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Research Article

Stabilizing Mutations Enhance Evolvability of BlaC β-lactamase by Widening the Mutational Landscape

Marko Radojković, Anouk Bruggeling van Ingen, Monika Timmer, and Marcellus Ubbink *

Leiden Institute of Chemistry, *Leiden University, Einsteinweg 55, 2333 CC Leiden, the Netherlands*

Correspondence to Marcellus Ubbink: *m.ubbink@chem.leidenuniv.nl (M. Ubbink)*
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Abstract

Antimicrobial resistance is fueled by the rapid evolution of β-lactamases. However, a gain of new enzyme activity often comes at the expense of reduced protein stability. This evolutionary constraint is often overcome by the acquisition of stabilizing mutations that compensate for the loss of stability invoked by new function mutations. Here, we report three stabilizing mutations (I105F, H184R, and V263I) in BlaC, a serine β-lactamase from *Mycobacterium tuberculosis*. Using a severely destabilized variant as a template for random mutagenesis and selection, these three mutations emerged together and were able to fully restore resistance toward the antibiotic carbenicillin. *In vitro* characterization shows that all three mutations increase chemical and thermal stability, which leads to elevated protein levels in the periplasm of *Escherichia coli*. We demonstrate that the introduction of stabilizing mutations substantially enhances the evolvability of the enzyme. These findings illustrate the important role of stabilizing mutations in enzyme evolution by alleviating function-stability trade-offs and broadening the accessible evolutionary landscape.

Introduction

Most amino acid substitutions have detrimental consequences for the phenotype, because of adverse effects on folding, stability, or function.[1–3] Thus, mutational robustness is a feature that strongly influences protein evolvability. A plethora of computational and directed evolution studies have been conducted with the aim of elucidating factors that define evolvability.[4–9] Enzyme evolution is of importance, particularly in antimicrobial resistance, an escalating global issue that in 2019 alone led to 1.27 million deaths globally.[10] At the forefront of this development is the resistance against β-lactam antibiotics, which constitute around 60% of all prescribed antibiotics worldwide.[10] The primary resistance mechanism involves the production of β-lactamases, enzymes that break down the β-lactam ring common to these drugs. Based on sequence identity, β-lactamases are grouped into four classes (classes A-D), of which class A is the largest and most diverse.[11–13] The extensive use of β-lactams has driven divergent evolution of β-lactamases, leading to the continual emergence of enzyme variants.[14–18] This was particularly the case after the introduction of new generations of cephalosporins and β-lactam/β-lactamase inhibitor combinations, which gave rise to highly resistant extended-spectrum β-lactamases (ESBLs), capable of hydrolyzing these antibiotics or evading inhibition.[19–22]

However, evolution of new function is often accompanied by reduction in stability, a trade-off which was observed in several β-lactamases.[23–26] Enzymes are only marginally stable entities and have limited buffering capacity to tolerate new

mutations. Therefore, acquisition of global stabilizing mutations, which compensate for stability defects incurred by function-altering mutations, is often necessary to maintain protein fitness and promote evolvability.[6,27,28] Among class A β-lactamases, several stabilizing mutations have been identified in clinical isolates of variants with increased resistance to β-lactamase inhibitors or cephalosporins.[29–32] M182T in TEM-1 β-lactamase is one of the most frequently occurring mutations that compensates for stability and folding defects caused by substitutions that improve catalytic activity toward cefotaxime and ceftazidime.[1,27,33] Additional mutations, such as A184V, T265M, R275Q, and N276D were proven to act similarly and found in clinical isolates.[34] The A77V mutation is a common global stabilizing mutation in the CTX-M group of β-lactamases, in which it cancels negative effects of P167S and D240G mutations that allow for easier accommodation and hydrolysis of ceftazidime.[35,36]

BlaC, the β-lactamase from *Mycobacterium tuberculosis* (Mtb), is a broad-spectrum Ambler class A serine β-lactamase encoded by a chromosomal gene. Directed evolution studies demonstrated that variants with improved inhibitor resistance can be readily obtained.[37,38] We recently showed that loss of activity caused by inhibitor-resistant mutations can be easily compensated for by a variety of substitutions at position 105,[39] which is referred to as "gatekeeper residue".[40] However, the nature of this compensatory mechanism was not further investigated, and the fitness compensation likely stems, at least in part, from the improvement in catalytic efficiency of the enzyme.[40]

In this study, we wanted to test experimentally if stabilizing mutations promote BlaC evolution and lead to expansion of the mutational landscape. To that end, we identified stabilizing mutations from the ground up by performing laboratory evolution and selection of mutations that increase the stability of a highly destabilized enzyme variant and restore resistance to a penicillin antibiotic. After a single round of selection, a clone carrying three additional mutations I105F, H184R, and V263I was identified. Collectively, these three mutations augmented MIC of the destabilized template by 100-fold, reaching a wild-type level of resistance. The triple mutant variant showed increased thermal and chemical stability, compared to the wild-type. Furthermore, we show that the stabilizing mutations enable the evolution of activity for ceftriaxone hydrolysis through diverse mutational pathways that are not accessible to the wild-type enzyme. Interestingly, some of the new function mutations did not severely cripple resistance to penicillin antibiotics, leading to a highly resistant bacterial phenotype. Structural implications on enzyme stability are discussed.

Results

Random mutagenesis and selection for phenotype restoration

To identify stabilizing mutations, we used the phenotype restoration approach, in which a highly destabilized enzyme with low fitness is used as a template for mutagenesis to screen for mutations that enable the phenotype to return to wild-type. For choosing the right template, we applied the following criteria: (1) the residue is not in the active-site or indirectly involved in catalytic activity; (2) the mutation is not essential for correct folding; (3) the mutation leads to reduced enzyme stability; (4) when introduced into *Escherichia coli*, the mutated enzyme variant results in a lower minimal inhibitory concentration (MIC) value. We opted for two mutations, F66Y and G156A, which were previously identified as third-shell residue substitutions that lead to lower stability of the enzyme.[41] These two mutations dramatically reduce resistance to carbenicillin, from 1000 μg/mL to 20 μg/mL.[41] To maximize the likelihood of positive hits and to avoid the emergence of collateral mutations, we chose to introduce them in tandem.

The gene of the highly destabilized variant carrying the F66Y and G156A mutations was used as a template for error-prone PCR and the products were ligated to a vector via Gibson Assembly method.[42] The plasmid library was used to transform *E. coli* cells. The cells were screened for growth on agar plates containing 500, 250, or 100 μg/mL carbenicillin. Approximately 50 million CFU were screened for each carbenicillin concentration, and several mutants were identified after sequencing plasmid DNA from 8 colonies (Table 1).

The only variant that was able to grow on the highest carbenicillin concentration had three new mutations (I105F, H184R, and V263I), in addition to the template mutations (F66Y-G156A). Surprisingly, screening on lower carbenicillin concentrations did not yield a considerably higher number of colonies or expand mutant variety. In three instances, Y66 mutated back to its original residue (phenylalanine), whereas mutation of A156 back to glycine was not observed. Noticeably, mutation of residue 105 appeared in all clones, which was to be expected, given that there is a strong preference for an aromatic residue at this position in β-lactamases with high penicillinase activity.[43,44] Since only one variant grew at the highest carbenicillin concentration, we decided to analyze it in more detail and determine if all new mutations are necessary to reach this level of resistance. For that reason, we introduced all single and double mutations in the F66Y-G156A template and determined the MIC values for carbenicillin.

Table 1 BlaC variants identified after random mutagenesis of BlaC F66Y-G156A template and screening at three carbenicillin concentrations.

Variant	Carbenicillin concentration (μg/mL)
F66Y, G156A, I105F, H184R, V263I	500
Y66F, G156A, I105F	250
Y66F, G156A, I105Y	
F66Y, G156A, I105Y, H184R	100
Y66F, G156A, I105F	

Table 2 Carbenicillin MIC of various BlaC variants and negative control.

Variant	MIC carbenicillin (μg/mL)
pUK21 (negative control)[a]	10
Wild-type	1000
F66Y-G165A	10
F66Y-G165A + I105F	50
F66Y-G165A + H184R	25
F66Y-G165A + V263I	50
F66Y-G165A + H184R + V263I	250
F66Y-G165A + I105F + V263I	250
F66Y-G165A + I105F + H184R	500
F66Y-G165A + I105F + H184R + V263I	1000

[a] pUK21 vector without *blaC* gene.

As shown in Table 2, none of the single mutations can substantially increase resistance against carbenicillin, whereas out of double mutant combinations, I105F-H184R displays the strongest phenotype. The highest resistance is observed when all three mutations are present together, which represents a 100-fold increase compared to the F66Y-G156A template. We also determined the carbenicillin MIC for all variants from Table 2, but without F66Y-G156A mutations in the background (Table S1).

Enzyme kinetics parameters of various BlaC mutants

Next, we sought to determine the effects of the new mutations on enzyme activity. To that end, several BlaC variants were produced and purified and the steady-state kinetic parameters for two standard substrates, nitrocefin and ampicillin, were determined (Table 3). The latter was used as an alternative for carbenicillin, for which conversion could not be followed due to a very small difference in the extinction coefficient of substrate and product.

We corroborated the high penicillinase activity of I105F BlaC,[40] which was also preserved in the triple and quintuple mutants. On the other hand, mutations H184R and V263I had a slightly negative impact on nitrocefin activity, whereas the same effect was a bit more pronounced for ampicillin, resulting in roughly 1.5-fold and 2-fold decrease in catalytic efficiency, respectively. This modest decline in activity was completely compensated for by the I105F mutation, which led to catalytic improvement for both substrates in the triple mutant. Interestingly, destabilizing mutations F66Y and G156A even marginally improved this effect in the quintuple variant (2.7-fold vs. 2.4-fold, and 4.6-fold vs. 4.0-fold, for nitrocefin and ampicillin, respectively). These results imply that the MIC value of the quintuple mutant (Table 2) is greatly influenced by activity improvement conferred by the I105F mutation.

Thermal and chemical stability are promoted

The effect of the mutations on enzyme stability was assessed with thermal and chemical denaturation assays. Global stabilizing mutations are known to increase stability of the protein even without destabilizing mutations.[33,45] Thermal unfolding was followed in the presence of SYPRO orange dye, which binds to hydrophobic pockets that are exposed during denaturation. The negative first derivative of the fluorescence signal and corre-

Table 3 Steady-state kinetic parameters determined for wild-type (WT) BlaC and various mutants. All measurements were done in phosphate buffer (100 NaPi, pH 6.4) at 25 °C. Errors represent one standard deviation of the mean of triplicate measurements. The K_M is indicated as apparent because regular Michaelis-Menten kinetics do not apply to the two-step hydrolysis reaction.[26] Michaelis-Menten plots for both substrates are shown in Figure S1.

	Nitrocefin				Ampicillin			
	K_M^{app} (μM)	k_{cat} (s^{-1})	k_{cat}/K_M^{app} (μM^{-1} s^{-1})	k_{cat}/K_M^{app} over WT	K_M^{app} (μM)	k_{cat} (s^{-1})	k_{cat}/K_M^{app} (μM^{-1} s^{-1})	k_{cat}/K_M^{app} over WT
WT	131 ± 2	86 ± 2	0.65 ± 0.01	1	80 ± 5	17 ± 1	0.21 ± 0.01	1
I105F	31 ± 4	45 ± 1	1.46 ± 0.08	2.3	42 ± 1	37 ± 1	0.87 ± 0.03	4.1
H184R	138 ± 2	78 ± 2	0.57 ± 0.02	0.9	101 ± 1	13 ± 1	0.13 ± 0.01	0.7
V263I	132 ± 5	69 ± 2	0.52 ± 0.03	0.8	102 ± 9	11 ± 1	0.11 ± 0.01	0.5
Triple[a]	30 ± 1	46 ± 1	1.57 ± 0.02	2.4	61 ± 5	50 ± 1	0.83 ± 0.07	4.0
Quintuple[b]	29 ± 1	51 ± 1	1.78 ± 0.06	2.7	53 ± 7	50 ± 2	0.97 ± 0.13	4.6

[a] I105F-H184R-V263I.
[b] F66Y-G165A-I105F-H184R-V263I.

sponding melting temperatures (T_m) are shown in Figure 1.

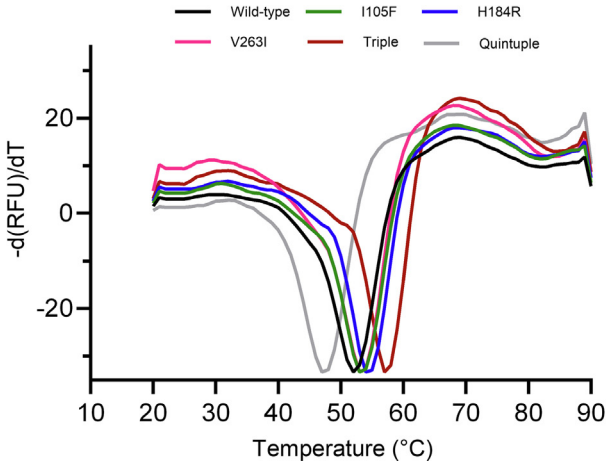
All three single mutations lead to elevated thermal stability of the enzyme, of which H184R has the biggest effect and increases T_m by 2.3 °C. Stability improvements of the single mutants are additive, which results in a T_m that is 5 °C higher for the triple mutant than for the wild-type. By comparing the T_m values of the triple and quintuple mutants, it can be inferred that the F66Y and G156A mutations severely undermine the stability and reduce the T_m by 9.7 °C, if the effects are additive.

Resistance to chemical denaturation was examined by measuring intrinsic tryptophan fluorescence in the presence of different urea concentrations. Similar to thermal denaturation, the bending point in the curvature of the tryptophan fluorescence signal marks the concentration of denaturant (C_m) at which the native and denatured states are equally populated. Increased resistance to urea denaturation was observed for all three single mutants (Figure 2). Contrary to the observation from melting temperatures, the individual resistance improvements do not add up, as the triple mutant and I105F have the same level of denaturant resistance (C_m = 6.1 M). Introduction of the destabilizing mutations significantly impairs enzymes stability, resulting in a 0.8 M decrease in C_m, even with three stabilizing mutations in the background.

Additionally, we evaluated the effect of each stabilizing mutation on Gibbs free energy computationally, using the Rosetta Online server.[46] Negative values upon substitution indicate a favorable effect on the stability. As depicted in Figure S2, each of the three mutations promotes stability, with H184R being the most prominent.

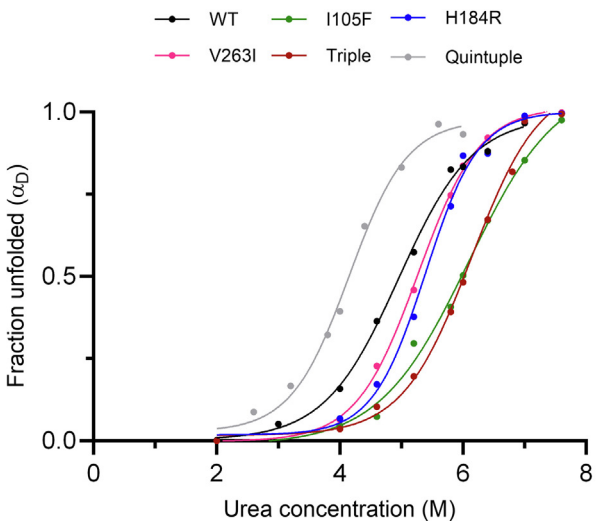
Effect of stabilizing mutations on cellular protein levels

Increased cellular amounts of the β-lactamases often lead to increased resistance against β-lactam antibiotics.[34,45] On the contrary, unstable enzymes are prone to proteolysis, resulting in lower protein levels and consequently lower resistance.[27,33] To establish if observed stability improvements translate to elevated protein levels in bacterial cells, periplasmic fractions of *E. coli* cells producing different BlaC variants were analyzed with Western blot and anti-His monoclonal antibodies (Figure 3). The same construct was used in evolution experiments, in which BlaC is transported to the periplasm via the twin-arginine transporter (Tat) system. The highly deleterious effect of combined F66Y and G156A mutations led to no detectable enzyme amounts, likely indicating that these two mutations result in accumulation of misfolded enzyme which cannot be transported via the Tat pathway. Among single mutants, mutation V263I showed the highest impact on periplasmic protein concentration resulting in a 3.0 times increase relative to the wild-type. The effect of the other two mutations was less pronounced and led to 1.5 and 2.1-fold improvement, for I105F and H184R, respectively. This observation suggests that the V263I mutation has a greater impact on cellular protein levels and might compensate better for stability defects than the other two. Expectedly, all three mutations jointly conferred the highest improvement in the enzyme amount (4.2-times). However, when destabilizing mutations are present in the background (Quintuple mutant), the protein level drops dramatically to merely 0.1 of the wild-type amount, confirming highly detrimental effect of F66Y and G156A mutations.



BlaC variant	T_m (°C)	ΔT_m (°C)
Wild-type	52.0 ± 0.1	/
I105F	53.0 ± 0.1	+1
H184R	54.3 ± 0.5	+2.3
V263I	53.0 ± 0.1	+1
Triple[a]	57.0 ± 0.1	+5
Quintuple[b]	47.3 ± 0.5	-4.7

Figure 1. Melting curves of several BlaC mutants and wild-type. Left – the negative first derivative of the normalized fluorescence signal; right – melting temperatures determined for each variant. Errors represent one standard deviation of the mean of triplicate measurements. [a]I105F-H184R-V263I; [b]F66Y-G165A-I105F-H184R-V263I.



BlaC variant	C_m (M)	ΔC_m (M)
Wild-type	5.0 ± 0.6	/
I105F	6.1 ± 0.4	+1.1
H184R	5.4 ± 0.2	+0.4
V263I	5.3 ± 0.4	+0.3
Triple[a]	6.1 ± 0.4	+1.1
Quintuple[b]	4.2 ± 0.1	-0.8

Figure 2. Chemical denaturation curves of BlaC wild-type and several variants determined at 35 °C. Left – the fraction of unfolded protein is plotted as a function of the urea concentration; right – the concentration of denaturant at which populations of native and denatured states are equal. Errors represent one standard deviation of the mean of triplicate measurements. The graphs of the intrinsic tryptophane fluorescence signal from which the unfolded fraction (α_D) is calculated are shown in Figure S7. [a]I105F-H184R-V263I; [b]F66Y-G165A-I105F-H184R-V263I.

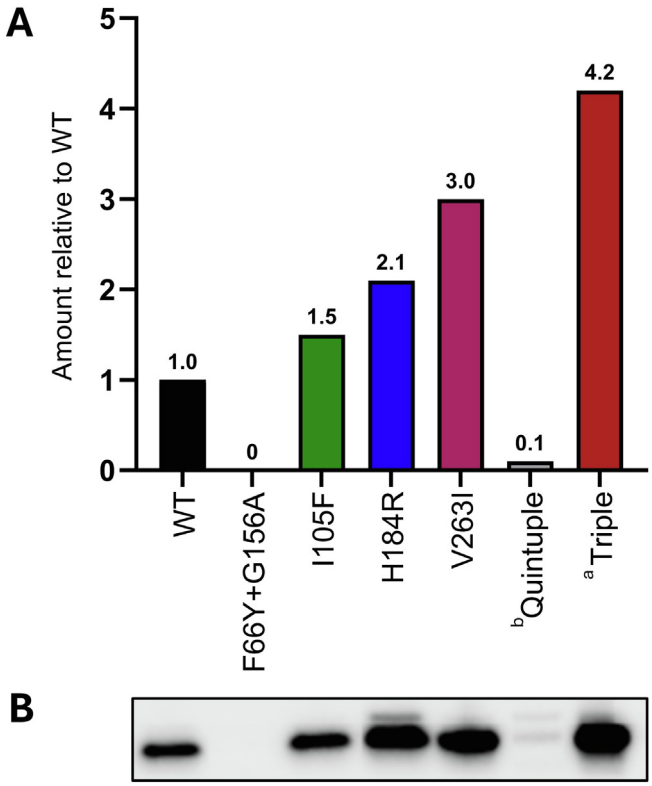


Figure 3. Protein levels of wild-type and mutant BlaC enzymes in *E. coli* periplasm. A – the intensity of the bands from Western blot quantified with imaging software and expressed relative to the wild-type enzyme. The band intensity was corrected with respect to the signal of the band from SDS-PAGE gel belonging to an *E. coli* protein around 55 kDa (Figure S3A); B – Western blot analysis of periplasmic fractions of *E. coli* containing pUK21 constructs encoding different BlaC variants. A full picture of the Western blot is shown in Figure S3B. [a]I105F-H184R-V263I; [b]F66Y-G165A-I105F-H184R-V263I.

Stabilizing mutations exhibit global compensatory potential

To test whether identified stabilizing mutations have the potential to curb stability defects in different backgrounds, and are not only related to F66Y-G156A mutations, we combined them with two additional destabilizing mutations and investigated their effect on bacterial resistance. Substitutions G144L and L199V undermine protein stability and drastically reduce the MIC value of *E. coli* harboring these variants.[41] Each of the stabilizing mutations has increased resistance to carbenicillin in both destabilizing backgrounds, although to a different extent. (Table 4). Generally, it appears that mutation L199V has a more detrimental effect on carbenicillin resistance, since none of the stabilizing mutations could increase the MIC more than 4-fold. On the other hand, the effect of mutation G144L seems to be less severe and is completely neutralized by the H184R mutation alone (20-fold increase in MIC).

Table 4 MIC values of wild-type BlaC, single, and double mutant variants with different pairs of stabilizing and destabilizing substitutions.

Variant	MIC carbenicillin (μg/mL)
pUK21 (negative control)[a]	10
Wild-type	1000
G144L	50
G144L + I105F	250
G144L + H184R	1000
G144L + V263I	250
L199V	25
L199V + I105F	100
L199V + H184R	100
L199V + V263I	50

[a] pUK21 vector without *blaC* gene.

Evolution of cephalosporin resistance is facilitated in the presence of stabilizing mutations

Mutations that improve cephalosporinase activity of β-lactamases often come at the expense of reduced stability.[1,23,26] We wondered if using an enzyme reinforced by stabilizing mutations as starting point for random mutagenesis would facilitate evolution of resistance against ceftriaxone, a third-generation cephalosporin β-lactam. For that purpose, we used the I105F-H184R-V263I variant as a template for error-prone PCR and subsequent screenings. A control experiment using wild-type BlaC as a template was done in parallel. Screenings were done at 2x MIC (0.4 μg/mL of ceftriaxone), which was the same value for both wild-type and triple mutant, indicating that stabilizing mutations themselves do not improve resistance against ceftriaxone. Nine clones with increased ceftriaxone resistance were identified using the stabilizing template (among 3.5 million CFU) and none for the wild-type, despite screening three times more CFU (12.1 million). Each identified variant had all three stabilizing mutations in the background, thereby confirming their neutral effect on ceftriaxone resistance (Table 5).

Generally, we observed up to 16-fold increase in ceftriaxone MIC, whereas carbenicillin MIC dropped 2- to 16-times in comparison to the triple mutant template (Table 5). Interestingly, besides variants with mutations in the Ω-loop (residues 161–179, Figure 4), which are common for the evolution of third-generation cephalosporinase activity,[47–51] we also identified variants with substitutions on positions that are not associated with increased resistance toward cephalosporins (N136Y, F148L, D202G, and K230R). In one such variant, both mutations N136Y and K230R lie outside of the Ω-loop (Figure 4) and were not reported in the literature before. To establish if both substitutions are necessary for the displayed phenotype, we probed their individual effects and found that N136Y alone is sufficient to reach the same magnitude of ceftriaxone resistance (Table S2). Two single mutant variants with the strongest ceftriaxone phenotype (L169P and D179G) were analyzed more in detail, by determining kinetic steady-state parameters (Table S3) and melting temperatures of the purified enzymes (Table S4). Both mutations severely cripple the ability of the enzyme to hydrolyze nitrocefin, with L169P being slightly more detrimental (Table S3). For the substrate ceftriaxone, the apparent K_M of all tested enzymes is high and only k_{cat}/K_M could be determined (Figure S1), except for the D179G variant with the stabilizing mutations, whose kinetic parameters were not reproducible. The L169P mutation provided an 8.2 and 5.5-fold increase in activity for ceftriaxone, compared to the wild-type and I105F-H184R-

Table 5 MIC values for ceftriaxone and carbenicillin of wild-type, triple mutant, and other variants identified after ceftriaxone screenings.

Variant	MIC ceftriaxone (μg/mL)	MIC carbenicillin (μg/mL)
pUK21 (negative control)[a]	0.1	10
Wild-type	0.2	1000
Triple[b]	0.2	8000
Triple + D176G	1.6	4000
Triple + D163G	3.2	4000
Triple + L169P	3.2	500
Triple + D179G	3.2	2000
Triple + N136Y, K230R	1.6	500
Triple + D179E	1.6	500
Triple + N170T, D176G	3.2	1000
Triple + L169Q, P174S	3.2	500
Triple + D179G, D202G	3.2	1000

[a] pUK21 vector without *blaC* gene.
[b] I105F-H184R-V263I.

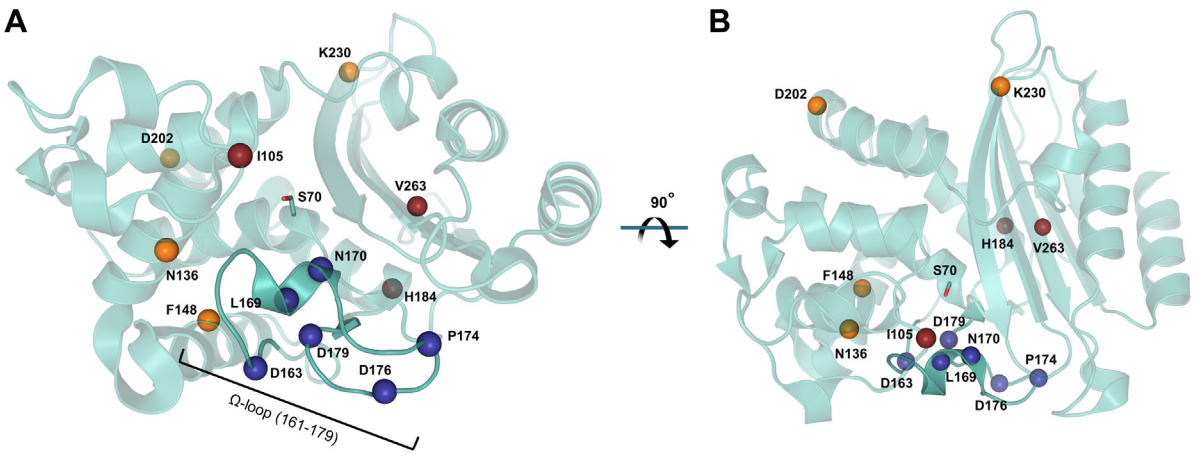


Figure 4. Positions of mutations identified after ceftriaxone screenings together with stabilizing mutations. (A–B) Front and top view of the BlaC structure. Positions of mutations are shown as spheres. Mutations in the Ω-loop are in blue, and others in orange. The stabilizing mutations are shown in ruby. The catalytic residue S70 is shown in sticks (PDB 2GDN[52]).

V263I enzymes, respectively, in line with the increased MIC. Analysis of melting curves indicated stability loss conferred by the mutations L169P and D179G, leading to a 4 °C and 3 °C reduction in T_m of the stabilizing template (Table S4), which may explain why these mutations were only found in the stabilized background and not in the wild-type BlaC.

Discussion

Mutations that alter the functionality of the proteins often have negative implications on other protein features, such as stability, dynamics or substrate affinity[23,53,54] and can even lead to a deleterious phenotype. However, loss of fitness caused by one mutation can be compensated by another mutation.[55–57] Because the majority of function-switching mutations compromise stability, stabilizing mutations with global compensatory potential are thought to play a crucial role in protein evolution.[6,58–60]

Here, we experimentally probed the hypothesis that global stabilizing mutations enhance evolvability and allow for a wider search of the evolutionary landscape, using BlaC as a model enzyme. β-Lactamases are highly evolvable enzymes and the leading cause of antimicrobial resistance, which makes them attractive targets for various studies. Since β-lactam antibiotics are being re-evaluated for the treatment of tuberculosis,[61–64] compensatory potential is an important factor to consider when developing long-term strategies of antibiotic therapies. To this date, we did not find any studies describing stabilizing mutations in BlaC.

As shown in Table 1, we identified three mutations, which jointly completely neutralized the negative effects of destabilizing mutations and conferred wild-type level of resistance. The I105F mutation was previously found in a directed evolution study and exhibited 2-fold and 3-fold higher *in vitro* activity for ampicillin and nitrocefin, respectively, compared to the wild-type BlaC.[40] Our kinetic data are in good agreement with previous observations, as we also report improvement in catalytic efficiency for both ampicillin and nitrocefin, although to a different extent (4-fold and 2-fold increase, Table 3).

To seek the structural explanation of enhanced activity, we tried to solve the crystal structure of the I105F-H184R-V263I mutant. However, despite several rounds of seeding and optimization, the protein crystals with favorable diffraction properties could not be obtained. As an alternative, we modelled each mutation *in silico*. The activity improvement of mutation I105F likely arises from an enlarged active site cavity (Figure 5C). The phenyl ring of Phe is pointing outwards, away from the active site, which could allow for easier accommodation of substrate molecule. Furthermore, the I105F mutation promotes thermal and chemical stability (Figures 2 and 3, respectively). Interestingly, the I105F variant showed higher resistance to urea denaturation than BlaC H184R and V263I (Figure 3). We conclude that the compensatory potential of I105F mutation stems from both stability and activity improvements, although the latter observation primarily pertains to penicillin substrates.

The V263I substitution was identified together with I105F and T145dI mutations.[40] However, this variant was not further characterized, as authors shifted their focus to I105F. The V263I mutation led to a 3-fold increase in periplasmic protein levels

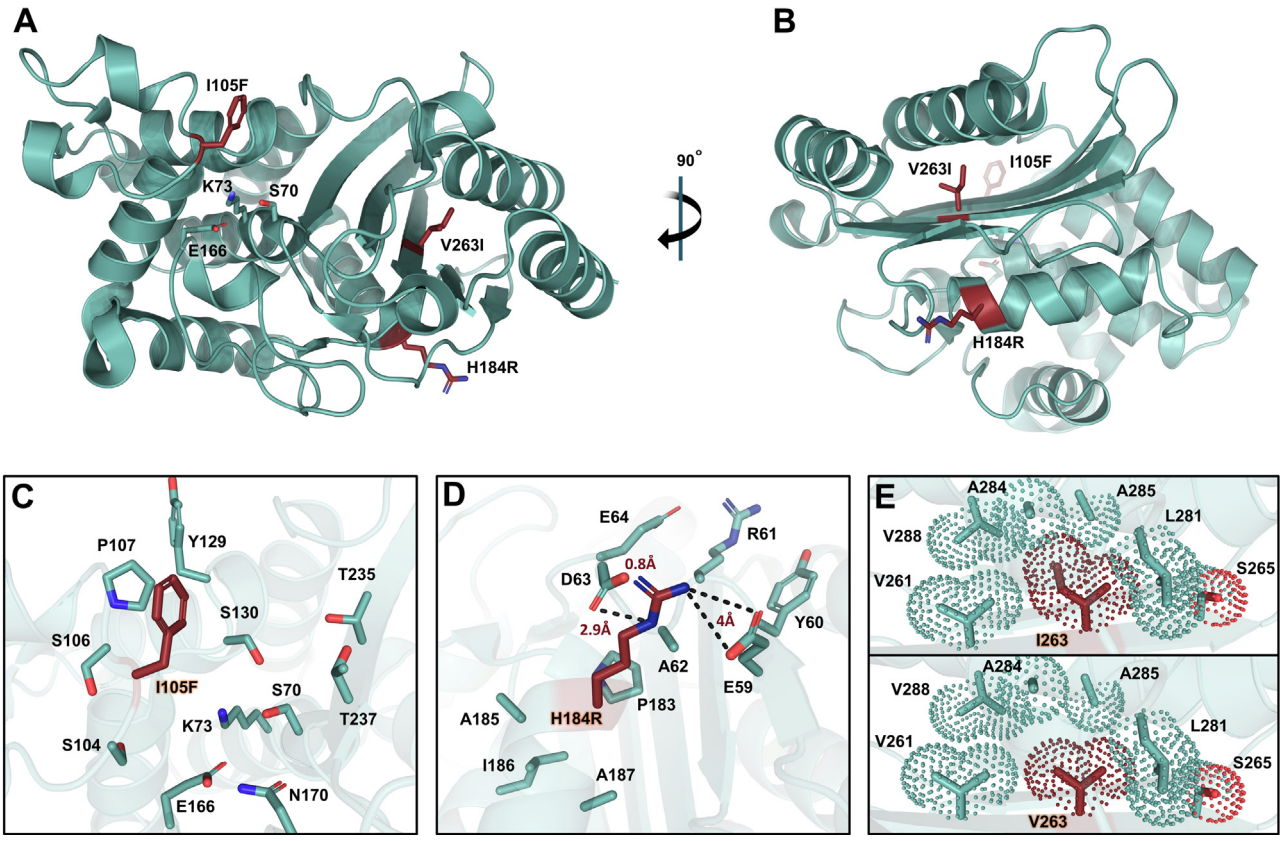


Figure 5. BlaC structure with modelled I105F, H184R and V263I mutations. (A-B) Full enzyme structure shown from two different angles. Active-site residues are shown as sticks. Mutated residues are shown as sticks and colored ruby. (C-E) The local environment of each mutated residue, I105F (C); H184R (D); I263 – top, V263 – bottom (E). Dashed lines represent distances and possible interactions with neighboring residues. The van der Waals radius of the side chain atoms is illustrated with dots in (E), (PDB 2GDN[52]).

(Figure 3), which correlated with increased T_m (Figure 1) and C_m values (Figure 2). Position 263 is located in a β-strand, capped by an α-helix (Figures 5A and 5B). The mutation to Ile likely improves packing within the local hydrophobic pocket formed by other non-polar residues, such as V261, L281, A285, and V288 (Figure 5E), thereby improving stability. Steady-state kinetic parameters suggest that the reduced catalytic efficiency for ampicillin originates from an increase in the K_M value, rather than from a lower turnover number (Table 3). Nevertheless, we presume that this modest decline in activity does not significantly affect bacterial resistance and is overcompensated by increased stability and consequently higher enzyme levels.

Our data indicate that mutation H184R has the biggest impact on thermostability (Figure 2). Based on the modelled structure, this stability enhancement could be explained by multiple favorable interactions of the arginine side chain with the neighboring residues (Figure 4D). The carboxylate group of D63 likely forms strong electrostatic interactions with the guanidinium group of arginine. In addition, another salt bridge could be formed with the E59, which is located on the opposite side (Figure 4D). Interestingly, mutation of Ala to Val at the same position (A184V) in TEM-1 has also been assigned a role of global stabilizing mutation and is frequently detected in clinical isolates of ESBLs.[34]

We have shown that the emergence of a new function was only attainable in the presence of compensatory mutations. Despite screening a notably higher number of colonies with a wild-type template, the stability toll imposed by new function mutations is likely too detrimental in the wild-type background to lead to a higher level of ceftriaxone resistance (Table S2). Conversely, this evolutionary trade-off was greatly alleviated in the presence of I105F, H184R and V263I mutations, yielding a plethora of variants with enhanced ceftriaxone resistance. A remarkable 16-fold increase was observed for three single mutations: D163G, L169P, and D179G (Table 5). Surprisingly, the D163G mutation did not lead to a dramatic loss of carbenicillin resistance (only a 2-fold decrease compared to the template), suggesting that acquisition of a new function does not always severely compromise original activity. It remains to be seen if the effect of substitution

D163G is only relevant in the context of BlaC, or whether it extends to other class A β-lactamases. *In vitro* characterization of the other two variants (L169P and D179G) confirmed the function-stability trade-off because increased activity for ceftriaxone was accompanied by reduced stability (Tables S3 and S4, respectively). Intriguingly, the buffering capacity of stabilizing mutations seems to be more potent than one would expect from the additive effects, as D179G was previously found to decrease T_m of the wild-type BlaC by 7 °C,[65] in contrast to the 3 °C loss in stabilizing background (Table S4). Among the variants with substitutions outside of the Ω-omega loop, we found that N136Y alone leads to the 8-fold increase in ceftriaxone MIC (Table S2). Replacement of Asn with Tyr leads to loss of the H-bonds between side chain amide group of N136 and backbone amino and carbonyl groups of E166 (Figure S4), and we hypothesize that this alteration triggers increased mobility of the Ω-omega loop and possibly ameliorated binding of ceftriaxone molecule.

Within the existing literature, stabilizing mutations were most frequently described in the context of TEM-1 β-lactamase.[27,34,56] The observed numbers of stabilizing mutations were notably higher (5 and 8 mutations) than we found.[34,56] Stability improvements bestowed by substitutions I105F, H184R, and V263I (Figure 1) are comparable to the values obtained for stabilizing mutations in TEM-1 (1–2 °C increase in T_m), except for M182T, which raises T_m of TEM-1 by as much as 6.5 °C.[34] Taken together, we speculate that TEM-1 might have better compensatory potential and thus, higher evolvability than BlaC. Nevertheless, if β-lactams will be used more in the future to treat tuberculosis, adaptation by mutations identified in this study can be foreseen.

Materials and Methods

Random library generation and selection

Random mutagenesis was conducted via error-prone PCR using the *blaC* F66Y-G156A gene on a pUK21 vector as a template. The sequence comprised the *blaC* gene encoding the soluble domain of BlaC (Uniprot sequence P9WKD3, residues 43 – 307) with an N-terminal twin-arginine translocation signal peptide and a C-terminal 6xHis tag (Figure S5). The reaction was performed using DreamTaq DNA Polymerase (Thermo Fischer Scientific) under non-optimal conditions (2 mM $MgCl_2$ and 0.2 mM $MnCl_2$) and an unbalanced ratio of nucleotides (0.2 mM dATP, 0.2 mM dGTP, 0.52 mM dCTP, and 0.52 mM dTTP) to increase the error rate during DNA amplification. Mutations were only introduced in the sequence of the mature enzyme, not the signal peptide or His tag. This product was subsequently ligated into the pUK21 vector using Gibson Assembly.[42] The reaction was done at 50 °C for 1 h and the product was purified with a Monarch PCR & DNA cleanup kit (New England Biolabs). The resulting plasmid library was introduced in *E. coli* KA797[66] cells by electroporation, which were plated on selective lysogeny broth (LB) agar plates containing 50 μg/mL kanamycin (to maintain the vector) and 500, 250 or 100 μg/mL of carbenicillin. Plates were incubated for 16 h at 37 °C. Colonies that grew on selection plates were used to inoculate 2 mL of LB media, which were incubated overnight at 37 °C. The next day, plasmid DNA was harvested using GeneJET Plasmid Miniprep Kit (Thermo Fischer Scientific), re-transformed into fresh KA797 cells, and plated again on selective plates to confirm that resistance originated from substitutions in the *blaC* gene, not elsewhere in the *E. coli* genome. Mutations from positive hits were identified with Sanger sequencing. Laboratory evolution of ceftriaxone resistance was performed in the same manner but using different *blaC* templates (wild-type and I105F-H184R-V263I) for error-prone PCR. Plasmid libraries generated from both templates were used for selection on LB agar plates containing 50 μg/mL kanamycin and 0.4 μg/mL ceftriaxone (2x the MIC of wild-type and I105F-H184R-V263I). Positive hits were identified and sequenced as described above.

Site-directed mutagenesis

Individual mutations were introduced into different variants of *blaC* gene by the QuikChange method (Agilent) and verified by Sanger sequencing. All mutagenic primers are listed in Table S5.

Minimum inhibitory concentration determinations

The MIC was determined for *E. coli* KA797 cells carrying various pUK21-*blaC* plasmids with the *lac* promotor upstream of the *blaC* gene. Overnight cultures of *E. coli* were inoculated into fresh LB medium with 50 μg/mL kanamycin and incubated at 37 °C until OD_{600} reached 2. Subsequently, cells were pipetted into a honeycomb 100-well plate with the selection antibiotic (carbenicillin or ceftriaxone) to an OD_{600} = 0.01. The concentration of the selection antibiotic was increased in 2.0- or 2.5-fold increments. The plate was incubated for 16 h at 37 °C in a Bioscreen C Pro plate reader with continuous shaking and the OD_{600} was recorded every 15 min. The MIC was defined as the concentration of the selection antibiotic at which no increase in OD_{600} was observed.

Periplasmic fraction isolation and Western blot analysis

The amount of soluble BlaC in periplasmic space was analyzed with Western blotting. Overnight cultures of *E. coli* KA797 cells carrying various

pUK21-*blaC* plasmids were inoculated into fresh 250 mL of LB medium with 50 μg/mL kanamycin and grown until OD_{600} reached ~3. Cells were harvested by centrifugation (4000 g, 20 min and 4 °C), resuspended in 15 mL of ice-cold TSE buffer (30 mM Tris, 20% w/v sucrose, 1 mM EDTA, pH 8) and incubated on a roller mixer at 4 °C for at least 15 min. For subcellular analysis, the cell periplasm was extracted and fractionated according to the ice-cold osmotic shock method.[67] Proteins from the periplasmic space were separated by SDS-PAGE using a 4–15% polyacrylamide gel (Bio-Rad). The amount of loaded protein for each BlaC variant was normalized according to the OD_{600} value relative to the wild-type. Proteins were transferred to a PVDF membrane using a semi-dry protocol and a Trans-Blot Turbo Transfer System (Bio-Rad). The membrane was blocked with a PBST (PBS + 0.05% Tween 20) solution containing 2% BSA, washed with PBST, incubated with anti-His monoclonal antibody conjugated with HRP enzyme (Sigma-Aldrich) and washed with PBST again. The chemiluminescence reaction was initiated by mixing 1 mL of the stable peroxide solution with 1 mL of enhanced luminol solution according to the manufacturer's instructions (Thermo Fischer Scientific) and the light signal was captured by Gel Doc XR+ (Bio-Rad). The intensity of the bands corresponding to the amounts of soluble BlaC in the periplasm was analyzed with an ImageLab 6.0.1 (Bio-Rad). Chemiluminescence signal of each variant was normalized relative to the signal of the wild-type variant and band from *E. coli* protein around 55 kDa from SDS-PAGE analysis (Figure S3).

Protein production and purification

Recombinant proteins were produced in *E. coli* BL21 (DE3) pLysS cells transformed with pET28a plasmids carrying the *blaC* gene with an N-terminal His tag and TEV cleavage site (Figure S6). Protein production and purification were performed as described previously,[68] except for the size exclusion chromatography step. The purity of protein samples was assessed with SDS-PAGE (≥95%). Pure protein samples were aliquoted, flash frozen in liquid nitrogen and stored at −80 °C in 100 mM sodium phosphate buffer (pH 6.4).

Enzyme kinetics

Steady-state kinetic parameters were determined using nitrocefin (BioVision) and ampicillin (Serva) as substrates. Measurements were performed in triplicate in a 10 mm and 5 mm QS High Precision cell (Hellma Analytics), using a PerkinElmer Lambda 1050 + UV–Vis spectrometer thermostated at 25 °C. Nitrocefin conversion was monitored at 486 nm ($\Delta\varepsilon$ = 11,300 M^{-1} cm^{-1}) for 3 min in the presence of 2 nM BlaC. Ampicillin hydrolysis was followed at 235 nm ($\Delta\varepsilon$ = 861 M^{-1} cm^{-1}) for 3 min in the presence of 10 nM BlaC. Ceftriaxone degradation was recorded at 256 nm ($\Delta\varepsilon$ = 9 541 M^{-1} cm^{-1}) for 5 min in the presence of 100 nM enzyme (wild-type and I105F-H184R-V263I), or 20 nM enzyme (I105F-H184R-V263I + L169P). The lower concentration of the latter variant was selected due to the unstable signal and possible aggregation at higher enzyme concentrations. Substrate concentration varied from 10 to 300 μM. All measurements were done in 100 mM phosphate buffer (pH 6.4). GraphPad Prism 8.0 was used to fit initial reaction velocities to the Michaelis-Menten equation (Eq. (1)):

$$v_i = \frac{k_{cat}[E][S]}{K_M + [S]} \quad (1)$$

where v_i is the initial reaction velocity, [S] is the initial substrate concentration, [E] is the total enzyme concentration, and k_{cat} and K_M are the standard Michaelis-Menten parameters. In the case of two-phased kinetic behavior, slopes of the first phase were fitted to the Michaelis-Menten equation. Michaelis-Menten plots of all analyzed variants for all three substrates are shown in Figure S1.

Thermal shift assay

The thermal stability of the proteins was assessed with a thermal shift assay using a CFX96 Touch Real-time PCR Detection System (Bio-Rad). The fluorescence signal of SYPRO Orange dye was followed in the presence of 10 μM BlaC in 100 mM phosphate buffer (pH 6.4). The temperature was increased from 20 to 90 °C with a 1 °C increment, and the samples were incubated for 1 min at each temperature before detection of the fluorescence signal. Melting temperatures (T_m) were determined from the derivatives of the obtained curves. Errors in the reported values represent one standard deviation of the mean of triplicate measurements.

Urea induced unfolding

The equilibrium unfolding transition of BlaC enzymes was followed with a Tycho NT.6 (Nanotemper) by measuring intrinsic tryptophane fluorescence at 35 °C. The measurements were done in 100 mM phosphate buffer with urea concentrations varying from 0 M to 9 M and 5 or 10 μM BlaC. The data were analyzed according to a two-state model of unfolding.[69] GraphPad Prism 8.0 was used to fit the calculated fraction of the unfolded state to a sigmoidal function. The IC_{50} value of each curve fit represents the midpoint of the transition (C_m). Errors in the reported values are one standard deviation of the mean of triplicate measurements. The graphs of the intrinsic tryptophane fluorescence signal from which the unfolded fraction (α_D) is calculated are shown in Figure S7.

Computational prediction of stabilizing mutations

The effect of I105F, H184R and V263I mutations on protein stability was assessed using Rosetta Online Server and point mutation (PM) protocol.[46] The wild-type BlaC structure (PDB 2GDN[52]) was used as a starting point and relaxed to a low-energy state prior to mutagenesis.

At selected amino acid positions, the Rosetta energy function approximates the Gibbs free energy of folding upon mutation to all other 19 residues. The $\Delta\Delta G$ values are reported in Rosetta Energy Units (REU), which are parameterized to be on a similar scale as kcal/mol.[70]

Molecular modelling

I105F, H184R and V263I mutations were modelled with PyMOL, using the wild-type BlaC structure as a template (PDB 2GDN[52]). For each mutation, the side-chain rotamer with highest probability was selected.

CRediT authorship contribution statement

Marko Radojković: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anouk Bruggeling van Ingen:** Investigation, Formal analysis. **Monika Timmer:** Investigation. **Marcellus Ubbink:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

DATA AVAILABILITY

Data will be made available on request.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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