast majority of these mutations showed namely undetectable effects, which did not depend on the environment.

Overall, we were able to confirm the expectation of (1) the possible existence of beneficial mutations in *rpoB* in both stressful environments (Hall and MacLean 2011), (2) an increased deleterious effect of mutations in *rpsL* under high temperature, and (3) the weak effect of *cycA* mutations. The anticipated potential advantage of the Nal^r mutants at high temperature was not observed. In sum, predicting a trend for the effect of stress remains very difficult.

We found support for a strong effect of stress in increasing the variance of mutation effects, as well as changing the sign of some mutations (20% became beneficial under stress). Recently, an interesting survey of the effect of almost 4000 mutants, involving mainly deletions of nonessential genes of *E. coli*, in 324 environments was done (Nichols et al. 2011). Using this publicly available dataset, we can ask about the nature of GxE interactions of deletions at a genome wide level. Interestingly, in 15% of the environments the mean effect of deletions was positive. We also asked if stress increased the strength of selection by considering environments with increasing levels of stress. We found that in most of the cases tested (82%), the strength of selection increased. Importantly, 85% of the stresses led to increased variance, just as we found here. In 75% of the cases, both mean and variance increased. Given that this set of mutations is representative at the genome level, the results of this analysis suggest that stress tends to increase both the mean and the variance of s.

A recent study performed in *P. fluorescens* on the effects of mutations conferring resistance to nalidixic acid, across many different environments, found that FGM could provide a reasonable framework to describe the DFE among the mutations that were beneficial. The data on *P. fluorescens* also suggested that under FGM the effective number of traits, n_e, is small (two to four in the majority of environments, but >10 in some of the conditions tested) (Bataillon et al. 2011). This suggestion is strikingly similar with the value of n_e estimated in our data (in LB, MM, and MM40 $n_e \sim 3$). The effective number of traits under stabilizing selection can also be estimated based on mutation accumulation experiments (MA) namely, from the rate of decline in mean fitness and rate of increase in variance among independent lines

accumulating many deleterious mutations (Martin and Lenormand 2006a). These MA in *E. coli* (Kibota and Lynch 1996; Loewe et al. 2003; Trindade et al. 2010) typically lead to very low estimates of n_e (Martin and Lenormand 2006a). In contrast, studies of adaptation toward compensation of costs of resistance in *E. coli* suggest n_e bigger than 10 (Sousa et al. 2012). Given the simplicity of Fisher model, the theoretical predictions currently made on its extensions, and its apparent adequacy in describing several pieces of empirical data, FGM is becoming a model which stimulates future empirical tests and novel theoretical predictions (Orr 2005; Waxman and Welch 2005; Martin et al. 2007; Gros et al. 2009; Lourenço et al. 2011).

AKNOWLEDGMENTS

This work was supported by ERC grant ECOADAPT-260421 to IG and AS and grant PTDC/BIA-EVF/114622/2009 and SFRH/BD/40162/2007 to ST financed by Fundação para a Ciência e Tecnologia.

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Associate Editor: M. Johnston

Supporting Information

The following supporting information is available for this article:

Figure S1. Correlation of mutation effects across environments.

Figure S2. Distribution of parameters β, α, and s_0 of the shifted gamma distribution, for the effects of mutations in LB, MM, and MM40.

Table S1. Selection coefficients for the 25 mutants in the three studied environments.

Supporting Information may be found in the online version of this article.

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