Adaptive Processes Change as Multiple Functions Evolve

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ABSTRACT Epistasis influences the gene-environment interactions that shape bacterial fitness through antibiotic exposure, which can ultimately affect the availability of certain resistance phenotypes to bacteria. The substitutions present within *bla*_{TEM-50} confer both cephalosporin and β -lactamase inhibitor resistance. We wanted to compare the evolution of *bla*_{TEM-50} with that of another variant, *bla*_{TEM-85}, which differs in that *bla*_{TEM-85} contains only substitutions that contribute to cephalosporin resistance. Differences between the landscapes and epistatic interactions of these TEM variants are important for understanding their separate evolutionary responses to antibiotics. We hypothesized the substitutions within *bla*_{TEM-50} would result in more epistatic interactions than for *bla*_{TEM-85}. As expected, we found more epistatic interactions between the substitutions present in *bla*_{TEM-50} than in *bla*_{TEM-85}. Our results suggest that selection from many cephalosporins is required to achieve the full potential resistance to cephalosporins but that a single β -lactam and inhibitor combination will drive evolution of the full potential resistance phenotype. Surprisingly, we also found significantly positive increases in growth rates as antibiotic concentration increased for some of the strains expressing *bla*_{TEM-85} precursor genotypes but not the *bla*_{TEM-50} variants. This result further suggests that additive interactions more effectively optimize phenotypes than epistatic interactions, which means that exposure to numerous cephalosporins actually increases the ability of a TEM enzyme to confer resistance to any single cephalosporin.

KEYWORDS adaptive landscape, beta-lactamase, drug resistance evolution

Antibiotic resistance is a powerful model for studying evolutionary dynamics. While much evolutionary fine-tuning of antibiotic resistance is modern, it is an ancient phenomenon predating the human production and consumption of antibiotics (1–3). The resistance gene encoding the TEM β -lactamase is broadly distributed in *Enterobacteriaceae* around the world (4–7) and has been found in ancient oceanic cold seep sediments (8). It appears that the TEM gene has been under stabilizing selection for at least thousands of years before the discovery of antibiotics in the 1940s. In modern times, the widespread consumption of various β -lactam antibiotics has imposed enormous selective pressure upon bacteria, resulting in the rapid evolution of the TEM gene and its transmission through plasmids, integrons, and transposable elements within bacteria spanning many environments (9–17). Additionally, TEM has accumulated many mutations that have adaptive benefits.

The therapies that combine β -lactam antibiotics with β -lactamase inhibitors select mutations at different sites than a single cephalosporin therapy would (18–20). Additionally, resistance to combination therapies often results in antagonistic effects to penicillin and cephalosporin resistance phenotypes (19, 20). Substitutions that result in cephalosporin resistance typically open the binding pocket, and those that result in β -lactamase inhibitor resistance typically tighten it (21).

Epistasis, or nonadditive interactions between resistance genes (22, 23) and amino

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acid substitutions, has been found to play a major role in the selection of these genes and is present among mutations within TEM-1 (24–27). Epistatic interactions depend on which combinations of the mutations are present (23, 28) and which antibiotic is used as the selective agent (29). One way to study epistasis is through fitness landscapes, two- and three-dimensional visualizations that represent the strength of epistasis by peaks and valleys (30). It has been suggested that the more rugged a fitness landscape, the less predictable the evolutionary outcomes and the more likely the fitness maxima will be reached (23, 31). Empirical analysis of reconstructed fitness landscapes and theoretical models are now being used to predict the directionality and outcomes of evolution (23, 32). In this study, we have solved two subsets of the TEM β -lactamase landscape.

We compared the effects of sublethal concentrations of commonly used β -lactam antibiotics (similar to concentrations of other antibiotics identified in the environment) on the evolution of a susceptible wild type, bla_{TEM-1} , and two TEM variants, bla_{TEM-50} and bla_{TEM-85} (33–36). bla_{TEM-85} has evolved the ability to confer resistance to cephalosporins, while bla_{TEM-50} has evolved the ability to confer resistance to both cephalosporins and β -lactamase inhibitors (20). β -Lactamase inhibitor resistance and cephalosporin resistance typically do not co-occur (37) because of the opposing effects the involved substitutions have on the TEM enzyme. Although previous studies focused on individual evolutionary landscapes within the TEM variants bla_{TEM-50} and bla_{TEM-85} (29), we wanted to investigate distinctive parts of these landscapes and compare the changes in adaptive strategies that result from different selective pressures in the evolution of both bla_{TEM-50} and bla_{TEM-85} . Based on the opposing effects of the substitutions present in bla_{TEM-50} and bla_{TEM-85} , we correctly hypothesized that the landscapes showing the fitness peaks and valleys between bla_{TEM-1} and bla_{TEM-50} would be more rugged than those landscapes between bla_{TEM-1} and bla_{TEM-85} . Upon confirmation of our hypothesis, we then investigated the differences this ruggedness has on evolutionary outcomes. In this study, we sought to understand how evolutionary processes, specifically the structure of an adaptive landscape and collateral sensitivity, are affected by these different types of antibiotic treatments, with a long-term hope of implementing improved therapeutic approaches that hinder the evolution of antibiotic resistance.

RESULTS

To reconstruct fitness landscapes, we measured growth rates for the 16 possible TEM variants generated from four mutations of bla_{TEM-85} . Those variants were expressed from the pBR322 plasmid in *Escherichia coli* (Table 1). We measured their growth rates in the presence of 13 separate antibiotics, most antibiotics at three sublethal concentrations (one antibiotic was evaluated at only two concentrations), resulting in a total of 38 separate antibiotic treatments (Table 2). We used bacterial growth rates as a limited proxy for fitness by considering only the exponential growth phase. There may be other additional effects from the concentrations that are used at other stages of bacterial growth and survival. For example, an extended lag phase may aid in the ability to persist over time in the presence of sublethal antibiotic concentrations yet result in a lower growth rate.

After calculating the exponential growth rates, we then reconstructed fitness landscapes based on mean growth rate values for each antibiotic treatment, as was previously done for bla_{TEM-50} (29).

These fitness landscapes compare the mean growth rates of adjacent genotypes, which differ by a single amino acid substitution. In each case, the arrow direction points toward the genotype with the higher mean growth rate. The arrow direction can signify either selection for new substitutions or can signify selection for reversions. Phenotypic differences in fitness landscapes were tested for statistical significance using one-way analysis of variance (ANOVA), where solid arrows represent a significant difference between growth rates (P value < 0.05) and dashed arrows represent non-significant differences between growth rates (P value ≥ 0.05).

TABLE 1 Constructs containing all of the possible combinations of substitutions in *bla*_{TEM-85} and *bla*_{TEM-50}^a

Binary code	TEM-50			TEM-85		
	Substitution	Isolated	Yr	Substitution	Isolated	Yr
0000	TEM-1	TEM-1	1965	TEM-1	TEM-1	1965
1000	M69L	TEM-33	1999	L21F	TEM-117	2003
0100	E104K	TEM-17	2000	R164S	TEM-12	1999
0010	G238S	TEM-19	1990	E240K	TEM-191	2011
0001	N276D	TEM-84	2000	T265M	TEM-168	2009
1100	M69L/E104K			L21F/R164S	TEM-53	1999
1010	M69L/G238S			L21F/E240K		
1001	M69L/N276D	TEM-35	1994	L21F/T265M	TEM-110	2002
0110	G238S/E104K	TEM-15	1990	R164S/E240K	TEM-10	1989
0101	G238S/N276D			R164S/T265M		
0011	N276D/E104K			E240K/T265M		
1110	M69L/E104K/N276D			L21F/R164S/E240K		
1101	M69L/E104K/G238S			L21F/R164S/T265M	TEM-102	2003
1011	G238S/N276D/E104K			L21F/E240K/T265M		
0111	G238S/N276D/M69L			R164S/E240K/T265M		
1111	M69L/E104K/G238S/N276D	TEM-50	1997	L21F/R164S/E240K/T265M	TEM-85	2005

^aThe leftmost column shows the binary allelic code we used to represent the variants in fitness landscapes. The number "1" represents the presence and a "0" represents the absence of a substitution at a specific location. The second and fifth columns list the substitutions, with the first letter representing the amino acid that was replaced, followed by the position in the protein and then the new amino acid. For example, L21F corresponds to the substitution of leucine (L) with phenylalanine (F) at the 21st amino acid position. If the variant has been clinically identified, the name is listed in the third and sixth columns along with the year of isolation (37) in the fourth and seventh columns.

Figure 1 shows *bla*_{TEM-85} fitness landscapes for one antibiotic at three concentrations. Using the CompareGrowthRates (CGR) program (38), we measured the reproducibility and stability of these landscapes across the variance in replicates by using the bootstrapping method. There was a 90.6% to 100% consensus across all 38 treatments. Percent similarity values can be found in Table S1 in the supplemental material, and a complete set of fitness landscapes is provided in Fig. S1 to S13.

In each fitness landscape, we identified the fitness maximum or the genotype with the greatest growth rate within the same treatment. To identify the genotypes that contribute the most and least to resistance across the antibiotics we tested, we plotted the frequencies of the three fastest- and slowest-growing genotypes within each treatment (Fig. 2). Overall, the triplet 1101 (L21F/R164S/T265M, *bla*_{TEM-102}) appeared most frequently in the top three fastest-growing genotypes (30 out of 38 treatments), followed by 1111 (*bla*_{TEM-85}), which appeared 25 times among the top three fastest-growing genotypes. These two genotypes have been clinically identified, and both contain the strongly

TABLE 2 List of the β -lactam antibiotic treatments used^a

β -Lactam generation	Antibiotic name	Abbreviation	Concns (μ g/ml)
Penicillin	Amoxicillin	AMX	256, 512
Penicillin	Ampicillin	AMP	1,024, 2,048, 3,072
3	Ceftazidime	CAZ	0.125, 0.25, 0.5
2	Cefaclor	CEC	2, 4, 8
3	Cefprozil	CPR	8, 12, 16
3	Ceftriaxone	CRO	0.025, 0.05, 0.1
2	Cefotetan	CTT	0.063, 0.125, 0.25
3	Cefotaxime	CTX	0.03, 0.06, 0.123
2	Cefuroxime	CXM	2.25, 3, 4
4	Cefepime	FEP	0.0312, 0.0625, 0.125
Penicillin + inhibitor	Sulbactam + ampicillin	SAM	8, 16, 32
Penicillin + inhibitor	Tazobactam + piperacillin	TZP	32, 64, 128
3	Ceftizoxime	ZOX	0.0078, 0.0156, 0.03

^aFor the treatments SAM and TZP, the β -lactamase inhibitors (sulbactam and tazobactam) were used at a constant 8 μ g/ml and the concentration of the penicillin changed (listed). Treatments in bold were used for both *bla*_{TEM-50} and *bla*_{TEM-85}. We needed antibiotic concentrations where most of the genotypes were able to grow but had detectable inhibition. The concentrations used were selected based on the MIC values of each antibiotic to *bla*_{TEM-1}.

FIG 1 Fitness landscapes for *bla*_{TEM-85} treated with cefotaxime (CTX) at three concentrations: 0.03 µg/ml (A), 0.06 µg/ml (B), and 0.123 µg/ml (C). Arrows connect genotypes that differ by only one amino acid substitution and point in the direction of the greater mean growth rate. Solid arrows represent significance with a *P* value of ≤ 0.05 using one-way ANOVA. Dashed arrows represent nonsignificance (*P* value > 0.05). The genotype that has the highest mean growth rate in each treatment, the global maximum, is in bold. The binary code reflects the amino acid substitution locations on the *bla*_{TEM-85} gene. The first amino acid position (1000) represents L21F, the second amino acid position (0100) represents R164S, the third amino acid position (0010) represents E240K, and the fourth amino acid substitution (0001) represents T265M. All possible combinations of these amino acid substitutions are shown using this layout.

selected substitution R164S, which increases the enzymatic activity of TEM toward cephalosporins (21). This agreement between our results and the clinical record suggests that we may have captured natural phenomena that have shaped TEM evolution.

However, genotype 0011 (E240K/T265M), which has not been clinically isolated, is the third fastest growing (Fig. 2). This observation suggests that there are factors affecting evolution that are not fully understood or that there may be important lapses in the clinical record. Additionally, *bla*_{TEM-1} (0000) appeared as the fastest growing in the treatment consisting of cefprozil at 16 µg/ml and four times as the second fastest growing in the treatments consisting of amoxicillin at 512 µg/ml, sulbactam plus ampicillin at 32 µg/ml, cefuroxime at 3 µg/ml, and ceftazidime at 0.125 µg/ml. *bla*_{TEM-1} is efficient at hydrolyzing penicillins, so it is expected to perform well in penicillin treatments. It was surprising that it appeared as the fitness maximum in the presence of a cephalosporin treatment. We suspect that mutations arose in some of the biological replicates ($n = 12$) that expressed *bla*_{TEM-1}, and these mutations enhanced the populations' ability to hydrolyze cephalosporins, therefore resulting in an increased mean growth rate. The three genotypes that appeared most frequently as the slowest growing across all treatments were 1110 (L21F/R164S/E240K), 0111 (R164S/E240K/T265M), and 1010 (L21F/E240K). None of these has been clinically isolated; thus, these outcomes are consistent with laboratory measurements.

Pathways throughout the fitness landscapes. Using our fitness landscapes, we assumed the strong selection, weak mutation (SSWM) model (39) and identified pathways where adaptation occurs through new substitutions. Beginning at *bla*_{TEM-1} (0000), we found that only 58% of the fitness landscapes had adaptive pathways leading from the wild type (*bla*_{TEM-1}) to the global maximum. This contrasts our previously published study of *bla*_{TEM-50} landscapes, in which 97% (29/30) of the fitness landscapes had

FIG 2 Fastest- and slowest-growing *bla*_{TEM-85} genotypes. The frequency of *bla*_{TEM-85} genotypes among the three highest growth rates (dark gray) and three lowest growth rates (light gray) across all 38 antibiotic treatments are shown. Asterisks appear next to the genotypes that have been clinically identified. The genotype 1101, with the substitutions L21F, R164S, and T265M (*bla*_{TEM-102}), is the genotype that results in the fitness maximum most frequently, followed by *bla*_{TEM-85} and *bla*_{TEM-10} (R164S and E104K). The binary code reflects the amino acid substitution locations on the *bla*_{TEM-85} gene. The first amino acid position (1000) represents L21F, the second amino acid position (0100) represents R164S, the third amino acid position (0010) represents E240K, and the fourth amino acid substitution (0001) represents T265M. All possible combinations of these amino acid substitutions are shown using this layout.

pathways to the global maximum (29). This result highlights the effect that many randomly varied treatments can have on accelerating the evolution of resistance.

Enzymatic optimization of *bla*_{TEM-85}. Across all growth rates, we expected to see decreases in growth rate as antibiotic concentration increased. However, we found that some growth rates for the *bla*_{TEM-85} landscapes actually increased as the antibiotic concentration increased for some antibiotic treatments. We found this trend to be true mainly for the genotypes that have been clinically identified. To verify this trend and establish significance, we plotted the growth rate of each genotype against the concentration of each treatment (Fig. 3).

For 10 (out of the 13) antibiotics, we found up to 11 genotypes that had a positive increase in growth rate (in at least one of the two experimental reruns) (Table 3). This means that the highest antibiotic concentration had significantly higher growth rates than any or all of the lower antibiotic concentrations.

Cefaclor and cefepime treatments had the most genotypes with significant increases in growth rates (11 and 8, respectively). Of particular interest, seven out of the eight genotypes for the cefepime treatment contained the substitution R164S (Fig. 3), which is the most common substitution found in TEM variants and contributes to the efficient hydrolyzation of cephalosporins (21). Not all of the variants resulted in a significant increase in growth rate, indicating a definitive difference across the variant *bla*_{TEM} genotypes. Boxplots for all *bla*_{TEM-85} treatments can be found in the supplemental material (*bla*_{TEM-85r} Fig. S14 to S36, and *bla*_{TEM-50r} Fig. S37 to S45). While the increase in bacterial growth rate as antibiotic concentration increases has previously been shown in many soil bacteria, including *Streptomyces* (40), *Pseudomonas*, *Enterobacteriaceae*, actinomycetes (41), and others (42), our results show contemporary optimization of the TEM β -lactamase enzyme that is in line with modern antibiotic consumption.

We hypothesized this increase in growth rate would hold true for *bla*_{TEM-50} landscapes. However, our results indicated lower overall fitness achieved during the course of *bla*_{TEM-50} evolution. This is likely due to physical interference of co-occurring

FIG 3 Boxplots of growth rates versus cefepime concentration (0, 0.0312, 0.0625, and 0.125 $\mu\text{g/ml}$) of all of the precursor genotypes leading up to *bla*_{TEM-85}. Each panel represents one genotype and each boxplot within the panels represents one experiment. Growth rates that are significantly different are indicated by connecting brackets, with *P* values shown.

substitutions (such as those present in *bla*_{TEM-50}), increasing epistasis while simultaneously decreasing the magnitude of each substitution.

Selection for new substitutions depends on antibiotic type. We hypothesized that the evolution of *bla*_{TEM-50} and *bla*_{TEM-85} has been driven by acquisition of new sub-

TABLE 3 List of antibiotic treatments and *bla*_{TEM-85} genotypes for which a significant positive increase in growth rate occurred as antibiotic concentration increased^a

Antibiotic	No. of genotypes	<i>bla</i> _{TEM-85} genotype(s)
Amoxicillin	1	1110
Ampicillin	3	1001 , 0011, 1100
Ceftazidime	3	1100 , 0110 , 0101
Cefaclor	11	1010, 1011, 1100 , 0011, 1110, 1101 , 0100 , 0110 , 0111, 0101, 1111
Cefprozil	4	0000 , 1000 , 0100 , 0010
Ceftriaxone	6	0100 , 0110 , 0101, 1101 , 0111, 1111
Cefotaxime	1	1111
Cefepime	8	0100 , 1100 , 0110 , 0101, 1101 , 0111, 1001 , 1111
Tazobactam + piperacillin	3	1000 , 0001 , 1111
Ceftizoxime	1	1101

^aClinically identified genotypes are highlighted in bold typeface. The binary code reflects the amino acid substitution locations on the *bla*_{TEM-85} gene. The first amino acid position (1000) represents L21F, the second amino acid position (0100) represents R164S, the third amino acid position (0010) represents E240K, and the fourth amino acid substitution (0001) represents T265M. All possible combinations of these amino acid substitutions are shown using this layout.

FIG 4 The frequency of the ratios of new substitutions: reversions within the fitness landscapes of bla_{TEM-50} (left) and bla_{TEM-85} (right). The ratios were calculated based on the number of forward arrows (N, new substitutions) to reverse arrows (R, reversions). If this ratio was greater than 1, then new substitutions were favored ($N > R$). If this ratio was less than 1, then reversions were favored ($N < R$). If the number of new substitutions was equal to the number of reversions, then $N = R$.

stitutions rather than reversions. We enumerated new substitutions and reversions selected in each fitness landscape and calculated the ratio between them. When this ratio was greater than 1, it indicated stronger selection for new substitutions (Fig. 4). We found that for both bla_{TEM-85} and bla_{TEM-50} , new substitutions were favored in cephalosporin treatments. However, new substitutions were not favored for bla_{TEM-50} in treatments with penicillin plus β -lactamase inhibitor.

The fitness landscapes of bla_{TEM-50} are more rugged than the fitness landscapes of bla_{TEM-85} . We hypothesized that because of the opposing functions of the substitutions found in bla_{TEM-50} , the associated landscapes would contain more epistatic interactions than bla_{TEM-85} . We compared the relative frequencies of epistatic interactions within the bla_{TEM-85} and bla_{TEM-50} landscapes. As suspected, we observed greater pairwise epistasis means (43) in bla_{TEM-50} for 11 out of the 16 treatments, which suggests that the fitness landscapes for bla_{TEM-50} were more rugged (i.e., there were more epistatic interactions between the amino acid substitutions present in bla_{TEM-50}) than the landscapes for bla_{TEM-85} . However, we found that bla_{TEM-85} did have more epistatic interactions in both amoxicillin treatments, two cephalosporin treatments (cefotetan at $0.063 \mu\text{g/ml}$ and ceftizoxime at $0.03 \mu\text{g/ml}$), and one treatment with penicillin plus β -lactamase inhibitor (tazobactam plus piperacillin at $128 \mu\text{g/ml}$) (Fig. 5). For each of these treatments, both of the bla_{TEM-85} replicates had a slightly greater pairwise epistasis mean than bla_{TEM-50} . These results show that in the presence of some β -lactam antibiotics, substitutions present in bla_{TEM-85} yield more epistatic interactions than those substitutions present in bla_{TEM-50} .

DISCUSSION

Our work shows that epistasis is a common phenomenon during the adaptation of the TEM β -lactamase to modern antibiotic consumption (15, 44–46). Comparison of the epistatic interactions of the individual substitutions that produce the highly evolved TEMs (up to four amino acid substitutions) bla_{TEM-50} (29) and bla_{TEM-85} highlights the impacts of epistasis on the availability of fitness maxima and the overall heights of those maxima.

We found that bla_{TEM-85} is similar to bla_{TEM-50} in that changing either the type or concentration of β -lactam antibiotics selects for almost every genotype within the fitness landscape. This demonstrates the pliable nature of TEM during exposure to varied

FIG 5 Pairwise epistasis means for the 16 identical treatments for *bla*_{TEM-50} (29) and *bla*_{TEM-85}. Concentrations are listed next to the name of the antibiotic abbreviation and are in micrograms per milliliter, also shown in bold Table 2. For the treatments with penicillin plus β -lactamase inhibitor, the β -lactamase inhibitor concentrations are all at 8 μ g/ml. Both *bla*_{TEM-85} replicates are shown. The replicate experiments were performed for *bla*_{TEM-85} and these values are similar, which confirms their repeatability.

antibiotics and how the presence of sublethal concentrations can increase the diversity of resistance genes present in a bacterial population.

We also found that the topography of the fitness landscapes of *bla*_{TEM-50} is more rugged, i.e., contains more epistatic interactions between amino acid substitutions, than the topography of the fitness landscapes of *bla*_{TEM-85}. This is consistent with evolutionary theory that states that sign epistasis contributes to multi peaked fitness landscapes (47). It is also consistent with experimental work showing that stronger pleiotropy is associated with stronger epistatic interactions (22).

Quantification of the number of pathways leading to the global maxima suggests that increased landscape ruggedness (as seen for *bla*_{TEM-50}) increases the accessibility of adaptive peaks (46). This means that for complex resistance phenotypes, a single β -lactam that is combined with an inhibitor can likely drive evolution to its maximum potential. In contrast, the connection between increasing antibiotic concentration and increasing fitness shows that smoother landscapes with less epistasis have higher fitness peaks, though exposure to more antibiotics is likely required. Furthermore, the genotypes within *bla*_{TEM-85} landscapes are more likely to be found among clinical isolates, demonstrating that these higher fitness peaks have real-world consequences on TEM evolution and that the multiple cephalosporin exposures required for their selection are prevalent. We propose that additivity within landscapes increases the height of fitness maxima and that epistatic interactions increase the availability of fitness maxima. The clinical implications of these predictions are that combination therapies will drive evolution to its limits when only one combination is used but that cephalosporin resistance will be pushed to evolutionary limits by the use of many cephalosporins. This suggests that using fewer cephalosporins will actually slow and suppress the evolution of cephalosporin resistance.

Our results are limited to *E. coli*, and there are other important Gram-negative bacilli that express variants of the *bla*_{TEM} gene, such as *Klebsiella pneumoniae* (48). It will be

informative to perform the same assays on other species expressing *bla*_{TEM}. In future studies, we will investigate the important matter of how these results compare with those for other species.

Our results agree with previous studies showing that the multitude of sublethal concentrations of antibiotics present in the environment increases the selection and diversification of antibiotic resistance genes (2, 33, 34, 49–51). Our work further suggests that fitness landscapes provide insight into what genotypes have appeared, or can appear, in the clinic. Solving the fitness landscapes of antibiotic resistance genes brings us ever closer to predicting the evolutionary trajectories of microbes (23) and limiting the occurrence of antibiotic resistance. As we expand these assays to other species, we also make these results more generalizable.

MATERIALS AND METHODS

Fitness assays. Materials and methods for fitness assays were taken from reference 29. Briefly, we expressed all 16 TEM variant genotypes from the pBR322 plasmid in *E. coli* strain DH5 α -E. We incubated the cells in 5 ml of Luria broth with tetracycline (15 mg/liter) overnight in oxygen-limited cultures and then diluted them to a concentration of 1.9×10^5 per ml. The cells of the 16 variant genotypes were then aliquoted down the 16 rows of a 384-well plate. We treated the first 12 columns with one antibiotic at one concentration, and the last 12 columns served as a control, with no antibiotic treatment. The optical density (OD) was measured over a 22-h period at 25.1°C. We used this temperature rather than 37°C because many β -lactam antibiotics break down too quickly at 37°C to obtain consistent measurements for 22 h. Two biological replicates were run for *bla*_{TEM-85} and associated genotypes. The growth rates and statistical analyses were computed using the programs Growthrates (52) and CompareGrowthRates (CGR) (38).

Fresh antibiotic stock solutions (10.2 g/ml) were made for each antibiotic prior to the start of each set of experiments and stored in 4°C until use. The concentration of antibiotics used for each experiment was initially based on the MIC values of each of the 32 TEM variants (Table S2) to identify a range of concentrations further fine-tuned through growth rate assays. All raw OD readings for each experiment can be found in Data Set S3, a tab-delimited file with each tab representing the individual experimental runs.

Creating adaptive landscapes and statistical analysis. Based on results from CGR (38), we confirmed that the amount of variation in the growth rate data was minimal and the intervals between readings were optimal (60 min). We reanalyzed the *bla*_{TEM-50} data (29) using CGR with the optimal intervals (60 min) to create the fitness landscapes we compared with *bla*_{TEM-85}. The CGR package also allows bootstrap analysis of the fitness landscapes to measure reproducibility. CGR uses 10,001 bootstraps and recreates fitness landscapes with the probability values of each transition within the fitness landscapes. We measured the percent similarity of arrow direction in the bootstrap landscapes to our fitness landscapes.

To create the fitness landscapes, we used one-way analysis of variance (ANOVA) to compare the means of the growth rates we obtained and to identify significant differences between the growth rates of each genotype. We compared adjacent genotypes (differing by a single amino acid substitution), going from the wild type, *bla*_{TEM-1*} to *bla*_{TEM-85}. We used a 95% confidence interval, which translates to a *P* value of ≤ 0.05 . Arrows pointed in the direction of the greater mean growth rate. Solid arrows represent significant differences between the growth rates ($P \leq 0.05$), and dashed arrows represent insignificant differences ($P > 0.05$) (see also Fig. 1). While insignificant differences may not deviate from neutrality, we have elected to show the direction of the arrows to avoid incorrectly biasing our results toward neutrality. A complete set of growth rate data for each of the treatments is provided in Data Sets S1 and S2.

To calculate the number of new substitutions compared to reversions across antibiotic treatments, we summed the number of forward arrows (new substitutions) and the number of backward arrows (reversions) and calculated this ratio (new substitutions/reversions). If this ratio was greater than 1, then new substitutions were favored. If this ratio was less than 1, then reversions were favored. We then summarized these values in Fig. 4 for the identical antibiotic treatments used for *bla*_{TEM-50} and *bla*_{TEM-85}.

Visualizing the change in growth rate as concentration increased. To visualize how growth rates changed as antibiotic concentration increased, we plotted our data using boxplots and confirmed significant changes using the Tukey HSD (honestly significant difference) test. The Tukey HSD test computes all pairwise *P* values between all concentrations. Each boxplot shown represents all 12 replicate data points for each genotype and each concentration (Fig. 3 and Fig. S14 to S45). All growth rate values can be found in Data Sets S1 and S2.

Calculating epistasis. The pairwise epistasis mean (PWEM) is defined as the average epistasis between all possible pairs of substitutions and calculated as follows:

$$\text{PWEM} = \log_{10} \frac{W_{ab} \times W_0}{W_a \times W_b}$$

where W_0 is the mean growth rate of the unmutated genotype, *bla*_{TEM-1*}, W_a and W_b are the mean growth rates of the genotypes with single substitutions, and W_{ab} is the mean growth rate of the genotype with both substitutions (43). We plotted these values across all identical treatments for both *bla*_{TEM-50} and *bla*_{TEM-85} to visualize the influence epistatic interactions have on landscape ruggedness.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 3.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 2 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.4 MB.

SUPPLEMENTAL FILE 4, XLSX file, 7.9 MB.

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