Essentiality of conserved amino acid residues in β-lactamase

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Chapter 2
The roles of highly conserved, non-catalytic residues in class A β-lactamases

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Abstract

Evolution minimizes the number of highly conserved amino acid residues in proteins to ensure evolutionary robustness and adaptability. The roles of all highly conserved, non-catalytic residues, 11% of all residues, in class A β-lactamase were analyzed by studying the effect of 146 mutations on *in vivo* and *in vitro* activity, folding, structure and stability. Residues around the catalytic residues (second shell) contribute to fine-tuning of the active site structure. Mutations affect the structure over the entire active site and can result in stable but inactive protein. Conserved residues farther away (third shell) ensure a favorable balance of folding vs. aggregation or stabilize the folded form over the unfolded state. Once folded, the mutant enzymes are stable and active and show only localized structural effects. These residues are found in clusters, stapling secondary structure elements. The results give an integral picture of the different roles of essential residues in enzymes.

Introduction

In the cause of evolution, organisms need to adapt to changing environmental conditions, so new enzyme functions must evolve, requiring changes in amino acid sequences. If such changes have a high chance of causing loss of function, the organism is less likely to adapt. Thus, evolution not only selects for functionality but also for evolvability. The potential to gain a new function is limited by the adaptability of the amino acid sequence. It is evolutionary disadvantageous to have many essential amino acid residues, because mutations in these residues will often render a protein inactive. Therefore, evolution forces tend to minimize the number of essential residues.

Conservation of amino acid residues within or among protein families serves as a proxy for essentiality[5,34,133,134]. If an amino acid residue is highly conserved, it is assumed that evolution has selected against its substitution to prevent loss of function of an enzyme[34–37]. Such a loss can have many causes, including impaired folding, enhanced misfolding, translocation problems, poor stability, enhanced breakdown, and poor function. Given all these possible causes of function loss, the number of highly conserved residues in enzymes is surprisingly low. Broadly, three groups of highly conserved residues can be distinguished, based on their location. The first-shell residues are in the active site and involved in the catalytic function of the enzyme, so substrate binding and catalysis. Substitution of these residues will affect enzymatic function. Second-shell residues surround active site but are not directly involved in catalysis. It is assumed that these residues are important to ensure the exact positioning, to sub-Ångström precision, of the first-shell residues, which is required to enable catalysis of a chemical reaction. Third-shell residues are farther from the active site and, thus, are less likely to affect activity, except in the case of allosteric interactions. Conservation of such residues is expected to be related to a role in folding, prevention of misfolding and/or stability of the three-dimensional structure.

β-Lactamases, enzymes responsible for the breakdown of β-lactam antibiotics[135], exhibit high evolvability[136]. Over the past decades bacteria managed to respond to the high and constantly changing antibiotic selection pressures by evolving β-lactamases able to degrade novel antibiotics. New β-lactam resistant strains are frequently reported, with sometimes only one point mutation in the enzyme leading to a new catalytic function[82,137–139]. This room for evolution in β-lactamases is reflected in the low amino acid conservation. A sequence alignment of the β-lactamase from *Mycobacterium tuberculosis* (Mtb), BlaC, the object of this study, with 493 other class A β-lactamases shows 49% - 81% identity[108,109,140]. Fifteen percent of the residues is extremely conserved, with the same residue type among more than 92% of the sequences. Figure 2.1 shows the degree of conservation mapped on the crystal structure of BlaC. Highly conserved residues are found not only in the active site but widely

spread over the three-dimensional structure. While the roles of the conserved residues from the first shell have been studied extensively[81,83,84,118,141–144], the residues from the other two shells received less attention[124,131,145,146]. In this study, our aim was to establish the roles of the highly conserved residues in the second and third shells of BlaC and to test the general idea that conserved second-shell residues ensure the precise formation of the active site, whereas conserved third-shell residues are important in folding and stability. Evolvability against new antibiotics and β-lactamase inhibitors is related to the conservation of residues, so knowledge of which roles highly conserved residues play will aid in understanding evolutionary pathways toward new enzymatic functions.

We introduced single point mutations for all these conserved residues and characterized the activity, stability, and structure of the enzyme variants. The results of this study support the models formulated above and allow us to assign roles to the highly conserved residues. The structural analysis shows an interesting difference between second and third shell residues in the extend of structural changes caused by the mutation. Surprisingly, for some variants folding, stability and activity are not affected, raising the question as to why these residues are conserved.

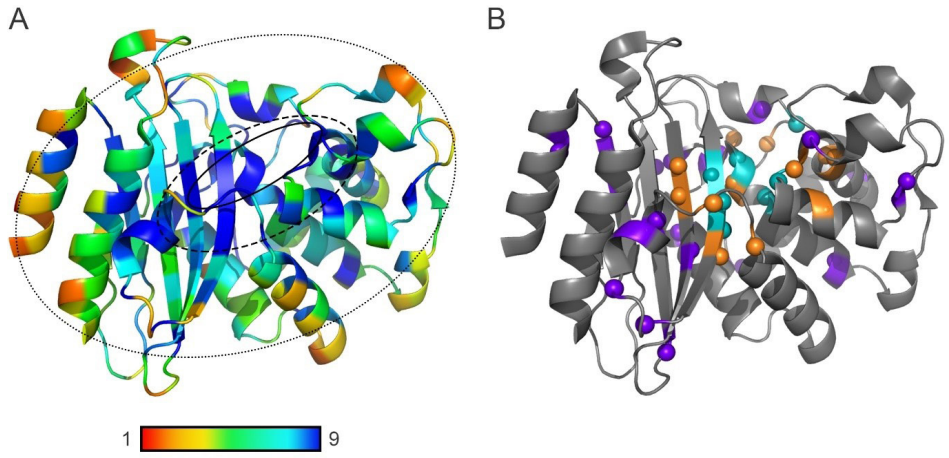

Figure 2.1. (A) Structure of β-lactamase from Mtb (PDB entry 2GDN[101]) colored by sequence conservation as determined by ConSurf[108,109,140] with 1 being highly variable and 9 being highly conserved. Solid line, dashed line and dotted line show the area of the first, second and third shells, respectively; (B) Highly conserved residues (>92% with conservation score 9) in BlaC (PDB entry 2GDN). Residues of the first, second and third shell are colored cyan, orange or purple, respectively; backbone nitrogens are shown as spheres.

Results

Several highly conserved residues can be mutated without activity loss.

An overlay of the amino acid sequences of 494 class A β-lactamases revealed 40 (15%) amino acid residues to be conserved in more than 92% of the sequences with the conservation score 9 (Figure 2.1, Table S2.1). Conserved residues in BlaC were divided in first, second and third shell based on their proximity and association with the positioning of the active site (Figure 2.1). The first shell comprises eight conserved residues involved in catalysis and substrate binding. Residues that have at least one atom located within 4 Å of a first-shell residue, were considered second shell and included 8 residues. Residues that have at least one atom of their side chain within 4 Å of those second-shell residue were also considered second shell and counted 4 residues. The other 19 conserved residues that are located outside this sphere of direct influence on the positioning of the active site residues are defined as third shell. The Cα of third-shell conserved residues were found up to 25 Å away from the Cα of active site residue Ser70.

To establish the roles of the conserved residues in β-lactamases, the second and third shell residues were mutated separately to several amino acid types, and the variant enzymes were extensively characterized, both *in vivo*, using an *Escherichia coli* expression system, and *in vitro*, using cell lysates with overexpressed BlaC. To make the number of mutants manageable, the variants were selected judiciously to vary the size, charge, and polarity of the side chain (Table S2.2), resulting in 146 variants for 31 highly conserved residues (11% of all residues) in the second and third shells. Two residues, at positions 132 and 164, were not included in the study, as BlaC is exceptional in that it carries non-conserved amino acid residues at these positions.

In vivo β-lactamase activity was tested on LB-agar plates with ampicillin and carbenicillin using a construct in which wild type BlaC is produced in low quantity and transported to the periplasm of *E. coli*, to mimic the location in Mtb[147]. Compared to ampicillin, carbenicillin only carries an extra carboxyl group. Despite this subtle change in structure, minimal inhibitory concentrations (MIC) of ampicillin and carbenicillin differ by ten-fold for wild type BlaC in *E. coli*, being 100 μg/mL and 1000 μg/mL respectively. Most mutants exhibit decreased or no activity for both substrates (Figure 2.2a), but twelve mutants show activity comparable to that of wild type BlaC. Interestingly, these comprise mutations to another of the five amino acid groups (non-conservative mutations), including T71V, T71L, N214A, N214S, and N245H, and some positions were able to tolerate all mutations that were introduced. Moreover, mutants D179N and N245H displayed MICs higher than for wild type BlaC.

Many of the conserved residues are critical to obtain folded protein.

The enzyme production level in a cytoplasmic *E. coli* overexpression system was monitored using gel electrophoresis. Production levels of the mutants were found to be close to the wild type, however, for more than half of the mutants, most protein was not soluble. These mutants also show no or very low ampicillin resistance in the low-level expression, periplasmic system (Figure 2.2, panels a and b), indicating that both systems are at least to some extent comparable. It is noted that the periplasmic system is based on Tat-translocation, in which the folded protein is moved towards the periplasm. Thus, low solubility is likely to affect also the quantity of enzyme in the periplasm. This finding suggests that almost half of the conserved residues have a role in correct folding or avoiding misfolding.

All soluble mutants were further analyzed for their fold, stability, and activity. Because of the overproduction systems, these assays could be performed on cell lysates, not requiring the purification of each separate enzyme. Circular dichroism (CD) spectroscopy was used to determine the amount of folded enzyme relative to a wild type BlaC cell lysate and this value was used in determining the activity of the mutant relative to that of wild type BlaC. Example data are shown in Figure S2.2. The chromogenic substrate nitrocefin was used to assay activity (Figures S2.3-S2.4). For some mutants little or no activity was detected due to the protein being unfolded, which was confirmed with both CD and NMR spectroscopies. Interestingly, most mutants for which soluble protein was obtained showed nitrocefin activity comparable to that of wild type BlaC (Figure 2.2c). That was observed also for the mutations that greatly affected the amount of folded protein. Correct folding was hindered by the mutation but once folded, the enzyme behaves mostly as wild type enzyme. For others, such as N136, all variants gave protein yields comparable or better than that for wild type BlaC, yet each had lost activity, as expected for second-shell residues. A thermal shift assay was used to evaluate thermal stability of mutant proteins. The majority of mutants show a slight decrease of melting temperature of 2-3 ˚C (Figure 2.2c, Figure S2.5). BlaC N245Q showed the largest decrease, by 7 ˚C. Some mutations increased the thermostability slightly, and for most of these activity was still close to that of wild type BlaC. This observation indicates again that, once folded, the enzyme resembles wild type BlaC, although many residues that are important for folding also contribute to the thermal stability of the folded enzyme.

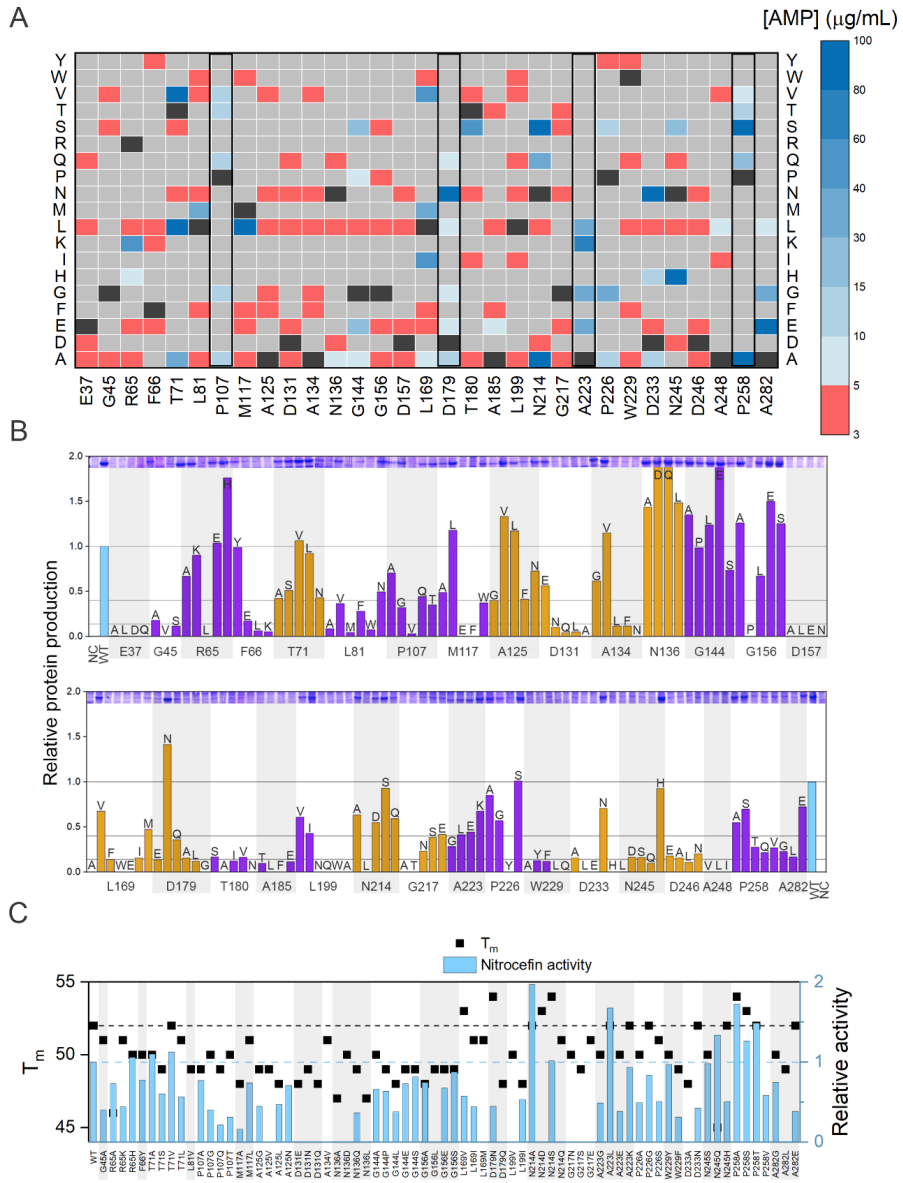

Figure 2.2. Activity, stability, and amount of soluble enzyme of BlaC variants. (A) MICs based on an ampicillin activity plate assay, with substitutions shown vertically. Wild type BlaC is indicated with a black box in each column and grey represents mutations that were not generated. Black rectangles show residues for which all generated mutations were tolerable; (B) Amount of BlaC found in soluble cell fraction of an *E. coli* cytoplasmic overexpression system relative to wild type BlaC, by comparison of the gel band intensity (shown on top of the histograms, full gels can be found in Figure S2.1). Each BlaC band was scaled to the *E. coli* protein band at 40 kDa from the same culture. NC, negative control, the vector without the blaC gene. The third-shell mutants are shown in purple, the second-shell mutants are shown in orange. The horizontal bars indicate cutoffs for good/poor/no soluble protein production; (C) Melting temperatures and relative activity in nitrocefin conversion of soluble mutants in cell lysate (see Materials and Methods for details). Horizontal dashed lines show the wild type BlaC values. The precision of the T_m is 0.5 degree. Mutants that did not yield folded enzyme sufficient for T_m and activity determination are not shown, mutants with a T_m but lacking an activity bar show no detectable activity for nitrocefin.

Second-shell variants cause more wide-spread structural changes than third-shell.

NMR TROSY spectra of the soluble fraction of the lysates were acquired to evaluate the fold of BlaC variants and the changes in structure that occur upon mutation. For many soluble BlaC variants the intensities of well-dispersed peaks were low, pointing to a low amount of folded protein, and often intense signals in the region from 8.0 to 8.5 ppm indicated the presence of a fraction of unfolded protein (Figure 2.3a). For other mutants, the spectra were suitable for tentative peak assignment, which was done by comparison to a spectrum of wild type BlaC in lysate. Figure 2.3a shows an overlay of the spectra of His-tagged enzyme in lysate and purified and His-tag cleaved BlaC. Most resonances derived from BlaC nuclei can readily be identified[148]. In general, NMR spectra of second-shell mutants aligned worse with that of wild type BlaC than spectra of third-shell mutants (Figure 2.3a). Significant chemical shift perturbations (CSPs), defined here as > 0.03 ppm, were found for 17% and 6% of assigned peaks for second-shell and third-shell variants, respectively (Figure 2.3b). For second-shell variants, CSPs were mostly found for the same set of residues, spread throughout and around the active site (Figure 2.4). The CSPs were also observed for mutants exhibiting only moderate change in activity or stability. For the third shell residues, CSPs were mostly localized around mutation site. This interesting observation is general for all variants with good NMR spectra and suggests that the interactions that third-shell residues make have low correlation with those further away in the protein (local structural effects only), whereas second-shell mutations cause structural effects that are felt throughout the active site, showing correlation over a longer distance. The effects of the second-shell mutations were similar for both core and surface localized residues (Figure S2.8). Only for two second-shell residues in Ω-loop, L169 and D179, CSPs were found to be spread somewhat less extensively than for the rest of the second-shell residues, however, changes still involved active site and regions around it (Figure 2.4b).

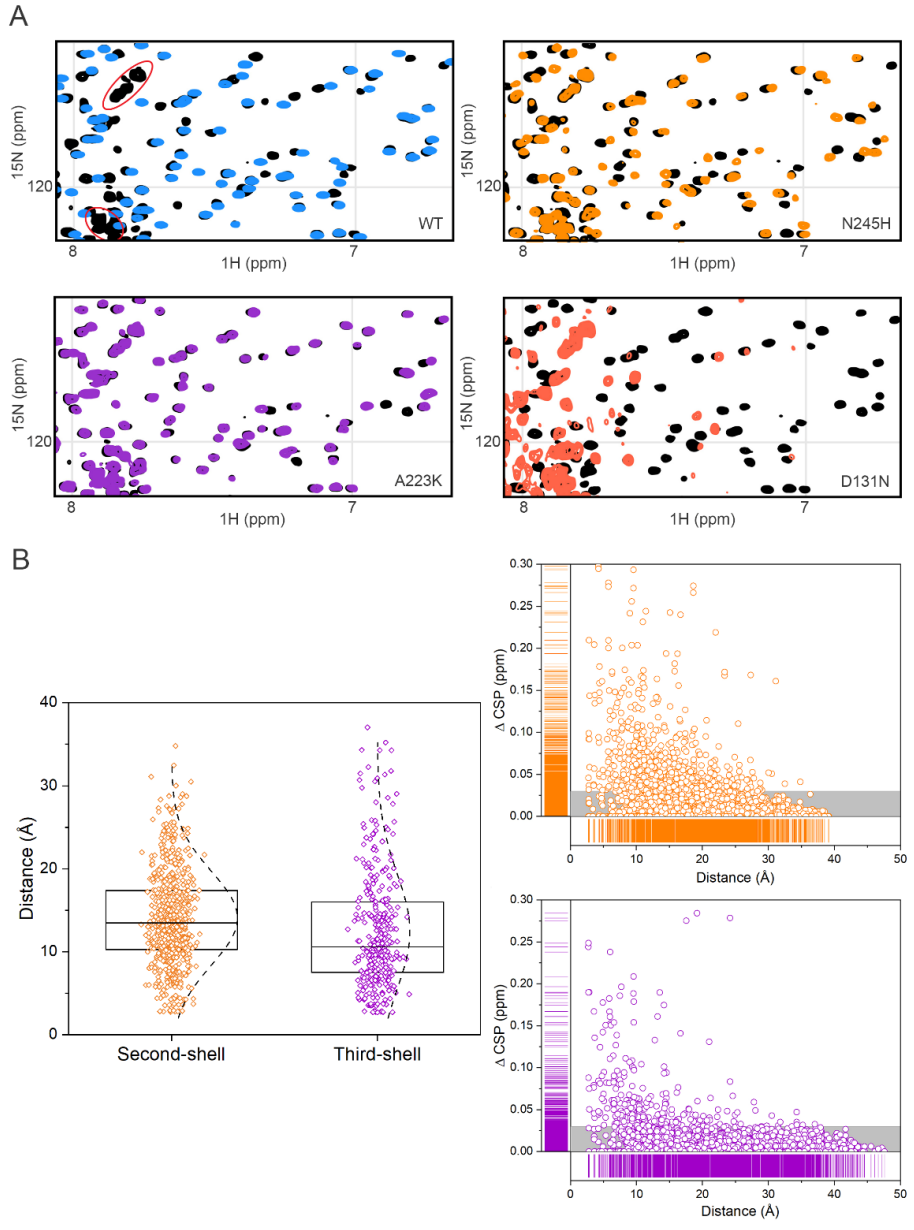

Figure 2.3. NMR TROSY spectra and CSPs. (A) Examples of details of NMR spectra (full spectra in Figures S2.6-S2.7). Wild type BlaC spectrum from whole lysate is shown in black; purified wild type BlaC is shown in blue; spectra of well-folded second-shell (N245H) and third-shell (A223K) mutants are shown in orange and purple respectively; the spectrum of a poorly folded mutant (D131N) is shown in salmon. Red ovals indicate peaks from *E. coli* proteins. (B) All CSPs from all mutant spectra from second (orange) and third (purple) shell are presented on the right against distance from the mutation site (backbone amide to amide). Grey bars represent insignificant CSP (< 0.03 ppm). On the left CSPs above 0.03 ppm are shown for all assigned peaks of all mutants against the distance of the amide nitrogen relative to the Cα atom of the mutated residue. The boxes represent 25th-75th percentile, lines inside the boxes represent the medians, dashed lines represent distribution by count. The median of the second-shell residues is significantly larger ($p < 0.01$) than that of the third-shell residues.

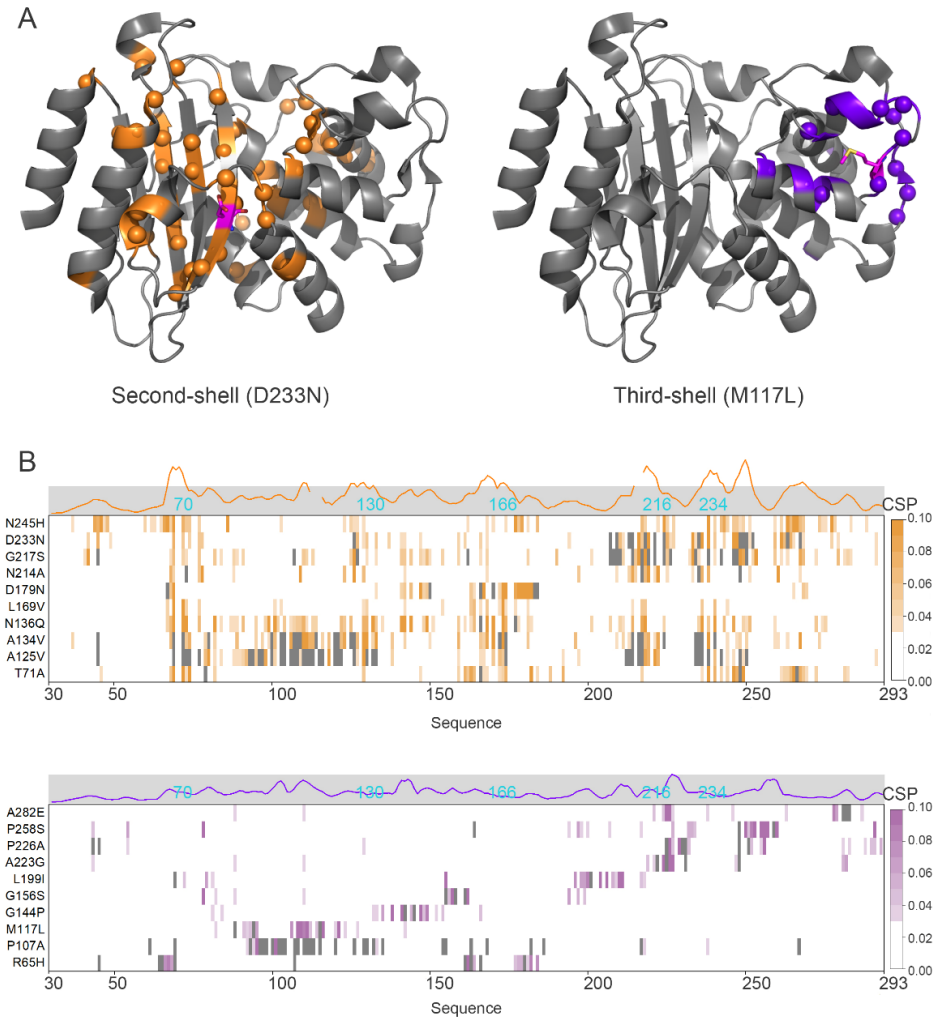

Figure 2.4. Changes in structure observed upon mutations. (A) Examples of the location of nuclei with significant CSP in a second-shell (left) and third-shell mutant (right). CSPs are spread far from mutation site in the second-shell mutant D233N and are localized around the mutation site in the third-shell mutant M117L. The mutated residue is shown in magenta sticks. PDB entry 2GDN[101]. (B) CSPs of some mutants plotted on the amino acid sequence. Residues for which no data are available are shown in grey. The positions of several first-shell residues are indicated in cyan. For most second-shell mutants large CSPs are found for the same residues, in regions around residues 67-72, 110-112, 125-132, 162-169, 213-217, 232-238, 242-248, 261-266. Third-shell mutants show CSP mostly close to the site of mutation. Traces above the graph represent averaged CSPs for all mutants, curves are smoothed using Savitzky-Golay filter to aid the visualization.

Conservative mutations can be more detrimental than non-conservative ones.

Conservative mutations of conserved third-shell residues always had an equal or less detrimental effect on BlaC, as compared to non-conservative mutations. For several variants of second-shell residues, however, conservative mutations performed worse than non-conservative ones, indicating that slight changes in the functional group have a more negative effect than replacement with a side chain that cannot maintain any interaction. Examples of such residues are Thr71 and Asn214. The conservative mutant T71S displayed *in vivo* activity, thermal stability, as well as the quality of NMR spectrum that was worse than those of T71 substituted by Leu, Val or Ala. Mutants N214D and N214Q performed much worse *in vivo* than mutants N214A or N214S (Figure 2.5, Table S2.2).

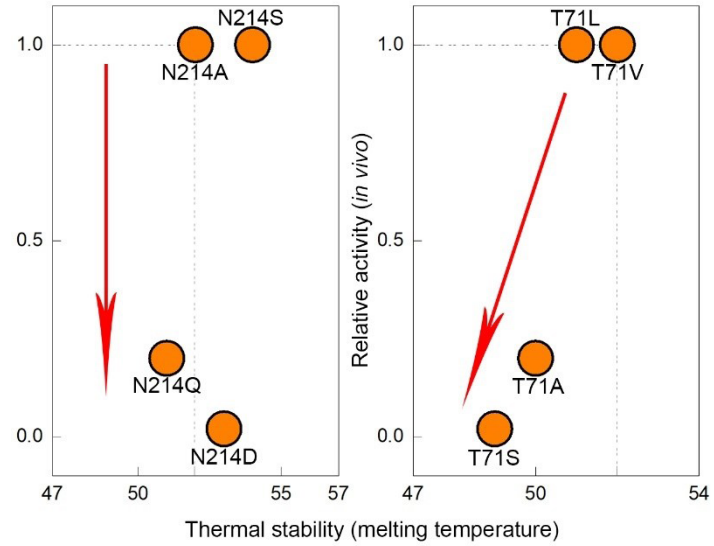

Figure 2.5. Schematic representation of the effect of mutations in residues Thr71 and Asn214 relative to WT. Dashed lines show the WT values. The red arrows show worsening of the mutation effect. For residue Asn214 activity is affected more with conservative substitutions to Gln and Asp compared to non-conservative Ala and Ser. For residue Thr71 conservative substitution to Ser displays the worst activity and stability.

Discussion

In general, we observed three types of mutation effects. Mutations in group A result in strongly reduced amounts of soluble protein and proportionally more in the insoluble fraction. The proteins in the soluble fraction of these BlaC variants exhibit near normal stability, structure and activity. These observations suggest that for these proteins, the mutation has shifted the balance between folding and formation of aggregates to the latter. Considering that folding into the soluble three-dimensional structure is kinetically driven, it implies that the mutation either slows the folding rate or accelerates the aggregation rate (Figure 2.6, blue effect). Thus, these residues were most likely conserved by the evolutionary process to select against misfolding[149]. The second group (B) concerns variants that yield substantial amounts of soluble protein of which a fraction remains unfolded, as demonstrated by the NMR spectra. The mutants in this group often have a reduced melting temperature. We hypothesize that in this case the thermodynamic balance between folded and unfolded protein is affected. That implies that the conserved residue contributes to the stability of the folded form, relative to the unfolded state (Figure 2.6, red effect). The third group (C) are mutations that do not affect the production of a folded and stable enzyme but yield a non-native form of the enzyme with reduced activity (Figure 2.6, magenta effect). We conclude that in this case, the conserved residue is critical to obtain the active conformation, within the ensemble of conformations that the enzyme can visit within the folded form. The major effect of mutations on protein production, stability of the native form or activity was also demonstrated for other proteins. The mutational studies on two PER-ARNT-SIM domain proteins showed that many of conserved residues contributed to the production of soluble protein[150,151]. For glutathione-S-transferase mutations in conserved motifs resulted in decreased stability and refolding rate, as well as in altered activity[152]. The destabilizing effect of mutations in conserved positions was also demonstrated for acyl-coenzyme A binding protein[40].

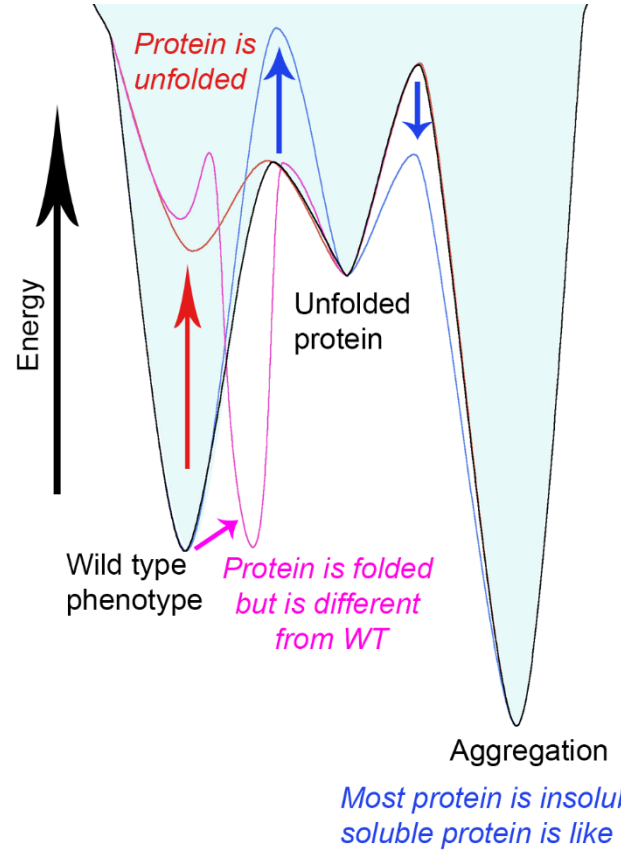

Figure 2.6. The free energy landscape of protein forms. The free energy landscape for wild type BlaC is shown with the black line. The effects of mutations described in the text for groups A, B and C are shown in blue, red and magenta lines, respectively.

The effect of group A is mainly represented by conserved residues far from the active site (third-shell residues). They are important for obtaining the correct three-dimensional fold, because for many of them mutation leads to a strong reduction in the amount of soluble protein (Figure 2.2, Table S2.2). Many are localized near the edges of the secondary structure elements (Figure 2.7a) and have interactions with nearby conserved residues, creating clusters. Such clusters are critical for the three-dimensional structure by "stapling" the secondary structure elements together. Mutations diminish the folding efficiency and protein stability, but the fraction that is folded resembles the wild type enzyme structurally and kinetically[145]. Protein stability and ability to fold are a well-known selection forces[153–155].

Mutations elsewhere outside from the active site area in most cases are not detrimental as long as the secondary structure elements are formed and stapled by these essential residues. This principle results in a low number of essential residues outside the active site and thus a high evolutionary robustness of the protein. The effect of conserved residues clustering was shown for different folds[42], where such clusters play a role in ensuring native form integrity of enzymes.

The group B effect was shown for some second-shell residue mutants. The soluble protein coexisted with the unfolded form, which could be detected with NMR (Figure 2.3a, Figure S2.7). Highly rigid proteins tend to unfold slowly, and active sites have a destabilizing effect on proteins because they require a degree of dynamics or expose hydrophobic interaction sites[156,157]. Mutation of these group B residues thus could increase the unfolding rate and shift the balance of folding and unfolding toward the latter by destabilizing the folded form.

The activity change observed for group C mutants was evident to different degrees. Subtle effects (group C1) were observed for a group of third-shell residues containing glycines, prolines and alanines, found mostly in solvent-exposed loops (Table 2.1). These residues might be important for protein dynamics[158,159]. Very low activity (group C2) was displayed by 12 well-folded and stable mutants of second-shell residues. These residues are located in places that ensure exact positioning of the residues involved in catalysis. Examples of such residues can be seen in figure 7b. Residue Ala134 is located in α-helix 8 (Figure S2.9) and its side chain points towards α-helix 7. Introducing a larger side chain is likely to interfere with the relative positions of these helices, dislocating active site residue Ser130. Residue Asn136 is also found in α-helix 8, making two hydrogen bonds to backbone atoms of catalytic residue Glu166 and ensuring the cis conformation of the peptide bond between Glu166 and Pro167[124].

An interesting observation was made for a group of second-shell residues for which the effect of the mutation is dependent on the nature of the new side chain. For these residues, variants with substitutions that remove the functional group are performing better than mutants with conservative substitutions. This observation emphasizes that the functional enzyme, especially around the active site, consists of a complex and extensive web of interactions. The presence of such a web is also supported by the NMR data, showing CSPs spreading far from the mutation site for the second shell residues. CSP can represent minute structural changes that are not easily picked up in crystal structures yet may have catalytic consequences if they cause small changes in the atom positions of catalytic residues or polarity changes in the active site. Mutations that influence the interactions of the side chain cause changes in the whole web of interacting residues and result in a dysfunctional protein. Substitutions that simply eliminate the interactions of the side chain apparently make it possible for other

residues to compensate for the lost interaction. For example, residue Thr71 probably aids the precise positioning of catalytic residue Ser70, by providing a H-bond between the Oγ1 and the carbonyl oxygen of Phe68 (Figure 2.7c). Substitution T71S, which is likely to modify the length of this H-bond, yields less stable protein with somewhat decreased activity against nitrocefin *in vitro* and no activity against ampicillin and carbenicillin *in vivo*. However, substitutions to Leu, Val or Ala, which eliminate this H-bond completely, result in protein that resembles wild type BlaC better.

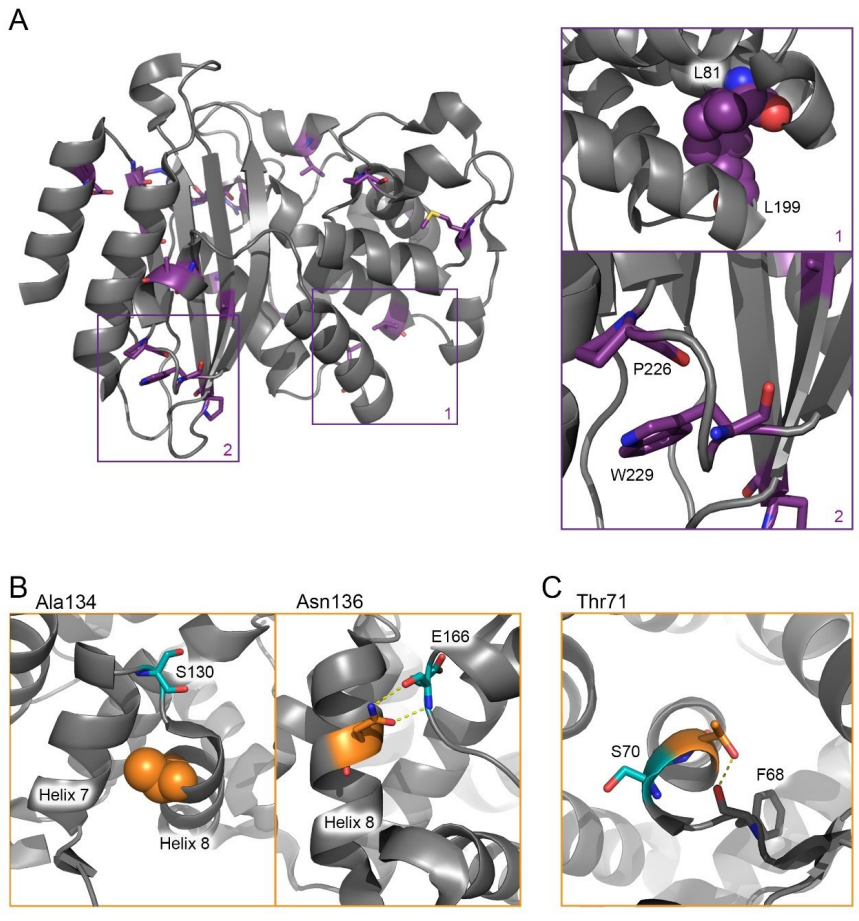

Figure 2.7. (A) Third-shell residues localized around the edges of the secondary structure elements (shown in purple sticks or spheres) and zoom-in of examples of interacting residues; (B) Examples of second-shell residues (shown in orange) influencing the position of the first-shell residues (shown in cyan) by ensuring the tight packing (Ala134) or making direct bonds to an active site residue (Asn136); (C) Environment of the second-shell residue Thr71 (in orange).

All analyzed residues had conservation higher than 92%. Such high conservation of a residue suggests that the exact nature of the side chain is extremely important. Nevertheless, a few mutants demonstrated behavior close to that of wild type BlaC. Two mutants, BlaC N245H and D179N exhibit in fact *in vivo* activity higher than wild type BlaC, accompanied by a melting temperature that is the same or higher. One would expect to find these residues in natural variants of β-lactamases as often as the conserved residues, yet the extreme conservation shows the preference towards the native residue. A few explanations can be given for this phenomenon. First, our experiments could only characterize a limited set of protein properties, activity against two specific antibiotics and nitrocefin, protein production, thermal stability and structural changes. It is possible that these mutants have lost a trait not tested within this research. One such trait is the evolvability of the protein[15,23,160,161]. Microorganisms are under strong selection pressure of β-lactam antibiotics, so β-lactamases must be highly evolvable. Counterintuitively, it is possible that some residues might be conserved to preserve this trait, allowing residues around them to be able to undergo changes. In this vein, mutations in these residues could be a "epistatic dead end" by making other residues less evolvable. A second explanation is that not all amino acid substitutions can appear equally often. The transition/transversion bias can contribute to the lower chance of some mutations to occur in nature[162]. One example of such substitution can be the mutant N245H, which requires a change from adenine to cytosine. Furthermore, some amino acid substitutions require two nucleotide changes. For example, substitutions T71V or T71L that happen via consecutive point mutations can only occur via T71P, T71A, T71M or T71I. We have no data about the effects of T71P, T71I and T71M on the protein, but based on the position of Thr71 it is possible to assume that at least substitutions to Pro and Met will have a negative effect on the protein function. A third explanation is that a residue is essential in most β-lactamases but not in BlaC. In that case, the residue in BlaC is an evolutionary rudiment. For example, the variant D179N outperforms wild type BlaC in terms of *in vivo* activity, stability and protein level (*in vitro* activity is somewhat reduced). However, in TEM-1 this variant shows reduced fitness against ampicillin selection pressure[128,129]. Thus, it can be that Asp179 is essential for many class A β-lactamases but for BlaC a mutation to Asn would not be detrimental, perhaps even an improvement for the fitness.

The results of this study led us to formulate a hypothetical function for each conserved second and third shell residue in BlaC (Table 2.1).

In conclusion, this work offers an explanation of general trends of conservation in serine β-lactamases. Conserved residues of the first shell form a catalytic center of a protein, conserved residues of the second shell establish a functional core of the protein and the third-shell residues ensure the overall fold. For the third-shell residues, the interactions they make in a final form of a protein or during folding process are extremely important. For that reason, many of them do not tolerate any mutations and others can only undergo conservative substitutions. Residues of the third-shell contribute to a high evolutionary robustness of the protein by limiting conservation to a few clusters that are essential to staple secondary structure elements correctly. The second-shell residues form a web of interactions around the active site, fine-tuning the structure of the active site. In that way residues of the second shell contribute to a high evolvability of the protein. The conserved residues of the second shell ensure overall integrity of the active site, while random mutations can accumulate around the active site, potentially leading to a new trait.

Table 2.1. Effect of mutations and possible functions of conserved residues of the second and third shells.

Residue	Conservation among 494 sequences (%)	Other natural variants (%)	Effect of mutations (Group)	Hypothetical role	Shell
E37	99.2	A, D, Q, S (0.2)	Mutations cause loss of *in vivo* activity due to production of either insoluble or soluble unfolded protein; for some residues conservative mutations lead to production of soluble protein in low quantities, with a phenotype comparable to wild type. (A/B)	Ensures correct folding by priming the position of the first β-strand[a]	III
G45	100			Allows the side chain of conserved residue F66 to occupy its position	III
R65	96.2	A (1.6), T, P (0.6), L (0.4), H, K, C (0.2)		Stapling function, stabilizes the position of Ω-loop via interaction with conserved residue T180	III
F66	99.8	Y (0.2)		Locks β6 on β1 via interaction with residue 43	III
L81	99.2	M, V (0.4)		Is involved in hydrophobic interactions between α3, α7 and α13	III
M117	92.5	L (6.8), P (0.6), I (0.4)		Fills hydrophobic cavity between α6, α7 and α8	III
D157	99.2	N (0.8)		Is involved in stabilization of Ω-loop	III
T180	99.6	S, I (0.2)		Stabilizes the Ω-loop via interaction with conserved residue R65	III
A185	94.3	S (2.2), T (1.2), V, E (0.6), Q (0.4), R, N, G (0.2)		Fills small space between α12 and a loop, allowing for correct positioning of side chain of conserved residue D157	III
L199	100			Keeps bend between α12 and α13 inside the hydrophobic cavity	III
W229	98	S (0.8), A (0.6), Y, C, F (0.2)		Contributes many hydrophobic and stacking interactions	III
A248	74.5	G (25.5)		Fills small space inside the core of the protein	III
P107	98.6	A (0.8), V, Q, T (0.2)		Is involved in a formation of a loop. Can be important for protein dynamics.	III
G144	99	D (0.6), S, N (0.2)		Is involved in a formation of a loop. Can be important for protein dynamics.	III
G156	97.8	N, D (0.6), E, S (0.4), R (0.2)		Makes a turn allowing for correct position of conserved residue D157	III
A223	97	K (1.2), V, S (0.4), E, R, M, T, G (0.2)	Slight effect on stability and/or activity. (C1)	Points in the solvent together with conserved residue A282, might be involved in protein-membrane interaction	III
P226	98.4	Q, A (0.6), L, D (0.2)		Is involved in stapling the turn between α13 and β7 via interaction with conserved residue W229	III
P258	98	A, S, T (0.6), Q (0.2)		Allows for a bend between β8 and β9	III
A282	94.2	E (1.3), S, T (1.1), K (0.9), G (0.7), V (0.4), R (0.2)		Points in the solvent together with conserved residue A223, might be involved in protein-membrane interaction	III
A134	99.4	S (0.4), G (0.2)	*In vitro* activity loss with or without effect on folding. (C2)	Fills small space between α6, α7 and α8 together with conserved residue A125, allowing for correct positioning of active site Ser130	II
N136	100			Reduces mobility of the first-shell residue E166 via two H-bonds; stabilizes *cis* bond between E166 and P167[c]	II
G217	94.3	S (5), N (0.4), T (0.2)		Makes a turn aiding the correct position of the first-shell residue T216	II
D246	96.4	N (1.4), T (1), A (0.6), I (0.4), E (0.2)		Pulls α14 to β8; might orient conserved residue D233 for its bond with conserved residue N214	II
T71	98.2	S (0.6), A, V, L (0.4)	Various effects on protein stability and/or activity; effect is dependent on type of substitution. (B/C)	Reduces mobility of the active site residue S70 via H-bond to residue 68	II
A125	99.8	G (0.2)		Fills small space between α6, α7 and α8 together with conserved residue 134, allowing for correct positioning of active site Ser130	II
L169	95	I (2.6), M (1.6), V (0.8)			II
N214	92.9	S (3.6), T (1.4), C (1), A, G (0.4), V (0.2)		Stabilizes position of the first-shell residue T216 via bond to a conserved residue D233; affects active site residue K234 via water	II
D233	96.8	E (2), G, H (0.4), S (0.2)		Interacts with conserved residues D246 and N214, influencing the first-shell residues T216 and K234	II
N245	92.5	A (1.8), H, L, G (1.6), S (0.6), I (0.2)		Makes an interaction with residue 68, allowing it to position precisely for an interaction with conserved residue T71	II
D131	100		All mutations caused production of unfolded or partly folded protein. (B)	Plays role in protein stability, holds together α6, α7 and α8, positioning active site S130, via bonds to residues V108, A109, A125, T133, A134, A135[b]	II
D179	99.8	G (0.2)	Mutation D179N had a positive effect on stability, the other mutations affected protein folding and activity. (B/C)	Stabilizes the Ω-loop via bonds with residues D172, D163, A164; might be involved in stabilization of active site S70 via bond with residue 68	II

[a] Chikunova, A. *et al.* Conserved residues Glu37 and Trp229 play an essential role in protein folding of β-lactamase. *FEBS J.* **288**, 5708–5722 (2021)

[b] Swarén, P. *et al.* Electrostatic analysis of TEM1 β-lactamase: effect of substrate binding, steep potential gradients and consequences of site-directed mutations. *Structure* **3**, 603-13 (1995)

[c] Banerjee, S. *et al.* Probing the non-proline cis peptide bond in β-lactamase from Staphylococcus aureus PC1 by the replacement Asn136 → Ala. *Biochemistry* **36**, 10857–10866 (1997).

Materials & Methods

Conservation analysis

Sequence conservation among β-lactamases was determined using the ConSurf server[109]. The BlaC sequence from Wang, Cassidy & Sacchettini[101] (PDB ID: 2GDN) was used to find homologs using a PSI-BLAST algorithm with five iterations. Parameters for minimum and maximum sequence homology were set to 35 and 95%. An alignment of 494 sequences[163] was generated using the MAFFT algorithm[164] and the level of conservation of each amino acid residue position was calculated.

Mutagenesis

Site-directed mutagenesis was performed using two vectors as template. For *in vivo* studies mutants were created in a pUK21 (pUC21 based plasmid with kanamycin resistance) plasmid carrying the *blaC* gene, encoding a Tat signal peptide for transmembrane transport[147,165]. The plasmid pET28a+ carrying the *blaC* gene with the code for a cleavable N-terminal His(6)-tag[166] was used as overproduction system and to determine the amount of soluble protein. Mutagenesis was performed using the QuikChange (Agilent) method. The presence of the mutations was confirmed by sequencing. Primer sequences can be found in Table S2.3.

To establish the localization of mature protein BlaC WT with the signal peptide for Tat-system was overexpressed in pET28a+ vector confirming that the protein was in membrane, while most protein was found in cytoplasm for BlaC WT expressed without Tat-system signal peptide (Figure S2.10).

In vivo activity studies

The survival of the *E. coli* cells carrying pUK-based plasmids with wild type or mutant *blaC* genes was tested on LB-agar plates with antibiotics. All plates contained 50 μg mL^{-1} kanamycin and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were plated as 10 μL drops with OD_{600} values of 0.3. In the first round of *in vivo* studies, the plates contained ampicillin (3 μg mL^{-1} or 15 μg mL^{-1}) or carbenicillin (20 μg mL^{-1} or 200 μg mL^{-1}). Mutants that did not grow on the lowest concentrations of antibiotics were not used in the following test rounds; growth of mutants surviving the lowest concentrations but not the highest was tested on intermediary concentrations of ampicillin (5 μg mL^{-1} and 10 μg mL^{-1}) and carbenicillin (50 μg mL^{-1} and 100 μg mL^{-1}). For mutants surviving the highest concentrations, growth was tested on plates with 30, 60 and 100 μg mL^{-1} ampicillin and 500, 1000 and 2000 μg mL^{-1} carbenicillin. All tests were performed with two biological replicates. In case of disagreement between replicates mutants were tested separately again. *E. coli* cells expressing the gene of BlaC S70A was used as negative control, because these cells produce intact but inactive BlaC.

BlaC presence in E. coli cells

For recombinant production of mutant proteins, *E. coli* strain BL21pLysS (DE3) was used in combination with the pET28a+ based plasmids. Cells were cultured in LB medium at 37 ˚C until the OD_{600} reached 0.6-1.0, at which point protein production was induced with 1 mM IPTG, followed by incubation of the cultures at 18 ˚C for 16 hours. Of overnight bacterial cultures 100 μL were tested for nitrocefin activity and 500 μL were centrifugated and resuspended in 50 μL B-PER (Thermo Scientific) for lysis. After 30 minutes incubation at room temperature, 20 μL was treated with SDS-PAGE cracking buffer (20 mM Tris/HCl pH 6.8, 5 mM EDTA, 0.5% SDS, 0.1% β-mercaptoethanol) and the rest was centrifugated for separation of soluble and insoluble fractions. The soluble fraction was tested for nitrocefin activity and treated with SDS-PAGE cracking buffer. Whole lysate and soluble fraction samples were analyzed using SDS-PAGE and gels were stained with Coomassie Brilliant Blue. The signal intensity of the band corresponding to BlaC for each mutant was compared to signal intensity of the *E. coli* protein at 40 kDa using ImageLab software (BioRad). The experiment was done with four replicates and the gels were compared to each other, only one replicate was used to calculate the relative expression.

Mutants that showed a clear band on the gel for the soluble fraction were analyzed *in vitro*. Cultures of 10 mL were induced and incubated overnight at 18 ˚C and lysed with B-PER for 30 min at room temperature. After centrifugation the supernatant containing the soluble protein fraction of the lysate was diluted 100-fold.

Thermal stability

Thermal stability of the proteins was analyzed with a thermal shift assay with SYPRO Orange dye (Invitrogen). The measurements were performed in triplicate using the CFX 96 Touch Real-Time PCR Detection System (Bio-Rad) with the temperature range 20-80 ˚C. Melting temperatures were determined from averaged signal of three measurements. The technical error is 0.5 ˚C.

Circular dichroism

CD profiles were recorded at 25 ˚C using a Jasco J-815 spectropolarimeter with a Peltier temperature controller (Jasco, MD). Spectra were acquired in 1 mm quartz cuvette at a scan rate of 50 nm/min. The negative control spectrum was used as a background spectrum of *E. coli* proteins and subtracted from all mutant and wild type spectrum (Figure S2.2). The CD signal at 222 nm was then used as a measure for the amount of folded BlaC for calculating activity of mutants relative to wild type BlaC.

Kinetics

Determination of the Michaelis-Menten kinetic constants was done by measuring the absorption change at 486 nm for nitrocefin ($\Delta\varepsilon$ = 25.7 mM^{-1} cm^{-1} based on concentration calibration using quantitative NMR against 2.0 mM trimethylsilylpropanoic acid) with a TECAN Infinite® M1000PRO plate reader thermostated at 25 ˚C. All measurements were performed in triplicate. Initial rates of the hydrolysis were plotted against concentration of substrate and Michaelis-Menten curves were fitted to the Michaelis-Menten equation 2.1 using OriginPro 9.1,

$$v_0 = \frac{V_{max}[S]_0}{[S]_0 + K_M} \quad (2.1)$$

where v_0 is the initial reaction rate, $[S]_0$ the initial substrate concentration, V_{max} the maximum reaction rate and K_M the Michaelis-Menten constant. v_0 and $[S]_0$ are the dependent and independent variables, respectively, and K_m and V_{max} are the fitted parameters. The lower limit for K_M determination was set to 10 µM, below which the activity was considered as not determined. The V_{max}/K_M parameters were calculated and averaged, the values can be found in Table S2.2. The ratio of signals at 222 nm derived from CD spectra was used for normalization of activities relative to wild type.

NMR spectroscopy experiments

For the production of isotope labeled proteins for NMR experiments, M9 medium was used with ^{15}N ammonium chloride as the sole nitrogen source (components can be found in Table S2.4). Cultures (100 mL) were spun down and resuspended in B-PER and after 30 min incubation at room temperature, samples were centrifuged, and TROSY-HSQC spectra of soluble fraction were recorded on a AVIII HD 850 MHz spectrometer (Bruker) at 25 ˚C with 6% D_2O. Data were processed in Topspin 4.1 (Bruker). Spectra were analyzed with CCPNmr Analysis V3[167] software. Peaks of the mutant spectra were assigned where possible by comparison to those in the wild type BlaC spectrum and average chemical shift perturbations (CSP), $\Delta\delta$, of the ^1H ($\Delta\omega_1$) and ^{15}N ($\Delta\omega_2$) resonances of backbone amides were calculated using equation 2.2.

$$\Delta\delta = \sqrt{\frac{1}{2}\left(\Delta\omega_1^2 + \left(\frac{\Delta\omega_2}{5}\right)^2\right)} \quad (2.2)$$

Supporting data

Supplementary figures

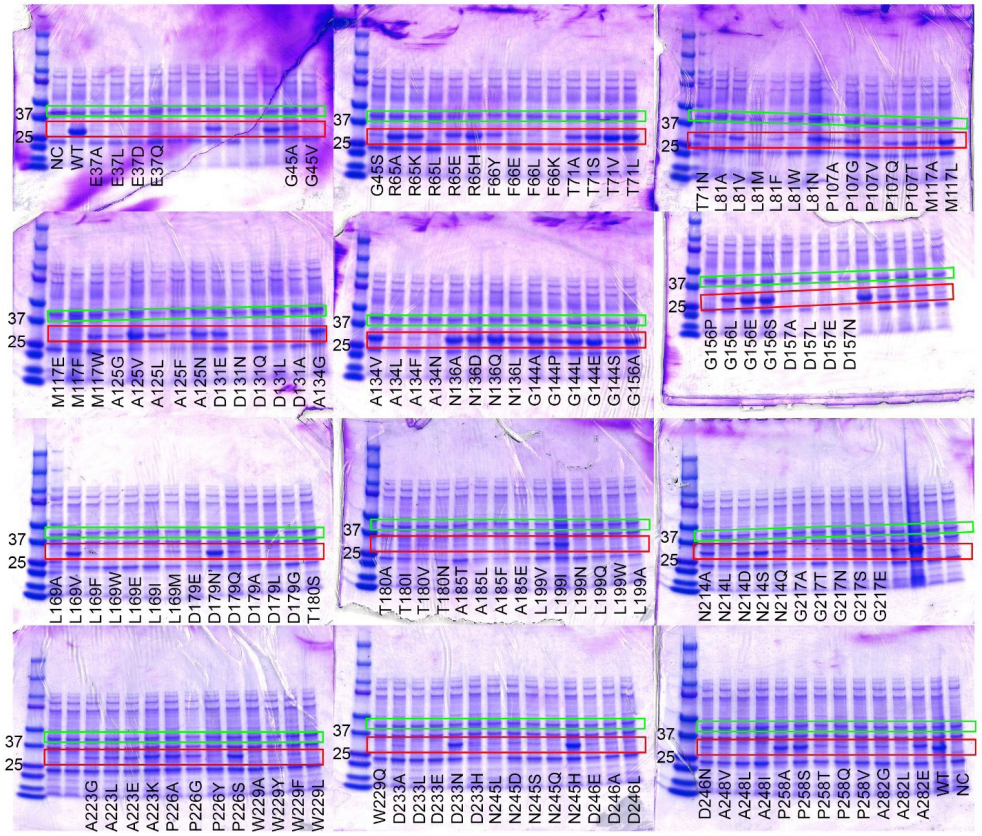

Figure S2.1. Gels displaying soluble fractions of cell cultures expressing WT and mutant BlaC. NC – negative control, cells containing empty vector. Red frame shows the position of BlaC at 31 kDa, green frame shows the position of *E. coli* protein that was used for signal scaling. Non-labeled lanes contain mutants of residues with lower percent conservation, thus not discussed within this study.

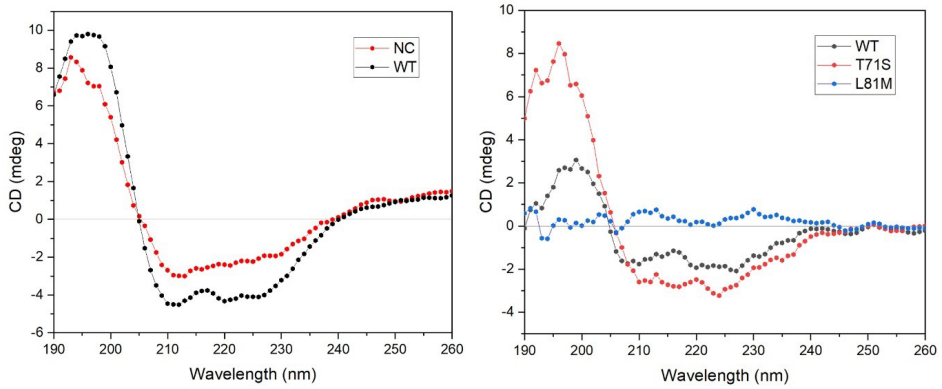

Figure S2.2. Example of CD spectra acquired for negative control (NC) and wild type (WT) (left panel); spectra of wild type, a poorly produced mutant (L81M) and a folded mutant (T71S) after background correction (right panel

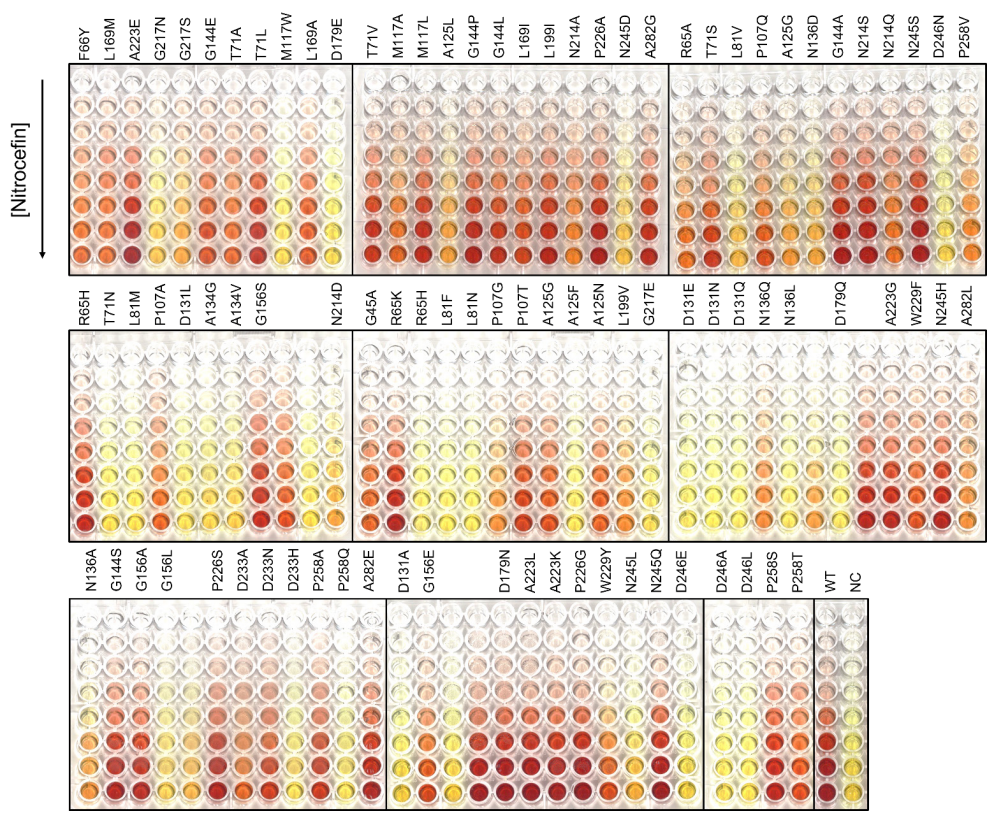

Figure S2.3. Plates with the range of nitrocefin concentration and whole lysates containing BlaC variants after 5 minutes of reaction. Non-labeled wells contain mutants of residues with lower percent conservation, thus not discussed within this study.

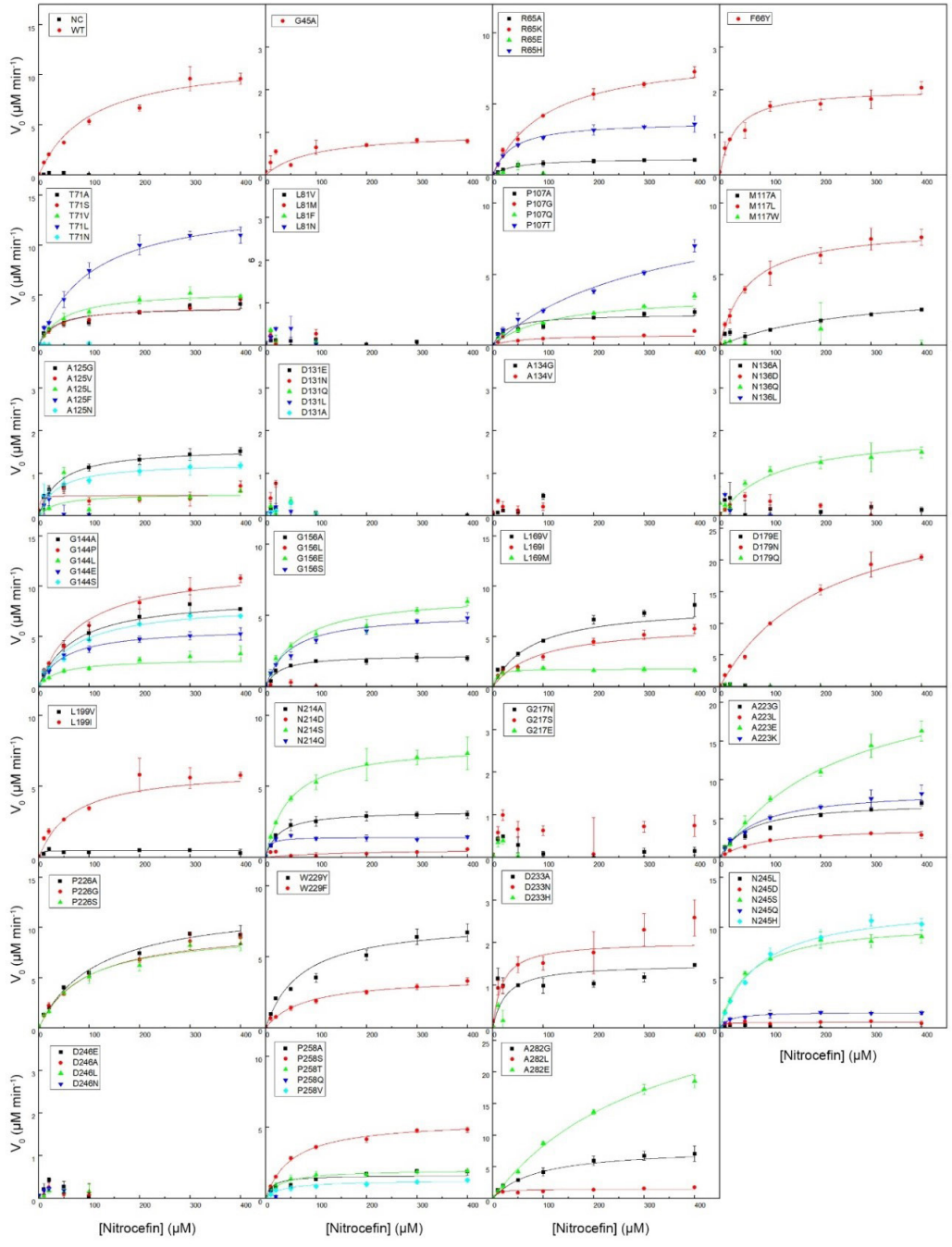

Figure S2.4. Nitrocefin kinetic curves. Error bars represent the standard deviation of a triplicate measurement. Lines represent the Michaelis-Menten fit.

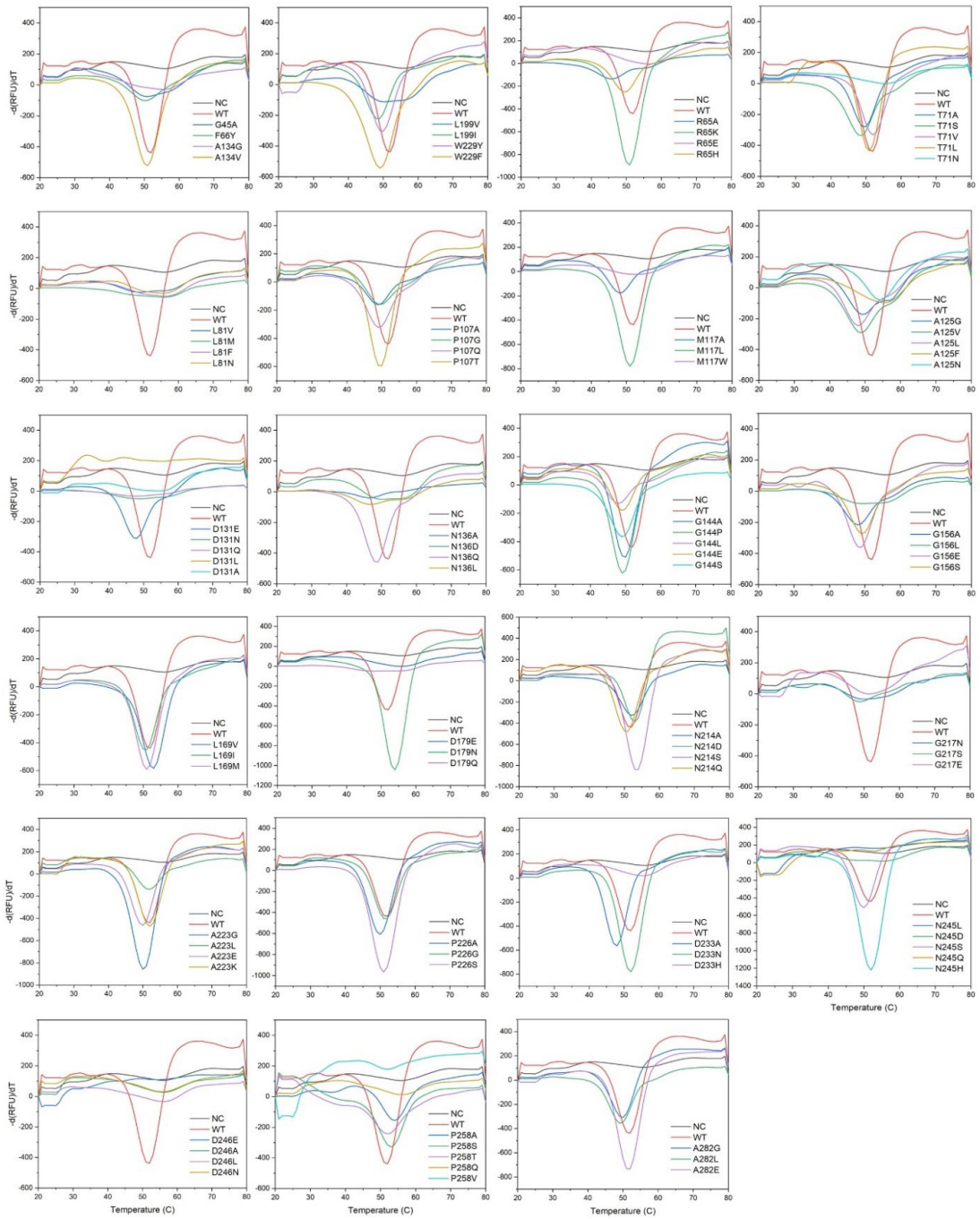

Figure S2.5. Negative derivative of the RFU signal from thermal shift assay for WT and mutant BlaC.

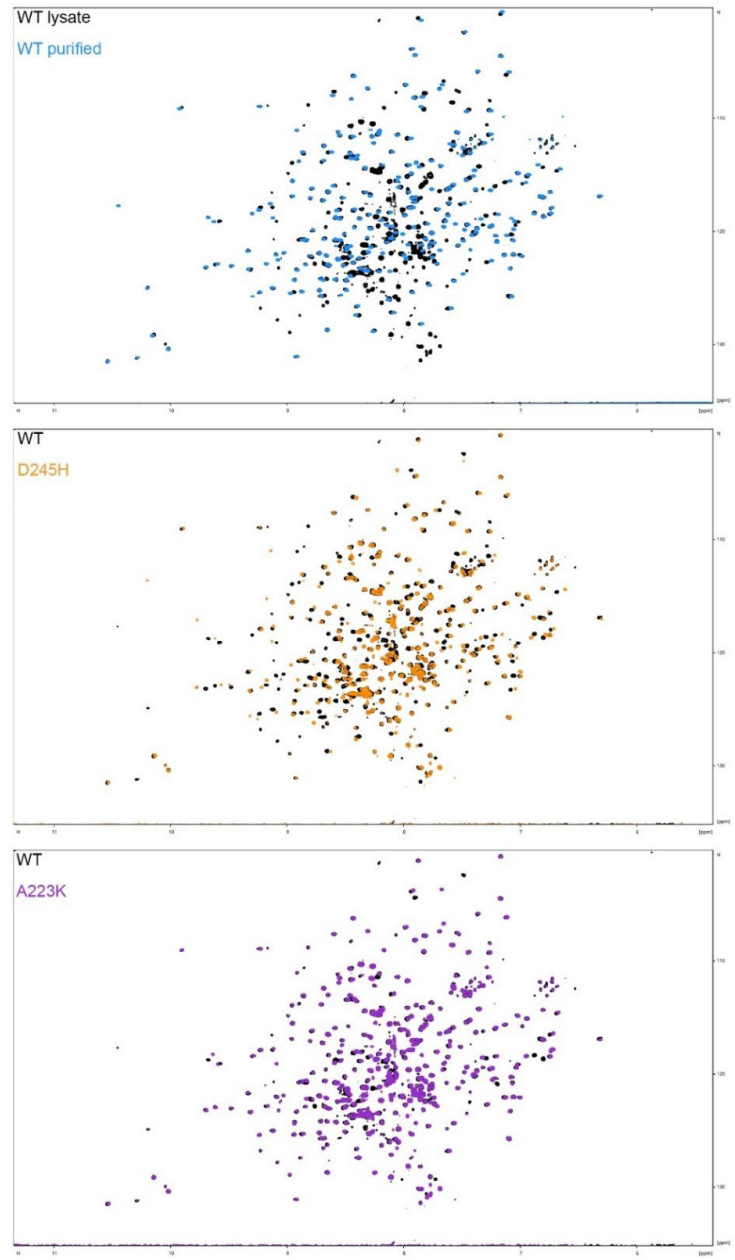

Figure S2.6. Examples of NMR spectra of cell lysates. The upper panel displays overlay of purified wild type in phosphate buffer pH 6.4 (in blue) and the soluble fraction of cell lysate of a culture expressing the wild type blaC gene (in black). The middle and bottom panels display the overlays of the cell lysate spectra of the second-shell mutant D245H (in orange) with wild type BlaC (in black) and of the third-shell mutant A223K (in purple) with wild type BlaC (in black).

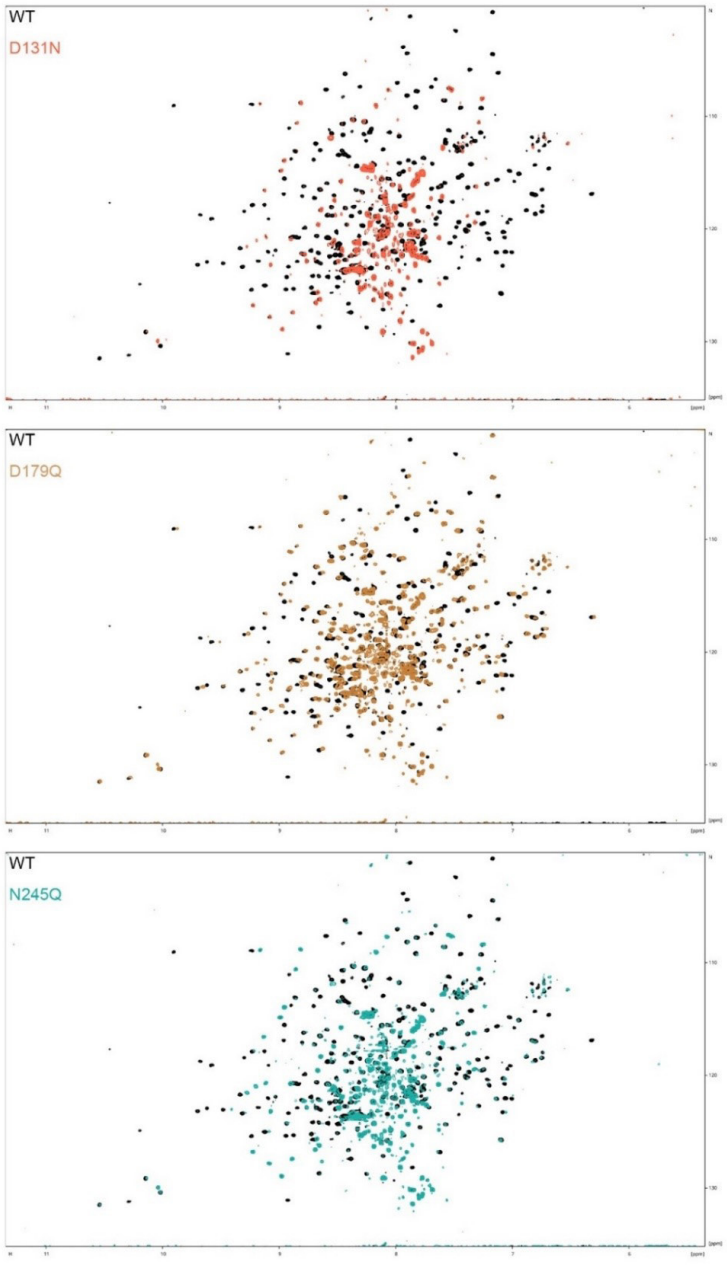

Figure S2.7. Examples of NMR spectra of cell lysates with unfolded protein. The panels display the overlays of spectra of the soluble fraction of cell lysates of mutants D131N (in pink, top), D179Q (in brown, middle) and N245Q (in turquoise, bottom) with that of wild type BlaC (in black).

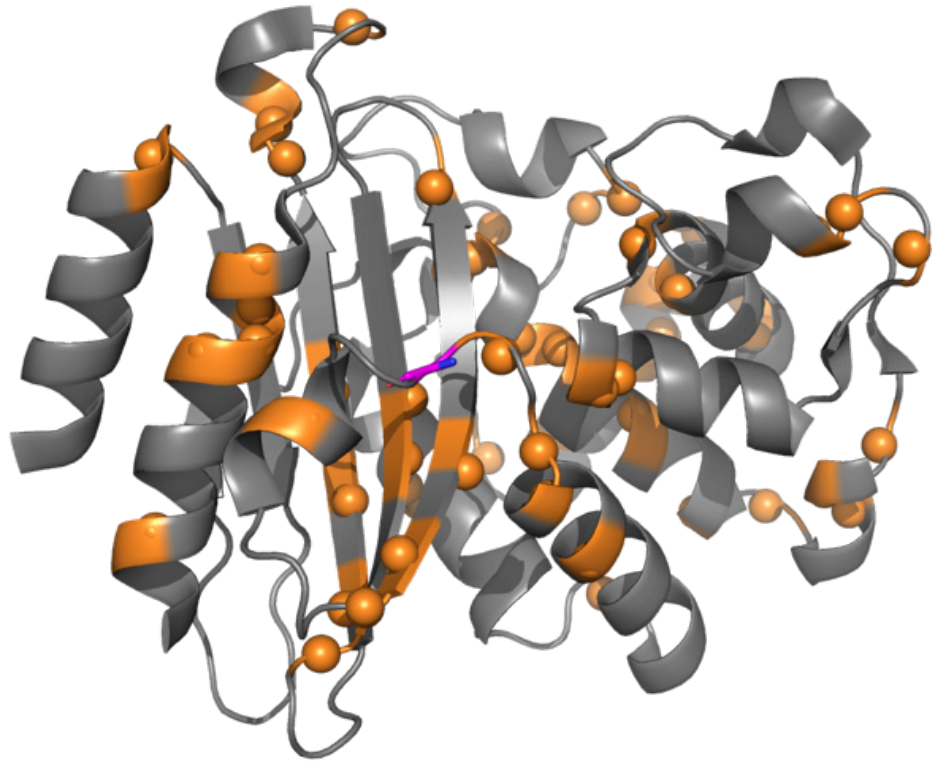

Figure S2.8. Example of the location of nuclei with significant CSP in a second-shell mutant (G217S) located on the protein surface. The mutated residue is shown in magenta sticks.

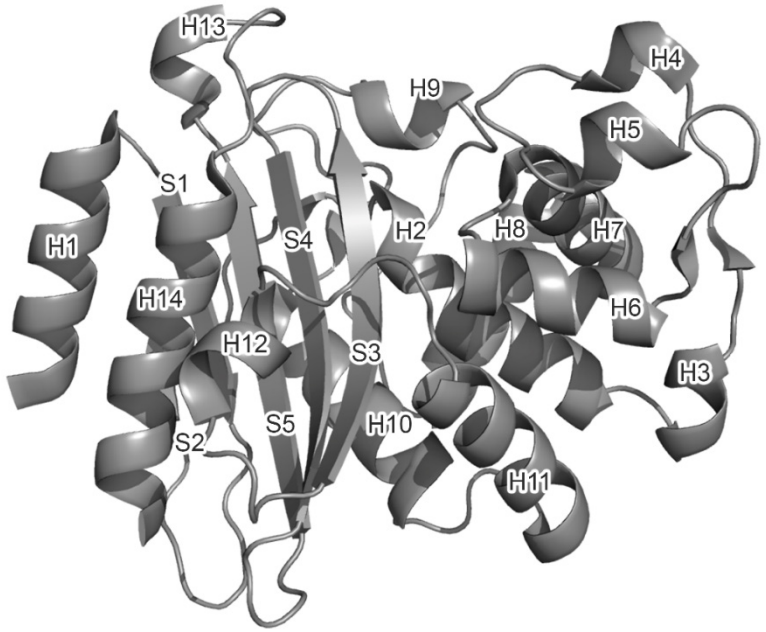

Figure S2.9. BlaC structure (2GDN[101]) with all α-helices and β-strands labeled.

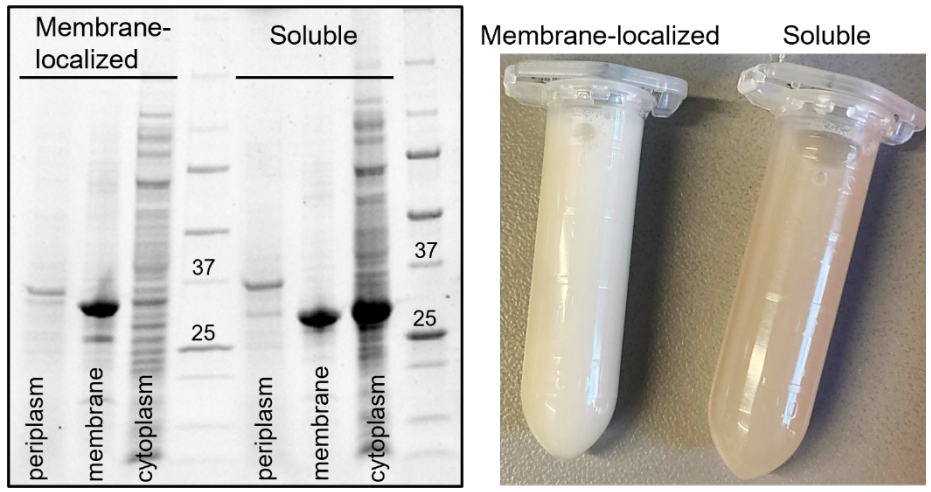

Figure S2.10. Detection of BlaC location after overexpression with and without a signal peptide for membrane localization. Most protein is found in membrane in construct with the signal peptide, which is shown on gel (left) and by the lighter color of the cells (right). Most protein is found in cytoplasm after expression in construct without the signal peptide with some protein detected in the membrane fraction, which can be possibly attributed to inclusion bodies formation due to overexpression (membrane fraction and inclusion bodies were not separated).

Supplementary tables

Table S2.1. BlaC sequence with ConSurf grades and percentages of the most occurring residue per position.

Position (Ambler)	ConSurf Grade	Residue in BlaC	The most occurring residue (%)	Position (Ambler)	ConSurf Grade	Residue in BlaC	The most occurring residue (%)
28	1	Asp	D (29.48)	65	9	Arg	R (96.154)
29	1	Leu	L (40.417)	66	9	Phe	F (99.798)
30	1	Ala	DE (16.242)	67	7	Ala	A (57.895)
31	1	Asp	R (17.812)	68	6	Phe	Y (29.96)
32	1	Arg	E (21.495)	69	6	Cys	C (54.453)
33	5	Phe	F (50.947)	70	9	Ser	S (100)
34	1	Ala	A (44.05)	71	9	Thr	T (98.178)
35	1	Glu	E (24.33)	72	7	Phe	F (71.862)
36	8	Leu	L (88.525)	73	9	Lys	K (100)
37	9	Glu	E (99.187)	74	4	Ala	A (51.215)
38	1	Arg	R (29.268)	75	5	Pro	L (65.182)
39	1	Arg	K (23.984)	76	7	Leu	A (68.016)
40	2	Tyr	F (32.657)	77	6	Val	A (65.385)
41	2	Asp	G (43.205)	78	6	Ala	A (55.466)
42	8	Ala	A (55.263)	79	6	Ala	A (73.684)
43	8	Arg	R (89.879)	80	5	Val	V (60.324)
44	8	Leu	L (92.308)	81	9	Leu	L (99.19)
45	9	Gly	G (100)	82	2	His	Q (21.457)
46	8	Val	V (87.247)	83	4	Gln	R (37.854)
47	5	Tyr	Y (48.583)	86	5	Asn	S (21.837)
48	7	Val	A (69.636)	87	5	Pro	S (25.562)
49	2	Pro	L (38.945)	88	1	Leu	L (20.654)
50	7	Ala	D (76.316)	89	2	Thr	E (33.266)
51	7	Thr	T (85.366)	90	2	His	L (21.053)
52	1	Gly	G (54.268)	91	7	Leu	L (85.83)
53	5	Thr	T (57.317)	92	1	Asp	D (49.798)
54	6	Thr	G (66.667)	93	3	Lys	R (35.223)
55	2	Ala	R (39.676)	94	5	Leu	R (46.154)
56	3	Ala	T (37.652)	95	5	Ile	I (56.275)
57	3	Ile	V (46.356)	96	1	Thr	T (39.676)
59	1	Glu	A (32.186)	97	7	Tyr	Y (82.591)
60	4	Tyr	Y (56.68)	98	1	Thr	T (33.603)
61	8	Arg	R (85.02)	99	1	Ser	K (30.567)
62	3	Ala	A (45.344)	100	1	Asp	D (32.996)
63	4	Asp	D (59.312)	101	7	Asp	D (83.401)
64	8	Glu	E (89.069)	102	7	Ile	L (81.174)

103	6	Arg	V (69.636)
104	1	Ser	T (22.065)
105	4	Ile	Y (64.372)
106	8	Ser	S (77.53)
107	9	Pro	P (98.583)
108	5	Val	V (45.749)
109	8	Ala	T (88.462)
110	6	Gln	E (71.255)
111	4	Gln	K (59.919)
112	7	His	H (81.579)
113	5	Val	V (69.98)
114	1	Gln	D (35.628)
115	1	Thr	T (50.405)
116	8	Gly	G (94.13)
117	9	Met	M (92.51)
118	6	Thr	T (76.316)
119	6	Ile	L (52.227)
120	3	Gly	A (32.794)
121	5	Gln	E (61.538)
122	7	Leu	L (80.567)
123	5	Cys	C (69.636)
124	6	Asp	D (38.259)
125	9	Ala	A (99.798)
126	7	Ala	A (71.862)
127	4	Ile	I (41.498)
128	8	Arg	R (47.773)
129	3	Tyr	Y (63.968)
130	9	Ser	S (100)
131	9	Asp	D (100)
132	9	Gly	N (92.308)
133	8	Thr	T (81.579)
134	9	Ala	A (99.393)
135	6	Ala	A (49.19)
136	9	Asn	N (100)
137	8	Leu	L (87.045)
138	7	Leu	L (76.721)
139	6	Leu	L (61.741)
140	2	Ala	K (24.899)
141	2	Asp	E (23.887)
142	4	Leu	L (62.753)
143	8	Gly	G (95.344)
144	9	Gly	G (98.988)
145	8	Pro	P (94.737)
145A	1	Gly	A (31.377)
145B	1	Gly	G (53.239)
145C	6*	Gly	G (100)
145D	6*	Thr	T (100)
146	6*	Ala	A (100)
147	6*	Ala	A (100)
148	5	Phe	L (42.915)
149	7	Thr	T (55.061)
150	3	Gly	A (44.332)
151	3	Tyr	F (35.02)
152	6	Leu	L (55.668)
153	7	Arg	R (82.794)
154	2	Ser	S (37.247)
155	4	Leu	I (46.964)
156	9	Gly	G (97.773)
157	9	Asp	D (99.19)
158	1	Thr	D (20.445)
159	1	Val	V (50.405)
160	8	Ser	T (63.968)
161	7	Arg	R (67.004)
162	7	Leu	L (53.846)
163	7	Asp	D (75.101)
164	9	Ala	R (92.713)
165	1	Glu	W (23.887)
166	9	Glu	E (99.595)
167	6	Pro	P (61.134)
168	3	Glu	E (51.417)
169	9	Leu	L (94.939)
170	9	Asn	N (87.449)
171	4	Arg	E (37.045)
172	7	Asp	A (61.943)
173	1	Pro	I (26.978)
174	8	Pro	P (94.523)
175	6	Gly	G (87.652)
176	8	Asp	D (81.579)
177	1	Glu	P (39.474)
178	8	Arg	R (88.057)
179	9	Asp	D (99.798)
180	9	Thr	T (99.595)
181	8	Thr	T (58.3)
182	8	Thr	T (89.271)
183	8	Pro	P (92.308)
184	2	His	R (35.83)
185	9	Ala	A (94.332)
186	7	Ile	M (48.178)
187	6	Ala	A (71.66)
188	1	Leu	A (24.089)
189	5	Val	S (41.296)
190	6	Leu	L (74.291)
191	4	Gln	R (38.866)
192	3	Gln	A (39.271)
193	4	Leu	L (47.976)
194	3	Val	V (35.425)
195	6	Leu	L (75.709)
196	6	Gly	G (87.247)
197	3	Asn	D (62.955)
198	3	Ala	A (57.49)
199	9	Leu	L (100)
200	1	Pro	P (34.615)
201	1	Pro	A (24.494)
202	1	Asp	A (29.352)
203	7	Lys	K (31.377)
204	8	Arg	R (90.283)
205	1	Ala	A (30.972)
206	4	Leu	Q (39.676)
207	8	Leu	L (91.093)
208	1	Thr	T (35.425)
209	1	Asp	D (41.7)
210	8	Trp	W (91.903)
211	6	Met	L (62.955)
212	2	Ala	K (29.96)
213	5	Arg	G (42.105)
214	9	Asn	N (92.915)
215	6	Thr	T (63.968)
216	9	Thr	T (94.737)
217	9	Gly	G (94.332)
218	2	Ala	D (57.085)
219	1	Lys	A (23.732)
220	8	Arg	R (45.842)
221	8	Ile	I (72.672)
222	8	Arg	R (92.713)
223	9	Ala	A (96.964)
224	8	Gly	G (92.51)
225	6	Phe	V (50.405)
226	9	Pro	P (98.381)
227	1	Ala	A (26.735)
228	1	Asp	G (57.287)
229	9	Trp	W (97.976)
230	1	Lys	V (19.028)
231	8	Val	V (88.664)
232	6	Ile	G (66.802)
233	9	Asp	D (96.761)
234	9	Lys	K (98.178)
235	9	Thr	T (85.223)
236	9	Gly	G (100)
237	5	Thr	T (35.425)
238	7	Gly	G (87.652)
240	2	Asp	D (31.984)
241	6	Tyr	Y (77.393)
242	8	Gly	G (95.142)
243	7	Arg	T (67.206)
244	6	Ala	R (41.903)
245	9	Asn	N (92.51)
246	9	Asp	D (96.356)
247	7	Ile	I (68.826)
248	8	Ala	A (74.494)
249	6	Val	V (67.004)
250	3	Val	V (37.652)
251	7	Trp	W (93.725)
252	8	Ser	P (85.425)
254	4	Pro	P (74.696)
255	1	Thr	D (22.267)
256	2	Gly	R (42.105)
257	4	Val	A (49.393)
258	9	Pro	P (97.976)
259	4	Tyr	I (56.68)
260	6	Val	V (65.385)
261	4	Val	L (40.688)
262	6	Ala	A (71.197)
263	5	Val	V (51.619)
264	7	Met	Y (42.51)

Table S2.2 Data from *in vivo* and *in vitro* experiments. The orange and purple color represent the second and the third shell respectively. Color coding for the experimental data is as follows: green – (almost) as good as wild type, yellow – somewhat worse than wild type, red – considerably worse than wild type. ND – not detected.

Conserved residue	Mutations	MIC (μg mL^{-1})		Protein on gel		Nitrocefin reaction in cells		T_m	NMR	V_{max}/K_M in cell lysate (10^{-2} min^{-1})	CD signal at 222 nm relative to WT	Relative activity
		Ampicillin	Carbenicillin	Lysate	Supernatant (Relative to WT)	Whole cell (within 10 min)	Supernatant (first minutes)					
	WT	100	1000		1			52	Folded protein	8.9 ± 0.3	1	1
	NC	3	20		ND			ND	Only *E. coli* protein peaks	ND	Used as background	ND
Glu37	E37A	3	20		0.01							
	E37L	3	20		ND							
	E37D	3	20		ND							
	E37Q	3	20		ND							
Gly45	G45A	3	20		0.18			51	Low signal	3.5 ± 1.8	1.0	0.4
	G45V	3	20		ND							
	G45S	3	20		0.12							
Arg65	R65A	3	20		0.67			46	Folded protein in low quantity	2.9 ± 0.9	0.4	0.7
	R65K	40	200		0.90			51	Folded protein	7.9 ± 0.7	2.0	0.4
	R65L	3	20		ND							
	R65E	3	20		1.04			ND	Low signal	ND	1.1	ND
	R65H	5	50		1.76			50	Folded protein	9.3 ± 0.9	1.0	1.1
Phe66	F66Y	3	20		0.99			50	Low signal	6.5 ± 1.2	0.9	0.8
	F66E	3	20		0.17							
	F66L	3	20		0.07							
	F66K	3	20		0.05							
Thr71	T71A	30	200		0.42			50	Folded protein	9.0 ± 0.2	0.9	1.1
	T71S	3	20		0.51			49	Folded protein in low quantity	8.2 ± 0.7	1.5	0.6
	T71V	80	1000		1.06			52	Folded protein	10.0 ± 0.2	1.0	1.1
	T71L	80	1000		0.92			51	Folded protein	15.6 ± 0.3	3.1	0.6
	T71N	3	20		0.43			ND	Folded and unfolded protein	ND	0.5	ND

Leu81	L81A	3	20		0.09							
	L81V	3	20		0.37			49	Folded and unfolded protein	ND	0.4	ND
	L81M	30	200		0.05			ND	Unfolded protein	ND	ND	ND
	L81F	3	20		0.28			ND	Low signal	ND	ND	ND
	L81W	3	20		0.07							
	L81N	3	20		0.50			ND	Low signal	ND	ND	ND
Pro107	P107A	10	200		0.71			49	Folded protein	5.8 ± 0.2	0.8	0.8
	P107G	10	200		0.32			50	Low signal	3.4 ± 0.1	0.9	0.4
	P107V	10	200		0.03							
	P107Q	10	200		0.45			49	Folded protein	2.7 ± 0.05	1.4	0.2
	P107T	10	200		0.35			50	Folded protein	2.9 ± 0.2	1.0	0.3
Met117	M117A	3	20		0.49			48	Folded protein in low quantity	2.4 ± 0.3	1.7	0.2
	M117L	80	1000		1.18			51	Folded protein	14.0 ± 1.9	2.1	0.7
	M117E	3	20		ND							
	M117F	3	20		ND							
	M117W	3	20		0.38			ND	Low signal	ND	0.7	ND
Ala125	A125G	3	20		0.40			49	Low signal	3.7 ± 0.6	0.9	0.4
	A125V	3	20		1.33			49	Folded protein	ND	1.3	ND
	A125L	3	20		1.17			48	Folded protein	4.7 ± 1.2	1.1	0.5
	A125F	3	20		0.41			ND	Low signal	ND	0.2	ND
	A125N	3	20		0.73			ND	Folded and unfolded protein	3.9 ± 0.8	0.6	0.7
Asp131	D131E	3	20		0.56			48	Folded and unfolded protein	ND	1.0	ND
	D131N	3	20		0.10			49	Folded and unfolded protein	ND	ND	ND
	D131Q	3	20		0.04			48	Low signal	ND	ND	ND
	D131L	3	20		0.05			ND	Low signal	ND	ND	ND
	D131A	3	20		ND			ND	Low signal	ND	ND	ND
Ala134	A134G	3	20		0.62			ND	Folded protein in low quantity	ND	0.7	ND
	A134V	3	20		1.15			51	Folded protein	ND	2.0	ND
	A134L	3	20		0.12							
	A134F	3	20		0.12							
	A134N	3	20		ND							
Asn136	N136A	5	200		1.44			47	Folded protein	ND	ND	ND
	N136D	3	20		1.92			50	Folded and unfolded protein	ND	0.9	ND
	N136Q	3	100		2.10			49	Folded protein	2.3 ± 0.3	0.7	0.4
	N136L	3	20		1.48			47	Low signal	ND	ND	ND
Gly144	G144A	5	200		1.35			50	Folded protein	13.0 ± 2.0	2.2	0.7
	G144P	5	100		0.98			49	Folded protein	12.0 ± 0.7	2.1	0.6
	G144L	3	20		1.24			48	Folded protein	5.0 ± 1.4	1.5	0.4
	G144E	15	200		1.90			49	Folded protein	12.0 ± 3.0	1.8	0.7
	G144S	15	200		0.73			49	Folded protein	10.0 ± 1.3	1.4	0.8
Gly156	G156A	3	20		1.26			48	Folded protein	6.7 ± 1.1	1.0	0.7
	G156P	3	20		ND							
	G156L	3	20		0.67			49	Low signal	ND	0.3	ND
	G156E	3	20		1.50			49	Folded protein	10.0 ± 2.4	1.7	0.7
	G156S	3	20		1.25			49	Folded protein	8.4 ± 1.0	1.1	0.9
Asp157	D157A	3	20		ND							
	D157L	3	20		ND							
	D157E	3	20		ND							
	D157N	3	20		ND							
Leu169	L169A	5	20		ND							
	L169V	40	200		0.68			53	Folded and unfolded protein	10.0 ± 2.0	1.9	0.6
	L169F	3	20		0.14							
	L169W	3	20		ND							
	L169E	3	20		ND							
	L169I	40	200		0.16			51	Folded protein	6.0 ± 2.0	1.5	0.4
	L169M	30	200		0.47			51	Folded protein	ND	2.2	ND
Asp179	D179E	5	20		0.14			ND	Folded and unfolded protein	ND	0.5	ND
	D179N	100	2000		1.41			54	Folded protein	14.0 ± 1.1	3.5	0.4
	D179Q	5	20		0.36			48	Folded and unfolded protein	ND	ND	ND
	D179A	10	200		0.16							
	D179L	5	20		0.12							
	D179G	5	100		ND							
Thr180	T180S	40	500		0.17							
	T180A	3	20		ND							
	T180I	3	20		0.13							
	T180V	3	20		0.16							
	T180N	3	20		ND							
Ala185	A185T	3	20		0.10							
	A185L	3	20		ND							
	A185F	3	20		ND							
	A185E	5	100		0.12							

Leu199	L199V	3	20		0.61			50	Folded and unfolded protein	ND	ND	ND
	L199I	3	50		0.43			48	Folded protein	10.0 ± 0.6	2.1	0.5
	L199N	3	20		ND							
	L199Q	3	20		ND							
	L199W	3	20		ND							
	L199A	3	20		ND							
Asn214	N214A	100	1000		0.63			52	Folded protein	14.0 ± 1.5	0.8	2.0
	N214L	3	20		ND							
	N214D	3	20		0.55			53	Folded protein	ND	2.3	ND
	N214S	100	1000		0.93			54	Folded protein	17.0 ± 2.9	1.9	1.0
	N214Q	30	200		0.59			51	Folded protein	ND	2.0	ND
Gly217	G217A	3	20		ND							
	G217T	3	20		ND							
	G217N	3	20		0.23			50	Folded protein	ND	0.8	ND
	G217S	3	20		0.39			49	Folded protein	ND	1.0	ND
	G217E	3	20		0.42			51	Folded protein	ND	0.6	ND
Ala223	A223G	30	500		0.29			50	Folded protein	8.5 ± 1.1	2.0	0.5
	A223L	30	500		0.41			52	Folded protein	5.1 ± 0.5	0.3	1.7
	A223E	30	500		0.45			50	Folded protein	10.0 ± 1.4	2.9	0.4
	A223K	60	1000		0.68			52	Folded protein	11.0 ± 1.4	1.3	0.9
Pro226	P226A	10	200		0.85			50	Folded protein	11.0 ± 0.8	2.5	0.5
	P226G	30	500		0.57			52	Folded protein	9.8 ± 0.5	1.3	0.8
	P226Y	3	20		ND							
	P226S	10	200		1.01			51	Folded protein	9.7 ± 0.6	2.2	0.5
Trp229	W229A	3	20		ND							
	W229Y	3	50		0.13			50	Folded protein in low quantity	8.0 ± 0.3	0.9	1
	W229F	3	50		0.12			49	Folded protein in low quantity	4.3 ± 0.5	1.5	0.3
	W229L	3	20		ND							
	W229Q	3	20		ND							
Asp233	D233A	10	200		0.16			48	Folded protein	ND	2.2	ND
	D233L	3	20		ND							
	D233E	3	20		ND							
	D233N	100	500		0.71			52	Folded protein	9.3 ± 2.4	2.4	0.4
	D233H	10	200		ND			ND	Folded protein in low quantity	ND	0.7	ND

Asn245	N245L	3	20		ND			ND	Unfolded protein	ND	ND	ND
	N245D	3	20		0.16			ND	Folded protein	ND	0.8	ND
	N245S	15	200		0.16			50	Folded protein	21.0 ± 1.0	2.4	1.0
	N245Q	3	20		0.10			45	Folded and unfolded protein	7.8 ± 0.6	0.7	1.3
	N245H	100	2000		0.93			52	Folded protein	16.0 ± 0.5	3.6	0.5
Asp246	D246E	3	20		0.18			ND	Folded protein in low quantity	ND	0.2	ND
	D246A	3	20		0.16			ND	Folded protein in low quantity	ND	0.6	ND
	D246L	3	20		0.11			ND	Low signal	ND	0.5	ND
	D246N	3	20		0.20			ND	Folded protein in low quantity	ND	0.9	ND
Ala248	A248V	3	20		ND							
	A248L	5	50		ND							
	A248I	3	20		ND							
Pro258	P258A	80	1000		0.55			54	Folded protein in low quantity	5.5 ± 1.3	0.4	1.7
	P258S	80	1000		0.70			53	Folded protein	11.0 ± 0.1	1.0	1.3
	P258T	10	200		0.28			52	Low signal	8.7 ± 2.0	0.7	1.5
	P258Q	15	200		0.22			ND	Low signal	ND	0.3	ND
	P258V	5	50		0.27			ND	Low signal	3.4 ± 1.1	0.7	0.6
Ala282	A282G	30	500		0.23			50	Folded protein in low quantity	9.7 ± 0.6	1.5	0.7
	A282L	5	50		0.17			49	Folded protein in low quantity	ND	0.9	ND
	A282E	80	1000		0.72			52	Folded protein	11.0 ± 0.5	3.2	0.4

Table S2.3. Primers used to introduce mutations to the *blaC* gene. Only forward primer is presented.

Mutation	Forward primer 5'-3'	Mutation	Forward primer 5'-3'
E37A	G GCA GAT CGT TTT GCA GAA CTG GCG CGT CGT TAT GAT GCA CG	L169A	GAT GCA GAA GAA CCG GAA GCG AAT CGT GAT CCG CCT GGT
E37D	G GCA GAT CGT TTT GCA GAA CTG GAT CGT CGT TAT GAT GCA CG	L169V	GAT GCA GAA GAA CCG GAA GTG AAT CGT GAT CCG CCT GGT
E37L	G GCA GAT CGT TTT GCA GAA CTG CTG CGT CGT TAT GAT GCA CG	L169F	GAT GCA GAA GAA CCG GAA TTC AAT CGT GAT CCG CCT GGT
E37Q	G GCA GAT CGT TTT GCA GAA CTG CAG CGT CGT TAT GAT GCA CG	D179E	G CCT GGT GAT GAA CGT GAA ACC ACC ACA CCG CAT GCC ATT
G46A	CGT CGT TAT GAT GCA CGT CTG GCG GTT TAT GTT CCG GCA ACC	D179N	G CCT GGT GAT GAA CGT AAT ACC ACC ACA CCG CAT GCC ATT
G46V	CGT CGT TAT GAT GCA CGT CTG GTG GTT TAT GTT CCG GCA ACC	D179Q	G CCT GGT GAT GAA CGT CAG ACC ACC ACA CCG CAT GCC ATT
G46S	CGT CGT TAT GAT GCA CGT CTG TCT GTT TAT GTT CCG GCA ACC	D179A	G CCT GGT GAT GAA CGT GCA ACC ACC ACA CCG CAT GCC ATT
R65A	GCA ATT GAA TAT CGT GCA GAT GAA GCG TTT GCA TTT TGC AGC ACC	D179L	G CCT GGT GAT GAA CGT CTT ACC ACC ACA CCG CAT GCC ATT
R65K	GCA ATT GAA TAT CGT GCA GAT GAA AAA TTT GCA TTT TGC AGC ACC	D179G	G CCT GGT GAT GAA CGT GGT ACC ACC ACA CCG CAT GCC ATT
R65L	GCA ATT GAA TAT CGT GCA GAT GAA CTG TTT GCA TTT TGC AGC ACC	T180S	G CCT GGT GAT GAA CGT GAT AGC ACC ACA CCG CAT GCC ATT
R65E	GCA ATT GAA TAT CGT GCA GAT GAA GAA TTT GCA TTT TGC AGC ACC	T180A	G CCT GGT GAT GAA CGT GAT GCA ACC ACA CCG CAT GCC ATT
R65H	GCA ATT GAA TAT CGT GCA GAT GAA CAT TTT GCA TTT TGC AGC ACC	T180I	G CCT GGT GAT GAA CGT GAT ATT ACC ACA CCG CAT GCC ATT
F66Y	CGT GCA GAT GAA CGT TAC GCA TTT TGC AGC ACC TTT AAA GCA CCG C	T180V	G CCT GGT GAT GAA CGT GAT GTT ACC ACA CCG CAT GCC ATT
F66E	CGT GCA GAT GAA CGT GAG GCA TTT TGC AGC ACC TTT AAA GCA CC	T180N	G CCT GGT GAT GAA CGT GAT AAT ACC ACA CCG CAT GCC ATT
F66L	CGT GCA GAT GAA CGT CTC GCA TTT TGC AGC ACC TTT AAA GCA CC	A185T	GAT ACC ACC ACA CCG CAT ACC ATT GCA CTG GTT CTG CAG
F66K	CGT GCA GAT GAA CGT AAG GCA TTT TGC AGC ACC TTT AAA GCA CCG C	A185L	GAT ACC ACC ACA CCG CAT CTG ATT GCA CTG GTT CTG CAG
T71A	CGT TTT GCA TTT TGC AGC GCG TTT AAA GCA CCG CTG GTT GCA GCC	A185F	GAT ACC ACC ACA CCG CAT TTC ATT GCA CTG GTT CTG CAG
T71S	CGT TTT GCA TTT TGC AGC TCT TTT AAA GCA CCG CTG GTT GCA GCC	A185E	GAT ACC ACC ACA CCG CAT GAA ATT GCA CTG GTT CTG CAG
T71V	CGT TTT GCA TTT TGC AGC GTG TTT AAA GCA CCG CTG GTT GCA GCC	L199V	CTG GTT CTG GGT AAT GCA GTG CCT CCG GAT AAA CGT GCA
T71L	CGT TTT GCA TTT TGC AGC CTG TTT AAA GCA CCG CTG GTT GCA GCC	L199I	CTG GTT CTG GGT AAT GCA ATC CCT CCG GAT AAA CGT GCA
T71N	CGT TTT GCA TTT TGC AGC AAC TTT AAA GCA CCG CTG GTT GCA GCC	L199N	CTG GTT CTG GGT AAT GCA AAC CCT CCG GAT AAA CGT GCA
L81A	CTG GTT GCA GCC GTT GCG CAT CAG AAT CCG CTG ACC CAT CTG	L199Q	CTG GTT CTG GGT AAT GCA CAG CCT CCG GAT AAA CGT GCA
L81V	CTG GTT GCA GCC GTT GTG CAT CAG AAT CCG CTG ACC CAT CTG	L199W	CTG GTT CTG GGT AAT GCA TGG CCT CCG GAT AAA CGT GCA
L81M	CTG GTT GCA GCC GTT ATG CAT CAG AAT CCG CTG ACC CAT CTG	L199A	CTG GTT CTG GGT AAT GCA GCG CCT CCG GAT AAA CGT GCA
L81F	CTG GTT GCA GCC GTT TTT CAT CAG AAT CCG CTG ACC CAT CTG	N214A	CTG ACC GAT TGG ATG GCA CGT GCG ACC ACC GGT GCC AAA CGT
L81W	CTG GTT GCA GCC GTT TGG CAT CAG AAT CCG CTG ACC CAT CTG	N214L	CTG ACC GAT TGG ATG GCA CGT CTG ACC ACC GGT GCC AAA CGT
L81N	CTG GTT GCA GCC GTT AAC CAT CAG AAT CCG CTG ACC CAT CTG	N214D	CTG ACC GAT TGG ATG GCA CGT GAT ACC ACC GGT GCC AAA CGT
P107A	GAT ATC CGT AGC ATT AGT GCG GTT GCA CAG CAG CAT G	N214S	CTG ACC GAT TGG ATG GCA CGT TCT ACC ACC GGT GCC AAA CGT
P107G	GAT ATC CGT AGC ATT AGT GGC GTT GCA CAG CAG CAT G	N214Q	CTG ACC GAT TGG ATG GCA CGT CAG ACC ACC GGT GCC AAA CGT
P107V	GAT ATC CGT AGC ATT AGT GTG GTT GCA CAG CAG CAT G	G217A	CTG ACC GAT TGG ATG GCA CGT AAT ACC ACC GCG GCC AAA CGT
P107Q	GAT ATC CGT AGC ATT AGT CAG GTT GCA CAG CAG CAT G	G217T	CTG ACC GAT TGG ATG GCA CGT AAT ACC ACC ACC GCC AAA CGT
P107T	GAT ATC CGT AGC ATT AGT ACC GTT GCA CAG CAG CAT G	G217N	CTG ACC GAT TGG ATG GCA CGT AAT ACC ACC AAC GCC AAA CGT
M117A	CAG CAG CAT GTT CAG ACC GGT GCG ACC ATT GGT CAG CTG	G217S	CTG ACC GAT TGG ATG GCA CGT AAT ACC ACC TCT GCC AAA CGT
M117L	CAG CAG CAT GTT CAG ACC GGT CTG ACC ATT GGT CAG CTG	G217E	CTG ACC GAT TGG ATG GCA CGT AAT ACC ACC GAA GCC AAA CGT
M117E	CAG CAG CAT GTT CAG ACC GGT GAA ACC ATT GGT CAG CTG	A223G	ACC ACC GGT GCC AAA CGT ATT CGT GGC GGT TTT CCG GCA GAT
M117F	CAG CAG CAT GTT CAG ACC GGT TTC ACC ATT GGT CAG CTG	A223L	ACC ACC GGT GCC AAA CGT ATT CGT CTG GGT TTT CCG GCA GAT
M117W	CAG CAG CAT GTT CAG ACC GGT TGG ACC ATT GGT CAG CTG	A223E	ACC ACC GGT GCC AAA CGT ATT CGT GAA GGT TTT CCG GCA GAT
A125G	GGT ATG ACC ATT GGT CAG CTG TGT GAT GGC GCA ATT CGT	A223K	ACC ACC GGT GCC AAA CGT ATT CGT AAA GGT TTT CCG GCA GAT
A125V	GGT ATG ACC ATT GGT CAG CTG TGT GAT GTG GCA ATT CGT	P226A	CGT GCA GGT TTT GCG GCA GAT TGG AAA GTT ATT GAT AAA ACC
A125L	GGT ATG ACC ATT GGT CAG CTG TGT GAT CTG GCA ATT CGT	P226G	CGT GCA GGT TTT GGC GCA GAT TGG AAA GTT ATT GAT AAA ACC
A125F	GGT ATG ACC ATT GGT CAG CTG TGT GAT TTC GCA ATT CGT	P226Y	CGT GCA GGT TTT TAC GCA GAT TGG AAA GTT ATT GAT AAA ACC
A125N	GGT ATG ACC ATT GGT CAG CTG TGT GAT AAC GCA ATT CGT	P226S	CGT GCA GGT TTT TCT GCA GAT TGG AAA GTT ATT GAT AAA ACC
D131E	GCA ATT CGT TAT AGT GAA GGC ACC GCA GCC AAT CTG CTG CTG	W229A	CGT GCA GGT TTT CCG GCA GAT GCG AAA GTT ATT GAT AAA ACC
D131N	GCA ATT CGT TAT AGT AAC GGC ACC GCA GCC AAT CTG CTG CTG	W229Y	CGT GCA GGT TTT CCG GCA GAT TAT AAA GTT ATT GAT AAA ACC
D131Q	GCA ATT CGT TAT AGT CAG GGC ACC GCA GCC AAT CTG CTG CTG	W229F	CGT GCA GGT TTT CCG GCA GAT TTC AAA GTT ATT GAT AAA ACC
D131L	GCA ATT CGT TAT AGT CTG GGC ACC GCA GCC AAT CTG CTG CTG	W229L	CGT GCA GGT TTT CCG GCA GAT CTG AAA GTT ATT GAT AAA ACC
D131A	GCA ATT CGT TAT AGT GCG GGC ACC GCA GCC AAT CTG CTG CTG	W229Q	CGT GCA GGT TTT CCG GCA GAT CAG AAA GTT ATT GAT AAA ACC
A134G	GCA ATT CGT TAT AGT GAT GGC ACC GGC GCC AAT CTG CTG CTG	D233A	CCG GCA GAT TGG AAA GTT ATT GCT AAA ACC GGT ACG GGT G
A134V	GCA ATT CGT TAT AGT GAT GGC ACC GTG GCC AAT CTG CTG CTG	D233E	CCG GCA GAT TGG AAA GTT ATT GAA AAA ACC GGT ACG GGT G
A134L	GCA ATT CGT TAT AGT GAT GGC ACC CTG GCC AAT CTG CTG CTG	D233H	CCG GCA GAT TGG AAA GTT ATT CAT AAA ACC GGT ACG GGT G

A134F	GCA ATT CGT TAT AGT GAT GGC ACC TTC GCC AAT CTG CTG CTG
A134N	GCA ATT CGT TAT AGT GAT GGC ACC AAC GCC AAT CTG CTG CTG
N136A	CGT TAT AGT GAT GGC ACC GCA GCC GCG CTG CTG CTG GCC
N136D	CGT TAT AGT GAT GGC ACC GCA GCC GAT CTG CTG CTG GCC
N136Q	CGT TAT AGT GAT GGC ACC GCA GCC CAG CTG CTG CTG GCC
N136L	CGT TAT AGT GAT GGC ACC GCA GCC CTG CTG CTG CTG GCC
G144A	CTG GCC GAT CTG GGT GCG CCG GGT GGT GGT ACA GCA GCC TTT
G144P	CTG GCC GAT CTG GGT CCG CCG GGT GGT GGT ACA GCA GCC TTT
G144L	CTG GCC GAT CTG GGT CTG CCG GGT GGT GGT ACA GCA GCC TTT
G144E	CTG GCC GAT CTG GGT GAA CCG GGT GGT GGT ACA GCA GCC TTT
G144S	CTG GCC GAT CTG GGT TCT CCG GGT GGT GGT ACA GCA GCC TTT
G156A	GGT TAT CTG CGT AGC CTG GCG GAT ACC GTT AGC CGT CTG GAT
G156P	GGT TAT CTG CGT AGC CTG CCG GAT ACC GTT AGC CGT CTG GAT
G156L	GGT TAT CTG CGT AGC CTG CTG GAT ACC GTT AGC CGT CTG GAT
G156E	GGT TAT CTG CGT AGC CTG GAA GAT ACC GTT AGC CGT CTG GAT
G156S	GGT TAT CTG CGT AGC CTG TCT GAT ACC GTT AGC CGT CTG GAT
D157A	GGT TAT CTG CGT AGC CTG GGT GCG ACC GTT AGC CGT CTG GAT
D157L	GGT TAT CTG CGT AGC CTG GGT CTG ACC GTT AGC CGT CTG GAT
D157E	GGT TAT CTG CGT AGC CTG GGT GAA ACC GTT AGC CGT CTG GAT
D157N	GGT TAT CTG CGT AGC CTG GGT AAC ACC GTT AGC CGT CTG GAT
L169W	GAT GCA GAA GAA CCG GAA TGG AAT CGT GAT CCG CCT GGT
L169E	GAT GCA GAA GAA CCG GAA GAA AAT CGT GAT CCG CCT GGT
L169I	GAT GCA GAA GAA CCG GAA ATC AAT CGT GAT CCG CCT GGT
L169M	GAT GCA GAA GAA CCG GAA ATG AAT CGT GAT CCG CCT GGT
D233L	CCG GCA GAT TGG AAA GTT ATT CTG AAA ACC GGT ACG GGT G
D233N	CCG GCA GAT TGG AAA GTT ATT AAC AAA ACC GGT ACG GGT G
N245A	ACG GGT GAT TAT GGT CGT GCA GCG GAT ATT GCA GTT GTT TGG
N245L	ACG GGT GAT TAT GGT CGT GCA CTG GAT ATT GCA GTT GTT TGG
N245D	ACG GGT GAT TAT GGT CGT GCA GAT GAT ATT GCA GTT GTT TGG
N245S	ACG GGT GAT TAT GGT CGT GCA TCT GAT ATT GCA GTT GTT TGG
N245Q	ACG GGT GAT TAT GGT CGT GCA CAG GAT ATT GCA GTT GTT TGG
N245H	ACG GGT GAT TAT GGT CGT GCA CAT GAT ATT GCA GTT GTT TGG
D246A	GGT GAT TAT GGT CGT GCA AAT GCT ATT GCA GTT GTT TGG AGC
D246E	GGT GAT TAT GGT CGT GCA AAT GAA ATT GCA GTT GTT TGG AGC
D246L	GGT GAT TAT GGT CGT GCA AAT CTG ATT GCA GTT GTT TGG AGC
D246N	GGT GAT TAT GGT CGT GCA AAT AAC ATT GCA GTT GTT TGG AGC
A248V	GGT CGT GCA AAT GAT ATT GTG GTT GTT TGG AGC CCG ACC
A248L	GGT CGT GCA AAT GAT ATT CTG GTT GTT TGG AGC CCG ACC
A248I	GGT CGT GCA AAT GAT ATT ATC GTT GTT TGG AGC CCG ACC
P258A	GTT TGG AGC CCG ACC GGT GTT GCG TAT GTT GTT GCA GTT ATG
P258S	GTT TGG AGC CCG ACC GGT GTT TCC TAT GTT GTT GCA GTT ATG
P258T	GTT TGG AGC CCG ACC GGT GTT ACC TAT GTT GTT GCA GTT ATG
P258Q	GTT TGG AGC CCG ACC GGT GTT CAG TAT GTT GTT GCA GTT ATG
P258V	GTT TGG AGC CCG ACC GGT GTT GTG TAT GTT GTT GCA GTT ATG
A282G	CGT GAA GCA CTG CTG GGC GAA GCA GCA ACC TGT GTT GCC
A282L	CGT GAA GCA CTG CTG CTG GAA GCA GCA ACC TGT GTT GCC
A282E	CGT GAA GCA CTG CTG GAA GAA GCA GCA ACC TGT GTT GCC

Table S2.4. M9 medium composition per liter.

KH_2PO_4	13.0 g
K_2HPO_4	10.0 g
Na_2HPO_4	9.0 g
Na_2SO_4	2.4 g
$^{15}NH_4Cl$	0.3g
Glucose	4.0 g
Thiamine	30 mg
$MgCl_2$	2 g
Biotin	1 mg
Choline chloride	1 mg
Folic acid	1 mg
Niacinamide	1 mg
D-pantothenate	1 mg
Pyridoxal	1 mg
Riboflavin	0.1 mg
$FeSO_4*7H_2O$	10 mg
$CaCl_2*2H_2O$	60 mg
$MnCl_2*4H_2O$	12 mg
$CoCl_2*6H_2O$	8 mg
$ZnSO_4*7H_2O$	7 mg
$CuCl_2*2H_2O$	3 mg
H_3BO_3	0.2 mg
EDTA	50 mg