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

Katarzyna Walkiewicz1,2,1, Andres S. Benitez Cardenas3,1, Christine Sun2, Colin Bacorn2, Gerda Saxer3, and Yousif Shamoo4,2,2

Departments of 1Biochemistry and Cell Biology and 2Ecology and Evolutionary Biology, Rice University, Houston, TX 77005-1892
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 identifying the physicochemical properties most correlated with organismal fitness. Surprisingly, small changes in the $K_{\text{cat}}$ of TetX2, 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 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)$_{7280A}$ and tet(X2)$_{3371I}$. The structure of the most efficient variant, TetX2$_{7280A}$, 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 can, in turn, greatly increase the fitness of an organism during natural selection.

K.W. and A.S.B.C. contributed equally to this work.

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., G.S., and Y.S. analyzed data; and K.W., A.S.B.C., G.S., and Y.S. wrote the paper.

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).

1K.W. and A.S.B.C. contributed equally to this work.

2To whom correspondence should be addressed. E-mail: shamoo@rice.edu.

This article contains supporting information online at www.pnas.orglookup/suppl/doi:10.1073/pnas.1209335110/-/DCSupplemental.
Changes in growth rates of mutant alleles are relative to the wild-type T280S 30 ± K64R 36 F235Y 54 ± N371T 24 ± N371I 18 (centrations (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).

The growth rates of the wild-type tet(X2) and all of the tet(X2) mutants were significantly lower than the growth rates of the host BW25113 without tet(X2), indicating a fitness cost of 4.6% to carrying a tet(X2) allele (planned comparison between host without and strains with tet(X2) alleles: F_{2,31} = 25.9, P < 0.0001).

As expected, with increasing MCN concentration, the growth rates of all tet(X2) mutants decreased (Fig. 2). Three mutants, tet(X2)_{F235Y}, tet(X2)_{S326I}, and tet(X2)_{N371T}, exhibited intermediate fitness compared with the most successful mutant, tet(X2)_{T280A}. At 32 µM MCN, the breakpoint for clinical resistance, tet(X2)_{T280A} was 1.7, 1.6, and 1.2 times fitter than tet(X2)_{F235Y}, tet(X2)_{S326I}, and tet(X2)_{N371T}, respectively. Tet(X2)_{N371T} and tet(X2)_{F235Y} had the

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

<table>
<thead>
<tr>
<th></th>
<th>Km(MCN)</th>
<th>Km(NADPH)</th>
<th>kcat</th>
<th>(Vmax/Etotal)</th>
<th>kcat/Km(MCN)</th>
<th>km-1 s-1</th>
<th>kcat/Km(NADPH)</th>
<th>km-1 s-1</th>
<th>Δ growth rate at 32 µM MCN</th>
<th>%*</th>
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<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>
<td>75 ± 4.1</td>
<td>0.34 ± 0.01</td>
<td>0.010 ± 0.0006</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>
<td>18 ± 1.1</td>
<td>0.43 ± 0.01</td>
<td>0.024 ± 0.0013</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>
<td>64 ± 6.3</td>
<td>0.37 ± 0.02</td>
<td>0.020 ± 0.0024</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>
<td>130 ± 11</td>
<td>0.40 ± 0.02</td>
<td>0.017 ± 0.0017</td>
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<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>
<td>73 ± 5.5</td>
<td>0.36 ± 0.01</td>
<td>0.010 ± 0.0008</td>
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<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>
<td>99 ± 11</td>
<td>0.32 ± 0.06</td>
<td>0.006 ± 0.0013</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>
<td>110 ± 15</td>
<td>0.32 ± 0.03</td>
<td>0.009 ± 0.0014</td>
<td>86</td>
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<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>
<td>100 ± 14</td>
<td>0.18 ± 0.01</td>
<td>0.006 ± 0.0008</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. 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.
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 $K_m(MCN)$, $k_{cat}(NADPH)$, and $K_m(NADPH)$ for each purified mutant enzyme were determined. Overall, the adaptive mutants exhibited similar mechanistic behaviors. As shown in Fig. 3A, the initial velocities of the enzyme (here, TetX2T280A) at different MCN concentrations increased with increasing NADPH, which, in turn, resulted in an increase in $V_{max}(MCN)$. In addition, the $K_m(MCN)$ also increases with increasing NADPH concentrations, which suggests that only binary complexes of the enzyme with these substrates can occur and that MCN and NADPH cannot be bound simultaneously in the active site (i.e., no ternary TetX2:MCN:NADPH complex forms). Lineweaver–Burke plots of initial velocities vs. MCN concentration strongly support a ping-pong mechanism for TetX2 inactivation of MCN (Fig. 3B). Therefore, the kinetic parameters $K_{m(MCN)}$, $K_{m(NADPH)}$, and $k_{cat}$ ($V_{max} = k_{cat} \times [E]$, 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 [$K_{m(MCN)} = 35 μM$, $K_{m(NADPH)} = 75 μM$]. All seven TetX2 mutants isolated from in vitro mutagenesis had a measured $k_{cat}$ comparable to that of wild-type TetX2 ($k_{cat} = 0.34 s^{-1}$) 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). TetX2T280A had no significant change in the $K_m(MCN)$ but a higher $K_m(NADPH)$ 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 TetX2T280A 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 TetX2F235Y were very similar to those of the wild-type enzyme, showing no significant changes in the $K_m(MCN)$ and $k_{cat}$ and only a slight increase in the $K_m(NADPH)$, suggesting that steady-state enzymatic parameters alone could not account for our 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 fastest 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).

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 $K_m(MCN)$, $k_{cat}(NADPH)$, and $K_m(NADPH)$ for each purified mutant enzyme were determined. Overall, the adaptive mutants exhibited similar mechanistic behaviors. As shown in Fig. 3A, the initial velocities of the enzyme (here, TetX2T280A) at different MCN concentrations increased with increasing NADPH, which, in turn, resulted in an increase in $V_{max}(MCN)$. In addition, the $K_m(MCN)$ also increases with increasing NADPH concentrations, which suggests that only binary complexes of the enzyme with these substrates can occur and that MCN and NADPH cannot be bound simultaneously in the active site (i.e., no ternary TetX2:MCN:NADPH complex forms). Lineweaver–Burke plots of initial velocities vs. MCN concentration strongly support a ping-pong mechanism for TetX2 inactivation of MCN (Fig. 3B). Therefore, the kinetic parameters $K_{m(MCN)}$, $K_{m(NADPH)}$, and $k_{cat}$ ($V_{max} = k_{cat} \times [E]$, 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).

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Adaptive TET2 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 TET2 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). A comparison 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 − \frac{\text{MCN}}{\text{EC}_{50} + \text{MCN}} \text{,}

(Eq. S2). At steady state, degradation of MCN by TET2 equals the rate of diffusion of minocycline into the cell. The bisubstrate 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 TET2 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 TET2 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)F235Y, tet(X2)T280S, and tet(X2)T280A could be predicted reasonably only if their steady-state protein concentration was at least of the wild-type TET2 (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 bisubstrate 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 plateau 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 μM 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 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) (16). 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)T280S were not observed. Unexpectedly, tet(X2)T280A was observed in 2 populations despite data showing that the growth rates of tet(X2)T280A were equal to those of tet(X2).
in a similar conformation in the active site to 7-ITc with the hydrophilic portion of the substrate oriented toward the isoxalazine 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 TetX2T280A 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 TetX2T280A:MCN complex (Fig. 5) showed that all of the mutated residues, except TetX2K64R and TetX2S326A, 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 Waals contacts within a hydrophobic pocket (Fig. 5C) but the polar hydroxyl group of Tyr235 in TetX2F235Y could readily satisfy a new hydrogen bond to Thr281, potentially stabilizing the protein. In contrast to position 235, the side chain of Asn371 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 Kcat(MCN) and modest increases in kcat. 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 Kcat, enzyme expression, and Vmax 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 exquisitely 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, TetX2T280A, TetX2N371A, and TetX2N371T produced at most a twofold, a slight increase in Kcat, and, at most, a slight increase in kcat. Even though the Kcat for 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 TetX2K64R, which had growth rates and kinetics comparable to those of wild-type TetX2. In vivo performance of TetX2T280A and TetX2T280S 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 small changes on fitness are readily evident when overall activity is plotted as a function of Kcat(MCN) and kcat(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 net evolutionary 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)T280A and tet(X2)N371A. Of the 7 populations where adaptive mutations to tet(X2) were observed, 5 contained tet(X2)T280A and tet(X2)N371A whereas 2 showed the presence of an allele that would be close to neutral, tet(X2)T280S. The remaining four tet(X2) alleles

**Fig. 5.** Structure of the most efficient variant, TetX2T280A, in complex with MCN at 2.7 Å resolution. (A) Ribbon representation of TetX2T280A structure with MCN shown in yellow and FAD in orange as sticks. The position of each adaptive point mutation is indicated as a sphere: 280 (red), 371 (orange), 326 (green), 235 (blue), and 64 (purple). (B) Active site of TetX2T280A, illustrating residues interacting with MCN with 2Fo-Fc SIGMAA-weighted electron density map contoured at 1σ. (C) View of the active site with conserved nonpolar residues shown in purple.
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 large consequences to organismal fitness during natural selection.

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 MCN. DNA sequencing of 35 colonies identified seven tet(X2) mutants: tet(X2)T280A, tet(X2)K64R, tet(X2)K637T, tet(X2)S326I, tet(X2)T280F, and tet(X2)664R.

Construction of Recombinant Strains. Integration of tet(X2) or variants into the chromosome of E. coli strain BW25113 was performed using a short homology recombinating approach (12). Briefly, tet(O2) was amplified from tet(O2)pUC19 and used as a template for recombination into the spa operon of E. coli between prlA (SecY) and rmpl (L36) to generate BW25113<sub>prlA</sub> (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(O2) variants were used to inoculate overnight cultures and subsequently diluted to a final OD<sub>600</sub> of 0.01. Growth at OD<sub>600</sub> 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 Histag and purified as described previously (SI Materials and Methods).

Kinetic Studies. Steady-state kinetic parameters were measured using a previously described, but modified TetX2 activity assay (14) (SI Materials and Methods). Steady-state kinetic parameters were determined by fitting initial reaction rates (v<sub>i</sub>) to a ping-pong mechanism (Eq. S1).

TetX2 Activity from Cell Extracts. In vivo TetX2-mediated inactivation of MCN was estimated from cell extracts prepared from each of the E. coli BW25113 cell lines expressing chromosomal copies of wild-type and mutant tet(O2). Background levels of MCN inactivating activity were determined from extracts of BW25113 without tet(O2). Cells were grown in lysogeny broth (LB) without MCN at 37 °C and harvested by centrifugation at an OD of ~0.6-0.8 before lysis by sonication. The steady-state TetX2 activity for inactivation of MCN by the soluble crude extracts was assayed using the previously described in vitro assay. TetX2 activity was measured at 25, 50, and 200 μM MCN at 200 μM of NADPH. All measurements were done in triplicate. The total amount of protein in each cell lysate was estimated by Bradford assay to account for variability in the efficiency of cell lysis. Relative enzyme concentrations were calculated from Eq. S1, where V<sub>max</sub> = k<sub>cat</sub>[E]<sub>0</sub> and E<sub>0</sub> = total enzyme concentration.

Structure Determination and Refinement. Crystallization of TetX2<sub>664A</sub> was performed under similar conditions to those of the wild-type TetX2 (26). The structure was solved by molecular replacement, using 3P9U as a search model (SI Materials and Methods). The structure coordinates and amplitudes were deposited as 3V3N.

Serial-Passage Evolution Experiment. The MIC of E. coli BW25113<sub>prlA</sub> in liquid culture was determined to be 16 μg/mL MCN in LB at 37 °C. To determine when changes to tet(O2) could be observed, serial passage experiments were initiated at 10 μg/mL MCN and increased daily as 10, 16, 24, 36, 52, 80, 120, 180, 300, and 320 μg/mL MCN. DNA samples for deep sequencing of 10 independent replicates was obtained from populations at 10, 16, and 24 μg/mL MCN. Each day 50 μL of the population was transferred to a new condition and 1-mL samples were frozen at ~80 °C for DNA sequencing and characterization.

DNA Barcoding. We received 81 barcode plasmids containing distinct barcode sequences as a gift from the Marx Laboratory at Harvard University (Cambridge, MA) (16) (SI Materials and Methods).

ACKNOWLEDGMENTS. The authors thank Jay Nix for collecting X-ray data at Advanced Light Source. We also thank Drs. John S. Olson and Matthew R. Bennett for their advice and Roy Lohaus for help with the growth curve analyses. This work was supported by National Institutes of Health Grant R01AI080714 (to Y.S.). The Rice University Crystallographic Core Facility is supported by a Kreega Science Initiative grant.

Supporting Information

Walkiewicz et al. 10.1073/pnas.1209335110

SI Materials and Methods

Construction of the *tet(X2)* Mutant Library. The library of single-point mutants was generated using a commercially available GeneMorphII Random Mutagenesis Kit (Agilent Technologies). The mutational rate was adjusted by varying the template concentrations in the PCR step. The target mutational rate for the library of one to two single-point mutations per gene was achieved when 20 ng of a template ([tet(X2)/pUC19]) was used in each reaction. Mutated *tet(X2)* PCR products were ligated into a pUC19 backbone, between BamHI and EcoRI restriction sites where expression of *tet(X2)* is driven by the constitutive lacZ promoter. The ligation products were transformed into *Escherichia coli* DH10B cells and plated on 100 μg/mL of ampicillin to estimate the library size and mutational rate. Before selection, we estimated that to sample 95% of all possible single-point mutants, a library of 10,000–15,000 transformants had to be made and subjected to selection at a drug concentration at which *tet(X2)* was amplifiable by site-directed mutagenesis, using a primer based on sequence homology with an expression vector, pET-28b(+) containing *Bacteroides thetaiotaomicron* *tet(X2)*. DH10B cells were grown in 30 °C to 37 °C overnight. As expected, cells grown at nonpermissive temperatures for the replication of the vector pKD46 harboring bla lost the ability to grow on ampicillin. Glycerol stocks of BW25113 cells carrying a chromosomal copy of the gene of interest were prepared by mixing 50% sterile glycerol and overnight liquid cultures in 1:2 ratios and were stored at −80 °C. All mutants examined in this study were integrated into the chromosome, using the same primers and procedures as used for the wild-type *tet(X2)* gene.

Generation of Constructs for Expression of Mutant Enzymes. The original expression vector harboring wild-type *tet(X2)* was a generous gift from G. D. Wright (McMaster University, Hamilton, ON, Canada). All mutant enzymes examined in this study were introduced into *tet(X2)* by site-directed mutagenesis, using a QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies) with an expression vector, pET-28b(+) containing *Bacteroides thetaiotaomicron* *tet(X2)* (residues 11–388) as a template. Primers for the mutants are listed below.

**Primer pair for T280S.**

Forward: 5′ CTCAAAAGAATGGATTCATCGACGTTG-TCAATTTATGG 3′

Reverse: 5′ CCTTACATGACGATGCATGATTTTGAATTCAATTTGAGATG 3′

**Primer pair for F235Y.**

Forward: 5′ CGCGTAAATTTCTCTAGGGAAGACGACAGAA-CATTTTTTACCTTCGTATCTCTTATATCATCTTT AACAATTGCCT 3′

Reverse: 5′ CGCGTAAATTTCTCTTAGGAAGACGACAGAA-CATTTTTTACCTTCGTATCTCTTATATCATCTTT AACAATTGCCT 3′

Recombinants were isolated from LB agar plates containing 10 μg/mL tetracycline. Confirmation PCR was performed using genomic DNA isolated from colonies grown at 10 μg/mL of tetracycline, using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories) with primers aligning to the chromosome outside of the integration site (5′ ATGGCTAAACCGG-GATTATATTTAAGTGCCC 5′ and 5′ GCATCTTACCAGCATGGATATGG 3′). Using these primers, successful recombination was confirmed by a 3-kb PCR amplicon, compared with a shorter 1.8-kb ampicillin without the gene insert, and sequencing of the 3-kb ampicillin (SeqWright). To cure the temperature-sensitive helper vector pKD46, cells were grown at 37 °C overnight. As expected, cells grown at nonpermissive temperatures for the replication of the vector pKD46 harboring *bla* lost the ability to grow on ampicillin. Glycerol stocks of BW25113 cells carrying a chromosomal copy of the gene of interest were prepared by mixing 50% sterile glycerol and overnight liquid cultures in 1:2 ratios and were stored at −80 °C. All mutants examined in this study were integrated into the chromosome, using the same primers and procedures as used for the wild-type *tet(X2)* gene.
Mutagenesis reactions were performed following the manufacturer’s protocol. The reaction products were transformed into chemically competent XL-1 Blue cells (Agilent Technologies) and subsequently plated on LB-agar plates containing 50 μg/mL kanamycin. Mutations were verified by DNA sequencing.

**Minimum Inhibitory Concentration Determination.** Minimum inhibitory concentrations (MICs) were determined by the agar-dilution method (3) and E-test strips (Tetet; AB Biodisk) on cation-adjusted Mueller–Hinton agar. In addition, the initial antibiotic concentrations for serial-passage experiments were determined by a modified MIC test in liquid media, where single colonies were used to inoculate 10 mL LB media grown for 8 h. Each of these cultures (10 μL) was then used to inoculate 10 mL LB liquid media containing MCN concentrations ranging from 0 to 100 μg/mL for BW25113(tetX2). The highest MCN concentration that permitted growth to stationary phase in 12 h was picked as the starting MCN concentration for the serial passage experiment (10 μg/mL).

**Growth assay.** Fitness was measured as the maximal growth rate by measuring absorbance at OD_{600} over a 24-h growth cycle. To analyze the growth curves, we followed the method developed by Friesen et al. (4), which assumes that all wells were inoculated at the same density of cells and maximum growth rate is determined as the maximal slope through a moving window. We analyzed the data by allowing for the possibility of a two-phase growth, commonly seen in diauxic growth, and measured the maximal growth rate during the two phases individually (Fig. S2), but considered only the maximal growth rate during the first phase for further analyses. A faster second growth occurring >6–8 h after the initial growth rate could occur from one of four likely possibilities: (i) a small number of cells that have a higher growth rate that take a few hours to finally surpass the original bulk population, (ii) a change in the population that is the result of transcriptional optimization in response to drug, (iii) the rise of a new mutant, or (iv) degradation of the drug with time (by TetX2 or by oxidation in the media). As the drug is being inactivated by oxidation and the action of TetX2 in all of the wells (except 0 drug where there is no observable second phase), the most likely scenario is a simple degradation of the drug over time due to oxidation and the low levels of TetX2 activity eventually allowing the cell to grow. Most importantly, in all these cases the growth rate of the major population is not the second phase and that is why we do not use it. Maximal growth rates were determined using R.

**TetX2 Kinetic Assay.** Changes in absorbance corresponding to MCN hydroxylation by TetX2 or mutant enzymes were monitored at 400 nm (MCN κ_{400} = 12,000 M^{-1} cm^{-1}) over 5 min. To determine the steady-state kinetics parameters for each enzyme, we measured initial velocities of MCN inactivation at various MCN (0–400 μM) and NADPH (12.5–200 μM) concentrations at 37 °C. Assay conditions were 5 mM MgCl2 (activity of the enzymes is strongly dependent on magnesium), 0.2 μM protein, 0–400 μM MCN, and 12.5–200 μM NADPH in 20 mM Tris, pH 8.0. All assays were performed in triplicate. Steady-state kinetic parameters were determined by fitting initial reaction rates (v_{0}) to a ping-pong mechanism (Eq. S1 and Scheme S1) (5), where A = MCN, B = NADPH, V_{max} is maximal initial velocity, and K_{m(A)} and K_{m(B)} are substrate concentrations at half V_{max} when either B or A is saturating, respectively.

\[
V_{0} = \frac{V_{\text{max}}[A]}{1 + \frac{K_{m(B)}}{[B]}}, \quad v_{0} = \frac{K_{m(A)}[A]}{1 + \frac{K_{m(B)}}{[B]}}.
\]  

(S1)

The adaptive mutations could potentially affect interactions of the protein with any of the substrates, so we determined the Michaelis–Menten constants and steady-state catalytic rates as a function of both [MCN] and [NADPH]. Ideally, the third substrate, oxygen should also be taken into account by varying concentrations of oxygen. However, this is experimentally very challenging and studies of the catalytic mechanism of PHBH have shown that the rate of oxygen binding is very fast and is not a rate-limiting step (6). Moreover, the oxygen concentration during bacterial growth is held constant for all our fitness experiments. Therefore, we focused our efforts on understanding the interactions of the proteins with MCN and NADPH and their effects on the rates of catalysis. In the ping-pong complex mechanism (Schemes S1 and S2), the substrate-free enzyme in a reduced form (E) reacts with the first substrate A (MCN) with an equilibrium dissociation constant defined by K_{m(A)} to form the first binary complex (EA). This complex then reacts rapidly with O_{2}, which is held at a fixed concentration. In our simplified analysis, this hydroxylation step is represented by a simple pseudo–first-order process defined by k_{1}. The net result is the formation of the oxidized enzyme and the dissociation of the hydroxylated and inactivated MCN. This step should, in principle depend on [O_{2}]. The second reaction involves formation of a binary complex between the oxidized enzyme (E) and the reductant B (NADPH) to regenerate the enzyme to its active form (E). The binding of NADPH to E is defined by the equilibrium constant K_{m(B)}. As shown in Fig. 3B, the slopes of Lineweaver–Burk plot are similar because the K_{m(obs)} for MCN shows the same increase with [NADPH] as does the V_{max(obs)} which is characteristic for ping-pong mechanisms, where the two substrates compete for the same, or overlapping, sites on the enzyme. This mechanism suggests that both A and B compete for the same active site on the enzyme and assumes that the binding of MCN and NADPH is much faster than the hydroxylation and electron transfer steps. It is likely that a more complex mechanism applies with respect to these assumptions. However, in all cases, parallel lines will be observed if only binary complexes occur.

**Scheme S1:**

\[
\begin{align*}
\text{A} & \quad \text{E} \quad \text{P} \quad \text{B} \quad \text{Q} \\
\text{E} & \quad (\text{EA} \leftrightarrow \text{E}'P) \quad \text{E}' \quad (\text{E}'B \leftrightarrow \text{EQ}) \quad \text{E} \\
\end{align*}
\]

**Scheme S2:**

\[
\begin{align*}
\text{TetX2}^{\text{new}} + \text{MCN} & \quad \text{TetX2}^{\text{new}} \cdot \text{MCN} \quad \text{TetX2}^{\text{new}} + \text{MCN}^* \\
& \quad \text{TetX2}^{\text{new}} \cdot \text{MCN} \quad \text{TetX2}^{\text{new}} + \text{MCN}^* \\
& \quad \text{O}_2 \quad \text{O}_2 \\
\end{align*}
\]

where \( k_{1} = \frac{k_{m(O_2)}}{k_{m(O_2)} + [O_2]} \).
Physiological Model for TetX2-Mediated Inactivation of MCN. We developed a mathematical model based on experimental data to quantitatively describe the success and failure of each adaptive mutation over a range of MCN concentrations from their in vitro properties (Fig. 4A).

As shown in Fig. 4, growth rates for *E. coli* BW25113 carrying variants of tet(X2) could be predicted accurately from experimentally derived kinetics and protein expression levels. Inhibition of bacterial growth rates by cytosolic MCN was determined by fitting the growth rate dependence of *E. coli* to MCN with Eq. S2. The growth rate is predicted from the cytosolic concentration of minocycline [MCNₐ], using the Hill equation with parameters A and B:

$$GR = 1 - \left( \frac{[MCNₐ]^A}{A + [MCNₐ]^A} \right)$$  \[S2\]

In this cell line, endogenous MCN export was assumed to be negligible compared with degradation by TetX2 such that at steady state the rate of diffusion of minocycline equals the degradation rate of MCN by TetX2 (Eq. S3):

$$\frac{V_{max}}{1 + \frac{K_{m(NADPH)}[NADPH]}{[MCNₐ]}} = D\left([MCNₐ] - 0.25[MCNₐ]\right).$$  \[S3\]

The bisubstrate kinetics equation (Eq. S1) and Fick’s law were used to calculate the steady-state cytosolic MCN concentration from the concentration of MCN in the media (Eq. S4):

$$[MCNₐ] = 2\left(\left([MCNₐ] - 0.25\frac{K_{m(MCN)}}{1 + \frac{K_{m(NADPH)}[NADPH]}{D}}\right)\right) + \frac{K_{m(MCN)}}{1 + \frac{K_{m(NADPH)}[NADPH]}{D}}[MCNₐ].$$  \[S4\]

We used the overall model for tetracycline diffusion proposed by Thanassi et al. (7). Tetracyclines are predicted to cross the outer membrane by passive diffusion through OmpF as a complex with Mg²⁺. Because of differences in concentration of Mg²⁺ in the periplasm compared with the media, the effective concentration of MCN is determined by the Donnan potential and predicted to be one-half the actual MCN concentration (Eq. S5) (7). Similarly, MCN equilibrium across the inner membrane is affected by the differences in pH between the cytosol and the periplasm. The charged form of MCN cannot cross the inner membrane, such that the effective concentration of MCN in the cytosol is again one-half of the actual concentration (Eq. S6) (7). The concentration difference across both membranes is defined by equation Eq. S7:

$$J_I = D_I\left([MCNₐ] - 0.5[MCNₐ]\right)$$  \[S5\]

$$J_O = D_O\left([MCNₐ] - 0.5[MCNₐ]\right)$$  \[S6\]

$$J = D\left([MCNₐ] - 0.25[MCNₐ]\right).$$  \[S7\]

Growth rate inhibition by MCN is predicted to be dependent on the ratio of uninhibited ribosomes to total ribosomes. The Hill function (Eq. S2) is a general biochemical function for substrate binding that can be used to calculate the fraction of uninhibited ribosomes and the fraction of ribosomes that are being inhibited by binding MCN. The Hill 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. 4 AII), and fitted using the measured values of $K_{m(MCN)}$, $K_{m(NADPH)}$, and $k_{cat}$ (Fig. S3). The parameter A corresponds to the apparent dissociation constant $K_D$ for minocycline. $K_D$ in the context of the model represents interactions between MCN and the ribosome but is also affected by any other MCN interactions within the cytosol that would reduce or alter its effective concentration. Despite this caveat, the value of A was estimated to be about 26 μM, which is in good agreement with the expectation of MCN binding to the ribosome. The parameter B is the Hill coefficient that represents the effect of MCN binding to the ribosome on growth rates at exponential phase.

Parameters for A, B, and the combined diffusion rate D were fitted using a least-squares method to find the best fit for estimating growth rates (Fig. S3). The mathematical fits were done using the R programming language and the optim function with standard parameters (8). The A parameter and the accumulation of MCN inside the cytoplasm are correlated because decreasing A can roughly compensate for an increase in MCN accumulation. Although this has no effect on the fits, it is important to note that the accumulation of a particular substrate species may be different for different bacteria that have significant changes to their membrane chemistry or transporters.

To validate the accuracy of the mathematical fit the model parameters were used to back calculate the $K_{m(MCN)}$ and $V_{max}$ for each of the mutants from the growth rate curves. Each mutant growth rate was fitted independently, using a two-step approach. An initial fit was done using a wide range of starting conditions for $V_{max}$ and activity ($V_{max}K_{m(MCN)}$). The result was a set of solutions that share a similar $V_{max}K_{m(MCN)}$ but could vary widely in absolute values of $V_{max}$ and $K_{m(MCN)}$. As shown in Fig. S3A, however, the agreement on the ratio of $V_{max}K_{m(MCN)}$ was excellent. Solutions were then sorted by calculating the slope of the growth rate curve at the midpoint, using five points and comparing the slope of the first set of solutions at the same [MCN]...
concentrations. As shown in Fig. S3B, the shape of growth curves generated from an ensemble of equal \( \frac{V_{\text{max}}}{K_{\text{m(MCN)}}} \) ratios of varying absolute magnitudes of \( V_{\text{max}} \) and \( K_{\text{m(MCN)}} \) produces different growth rate responses that can be used as the second stage of fitting. The ratio \( \frac{V_{\text{max}}}{K_{\text{m(MCN)}}} \) of the solution from the first set that best fits the slope of the growth rates (Fig. S3B) is used as a constraint in a second fit that is used to estimate \( K_{\text{m(MCN)}} \) and \( V_{\text{max}} \) (Table S3). We did not observe a significant difference between the measured and estimated values of \( \frac{V_{\text{max}}}{K_{\text{m(MCN)}}} \) (paired t test: df = 7, \( P = 0.2 \)).

A statistical jackknife method was used to test the variance of the model fit for each of the mutants. Each of the variants of TetX2 was removed from the dataset sequentially and fits of the parameters \( A, B, \) and \( D \) were calculated for the remaining seven variants. The variance of the predicted values was measured by calculating the mean and the SD of the \( A, B, \) and \( D \) predicted values from each set of parameters (Table S4). With the exception of F23S5, removing any of the mutants did not have a large effect on the predicted parameters.

In five of the seven mutants, the growth rate and consequent evolutionary dynamics are accounted for largely by kinetic parameters (Table S3). It is difficult to experimentally estimate the \( K_{\text{m(MCN)}} \) when values are <20 \( \mu \)M and thus the experimental data at the lowest values of \( K_{\text{m(MCN)}} \) may be an overestimate. The model fit for TetX2 growth rates is most sensitive to \( K_{\text{m(MCN)}} \) as it is not as sensitive to \( K_{\text{cat}} \) and \( V_{\text{max}} \). The concentration of MCN, \( K_{\text{m(MCN)}} \) affects the initial plateau where there is little effect on growth rate when increasing MCN. The measured \( K_{\text{cat}} \) 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 activity and changes in active protein concentration because these two variables affect the shape of the growth curve in the same way. Thus, the best estimate of the model from growth rates alone is for \( \frac{V_{\text{max}}}{K_{\text{m(MCN)}}} \). Using growth rates alone, the model is able to robustly estimate \( \frac{V_{\text{max}}}{K_{\text{m(MCN)}}} \). Which, in turn, can be used to estimate \( K_{\text{m}} \).

The MCN concentration where the most successful adaptive mutant (TetX2\text{280A}) was first observed by experimental evolution (32 \( \mu \)M) is very near the range of \( K_{\text{m}} \) for the most successful adaptive TetX2 mutants identified in vitro (Table 1).

**Stability of TetX2 and TetX2\text{280A}**

To assess the effect of the adaptive mutation T280A on the amount of folded protein, the in vitro stabilities of wild type and TetX2\text{280A} were determined by thermal denaturation monitored by circular dichroism (CD). CD experiments were performed using a Jasco J-815 spectrophotometer. Spectra were recorded at 200–250 nm at 20 °C. The thermal stability of TetX2 and the mutant TetX2\text{280A} was determined by monitoring absorption at 220 nm at the temperature range 20–90 °C, using a scanning rate of 60 °C/h in triplicate. Protein samples were prepared to a concentration of 20 \( \mu \)M in 20 mM Tris, pH 8.0.

The stability data show that both TetX2 and TetX2\text{280A} have nearly identical unfolding profiles (Fig. S4A). However, only the thermal unfolding midpoint (\( T_{m} \)) could be approximated as protein denaturation was largely irreversible and at least two transitions were observed. The large transition occurs at 71.7 ± 0.5 °C and 72.6 ± 0.5 °C, for wild-type and TetX2\text{280A}, respectively (Fig. S4A). In addition, before the sharp transition, a gradual decrease in the CD signal of about 5 mdeg is observed between 25 °C and 45 °C (Fig. S4B). Chemical unfolding in the presence of guanidinium chloride shows that the change in signal for fully unfolded TetX2 is expected to be significantly larger than what is observed at lower temperature (Fig. S4C). Even though we were not able to assess the fraction of folded protein using CD thermal unfolding, we concluded that the T280A mutation does not dramatically alter the stability of the enzyme. Because the changes in signal for TetX2 and TetX2\text{280A} both in the pretransition (first phase) and in the later large transition are comparable, there is no evidence that the mutation has a significantly altered stability at 37 °C. The strongly irreversible unfolding of TetX2 involves at least two transition states and precluded an accurate estimation of thermodynamic stability. To assess overall expression of steady-state levels of the TetX2 variants in vivo we measured activity from cell extracts as described previously.

**Structure Determination and Refinement of the TetX2\text{280A}–MCN Complex at 2.7 Å Resolution.** Native crystals were soaked in various MCN concentrations 16 h before cryoprotection. Crystals were cryoprotected in 25% glycerol (vol/vol) and flash frozen in liquid nitrogen. Single-wavelength data were collected at the Advanced Light Source beamline 4.2.2, using the NOIR 1 MBC detector. The diffraction data were processed using HKL2000 software in space group P1 to 2.7 Å resolution (9) (Table S2). Molecular replacement (MR) as implemented in phenix.autosol (10) was used for the initial structure determination, using the wild-type TetX2 structure excluding the flavin cofactor [Protein Data Bank (PDB) ID 3P9U] as a search model. The electron density for flavin adenine dinucleotide (FAD) was clearly visible in all four molecules of the asymmetric unit. FAD was built manually in COOT (11) and was used in the refinement of the structure. In addition, unoccupied electron density corresponding to MCN molecules was identified near the FAD isoisalloxazine ring in a (2Fo-Fc) SIGMAA weighted composite omit map. MCN molecules were fitted manually in COOT and were included in final stages of refinement. The final \( R_{\text{work}} = 22.1% \) and \( R_{\text{free}} = 27.6% \) are included with additional data collection and refinement statistics in Table S2. On the basis of the initial MR solution, four molecules were positioned in the asymmetric unit. The initial model was submitted for automated building and refinement to phenix.autobuild and phenix.refine (10), respectively. The final model had an \( R_{\text{free}} = 27.6% \) and \( R_{\text{work}} = 22.08% \) (Table S2) and was deposited in the PDB as 3V3N.

**Serial-Passage Evolution Experiment to Identify Concentrations of MCN Required to Generate Mutations in TetX2.** Initially, two populations were used to identify the appropriate range of MCN concentrations that would favor mutations to TetX2. The MIC of \( E. coli \) BW25113 tet(X2) in liquid culture was determined to be 16 \( \mu \)g/mL (32 \( \mu \)M) MCN in LB broth at 37 °C. Serial passage experiments were initiated at 10 \( \mu \)g/mL (20 \( \mu \)M) MCN and MCN concentration increased daily according to the following schedule: 10, 16, 24, 36, 52, 80, 120, 180, 300, and 320 \( \mu \)g/mL (or 20, 32, 48, 72, 104, 160, 240, 360, 600, and 640 \( \mu \)M). Each day, 50 \( \mu \)L of the population was transferred to a new condition and a 1-mL sample of each population was frozen at −80 °C for DNA sequencing and characterization.

Every day, we isolated 5–10 individual colonies on media containing MCN corresponding to the particular day of the serial passage experiment and sequenced the tet(x2) gene. In both populations, TetX2\text{280A} could be isolated readily by days 2–3. Beyond day 3 (24 \( \mu \)g/mL), no additional or new mutations to tet(x2) could be identified despite increasing MIC of the evolved strains, suggesting a strong role for changes outside tet(x2), presumably chromosomal changes. Interestingly, we were unable to generate high resistance in \( E. coli \) BW25113 without tet(x2), suggesting an important role for tet(x2) in allowing BW25113 to tolerate low MCN concentrations and enabling the fixation of subsequent chromosomal changes in a highly epistatic manner. Although expression of tet(x2) is linked epistatically to subsequent increases in resistance via chromosomal changes, it is not essential for beneficial mutations to accumulate in tet(x2). For example, constitutive high-level expression of tet(x2) from the lac promoter form of pUC19 obviated the need for any changes to tet(x2). For the purposes of this work on adaptation via tet(x2),
changes to the chromosome that fix within the population are not relevant. Whereas mutations outside tet(X2) were clearly able to generate very high resistance, their level of resistance is well beyond the range of MCN concentrations used clinically (3).

**DNA Barcoding of tet(X2) SNPs in 10 Replicate Populations During Serial Passage (FREQ-SEQ).** Although we identified seven tet(X2) mutants with equal or higher MIC than tet(X2) using error-prone PCR, only tet(X2)T280A could be isolated from two evolved populations. To test whether other tet(X2) mutants can evolve during the selection experiment, we evolved 10 replicated populations for 3 d following the same MCN concentration scheme as before and used FREQ-SEQ (12), a DNA barcode sequencing technology, to follow the frequency of each possible mutant in every population over the course of the experiment. Generation of samples suitable for such deep sequencing required three steps. First, a barcoded primer is amplified from a plasmid (gift of C. Marx, Harvard University, Cambridge, MA), using an Illumina forward primer and an M13 primer. Second, a 100-nt fragment containing a mutation site is amplified with primers containing the same M13 recognition site on the forward primer and the Illumina primer for the reverse reaction on the reverse primer. Finally, a unique barcode is added by amplification of the sample prepared in step 2 with primers generated in step 1, to create an oligonucleotide containing Illumina primers, a barcoded sequence, an M13 sequence, and our target sequence. Primers are listed below:

**Illumina forward primers.**

K64/A191: GTAAAAACGACGGCCAGTGTGGAACCC- TTAGCTTACA
F235/T704: GTAAAAACGACGGCCAGTGTGGAACCC- TTAGCTTACA
T280/A838: GTAAAAACGACGGCCAGTGTGGAACCC- TTAGCTTACA
S326/G977: GTAAAAACGACGGCCAGTGTGGAACCC- TTAGCTTACA
N371/A1112: GTAAAAACGACGGCCAGTGTGGAACCC- TTAGCTTACA

**Illumina reverse primers.**

K64/A191: CAACGAGAAGACGGCAGTACGAGCTTCTC- CGATCTCCACGTCATCACGAGATCCAG
F235/T704: CAACGAGAAGACGGCAGTACGAGCTTCTC- CGATCTCCACGTCATCACGAGATCCAG
T280/A838: CAACGAGAAGACGGCAGTACGAGCTTCTC- CGATCTCCACGTCATCACGAGATCCAG
S326/G977: CAACGAGAAGACGGCAGTACGAGCTTCTC- CGATCTCCACGTCATCACGAGATCCAG
N371/A1112: CAACGAGAAGACGGCAGTACGAGCTTCTC- CGATCTCCACGTCATCACGAGATCCAG

**Primers for barcode adapter amplification from plasmid.**

Forward: AATGATACGGGCGACCAC Reverse: ACTGGCCGTCGTTTAC

**Universal primers for amplification of barcoded final product.**

Forward: AATGATACGGGCGACCAC Reverse: CAACGAGAAGACGGCAGTACGAGATCCAG

On the basis of the earlier serial passage experiments, we focused on the first 3 d and examined the occurrences of tet(X2)T280A, tet(X2)N371I, tet(X2)N371T, tet(X2)P235Y, tet(X2)S326I, tet(X2)T280S, and tet(X2)G64R in the 10 replicate populations, using a unique barcode for every combination of day and population. Primer sequences were used to distinguish the different SNP loci. On average, the SNP site was covered by $2.4 \times 10^5$ reads with a 95% confidence interval (CI) of $3.7 \times 10^4$. Three barcodes returned relatively low numbers of reads for all SNP sites. Excluding those three barcodes increased the average coverage to $2.7 \times 10^4$ reads per site. On average, 72.4% ($\pm 1.2\%$, 95% CI) of the reads of each barcode had a perfect match over the length of the primer, the mutation (including A, C, G, or T at the mutation site), and the 2 nt following the mutation site. These reads were used to calculate the allele frequencies. The frequency of an allele with an A at the SNP site in a given population at a certain day was determined as (number of reads with nucleotide A at SNP site)/(sum of number of reads with A, C, G, or T at SNP site). Any read that was included in this analysis matched the primer sequence up to the SNP site and the 2 nt 3' of the SNP site perfectly. To test whether the combined error rate of PCR polymerase and sequencing was below the frequency of our mutant alleles, we determined the frequency of reads that did not have the wild-type base at the two sites 5' and 3' of the SNP site [error rate was calculated as the average of the frequencies of the three non–wild-type nucleotides at a given site or (1 – frequency of wild type)/3, where wild type refers to the base found in tet(X2) at that particular site and does not refer to a wild-type allele covering the whole gene. Because we were interested in the frequency of a particular nucleotide, we used our error rate calculation equally.] The overall error rate averaged across position, loci, days, and populations is 0.0024 for a single nucleotide, with a 95% CI ranging from 0.0023 to 0.0025. On the basis of this error rate, we decided that we could distinguish true allelic frequencies from sequencing errors at the level of 0.5%. As summarized in Fig. S5, tet(X2)T280A and tet(X2)N371I were identified in seven populations in good agreement with growth rate studies, in vitro enzyme kinetics, and cell extract assays that suggested that tet(X2)T280A and tet(X2)N371I were the most advantageous tet(X2) alleles. Surprisingly, tet(X2)T280S appeared in two populations despite growth rate studies, MIC values, enzyme kinetics, and cell extract properties more consistent with having no increase over the performance of wild-type tet(X2) and although it decreased in frequency by day 3 in one population, it reached relatively high frequency in the other populations. This increase could be due to hitchhiking of tet(X2)T280S with other beneficial mutations in the chromosome. Tet(X2)T280S is caused by a substitution of an A to a T. To test for an A to T bias in our reads, we identified silent A to T mutations for each locus and tested for an A to T bias by comparing the frequency of A to T mutations to the average frequency of A to G and A to C mutations. A to T mutations were significantly less common at every locus (that holds true even after sequential Bonferroni correction: paired t-test, A191, $P = 0.005$; T704, $P = 0.005$; and A838, G977, and A1112, $P < 0.0001$, with df = 30 for all loci). Because the silent mutations were at different positions in the reads, we also tested for an effect of location, i.e., position of the silent site in the read as bases toward the end of a read are more prone to errors: A fixed-factor ANCOVA on the difference between the average frequency of A to G and A to C mutations and the frequency of A to T mutations with the position of the base as a covariate showed a slight difference among barcodes ($F_{30,123} = 1.57, P = 0.045$) and a highly significant effect of position in the read ($F_{1,123} = 31.43, P < 0.0001$).


Fig. S1. Distribution of mutations in the error-prone library of tet(X2) mutants. The average mutation rate of the tet(X2) library was estimated at 1.37 mutations/kb. Most sequences contained zero or one mutation in the tet(X2) gene.

Fig. S2. Maximal growth rates of TetX2 and its mutants at various MCN concentrations. 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 the largest slope through a moving window. At higher MCN concentrations, growth occurred in two phases. In those instances, only the first growth phase was considered (details in SI Materials and Methods). In cases like well B12, we analyzed the well individually, by testing the data only from the first 12 h of growth to get the first maximal growth rate and not the second, as shown.
Fig. S3. Description of the two-stage mathematical fit to estimate $V_{\text{max}}/K_{\text{m(MCN)}}$. (A) An initial fit is done to determine the $V_{\text{max}}/K_{\text{m(MCN)}}$ ratio that provides the best fit for each TetX2 mutant. Fitting with multiple starting conditions results in a series of solutions. The fits with the lowest residuals share the same ratio. (B) The same ratio of $V_{\text{max}}/K_{\text{m(MCN)}}$ results in a series of curves with distinct effects on predicted growth rates. (C) The solution that matches the slope of the growth curve best at the midpoint is used to determine the appropriate $V_{\text{max}}/K_{\text{m(MCN)}}$. (D) The growth curve is fitted again using the $V_{\text{max}}/K_{\text{m(MCN)}}$ value determined in the previous step to estimate $V_{\text{max}}$ and $K_{\text{m(MCN)}}$. 
**Fig. S4.** T280A does not significantly alter the in vitro stability of TetX2. Thermal stability of TetX2<sub>wt</sub> and TetX2<sub>T280A</sub> was monitored by change in circular dichroism as a function of temperature. (A) Thermal denaturation midpoint (Tm) of unfolding is estimated at 71.7 °C for TetX2<sub>wt</sub> (black solid circles) and at 72.6 °C for TetX2<sub>T280A</sub> (red solid triangles) for the sharp transition. (B) Small change in CD signal during thermal unfolding of TetX2 occurs between 30 °C and 45 °C and precedes sharp transition at higher temperature. (C) Chemical unfolding in the presence of guanidinium chloride (GuHCl) of TetX2 shows a large change in CD between folded (0 M GuHCl) and unfolded protein (3 M GuHCl).

**Fig. S5.** Deep sequencing reveals that TetX2<sub>T280A</sub> and TetX2<sub>N371I</sub> are the two most successful alleles during adaptation to MCN. Deep sequencing was used to monitor the frequencies of TetX2 variants (identified by error-prone mutagenesis) in 10 individual populations of E. coli BW25113 tet(X2) evolved for 3 d to increasing MCN concentrations (10, 16, and 24 μg/mL). As predicted by the model, mutation TetX2<sub>T280A</sub> is the most successful and is present in 5 (of 10) populations, whereas TetX2<sub>N371I</sub> is found in 2 populations and reaches fixation in 1 of them (population 8). An unexpected neutral allele TetX2<sub>T280S</sub> is present in 2 populations. The remaining mutants do not evolve or remain at very low frequencies.
Table S1. Relative maximal growth rates of ancestral strain and E. coli expressing chromosomal copies of wild-type and adaptive mutations

<table>
<thead>
<tr>
<th>MCN, μM</th>
<th>Ancestor E. coli&lt;sub&gt;bw25113&lt;/sub&gt;</th>
<th>TetX2</th>
<th>TetX2&lt;sub&gt;T280A&lt;/sub&gt;</th>
<th>TetX2&lt;sub&gt;N371I&lt;/sub&gt;</th>
<th>TetX2&lt;sub&gt;N371T&lt;/sub&gt;</th>
<th>TetX2&lt;sub&gt;S326I&lt;/sub&gt;</th>
<th>TetX2&lt;sub&gt;F235Y&lt;/sub&gt;</th>
<th>TetX2&lt;sub&gt;K64R&lt;/sub&gt;</th>
<th>TetX2&lt;sub&gt;T280S&lt;/sub&gt;</th>
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<tbody>
<tr>
<td>0</td>
<td>1.00 ± 0.01</td>
<td>1.00 ± 0.01</td>
<td>1.00 ± 0.01</td>
<td>1.00 ± 0.01</td>
<td>1.00 ± 0.01</td>
<td>1.00 ± 0.01</td>
<td>1.00 ± 0.01</td>
<td>1.00 ± 0.01</td>
<td>1.00 ± 0.01</td>
</tr>
<tr>
<td>4.1</td>
<td>0.59 ± 0.01</td>
<td>1.01 ± 0.01</td>
<td>0.97 ± 0.01</td>
<td>0.98 ± 0.01</td>
<td>0.98 ± 0.01</td>
<td>0.95 ± 0.01</td>
<td>0.95 ± 0.01</td>
<td>0.97 ± 0.01</td>
<td>1.00 ± 0.01</td>
</tr>
<tr>
<td>8.1</td>
<td>0.39 ± 0.01</td>
<td>0.94 ± 0.03</td>
<td>0.99 ± 0.01</td>
<td>0.98 ± 0.01</td>
<td>0.95 ± 0.01</td>
<td>0.96 ± 0.01</td>
<td>0.96 ± 0.01</td>
<td>0.91 ± 0.05</td>
<td>0.92 ± 0.05</td>
</tr>
<tr>
<td>12.2</td>
<td>0.24 ± 0.01</td>
<td>0.73 ± 0.03</td>
<td>1.00 ± 0.01</td>
<td>0.99 ± 0.01</td>
<td>0.98 ± 0.01</td>
<td>0.93 ± 0.02</td>
<td>0.94 ± 0.01</td>
<td>0.69 ± 0.03</td>
<td>0.73 ± 0.03</td>
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<tr>
<td>16.2</td>
<td>0.09 ± 0.05</td>
<td>0.56 ± 0.03</td>
<td>1.00 ± 0.01</td>
<td>0.99 ± 0.01</td>
<td>0.96 ± 0.01</td>
<td>0.87 ± 0.02</td>
<td>0.85 ± 0.03</td>
<td>0.49 ± 0.03</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td>20.3</td>
<td>0.04 ± 0.01</td>
<td>0.41 ± 0.01</td>
<td>1.00 ± 0.02</td>
<td>0.97 ± 0.07</td>
<td>0.95 ± 0.07</td>
<td>0.79 ± 0.02</td>
<td>0.77 ± 0.02</td>
<td>0.29 ± 0.02</td>
<td>0.30 ± 0.02</td>
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<tr>
<td>24.3</td>
<td>ND</td>
<td>0.27 ± 0.01</td>
<td>1.00 ± 0.01</td>
<td>0.95 ± 0.04</td>
<td>0.87 ± 0.04</td>
<td>0.70 ± 0.02</td>
<td>0.67 ± 0.02</td>
<td>0.20 ± 0.01</td>
<td>0.21 ± 0.02</td>
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<tr>
<td>32.4</td>
<td>ND</td>
<td>0.17 ± 0.01</td>
<td>0.92 ± 0.02</td>
<td>0.89 ± 0.01</td>
<td>0.75 ± 0.01</td>
<td>0.58 ± 0.01</td>
<td>0.54 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.11 ± 0.02</td>
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<tr>
<td>40.6</td>
<td>ND</td>
<td>0.09 ± 0.01</td>
<td>0.78 ± 0.04</td>
<td>0.77 ± 0.01</td>
<td>0.62 ± 0.01</td>
<td>0.44 ± 0.01</td>
<td>0.40 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.01</td>
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<tr>
<td>60.8</td>
<td>ND</td>
<td>ND</td>
<td>0.37 ± 0.05</td>
<td>0.55 ± 0.01</td>
<td>0.34 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>101.4</td>
<td>ND</td>
<td>ND</td>
<td>0.07 ± 0.04</td>
<td>0.23 ± 0.02</td>
<td>0.12 ± 0.02</td>
<td>0.10 ± 0.02</td>
<td>0.03 ± 0.02</td>
<td>ND</td>
<td>ND</td>
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</table>

Growth rates are normalized for each strain tested to the growth rate determined at 0 μM MCN. ND, growth not detected.

Table S2. Summary of data collection and refinement statistics

<table>
<thead>
<tr>
<th>TetX2&lt;sub&gt;T280A&lt;/sub&gt;-MCN</th>
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<tbody>
<tr>
<td>Data collection</td>
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<tr>
<td>Wavelength, Å</td>
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<td>Resolution, Å*</td>
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<td>Space group</td>
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<td>Unit Cell, Å</td>
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<td></td>
</tr>
<tr>
<td>Total no. reflections</td>
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<tr>
<td>Unique reflections</td>
</tr>
<tr>
<td>Average redundancy*</td>
</tr>
<tr>
<td>Completeness, %*</td>
</tr>
<tr>
<td>R&lt;sub&gt;merge&lt;/sub&gt;, %*</td>
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<tr>
<td>Output &lt;I/sigI&gt;*</td>
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<tr>
<td>Refinement</td>
</tr>
<tr>
<td>R&lt;sub&gt;work&lt;/sub&gt;, %</td>
</tr>
<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt;, %</td>
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<tr>
<td>rmsd from ideality Bonds, Å</td>
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<tr>
<td>Angles, °</td>
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<tr>
<td>Average B-factor, Å²</td>
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<tr>
<td>Ramachandran</td>
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<td>Favored, %</td>
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<tr>
<td>Outliers, %</td>
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<tr>
<td>PDB accession no.</td>
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</tbody>
</table>

*Values for the last shell are in parentheses.

<sup>1</sup>R<sub>merge</sub> = ∑<sub>j</sub> I<sub>j</sub> – <I>/<I>∑<sub>j</sub> I<sub>j</sub> where I<sub>j</sub> is measured intensity for reflections with indexes of hkl.

<sup>2</sup>R<sub>work</sub> = ∑<sub>j</sub> |F<sub>o</sub> – F<sub>c</sub>|/|F<sub>o</sub>| for all data with F<sub>o</sub> > 2σ(F<sub>o</sub>) excluding data to calculate R<sub>free</sub>.

<sup>3</sup>R<sub>free</sub> = ∑<sub>j</sub> |F<sub>o</sub> – F<sub>c</sub>|/|F<sub>o</sub>| for all data with F<sub>o</sub> > 2σ(F<sub>o</sub>) excluded from refinement.

<sup>R</sup> Root mean square deviation.

<sup>1</sup>Calculated by using MolProbity.
Table S3. Comparison of predicted and calculated steady-state kinetic parameters for wild-type TetX2 and adaptive mutants

<table>
<thead>
<tr>
<th></th>
<th>Measured $V_{\text{max}}/K_{\text{m(MCN)}}$ $\mu$M$^{-1}$s$^{-1}$</th>
<th>Predicted $V_{\text{max}}/K_{\text{m(MCN)}}$ $\mu$M$^{-1}$s$^{-1}$</th>
<th>Measured $K_{\text{m(MCN)}}$ $\mu$M</th>
<th>Predicted $K_{\text{m(MCN)}}$ $\mu$M</th>
<th>Measured $V_{\text{max}}$ s$^{-1}$</th>
<th>Predicted $V_{\text{max}}$ s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TetX2</td>
<td>0.005 ± 0.0003</td>
<td>0.004</td>
<td>35 ± 1.9</td>
<td>25.7</td>
<td>0.16 ± 0.005</td>
<td>0.10</td>
</tr>
<tr>
<td>T280A</td>
<td>0.011 ± 0.0006</td>
<td>0.019</td>
<td>18 ± 0.9*</td>
<td>5.1</td>
<td>0.19 ± 0.004</td>
<td>0.10</td>
</tr>
<tr>
<td>N371I</td>
<td>0.013 ± 0.0018</td>
<td>0.013</td>
<td>18 ± 1.9</td>
<td>39.9</td>
<td>0.23 ± 0.015</td>
<td>0.54</td>
</tr>
<tr>
<td>N371T</td>
<td>0.008 ± 0.0011</td>
<td>0.011</td>
<td>24 ± 2.1</td>
<td>26.3</td>
<td>0.20 ± 0.013</td>
<td>0.30</td>
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<tr>
<td>S326I</td>
<td>0.007 ± 0.0005</td>
<td>0.007</td>
<td>37 ± 2.8</td>
<td>59.5</td>
<td>0.25 ± 0.007</td>
<td>0.44</td>
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<tr>
<td>F235Y</td>
<td>0.005 ± 0.0009</td>
<td>0.006</td>
<td>54 ± 6.1</td>
<td>64.9</td>
<td>0.28 ± 0.041</td>
<td>0.42</td>
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<tr>
<td>K64R</td>
<td>0.004 ± 0.0006</td>
<td>0.004</td>
<td>36 ± 4.6</td>
<td>13.3</td>
<td>0.16 ± 0.013</td>
<td>0.06</td>
</tr>
<tr>
<td>T280S</td>
<td>0.005 ± 0.0008</td>
<td>0.004</td>
<td>30 ± 3.4</td>
<td>13.5</td>
<td>0.16 ± 0.009</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*T280A experimental measurement of $K_{\text{m(MCN)}}$ may be underestimated as the assay cannot easily distinguish $K_{\text{m(MCN)}} < 20 \mu$M.

Table S4. Error estimates of model fits

<table>
<thead>
<tr>
<th></th>
<th>$A^*$</th>
<th>$B^*$</th>
<th>$D^*$</th>
<th>$SS^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>All mutants</td>
<td>25.8</td>
<td>2.5</td>
<td>6.7E-04</td>
<td>1.842</td>
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<tr>
<td>TetX2</td>
<td>57.5</td>
<td>2.3</td>
<td>9.6E-04</td>
<td>0.173</td>
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<tr>
<td>T280A</td>
<td>40.1</td>
<td>2.2</td>
<td>9.5E-04</td>
<td>0.346</td>
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<tr>
<td>N371I</td>
<td>51.5</td>
<td>2.4</td>
<td>9.0E-04</td>
<td>0.024</td>
</tr>
<tr>
<td>N371T</td>
<td>71.4</td>
<td>2.3</td>
<td>10.0E-04</td>
<td>0.052</td>
</tr>
<tr>
<td>S326I</td>
<td>21.8</td>
<td>2.6</td>
<td>6.1E-04</td>
<td>0.095</td>
</tr>
<tr>
<td>F235Y</td>
<td>0.1</td>
<td>3.2</td>
<td>1.2E-04</td>
<td>0.509</td>
</tr>
<tr>
<td>K64R</td>
<td>50.3</td>
<td>2.3</td>
<td>8.9E-04</td>
<td>0.105</td>
</tr>
<tr>
<td>T280S</td>
<td>37.2</td>
<td>2.4</td>
<td>7.3E-04</td>
<td>0.538</td>
</tr>
<tr>
<td>Average</td>
<td>41.2</td>
<td>2.5</td>
<td>7.7E-04</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>22.2</td>
<td>0.3</td>
<td>2.9E-04</td>
<td></td>
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</tbody>
</table>

*A* Error estimates when removing each mutant were done using a jackknife estimator approach.

$^1$Sum of squared residuals ($SS$) was measured for each of the individual curves from the mathematical fit using all eight mutants.