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Genetic and Structural Characterization of an L201P Global Suppressor Substitution in TEM-1 β-Lactamase

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TEM-1 β-lactamase confers bacterial resistance to penicillin antibiotics and has acquired mutations that permit the enzyme to hydrolyze extended-spectrum cephalosporins or to avoid inactivation by β-lactamase inhibitors. However, many of these substitutions have been shown to reduce activity against penicillin antibiotics and/or result in loss of stability for the enzyme. In order to gain more information concerning the tradeoffs associated with active site substitutions, a genetic selection was used to find second site mutations that partially restore ampicillin resistance levels conferred by an R244A active site TEM-1 β-lactamase mutant. An L201P substitution distant from the active site that enhanced ampicillin resistance levels and increased protein expression levels of the R244A TEM-1 mutant was identified. The L201P substitution also increases the ampicillin resistance levels and restores expression levels of a poorly expressed TEM-1 mutant with a core-disrupting substitution. *In vitro* thermal denaturation of purified protein indicated that the L201P mutation increases the T_m value of the TEM-1 enzyme. The X-ray structure of the L201P TEM-1 mutant was determined to gain insight into the increase in enzyme stability. The proline substitution occurs at the N-terminus of an α-helix and may stabilize the enzyme by reducing the helix dipole, as well as by lowering the conformational entropy cost of folding due to the reduced number of conformations available in the unfolded state. Collectively, the data suggest that L201P promotes tolerance of some deleterious TEM-1 mutations by enhancing the protein stability of these mutants.

Keywords: antibiotic resistance; global suppressor; protein stability; enzyme structure; random mutation

Introduction

The most prevalent plasmid-encoded β-lactamase in Gram-negative bacteria, TEM-1, provides resistance to penicillins and early-generation cephalosporins.[1] TEM-1 β-lactamase has evolved in response to the introduction of extended-spectrum β-lactams and β-lactamase inhibitors by acquiring substitutions that modify the substrate profile of the enzyme to match the new selection environment. Many of these active site substitutions alter the substrate profile of the TEM-1 enzyme at the cost of reduced hydrolysis of penicillin β-lactams such as ampicillin, amoxicillin, and benzylpenicillin.[2–9]

In addition to sacrificing high levels of catalytic efficiency against penicillin β-lactams, substitutions near the active site of TEM-1 frequently result in a loss of stability of the enzyme.[10] For example, a G238S substitution confers extended-spectrum β-lactamase activity but reduces the protein's melting temperature ($\Delta T_m = -4.5$ relative to wild-type TEM-1), translating into a 1.94-kcal/mol loss of stability.[10] Likewise, the R164S extended-spectrum β-lactamase mutation results in a reduced melting temperature ($\Delta T_m = -1.7$) and corresponding loss of stability ($\Delta\Delta G_m = -0.73$ kcal/mol).[10] The stability of M69

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Abbreviations used: MIC, minimum inhibitory concentration; TA, tetrazolium agar.

mutants conferring inhibitor resistance is dependent upon the substituting residue. The M69I substitution results in a loss of stability ($\Delta\Delta G_m = -1.3$), while the M69L TEM-1 mutant is actually more thermodynamically stable than wild-type TEM-1 ($\Delta\Delta G_m = +1.0$).[10]

One substitution distant from the active site, M182T, has arisen repeatedly among antibiotic-resistant isolates in the context of active site substitutions that reduce stability†. A genetic selection for suppressors of a destabilized TEM-1 β-lactamase led to the identification of the M182T mutation as a general suppressor of folding and/or stability defects.[11,12] The M182T substitution has since been repeatedly identified in clones isolated from directed evolution experiments of TEM-1, consistent with its importance in correcting enzyme stability defects associated with other substitutions.[13–19] In restoring stability to TEM-1 variants, the M182T substitution mitigates stability tradeoffs associated with amino acid substitutions that provide for extended-spectrum cephalosporin hydrolysis and inhibitor resistance.[10]

Several TEM-1 variants with substitutions at position 244 have been found in clinical isolates resistant to certain β-lactam/inhibitor combinations.[6,7,20–25] The arginine at position 244, however, also plays an important role in substrate binding and catalysis. The X-ray structure of TEM-1 β-lactamase with an acylated penicillin in the active site indicates that the positive charge of this arginine residue interacts, via a bridging water molecule, with the C3 carboxylate group that is common to all classes of β-lactam antibiotics.[26] Moreover, substitutions at position 244 increase the K_m value for the hydrolysis of penicillin and, to a lesser extent, cephalosporin antibiotics.[7,9] Yet, substitutions that remove the positive charge from position 244 reduce inactivation by inhibitors as a result of the missing interaction with the C3 carboxylate group.[6,7,20–25] Thus, the normally high level of catalytic efficiency towards penem substrates is sacrificed in these mutants in exchange for inhibitor resistance, due to the increase in K_m value for the hydrolysis of β-lactam antibiotics associated with these substitutions.

TEM-1 β-lactamase is a member of a gene family called class A β-lactamases based on primary amino acid sequence homology.[27,28] The class A β-lactamases include a large number of enzymes from both Gram-positive and Gram-negative bacteria.[29] Several class A β-lactamases do not encode an arginine at position 244, but rather provide the positive charge that interacts with the C3 carboxylate from another position near the active site.[30] For example, the SME-1 and *Streptomyces albus* G β-lactamases provide a positive charge from R220, while the Toho-1 β-lactamase has an arginine at position 276. The structures of these enzymes indicate that the guanidinium group from the arginine occupies a similar position in the active site, whether it is provided by R220, R244, or R276.[31–34] Therefore, it appears that there is structural and evolutionary plasticity in the placement of the arginine that provides the positive charge for substrate binding. To further investigate the loss of catalytic activity associated with substitutions at position 244 and the plasticity with regard to the positive charge, an R244A mutant of TEM-1 β-lactamase was generated. A selection for mutations that increased the ampicillin resistance of *Escherichia coli* containing the active site mutant R244A was designed to identify second site mutations that restore the positive charge—and thereby the catalytic efficiency—of the enzyme for ampicillin hydrolysis. The selection, however, resulted in the isolation of an L201P substitution that improves fitness by increasing stability and the steady-state protein levels of the R244A enzyme. Suppressor function was compared between the L201P substitution and the established stabilizing M182T substitution by combining each with other primary substitutions with decreased function. Both L201P and M182T compensate for several, but not all, primary substitutions and exhibit an overlapping, but not identical, pattern of suppression. Therefore, these suppressors are "global" in that they act on many different primary substitutions, but they may not act universally to compensate for all defects associated with primary substitutions.

† http://www.lahey.org/studies

Results and Discussion

Genetic selection of a second site suppressor of the TEM-1 R244A mutant

In TEM-1 β-lactamase, the side chain of arginine at position 244 is directed towards the active site pocket, where it interacts with the C3 carboxylate group of a β-lactam substrate.[9,10,26] In order to initiate study of this position, the arginine side chain was replaced with alanine via site-directed mutagenesis in the pET-TEM-1 expression vector.[35] The pET-TEM-1 vector expresses large quantities of soluble TEM-1 β-lactamase via transcription from a T7 promoter and encodes an ompA leader fused to the mature portion of the TEM-1 enzyme.[35] This bla_{TEM-1} gene has subsequently been shown to contain a E28G substitution in mature TEM-1 near the signal cleavage site.[35,36] The mature ompA-TEM-1 E28G β-lactamase is properly processed as determined by N-terminal protein sequencing and does not exhibit measurable changes in catalytic properties relative to wild-type TEM-1 with its native signal sequence.[36] The R244A substitution results in a fourfold to eightfold loss of ampicillin resistance relative to TEM-1 E28G β-lactamase, as determined by the minimum inhibitory concentration (MIC) of this β-lactam antibiotic. The R244C mutant of TEM-1 has been shown to have a similar effect on amoxicillin resistance levels.[20] The loss of activity seen with these arginine 244 substitutions could be the result of the displacement of an active site water molecule that normally interacts with the β-lactam carboxylate, as

suggested by the crystal structure of the R244S mutant of TEM-1.[10]

To study the plasticity of the positive charge in this region of the active site, a search for mutations that partially restore ampicillin resistance to the R244A mutant was made. This was accomplished by constructing a random library of point mutations of the TEM-1 E28G:R244A gene encoded in the pET-TEM-1 vector by error-prone PCR. From the E28G:R244A library, a suppressor distant from the active site was isolated with a single nucleotide substitution, resulting in an L201P mutation. Interestingly, the L201P substitution has been observed previously upon selection for inhibitor-resistant TEM-1 β-lactamases from an error-prone library of TEM-1 enzymes.[37] In addition, the L201P substitution was recently identified as a suppressor among populations of highly mutagenized TEM-1 mutants propagated under conditions of neutral drift.[38]

In order to ensure separation of function from potential mutations found elsewhere in the pET-TEM-1 plasmid or from the E28G substitution, the L201P:R244A double mutants and R244A single mutants were reconstructed in the original pET-TEM-1 plasmid, and the E28G substitution was reverted to wild type by site-directed mutagenesis. The E28G:R244A and R244A mutants in pET-TEM-1 exhibited the same ampicillin MIC (MIC = 512 μg/ml). In addition, the pBG66 plasmid that encodes wild-type TEM-1 β-lactamase was used to create both the R244A and the L201P:R244A TEM-1 mutants by site-directed mutagenesis. Unlike the T7 promoter-driven expression of $bla_{\text{TEM-1}}$ from the pET-TEM-1 expression vector, $bla_{\text{TEM-1}}$ is constitutively transcribed by its native promoter in the pBG66 plasmid.[39] The reconstructed pET-TEM-1 L201P:R244A in *E. coli* BL21(DE3) exhibits similar ampicillin resistance (MIC = 512 μg/ml) relative to the R244A mutant, as determined by twofold antibiotic dilutions. However, a difference in resistance levels was apparent when additional ampicillin concentrations were examined near the MIC value where R244A exhibited an MIC of 358 μg/ml, while the L201P:R44A had an MIC of 512 μg/ml.

Table 1. Influence of L201P substitution on TEM-1 β-lactamase-mediated MIC levels

	Ampicillin (μg/ml)[a]	Amoxicillin + clavulanic acid (μg/ml)[b]
TEM-1	2048	24
L76N	32	6
M182T	2048	24
L201P	2048	24
R244A	256	32
M182T:L201P	2048	16
L201P:R244A	256	64
L76N:M182T	512	16
L76N:L201P	64	12
L76N:M182T:L201P	1024	16

[a] Ampicillin using broth dilution and pBG66 plasmid.
[b] Amoxicillin with clavulanic acid in 2:1 ratio using E-test strip and pBG66 plasmid.

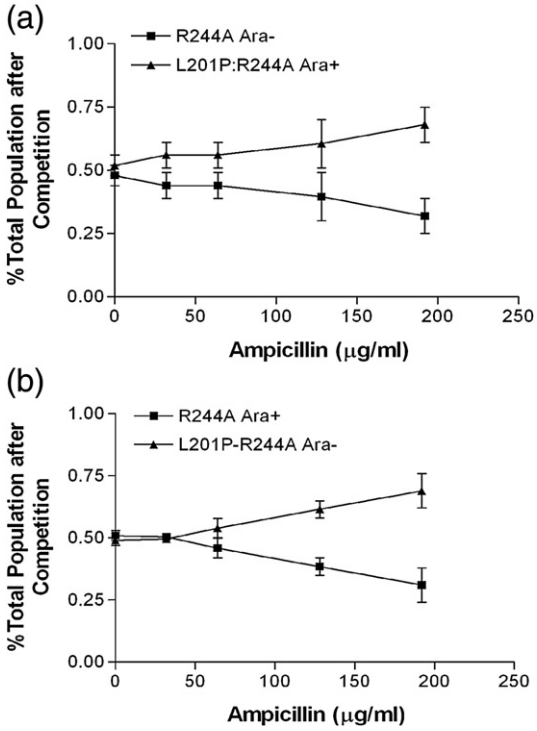

Fig. 1. Competition experiment between *E. coli* B cells containing pBG66-R244A and pBG66-L201P:R244A plasmids. (a) Equal volumes of overnight cultures of *E. coli* Ara^+ pBG66 R244A and *E. coli* Ara^- pBG66 L201P:R244A cells were mixed and allowed to compete for growth overnight in LB medium containing the concentration of ampicillin indicated on the *x*-axis. The *y*-axis indicates the percentage of the total culture of *E. coli* Ara^+ pBG66 R244A and *E. coli* Ara^- pBG66 L201P:R244A cells after the competition. (b) Reciprocal competition experiment of *E. coli* Ara^- pBG66 R244A and *E. coli* Ara^+ pBG66 L201P:R244A strains.

Similarly, the ampicillin MIC for pBG66-R244A and pBG66-L201P:R244A was 256 μg/ml, as determined by twofold ampicillin dilutions (Table 1). MIC determinations using commercially available E-test strips containing amoxicillin and a β-lactamase inhibitor, however, indicated that the pBG66-L201P:R244A mutant exhibits an increased level of resistance relative to the pBG66-encoded R244A mutant (Table 1).

In order to further examine the fitness advantage conferred upon R244A by the L201P substitution, competition experiments were performed between *E. coli* strains containing the pBG66 plasmid with either the R244A or the L201P:R244A β-lactamase genes. These experiments made use of otherwise isogenic Ara^+ and Ara^- *E. coli* B strains that, when grown on tetrazolium agar (TA) plates, yield pink and red colonies, respectively.[40] The R244A- and L201P-R244A-encoding plasmids were introduced into the Ara^+ and Ara^- strains, and a culture of each was grown to saturation in the absence of ampicillin. Equal volumes of the cultures were then diluted into fresh media containing increasing concentrations of ampicillin and then grown to saturation.

The cultures were then spread on TA plates, and the numbers of pink and red colonies were determined. The results in Fig. 1 clearly indicate that the L201P:R244A mutant outcompetes the R244A mutant as ampicillin concentrations are increased, indicating that the L201P substitution provides a fitness advantage.

As a further test of the relative fitness of the R244A mutant *versus* the relative fitness of the L201P:R244A mutant, bacterial cultures harboring each mutant on the pBG66 plasmid were spread on agar plates containing increasing concentrations of ampicillin, and the number of colonies under each condition was counted to generate a survival curve for each mutant (Fig. 2a). Data from the survival curve experiments were also used to assign ampicillin IC_{90} values to each TEM-1 mutant, defined here as the concentration of ampicillin required to reduce the viability (colony-forming units) of TEM-1-expressing bacteria by $\geq$90% (Table 2). The results indicate that the L201P:R244A mutant exhibits enhanced survival relative to the R244A mutant at increasing ampicillin concentrations. Expanded survival curves to an ampicillin concentration of 950 μg/ml are shown in Supplementary Fig. 1a. Taken together, the MIC determinations, competition experiments, and survival curves indicate that the L201P substitution confers enhanced ampicillin resistance to the R244A TEM-1, independent of plasmid and promoter context.

Table 2. Effect of stabilizer substitutions on TEM-1 β-lactamase-mediated IC_{90} levels

	Ampicillin (μg/ml)
TEM-1	>960
M182T	>960
L201P	>960
L76N	30
L76N:L201P	120
L76N:M182T	960
R244A	120
L201P:R244A	240
M182T:R244A	120
I47Y:E48C	240
I47Y:E48C:L201P	480
I47Y:E48C:M182T	480
H158S:V159S:T160H	30
H158S:V159S:T160H:L201P	30
H158S:V159S:T160H:M182T	960

IC_{90}, defined as the concentration of ampicillin that reduces colony-forming units per milliliter of culture by $\geq$90%, is derived directly from data measurements reported in Fig. 2.

It is noteworthy that the M182T suppressor mutation was not identified among the suppressors of the

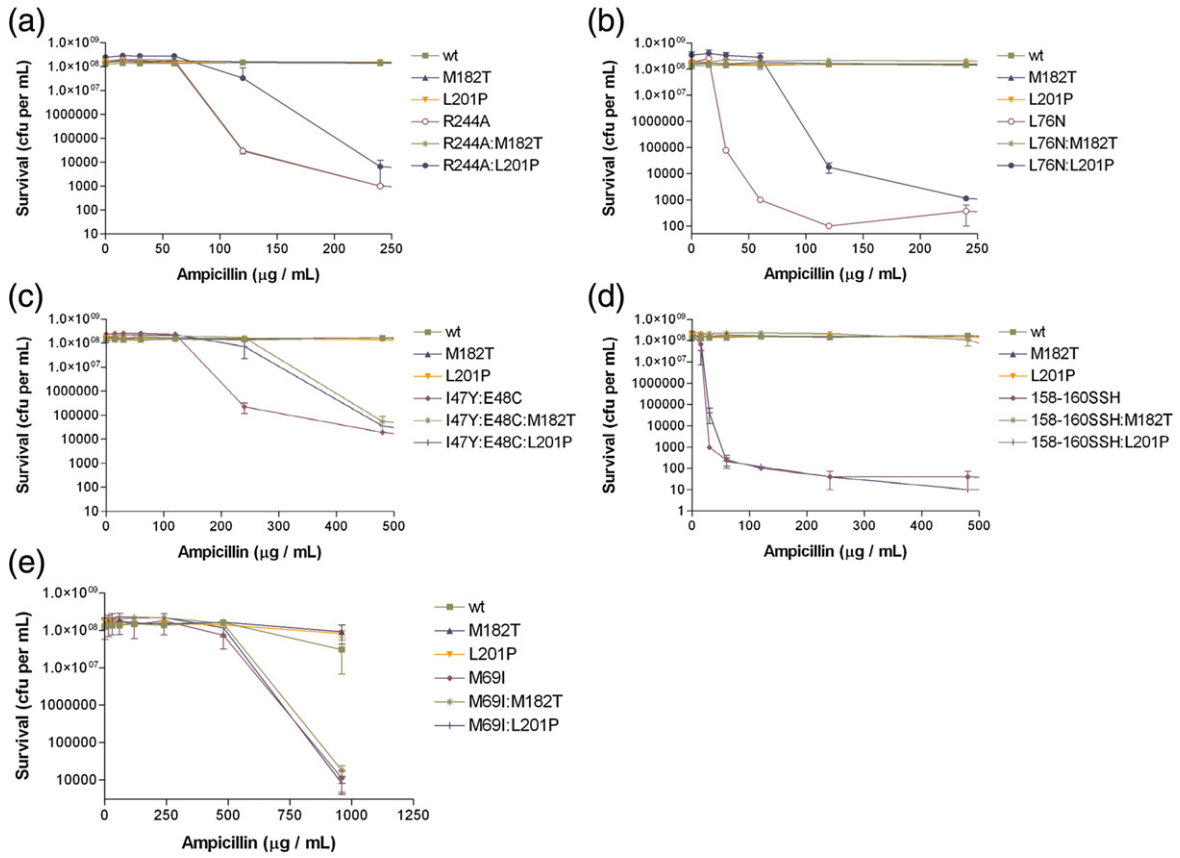

Fig. 2. Survival curves of *E. coli* with the pBG66 plasmid encoding TEM-1 β-lactamase mutants. (a) Colony-forming units on agar plates containing increasing concentrations of ampicillin for *E. coli* containing R244A, M182T, and L201P single and double mutants. (b) Colony-forming units for *E. coli* containing L76N, M182T, and L201P single and double mutants. (c) Colony-forming units for *E. coli* containing I47Y:E48C, M182T, and L201P mutant combinations. (d) Colony-forming units for *E. coli* containing H158S:V159S:T160H, M182T, and L201P mutant combinations. (e) Colony-forming units for *E. coli* containing M69I, M182T, and L201P single and double mutants.

R244A substitution, despite the fact that M182T has been identified previously as a suppressor of folding and/or stability defects of several different primary mutations.[13–19] One explanation is that the library of mutants constructed by PCR contained 1.8×10^5 clones, and the M182T mutant may not have been in this collection. Another possibility is that the M182T substitution does not enhance ampicillin resistance of the R244A primary mutant. To test this possibility, the M182T:R244A double mutant was constructed, and an ampicillin survival curve was generated. As seen in Fig. 2a, the survival curves of the R244A and M182T:R244A are superimposable, indicating that, at the resolution of this experiment, M182T does not enhance the ampicillin resistance of the R244A mutant. Therefore, the M182T mutation was not found among the R244A suppressor mutants because it does not alter the ampicillin resistance of R244A.

That the M182T suppressor substitution apparently does not act on the R244A enzyme suggests that the L201P mutation may be allele-specific (i.e., the mutation may increase ampicillin resistance only in combination with R244A). In order to investigate this possibility, double-mutant constructs were made with the β-lactamase L76N substitution.[11] In TEM-1 β-lactamase, the completely buried leucine at position 76 is part of a hydrophobic core of the enzyme.[26,41] The L76N mutation has a modest effect on the catalytic properties of the enzyme *in vitro*, but results in poor protein expression *in vivo.* This translates into a significant loss of resistance towards ampicillin.[12] Because of these qualities, the L76N mutant can be utilized as a sensitive test of the ability of second site amino acid substitutions to correct protein folding and/or stability defects.[11,12] In addition, L76N was the original primary mutant used to identify M182T as a suppressor of folding and/or stability defects.[11]

Both MIC and survival curve experiments, with *E. coli* containing the wild-type, M182T, L201P, L76N, L76N:M182T, and L76N:L201P mutants encoded on the pBG66 plasmid, were performed. The addition of the M182T substitution restores the ampicillin resistance (MIC) of the pBG66-encoded L76N mutant to high levels (Table 1). The pBG66-L76N:L201P mutant also displays a twofold increase in ampicillin resistance relative to that of pBG66-L76N alone (Table 1). Moreover, the results in Fig. 2b and Table 2 show that the L201P substitution increases the survival of the L76N mutant in ampicillin. These data indicate that L201P can act on substitutions other than R244A. Because the L76N mutant enzyme is unstable, the result also suggests that L201P acts to increase stability or to improve the folding of primary mutants. In addition, the M182T substitution acted as a suppressor of L76N, consistent with previous observations (Fig. 2b).[11]

Several additional mutant combinations were constructed to examine the specificity of action of the M182T and L201P substitutions. The I47Y:E48C mutant was isolated from a library of random substitutions encompassing residues 46–48 of β-lactamase.[39] This mutant exhibits reduced ampicillin resistance and lower protein expression levels relative to those of wild-type TEM-1, and was used previously to demonstrate that M182T can suppress the folding and/or stability defects of mutations other than L76N.[11] As seen in Fig. 2c and Table 2, *E. coli* containing either the I47Y:E48C: M182T or I47Y:E48C:L201P mutants exhibited increased survival on agar plates containing ampicillin compared to the I47Y:E48C parent mutant. This finding indicates that both M182T and L201P improve the activity of the I47Y:E48C enzyme *in vivo*.

The H158S:V159S:T160H TEM-1 β-lactamase mutant was isolated from a library of random substitutions encompassing residues 158–160.[39] This mutant was also chosen to test the suppression specificity of M182T and L201P based on it's reduced ampicillin resistance and low protein expression levels. It was found that the M182T substitution strongly increased the ampicillin resistance levels of *E. coli* containing the H158S:V159S:T160H mutant, whereas the L201P substitution had no detectable effect on the resistance levels of *E. coli* containing the mutant (Fig. 2d, Table 2).

Finally, neither M182T nor L201P increased the ampicillin resistance levels of the M69I mutant (Fig. 2e). It was previously shown that the effect of the M182T substitution on the M69I enzyme could only be detected by measuring the specific activity of enzyme lysates at increasing temperatures.[11]

The results of the double-mutant experiments described above indicate that the TEM-1 L201P substitution can increase the ampicillin resistance of *E. coli* containing TEM-1 with primary mutations at different locations on the enzyme. This finding is consistent with L201P acting as a global suppressor mutant. However, it was also found that the addition of L201P did not result in detectable changes in ampicillin resistance for the H158S:V159S:T160H or M69I mutants. Similarly, the addition of the M182T substitution increased the ampicillin resistance conferred by several β-lactamase mutants but not the M69I or R244A mutants. The failure to observe a change in resistance levels when M182T or L201P is combined with certain mutants could be due to the failure of the assay to detect subtle effects. For example, *E. coli* containing either M69I:M182T or M69I:L201P did not exhibit increased resistance relative to the M69I parent (Fig. 2e). However, previous studies examining temperature sensitivity have shown that the M69I:M182T enzyme is more active than the M69I parent enzyme at elevated temperatures.[11] Therefore, the failure to observe a change in ampicillin resistance levels does not rule out an effect on the stability of the enzyme. Nevertheless, the patterns of suppression of the M182T and L201P substitutions display differences. In general, the M182T substitution is a stronger suppressor than L201P, given that M182T confers higher levels of ampicillin resistance to most of the β-lactamase mutants tested. The notable exception is the failure of M182T to enhance the ampicillin resistance of the R244A mutant.

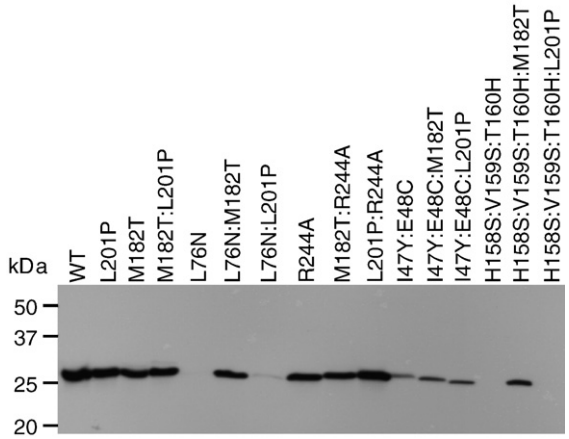

Fig. 3. Steady-state protein levels of wild-type and mutant TEM-1 β-lactamases, as determined by immunoblot of *E. coli* periplasmic protein. Positions are as follows: (1) wild type TEM-1; (2) L201P; (3) M182T; (4) M182T:L201P; (5) L76N; (6) L76N:M182T; (7) L76N:L201P; (8) R244A; (9) M182T:R244A; (10) L201P:R244A; (11) I47Y:E48C; (12) I47Y:E48C:M182T; (13) I47Y:E48C:L201P; (14) H158S:V159S:T160H; (15) H158S:V159S:T160H:M182T; (16) H158S:V159S:T160H:L201P.

Immunoblot analysis of steady-state levels of β-lactamase mutants

The effect of the L201P substitution on steady-state protein levels of several TEM-1 mutants was quantified by immunoblot analysis of the periplasmic contents of the cell. The protein core-disrupting L76N substitution greatly reduces expression of TEM-1 β-lactamase (Fig. 3 and Huang and Palzkill[11]). A previous study found that the reduced expression level of the L76N mutant is due to periplasmic degradation in that the L76N mutant expression levels are similar to those of wild-type TEM-1 when expressed in an *E. coli* strain that is deficient in four periplasmic proteases.[11] Because unstable proteins are rapidly proteolyzed in *E. coli*, steady-state levels of expression relative to those of wild type can be used as an indicator of the effect of a mutation on protein stability.[42]

The levels of protein expression of TEM-1 β-lactamase wild type and mutants were monitored by immunoblots of the periplasmic contents of *E. coli* expressing the enzymes from the pBG66 plasmid using anti-β-lactamase polyclonal antisera.[11] Consistent with previous results, the introduction of the M182T substitution rescues steady-state protein levels of the L76N mutant (Fig. 3).[11] The L201P substitution also improves steady-state L76N protein expression levels, consistent with it acting as a stability-enhancing mutation similar to M182T (Fig. 3) and also consistent with the increased ampicillin resistance observed in the survival curve (Fig. 2b). The R244A mutation had little effect upon steady-state protein levels, suggesting that any effect upon protein folding associated with this active site substitution is modest in comparison to the effect of the L76N mutation (Fig. 3). However, the L201P:R244A clone displayed a higher level of protein expression relative to the R244A clone (Fig. 3, lane 10 *versus* lane 8), while the M182T:R244A clone displayed similar levels as the R244A clone (Fig. 3, lane 9 *versus* lane 8). These observations are consistent with the results of the survival curve experiments with M182T:R244A and L201P:R244A. The higher level of protein expression of L201P:R244A may explain the increased ampicillin resistance that resulted in the isolation of this mutant in the second site mutation selection. The ability of L201P to increase the expression levels of both the R244A and the L76N mutants of TEM-1 β-lactamase provides evidence that L201P can act on more than one primary mutation in the enzyme.

The results of the survival curve experiments in Fig. 2 suggest that L201P can suppress defects associated with the I47Y:E48C mutant enzyme, but not defects associated with the H158S:V159S:T160H enzyme, while the M182T substitution can act on both mutants (Fig. 2c and d, Table 2). The immunoblotting results correlate well with these findings in that the I47Y:E48C:L201P enzyme exhibits increased expression relative to the parent enzyme, while the H158S:V159S:T160H:L201P enzyme levels are not altered relative to the parent enzyme (Fig. 3). In addition, the expression levels of the I47Y:E48C:M182T and H158S:V159S:T160H:M182T enzymes are higher than those of the parent enzymes (Fig. 3). Taken together, the survival curve and immunoblotting analyses indicate that both the M182T and the L201P substitutions can act on more than one primary mutation, and that the sets of mutants that can be suppressed by each overlap but are not identical.

Finally, both M182T and L201P substitutions increase the protein expression levels of other mutants; therefore, it was of interest to examine the effect of the combination of M182T and L201P on a double mutant. It was observed that the M182T:L201P double mutant has expression levels similar to those of the wild type or the M182T or L201P single mutants (Fig. 3).

Table 3. Enzyme kinetic parameters for TEM-1 β-lactamase and mutant derivatives for ampicillin hydrolysis

	TEM-1	L201P	R244A	R244A-L201P
K_m (μM)	55.2±5.0	58.9±7.0	2704±492	3076±649
k_{cat} (s^{-1})	641±51	844±8	1747±359	1706±383
k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	11.735±2.091	14.480±1.890	0.645±0.029	0.554±0.016

Enzyme kinetic parameters of TEM-1 β-lactamase mutants

The M182T substitution rescues β-lactamases containing destabilizing mutations, but has little influence on enzyme catalytic activity.[10,12] Although ~25 Å distant from the active site, the L201P suppressor could increase enzymatic activity by altering the active site via a long-range structural change propagated down helix α9. To address this issue, the R244A and L201P:R244A enzymes were expressed and purified for kinetic analysis, with ampicillin as substrate. No major differences in k_{cat} or K_m values were found between R244A and L201P:R244A (Table 3). However, as stated above, the L201P substitution is able to increase *in vivo* ampicillin or amoxicillin–clavulanic resistance levels of the R244A TEM-1 mutant in both pET-TEM-1 and pBG66 genetic contexts. The finding that the L201P substitution does not enhance enzyme activity via a change in catalytic efficiency but does restore function of both the L76N and the R244A mutants *in vivo* supports the hypothesis that the L201P substitution acts by increasing the stability of the TEM-1 enzyme and thereby increases steady-state enzyme levels.

Thermodynamic stability of TEM-1 β-lactamase mutant enzymes

To directly address the effect of the L201P substitution upon TEM-1 stability, the L201P, R244A, L201P:R244A, and M182T:L201P TEM-1 enzymes were purified to obtain thermal denaturation curves. Measuring the helical signal by circular dichroism, all mutant proteins denatured reversibly in an apparently two-state manner, as previously observed for the wild-type enzyme.[43] The TEM-1 L201P enzyme exhibited an increased melting temperature relative to wild-type TEM-1 (by 1.9 °C), indicating increased stability at higher temperatures[10,43] (Table 4). Typically, such a substantial change in the T_m may be interpreted as an increase in the stability of the protein across its folded temperature range. Using the method of Becktel and Schellman, which analyzes stability changes in the area of the T_m, L201P would be 0.8 kcal/mol more stable than the wild-type enzyme.[44] However, it is worth noting that the van't Hoff enthalpy of denaturation for L201P (111.4 kcal/mol) is lower than that of the wild-type enzyme (139.5 kcal/mol), which usually cautions against extrapolating changes in stability around the T_m back to lower temperatures where the enzyme is maximally stable. What we can certainly say is that L201P is more stable at higher temperatures than wild-type TEM-1.

We also determined the T_m value of the R244A enzyme, which was 2.5 °C higher than that of wild type and therefore even more stable than that of L201P, at least at the melting temperature. However, the van't Hoff enthalpy of denaturation for R244A (78.1 kcal/mol) is much more depressed than that of L201P, suggesting that this increase in stability can only be trusted at the melting temperature, and that the relative stabilities might well be reversed at lower temperatures. Moreover, the L201P:R244A double mutant has a T_m value that is 0.7° higher than that of R244A, and so the L201P substitution does further stabilize the R244A enzyme. In this double mutant, moreover, the van't Hoff enthalpy of denaturation is partly restored towards its wild-type value, consistent with stabilization throughout the temperature range of folded protein—and not only at higher temperatures—relative to R244A. This result is consistent with the MIC and survival curve results, and with increased steady-state periplasmic expression levels of the L201P:R244A enzyme compared to the

Table 5. Data collection and refinement statistics

Data collection	
Space group	$P2_12_12_1$
Cell dimensions	
a, *b*, *c* (Å)	41.218, 59.140, 87.802
α, β, γ (°)	90.00, 90.00, 90.00
Resolution (Å)	50.00–1.92 (1.99–1.92)[a]
R_{merge} (%)	6.1 (35.4)
$I/\sigma I$	14.0 (2.48)
Completeness (%)	92.0 (83.3)
Refinement	
R_{work}/R_{free} (%)	19.6/24.2
RMSD	
Bond lengths (Å)	0.013
Bond angles (°)	1.473
Ramanchandran plot[b]	
Most favored region (%)	93.0
Additionally allowed region (%)	6.6
Generously allowed region (%)	0.4

[a] Values in parenthesis are for the highest-resolution shells.
[b] Calculated by PROCHECK, excluding glycine and proline.[46]

Table 4. Thermodynamic parameters for TEM-1 β-lactamase mutations affecting stability

	T_m[a] (°C)	ΔT_m (°C)	ΔH (kcal/mol)	$\Delta\Delta G_u$ (kcal/mol)	ΔS_u (kcal/mol K)
TEM-1[b]	51.5±0.1	—	139.5±7.9	—	0.43
M182T[c]	57.7±0.1	6.2±0.2	160.3±4.3	2.67±0.13	0.48
L201P	53.4±0.1	1.9±0.2	111.4±0.7	0.80±0.08	0.34±0.002
L201P:M182T	58.0±0.2	6.5±0.3	96.9±5.9	2.80±0.13	0.29±0.01
R244A	54.0±0.3	2.5±0.4	78.1±5.5	1.08±0.17	0.24±0.02
L201P:R244A	54.7±0.3	3.2±0.4	85.8±1.7	1.38±0.17	0.26±0.004

[a] Relative to TEM-1.
[b] Data from Wang *et al.*[43]
[c] Data from Wang *et al.*[10]

R244A enzyme. The reduced hydrolytic activity associated with the R244A substitution results in a significant reduction in ampicillin resistance. The reduction in resistance levels leads to ampicillin MICs being more sensitive to small changes in β-lactamase stability and expression levels.[45] This may have provided the sensitivity necessary for the ampicillin selection to identify a suppressor that enhanced steady-state expression levels via an increase in the stability of the enzyme.

M182T and L201P each stabilize TEM-1 β-lactamase. Therefore, it was of interest to assess the effects of a double mutant containing both substitutions on enzyme stability. The M182T:L201P double mutant exhibits a 6.5° increase in T_m relative to that of wild type, and is marginally more stable than M182T alone, at least around the melting temperature. Still, the increase in stability of the double mutants relative to that of the single mutants is much less than additive, which is surprising given how distant the two substitutions are in space in the structure. On closer inspection, residues 201 and 182 both sit at the N-termini of sequential helices, which are connected by a four-residue loop. Thus, whereas each of these substitutions can act independently as stabilizers of destabilizing substitutions, together, the two substitutions exhibit negative additivity. This, in turn, suggests that the two helices on which

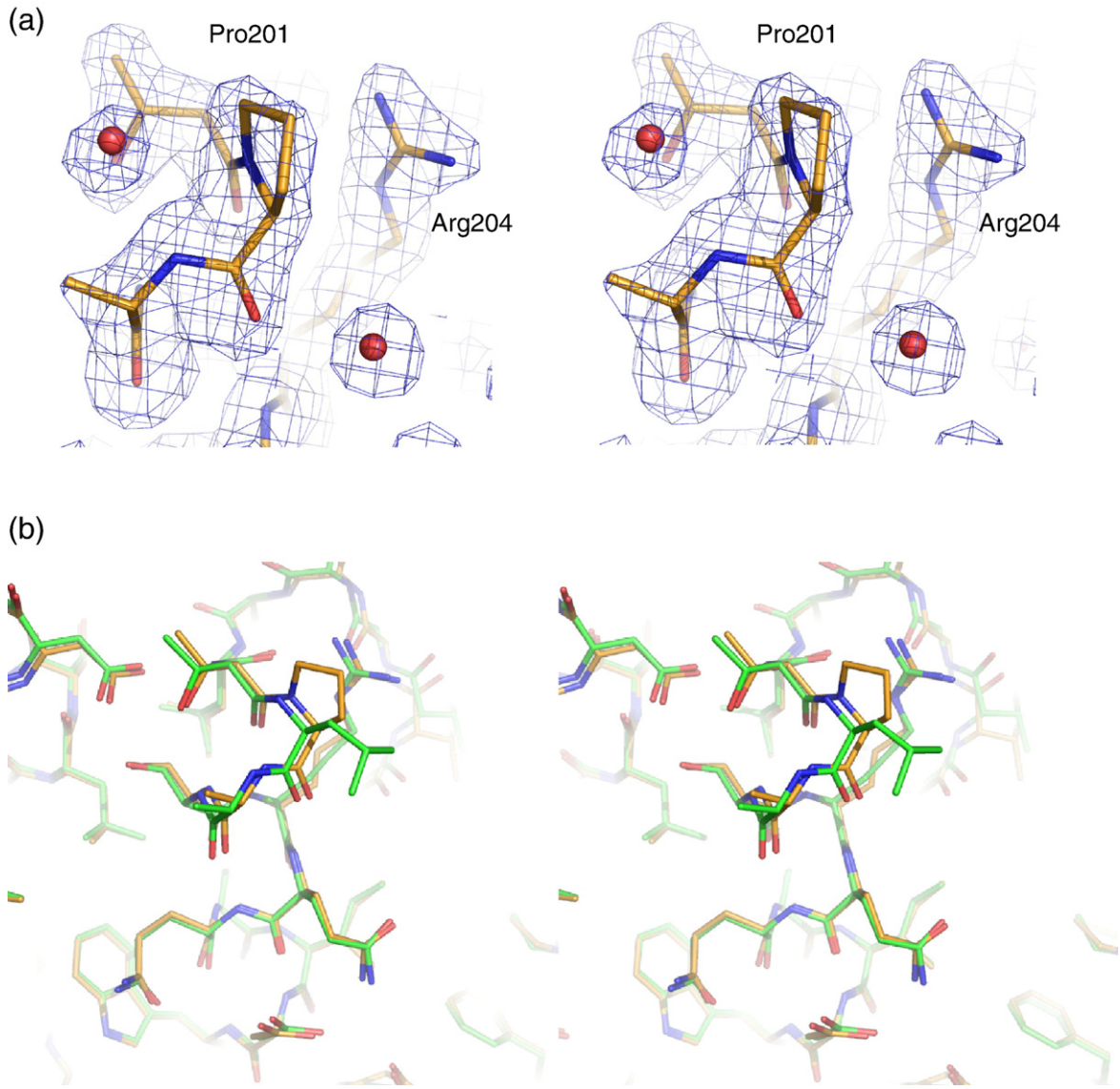

Fig. 4. Structure of the L201P mutant of TEM-1 b-lactamase. (a) $2F_o - F_c$ electron density map, contoured at 1σ, shows the mutated residue 201 and the surrounding environment, including two ordered water molecules (red spheres) that interact with the protein backbone. Carbon atoms are shown in gold, oxygen atoms are shown in red, and nitrogen atoms are shown in blue. (b) Overlay of the TEM-1 wild-type structure (carbon atoms in green) and of the L201P mutant (carbon atoms in gold) in the region of the Leu201 → Pro substitution. (c) Wild-type TEM-1 (1AXB) helix α9 is shown next to α9 of the L201P structure. The residue at position 201 is presented in stick format, and the solvent-exposed surface associated with residue 201 is presented in each structure.

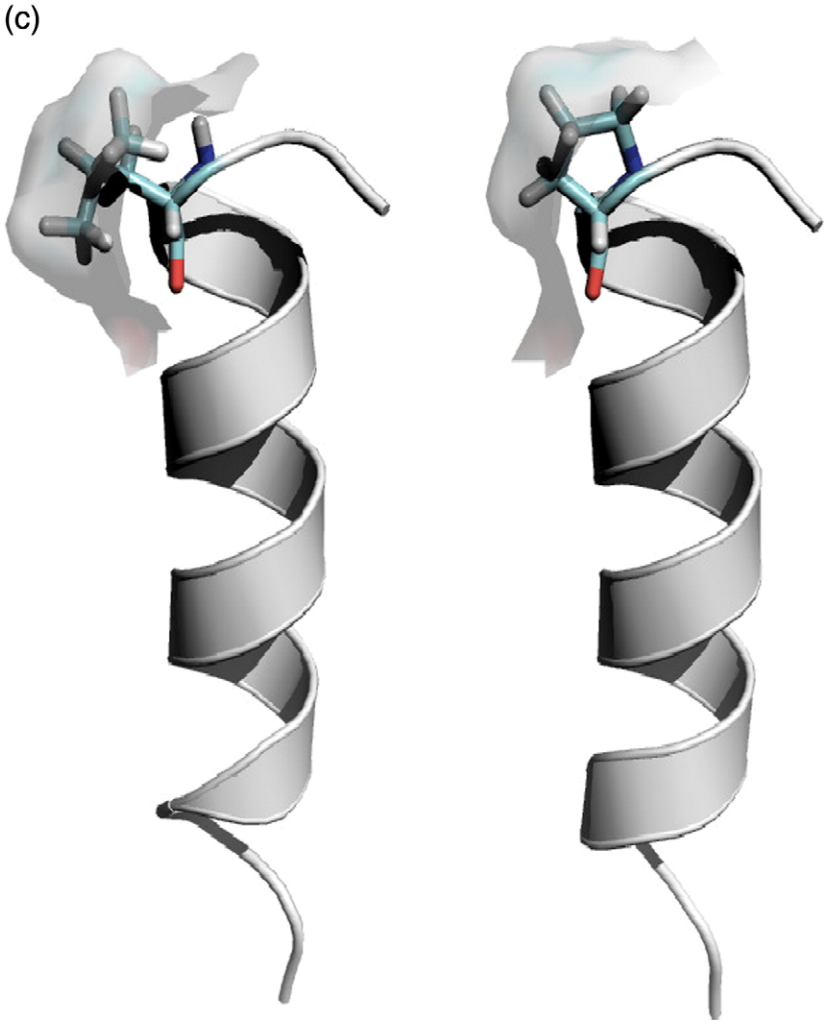

Fig. 4 (*legend on previous page*)

they reside may affect one another. To better understand this observation and to investigate the effects of Leu201 → Pro at atomic resolution, we determined the structure of L201P by X-ray crystallography.

Structure determination of L201P β-lactamase

The L201P TEM-1 enzyme was crystallized under conditions similar to those of native TEM-1. Its structure was determined to 1.92 Å by X-ray, and the structure was modeled and refined with good statistics (Table 5). The electron density at and around the point of substitution (Leu201 → Pro) was unambiguous, allowing us to compare the protein environment in the native and mutant enzymes (Fig. 4a). Residue 201 is at the N-terminus of the α9 helix in TEM-1; in both the native and the mutant structures, the residue is surface-exposed, making only van der Waals contacts with Arg204 in both structures. We compared the structure of the L201P mutant to two native structures (Protein Data Bank IDs 1AXB and 1XPB). For both, the mutant overlapped closely in the region of substitution (see Fig. 4b for the superposition with 1AXB), with the overall C^{α} RMSD between the mutant and 1AXB being only 0.31 Å; in the local region of the substitution (residues 197–205), it was 0.15 Å (the values for the superposition with 1XPB were 0.34 and 0.18 Å, respectively). Thus, the increased temperature stability of the mutant enzyme is not easily explained by changes in interactions in the folded form of the enzyme. Rather, the proline substitution removes the uncompensated main-chain amide proton of Leu201, lying as it does at the N-terminus of helix α9, replacing it with a nonpolar carbon of the proline (Fig. 4c). This reduces the cost of desolvating this group on folding. Consistent with such a role, there is a high propensity for proline at the first (N1) position of α helices.[47] Also, the reduced flexibility of the proline side chain and main chain will increase the free energy of the unfolded form, further increasing the relative stability of the folded enzyme. This entropic effect is thought to be responsible for a ~1-kcal/mol increase in stability observed with proline substitutions in T4 lysozyme and ribonuclease Sa.[48] Thus, the L201P substitution may enhance the stability of TEM-1 β-lactamase via both helix dipole and entropic mechanisms.

Conclusions

The frequent appearance of the M182T substitution, in combination with destabilizing active site mutations in TEM-1 β-lactamase variants from clinical isolates, provides a medically relevant example of evolutionary pressures acting upon protein stability‡.[10,11,16] The L201P substitution was previously identified during an *in vitro* selection experiment for inhibitor-resistant TEM-1 mutants,[37] and it was also recently identified as a suppressor from selection experiments with populations of highly mutagenized TEM-1 genes.[38] Unlike the M182T substitution, however, the L201P change has not been identified in natural isolates. The L201P substitution exhibits properties similar to those of M182T in that it increases stability and steady-state protein expression levels and is not strongly allele-specific (i.e., it can increase the stability of enzymes with different primary substitutions). The results also indicate, however, that both M182T and L201P display some specificity with regard to their ability to stabilize enzymes containing destabilizing primary mutations. For example, the M182T substitution had no detectable impact on the R244A mutant, and the L201P substitution had no detectable effect on the H158S:V159S:T160H triple mutant.

Both M182T and L201P increase the thermodynamic stability of the wild-type enzyme. Therefore, M182T and L201P could generally increase stability ($-\Delta\Delta G$) and act additively with the primary mutations such that the double mutant has increased stability compared to the enzyme containing the primary mutation. The cases where either the M182T or the L201P substitutions do not enhance stability could be explained as nonadditivity with the primary substitution. By this view, the degree of allele specificity of a suppressor mutation (substitution) is a function of its additivity relationships with various primary mutations. The exact mechanisms by which M182T and L201P substitutions act and how they communicate with other residue positions in terms of additivity are currently unknown. It is possible that global suppressors actually act on subsets of residue positions, and these subsets may overlap completely, partially (as for the targets of M182T and L201P), or not at all. The exact physical mechanism by which the substitutions act on the enzyme would determine additivity relationships. For example, the global stabilizer substitutions could differ with regard to acting upon the native folded protein or certain intermediates on the β-lactamase folding pathway, which, in turn, could influence proteolysis or aggregation of the intermediates *in vivo*. If stabilizer substitutions acted in the same way, they would exhibit a similar pattern of suppression of primary mutations, whereas a different pattern of suppression would be observed for stabilizer substitutions that act by different mechanisms. Further experiments are required to clarify these questions. Nevertheless, it is clear from these experiments and other recent observations[38] that the Leu201→Pro substitution provides an important additional example of a stabilizer in TEM β-lactamase that acts on multiple primary mutations and suggests that multiple single-point restabilizing substitutions may be accessible as a protein evolves new activity, increasing its ability to do so without catastrophic loss of stability.

‡ http://www.lahey.org/studies

Materials and Methods

Bacterial strains and plasmids

E. coli XL1-Blue strain (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′proAB lacI^{q}ZΔM15 Tn10 (Tet^r)]) was obtained from Stratagene (La Jolla, CA) and utilized in site-directed mutagenesis and determination of steady-state protein expression levels. The *E. coli* BL21 (DE3) strain (F$^-$ *ompT gal [dcm] [lon] hsdSB* (r_B^- m_B^-) (λ DE3)) was employed in MIC determination and protein purification. The pET-TEM-1 vector encodes an ompA leader–TEM-1 fusion gene driven by the T7 promoter.[35] The ompA–TEM-1 fusion in pET-TEM-1 also contains an E28G substitution in TEM-1 near the signal cleavage site. The pET-TEM-1 E28G construct was used as template for library construction and for selection of the L201P suppressor mutation. Although no phenotypic changes have been described for the E28G substitution, the substitution was reverted by site-directed mutagenesis prior to subsequent purification of proteins used in enzyme kinetics, as well as thermal denaturation experiments and crystallization of the L201P mutant. The pBG66 plasmid encodes wild-type TEM-1 β-lactamase under the transcriptional control of its native promoter. Mutations were made in the pBG66 background by site-directed mutagenesis and used in MIC determinations and immunoblot analysis of steady-state protein levels.

Site-directed mutagenesis

All TEM-1 β-lactamase site-directed mutants were created using Stratagene's QuikChange kit in accordance with the manufacturer's protocol. Primers were obtained from Integrated DNA Technologies (Coralville, IA). The following top strand primers were used to introduce mutations (underlined) into the TEM-1 gene: M182T (5′-CGAGCGTGACACCACGACGCCTGCAGCAATGGC-3′), L201P (5′-GGCGAACTACTTACTCCGGCTTCCC-3′), and R244A (5′-GCGTGGGTCTGCTGGTATCATTGCAGCACTGGG-3′). The L201P and R244A containing pBG66 TEM-1 mutants were constructed upon the previously described wild-type, M69I, I47Y:E48C, H158S:V159S:T160H, L76N, M182T, and L76N:M182T mutants.[11] Constructs were sequenced in-house using an Applied Biosystems Instruments (Foster City, CA) Prism Big Dye DNA sequencing kit with an ABI 3100 automated sequencing instrument or at Lonestar Labs (Houston, TX). The pET-TEM-1 E28G mutation was corrected using the top strand primer 5′-CGTAGCGCAGGCCCACCCAGAAACGCTGGTGAAAGTAAAAGATGC-3′.

Library construction and selection

A library of random point mutations was constructed by error-prone PCR using the pET-TEM-1 E28G:R244A gene

as template. Mutagenic PCR via inclusion of $MnCl_2$ was performed with 5 U of *Taq* polymerase (Promega, Madison, WI), 0.2 mM dATP, 0.2 mM dCTP, 0.4 mM dGTP, 0.4 mM dTTP (dNTPs from Bioline, Randolph, MA), 1.5 mM $MgCl_2$, 1× *Taq* polymerase buffer (Promega), 100 ng of outside primers, and 45 ng of pET-TEM-1 E28G:R244A plasmid as template in a 100-μl reaction. A series of mutagenic PCRs was performed by utilizing either 0.0625, 0.125, or 0.25 mM $MnCl_2$ (three reactions performed in triplicate). The PCR parameters were as follows: 95 °C for 1 min; followed by 30 cycles of 95 °C for 1 min, 50 °C for 2 min, and 72 °C for 3 min; ending with 72 °C for 10 min. All nine mutagenic PCRs were pooled and column-purified using a Qiagen PCR purification kit (Qiagen, Inc., Valencia, CA) before digestion with restriction enzymes XhoI/NdeI/DpnI obtained from New England Biolabs (Ipswich, MA). Insert DNA was gel-purified and ligated into pET-TEM-1 digested with XhoI/NdeI that was also treated with calf intestinal alkaline phosphatase obtained from New England Biolabs. The DNA in the ligation reaction was extracted with phenol/chloroform, transformed into electrocompetent *E. coli* BL21(DE3), and spread onto LB plates containing kanamycin. A total of 1.8×10^5 colony-forming units were pooled. The library was subjected to selection on 350 μg/ml ampicillin. DNA was isolated from individual clones and sequenced to reveal a t→c mutation resulting in the L201P substitution. The L201P:R244A mutant was recovered and sequenced six times. It is not known whether the six mutants were independent, or whether they represent clonal expansion. In addition, the P62L:R244A, A86T:R244A, and I208L:R244A mutants were recovered one time each. Only the P62L:R244A mutant (and L201P:R244A) reproducibly improved MIC relative to R244A. The L201P:R244A mutant was pursued because it was detected in multiple clones and it exhibited the most significant increase in ampicillin resistance.

MIC determinations

MIC determinations for pET-TEM-1 plasmids

E. coli BL21(DE3) was utilized as the host strain for determining the ampicillin MIC of the pDM122 (pET-TEM-1-R244A), pDM123 (pET-TEM-1-R244A:L201P), pDM124 (pET-TEM-1-L201P), and pET-TEM-1 (wild type) constructs. Briefly, overnight cultures were grown at 37 °C in LB broth containing 25 μg/ml kanamycin to maintain the plasmid and 100 μM IPTG to induce expression from the T7 promoter. The overnight cultures were diluted $1{:}10^4$ ($\sim 10^5$ bacteria/ml), and 90 μl was inoculated into 1.75 ml of fresh LB broth containing 25 μg/ml kanamycin, 100 μM IPTG, and various concentrations of ampicillin. The concentrations of ampicillin tested were 179, 230, 256, 282, 307, 333, 358, 384, 410, 435, 461, 486, 512, 563, 666, 860, 1024, and 1106 μg/ml. The cultures were then incubated at 37 °C with shaking for ~18 h, and the MIC was determined by examining the MIC of ampicillin for bacterial growth.

MIC determinations for pBG66 plasmids

Overnight cultures were grown in LB broth containing 12.5 μg/ml chloramphenicol to maintain the pBG66 plasmid. The overnight cultures were diluted $1{:}10^4$ ($\sim 10^5$ bacteria/ml), and 90 μl was inoculated into 1.75 ml of fresh LB broth containing 12.5 μg/ml chloramphenicol and various concentrations of ampicillin. The concentrations of ampicillin tested were 4, 8, 16, 32, 64, 128, 256, 512, 1024, 1536, 2048, 4096, and 8192 μg/ml. The cultures were then incubated at 37 °C with shaking for ~18 h and then examined to determine the MIC of ampicillin for bacterial growth. For MIC determination using amoxicillin + clavulanic acid E-test strips (AB Biodisk, Solna, Sweden), a one-tenth dilution of an overnight culture of *E. coli* XL1-Blue transformed with a pBG66 construct was spread upon LB plates before placement of the E-test strip on the bacterial lawn. Plates were allowed to incubate overnight at 37 °C before scoring for growth. Each assay was conducted at least in duplicate using independent overnight cultures.

Competition experiments using *E. coli* B Ara^+ and Ara^- strains

Competition experiments were performed between *E. coli* B Ara^+ and Ara^- cells containing the pBG66 plasmid encoding either the TEM-1 R244A or the L201P:R244A β-lactamases. For these experiments, pBG66-TEM-1 R244A and pBG66-TEM-1 L201P:R244A plasmids were transformed into both *E. coli* B Ara^+ and Ara^- strains. Reciprocal experiments were performed (i.e., Ara^+ R244A *versus* Ara^- L201P:R244A, and Ara^+ L201P:R244A *versus* Ara^- R244A for each ampicillin concentration). For simplicity, the methods described below are for the Ara^+ R244A *versus* Ara^- L201P:R244A experiment, but they were the same for the reciprocal experiment. A culture of each strain was grown overnight in LB medium containing 12.5 μg/ml chloramphenicol to select for the presence of the pBG66 plasmid. Equal volumes of each overnight culture were mixed and diluted to a final concentration of 1×10^4 cells/ml in LB medium containing 12.5 μg/ml chloramphenicol with or without ampicillin. A zero time point was taken from this culture and spread on TA plates containing 12.5 μg/ml chloramphenicol. After overnight growth at 37 °C, Ara^+ R244A colonies were observed in white/pink, and Ara^- L201P:R244A colonies were observed in red. By counting pink *versus* red colonies, it was possible to estimate the number of Ara^+ R244A and Ara^- L201P:R244A cells in the culture. Competition experiments were performed by adding various concentrations of ampicillin to the Ara^+:Ara^- mixed starting culture and by allowing the cells to compete for growth overnight at 37 °C. The cultures were then spread on TA plates, and pink and red colonies were counted after overnight growth at 37 °C.

Bacterial cell survival on ampicillin agar

E. coli XL1-Blue containing the pBG66 plasmid that encodes TEM-1 or a TEM-1 β-lactamase mutant was grown overnight in LB broth containing 12.5 μg/ml chloramphenicol. Overnight cultures were diluted 1:100 into LB broth containing 12.5 μg/ml chloramphenicol and incubated for 4 h at 37 °C to mid-log phase ($OD_{600} = 0.4$–0.6). Ten-fold serial dilutions of each culture were made, and 100 μl of each dilution was spread onto LB agar plates containing 0, 15, 30, 60, 120, 240, 480, or 960 μg/ml ampicillin. After incubation for 24 h at 37 °C, colony-forming units on each plate were counted. These data were used to calculate the colony-forming units per milliliter of culture, or survival on a given concentration of ampicillin.

Immunoblot analysis

The effect of the M182T and L201P substitutions upon steady-state expression levels of TEM-1 β-lactamase was determined by immunoblot analysis of *E. coli* XL1-Blue

periplasmic contents as previously described.[49] Briefly, overnight cultures transformed with pBG66 encoding wild-type TEM-1 or a mutant thereof were diluted 1:50 in fresh LB media containing chloramphenicol and allowed to grow to an OD_{600} of ~0.3. A total of 1.5 ml of culture was pelleted by centrifugation, resuspended in 200 μl of spheroplast buffer [50 mM Tris–HCl (pH 8), 1 mM ethylenediaminetetraacetic acid, and 20% sucrose], and incubated on ice for 10 min. The cells were again pelleted by centrifugation and resuspeded in 200 μl of cold H_2O to release the periplasmic contents of the culture. The concentration of soluble protein in each sample was measured using the Bio-Rad Bradford protein assay reagent (Hercules, CA), and approximately 15 μl of sample (adjusted according to protein concentration to ensure equal loading between wells) was resolved on a 12% SDS-PAGE gel. *E. coli* proteins were electrotransferred to a nitrocellulose membrane (Amersham; GE Healthcare, Piscataway, NJ) using a Bio-Rad semidry transfer apparatus. The membrane was blocked overnight in 5% milk before probing with rabbit polyclonal anti-TEM-1 antibody. A donkey anti-rabbit horseradish peroxidase conjugate was utilized with the Amersham ECL chemiluminescent detection reagent (GE Healthcare) to visualize TEM-1 proteins. This procedure was performed in duplicate using independent cultures.

Protein purification

The L201P, R244A, M182T:L201P, and L201P:R244A β-lactamases were purified to ~90% homogeneity. *E. coli* BL21(DE3) cells transformed with the relevant mutant construct were grown in LB broth with 300 mM sorbitol, 250 mM betaine, and 25 μg/ml kanamycin to an OD_{600} of 0.8, and transcription was induced with 0.4 mM IPTG. The induced culture was grown overnight with shaking at room temperature. Cells were harvested by centrifugation, and the periplasmic contents were obtained by osmotic shock. The periplasmic fraction was dialyzed overnight at 4 °C before loading onto a Hi-Trap zinc-chelating column (Amersham; GE Healthcare) charged with $ZnCl_2$.[5,50] The β-lactamase-containing fraction was eluted by a pH gradient, and purity was determined by SDS-PAGE gel electrophoresis. Buffer exchange into 50 mM PO_4 (pH 7.0) was facilitated by use of a Centricon centrifugal filter (Millipore, Billerica, MA). Mutant β-lactamases L201P, R244A, M182T:L201P, and L201P:R244A used in thermal denaturation and crystallization procedures were further purified to ~99% purity by size-exclusion chromatography. Protein concentrations were determined with the Bio-Rad Bradford protein assay reagent.

Enzyme kinetics

Michaelis–Menten steady-state kinetic parameters were determined on a Beckman Coulter spectrophotometer model DU 800 (Beckman Coulter, Fullerton, CA). Ampicillin hydrolysis was monitored at 235 nm. Reactions were performed at 30 °C in 50 mM phosphate buffer (pH 7.0). K_m and k_{cat} values were determined by fitting initial velocity rates over a range of substrate concentrations to a Michaelis–Menten curve using SigmaPlot. Measurements were performed in triplicate.

Thermal denaturation

Thermal denaturation was carried out in 200 mM potassium phosphate (pH 7.0), as previously described.[43] The enzymes were denatured by raising the temperature in 0.1 °C increments at a ramp rate of 2 °C/min using a Jasco J-715 spectropolarimeter with a Jasco PTC-348WI Peltier-effect temperature controller, and an in-cell temperature probe and stir bar were used as previously described. All T_m and ΔH_{VH} values were calculated with the program EXAM;[51] the change in heat capacity upon denaturation (ΔC_p) was set to 3.8 kcal/mol K for each enzyme.[10,43] Denaturation was marked by an obvious transition in the far-UV CD signal, monitored at 223 nm. Reversibility was measured by the return of folded CD signal divided by the amount of signal lost on unfolding: all enzymes showed greater than 90% reversibility after denaturation. Values of $\Delta\Delta G_u$ were determined by the method of Becktel and Schellman, using the entropy of unfolding of the wild-type (WT) enzyme, as determined previously:[10,43,44]

$$\Delta\Delta G_u = \Delta T_m \Delta S_{WT-enzyme}$$

Structure determination of L201P β-lactamase

L201P was crystallized in 1.6 M potassium phosphate buffer (pH 8.7) using a hanging-drop method.[10] Diffraction data were collected at beamline 8.3.1 of Advanced Light Source at the Lawrence Berkeley National Laboratory (Berkeley, CA) to 1.92 Å resolution and processed with HKL2000.[52] An initial model was obtained through molecular replacement based on a TEM-1 M182T mutant structure (1JWP) using EPMR.[53] Refinement was performed with CCP4 up to a final R value of 19.6% and an R_{free} value of 24.2%.[54] Full statistics are provided in Table 5.

Accession code

The coordinates have been deposited in the Protein Data Bank with access code 3CMZ.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2008.09.009

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