Small changes in enzyme function can lead to surprisingly large fitness effects during adaptive evolution of antibiotic resistance

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Edited by Richard E. Lenski, Michigan State University, East Lansing, MI, and approved November 12, 2012 (received for review June 7, 2012)

In principle, evolutionary outcomes could be largely predicted if all of the relevant physicochemical variants of a particular protein function under selection were known and integrated into an appropriate physiological model. We have tested this principle by generating a family of variants of the tetracycline resistance protein TetX2 and identified the physicochemical properties most correlated with bacterial fitness. Surprisingly, small changes in the $K_{m(MCN)}$, less than twofold, were sufficient to produce highly successful adaptive mutants over clinically relevant drug concentrations. We then built a quantitative model directly relating the in vitro physicochemical properties of the mutant enzymes to the growth rates of bacteria carrying a single chromosomal copy of the tet(X2) variants over a wide range of minocycline (MCN) concentrations. Importantly, this model allows the prediction of enzymatic properties directly from cellular growth rates as well as the physicochemical-fitness landscape of TetX2. Using experimental evolution and deep sequencing to monitor the allelic frequencies of the seven most biochemically efficient TetX2 mutants in 10 independently evolving populations, we showed that the model correctly predicted the success of the two most beneficial variants tet(X2)T280A and tet(X2)J271T. The structure of the most efficient variant, TetX2T280A, in complex with MCN at 2.7 Å resolution suggests an indirect effect on enzyme kinetics. Taken together, these findings support an important role for readily accessible small steps in protein evolution that may, in turn, greatly increase the fitness of an organism during natural selection.

What determines success or failure of variants within a population undergoing selection? To answer this challenging question, experimental evolution, genomics, and biochemistry have proved to be powerful approaches to the formulation of specific and testable hypotheses that can be validated in a quantitative manner. Most recently, experimental evolution has provided a wealth of insights into epistasis (1–3), adaptive convergence among populations (4, 5), and the role of mutation supply (6, 7) in evolutionary outcomes over a range of selective conditions. In addition to physicochemical properties, interactions among different mutants in a population such as clonal interference, the stochasticity with which new mutations arise, and the fact that even beneficial mutations can be lost through drift when they are very rare affect evolutionary dynamics (11).

We tested two important principles that are the key to the hypothesis that a careful physicochemical analysis of potential adaptive mutants can be used to predict evolutionary dynamics within large asexual populations undergoing selection (Fig. 1): first, that the physicochemical analysis of potential adaptive mutants and the development of an appropriate physiological model can quantitatively predict growth rates of bacteria carrying these alleles over a broad range of selective conditions; and second, that these ex vivo predictions for growth would predict the evolutionary dynamics in a population undergoing selection.

To test these principles, we used in vitro error-prone mutagenesis to identify adaptive mutants of Bacteroides thetaiotamnon tet(X2) that confered similar or increased resistance to minocycline (MCN) within 1-nt change of the original sequence. From the TetX2 library, seven potential adaptive mutants were identified and characterized biochemically to determine their steady-state enzyme kinetics. Each adaptive tet(X2) allele was then introduced as a single chromosomal copy into the Escherichia coli spec operon that encodes 10 ribosomal proteins, and their growth rates were measured over a wide range of MCN concentrations. Using data from a combination of the in vitro kinetics and in vivo expression level measurements we were able to build a mathematical model that accurately and quantitatively predicted the bacterial growth rates from biochemical first principles such as $k_{cat}$, $K_{m(MCN)}$, and $K_{m(NADPH)}$ and steady-state expression levels. An unexpected finding from these studies was the critical role of very small changes in physicochemical parameters and their outsized effects on bacterial growth rates over the relevant MCN selection range. For example, less than a twofold change in $K_{m}$ and 20% change in $k_{cat}$ toward MCN for the best adaptive mutant [tet(X2)T280A] increased growth rate 240% at 10 μg/mL MCN. After establishing the relationship of biochemical parameters to growth kinetics of the seven most successful adaptive TetX2 mutants, experimental evolution was used to monitor adaptation in 10 bacterial populations undergoing selection to increasing amounts of MCN.

The broad success of the model in building a quantitative link between enzyme function and growth rates suggests that, under specific conditions, growth rates over a wide range of selective conditions can provide a robust and high-throughput means of estimating kinetic parameters for libraries of enzyme variants.

Author contributions: Y.S. designed research; K.W., A.S.B.C., C.S., C.B., and G.S. performed research; K.W., A.S.B.C., C.S., C.B., G.S., and Y.S. analyzed data; and K.W., A.S.B.C., G.S., and Y.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 3V3N).

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This article contains supporting information online at www.pnas.org/cgi/lookup/suppl/doi:10.1073/pnas.1209335110/DCSupplemental.
without arduous purification of the individual protein variants. This approach can further expand our understanding of protein evolution and the physicochemical principles underlying natural selection. Most importantly, these findings highlight the critical relationship of small changes in enzyme performance to highly relevant changes in organismal fitness during natural selection.

**Results**

**TetX2 Variants Expressed from the Chromosomal spc Operon of E. coli Directly Drive Fitness to Minocycline.** The success of cells with newly acquired mutations in an evolving population depends strongly on the fitness benefit conferred by the mutations under specific conditions of selection. Elucidating a physicochemical basis for evolutionary dynamics is made easier if relevant adaptive mutations have been identified and characterized. In vitro error-prone mutagenesis was used to identify potential single-point mutations of tet(X2) that could be candidates for natural selection in a bacterial population undergoing adaptation to increasing MCN concentrations (Fig. S1). Seven adaptive mutants that conferred equal or greater minimal inhibitory concentrations (MICs) to MCN than wild type tet(X2) were identified from the library (Table 1).

As MCN concentrations are increased, the ability of a particular tet(X2) variant to reduce cytoplasmic MCN concentrations to a point sufficient to maintain wild-type growth rates becomes increasingly difficult until TetX2 is unable to maintain a sufficiently low steady-state concentration of MCN, resulting in slower growth rates. Here, we use absolute growth rate as a measure for fitness because it provides a more accurate link between physicochemical properties of TetX2 and bacterial growth than MICs. We introduced each of the adaptive tet(X2) alleles as a single chromosomal copy into the spc operon of E. coli BW25113 by Red short-homology recombineering (12) and measured the growth rates of E. coli BW25113 and the eight recombineered strains over a range of MCN concentrations that correspond to Food and Drug Administration guidelines for the susceptibility testing of Enterobacteria at 37 °C (13) (Fig. S2 and Table S1).

> Fig. 1. Predicting evolutionary outcomes using experimental evolution and biochemistry. (A) Changes in physicochemical properties of adaptive mutants that correlate with changes in fitness (growth rates) are determined and used to model the in vivo performance of the organisms expressing mutant alleles. (B) The predictions based on the mathematical model are tested by monitoring allelic frequencies, using deep sequencing.

Table 1. Steady-state kinetic parameters for wild-type TetX2 and adaptive mutants

<table>
<thead>
<tr>
<th></th>
<th>$K_{\text{m(MCN)}}$ μM</th>
<th>$K_{\text{m(NADPH)}}$ μM</th>
<th>$k_{\text{cat}}$ (V_{\text{max}}/E_{\text{total}}), s$^{-1}$</th>
<th>$k_{\text{cat}}/K_{\text{m(MCN)}}$ μM$^{-1}$s$^{-1}$</th>
<th>$k_{\text{cat}}/K_{\text{m(NADPH)}}$ μM$^{-1}$s$^{-1}$</th>
<th>Δ growth rates at 32 μM MCN, %*</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>35 ± 1.9</td>
<td>75 ± 4.1</td>
<td>0.34 ± 0.01</td>
<td>0.010 ± 0.0006</td>
<td>0.004 ± 0.0003</td>
<td>100</td>
</tr>
<tr>
<td>T280A</td>
<td>18 ± 0.9</td>
<td>18 ± 1.1</td>
<td>0.43 ± 0.01</td>
<td>0.024 ± 0.0013</td>
<td>0.024 ± 0.0016</td>
<td>540</td>
</tr>
<tr>
<td>N371I</td>
<td>18 ± 1.9</td>
<td>64 ± 6.3</td>
<td>0.37 ± 0.02</td>
<td>0.020 ± 0.0024</td>
<td>0.006 ± 0.0006</td>
<td>530</td>
</tr>
<tr>
<td>N371T</td>
<td>24 ± 2.1</td>
<td>130 ± 11</td>
<td>0.40 ± 0.02</td>
<td>0.017 ± 0.0017</td>
<td>0.003 ± 0.0003</td>
<td>440</td>
</tr>
<tr>
<td>S326I</td>
<td>37 ± 2.8</td>
<td>73 ± 5.5</td>
<td>0.36 ± 0.01</td>
<td>0.010 ± 0.0008</td>
<td>0.005 ± 0.0004</td>
<td>340</td>
</tr>
<tr>
<td>F235Y</td>
<td>54 ± 6.1</td>
<td>99 ± 11</td>
<td>0.32 ± 0.06</td>
<td>0.006 ± 0.0013</td>
<td>0.003 ± 0.0007</td>
<td>320</td>
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<tr>
<td>K64R</td>
<td>36 ± 4.6</td>
<td>110 ± 15</td>
<td>0.32 ± 0.03</td>
<td>0.009 ± 0.0014</td>
<td>0.003 ± 0.0005</td>
<td>86</td>
</tr>
<tr>
<td>T280S</td>
<td>30 ± 3.4</td>
<td>100 ± 14</td>
<td>0.18 ± 0.01</td>
<td>0.006 ± 0.0008</td>
<td>0.002 ± 0.0003</td>
<td>65</td>
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*Changes in growth rates of mutant alleles are relative to the wild-type tet(X2) growth rate set at 100%.
Fig. 2. Similar growth rate profiles for tet(X2)280A and tet(X2)373I suggest that both mutants should have equal opportunity for success during selection. Shown are growth rates of the ancestral E. coli BW25113 strains expressing a chromosomal copy of tet(X2)wild type and seven variants. The clinical breakpoint for resistance (dashed line) to MCN is 16 μg/mL (32 μM). At lower MCN concentrations (2 and 4 μg/mL), growth rates (e.g., fitness) for all of the variants are comparable. At higher MCN concentrations, tet(X2)280A and tet(X2)373I exhibit the fastest growth rates, whereas the growth rates of tet(X2)228S and tet(X2)K318E are significantly lower and resemble the growth of wild-type tet(X2). Error bars correspond to the SD among measurements for independent growth assays for three individual colonies.

Adaptive Mutations to TetX2 That Determine Evolutionary Dynamics over a Clinically Relevant MCN Range Show Remarkably Small Changes in Kinetic Performance. The in vivo resistance of bacteria expressing each candidate tet(X2) mutant was directly linked to the in vitro catalytic properties of TetX2 through steady-state activity assays that measure the kinetics of MCN inactivation in vitro. The catalytic profiles exhibited by TetX2 adaptive mutants suggest that surprisingly small changes in TetX2 steady-state kinetic parameters have large consequences on the in vivo performance of the organisms in a population. TetX2 is a flavin-dependent monooxygenase that regioselectively hydroxylates tetracyclines in a reaction that requires both NADPH and molecular oxygen (14). The initial velocities of MCN inactivation were measured at various NADPH and MCN concentrations (Table 1), and the steady-state kinetic parameters \( K_m(\text{MCN}, \text{NADPH}) \) and \( k_{cat}(\text{V}_{max} = k_{cat} \times [E]) \), where \( E \) is total enzyme concentration) reported in Table 1 were calculated by fitting initial velocities to a simple, rapid equilibrium ping-pong model for the two substrates MCN and NADPH (Eq. S1 and Schemes S1 and S2) (15).

Surprisingly, the changes in \( K_m \) toward MCN and NADPH, as well as \( k_{cat} \), that determined the growth rates within the individual strains and their subsequent success or failure during experimental evolution within populations are remarkably small (Table 1). The most successful TetX2 mutant, TetX2T280A, had less than a twofold decrease in \( K_m \) (18 μM) for MCN and a fourfold decrease for NADPH (18 μM) compared with the wild-type enzyme \([K_m(\text{MCN}) = 35 \mu\text{M}, K_m(\text{NADPH}) = 75 \mu\text{M}]\). All seven TetX2 mutants isolated from in vitro mutagenesis had a measured \( k_{cat} \) comparable to that of wild-type TetX2 (\( k_{cat} = 0.34 \text{s}^{-1} \)) and although TetX2T280A had the highest \( k_{cat} \) (0.43 s\(^{-1}\)), the increase was quite modest (∼20%).

The efficiencies of the two catalytic steps, first, FAD reduction by NADPH and, second, hydroxylation of MCN, were altered by at most fivefold (TetX2T280A). TetX2K64R had no significant change in the \( K_m(\text{MCN}) \) but a higher \( K_m(\text{NADPH}) \) and no change in \( k_{cat} \), which is largely consistent with its in vivo growth rates.

The subtle changes in kinetic parameters of the most successful mutants TetX2T280A and TetX2S326I conferred a >500% fitness benefit over that of the wild-type enzyme at 32 μM MCN (Fig. 2 and Table 1). However, the enzymatic profiles of TetX2S326I and TetX2T280A were very similar to those of the wild-type enzyme, showing no significant changes in the \( K_m(\text{MCN}) \) and \( k_{cat} \) and only a slight increase in the \( K_m(\text{NADPH}) \) suggesting that steady-state enzymatic parameters alone could not account for their observed beneficial effects on growth rates.

Small Changes of in Vivo Steady-State Protein Levels Also Prove to be Important for Increased Fitness. Fitness of cells with newly acquired mutations in an evolving population depends not only on the fast growth rates at intermediate and high MCN concentrations, suggesting that both mutants would have comparable success in a population during selection at MCN concentrations that would be used clinically (13).
catalytic performance of a particular enzyme but also on its in vivo expression levels. Therefore, we quantified the expression levels of TetX2 mutants to test whether any of the variants might be present at higher steady-state concentrations in vivo. To test this hypothesis, TetX2 activity at three concentrations of MCN was measured from cell extracts of E. coli expressing chromosomally integrated tet(X2) variants (Fig. 3C). On the basis of the activities obtained from cell lysates, the relative enzyme concentrations of each mutant compared with those of the wild-type TetX2 were determined. As shown in Fig. 3D, the steady-state protein levels of TetX2S326I and TetX2T280A were approximately twofold higher than those of the wild type; also TetX2N371I, TetX2N371T, and TetX2S326D appear at ~50% higher levels. The differences in the steady-state protein levels among the mutants are consistent with the growth rate data, which suggested that strains expressing TetX2S326D and TetX2T280A were better at hydrolyzing MCN than wild-type TetX2. The in vitro kinetics together with the increased steady-state protein levels of these two mutants are consistent with the success of strains carrying tet(X2)S326D, tet(X2)T280A, and tet(X2)F235Y.

Adaptive TetX2 Mutants Identified and Characterized in Vitro Provide the Basis for Quantitative ex Vivo Modeling of Fitness and Evolutionary Dynamics. The in vitro kinetic properties of the TetX2 variants were used to construct a mathematical model to quantitatively describe the growth rates of each adaptive mutation over a range of MCN concentrations (Fig. 4). Inhibition of bacterial growth rates by cytosolic MCN was determined by fitting the growth rate dependence of E. coli to MCN to a Hill function: 1 + [MCN]/K_{Hill} (Eq. 52). At steady state, degradation of MCN by TetX2 equals the rate of diffusion of minocycline into the cell. The bistable kinetics formulation (Eq. S1) and Fick’s law were used to calculate the steady-state cytosolic MCN concentration from the total concentration of MCN (Eq. S4). The function determined by the inhibition of MCN on growth rates of E. coli was transformed onto the growth rates of the TetX2 variants, using the steady-state equation (Fig. 4A), and fitted using the measured values of K_{m(MCN)}, K_{m(NADPH)}, and k_{cat}. At higher concentrations of MCN, inhibition of cell growth is a consequence of the limited ability of TetX2 or a particular variant to inactivate MCN to tolerable levels. Growth rates for E. coli carrying tet(X2)T280A, tet(X2)N371I, tet(X2)N371T, and tet(X2)K64R could be predicted readily, considering largely kinetic parameters, whereas growth rates of tet(X2)S326I and tet(X2)T280A could be predicted reasonably only if their steady-state protein concentration was greater than that of the wild-type TetX2 (Fig. 4). This prediction was born out by in vivo activity measurements from cell extracts shown in Fig. 3C. As expected from the range of selection for MCN and bistable kinetics, the fit for growth rates of E. coli with tet(X2) is most sensitive to K_{m} for MCN and is not as sensitive to K_{m} for NADPH under these selection conditions (SI Materials and Methods). The combination of K_{m(MCN)} and k_{cat} determines the shape of the growth rate curves with the initial plateaus being particularly sensitive to K_{m(MCN)}. The measured k_{cat} values were similar for seven of the eight variants and therefore played a lesser role. The total activity affects the slope of the curve defining how severe the drop-off in growth rate responds to increasing MCN. The model cannot discriminate between changes in k_{cat} or changes in active protein concentration because these two variables affect the shape of the growth curve in the same way. The model underscores the strong correlation between the MCN concentration at which the most successful adaptive mutants, tet(X2)T280A and tet(X2)N371I, were observed by experimental evolution (20–32 mM MCN) and their k_{m} to MCN (Table S3).

DNA Barcoding to Quantify the Allelic Frequency of the Seven Most Successful in Vitro tet(X2) Mutants. On the basis of growth rate and biochemical assays, we would expect both T280A and N371I to evolve. To test the success of the mutants in evolving populations, we evolved 10 replicate populations of BW25113tet(X2) and tracked the rise and fall of the seven mutant alleles over the course of 3 days undergoing selection at clinically relevant MCN concentrations. Adaptive mutations to tet(X2) can be identified from 10 to 32 μg/mL MCN (days 1–3) during a serial transfer experiment. Using the Illumina HiSeq sequencing system and a family of DNA barcodes, we were able to track changes in allelic frequencies for each of the characterized tet(X2) mutation sites at a resolution sufficient to identify a frequency of 0.5% with each population (SI Materials and Methods) (10). On average, each SNP was covered by 2.4 × 10^{6} [95% confidence interval (CI): 3.7 × 10^{6}] reads (per population per day). As expected, tet(X2)T280A and tet(X2)N371I had the most success and appeared in 5 and 2 populations, respectively, whereas tet(X2)K64R, tet(X2)F235Y, tet(X2)N371T, and tet(X2)T280A were not observed. Unexpectedly, tet(X2)T280S was observed in two populations despite data showing that the growth rates of tet(X2)T280S were equal to those of tet(X2).

Crystal Structure of the Most Successful Adaptive TetX2 Mutant TetX2T280A Suggests an Indirect Effect on Protein Dynamics to Increase Enzyme Activity to MCN. To understand the role of the mutations in the mechanism of MCN hydroxylation, we determined the crystal structure of the most successful and kinetically efficient variant, TetX2T280A, in complex with MCN at 2.7 Å resolution (Fig. 5 and Table S2). TetX2 is a monomer consisting of two domains, a larger N-terminal domain harboring a Rossmann fold responsible for interactions with the adenosine monophosphate of FAD and a smaller C-terminal domain that surrounds the catalytic cavity of the enzyme. The overall structure of TetX2T280A is very similar to that of the wild-type TetX2 [rmsd = 0.34 Å; Protein Data Bank (PDB) ID 3P9U] and 7-iódotetracycline(7-ITc):TetX2 complex structures (rmsd = 0.24 Å; PDB ID 2Y6Q) (17). MCN was found...
in a similar conformation in the active site to 7-ITc with the hydrophilic portion of the substrate oriented toward the isooxazine ring of FAD (17). The structure clearly shows that position 280 is not directly involved in the catalytic mechanism of the enzyme and suggests that TetX2<sub>T280A</sub> alters kinetics indirectly perhaps through altered protein dynamics (SI Materials and Methods and Fig. S4).

Mapping of the other adaptive TetX2 mutations onto the crystal structure of the TetX2<sub>T280A:MCN</sub> complex (Fig. 5) showed that all of the mutated residues, except TetX2<sub>K64R</sub> and TetX2<sub>S326I</sub>, are located on the second domain of the protein, which is implicated largely in substrate recognition. Only residues at positions 235 and 371 are within 5 Å of MCN (Fig. 5A). The side chain of Phe235 is oriented 180° away from the substrate and makes van der Waal contacts within a hydrophobic pocket (Fig. 5C) but the polar hydroxyl group of Tyr235 in TetX2<sub>T280V</sub> could readily satisfy a new hydrogen bond to Thr281, potentially stabilizing the protein. In contrast to position 235, the side chain of Arg213 is oriented directly toward the D-ring of MCN (~4.7 Å) (Fig. 5A) at the putative entrance site for tetracyclines. Substitution of a nonpolar residue (Ile) or a shorter polar side chain (Thr) at position 371 near the apolar pocket (Fig. 5B) results in smaller K<sub>m</sub>(MCN) and modest increases in k<sub>cat</sub>. These findings clearly demonstrate that even mutations outside of the active site can have large effects on the fitness of the organisms.

**Discussion**

The molecular pathways accessible in protein evolution are defined by the mutation supply and the fitness effects of these mutations in the selective environment. In this study, we tested the hypothesis that on the basis of in vitro physicochemical properties of potential TetX2 adaptive mutants we could build a mathematical model that accurately predicts changes to fitness from biochemical first principles. In practice, this model is entirely reversible and permits the evaluation of specific changes to kinetic performance such as K<sub>m</sub>, enzyme expression, and V<sub>max</sub> from the growth rates of cells measured at varying substrate concentrations. Development of an accurate model relating growth rates to enzyme performance would also allow quantitative high-throughput screening of large libraries of enzyme variants without the need for arduous protein purification, facilitating investigation of enzyme adaptation in a highly systematic fashion (Fig. S4 and Table S3).

We chose the tetracycline-resistant enzyme, TetX2, as a model system to study the biophysical basis for adaptation to antibiotics. We identified a family of TetX2 variants and showed that growth rates of *E. coli* expressing chromosomal copies of the tet(X2) mutant alleles were exclusively sensitive to MCN concentration and were tightly correlated to catalytic performance and steady-state expression levels of the TetX2 variants. On the basis of these experimental results, we were able to construct a mathematical model that consolidated the classic Michaelis–Menten kinetics and the relative in vivo expression levels to accurately and quantitatively predict growth rates and population dynamics.

Our work shows that small changes in kinetic parameters and steady-state protein concentrations can have large consequences on organismal fitness and adds to the repertoire of mechanisms through which drug resistance evolves. The success of TetX2 mutants with small improvements in catalytic performance over the wild-type TetX2 was reflected by large fitness benefits conferred by these variants in vivo as shown by growth rate assays (Table 1 and Fig. 2). We observed that the three mutants with the fastest growth rates at intermediate drug concentrations, TetX2<sub>T280A</sub>, TetX2<sub>F235Y</sub>, and TetX2<sub>N371T</sub> produced at most a twofold decrease in K<sub>m</sub>(MCN) and, at most, a slight increase in k<sub>cat</sub>. Even though the K<sub>m</sub>(NADPH) varied more widely among these three mutants, the in vivo concentration of NADPH is 5–10 times higher than that of MCN, making TetX2 performance less dependent on the concentration of NADPH under these selection conditions (18). A similar trend was observed for the mutant TetX2<sub>K64R</sub>, which had growth rates and kinetics comparable to those of wild-type TetX2. The in vivo performance of TetX2<sub>Y235F</sub> and TetX2<sub>T280S</sub> was linked to at most twofold changes in steady-state protein levels, again emphasizing the relevance of small changes in activity over the relevant range of selection to fitness. These combined effects of fitness and activity are readily evident when overall activity is plotted as a function of K<sub>m</sub>(MCN) and k<sub>cat</sub> for TetX2 to build a physicochemical fitness landscape for TetX2 (Fig. 4B).

Why might small steps in enzyme performance be more common during selection? First, mutations that result in only modest changes to enzyme performance are more common than those with large changes (19, 20). As shown by our results, even very modest changes in physicochemical performance can have very strong fitness effects, depending on the context of selection. As long as a mutation alters protein performance sufficiently to meet the physiological needs of the cell, there is no advantage to an enzyme that is “better” in terms of in vitro performance than others (21, 22). In addition, as beneficial mutations accumulate there is a general trend toward negative epistasis that shapes the adaptive landscape (1, 23).

Second, mutational spectra can change with the inherent DNA replication, recombination, and repair efficiencies of an organism as well as with the manner in which selection conditions induce stress (24, 25). Whereas we often envision selection as a harsh filter on populations, niche invasion can be a more gradual series of events wherein cells can work at the periphery of a condition, using the smaller, more numerous, and therefore more accessible ensemble of molecular trajectories afforded by protein structure and function. Although there has been much interest in changes to enzyme function that dramatically alter kinetic parameters, it is more likely that much more modest changes in enzyme performance may represent the more common adaptive pathway.

Our work shows that on the basis of molecular properties of the adaptive mutants of TetX2, we can accurately predict the success of mutant alleles during adaptation to antibiotic. As shown in Fig. S5, serial passages of 10 populations showed that in vitro enzyme kinetics correctly predicted the most successful adaptive alleles tet(X2)<sub>T280A</sub> and tet(X2)<sub>N371T</sub>. Of the 7 populations where adaptive mutations to tet(X2) were observed, 5 contained tet(X2)<sub>T280A</sub> and tet(X2)<sub>N371T</sub> whereas 2 showed the presence of an allele that would be close to neutral, tet(X2)<sub>T280S</sub>. The remaining four tet(X2) alleles
predicted to have little or no success according to the in vitro data did not appear at our level of detection (≥0.5% of the overall population). Although it is possible that tet(X2)_{T280S} may have an as yet unidentified fitness benefit, this was not observed in growth rate of the chromosomally expressed gene, enzyme kinetics of the purified enzyme, MIC determination from the chromosomal clone, or assays of crude extracts. The unexpected success of tet(X2)_{T280S} is a cautionary tale and may support an important role for mutation supply, clonal interference, and epistasis in evolutionary dynamics (6, 7). Taken together, these studies show that physicochemical and structural properties of enzymes can be used to construct a quantitative prediction of fitness that can be used in conjunction with experimental evolution to explore the role of even the most modest changes in physical properties to larger consequences to organismal fitness.

Materials and Methods

Library Construction. Tet(X2) mutants were made using error-prone PCR following the manufacturer’s protocols (Genemorph Random Mutagenesis Kit; Agilent Technologies). An appropriate tet(X2) library with one to two mutations per reaction was generated and subcloned into pUC19 (Invitrogen) to generate tet(X2)pUC19 vector (SI Materials and Methods). Tet(X2) mutants that conferred the same or higher MIC relative to wild-type tet(X2) were isolated by plating at 4 μg/mL of MCN. DNA sequencing of 35 colonies identified seven tet(X2) mutants: tet(X2)_{T280A}, tet(X2)_{N371I}, tet(X2)_{H271T}, tet(X2)_{P235Y}, tet(X2)_{S226G}, tet(X2)_{T280S}, and tet(X2)_{S68R}.

Construction of Recombinant Strains. Integration of tet(X2) or variants into the chromosome of E. coli strain BW25113 was performed using a short homology recombining approach (12). Briefly, tet(X2) was amplified from tet(X2)pUC19 and used as a template for recombination into the spc operon of E. coli between prA (SecY) and rpmJ (L36) to generate BW25113_{tet(X2)} (SI Materials and Methods).

Growth Assays. Growth assays were performed in a 96-well plate format, using the Synergy 2 Multi-Mode Microplate Reader (BioTek Instruments). Single colonies of E. coli carrying different tet(X2) variants were used to inoculate overnight cultures and subsequently diluted to a final OD_{600} of 0.01. Growth at OD_{600} was monitored in 5-min intervals over 24 h at MCN concentrations ranging from 0 to 50 μg/mL. The fastest growth was defined as a slope of a tangent parallel to linear-log phase growth (SI Materials and Methods and Fig. S2).

Protein Expression and Purification. All proteins were overexpressed in E. coli BL21(DE3) Star (Agilent Technologies) with cleavable N-terminal 6xHis tag and purified as described previously (SI Materials and Methods) (26).