Published in final edited form as:

J Mol Biol. 2010 December 17; 404(5): 832–846. doi:10.1016/j.jmb.2010.10.008.

Multiple global suppressors of protein stability defects facilitate the evolution of extended-spectrum TEM β -lactamases

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Abstract

The introduction of extended-spectrum cephalosporins and β -lactamase inhibitors has driven the evolution of extended-spectrum TEM β -lactamases (ESBLs) with the ability to hydrolyze these drugs. The evolved TEM ESBLs from clinical isolates of bacteria often contain substitutions that occur in the active site and alter the catalytic properties of the enzyme to provide increased hydrolysis of extended-spectrum cephalosporins or resistance to inhibitors. These active site substitutions often result in a cost in the form of reduced enzyme stability. The evolution of TEM ESBLs is facilitated by mutations that act as global suppressors of protein stability defects in that they allow the enzyme to absorb multiple amino acid changes despite incremental losses in stability associated with the substitutions. The best studied example is the M182T substitution that corrects protein stability defects and is commonly found in TEM-type ESBLs or inhibitor-resistant variants from clinical isolates. In this study, a genetic selection for second site mutations that could partially restore function to a severely destabilized primary mutant enabled the identification of A184V, T265M, R275Q, and N276D, which are known to occur in TEM ESBLs from clinical isolates, as suppressors of TEM-1 protein stability defects. Further characterization demonstrated that these substitutions increased the thermal stability of TEM-1 and were able to correct the stability defects of two different sets of destabilizing mutations. The acquisition of compensatory global suppressors of stability costs that are associated with active site mutations may be a common mechanism for the evolution of novel protein function.

Keywords

Antibiotic resistance; global suppressor; beta-lactamase; protein evolution; protein stability

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Introduction

Proteins are considered the biochemical workhorses of the cell and perform a variety of functions to govern cellular processes. During evolution, the genes encoding proteins often acquire point mutations leading to amino acid substitutions that alter the protein's properties to increase the overall fitness of the organism.¹⁻³ Understanding the properties that contribute to a protein's evolvability has become an active area of research and a number of directed evolution and computational studies have been performed.⁴⁻⁹

In the case of antibiotics, the evolution of bacterial resistance is often associated with the acquisition of enzymes that destroy or modify the antibiotic. The β -lactam antibiotics, which include the penicillins, cephalosporins and carbapenems, represent 60% of all antibiotic usage worldwide.¹⁰ The most common mechanism of resistance to β -lactam antibiotics is the bacterial production of β -lactamase enzymes that hydrolyze the four-membered β -lactam ring common to these drugs.¹⁰⁻¹¹ β -lactamases are divided into four classes (A-D) based on primary sequence homology and are the most common cause of antibiotic resistance.¹²⁻¹³ TEM-1 is a class A β -lactamase that utilizes a catalytic serine for β -lactam hydrolysis. It is among the most common β -lactamases in gram-negative bacteria and is able to efficiently hydrolyze penicillins and first and second generation cephalosporins to provide high levels of resistance to these drugs.¹⁴⁻¹⁵

Extended-spectrum cephalosporins such as cefotaxime and ceftazidime and β -lactamase inhibitors such as clavulanic acid were introduced because of the widespread occurrence of β -lactamases such as TEM-1. The value of these newer drugs is that, even if the bacteria contain a TEM-1 β -lactamase, they are susceptible to the antibiotic because the enzyme is either not able to hydrolyze the extended-spectrum cephalosporin or is inhibited by clavulanic acid. The introduction of these drugs, however, introduced a strong selective pressure on bacteria, and one result of this pressure has been the evolution of variant enzymes of TEM that have acquired amino acid substitutions allowing them to hydrolyze the new cephalosporins or avoid the action of the inhibitors.¹⁶⁻¹⁷⁻¹⁸

It has been previously demonstrated in multiple enzymes, including β -lactamases, that amino acid substitutions that alter the catalytic function of an enzyme often simultaneously result in a decrease in protein stability.³⁻¹⁹⁻²⁰⁻²¹ Because most proteins are only marginally stable prior to the acquisition of mutations and will unfold at physiological temperatures if multiple functional substitutions are present, a further selection of compensatory mutations that stabilize the functionally altered enzyme can occur.²² These observations are consistent with the hypothesis that increasing the thermodynamic stability of a protein increases evolvability.⁵⁻²³ If the protein or enzyme is further stabilized, data suggests that it can tolerate multiple substitutions that alter function but destabilize the enzyme.⁵ This scenario has been observed for β -lactamases during the evolution of improved activity towards extended-spectrum cephalosporins.¹

Currently, there are over 170 TEM extended-spectrum β -lactamases (ESBLs) or inhibitor-resistant TEM (IRT) variants identified from clinical isolates (www.lahey.org). Each one contains primary sequence changes compared to wild-type TEM-1. Some substitutions are located in or around the active site and have been demonstrated to improve the catalytic activity of the enzyme towards extended-spectrum cephalosporins or are involved in inhibitor resistance.¹³⁻¹⁶ These mutations often decrease the thermodynamic stability of the enzyme.³ For example, the E104K:G238S substitutions (TEM-15) function synergistically to greatly increase the hydrolytic activity of TEM-1 towards the extended-spectrum cephalosporins, cefotaxime and ceftazidime. These mutations slightly rearrange the active site of TEM to accommodate the larger and bulkier R-groups of these

cephalosporins.³ However, this rearrangement carries a stability cost to the enzyme.³ The TEM-52 enzyme contains an additional substitution, M182T, that stabilizes the enzyme and thereby compensates for the loss associated with the E104K:G238S substitutions.³

To date, several mutations encoding stabilizing substitutions in TEM-1 have been identified, including two changes that have been shown to act as global suppressors, M182T and L201P.^{1; 24} Global suppressor substitutions act to correct folding or stability defects of primary substitutions located at different positions throughout the protein.^{1; 25; 26} Though they may not counteract all primary substitution defects, global suppressor mutations increase the protein's evolvability by reducing the negative effects of the stability-function tradeoff.^{3; 5; 25; 27; 28} However, the prevalence and significance of global suppressor mutations in the evolution of extended-spectrum TEM-1 enzymes is currently unclear and is a topic of active research.^{23; 24; 29; 30}

In this study, we sought to gain insight into the evolution of TEM-1 β -lactamase by selecting for second site mutations that increase the stability of a severely destabilized starting mutant of TEM-1. A triple amino acid substitution was introduced into TEM-1 (H158S:V159S:T160H) that drastically reduced the level of antibiotic resistance of *E. coli* harboring a plasmid encoding this mutant in comparison to that containing a plasmid encoding wild-type.^{1; 24} This construct was used to create random point mutations and isolate second site suppressor mutations that increase ampicillin resistance of *E. coli* clones containing the plasmid. Several of the identified second site mutations were characterized and shown to act as global suppressor mutations. Interestingly, several of the global suppressors including M182T, A184V, T265M, R275Q and N276D identified in these experiments are found in evolved extended-spectrum TEM enzyme from clinical isolates resistant to either extended-spectrum cephalosporins or β -lactam inhibitors. In fact, over a third of all of the listed TEM variants from clinical isolates contain one of these substitutions. These substitutions were further shown to increase both the *in vivo* and *in vitro* stability of TEM-1, illustrating the significance of these secondary antibiotic resistance mutations to the evolution of TEM-mediated resistance. Interestingly, the R275Q and N276D substitutions were previously shown to be involved in inhibitor resistance and are assigned an additional role as global suppressors of protein stability defects.¹⁷

Results and Discussion

Selection of second site suppressor substitutions in β -lactamase

The acquisition of substitutions into the TEM-1 active site that change catalytic parameters often also result in reduced protein stability.^{3; 31} Periplasmic proteins with folding or stability defects are typically degraded by periplasmic proteases.^{1; 25} TEM-1 has been shown to acquire a secondary antibiotic resistance mutation, M182T, to correct for the potential defects in folding and/or stability and subsequent proteolysis that is associated with primary mutations which increase activity towards extended-spectrum β -lactams.^{3; 28}

Previously, the H158S:V159S:T160H triple mutant was shown to drastically reduce the levels of TEM-1 enzyme in *E. coli* and consequently the levels of resistance to ampicillin (from 1000 μ g/mL to 15 μ g/mL).²⁴ Periplasmic proteases are known to target the TEM mutants with folding or stability defects.¹ For this reason, this triple mutant was used as the starting point to select for second site mutations that partially correct the misfolding and/or stability defects associated with this TEM mutant.

The pBG66 plasmid encoding the TEM H158S:V159S:T160H mutant was introduced into a DNA repair deficient strain of *E. coli* (XL1-Red) that accumulates point mutations at an approximately 5000-fold higher rate than wild type (Stratagene). Mutagenized plasmids

were then isolated and introduced into a repair competent strain and selected for growth on agar plates containing 500 µg/mL ampicillin. The plasmid DNA from ten colonies was isolated and the *bla*_{TEM} gene was sequenced and all ten clones contained the M182T mutation in addition to the starting H158S:V159S:T160H mutant. The M182T global suppressor substitution has previously been shown to increase the ampicillin MIC of the H158S:V159S:T160H mutant to near wild-type levels.²⁴

Since M182T was the only second site mutation identified among the surviving clones in the 500 µg/mL ampicillin selection we reasoned that the selection may be too stringent to identify other potential suppressors and therefore the mutagenized plasmid was introduced into *E. coli* and selected for growth on agar plates containing 50 µg/mL ampicillin. The resulting colonies were screened for growth on agar plates containing 500 µg/mL ampicillin since it was known such clones only contained the M182T second site mutation. Twenty clones that grew at the lower concentration (50 µg/mL) but did not survive at the higher concentration of ampicillin (500 µg/mL) were sequenced to identify the associated second site mutations. The sequencing results identified I47V, A184V, T188I, I208M, R241H, T265M, R275Q, and N276D as possible second site mutations that could increase the stability of TEM-1 H158S:V159S:T160H and thereby increase ampicillin resistance levels (Fig. 1).

As shown in Table 1, half of these mutations including A184V, T265M, R275Q, and N276D have been identified in TEM ESBLs from clinical isolates resistant to extended-spectrum cephalosporins or β-lactamase inhibitors. The finding that these mutations suppress the defects of the H158S:V159S:T160H laboratory created mutant and are also found in TEM-1 ESBL or IRT β-lactamases is unlikely to be a coincidence. In addition, several of these substitutions have been observed in directed evolution experiments ²³ ²³ ³² ³³ ³⁴. Therefore, these ESBL-associated mutations were hypothesized to function as global suppressors of TEM-1 stability or folding defects.

Test of second site mutations for global versus allele-specific suppression of stability defects

In order to ensure that the second-site mutations are responsible for the enhanced ampicillin resistance observed in the revertant mutants and not from potential mutations found elsewhere on the pBG66 plasmid, the mutations were created by site-directed mutagenesis in the original pBG66 plasmid containing wild type TEM-1 as well as pBG66 harboring the H158S:V159S:T160H TEM mutant. The plasmids containing the reconstructed mutants were introduced into *E. coli* and were tested for the ampicillin resistance levels of bacterial cultures harboring each TEM-1 mutant combination to assign an ampicillin IC₉₀ value, defined here as the concentration of ampicillin required to reduce viability (colony forming units) of TEM-1 expressing bacteria by ≥90%. The data indicate that each second site mutant isolated does indeed enhance the ampicillin resistance of cells containing the H158S:V159S:T160H triple mutant (Table 2, Fig. 2).

The TEM-1 β-lactamase L76N substitution is located in the hydrophobic core of the protein and is known to result in protein misfolding associated with extensive proteolysis *in vivo*.²⁸ To test whether the second site mutants only correct the defect associated with the H158S:V159S:T160H TEM mutant, or if the global suppressors are able to increase the stability of multiple different primary mutations, the ampicillin resistance levels of the TEM-1 L76N mutant alone and in combination with each of the second site mutations was determined and an ampicillin IC₉₀ value was assigned (Table 2, Fig. 3). The results indicate that each second site mutation, except for T188I, enhances the ampicillin resistance when combined with TEM-1 L76N compared to the L76N mutant alone. Collectively, the data show that all second site mutations isolated from the selection, except for T188I, suppress

the deleterious effects of two different types of primary mutations that are known to cause TEM-1 instability or protein folding defects. The ability to compensate for multiple mutations demonstrates that they are not allele-specific suppressor mutations. Therefore the data suggest that, except for T188I, all the second site mutants identified are global suppressors of TEM-1 instability.

Second-site suppressors enhance TEM-1 steady-state protein levels *in vivo*

As described above, unstable or misfolded periplasmic proteins are rapidly proteolyzed in *E. coli*.^{1, 24} The TEM-1 L76N and H158S:V159S:T160H mutants have previously been shown to exhibit reduced expression levels due to proteolysis.^{1, 24, 28} The steady state protein expression levels of several combinations of primary and second site mutations in TEM-1 were evaluated by immunoblot analysis and indicated an increase in expression levels and stability of the H158S:V159S:T160H mutant when it was combined with each of the global suppressor mutations.

The protein expression levels of TEM-1 β -lactamase wild-type and mutants encoded from the pBG66 vector were determined by immunoblot analysis of whole cell lysates with anti- β -lactamase polyclonal antisera (Fig. 4). The L76N and L76N:M182T mutants were included as controls to demonstrate the known restoration of protein levels by the M182T global suppressor.¹ Similar to that observed with the TEM-1 L76N enzyme, the protein levels of the H158S:V159S:T160H mutant are barely detectable in comparison to wild-type, indicating the reduction in ampicillin resistance is due in part to low steady-state protein levels. When the second site mutations were combined with the H158S:V159S:T160H mutant, the protein expression levels were increased suggesting that the higher ampicillin resistance levels observed for the mutant combinations is likely due to an increase in active protein levels. These data provide further support that the suppressor mutations correct folding and/or stability defects, thus allowing the enzyme to avoid degradation.

To date, the I47V, T188I, and R241H substitutions have not been observed in clinical isolates. However, these substitutions were previously isolated in other TEM-1 β -lactamase library selections indicating their significance to the stability of TEM-1.³⁰ These substitutions could potentially compensate for the destabilizing effects associated with substitutions that increase extended-spectrum cephalosporin hydrolysis but they have yet to be isolated. The A184V, T265M, R275Q and N276D substitutions were further characterized because of their frequent presence in TEM ESBL variants (Table 1).

Kinetic parameters of TEM-1 β -lactamase mutants

The M182T and L201P global suppressors previously identified in TEM-1 have little or no effect on the catalytic activity of the enzyme.^{1, 24, 25} Therefore, these substitutions presumably function only as stabilizing mutations to increase resistance. The kinetic parameters of TEM-1 containing the ESBL-associated suppressor mutations towards ampicillin were determined to evaluate if increased catalytic efficiency plays a role in the increased resistance or if the mutations are due exclusively to enhanced protein stability.

The M182T, A184V, I208M, T265M, R275Q, and N276D substituted β -lactamases as well as the wild-type enzyme were expressed and purified to homogeneity for kinetic analysis of substrate hydrolysis. Overall, the enzymes containing the suppressor substitutions exhibited kinetic parameters for ampicillin hydrolysis similar to wild-type (Table 3). The N276D enzyme and, to a lesser extent, the R275Q enzyme, however, demonstrated a reduction in catalytic efficiency because of an increase in K_m . This observation was made previously and is associated with its resistance towards β -lactam inhibitors.^{35, 36, 37, 38, 39} The small

effect of R275Q and N276D on catalytic activity further supports the hypothesis that the role of these suppressor mutations is to correct for destabilizing effects.

Despite several attempts, it was not possible to purify the H158S:V159S:T160H β -lactamase mutant due to its poor expression and/or stability. However, the H158S:V159S:T160:M182T enzyme was expressed and purified for kinetic analysis. This enzyme had the lowest catalytic activity towards ampicillin due to a reduction in k_{cat} (Table 3). The M182T substitution is functionally neutral with respect to catalytic efficiency and therefore the reduction in activity is believed to be caused by the H158S:V159S:T160H substitutions (Table 3). Collectively these results suggest that H158S:V159S:T160H substitutions may result in decreased catalysis as well as greatly decreased enzyme stability. The similar activity of the suppressor substitutions on the wild-type background suggests the mechanism by which the second site mutations increase enzyme function as revealed by ampicillin resistance levels for the L76N and H158S:V159S:T160H enzymes is not the result of increased catalytic efficiency for substrate hydrolysis but rather increased levels of active enzyme.

The substrate specificity of the TEM-1 enzymes containing the suppressor substitutions were further examined for hydrolysis of nitrocefin (a chromogenic cephalosporin) and cefotaxime (an extended-spectrum cephalosporin) to test whether the substitutions resulted in increased hydrolysis. The hydrolysis of cefotaxime was observed for TEM-1 wild-type and mutants; however, the V_{max} was not obtained due to the large K_m of TEM-1 mediated cefotaxime hydrolysis. The catalytic efficiency was determined using three or more initial velocities at the lower concentration ranges ($[S] \ll K_m$). Overall, the TEM-1 enzymes containing suppressor substitutions function similar to wild-type, except for the R275Q and N276D enzymes, which exhibit significant reductions in catalytic efficiency for nitrocefin and cefotaxime hydrolysis (Table 3). In addition, the H158S:V159S:T160H:M182T substituted enzyme exhibited a reduction in catalytic efficiency for both cephalosporin substrates resulting from a decrease in k_{cat} towards nitrocefin and potentially cefotaxime. The fact that the suppressors do not contribute to enhanced cephalosporin hydrolysis suggests that the function of the suppressor substitutions in the evolved TEM ESBL enzymes is in compensating for the stability costs of active-site mutations that enhance the catalytic activity towards extended-spectrum cephalosporins (eg., cefotaxime).

Consequences of global suppressor substitutions on TEM-1 thermostability

Global suppressors increase the stability of the protein in a manner independent of the structural effects of the primary destabilizing substitutions.^{1; 24; 28} To directly examine the stability effects associated with the potential global suppressor substitutions found in extended-spectrum β -lactamases, the TEM-1 wild-type, M182T, A184V, I208M, T265M, R275Q, N276D and H158S:V159S:T160H:M182T enzymes were expressed, purified and used to generate thermal denaturation curves (Fig. 5). The denaturation curves were obtained by measuring the α -helix ellipticity by circular dichroism while increasing the temperature. The TEM-1 wild-type β -lactamase denatured in a two-state manner as shown in Figure 5 and supplementary material Figure S1.^{40; 41; 42} However, the folding was not fully reversible as previously described due to precipitation of denatured enzyme at the high concentrations needed for the assay.^{40; 41; 42} The protein concentration used is approximately 5-fold higher than previous thermal denaturation of TEM-1.²⁹

The melting temperature (T_m) of the wild-type enzyme was determined to be 56.7°C. This melting temperature is higher than previously reported (51.5 °C).²⁴ However, this did not significantly affect the overall result of the experiment since the T_m changes of the mutants are evaluated relative to wild type and measured under identical conditions. As controls, the denaturation of the M182T and I208M mutants was performed. Both mutants demonstrated

a thermal stabilization relative to the wild type TEM-1 enzyme of 6.5 °C and 1.1 °C, respectively (Table 4). This is similar to previous experiments where the M182T and I208M mutants were shown increase the thermostability of TEM-1 by 6.2 °C and 1 °C, respectively.^{24; 29} The crystal structures of M182T (1JWP) and a multiply substituted enzyme that included the I208M substitution (3DTM) were previously determined to gain insights into how these substitutions stabilize the enzyme.^{3; 29} The M182T substitution is hypothesized to act by adding multiple hydrogen bonds that stabilize the N-terminal portion of the α -helix at the hinge region of the protein linking the α -domain and the α/β domain of TEM-1 (Fig. 6a).

Although, the I208M substitution has yet to be isolated in extended-spectrum or inhibitor-resistant TEM β -lactamases, this substitution was further characterized because of its repeated isolation in laboratory studies and as a control to ensure the sensitivity of the thermal denaturation experiments since it exhibits a small change in T_m .²⁹ The previous discovery of this substitution in studies selecting for compensatory stability mutations validates its identification here as a global suppressor.^{29; 34} The I208M enzyme structure, in conjunction with other stabilizing mutations, was found to create additional van der Waals contacts by adding a larger side chain to interact with F230 and P257 (Fig. 6c). These contacts are hypothesized to stabilize this region and increase the thermostability of the protein.²⁹

The denaturation curve for the H158S:V159S:T160H:M182T enzyme revealed a thermostability that was reduced relative to TEM-1 wild-type (Figure 5 and Table 4). A unique observation concerning the thermal denaturation curve of the H158S:V159S:T160H:M182T enzyme is the elongated transition between the folded and unfolded state in comparison to the wild type and other TEM-1 variants (Fig. 5). The less cooperative unfolding of the H158S:V159S:T160H:M182T enzyme suggests the unfolding is not the two-state mechanism that is commonly observed for TEM-1 thermal denaturation.^{3; 23; 29; 38} The denaturation curve was best fit to a two phase transition or three-state unfolding equation (Materials and Methods). This observation suggests that during the unfolding process of the H158S:V159S:T160H:M182T enzyme, an intermediate is formed during the transition from the folded to unfolded state. The melting temperatures for these two transitions were determined to be 51.7°C and 59.5°C (Table 4). The three-state unfolding and dramatically reduced T_m of the native state is due to the presence of the H158S:V159S:T160H substitutions, as it is not observed in the M182T denaturation. If the effect of the M182T stabilizing substitution is assumed to act additively (adding 6.5 °C to thermal stability), the stability of the H158S:V159S:T160H enzyme would be reduced 11.5°C relative to wild type (Table 4).

The suppressor substitutions including A184V, T265M, R275Q, and N276D, were also tested to investigate their effects on the thermal stability of wild-type TEM-1. Each of the substitutions tested increased the thermostability of the TEM-1 enzyme, which is consistent with their proposed role as global suppressors of stability defects in TEM ESBLs (Table 4). The A184V substitution is present in several TEM ESBLs (Table 1) and is also encoded in the *bla*_{TEM-1} gene in laboratory pUC-based plasmids. The A184V substitution increases the T_m of TEM-1 by 1.4 °C and is near the position of the M182T global suppressor substitution. The threonine side chain of M182T contacts the main chain of residue Ala184; however, the valine side chain associated with A184V is oriented in an altered direction and therefore is in a different local environment than the position 182 threonine. The valine side chain of A184V is in a hydrophobic pocket which results in improved packing compared to the wild type alanine (Fig. 6b, PDB ID 1BTL).^{3; 43} Residue 184 is also surrounded by other putative global suppressor substitutions such as I47V and T188I, suggesting that this hinge region between the two domains is a hotspot for stabilizing substitutions (Fig. 6b).

The frequent occurrence of the T265M substitution in evolved TEM ESBLs has been observed, and the impact of this mutation on catalytic efficiency was previously tested by enzyme kinetic analysis.⁴⁴ Increased hydrolysis of extended-spectrum cephalosporins due to the T265M substitution was not observed in previous experiments or in this study. The frequent occurrence of T265M in clinical isolates as well as its occurrence, along with other substitutions, in directed evolution experiments^{32, 33}, resulted in a recent prediction that it is a global suppressor substitution.³⁰ Its identification in the genetic selection presented here and further immunoblotting and temperature denaturation experiments strongly support the hypothesis that T265M is a global suppressor of stability defects. Threonine at position 265 leaves a buried cavity in an otherwise well-packed environment. Molecular modeling of a methionine residue at this position suggests the substitution results in improved packing and reveals the possibility of several hydrophobic interactions stabilizing both the first and last helices of TEM-1 (Fig. 6d).

The R275Q and N276D substitutions are found in inhibitor-resistant TEM enzymes from clinical isolates and are known to reduce the susceptibility of the enzyme to β -lactamase inhibitors.^{17, 35, 37, 39} Interestingly, the R275Q and N276D substitutions result in a thermal stabilization of the enzyme of 2.0 and 1.3 °C, respectively. The positioning of Arg275 in the wild-type structure is unfavorable. Specifically, the positively charged guanidinium group of arginine is partially buried within the structure and is positioned at the N-terminus of an α -helix (residues 272–290), resulting in an unfavorable interaction with the dipole of the helix. R275Q and R275L are two mutations found in TEM variants (www.lahey.org) and the R275L substitution has been shown to stabilize wild type TEM-1 β -lactamase.²⁹ The removal of the arginine residue at position 275 changes the electrostatic environment of the region and has been hypothesized to displace a water molecule involved in the inactivation chemistry of β -lactam inhibitors.⁴⁵ The crystal structure of a multiply substituted enzyme containing R275L (3DTM) has previously been determined because of its ability to stabilize TEM-1.²⁹ This structure revealed that a leucine at position 275 resulted in improved packing relative to the wild type arginine while also removing the unfavorable charge effects.²⁹ A molecular model of the R275Q substitution reveals the glutamine residue packs well in this region by filling the void created by replacing arginine. In addition, the amide group of glutamine is in a favorable hydrogen-bonding environment (Fig. 6e), as the amide group becomes involved in a hydrogen bonding network with the main chain oxygen of Gly242 and Ala270, as well as a water molecule and the Ser268 side chain oxygen. Overall, the glutamine residue is both well-packed and is well-suited to the local environment at position 275.

The crystal structure of the TEM-1 N276D mutant was determined previously because of its role in inhibitor resistance (1CK3).⁴⁵ The N276D substitution was also identified in a search for stabilizing mutations of a circularly permuted version of TEM-1 independent of a selection for inhibitor resistance, which led to a recent prediction of the substitution having a stabilizing effect.^{30, 34} Our current study indicates N276D stabilizes the TEM-1 enzyme and can function as a global suppressor. In the wild-type structure, the asparagine at position 276 hydrogen bonds with the Arg244 residue. The positive charge of Arg244 plays a role in substrate binding by interacting with the conserved carboxylate found in all β -lactam substrates. Substitutions at this position increase inhibitor resistance of both TEM and SHV (a related class A β -lactamase) enzymes.^{17, 35} The N276D substitution exhibits increased interactions with Arg244 due to an altered position that strengthens the hydrogen bond interactions and by forming a salt bridge between the positive charge of Arg244 and the negatively charged carboxylate group of aspartate (Fig. 6f). The negatively charged side chain is also favorable with respect to the positively charged dipole at the N-terminal portion of the helix (Fig. 6f).

The R275Q and N276D substitutions each stabilize the protein and also reduce the catalytic efficiency of the enzyme for β -lactam hydrolysis. However, these substitutions present an interesting situation where both changes in function, i.e., increased inhibitor resistance, and increased stability provide a selective advantage, rather than a change in function resulting in decreased stability as commonly observed for stability-function tradeoffs.^{3; 17; 19; 20; 21} When the organism is stressed with a β -lactam/ β -lactamase inhibitor combination such as amoxicillin and clavulanate, these inhibitor-resistant mutations provide increased fitness by reducing their susceptibility to inhibitors and stabilizing the protein, to facilitate the acquisition of additional substitutions that could alter function.

Conclusion

Proteins are required to maintain a minimal level of stability to both fold and function at physiological temperatures within the contents of the cell.²² There is no immediate fitness advantage for proteins to have an increased level of stability over that needed at physiological temperatures and, indeed, it potentially can have negative effects if the excess stability hinders protein turnover. From an evolutionary perspective, point mutations that increase protein stability and/or suppress instability defects promote the evolvability of a protein.^{5; 23} The excess stability could allow the protein to accommodate substitutions that alter function but simultaneously destabilize the protein.^{4; 5; 6; 8; 23}

The antibiotic resistance enzyme TEM-1 β -lactamase has evolved in response to the use of extended-spectrum β -lactams and β -lactamase inhibitors in antimicrobial therapy.^{13; 16; 30; 35} Active site substitutions that increase the hydrolytic activity of TEM-1 towards extended-spectrum cephalosporins tend to destabilize the protein.³ In response, global suppressors of protein instability can be selected as secondary antibiotic resistance mutations that stabilize the enzyme and facilitate evolution of TEM-type ESBLs.¹ The M182T substitution found in TEM ESBLs has been established as a global suppressor that stabilizes the enzyme.³ In addition, this study demonstrates that the A184V, T265M, R275Q, and N276D substitutions found in TEM ESBLs exhibit similar effects as M182T in that they can compensate for primary destabilizing substitutions that occur at multiple different sites in the enzyme. As outlined above, the A184V, T265M, R275Q, and N276D global suppressors are located at different positions in the enzyme and increase the stability of TEM-1 both *in vivo* and *in vitro*.

The R275Q and N276D substitutions are intriguing as they provide both a functional advantage in the form of resistance to inhibitor inactivation and an increase in the stability of TEM-1, but at the cost of a decrease in the catalytic efficiency of the enzyme for hydrolysis of β -lactam antibiotics. A reduction in catalytic efficiency is a common characteristic of inhibitor-resistant TEM β -lactamases.^{17; 39; 46} The inhibitor-resistant substitution S130G is less catalytically active than wild-type; however, like R275Q and N276D, the S130G substitution results in increased thermal stability of TEM-1.⁴⁰ It is unexpected that the substitutions associated with inhibitor resistance also exhibit increased stability. This could be a chance occurrence or possibly due to the wild type, Ser130, Arg275 and Asn276 amino acids being constrained by enzyme catalytic requirements for an amino acids that are not optimal with respect to enzyme stability.

Taken together, the M182T, A184V, T265M, R275Q, and N276D global suppressors are present in over a third of the TEM-type ESBL and IRT variants found in clinical isolates resistant to either extended-spectrum β -lactams or β -lactamase inhibitors (www.lahey.org). Various second site and global suppressors that compensate for protein instability and/or misfolding have been identified in other unrelated proteins such as P53, staphylococcal nuclease, cytochrome P450, DNA gyrase, and P22 tailspike endorhamnosidase.^{5; 25; 26; 47;}

48; 49; 50; 51 This widespread occurrence of global suppressors of instability indicates these substitutions play a critical role in the evolution of protein structure and function.

Materials and Methods

Bacterial strains and plasmids

E. coli XL1 Blue strain (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F'*proAB lacI^qZAM15 Tn10* (Tet^r)] and *E. coli* XL1 Red strain (*endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT Tn10* (Tet^r)) were obtained from Stratagene (La Jolla, CA). XL1 Blue cells harboring pBG66 or a pBG66 variant were used for survival assays.^{52; 53} This plasmid encodes wild type TEM-1 β -lactamase under the transcriptional control of its native promoter.⁵⁴ Mutations of the TEM-1 gene of pBG66 were constructed by site-directed mutagenesis. XL1-Red cells were used to introduce random point mutations into the pBG66 plasmid containing the TEM-1 H158S:V159S:T160H mutant. *E. coli* BL21(DE3) strain (F⁻*ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) λ (DE3 [*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*])) was utilized for β -lactamase expression and purification. The pET-TEM-1 plasmid encodes wild type TEM-1 β -lactamase under the transcription control of T7 promoter.^{24; 55} Mutants of TEM-1 were constructed by site-directed mutagenesis and purified after expression in *E. coli* utilizing the pET-TEM-1 vector.*

Site-directed mutagenesis

All TEM-1 β -lactamase site-directed mutants were created using Stratagene's QuickChange kit (La Jolla, CA) according to the manufacturer's instructions. Primers were obtained from Integrated DNA Technologies (Coralville, IA). The following primers were used to introduce mutations into the TEM-1 gene: I47V (5'-gtgcacgagtggttacgtcgaactgcatcaac-3' and 5'-gttgagatccagttcgacgtaaccactcgtgcac-3'), A184V (5'-gacaccagatgcctgtagcaatggcaacaacg-3' and 5'-cgttggtgccattgctacagggcatcgtggtgac-3'), T188I (5'-gatgcctgcagcaatggcaataacgttgccga-3' and 5'-tgcgcaacgttattgccattgctgcaggcatc-3'), I208M (5'-tagcttcccggcaacaattaatgactggatggag-3' and 5'-ctccatccagtcattaattgtgcccgggaagcta-3'), R241H (5'-gagccggtgagcatgggtctcgcgg-3' and 5'-ccgcgagaccatgctcaccggctc-3'), T265M (5'-ccgtatcgtatgtatcatatgacggggagtcag-3' and 5'-ctgactccccgtcatgtagataactacgatacgg-3'), R275Q (5'-caggcaactatggtgaacaaaatagacagatcgctgag-3' and 5'-ctcagcgatcgtctattttgttcacatagttgcctg-3'), and N276D (5'-gcaactatggtgaacgagatagacagatcgctgaga-3' and 5'-tctcagcgatcgtctatctcgttcacatagttgc-3'). DNA sequencing was performed by Lonestar Labs (Houston, TX) to verify the sequence of all site directed mutants as well as the selected second site mutants.

Random mutagenesis and selection of TEM-1 second site mutants

The pBG66 plasmid containing the H158S:V159S:T160H TEM-1 mutant was transformed into the *E. coli* XL1-Red mutator strain. A single colony was used to inoculate an overnight culture grown at 37°C to generate the mutant library. The plasmids were isolated using the QIAprep Spin Miniprep Kit (Valencia, CA) and transformed into *E. coli* XL1-Blue. The cells were spread on LB agar plates supplemented with 12.5 μ g/ml chloramphenicol and various concentrations of ampicillin.

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 were grown overnight in LB broth containing 12.5 μ g/mL chloramphenicol. Ten-fold serial dilutions of each overnight culture were made, and 100 μ L of each dilution was spread onto LB agar plates containing 0 μ g/mL, 15 μ g/mL, 30 μ g/mL, 60 μ g/mL, 120 μ g/mL, and in some assays, also 240 μ g/mL, 480 μ g/mL, or 960 μ g/mL of

ampicillin. After incubation for 24 hours at 37°C, colony forming units (cfu) on each plate were counted. This data was used to calculate the cfu per mL of culture indicative of survival on a given concentration of ampicillin.

Immunoblot analysis

E. coli XL1-Blue containing the pBG66 plasmid that encodes TEM-1 wild-type or mutant β -lactamase were grown overnight at 37°C in 2YT broth supplemented with 12.5 μ g/mL chloramphenicol. The overnight cultures were diluted 50-fold and grown at 37°C for 3–4 hours. The cells were isolated by centrifugation and the media was decanted. The pellet was resuspended in 1x SDS loading buffer containing 1.4 M β -mercaptoethanol. This allowed for the cell concentration to be normalized based on the OD₆₀₀ of the cultures to ensure equal loading. The cells were incubated at 90°C for 10 min and fractionated on an SDS-PAGE gel. The proteins were electrotransferred to a nitrocellulose membrane using a Bio-Rad semidry transfer apparatus. The membrane was blocked with 5% milk before probing with a rabbit polyclonal anti-TEM-1 antibody. The immunoblot was developed using a donkey anti-rabbit secondary antibody conjugated with horseradish peroxidase and the ECL-western blotting analysis system (GE Healthcare). The immunoblot analysis was performed in duplicate using independent cultures.

Protein purification and kinetic analysis

The TEM-1 β -lactamase and its mutants were purified to >90% homogeneity and the *in vitro* kinetic parameters for β -lactam substrate hydrolysis were determined as previously described.^{24, 56} In summary, a Zn-chelating column and, when necessary, an S75 gel filtration column (GE Healthcare) were used to purify TEM-1 β -lactamase and its mutants. After purification, the proteins were dialyzed into 50 mM sodium phosphate buffer and used for kinetic analysis. Bovine serum albumin was added to the buffer at a concentration of 1 mg/mL for the substrates nitrocefin and cefotaxime; however, it was not added for ampicillin due to an overlap in absorbance. Substrate hydrolysis was observed with a DU800 spectrophotometer at wavelengths 235 nm, 482 nm, and 264 nm for ampicillin, nitrocefin and cefotaxime, respectively. These experiments were performed at 30 °C. The initial velocities were measured and fitted to the Michaelis-Menton equation, $v = k_{cat}[S]/(K_m + [S])$, using GraphPad Prism5 to determine k_{cat} and K_m . When the TEM-1 wild-type or mutants had high K_m values that prevented determination of V_{max} , the catalytic efficiency (k_{cat}/K_m) was estimated using the equation: $v = k_{cat}/K_m [E][S]$ where $[S] \ll K_m$. The initial velocities were measured in at least duplicate and averaged to determine the kinetic parameters.

Molecular modeling

Molecular models of the T265M and R275Q substitutions on the TEM-1 A184V structure (Protein Data Bank code 1BTL) were made with the Phenix refinement program.^{43, 57} The A184V and wild-type TEM-1 structures are nearly identical in the local environment of the T265M and R275Q substitutions and is not believed to affect the modeling results. Local minimizations are essential in Phenix to determine the global minimum of the potential energy hypersurface and to optimize the geometry of the protein structure. The geometry optimization in Phenix positions the side chains with idealized bond lengths, bond angles, etc.⁵⁸

Thermostability determined by circular dichroism

The thermostability of TEM-1 β -lactamase and its mutants were determined by increasing the sample temperature from 20 °C to 80°C in 0.1 °C increments at a rate of 1°C/min while measuring the Far-UV CD signal at 223 nm with a Jasco J-815 CD spectropolarimeter

(Essex, UK) coupled to a Peltier PFD-425S/15 for temperature control. The heat-induced unfolding transitions were monitored for 20 μ M of enzyme in 200 mM of potassium phosphate (0.1 cm cuvette, 2 nm bandwidth). Thermal denaturation of each β -lactamase was performed in at least quadruplicate. The melting temperature (T_m) or temperature midpoint of protein unfolding was determined by fitting the transition from the folded to unfolded state to either a single or double Boltzmann model for either a two-state or three-state unfolding mechanism, respectively. The data was fitted to these equations using GraphPad Prism5.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a training fellowship from the Pharmacoinformatics Training Program of the Gulf Coast Consortia (National Institutes of Health Grant No. T90 DK070109-05), Biomedical Discovery Training Program of the Gulf Coast Consortia (National Institutes of Health Grant No. 1 T90 DA022885-04) and by National Institutes of Health Grant AI32956 to T.P. We thank Dr. Yousif Shamoo for use of the spectropolarimeter. We also thank Chuan Hong for his assistance with the purification of the β -lactamase enzymes and Corwin Miller for his assistance in using the spectropolarimeter.

Abbreviations

ESBL	extended-spectrum β -lactamases
IRT	inhibitor-resistant TEM

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Fig. 1. Diagram illustrating the structure of TEM-1 β-lactamase and identifying the amino acid substitutions relevant to this study. A cartoon representation of the overall and secondary structural elements is shown in gray with the position of the catalytic serine (S70) marked by a blue ball in the TEM-1 active site. The destabilizing substitutions and second-site suppressors examined in this study are shown as red and green sticks, respectively, with the mesh outlining the van der Waals radius of the atoms. The right panel view of TEM-1 (a) is an 180° degree rotation of the left (b). The amino acid positions are numbered according to the Ambler numbering scheme.⁵⁹

Fig. 2.

Survival curves of *E. coli* containing the pBG66 plasmid encoding the TEM-1 β -lactamase H158S:V159S:T160H (158-60SSH) mutants and secondary suppressor substitutions. (a) Colony forming units (cfu) on agar plates containing increasing concentrations of ampicillin for *E. coli* containing 158-60SSH or I47V single mutants or the 158-60SSH:I47V double mutant. (b) Cfus for *E. coli* containing 158-60SSH or A184V single mutants or the double mutant. (c) Cfus for *E. coli* containing T188I mutant combinations. (d) Cfus for *E. coli* containing I208M mutant combinations. (e) Cfus for *E. coli* containing R241H mutant combinations. (f) Cfus for *E. coli* containing T265M mutant combinations. (g) Cfus for *E. coli* containing R275Q mutant combinations. (h) Cfus for *E. coli* containing N276D mutant combinations.

Fig. 3. Survival curves of *E. coli* with pBG66 plasmid encoding TEM-1 β-lactamase L76N mutants and secondary suppressor substitutions. (a) Colony forming units (cfu) on agar plates containing increasing concentrations of ampicillin for *E. coli* containing L76N or I47V single mutants or the L76N: I47V double mutant. (b) Cfus for *E. coli* containing L76N and A184V mutant combinations as in (a). (c) Cfus for *E. coli* containing L76N and T188I mutant combinations. (d) Cfus for *E. coli* containing L76N and I208M mutant combinations. (e) Cfus for *E. coli* containing L76N and R241H mutant combinations. (f) Cfus for *E. coli* containing L76N and T265M mutant combinations. (g) Cfus for *E. coli* containing L76N and R275Q mutant combinations. (h) Cfus for *E. coli* containing L76N and N276D mutant combinations.

Fig. 4. Steady state protein expression levels of wild-type and mutant TEM-1 β-lactamases as determined by immunoblot analysis of whole cell lysates of *E. coli* cultures containing pBG66 plasmid encoding wild type or mutants. Lanes representing wild-type and mutants are labeled.

Fig. 5. Thermal denaturation curves of TEM-1 wild-type and selected β -lactamase variants. Fractional changes of the CD signal for the wild-type (black), M182T (red), H158S:V159S:T160H:M182T (green) and A184V (blue) β -lactamases are shown. The results are representative of the results for the other mutants which are shown in Figure S1. The T_m values (Table 4) were determined by fitting a sigmoidal function to the transition from the folded to unfolded state (see Materials and Methods).

Fig. 6. Diagrams of global suppressor substitutions in the TEM-1 structure. Superimposition of the wild-type (gray) and mutant (cyan) crystal or modeled structures are illustrated (see results). The local environment of the global suppressor substitutions (a) M182T, (b) A184V, (c) I208M, (d) T265M, (e) R275Q and (f) N276D are shown in comparison to wild-type (1XPB). The dashed black lines indicate hydrogen bonds and the red sphere are water molecules. The Protein Data Bank codes for the M182T, A184V, I208M, and N276D mutants are 1JWP3, 1BTL43, 3DTM29, and 1CK345, respectively.

Table 1

TEM ESBL variants from clinical isolates that contain the second-site suppressor substitutions identified in this study.

Suppressor substitution	TEM ESBL or IRT variants
M182T	TEM-20, 32, 43, 52, 63, 72, 87, 88, 91, 92, 93, 94, 106, 107, 113, 124, 126, 131, 135, 149, 159, 161, 165, 177
A184V	TEM-116, 157, 162
T265M	TEM-4, 9, 13, 25, 27, 42, 47, 48, 49, 68, 73, 74, 75, 85, 86, 94, 102, 110, 111, 118, 142, 167, 168
R275Q	TEM-45, 82, 83, 122, 163
N276D	TEM-35, 36, 37, 39, 50, 78, 80, 84, 125, 151, 152, 158

Table 2

Ampicillin resistance levels of *E. coli* containing TEM-1 β -lactamase and mutant combinations expressed as IC₉₀ values^a.

	Amp. (μ g/mL)
TEM-1 (WT)	960
L76N	15
H158S:V159S:T160H	15
M182T ^b	>960
L76N:M182T ^b	960
H158S:V159S:T160H:M182T ^b	960
I47V	960
I47V:L76N	60
I47V:H158S:V159S:T160H	60
A184V	960
L76N:A184V	60
H158S:V159S:T160H:A184V	30
T188I	960
L76N:T188I	15
H158S:V159S:T160H:T188I	30
I208M	960
L76N:I208M	60
H158S:V159S:T160H:I208M	30
R241H	960
L76N:R241H	120
H158S:V159S:T160H:R241H	60
T265M	960
L76N:T265M	60
H158S:V159S:T160H:T265M	60
R275Q	960
L76N:R275Q	60
H158S:V159S:T160H:R275Q	60
N276D	960
L76N:N276D	60
H158S:V159S:T160H:N276D	30

^aIC₉₀, defined as the concentration of ampicillin that reduces cfu per mL of culture by $\geq 90\%$, is derived directly from data measurements reported in ampicillin survival curves.

^bData from Marciano *et al.*²⁴

Table 3

Kinetic parameters for β -lactam hydrolysis by TEM-1 β -lactamase wild type and enzyme containing suppressor substitution.

Enzymes	Substrates		
	Ampicillin	Nitrocefin	Cefotaxime
WT			
k_{cat} (s^{-1})	1737 ± 59	1053 ± 27	$>2.1^a$
K_m (μM)	45 ± 5	84 ± 6	$>1000^a$
k_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)	39	12	2.1×10^{-3}
M182T			
k_{cat} (s^{-1})	1566 ± 85	939 ± 25	>1.3
K_m (μM)	47 ± 9	90 ± 7	>1000
k_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)	33	10	1.3×10^{-3}
A184V			
k_{cat} (s^{-1})	1928 ± 85	1172 ± 37	>2.0
K_m (μM)	38 ± 6	82 ± 8	>1000
k_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)	50	14	2.0×10^{-3}
I208M			
k_{cat} (s^{-1})	1828 ± 62	995 ± 16	>1.8
K_m (μM)	56 ± 6	74 ± 4	>1000
k_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)	33	13	1.8×10^{-3}
T265M			
k_{cat} (s^{-1})	1344 ± 44	842 ± 12	>1.5
K_m (μM)	60 ± 6	84 ± 3	>1000
k_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)	22	10	1.5×10^{-3}
R275Q			
k_{cat} (s^{-1})	1614 ± 121	985 ± 104	>0.13
K_m (μM)	63 ± 15	195 ± 47	>1000
k_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)	26	5	1.3×10^{-4}
N276D			
k_{cat} (s^{-1})	2363 ± 92	997 ± 43	>0.24
K_m (μM)	104 ± 13	218 ± 24	>1000
k_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)	23	5	2.4×10^{-4}
H158S:V159S:T160H:M182T			
k_{cat} (s^{-1})	267 ± 11	638 ± 25	>0.66
K_m (μM)	28 ± 5	103 ± 11	>1000
k_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)	10	6	6.6×10^{-4}

^aThe k_{cat}/K_m values were determined because V_{max} was not observed due to high K_m values (see results).

Table 4

Enzymes	T_m (°C)	ΔT_m (°C)
Wild-type	56.7 ± 0.02	---
M182T	63.2 ± 0.03	6.5
A184V	58.1 ± 0.02	1.4
I208M	57.8 ± 0.03	1.1
T265M	58.3 ± 0.03	1.6
R275Q	58.7 ± 0.03	2.0
N276D	58.0 ± 0.03	1.3
H158S:V159S: T160H:M182T	51.7 ± 0.05^a 59.5 ± 0.33	

^aThe two melting temperatures are from the fitting of the thermal denaturation data to a two transition or three-state unfolding model (see Results and Discussion).