Supporting Information
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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 \( \left[ \text{tet}(X2)/\text{pUC19} \right] \) 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) wild type formed colonies within 24 h. On the basis of the number of colonies counted (2 × 10^10 cfu/mL), it was estimated that this library had an eightfold coverage of the minimum requirement, suggesting that most single mutations were sampled in the experiment. The mutation rate for tet(X2) based on sequencing of plasmid DNA from colonies picked from nonselective conditions was determined to be 1.37 mutations/kb. The distribution of mutations in the library is shown in Fig. S1. Screening for active mutants was performed on lysogeny broth (LB) agar plates containing 4 μg/mL of minocycline (MCN) [tet(X2)/pUC19 grows up to 2 μg/mL MCN]. DNA from 35 colonies was isolated and the tet(X2) gene was sequenced.

Construction of Strains Used for Natural Experiment Experiments and Growth Studies. Integration of tet(X2) into the chromosome of E. coli strain BW25113 \( [\Delta(araD-araB)567, \Delta lacZ4787::rrnB-3], \) lambda-, rph-1, \( \Delta(phaD-phaB)568, \) hsdR514, pKD46 was performed using the short homology recombination approach with phage-λ. Red recombinase expressed from a low-copy plasmid (LB) agar plates containing 4 μg/mL of minocycline (MCN) [tet(X2)/pUC19 grows up to 2 μg/mL MCN]. DNA from 35 colonies was isolated and the tet(X2) gene was sequenced.

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′ CTACAAGAATTGATTCATCGAGCTTG-TCAATTGAGG 3′
Reverse: 5′ CTCAAAATGDAACAGTCGAATCA-ATTTTGAG 3′

**Primer pair for F235Y.**

Forward: 5′ CCAATAATAATGGTGCAATGGATTGATG-AAAAAGTAGTG 3′
Reverse: 5′ CAGTTTTTTTTTAACTTATTCCATATTGAC-AATGACCAATTATTTG 3′

**Primer pair for N371I.**

Forward: 5′ CACAAGAGAAATCAAATGTGAATGAACTTG-ATCAATGTTTACCCA 3′
Reverse: 5′ GGTAAAACATTTCAATTTCCGATTTTGAGT-TGATTCTTCGTG 3′

**Primer pair for K64R.**

Forward: 5′ CGGTTAAACATTTCAATTTCCGATTTTGAGTATTCTTCGTTG 3′
Reverse: 5′ GTAAAGATTGTTTAAAACCC 3′

**Primer pair for S326I.**

Forward: 5′ GAAGAAGGCGGAGCGTTACTGGAGGTTACGCCATGCTCAATTTTACATTTAAG-ACAAATTGCTG 3′
Reverse: 5′ GGCTGATTCATCGAGCTTG-TCAATTGAGG 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′ ATGGCTAAACCCGCGGTTAGATTTCCGTCATGTCG and 5′ GCGATTACGGCAT-GCTTTAGTACAGAGG 3′). Using these primers, successful recombination was confirmed by a 3-kb PCR amplicon, compared with a shorter 1.8-kb amplicon without the gene insert, and sequencing of the 3-kb amplicon (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.

**Generation of Constructs for Expression of Mutant Enzymes.**

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**Primer pair for F235Y.**

Forward: 5′ CCAATAATAATGGTGCAATGGATTGATG-AAAAAGTAGTG 3′
Reverse: 5′ CAGTTTTTTTTTAACTTATTCCATATTGAC-AATGACCAATTATTTG 3′

**Primer pair for N371I.**

Forward: 5′ CACAAGAGAAATCAAATGTGAATGAACTTG-ATCAATGTTTACCCA 3′
Reverse: 5′ GGTAAAACATTTCAATTTCCGATTTTGAGT-TGATTCTTCGTG 3′

**Primer pair for K64R.**

Forward: 5′ CGGTTAAACATTTCAATTTCCGATTTTGAGTATTCTTCGTTG 3′
Reverse: 5′ GTAAAGATTGTTTAAAACCC 3′

**Primer pair for S326I.**

Forward: 5′ GAAGAAGGCGGAGCGTTACTGGAGGTTACGCCATGCTCAATTTTACATTTAAG-ACAAATTGCTG 3′
Reverse: 5′ GGCTGATTCATCGAGCTTG-TCAATTGAGG 3′

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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 (Etest; 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 FormData. 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 by 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 \( K_{M(0)} = 12,000 \text{ M}^{-1} \text{cm}^{-1} \)) 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 MgCl\(_2\) (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 \( A = \text{MCN}, B = \text{NADPH}, V_{\text{max}} \) 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]}{K_{m(B)} [B] + [A]} \\

\]

\[

v_0 = \frac{K_{m(A)} [A]}{K_{m(B)} + [A]} \\

\]

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:**

```
\[ A \downarrow \quad P \downarrow \quad B \downarrow \quad Q \downarrow \quad E \]
\[ E \quad (E \leftrightarrow E') \quad 'E' \quad (E'B \leftrightarrow EQ) \quad E \]
```

**Scheme S2:**

\[ \text{TetX2}^{\text{red}} + \text{MCN} \quad \text{TetX2}^{\text{red}} \cdot \text{MCN} \quad \text{TetX2}^{\text{ox}} + \text{MCN} \ast \quad \text{O}_2 \]

\[ \text{TetX2}^{\text{ox}} + \text{NADPH} \quad \text{TetX2}^{\text{ox}} \cdot \text{NADPH} \quad \text{TetX2}^{\text{red}} + \text{NADP} \]

where \( k_1 = \frac{K_{m(O_2)}}{k_{02} + [O_2]} \)
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 [MCN], using the Hill equation with parameters A and B:

\[
GR = 1 - \left( \frac{[MCN]}{A + [MCN]} \right)^B
\]  

(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):

\[
\frac{V_{\text{max}}}{1 + \frac{K_{m(NADPH)}}{[NADPH]}} \frac{[TetX2][MCN]}{[MCN]_C} = D([MCN_M] - 0.25[MCN_C]).
\]

(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):

\[
[MCN_C] = 2 \left( \frac{[MCN_M] - 0.25}{1 + \frac{K_{m(NADPH)}}{[NADPH]}} \right) \frac{V_{\text{max}}}{1 + \frac{K_{m(NADPH)}}{[NADPH]}} \frac{[TetX2]}{D} + \frac{K_{m(MCN)}}{1 + \frac{K_{m(NADPH)}}{[NADPH]}} [MCN_M].
\]

(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 Mg<sup>2+</sup>. Because of differences in concentration of Mg<sup>2+</sup> 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 S7:

\[
J_I = D_I ([MCN_P] - 0.5[MCN_C])
\]

(S5)

\[
J_O = D_O ([MCN_M] - 0.5[MCN_P])
\]

(S6)

\[
J = D ([MCN_M] - 0.25[MCN_C]).
\]

(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. 4AIII), and fitted using the measured values of *K_<sub>m</sub>(MCN)<sup>+</sup>, K<sub>m</sub>(NADPH), and k<sub>cat</sub> (Fig. S3). The parameter A corresponds to the apparent dissociation constant *K<sub>D</sub>*, MCN 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_<sub>m</sub>(MCN)<sup>+</sup> and V<sub>max</sub> 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<sub>max</sub> and activity (V<sub>max</sub>*k<sub>cat</sub>*K<sub>m</sub>(MCN)). The result was a set of solutions that share a similar V<sub>max</sub>*K<sub>m</sub>(MCN) but could vary widely in absolute values of V<sub>max</sub> and K<sub>m</sub>(MCN). As shown in Fig. S3A, however, the agreement on the ratio of V<sub>max</sub>*K<sub>m</sub>(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 $V_{\text{max}}/K_m(\text{MCN})$ ratios of varying absolute magnitudes of $V_{\text{max}}$ and $K_m(\text{MCN})$ produces different growth rate responses that can be used as the second stage of fitting. The ratio ($V_{\text{max}}/K_m(\text{MCN})$) 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 $K_m(\text{MCN})$ and $V_{\text{max}}$ (Table S3). We did not observe a significant difference between the measured and estimated values of $V_{\text{max}}/K_m(\text{MCN})$ (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 F235Y, 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 $K_m(\text{MCN})$ when values are <20 μM and thus the experimental data at the lowest values of $K_m(\text{MCN})$ may be an overestimate. The model fit for TetX2 growth rates is most sensitive to $K_m(\text{MCN})$ and is not as sensitive to $K_\text{cat}$; the concentration of MCN, $K_m(\text{MCN})$ 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 $V_{\text{max}}/K_m(\text{MCN})$. Using growth rates alone, the model is able to robustly estimate $V_{\text{max}}/K_m(\text{MCN})$. Which, in turn, can be used to estimate $K_m$. The MCN concentration where the most successful adaptive mutant (TetX2T280A) was first observed by experimental evolution (32 μM) is very near the range of $K_m$ for the most successful adaptive TetX2 mutants identified in vitro (Table 1).

**Stability of TetX2 and TetX2T280A.** To assess the effect of the adaptive mutation T280A on the amount of folded protein, the in vitro stabilities of wild type and TetX2T280A 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 TetX2T280A 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 μM in 20 mM Tris, pH 8.0.

The stability data show that both TetX2 and TetX2T280A 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 TetX2T280A, 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 TetX2T280A 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 TetX2T280A-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.autosolve (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 isoalloxazine 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 $R_{work} = 22.1\%$ and $R_{free} = 27.6\%$ 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 $R_{free} = 27.6\%$ and $R_{work} = 22.08\%$ (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 tet(X2). The MIC of E. coli BW25113-tet(X2) in liquid culture was determined to be 16 μg/mL (32 μM) MCN in LB broth at 37 °C. Serial passage experiments were initiated at 10 μg/mL (20 μM) MCN and MCN concentration increased daily according to the following schedule: 10, 16, 24, 36, 52, 80, 120, 180, 300, and 320 μg/mL (or 20, 32, 48, 72, 104, 160, 240, 360, 600, and 640 μM). Each day, 50 μL 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, TetX2T280A could be isolated readily by days 2–3. Beyond day 3 (24 μg/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: GTAAACGACGCCAGTTGTTGGAACC-TTGACCTTACA
F235/T704: GTAAACGACGCCAGTTAACCCTCAA-TAATGTTGATGCA
T280/A838: GTAAACGACGCCAGCAGAAGCTAC-AAGAAATGTAC
S326/G977: GTAAACGACGCCAGCTTTCGAGCGGAGATTA
N371/A1112: GTAAACGACGCCAGCAAGAGAAGCAC-AAGGAGATCCTCAA

**Illumina reverse primers.**

K64/A191: CAGGACGAAGAGCGCGATACGACGTCTTC-CGATCTCCAAGCTTACATACCCAGGAC
F235/T704: CAGGACGAAGAGCGCGATACGACGTCTT-CGATCTCCAAGCTTACATACCCAGGAC
T280/A838: CAGGACGAAGAGCGCGATACGACGTCTT-CGATCTCCAAGCTTACATACCCAGGAC
S326/G977: CAGGACGAAGAGCGCGATACGACGTCTT-CGATCTCCAAGCTTACATACCCAGGAC
N371/A1112: CAGGACGAAGAGCGCGATACGACGTCTT-CGATCTCCAAGCTTACATACCCAGGAC

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

Forward: AATGTATCAGCCGCCACC
Reverse: AATGTATCAGCCGCCACC

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

Forward: AATGTATCAGCCGCCACC
Reverse: AATGTATCAGCCGCCACC

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)F235Y, tet(X2)S326I, tet(X2)T280S, and tet(X2)K64R 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 × 10^5 reads with a 95% confidence interval (CI) of 3.7 × 10^5. Three barcodes returned relatively low numbers of reads for all SNP sites, excluding those three barcodes increased the average coverage to 2.7 × 10^5 reads per site. On average, 72.4% ± 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 location, 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 chromosomes. 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 (F1,123 = 1.57, P = 0.045) and a highly significant effect of position in the read (F1,123 = 31.43, P < 0.0001).


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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>wild type</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>wild type</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 <i>E. coli</i> BW25113<sup>tet(X2)</sup> 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.

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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</th>
<th>E. coli BW25113</th>
<th>TetX2</th>
<th>TetX2T280A</th>
<th>TetX2N371I</th>
<th>TetX2N371T</th>
<th>TetX2S326I</th>
<th>TetX2F235Y</th>
<th>TetX2K64R</th>
<th>TetX2T280S</th>
</tr>
</thead>
<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 ± 0.03</td>
<td>1.00 ± 0.03</td>
<td>1.00 ± 0.02</td>
<td></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>0.97 ± 0.01</td>
<td>1.00 ± 0.01</td>
<td></td>
<td></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>0.91 ± 0.05</td>
<td>0.92 ± 0.05</td>
<td></td>
<td></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>
<td></td>
</tr>
<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>
<td></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>
<td></td>
</tr>
<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>
<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>
<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>
<td></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>
<td></td>
</tr>
</tbody>
</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>TetX2T280A-MCN</th>
<th>Wavelength, Å</th>
<th>1.00004</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution, Å*</td>
<td>49.3–2.70 (2.85–2.80)</td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>P1</td>
<td></td>
</tr>
<tr>
<td>Unit Cell, Å</td>
<td>a = 68.43, b = 80.10, c = 87.36</td>
<td></td>
</tr>
<tr>
<td>α = 111.11°, β = 90.10°, γ = 93.01°</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total no. reflections</td>
<td>72,314</td>
<td></td>
</tr>
<tr>
<td>Unique reflections</td>
<td>41,120</td>
<td></td>
</tr>
<tr>
<td>Average redundancy*</td>
<td>1.7 (1.5)</td>
<td></td>
</tr>
<tr>
<td>Completeness, %*</td>
<td>93.0 (84.7)</td>
<td></td>
</tr>
<tr>
<td>Rmerge, %†</td>
<td>7.4 (31.7)</td>
<td></td>
</tr>
<tr>
<td>Output &lt;I/sigI&gt;*</td>
<td>16 0.1 (2.0)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Refinement</th>
<th>Rwork, %‡</th>
<th>22.08</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rfree, %§</td>
<td>27.57</td>
<td></td>
</tr>
<tr>
<td>rmsd from ideality</td>
<td>Bonds, Å</td>
<td>0.009</td>
</tr>
<tr>
<td>Angles, °</td>
<td>1.235</td>
<td></td>
</tr>
<tr>
<td>Average B-factor, Å²</td>
<td>50.60</td>
<td></td>
</tr>
<tr>
<td>Ramachandran†</td>
<td>Favored, %</td>
<td>93.47</td>
</tr>
<tr>
<td>Outliers, %</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>PDB accession no.</td>
<td>3V3N</td>
<td></td>
</tr>
</tbody>
</table>

*Values for the last shell are in parentheses.
†Rmerge = Σj[Ij − <Ij>]/ΣIj, where Ij is measured intensity for reflections with indexes of hkl.
‡Rwork = Σ|Fo| − |Fc|/Σ|Fo| for all data with |Fo| > 2σ(|Fo|) excluding data to calculate Rfree.
§Rfree = Σ|Fo| − |Fc|/Σ|Fo| for all data with |Fo| > 2σ(|Fo|) excluded from refinement.
{Root mean square deviation.
†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 $V_{\text{max}}/K_m$ ($\text{MCN}$) ($\mu$M$^{-1}$s$^{-1}$)</th>
<th>Predicted $V_{\text{max}}/K_m$ ($\text{MCN}$) ($\mu$M$^{-1}$s$^{-1}$)</th>
<th>Measured $K_m$ ($\text{MCN}$) ($\mu$M)</th>
<th>Predicted $K_m$ ($\mu$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>
</tr>
<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>
</tr>
<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>
</tr>
<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 $K_m$ ($\text{MCN}$) may be underestimated as the assay cannot easily distinguish $K_m$ (< 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$^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TetX2</td>
<td>57.5</td>
<td>2.3</td>
<td>9.6 $\times$0.04</td>
<td>0.173</td>
</tr>
<tr>
<td>T280A</td>
<td>40.1</td>
<td>2.2</td>
<td>9.5 $\times$0.04</td>
<td>0.346</td>
</tr>
<tr>
<td>N371I</td>
<td>51.5</td>
<td>2.4</td>
<td>9.0 $\times$0.04</td>
<td>0.024</td>
</tr>
<tr>
<td>N371T</td>
<td>71.4</td>
<td>2.3</td>
<td>10.0 $\times$0.04</td>
<td>0.052</td>
</tr>
<tr>
<td>S326I</td>
<td>21.8</td>
<td>2.6</td>
<td>6.1 $\times$0.04</td>
<td>0.095</td>
</tr>
<tr>
<td>F235Y</td>
<td>0.1</td>
<td>3.2</td>
<td>1.2 $\times$0.04</td>
<td>0.509</td>
</tr>
<tr>
<td>K64R</td>
<td>50.3</td>
<td>2.3</td>
<td>8.9 $\times$0.04</td>
<td>0.105</td>
</tr>
<tr>
<td>T280S</td>
<td>37.2</td>
<td>2.4</td>
<td>7.3 $\times$0.04</td>
<td>0.538</td>
</tr>
<tr>
<td>Average</td>
<td>41.2</td>
<td>2.5</td>
<td>7.7 $\times$0.04</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>22.2</td>
<td>0.3</td>
<td>2.9 $\times$0.04</td>
<td></td>
</tr>
</tbody>
</table>

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

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