Hidden Randomness between Fitness Landscapes Limits Reverse Evolution

Longzhi Tan,* Stephen Serene,† Hui Xiao Chao, and Jeff Gore‡

Department of Physics, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

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In biological evolution, adaptations to one environment can in some cases reverse adaptations to another environment. To study this ''reverse evolution'' on a genotypic level, we measured the fitness of *E. coli* strains with each possible combination of five mutations in an antibiotic-resistance gene in two distinct antibiotic environments. While adaptations to one environment generally lower fitness in the other, we find that reverse evolution is rarely possible and falls as the complexity of adaptations increases, suggesting a probabilistic, molecular form of Dollo's law.

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Whether adaptations are reversible is a fundamental question in evolutionary biology. When an adaptation to one environment decreases fitness in another environment (causing a fitness trade-off), it may be possible for evolution in the second environment to reverse the previous adaptation [1–3]. Traditionally, reverse evolution has been defined as returning to an ancestral phenotype (physical characteristics like size or antibiotic resistance). In 1890, Dollo hypothesized that evolution never reverses complex phenotypic adaptations, because such a reversal would require many coincident simple reversals [1]. Recent work, however, has undermined this hypothesis. For example, reverse evolution of insects has been identified both in evolutionary histories and in laboratory experiments [2,4].

However, reverse evolution has been little studied with respect to genotypes (genome sequences). A genotypic approach is interesting because it captures the dynamics and complexity of adaptations that phenotypic measures cannot. Treatments of genotypic evolution rest on the notion of a fitness landscape, which specifies the fitness values of all genotypes in a biological system. Here, we study a small subset of this landscape, which considers n mutational sites, each with two allowed states. Thus, every genotype can be represented by an n-bit string. For small mutation rates, mutations generally occur one at a time, meaning that a population can change only one ''bit'' at a time, leading to an n-dimensional hypercubic graph of allowed adaptations with 2^n nodes (genotypes) and $n2^{n-1}$ edges. Finally, for large population sizes, only beneficial mutations are likely to reach fixation during evolution by natural selection, which directs the edges of the hypercube from lower to higher fitness.

Advances in biology have allowed quantitative experimental measurements of such fitness landscapes [5–8]. Experimental landscapes have revealed that many mutations are beneficial only on certain genetic backgrounds, leading to complex adaptive dynamics. In particular, on such rugged landscapes there may be no selective path between a low-fitness and a high-fitness genotype.

In this Letter, we use a bacterial antibiotic-resistance gene as an experimental model system to study genotypic reversibility. We consider the fitness landscapes for five mutations in two antibiotic environments, in which fitness is represented by antibiotic resistance. We call an adaptation reversible when its end points are connected by allowed paths on both landscapes (in opposite directions). By using a related approach, a recent study found that interactions between mutations block reverse evolution of a glucocorticoid receptor, even under selection for the ancestral function [3]. Instead of considering only one pair of genotypes, we analyze reversibility as a global, statistical feature of fitness landscapes. We find in both experiments and simulations that an adaptation's reversibility falls as the number of mutations it involves increases (a longer genetic distance on the landscape), suggesting a probabilistic form of Dollo's law for genotypic evolution. We believe this is the first experimental observation of such a decline. In addition, we find a measure of the local correlation between two landscapes that well predicts the rate of this decay.

β-lactam antibiotics, which kill bacteria by inhibiting cell wall synthesis, are both the oldest and most widely used class of antibiotics [9]. Bacteria can gain resistance to these antibiotics by expressing the enzyme β-lactamase, although different alleles (sequences) of the β-lactamase gene confer different levels of resistance against different drugs [10,11]. Previous work has identified five mutations that can occur in the reference allele of the TEM-family β-lactamase gene, which together confer high-level resistance to cefotaxime and other clinically important antibiotics [12]. Four of the mutations (A42G, E104K, M182T, and G238S, numbering as in Ref. [13]) are in the protein-coding region of the gene and therefore change the amino acid sequence of the protein. The other mutation (g4205a) is in the regulatory region of the gene and increases its expression level [14]. Weinreich *et al.* analyzed the evolutionary paths between the reference allele and the allele with all five mutations, finding that many of the mutations are beneficial only in the presence of some other mutations. Such interactions between mutations blocked many of those paths [5].

Given the "ruggedness" in this fitness landscape, we expected it to constitute an interesting model system for reversibility. To study reversibility, we sought a pair of environments for which fitness is negatively correlated. Following Weinreich *et al.*, as a proxy for fitness we use the minimum inhibitory concentration (MIC), defined as the minimum concentration of an antibiotic required to inhibit cellular growth [5,15]. Note that the relationship between MIC and fitness may be complicated (higher MIC may be costly, leading to a selective disadvantage in some environments). However, our general conclusions are robust to small changes in the experimental landscapes.

We first measured the landscapes in piperacillin, a common penicillin (abbreviated as environment P), and in cefotaxime (environment C; comparison with data published by Weinreich *et al.* [5] is shown in Ref. [16]), but we found no fitness trade-off between environments C and P [Fig. 1(a)] [16]. Since adaptation to one antibiotic environment rarely leads to loss of fitness in the other, reverse evolution is unlikely.

However, alleles highly resistant to cefotaxime have been reported to be sensitive to β-lactamase inhibitors [12], suggesting a possible trade-off between fitness in cefotaxime and fitness in piperacillin with a β-lactamase inhibitor. Clavulanic acid, one such inhibitor, can transiently or irreversibly inactivate β-lactamase [17]. We measured the landscape in piperacillin with 0.5 μg/ml clavulanic acid (abbreviated as environment $P + I$, 0.5 μg/ml is similar to clinical concentrations [18]) and observed a significant fitness trade-off between environments $P + I$ and C [Fig. 1(b)] [16]. In particular, none of the 32 alleles displayed high-level resistance to both antibiotic treatments. So a bacterial population that has adapted to environment C may return to its original genotype by adapting to $P + I$, exhibiting evolutionary reversibility at the genotypic level.

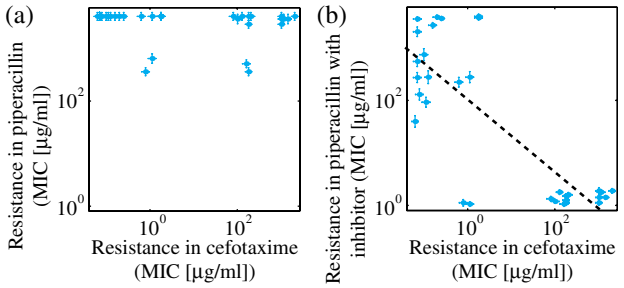

FIG. 1 (color online). Fitness trade-off between the cefotaxime and inhibitor environments. (a) Measurements of MIC show that, without an inhibitor, there is no fitness trade-off between the cefotaxime environment and the piperacillin environment. Increasing MIC values indicate increasing resistance. (b) A significant fitness trade-off exists between the cefotaxime environment and the piperacillin environment with a β-lactamase inhibitor. Linear regression on $\log_{10}$MIC yields slope $= -(0.69 \pm 0.09)$, $r^2 = 0.649$; $n = 32$. Alleles were constructed and transformed into *E. coli* strain DH5α by Weinreich *et al.* [5]. MIC was determined as the lowest concentration that ceased bacterial growth after culturing for 20 h at 37 °C.

Evolution involving only a single mutation will be reversible whenever that mutation causes a fitness trade-off. For example, for the allele that has only g4205a ($+ - - - -$; five $+$ or $-$ represent the presence or absence of, in order, g4205a, A42G, E104K, M182T, and G238S), G238S is beneficial in environment C but deleterious in $P + I$ [Fig. 2(a)]. Therefore, gaining G238S in this allele (evolving to $+ - - - +$) can be reversed by evolution in $P + I$. The G238S mutation enlarges the active site, increasing the enzyme's affinity to cefotaxime [19]. This opening of the active site also increases the affinity to β-lactamase inhibitors, making it more sensitive to their effect [17].

Evolution involving multiple mutations, however, may be irreversible even when there is a fitness trade-off between a pair of alleles (one is more fit in C, while the other is more fit in $P + I$), because there may not be an accessible evolutionary path in both directions. We consider a possible path to be blocked, or selectively inaccessible, whenever fitness (defined as median $\log_{10}$MIC [5]) falls or remains constant at any step along it, because only beneficial mutations are likely to take over a large population [20]. Our measurements suggest a fitness trade-off between the allele containing only the g4205a mutation ($+ - - - -$) and the allele containing g4205a, M182T, and G238S ($+ - - + +$) [Fig. 2(b)]. Starting from $+ - - - -$ in environment C, the population must first obtain G238S in order for M182T to be beneficial. M182T has been reported to reduce the rate of antibiotic hydrolysis but also to reverse the destabilizing effects of many mutations, including G238S [19]. Therefore, in C, only one of the two paths from $+ - - - -$ to $+ - - + +$ is accessible under natural selection.

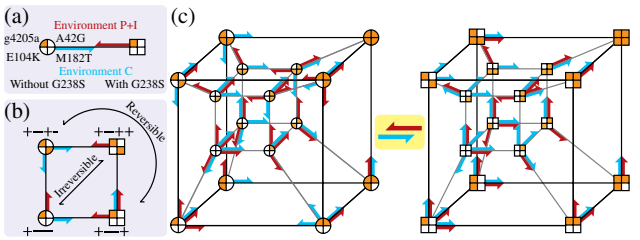

FIG. 2 (color online). Evolution follows paths through hypercubic directed graphs. (a) Evolution between alleles that differ by a single mutation (here, G238S) is reversible whenever there is a fitness trade-off between environments C and $P + I$. Each circle (without G238S) or square (with G238S) represents a genetic state, and each filled or blank quarter represents the presence or absence of g4205a, A42G, E104K, or M182T. Arrows show the direction of selection (blue in C, red in $P + I$). The lack of an arrow corresponds to a neutral mutation. (b) Considering a second mutation (M182T) gives two pairs that differ by two mutations. Here, both of these pairs have fitness trade-offs. Evolution between $+ - - + -$ and $+ - - - +$ is reversible, but evolution from $+ - - - -$ to $+ - - + +$ is irreversible. (c) Complete fitness landscapes in environments C and $P + I$, consisting of two four-dimensional hypercubes with all corresponding vertices (e.g., $+ - - - -$ and $+ - - - +$) connected via the G238S mutation (represented by the large middle arrows).

To determine whether evolution is reversible between these two alleles, we must determine whether there is a return path accessible in environment $P + I$. According to our measurements, M182T increases resistance in $P + I$ independent of G238S [Fig. 2(b)]. The $+ - - - -$ allele is therefore not accessible from the $+ - - + +$ allele in $P + I$, making evolution irreversible between this pair of alleles despite the fitness trade-off.

A central goal of this study is to analyze globally the experimental fitness landscapes, yielding statistical information about the fraction of adaptations that are reversible. We therefore consider all pairs of alleles with a fitness trade-off on our measured landscapes. For example, even on the partial landscape shown in Fig. 2(b), there is another pair of alleles that exhibits a fitness trade-off: The g4205a/G238S double mutant $(+ - - - +)$ is more fit in environment C, whereas the g4205a/M182T double mutant $(+ - - + -)$ is more fit in $P + I$. Evolution between these alleles is reversible because there exist paths connecting the states in both environments.

Considering all five mutations, we measured the fitness landscapes and determined the direction of selection for β-lactamase in environments C and $P + I$ [Fig. 2(c)]. Among all 496 pairs of alleles on these fitness landscapes, we first identify all pairs with a fitness trade-off and that are separated by more than one mutation (nonadjacent). If the two landscapes were uncorrelated, the expected number (with standard deviation) of nonadjacent pairs with trade-offs would be 184 ± 26 (obtained from 10 000 random permutations of fitness values across all alleles). For our experimental landscapes, the number is significantly larger (279), consistent with the global fitness trade-off [Fig. 1(b)]. Among these 279 pairs, 104 (37%) are connected by an accessible path in environment C and 82 (29%) in $P + I$ [Fig. 3(a)].

To predict the reversibility of evolution, we considered two extreme cases. If there were "perfect trade-offs" between the two environments (any beneficial mutation in $P + I$ is deleterious in C), then any path accessible in one environment would be reversible. In this case, there would be 82 reversible pairs. On the other hand, if the two landscapes were uncorrelated (no global trade-offs), the expected reversible fraction could be obtained by multiplying the accessible fractions at each distance and then summing them [16], yielding 34 ± 5 reversible pairs. Based on the global trade-off between environments C and $P + I$ [Fig. 1(b)], we expected the number of reversible pairs to be closer to 82 than to 34. Surprisingly, we found only 20 [Fig. 3(a)] (among them, two pairs are reversible only by gaining and subsequently losing an additional mutation to avoid a "fitness valley") [16]. Evolution is therefore generally not reversible between our experimental fitness landscapes.

The degree of reversibility is surprisingly low despite the fact that we do not require reverse evolution to be on the same trajectory as the original adaptation (if this is required, there will be only 12 reversible pairs [16]).

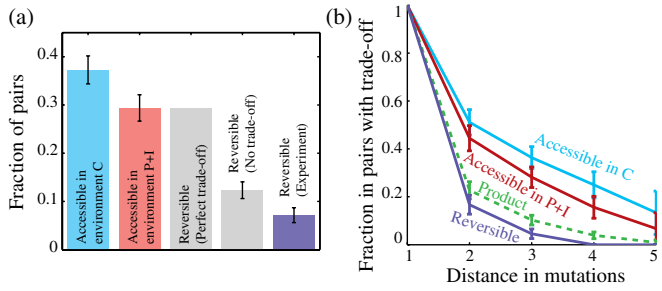

FIG. 3 (color online). Reverse evolution is rarely possible on the experimental fitness landscapes and falls with evolutionary distance. (a) Among all 279 pairs of alleles that have a fitness trade-off and are not adjacent, accessible evolutionary paths connect 104 in environment C and 82 in $P + I$. If there were perfect trade-offs between the two environments, then any path accessible in one environment would be reversible, yielding 82 reversible pairs. If the two landscapes were uncorrelated, there would be 34 ± 5 reversible pairs. Surprisingly, we find only 20 reversible pairs. Error bars, binomial error; $n = 279$. (b) Both accessibility (environment C in blue, $P + I$ in red) and reversibility (purple line) decline as the number of mutations between alleles increases. For distances greater than 1, the experimental reversibility is below the curve expected for uncorrelated landscapes (green dashed line). Error bars, binomial error; $n = 25, 90, 110, 64, 15$ for distance $= 1, 2, 3, 4, 5$.

Furthermore, allowing neutral mutations does not change our conclusion [16]. Genotypic reversibility is rare despite the fact that a globally optimal phenotype in either environment is accessible from every allele, yielding, in a sense, perfect phenotypic reversibility.

To further characterize evolution on our landscapes, we analyzed all pairs with a trade-off as a function of the number of mutations separating the start or end states [Fig. 3(b)]. We find the fraction of pairs connected by an accessible path in each environment to fall with mutational distance. If the two landscapes were uncorrelated, the expected reversibility as a function of mutational distance would be the product of the one-way accessibility curves [16]. Once again, we find the experimental reversibility curve below the curve expected for uncorrelated landscapes. To our knowledge, this is the first experimental estimate of evolutionary reversibility as a function of distance. No pairs are reversible for distances ≥ 4, suggesting a natural definition in this system for a complex adaptation in Dollo's law [1,21].

We therefore find that reverse evolution on our two experimental landscapes is very rare, despite the frequent fitness trade-offs between the two environments. We speculated that local positive correlation between the landscapes might limit reverse evolution while preserving the global negative correlation in fitness.

To test this idea, we analyzed how often gaining a given mutation has opposite effects in the two environments. A total of 80 edges (mutations on different genetic backgrounds) connect the 32 nodes (alleles) in our hypercubic fitness landscapes. Of the 80 edges, we found only 25

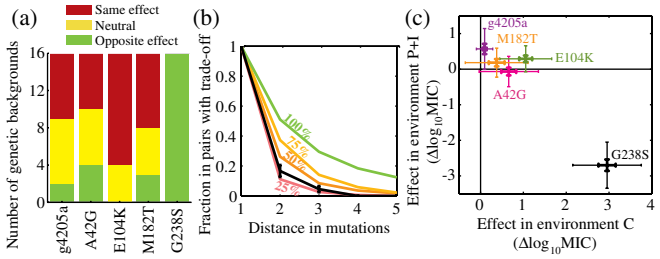

FIG. 4 (color online). One mutation dominates the fitness trade-offs. (a) Each mutation can appear on 16 genetic backgrounds. Although G238S is always reversible (has opposite effects in the two environments), the other four mutations are likely to have same effects in both environments, making many putative reverse paths inaccessible. (b) The reversibility of our experimental landscapes (with 31% reversible arrows) is plotted in black. Error bars are as in Fig. 3(b). Each colored curve shows the mean reversibility level of 200 random landscape pairs with 25%, 50%, 75%, or 100% reversible arrows. (c) The distribution of each mutation's effect across the 16 backgrounds. Although G238S is subject to greater mutation interactions than the other four mutations, these interactions are insufficient to change the sign of its large effect. Error bars are standard errors (thick line) and standard deviations (thin line), $n = 16$.

(31%) on which selection acts in opposite directions [Fig. 4(a)], corresponding to a reversible mutation [arrows pointing in opposite directions on the hypercubes in Fig. 2(c)]. Therefore, the global trade-off between the two environments is not reflected in the local structure of the landscapes. To further explore this discrepancy, we analyzed the effect of each mutation on all $2^4 = 16$ genetic backgrounds on which it can appear (all possible combinations of the four remaining mutations). One mutation (G238S) has opposite effects in the two environments on all backgrounds (consistent with the global negative correlation), but the other four more frequently than not have the same effect in both environments [in fact, E104K is always either beneficial or neutral in both environments; see Figure 4(a)]. Simulations on randomly generated landscapes showed that reversibility increases with the fraction of reversible edges. Our experimental reversibility is characteristic of landscapes with the same degree of local correlation [Fig. 4(b)] [16]. It is this local correlation that determines the rate at which evolutionary reversibility decays with genetic distance.

To understand the contrast between global trade-offs and local randomness, we looked at the magnitude of each mutation's effect over all genetic backgrounds [Fig. 4(c)]. G238S is located near the active site of the enzyme and primarily affects specificity, yielding large and opposite effects in environments C and $P + I$, thus dominating the global fitness trade-off. The other four mutations, which affect stability and expression, are compensatory mutations that lead to more random effects [22].

We find that global fitness trade-offs do not necessarily lead to frequent reversibility because these trade-offs can be dominated by one or a small number of mutations, leaving the remainder of the landscapes positively correlated. This situation may occur often in single-protein evolution where different environments require different binding specificities. To accurately predict the prevalence of reverse evolution between two landscapes, a measure of their correlation must capture local structure. This positive correlation then limits the maximal complexity of an adaptation that can be reversed by evolution.

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*Corresponding author.
tantan@mit.edu
†Corresponding author.
sserene@mit.edu
‡Corresponding author.
gore@mit.edu

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