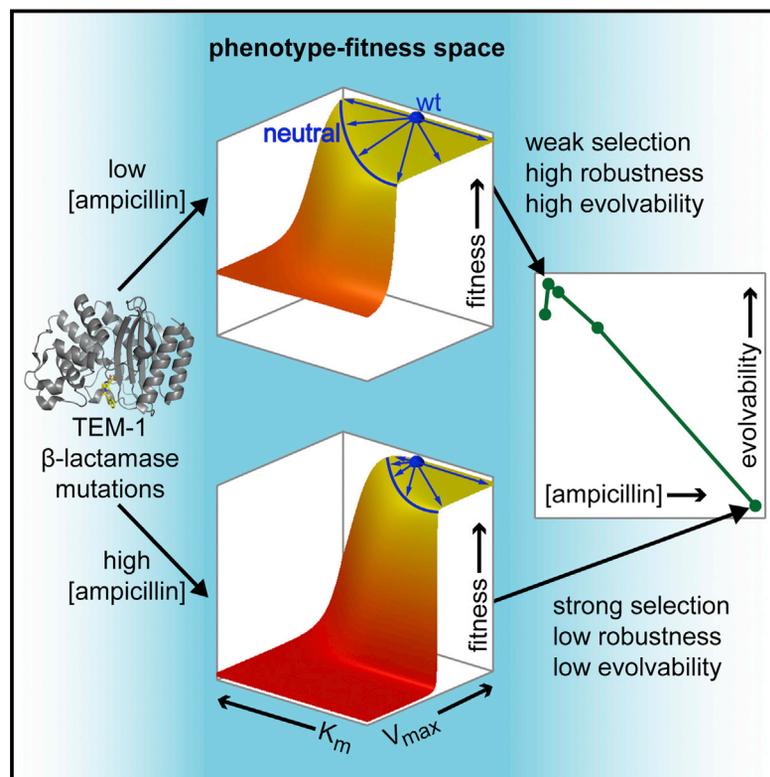


# Evolvability as a Function of Purifying Selection in TEM-1 $\beta$ -Lactamase

## Graphical Abstract



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## In Brief

The origins of evolvability are examined systematically by analyzing single amino acid mutants in an enzyme under selection for a wild-type function (ampicillin resistance) and for a new function (cefotaxime resistance). The findings indicate that fluctuating environments might select for enzymes with excess activity relative to the strength of selection.

## Highlights

- A study of how enzyme robustness and evolvability depend on selection strength.
- Fitness effects of all single amino acid mutations for wild-type and new function.
- Adaptation to new function is enhanced in a weak selection environment.
- A spatially distributed pattern of adaptive mutations in the protein structure.



# Evolvability as a Function of Purifying Selection in TEM-1 $\beta$ -Lactamase

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## SUMMARY

**Evolvability—the capacity to generate beneficial heritable variation—is a central property of biological systems. However, its origins and modulation by environmental factors have not been examined systematically. Here, we analyze the fitness effects of all single mutations in TEM-1  $\beta$ -lactamase (4,997 variants) under selection for the wild-type function (ampicillin resistance) and for a new function (cefotaxime resistance). Tolerance to mutation in this enzyme is bimodal and dependent on the strength of purifying selection in vivo, a result that derives from a steep non-linear ampicillin-dependent relationship between biochemical activity and fitness. Interestingly, cefotaxime resistance emerges from mutations that are neutral at low levels of ampicillin but deleterious at high levels; thus the capacity to evolve new function also depends on the strength of selection. The key property controlling evolvability is an excess of enzymatic activity relative to the strength of selection, suggesting that fluctuating environments might select for high-activity enzymes.**

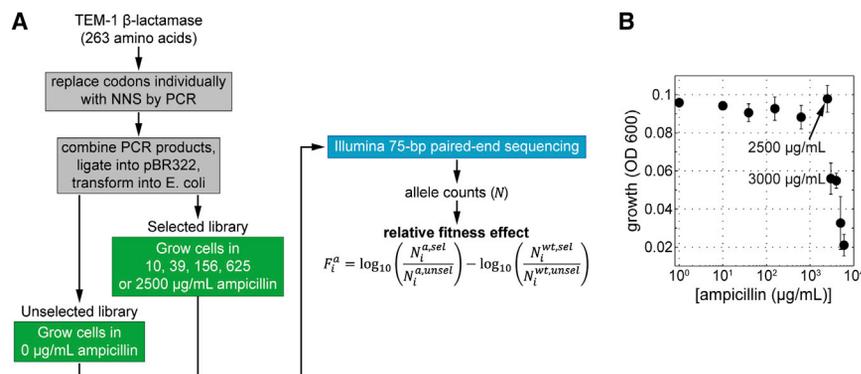
## INTRODUCTION

Biological systems are often regarded as remarkably tolerant of genetic perturbations. Mutational robustness has been observed at nearly all levels of biological organization, from protein structure and function (McLaughlin et al., 2012; Rennell et al., 1991; Suckow et al., 1996) to metabolic flux (Kacser and Burns, 1981) to regulation of gene expression (Wagner, 2005a) to development (Waddington, 1942, 1953). Because processes that buffer the effects of genetic variation inevitably have consequences for evolutionary outcomes, an understanding of both the causes and consequences of robustness is of central importance to biology (de Visser et al., 2003; Masel and Siegal, 2009; Wagner, 2005a). For example, recent studies (Draghi et al., 2010; Hayden et al., 2011; Payne and Wagner, 2014; Wagner, 2008) show that robustness can facilitate the adaptation of biological systems to environmental change—a property sometimes called “evolvability” (Kirschner and Gerhart, 1998). Besides being essential to basic evolutionary theory, understanding robustness

may also be important in the engineering of useful proteins that are more resilient to the effects of random mutations and for understanding the emergence of mutations that impact human health.

Due to their importance in defining phenotypes at the cellular and organismal level, and the ease with which large numbers of mutations can be introduced and assessed, proteins represent ideal model systems for studying robustness and evolvability. The tolerance of many proteins to mutations has been assessed in a number of important studies with high-throughput site-directed and random mutagenesis (Fowler et al., 2010; Guo et al., 2004; Huang et al., 1996; Jacquier et al., 2013; Loeb et al., 1989; McLaughlin et al., 2012; Melnikov et al., 2014; Rennell et al., 1991; Suckow et al., 1996). Overall, these studies suggest that the function of proteins is insensitive to the vast majority of amino acid changes (Bowie et al., 1990). By contrast, it is generally accepted that many missense mutations have measurable biophysical effects (e.g., on protein stability), supporting a view that most mutations are not neutral at the biochemical level (DePristo et al., 2005; Tokuriki and Tawfik, 2009). One possible explanation for this discrepancy is that robustness and evolvability are characteristics that ultimately refer to organismal fitness, a property that is difficult to assess and whose relationship to biochemical parameters of a protein could be complex and is generally unknown. Indeed, many comprehensive studies of mutational tolerance in proteins have assessed biochemical traits (e.g., protein-binding affinity; Fowler et al., 2010; McLaughlin et al., 2012) or other phenotypes (e.g., minimal inhibitory concentration of antibiotic, MIC; Firnberg et al., 2014; Jacquier et al., 2013); although much has been learned from these studies, the relationships between these properties and fitness are less clear.

More fundamentally, it is logical that the relationship between organismal fitness and biochemical parameters might vary significantly with the strength of selective pressure acting on the protein. If so, then robustness and evolvability must not be considered as absolute, invariant features of proteins but instead as properties that depend on environmental or experimental conditions that control purifying selection. To examine these ideas rigorously, we require (1) a quantitative mapping of the relationship between in vivo fitness and in vitro biochemistry in an appropriate model system, (2) a study of how both mutational sensitivity to existing function and the capacity to evolve new function depend upon selection pressure, and (3) a mechanistic principle for how these characteristics emerge from the properties of extant proteins.



**Figure 1. Experimental Scheme**

(A) Strategy for comprehensive assessment of the fitness effects of mutations in TEM-1 (Experimental Procedures and Extended Experimental Procedures). Each codon corresponding to the 263 amino acid positions of the mature TEM-1 protein was individually mutated to NNS (where N is a mixture of all four nucleotide bases, and S a mixture of C and G) using PCR. PCR products were combined in equimolar ratios, cloned into the pBR322 plasmid, and transformed into *E. coli*. The resulting whole-gene saturation mutagenesis library was then selected in ampicillin at either 0  $\mu\text{g/ml}$  (unselected library) or various concentrations (selected library). Illumina 75 bp paired-end sequencing was used to obtain allele counts  $N$  for

each amino acid mutation  $a$  at every position  $i$  and under each selection condition; relative fitness effect  $F_i^a$  is assessed as the logarithmic increase in allele counts in the selected library versus the unselected library, relative to the wild-type allele.

(B) Results of growth assays ( $n = 3$ ) for *E. coli* cells harboring wild-type TEM-1 under selection at various concentrations of ampicillin, indicating that growth is unaffected at ampicillin concentrations  $\leq 2,500 \mu\text{g/ml}$  (Extended Experimental Procedures). See also Figure S1.

The problem of how robustness and evolvability are conditional on selection strength can be investigated in proteins conferring antibiotic resistance by comprehensive experimental assessment of the fitness effects of mutations as a function of antibiotic concentration. Here, we focus on a powerful model system for protein evolution studies, TEM-1  $\beta$ -lactamase (Bershtein et al., 2006; Salverda et al., 2010; Weinreich et al., 2006). TEM-1 is an enzyme that hydrolyzes penicillin-class  $\beta$ -lactam antibiotics (e.g., ampicillin); the ability of bacteria to survive and reproduce (i.e., fitness) in the presence of these antibiotics relies solely on TEM-1 activity (Matagne et al., 1998; Medeiros, 1997). Using an application of deep sequencing (Fowler et al., 2010; McLaughlin et al., 2012), we determined the effects on organismal fitness of all single amino acid mutations in TEM-1 (4,997 mutations) under selection for ampicillin resistance (the wild-type function) and for resistance to cefotaxime (a new function). By assessing fitness under increasing concentration of ampicillin, we demonstrate that robustness indeed depends strongly and inversely on the strength of purifying selection. The pattern of mutational sensitivity is hierarchically organized in the atomic structure, building out from the active site as a function of ampicillin concentration in physically contiguous but anisotropic amino acid networks. Furthermore, we find that in TEM-1, evolvability is facilitated by robustness and therefore is also dependent on the strength of selection; mutations conferring cefotaxime resistance are predominantly neutral under selection at low to moderate ampicillin concentrations yet deleterious to fitness under high concentration. To mechanistically understand these properties, we propose a simple kinetic model that describes the fitness effects of all mutations in our study as a function of ampicillin concentration and intracellular  $\beta$ -lactamase activity. This model shows that, mechanistically, robustness and evolvability can be understood to arise from an excess of intracellular enzymatic activity relative to the strength of selection, a finding that suggests a role for fluctuating environments in the evolution of high-activity enzymes.

## RESULTS

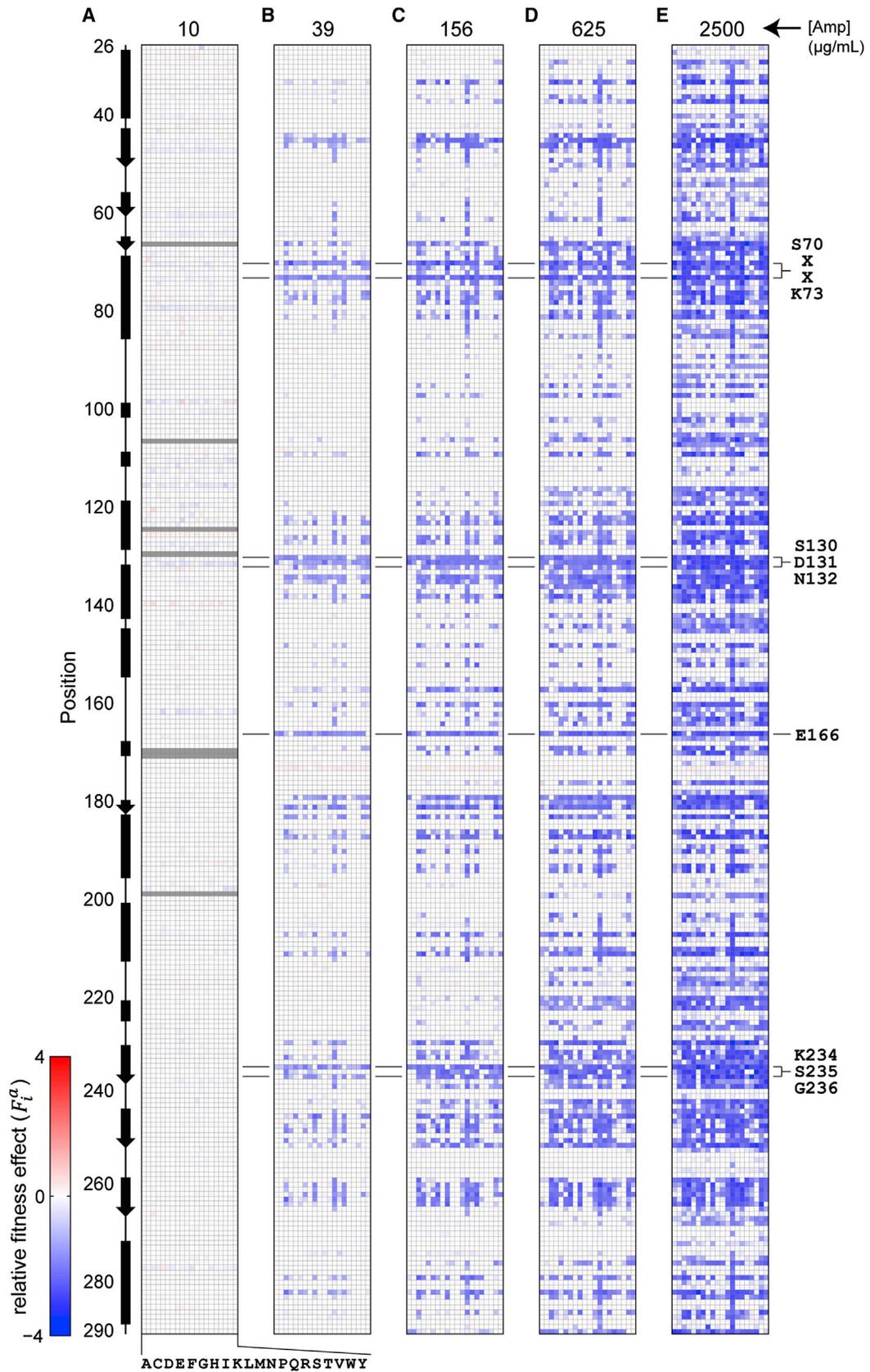
### Whole-Gene Saturation Mutagenesis and Fitness under Ampicillin Selection

We used site-directed mutagenesis to create a whole-gene saturation mutagenesis library comprising all 19 possible single-site amino acid point mutations at every position in the mature form of TEM-1  $\beta$ -lactamase (4,997 amino acid mutations total; Experimental Procedures and Extended Experimental Procedures; Figure 1A). To assess the effects of selection strength on robustness, the library was transformed into *E. coli* and selected at several concentrations of ampicillin ranging from zero to a concentration just below that at which cells encoding even wild-type TEM-1 decline considerably in fitness (2500  $\mu\text{g/ml}$  ampicillin; Extended Experimental Procedures; Figure 1B).

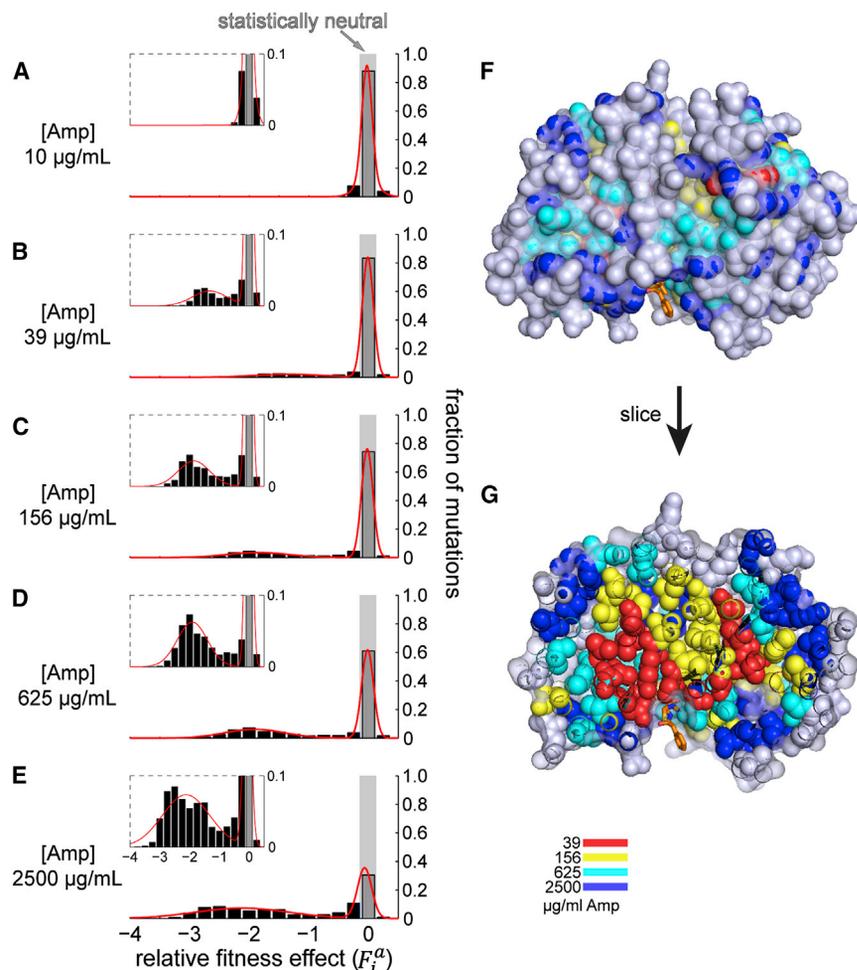
Illumina 75 bp paired-end sequencing was used to obtain counts for each mutant allele after selection at each ampicillin concentration; an average of 1,000 counts per amino acid mutation was obtained under conditions of no selection (0  $\mu\text{g/ml}$  ampicillin; Figure S1A). The relative fitness  $F_i^a$  of each amino acid mutation  $a$  at each position  $i$  is assessed as the logarithm of the allele counts in the selected population ( $N_i^{a,sel}$ ) versus the unselected population (0  $\mu\text{g/ml}$  ampicillin,  $N_i^{a,unsel}$ ), relative to the wild-type allele:

$$F_i^a = \log_{10} \left[ \frac{N_i^{a,sel}}{N_i^{a,unsel}} \right] - \log_{10} \left[ \frac{N_i^{wt,sel}}{N_i^{wt,unsel}} \right]. \quad (1)$$

Mutations that show no fitness effect have values of  $F_i^a$  close to that of wild-type ( $F_i^a \approx 0$ ), and those with an increase or decrease in fitness relative to wild-type have a positive or negative value of  $F_i^a$ , respectively, in proportion to their effect. The values of  $F_i^a$  are generally reproducible over two independent trials ( $r^2 = 0.91$  at 2500  $\mu\text{g/ml}$  ampicillin; Figure S1B), and effects due to codon bias under ampicillin selection appear to be small ( $r^2 = 0.96$  for relative fitness between all synonymous mutations at 2500  $\mu\text{g/ml}$  ampicillin; Figure S1C). Examination of the distribution of  $F_i^a$  for all mutations under no selection (0  $\mu\text{g/ml}$  ampicillin) provides a



(legend on next page)



**Figure 3. Distribution of Fitness Effects**

(A–E) Histograms of  $F_i^a$  values show that the distribution of the fitness effects (DFE) is bimodal and depends on the strength of purifying selection: (A) 10, (B) 39, (C) 156, (D) 625, and (E) 2,500  $\mu\text{g}/\text{mL}$  ampicillin. Red lines are heuristic fits to a bi-Gaussian function. The range of  $F_i^a$  that corresponds to a statistically neutral fitness effect is indicated in gray. Insets show DFEs enlarged over the range 0–0.1.

(F and G) The pattern of mutational sensitivity involves spatially heterogeneous yet physically connected networks of residues, building out from the active site and core as the strength of purifying selection is increased. Shown are (F) surface and (G) slice representations of the pattern of sensitivity to single-site amino acid point mutations at each ampicillin concentration mapped onto the structure of TEM-1 (PDB: 1FQG). Colored spheres indicate residues with a significant positional fitness effect (see Figure S2); different colors indicate results at each ampicillin concentration. Co-crystallized  $\beta$ -lactam (penicillin G) is shown as yellow stick bonds. See also Figure S2 and Table S2.

subset of more moderately conserved positions distributed within the protein core (Figures 3F and 3G). As the ampicillin concentration used for selection is further increased, the overall fitness cost of mutations dramatically increases—both in the number of mutations that show a fitness effect and in the degree of their effect relative to wild-type (Figures 2C–2E and 3C–3E). To some extent,

basis for defining cutoffs for  $F_i^a$  corresponding to statistically neutral effects on fitness (mean  $\pm$  two SD in  $F_i^a$ ; Figure S1D).

### Robustness Is Conditional on the Strength of Purifying Selection

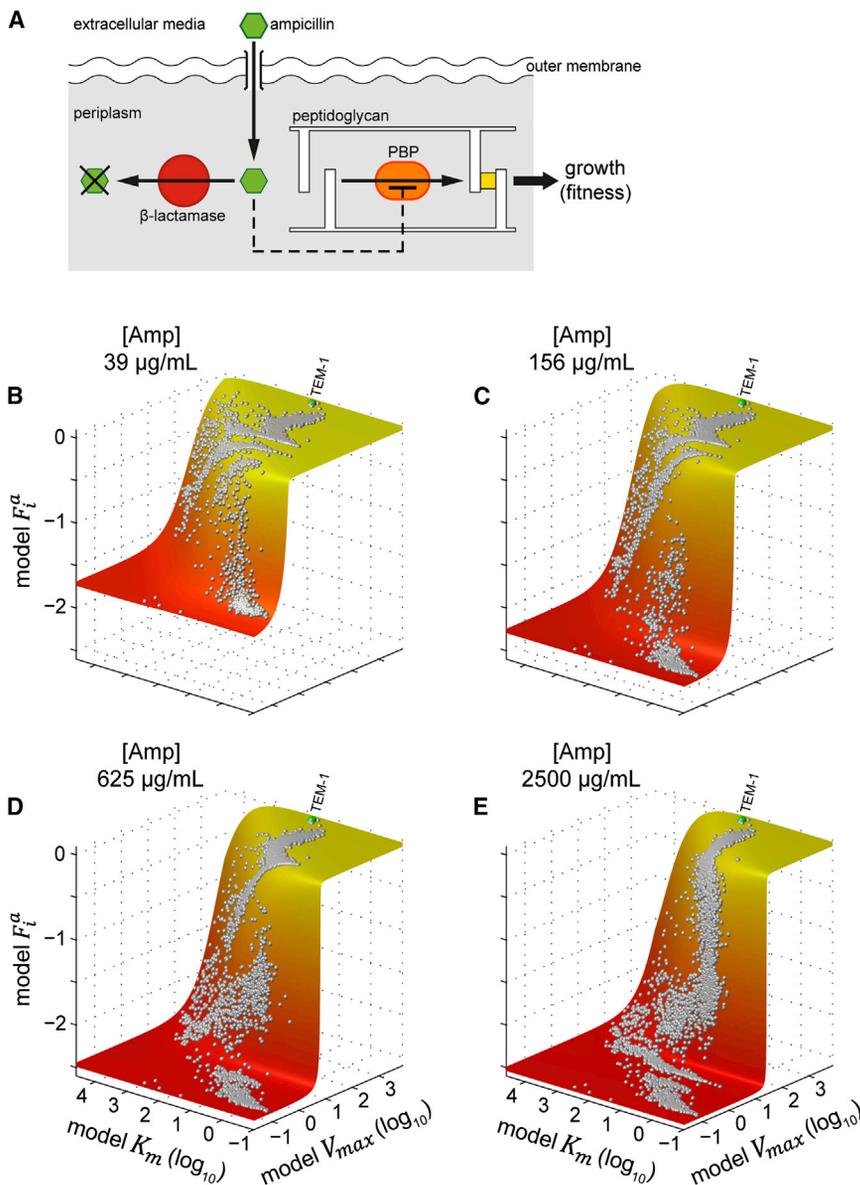
Figure 2 shows the fitness effect of all single amino acid mutations in TEM-1 at all ampicillin concentrations examined (10, 39, 156, 625, and 2500  $\mu\text{g}/\text{mL}$ ; see also Table S1). Under weak selection at a low ampicillin concentration (39  $\mu\text{g}/\text{mL}$ ; no selection was observed at 10  $\mu\text{g}/\text{mL}$ ; Figures 2A and 3A), the vast majority of mutations are statistically neutral ( $F_i^a \approx 0$ ), and only a small fraction of mutations significantly affect fitness (Figures 2B and 3B). These include highly conserved positions within the active site (S70, K73, S130, D131, N132, K234, and G236; numbering according to Ambler; Ambler et al., 1991) but also a

these results seem obvious; it is reasonable to expect that the fitness cost of mutations in an enzyme will depend on the strength of purifying selection for its function. In contrast, no single mutations at any concentration significantly increase fitness, indicating that TEM-1 occupies a local peak in its genotype-fitness landscape under the conditions of these experiments.

More interestingly, the distribution of the fitness effects of mutations (DFE) is decidedly bimodal, with one mode corresponding to mutations with significant deleterious effects on fitness, and the other mode comprising those with neutral or nearly neutral fitness effects (Figures 3A–3E and Table S2). The strength of ampicillin selection controls the fraction of mutations in these two modes; increasing antibiotic concentration causes the relative proportion of mutations in the mode with deleterious mutations to increase and in the mode with neutral/nearly neutral

**Figure 2. Fitness Effects of All Single Amino Acid Mutations in TEM-1 under Increasing Ampicillin Selection**

Shown are the data matrices containing the relative fitness effect for every amino acid mutation at every position in TEM-1 under selection with ampicillin at (A) 10, (B) 39, (C) 156, (D) 625, or (E) 2,500  $\mu\text{g}/\text{mL}$ . Rows within each matrix depict positions along the primary sequence of TEM-1, and columns indicate a mutation to one of the 20 amino acids (in alphabetical order by one-letter code indicated at bottom of A). Relative fitness effect is depicted colorimetrically with blue representing a deleterious fitness effect, red a positive fitness effect, and white no fitness effect relative to wild-type. Positions for which no data were obtained at 10  $\mu\text{g}/\text{mL}$  ampicillin are colored gray. The secondary structure of the wild-type sequence is indicated to left of (A). Several highly conserved motifs within the active site (S70-X-X-K73, S130-D131-N132, E166, and K234-S235-G236) are indicated to the right of (E). See also Table S1.



**Figure 4. A Phenotype-Fitness Landscape for TEM-1**

(A) Overview of the kinetic model describing fitness as a function of ampicillin concentration and intracellular  $\beta$ -lactamase activity (Experimental Procedures and Extended Experimental Procedures). The periplasmic concentration of ampicillin (green) is determined by the rate of passive diffusion across the outer membrane and the rate of inactivation (hydrolysis, denoted by X) by  $\beta$ -lactamase (red). Growth (i.e., fitness) is proportional to the rate of peptidoglycan strand-joining (yellow) by PBPs (orange); PBP activity, and thus fitness, is inhibited (dashed line) by unhydrolyzed ampicillin in the periplasm.

(B–E) Surface representation of the model revealing a non-linear relationship between phenotype (intracellular  $\beta$ -lactamase activity:  $K_m$  and  $V_{max}$ ) and fitness marked by a plateau at neutrality ( $F_i^a \approx 0$ ) that is dependent on the strength of selection: (B) 39, (C) 156, (D) 625, and (E) 2,500  $\mu\text{g/ml}$  ampicillin. Model estimated values of kinetic parameters and fitness for wild-type TEM-1 (green sphere) and all 4,997 single mutations (silver spheres) are shown.

See also Figure S3 and Table S3.

selection (39  $\mu\text{g/ml}$  ampicillin), mutation-sensitive positions comprise a physically contiguous but anisotropic network of residues buried within the protein core and extending out from the active site. As the level of selective pressure is increased, this main “functional core” acquires successive shells of mutation-sensitive residues that grow outward until nearly the whole protein core shows some degree of fitness cost upon mutation. Nevertheless, the heterogeneity in positional contribution to fitness persists even at the highest levels of ampicillin; the most mutationally sensitive positions at 2500  $\mu\text{g/ml}$  ampicillin are similar to

mutations to decrease. In addition, to different extents, both modes shift toward a greater average fitness cost as a function of ampicillin concentration (Table S2). Thus, these data (Figures 2 and 3) reveal that robustness in TEM-1 is strongly dependent on the strength of selection—under weak selection, TEM-1 is more robust as most mutations are neutral or nearly neutral in their fitness effects. But as selection is increased, robustness concomitantly decreases as more and more mutations become deleterious to fitness.

Mapping of the data onto an atomic structure of TEM-1 reveals a spatially anisotropic pattern of amino acid contributions to organismal fitness (Figures 3F and 3G). Here, we describe the average fitness cost of all mutations at each position ( $\langle F_i^a \rangle_a$ ) at each ampicillin concentration; for comparison, positions sensitive to mutation are defined based on the distribution of  $\langle F_i^a \rangle_a$  under selection at 2500  $\mu\text{g/ml}$  ampicillin (Figure S2). Under weak

the functional core defined at 39  $\mu\text{g/ml}$  ampicillin (Figure 7, compare panels D and F).

### Robustness as an Excess of Intracellular Enzymatic Activity Relative to the Strength of Purifying Selection

To study the mechanistic basis for both the dependency of robustness on selection strength and the bimodality of the DFE, we developed a simple kinetic model describing relative fitness as a function of ampicillin concentration and intracellular  $\beta$ -lactamase activity (Figure 4A, Experimental Procedures, and Extended Experimental Procedures). In this model, organismal fitness is proportional to the flux of peptidoglycan substrate through DD-carboxypeptidases and other penicillin-binding proteins (PBPs) involved in cell-wall biogenesis, with ampicillin acting as a competitive inhibitor of this process. The periplasmic concentration of ampicillin is dynamically set by the balance of

intracellular  $\beta$ -lactamase activity and passive diffusion of ampicillin across the outer membrane (Zimmermann and Rosselet, 1977). We determined values for the free parameters of our model by globally fitting the experimental data obtained for all 4,997 mutations (Extended Experimental Procedures and Table S3). The free parameters uniquely converge, and the model fits the data well with an overall  $r^2 = 0.98$ .

The main result of our model is the finding of a non-linear phenotype-fitness relationship in which fitness saturates at different levels of enzyme activity as a function of the concentration of applied ampicillin (Figures 4B–4E). The non-linearity arises from two sources: the steady state achieved between diffusion and hydrolysis of ampicillin and the competitive inhibition of the PBPs. This result provides a simple explanation for both the dependence of mutational robustness on selection strength and the bimodal nature of the DFE. In the model, enzyme activity of every mutant is defined by two parameters, the maximum intracellular rate of ampicillin hydrolysis ( $V_{\max}$ ) and the concentration of ampicillin that produces half-maximal rate ( $K_m$ ). Due to the high overall activity of TEM-1 for ampicillin, the wild-type enzyme resides well within the saturated regime (plateau) of the fitness-activity relationship. Mutational robustness emerges as a consequence because within the saturated region, changes in enzyme activity due to mutation have negligible effects on fitness (de Visser et al., 2003; Hartl et al., 1985; Kacser and Burns, 1981; Wagner, 2005a). The model is also consistent with the dependence of robustness on the strength of selection because fitness saturates at increasingly higher levels of activity as the ampicillin concentration increases (Figures 4B–4E, S3A, and S3B). That is, robustness collapses steadily with increasing selection pressure because the fitness-activity relationship depends on the steady-state levels of ampicillin in the periplasm. Finally, the bimodal nature of the fitness effects of mutations can be understood as a direct consequence of the steep non-linearity relating enzyme activity to fitness. For example, mutational variation in enzyme activity has basically two outcomes: to stay in front of the non-linearity and have a relatively negligible effect on fitness, or to cross the non-linearity “threshold” and display a profound effect on fitness (see Figures S3C–S3G for a simulation). Further contribution could come from inherent non-linearities in the effects of mutations on TEM-1 activity, but the model shows that such is not required for bimodality in the DFE. In short, the model shows that robustness and its dependency on the strength of selection arise from the high intracellular activity of TEM-1 and the ampicillin-dependent non-linear saturation relationship between enzyme activity and fitness.

A general model for mutational robustness in proteins has been previously proposed based on the thermodynamic stability of proteins. The idea is that the “extra” stability beyond that required to asymptotically populate the native state provides a thermodynamic basis for robustness by buffering the slightly destabilizing effects of most mutations (Bloom et al., 2005; Tokuriki and Tawfik, 2009; Wylie and Shakhnovich, 2011). We note that our conclusions are not inconsistent with this view; the overall rate of ampicillin hydrolysis in vivo is a combination of both the fraction of natively folded  $\beta$ -lactamase protein and the specific activity of the native state, and mutations could influence either

or both properties. More generally, we propose that robustness comes from an excess of intracellular enzymatic activity relative to the fitness threshold present at a particular strength of selection—a description that combines the probability of native-state folding with the biochemical parameters controlling catalytic power and accounts for the dependency of robustness on the strength of selection.

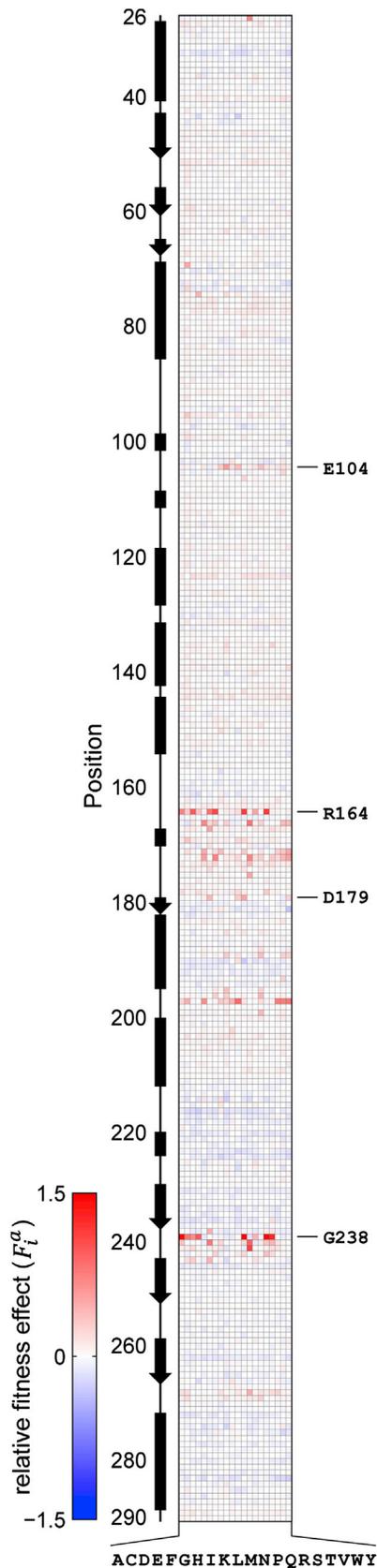
### Evolvability and Fitness under Cefotaxime Selection

Robustness implies invariance of organismal fitness upon mutation, which at first glance suggests that robust biological systems should have a decreased ability to evolve new phenotypes, or decreased evolvability. On the other hand, mutations that are neutral with regard to the current or wild-type function might be able to promote new functions; indeed, such “conditional neutrality,” whether with respect to genetic background or environmental factors, has been suggested to facilitate evolvability by permitting the accumulation of mutations that could be useful upon changes in selective pressure (de Visser et al., 2003; Kirschner and Gerhart, 1998, 2005; Masel and Trotter, 2010; Wagner, 2005a, b). To assess the relationship between robustness and evolvability in TEM-1, we performed selection on our whole-gene saturation mutagenesis library in the presence of a different  $\beta$ -lactam drug, cefotaxime. Cefotaxime is a poor substrate for TEM-1, with an approximately 1000-fold decrease in  $k_{cat}/K_m$  for cefotaxime versus ampicillin ( $\approx 10^4$  versus  $\approx 10^7$   $M^{-1}s^{-1}$ , respectively). As such, TEM-1 imparts no significant fitness advantage; the minimal inhibitory concentration (MIC) of cefotaxime for *E. coli* cells encoding wild-type TEM-1 is essentially unchanged from that of cells without  $\beta$ -lactamase (0.0625  $\mu g/ml$ ) (Hall, 2002). However, single amino acid changes in TEM-1 are known to increase resistance to cefotaxime both in nature and in the laboratory (Matagne et al., 1998; Salverda et al., 2010). Selection was performed at 0.15  $\mu g/ml$  cefotaxime (approximately double the MIC for TEM-1), and the fitness of each mutation relative to wild-type TEM-1 determined as described above.

Figure 5 shows the fitness effect of all single mutations under cefotaxime selection, and Figure S4 shows the corresponding DFE (see also Table S4). In contrast to the results obtained under ampicillin selection, no mutations show a significant fitness decrease relative to wild-type TEM-1, a result that simply reflects the already poor activity of TEM-1 on cefotaxime. However, a small number of mutations (106 total, or 2%) act to increase fitness; among these we observe alleles previously reported to impart an extended-spectrum phenotype in both TEM-1 and/or its homolog SHV-1 in clinical isolates (E104K, R164H, R164S, D179G, D179N, G238A, G238S) (Matagne et al., 1998; Salverda et al., 2010) and error-prone PCR libraries (Bershtein and Tawfik, 2008; Schenk et al., 2012).

### Robustness and Adaptation in TEM-1

A comparison of the relative fitness effects obtained for each mutation at each ampicillin concentration versus their respective effects under cefotaxime selection reveals how the robustness of TEM-1 under ampicillin selection (the current or wild-type function) relates to its evolvability toward cefotaxime resistance (a new function) (Figures 6A–6D). Under weak selection for ampicillin resistance (e.g., 39  $\mu g/ml$  ampicillin, Figure 6A), nearly all



**Figure 5. Fitness Effects of All Single Amino Acid Mutations in TEM-1 under Cefotaxime Selection**

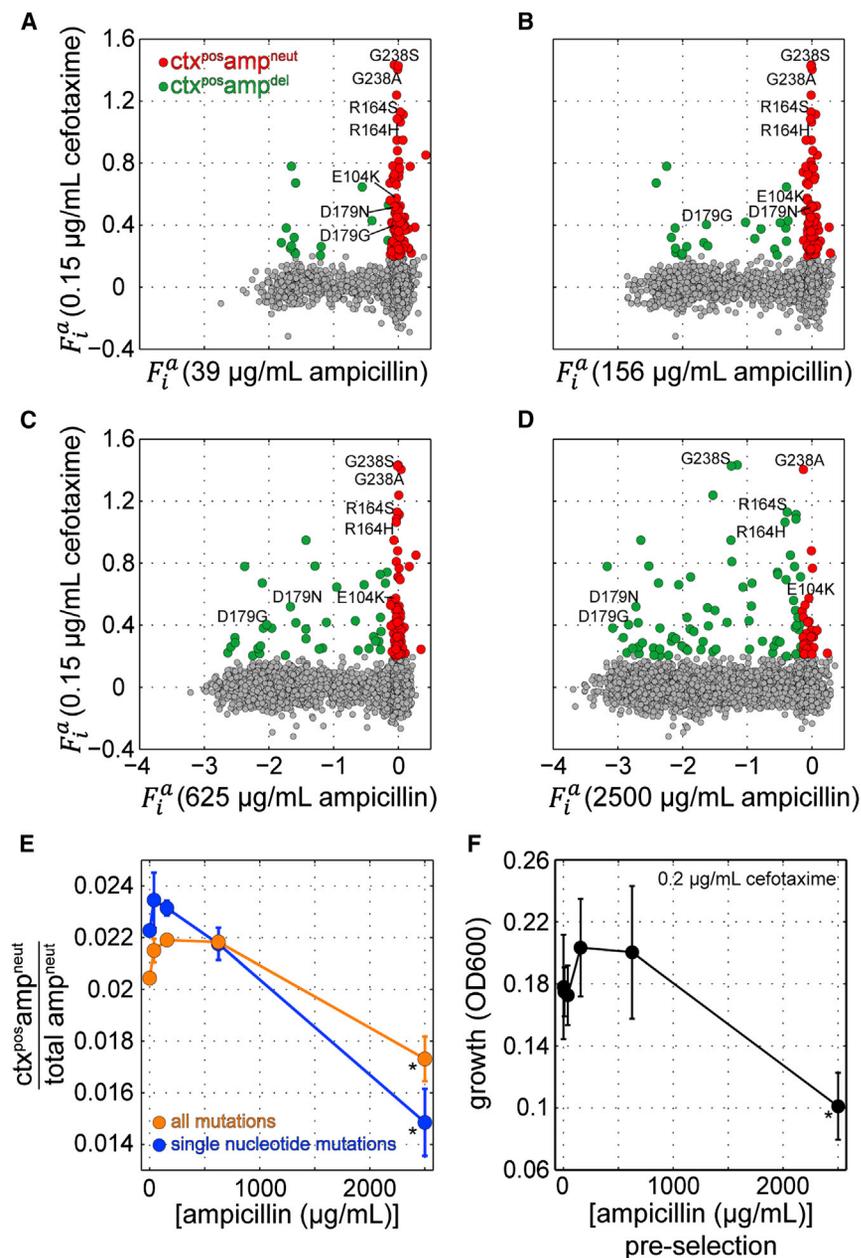
Shown is the data matrix depicting  $F_i^a$  for each amino acid mutation at each position in TEM-1 under selection with the non-optimal TEM-1 substrate cefotaxime at 0.15  $\mu\text{g}/\text{ml}$ . Labels and coloring are as in Figure 2. Several positions previously reported to confer increased cefotaxime resistance upon mutation in TEM-1 or its homolog SHV-1 are indicated at right (E104, R164, D179, and G238). See also Figure S4 and Table S4.

mutations conferring significant cefotaxime resistance are statistically neutral in their fitness effect in ampicillin; this includes all the above-stated mutations found in clinical isolates. These mutations are said to be conditionally neutral with respect to the environment (as their phenotypes depend on the condition of selection) and have been linked to the rate of evolution of new phenotypes (Draghi et al., 2011; Wagner, 2005b). However, under selection at increasing ampicillin concentration, mutations conferring cefotaxime resistance have progressively deleterious fitness effects in ampicillin; for example, the mutations R164H, R164S, D179G, D179N, and G238S, which are neutral at 39  $\mu\text{g}/\text{ml}$  ampicillin, now have significant deleterious fitness effects at 2,500  $\mu\text{g}/\text{ml}$  ampicillin (Figure 6D). That is, the neutrality of these mutations is itself conditional on the strength of selection, present at low levels of ampicillin and diminishing at higher levels. Thus, for TEM-1, more useful genetic variation (mutations conferring cefotaxime resistance) is proportionately available when robustness is high (weak ampicillin selection) than when robustness is low (strong ampicillin selection; Figure 6E).

To test this in an independent experiment, we created a library of TEM-1 variants by error-prone PCR (average of  $1 \pm 1$  mutations per gene, Extended Experimental Procedures), transformed into *E. coli*, and screened for growth of the population on cefotaxime (0.2  $\mu\text{g}/\text{ml}$ ) either with prior selection (Figures 6F, S5A, and S5B) or with co-selection (Figures S5C and S5D) on varying doses of ampicillin (Extended Experimental Procedures). The data show that in fact, the growth of the population of TEM-1 variants on cefotaxime is a function of ampicillin exposure and very nearly reflects the fraction of mutations that confer cefotaxime resistance but are statistically neutral for ampicillin in the comprehensive single-mutation library (Figure 6E). We conclude that in TEM-1, robustness enhances evolvability by permitting environmentally conditional neutral mutations that can confer cefotaxime resistance.

### The Spatial Distribution of Cefotaxime Adaptation in TEM-1

Mutations conferring initial cefotaxime resistance are distributed broadly throughout the tertiary structure of TEM-1 and include residues both in the core and on the surface of the protein (residues in dark gray, Figure 7). However, there is an interesting and physically informative pattern to the organization of these mutations. Few cefotaxime-adaptive mutations are directly within the functional core defined by residues with significant fitness effects at 39  $\mu\text{g}/\text{ml}$  ampicillin (residues in red, Figures 3G, 7A, and 7D), consistent with the finding that they are largely neutral in fitness effect under weak ampicillin selection (Figure 6A). But remarkably, the mutations conferring cefotaxime resistance are organized into sparse, physically connected networks of amino acids that connect a subset of surface positions to the functional



**Figure 6. Robustness and Evolvability of TEM-1**

(A–D) Comparison of  $F_i^\alpha$  values for all mutations under ampicillin (x axis) versus cefotaxime selection (0.15  $\mu\text{g/mL}$ ; y axis) shows that most mutations conferring significant cefotaxime resistance are statistically neutral in fitness effect at a low ampicillin concentration but deleterious at the highest ampicillin concentration. Mutations that confer cefotaxime resistance and are either statistically neutral ( $\text{ctx}^{\text{pos}}\text{amp}^{\text{neut}}$ ) or deleterious ( $\text{ctx}^{\text{pos}}\text{amp}^{\text{del}}$ ) in fitness effect under selection at the indicated ampicillin concentration are colored red and green, respectively: (A) 39, (B) 156, (C) 625, and (D) 2,500  $\mu\text{g/mL}$  ampicillin. Several mutations previously reported to impart an “extended-spectrum” phenotype in both TEM-1 and/or its homolog SHV-1 in clinical isolates are indicated.

(E) A comparison of the fraction of statistically neutral mutations that confer cefotaxime resistance at each ampicillin concentration as determined from the whole-gene saturation mutagenesis data. Results are shown as the mean and SD from two independent ampicillin selection experiments for all mutations (orange) or those obtainable by single-nucleotide mutation from TEM-1 (blue).

(F) Results of growth experiments ( $n = 6$ ) in which an error-prone PCR library of TEM-1 was subjected to pre-selection at several concentrations of ampicillin followed by cefotaxime selection (Extended Experimental Procedures). In (E) and (F), asterisks denote that results at 2,500  $\mu\text{g/mL}$  ampicillin are significantly different from those at all other ampicillin concentrations (one-way ANOVA and post-hoc Tukey test,  $p < 0.05$ ). See also Figure S5.

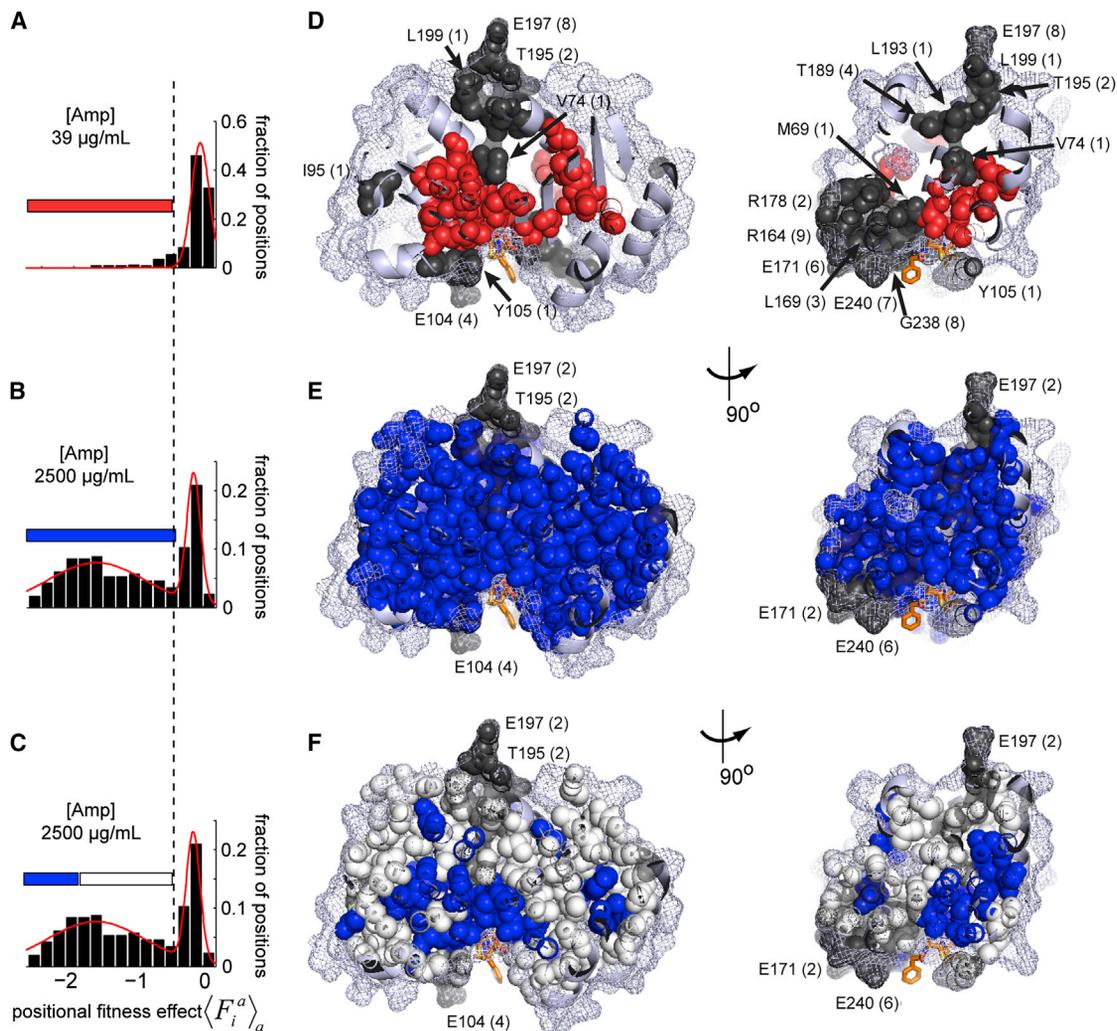
some surface positions (e.g., E104, T195, E197, E240) still maintain the capacity for initiation of cefotaxime resistance while remaining neutral over the full range of ampicillin concentrations examined here.

## DISCUSSION

In summary, these results show that both the robustness and evolvability of TEM-1

are not invariant properties of the protein but instead are dependent on the strength of purifying selective pressure applied. At low doses of ampicillin, the high activity of TEM-1 and the nonlinear dependence of fitness on enzyme activity render many mutations statistically neutral in their fitness effects under moderate selection conditions. Under these conditions, we find that the constraints on fitness are loaded in a small, physically connected network of residues in the TEM-1 tertiary structure—a functional core—that is built around and extending from the active site. Interestingly, a small fraction of positions showing neutral variation display the capacity to confer increased resistance to a new  $\beta$ -lactam drug, cefotaxime, upon single amino acid mutation. These positions comprise contiguous amino acid networks

core (Figure 7D). The physical interpretation of such “pathways” of amino acid connectivity remain to be established but suggest the possibility that these represent anisotropic collective modes in the protein structure that functionally connect a small set of surface sites to the protein active site (Lee et al., 2008; Reynolds et al., 2011). This architecture makes it so that about 50% of cefotaxime-adaptive single mutations (53/106 total) occur at surface positions far from the active site. The effect of selection under high levels of ampicillin is to reduce the set of mutations available for conferring cefotaxime resistance by reducing the likelihood of those that occur within the protein core and near to the active site (Figures 6D, 7B, 7C, 7E, and 7F). Nevertheless, the distributed architecture of adaptive mutations is such that



**Figure 7. Structural Relationship of Robustness and Evolvability**

(A–C) Shown are histograms of the average fitness effects of mutations at each position  $\langle F_i^a \rangle_a$  under selection at (A) 39  $\mu\text{g}/\mu\text{L}$  and (B and C) 2,500  $\mu\text{g}/\text{mL}$  ampicillin (see Figure S2). Red lines are fits to a double Gaussian function. The dashed line indicates a  $\langle F_i^a \rangle_a$  cutoff for positions with significant mutational sensitivity (see Figure S2), whereas colored bars indicate the range of  $\langle F_i^a \rangle_a$  for positions shown on structures in panels to the right (note the lower range in C).

(D–F) The spatial pattern of adaptability toward cefotaxime (dark gray spheres) in the context of mutational sensitivity under ampicillin selection at (D) 39  $\mu\text{g}/\mu\text{L}$  (red spheres) or (E and F) 2,500  $\mu\text{g}/\text{mL}$  (blue or white/blue spheres); shown are slices through the core of the TEM-1 protein, and the surface of TEM-1 is in mesh. The number of adaptive mutations per position is in parentheses (note that not all positions conferring cefotaxime resistance are labeled). The data show that adaptation to cefotaxime resistance in TEM-1 arises from different physically contiguous networks of residues that connect specific distal sites to the core catalytic and structural residues defined by mutational sensitivity at low levels of ampicillin selection (red, D). At high levels of ampicillin (B and E), most core positions display a significant fitness cost upon mutation, reducing the number of cefotaxime-adaptive but ampicillin-neutral mutations. The pattern of mutational sensitivity remains heterogeneous even at high ampicillin levels (C and F), with positions showing the largest fitness cost similar to those with a significant fitness cost at low ampicillin (compare D and F).

that extend from the functional core to connect the active site to a number of distantly positioned surface positions at which cefotaxime resistance can be acquired. The robustness of TEM-1, however, collapses at high levels of ampicillin, as more and more mutations are drawn into the regime where they affect organismal fitness. With the loss of robustness, the capacity for adaptation to cefotaxime is also much reduced as many adaptive mutations that were neutral at low ampicillin concentration now have a significant impact on fitness. It is important to note that these data address only the initial single mutation step toward

the evolution of new function and with only two substrates; it will be interesting to examine how paths of higher-order mutations and robustness and evolvability under different  $\beta$ -lactam selection regimes are related to the functional and adaptive architecture of TEM-1 described by the global saturation mutagenesis data presented here.

The key property underlying both robustness and evolvability of TEM-1 is the high activity of the enzyme *in vivo*—the enzyme sits far along the saturated region in the activity–fitness relationship (Figure 4). In much the same way that an excess of

thermodynamic stability has been associated with enhanced protein robustness and evolvability (Bloom et al., 2006; Bloom et al., 2005; Tokuriki and Tawfik, 2009), our results here make the more general point that an excess of activity in vivo (from high catalytic activity or in vivo enzyme concentration, or both) may underlie both of these properties.

Importantly, these findings suggest new testable hypotheses for how enzymes like TEM-1 could be selected to have such high enzymatic activity. TEM-1 is an example of a so-called perfect enzyme, catalyzing the hydrolysis of penicillin-class antibiotics near the diffusion-controlled limit (Matagne et al., 1998). Why should it be so highly active if organismal fitness can saturate at much lower levels of activity? One obvious hypothesis is simply that TEM-1 may have evolved a high activity phenotype because of direct selection under periods of high concentrations of penicillin class  $\beta$ -lactam compounds (e.g., ampicillin) or under conditions of high mutation rate (Wilke et al., 2001). However, the finding that cefotaxime resistance in TEM-1 predominantly emerges from mutations that are neutral under ampicillin selection at low to moderate doses suggests that high activity could also result indirectly due to selection for evolvability. In this scenario, ancestral TEM-1 also randomly encountered other non-optimal  $\beta$ -lactams (e.g., cephalosporins). Such conditions would favor evolvable variants, those by mutation capable of conferring resistance to non-optimal  $\beta$ -lactams while still maintaining high fitness in the presence of ampicillin. Given that the data show that high in vivo enzyme activity underlies robustness, and robustness in turn promotes new activities through harboring conditionally neutral mutations, it thus follows that an enzyme with high activity would be evolutionarily favored under fluctuations in the distribution of  $\beta$ -lactam substrates. Thus, mechanisms that promote evolvability could be selected as a result of their success under historical environmental fluctuations (Kirschner and Gerhart, 1998, 2005). Future work will experimentally address the notion that the history of environmental fluctuations fundamentally defines the robustness and evolvability of natural proteins.

## EXPERIMENTAL PROCEDURES

### Whole-Genome Saturation Mutagenesis Library Construction

A comprehensive whole-genome saturation mutagenesis library was constructed by an overlap extension PCR mutagenesis technique (Higuchi et al., 1988; McLaughlin et al., 2012). To permit full coverage of the *bla*<sub>TEM-1</sub> coding region (789 bp) with 80 base paired-end reads by Illumina sequencing, we split the sequence into ten subgroups (amino acid positions 26–51, 52–78, 79–104, 105–132, 133–156, 157–183, 184–209, 210–236, 237–264, and 265–290). The mutagenesis PCR products for the positions of each subgroup were mixed in equimolar ratios and ligated as a single library. Each subgroup was independently subject to selection in antibiotic and sample preparation for Illumina sequencing. A detailed description of the cloning procedure is provided in the [Extended Experimental Procedures](#).

### Antibiotic Selection

All selection experiments for the whole-genome saturation mutagenesis library were performed in *E. coli* MegaX DH10B T1 (Invitrogen). Selection was performed in 96-well deep-well plate format at 37°C in Luria-Bertani broth (Fisher Scientific) containing 12  $\mu$ g/ml tetracycline hydrochloride (Sigma). Wells contained either 0, 10, 39, 156, 625, or 2,500  $\mu$ g/ml of ampicillin or 0.15  $\mu$ g/ml cefotaxime at 25-fold concentration. The duration of growth (~2 hr) was chosen to obtain significant selection while maintaining sufficient population size

(~10<sup>8</sup> cells) relative to the library diversity and avoiding stationary growth. Details are provided in the [Extended Experimental Procedures](#).

### Illumina Sequencing

Samples for Illumina sequencing were prepared by PCR from libraries after antibiotic selection as previously described (McLaughlin et al., 2012). Addition of adaptor sequences for Illumina sequencing was performed in two rounds: the first round amplifies the mutated region of TEM-1, adds the annealing site for the Illumina paired-end sequencing primer, and incorporates a 4 bp barcode to indicate the concentration of antibiotic. The second round adds the remainder of the sequencing primer annealing site along with the annealing site for the Illumina flow cell. Primer sequences are available upon request. Illumina sequencing and determination of allele counts were performed as previously described (McLaughlin et al., 2012). Sequencing was performed at the UT Southwestern Genomics Core on an Illumina Genome Analyzer Ix using a version 4 paired-end PE-75 flow cell. Sequences from the Illumina base-caller were imported into CLC Genomics Workbench and trimmed for size and quality using a cutoff of 0.01 for the modified Mott algorithm. Custom scripts written in MATLAB were used to count the number of each allele under each selection condition and to determine relative fitness values. MATLAB scripts are available upon request.

### Mechanistic Model

A detailed description of the model describing relative fitness as a function of intracellular  $\beta$ -lactamase activity and ampicillin concentration is provided in the [Extended Experimental Procedures](#).  $\beta$ -lactam antibiotics inhibit bacterial growth through competitive inhibition of DD-carboxypeptidases and other PBPs involved in synthesis of the peptidoglycan layer of bacterial cell walls. In the model, relative fitness ( $F_i^p$ ) is proportional to the difference in PBP activity in the presence of mutant versus wild-type TEM-1. PBP activity is described according to Michaelis-Menten kinetics, modified to include competitive inhibition by ampicillin. The periplasmic concentration of ampicillin is determined by the equilibrium between the flux of antibiotic across the outer membrane and its rate of hydrolysis (inactivation) by  $\beta$ -lactamase (Zimmermann and Rosselet, 1977). We determined values for the free parameters of our model (total of 9,998 free parameters for 29,849 data points) by fitting the experimental data obtained for all 4,997 mutations using a Monte Carlo simulated annealing (MCSA) procedure; the model fits the data well with an overall  $r^2 = 0.9767$ .

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2015.01.035>.

## AUTHOR CONTRIBUTIONS

M.A.S. and R.R. developed the research plan and experimental strategy. M.A.S. performed all experiments. M.A.S. and D.R.H. developed the kinetic model. M.A.S., D.R.H., and R.R. interpreted the data and wrote the paper.

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