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Evolutionary adaptability of β -lactamase: a study of inhibitor susceptibility in various model systems

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General introduction

... we know what we are, but not what we may be.

- William Shakespeare, Hamlet



Protein evolution

Proteins are a product of evolution. Millions of years of small and gradual changes have resulted in proteins that are specialized in their function. This, however, is not where evolution stops. Constant wars between species and changes in their physical environment cause organisms to evolve and adapt. Mutations in the genetic material result in alterations in proteins and ultimately in phenotype. Selection then leads to the survival and propagation of the organisms with the fittest phenotype.

The fitness landscape of a protein is not simply a mountain with the protein being at the “top” of its evolutionary pathway but looks more like a mountain range. Changing selection pressures change the landscape, making some local optima less favorable and others more. However, going from one top to another requires a path through valleys of reduced stability or functionality. Protein fitness depends on a number of factors, such as the ability to fold into secondary and tertiary structures, the rate of folding, and stability.^{1,2} Fast folding is generally correlated with stable proteins and is thus preferred by evolution.^{3,4} For enzyme fitness several additional factors come into play, namely substrate specificity, affinity, and activity. It should be noted that higher specificity and activity are not necessarily desirable, and most enzymes are only moderately efficient.⁵ Evolution of an enzyme only occurs to such an extent that its activity affects the fitness of the organism. Without selection pressure, enzymes will not improve further. Two other important factors in evolution are evolutionary robustness and evolvability. Proteins that are optimal in their function, but for which many mutations render them useless, lack evolutionary robustness. In terms of the fitness landscape, these are on a high top surrounded by deep valleys. Evolutionary robust proteins can accommodate more mutations without or with minor loss of functionality.⁶ These are on a much broader top with weakly sloping sides. Evolvability reflects the ease for a protein to assume a different function upon accumulating mutations, for example, a change in substrate specificity of an enzyme.⁷ Such changes in function are required for significant changes in the phenotype of organisms. Many new enzymes have evolved after gene duplication and accumulation of mutations in one of the two copies of the gene. If a protein can assume new functions with few mutations, its evolvability is high. In the fitness landscape, it means that other tops are nearby and can be reached easily.

It should also be noted that evolution rates are not only affected by the inherent properties of the protein itself. Other properties, such as expression levels and translational robustness influence both the phenotype and evolutionary rates.⁸⁻¹³ Proteins that have more interactions with other proteins in a network are more likely to be well-conserved and evolve more slowly.¹⁴ Interacting proteins evolve at similar rates, as changes in one protein result in selection pressure for the partner.

The role of neutral mutations and epistasis

Three types of mutations are distinguished, beneficial, neutral, and deleterious. The majority is neutral and only up to 0.5% of mutations are beneficial.¹⁵ That does not mean that these neutral mutations are less important. As Zuckerkandl and Pauling described in 1965, a large improvement in the stability or function of a protein is not simply caused by a sum of mutations that each cause a small positive change, but the effect of each mutation is dependent on the genetic background.¹⁶ The importance of this concept, called epistasis, in evolution was first highlighted after an extensive analysis of protein sequences.^{16,17} Today, there are more and more indications that protein evolution is indeed supported by neutral, and even detrimental, mutations.¹⁷⁻¹⁹ An example of how these mutations aid proteins in traveling the fitness landscape was published by Bershtein and colleagues. They used the β -lactamase TEM-1 to demonstrate that the probability of new functions emerging increased when selection pressure was low and only variants harboring deleterious mutations were removed unless compensated for by other mutations.²⁰ This ability of some mutations to compensate for other mutations in TEM-1 was also demonstrated by Weinreich and colleagues, although the order in which a protein acquires these mutations is not inconsequential, and not every path is viable.²¹ One mutation enhancing hydrolysis rates, yet increasing aggregation, G238S, and a second mutation increasing stability at the cost of hydrolysis, M182T, could coexist, but the order in which these mutations were obtained was important. M182T was beneficial in the G283S variant, counteracting the aggregation, yet was not likely to occur first in the wild-type enzyme. Success rates of different evolutionary paths were also shown for the protein kinase PhoQ.²² During directed evolution experiments selecting for phosphatase activity, the evolutionary pathways of double mutants were analyzed. In only 23% of cases, multiple evolutionary paths led toward an improved phenotype, while in 46% of cases, only a single pathway was successful.^{22,23} This shows that epistasis is an important factor in evolution.

Directed evolution

Directed evolution is used to mimic natural evolution and selection in the laboratory, to improve the function or stability of a protein.¹⁵ It is based on several rounds of mutagenesis and screens to select for the desired phenotype. After each round, the fittest protein is selected, and its gene is used for a second round of mutagenesis. This method searches for an uphill path in the fitness landscape. One of the earliest examples of directed evolution is the acquisition of new functions for the Ebg enzyme, such as hydrolyzing lactose in a *lacZ*-deficient strain of *Escherichia coli*.^{24,25} In 2018, the Nobel Prize for chemistry was awarded to Frances Arnold for the engineering of enzymes using directed evolution. Cytochrome P450 enzymes are a large family of oxidases with high potential for industrial applications. Arnold and co-workers used a P450 variant that was involved in the hydroxylation of long-chain fatty acids and engineered it to efficiently convert propane to propanol.^{15,26} Directed evolution experiments are also an interesting tool to study and predict evolutionary adaptations of important proteins.

Beta-lactamases

β -Lactamases (EC 3.5.2.6) are enzymes that can hydrolyze β -lactam antibiotics, such as penicillins, cephalosporins, and carbapenems. This reaction renders the antibiotics inactive and causes bacteria that harbor these β -lactamases to have increased antibiotic resistance. The first β -lactamase was reported by Abraham and Chain in 1940 and was found in *Bacillus coli*.²⁷ The discovery of this enzyme, nowadays called *E. coli* AmpC, quickly led to the discovery of more “penicillinases”.^{28–31}

β -Lactam antibiotics have a four-membered 2-azetidinone ring and act by inhibiting D,D-transpeptidases (EC 3.4.16.4). These enzymes are responsible for the breaking of a D-alanyl-D-alanine bond, the final step of peptidoglycan cross-linking in cell wall synthesis. The antibiotic can block cell wall synthesis by irreversibly binding to a D,D-trans peptidase, which leads to cell death. For this reason, D,D-transpeptidases are commonly referred to as penicillin-binding proteins (PBPs).³² When β -lactamases catalyze the hydrolysis of the β -lactam ring, they prevent binding of the antibiotic to a PBP and therefore provide the bacteria with resistance against this antibiotic. Besides their shared capability to bind penicillin, these two enzyme families have a high structural similarity, including resembling active sites.^{33,34} Hence, it has been suggested that β -lactamases evolved from PBPs over two billion years ago.^{35–37} This is supported by the discovery of PBPs that demonstrate β -lactamase activity.³⁸

Ambler classification

In 1980, Ambler introduced a classification system to identify different types of β -lactamases.^{39,40} This system initially distinguished only between classes A and B based on sequence homology. Class A contains β -lactamases that use a serine for the hydrolysis of β -lactams, whereas class B consists of metalloenzymes that possess at least one Zn^{2+} ion in their active site. Class C and D, which were added later, contain the AmpC β -lactamases and the OXA β -lactamases, both serine β -lactamases that have no significant sequence similarity with class A and each other.^{41,42}

Another major classification system, introduced by Bush in 1989 and last revised in 2010, divides the enzymes into 3 groups based on functional characteristics.^{43,44} Group 1 β -lactamases are cephalosporinases that belong to Ambler class C. They mainly hydrolyze cephalosporins but are also active against benzylpenicillin and cephamycins. The serine β -lactamases that can be found in classes A and D of the Ambler classification system make up group 2. These enzymes are classified as extended-spectrum β -lactamases (ESBLs) and can hydrolyze a broad spectrum of antibiotics.^{45,46} Group 3 are the metallo- β -lactamases, functionally different from the other groups by the ability to hydrolyze carbapenems.^{43,44,47}

The first known Ambler class A β -lactamase was isolated in 1962. TEM-1 was found in *E. coli* and named after the patient in Athens from whom it was isolated (Temoniera).^{48,49} Other well-studied class A β -lactamases are sulfhydryl reagent variable (SHV-1), which was originally

found in *Klebsiella pneumoniae*, and cefotaxime-Munich (CTX-M), found in *E. coli*.^{50–52} The β -lactamase present in *Mycobacterium tuberculosis*, BlaC, was reported in 1965.⁴⁵ These class A enzymes are structural homologs, with important differences in sequence and function. For example, BlaC has approximately 40% sequence homology with other class A β -lactamases but has only minor differences in structure.^{45,53,54} The most studied class A β -lactamases TEM-1 and SHV-1 share 65% sequence homology.⁵⁴

BlaC

BlaC is a 307 amino acid protein encoded by the *blaC* gene on the chromosome of *M. tuberculosis* (Figure 1.1a).⁵⁵ *M. tuberculosis* is considered a Gram-positive organism based on 16S rRNA sequences but has two cell membranes characteristic of Gram-negative bacteria.⁵⁶ The membranes are separated by a peptidoglycan-arabinogalactan complex and the outer layer of the cells is formed by the capsule, which consists of polysaccharides.⁵⁷

After translation, the folded BlaC protein contains an N-terminal secretion signal with a twin-arginine motif, RRX, followed by two uncharged residues.^{58–60} It is transported to the periplasm of the cell by the twin-arginine translocation (Tat) system. It is unclear whether the signal sequence is cleaved off after transportation. In *E. coli* this sequence is removed by signal peptidase I encoded by the *lepB* gene, and *M. tuberculosis* has a homolog of this gene.^{61,62} However, there is evidence for the presence of a glycosylation site and a predicted lipid modification site at a cysteine present in the signal sequence, suggesting a role for this sequence after translocation.^{60,63,64}

The soluble part of BlaC (i.e. the part following the signal sequence) comprises two domains: an α -domain composed of 6 long and 4 short helices and an α/β -domain containing the N- and C-terminal helices, two short helices, and 5 β -strands in an anti-parallel β -sheet (Figure 1.1b).⁵³ Several motifs that are highly conserved among class A β -lactamases, are important for binding and hydrolysis of the substrate, the SXXK motif (residue 70-73 according to Ambler numbering⁶⁵), SDN loop (130-132), Ω -loop (161-179), and carboxyl binding site (234-237) (Figure 1.1).^{65–69} Many of these residues make up the active site, which contains residues from both the α - and α/β -domain and is located at the interface. While BlaC has many similarities with other β -lactamases, a few important amino acid substitutions broaden its substrate range. The asparagine in position 132 of the SDN loop is replaced by glycine, resulting in an SDG motif. Position 105, which is often occupied by a tyrosine or histidine, is an isoleucine, and 4 additional residues have been inserted between helix 7 and 8, just “behind” the active site. In the Ω -loop, position 164 is occupied by an alanine instead of an arginine, which removes a salt bridge with Asp179, potentially allowing more flexibility in the loop. These changes lead to an active site that is wider than those of other class A β -lactamases.⁵³

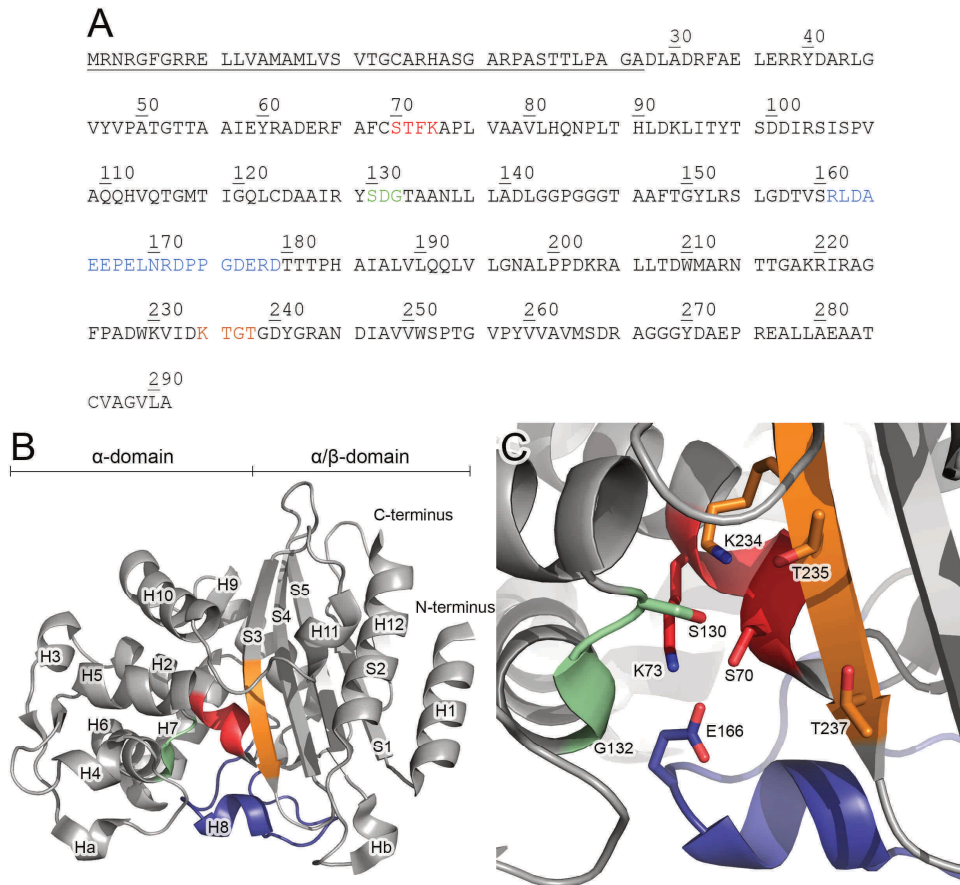


Figure 1.1. Sequence and crystal structure of *M. tuberculosis* BlaC. (A) Residues 28-291 of the amino acid sequence are numbered according to Ambler notation,⁶⁵ corresponding to residue numbers 43-307 of Uniprot entry P9WKD3. BlaC misses residues 58, 84, 85, 239, and 253 and has 6 additional residues: 145A, B, C, D, 269A, and B. Residues of the Tat-signal sequence are underlined. Indicated are the SXXK region (red), SDG loop (green), omega loop (blue), and carboxyl binding site (orange). (B) Three-dimensional structure of BlaC (PDB entry 2GDN⁵³). (C) Detail of the active site with selected residue in sticks. The colors match those in panel (A). Oxygen and nitrogen atoms of side chains are shown in red and blue, respectively.

Substrate hydrolysis

BlaC is a β -lactamase that can hydrolyze a broad spectrum of substrates, such as penicillins, cephalosporins, and carbapenems.^{45,46,53} The first step in the hydrolysis of the substrate is the activation of Ser70. It has been proposed that Glu166, a residue present in the flexible Ω -loop,⁷⁰ acts as a general base and deprotonates a water molecule after binding of the substrate, which activates Ser70.^{68,71-73} However, there is also evidence of Lys73 acting as the general base, accepting a proton directly from Ser70 (Figure 1.2a).⁷⁴⁻⁷⁷ Recent studies have proposed that in the presence of Glu166, Lys73 is expected to have a positive charge, which makes it less likely to act as a general base for some β -lactamases. In the absence of Glu166, the pK_a of Lys73

lowers, which will increase the probability of it acting as a general base.^{78,79} After deprotonation, the nucleophilic hydroxyl of Ser70 attacks the carbonyl group of the β -lactam antibiotic, and subsequent protonation of the nitrogen by Ser130 leads to the opening of the characteristic β -lactam ring and the formation of the acyl-enzyme (Figure 1.2b). To deacylate the enzyme, Glu166 activates a water molecule, which attacks the carbonyl leading to the lysis of the bond between the substrate and Ser70. Subsequently, Glu166 gets deprotonated by the amine of Lys73.^{53,80}

Several crystal structures show the presence of water molecules in the protein, one of them is part of a hydrogen network with Ser70 and Glu166.^{53,81} In addition to the residues mentioned before, multiple other residues that surround the active site are involved in stabilization of the substrate.⁵³ Ser130 in the SDN-loop is directly involved in hydrogen bonding interactions with substrates, as are Thr235 and Thr237. Lys234 acts as an anchor for the carboxylate of penicillins.⁸² The carbonyl oxygen of a β -lactam fits in the oxyanion hole that is formed between Ser70 and Thr237 (Figure 1.2c).^{77,80,83,84}

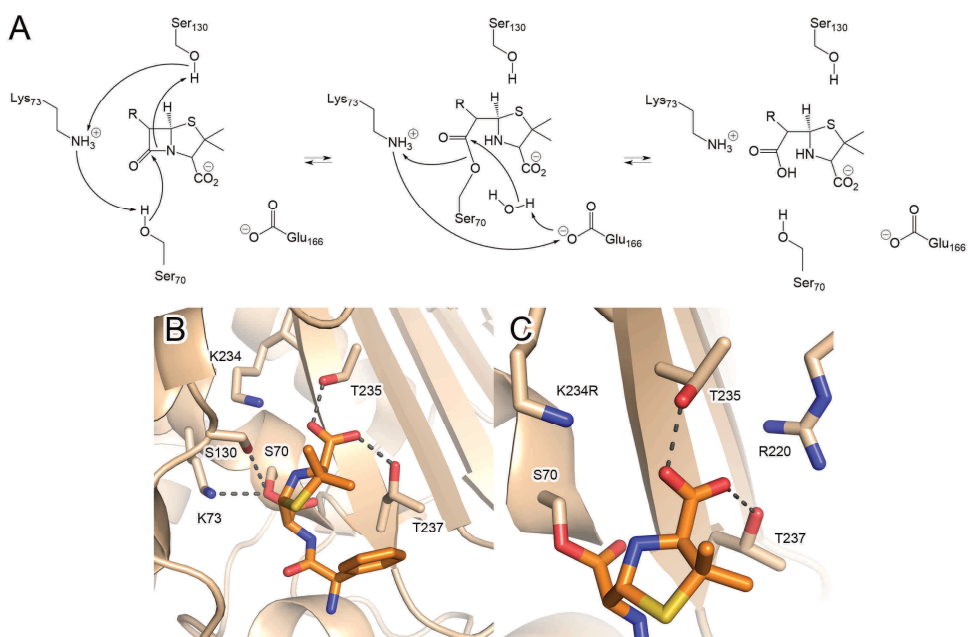
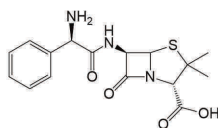


Figure 1.2. Interactions of BlaC with β -lactam substrates. (A) Proposed mechanism of hydrolysis of a β -lactam substrate by BlaC. Lys73 acts as a general base for acylation and Ser70 performs a nucleophilic attack. Glu166 activates a water molecule for deacylation and subsequent product release.^{85,86} (B, C) Crystal structure of BlaC E166A covalently bound to ampicillin (PDB entry 3N8L) showing the (B) active site and (C) the oxyanion hole. Black dotted lines indicate potential hydrogen-bonding interactions.

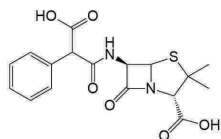
Beta-lactamase inhibitors

Some compounds act as β -lactamase inhibitors. They often resemble the structure of β -lactam substrates or the transition state of the (de)acylation yet are not as easily hydrolyzed. Inhibition can be achieved through several mechanisms, and the duration of inhibition differs. For example, if inhibition is reversible, the active site is blocked by the inhibitor for a fraction of the time depending on affinity and inhibitor concentration. The enzyme can also be inhibited irreversibly, when the covalent binding of the molecule to the enzyme causes a permanent change in the molecular structure of the enzyme, rendering it inactive. For example, in the case of β -lactamases, the residues Ser70 and Ser130 can be cross-linked.⁸⁷ Most inhibitors act as substrates and will be hydrolyzed, but at a low rate. Such inhibitors are commonly used in clinical settings to treat infections by Gram-negative bacteria harboring class A β -lactamases. Chemically, most of the available inhibitors can be divided into three categories, β -lactam based, diazabicyclooctane (DBO) based, and boronic acid-based inhibitors (Figure 1.3).

Penicillins

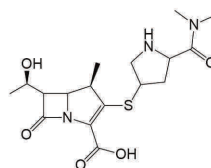


ampicillin



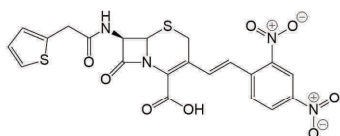
carbenicillin

Carbapenem

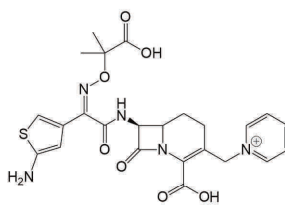


meropenem

Cephalosporins

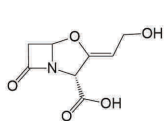


nitrocefin

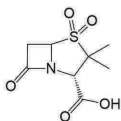


ceftazidime

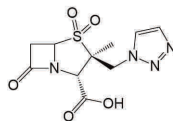
β -Lactam inhibitors



clavulanic acid

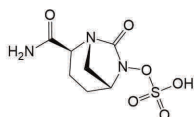


sulbactam

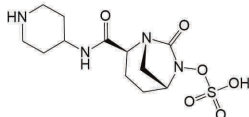


tazobactam

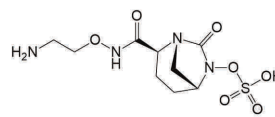
Diazabicyclooctane inhibitors



avibactam

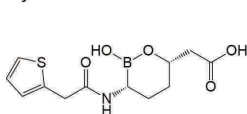


relebactam

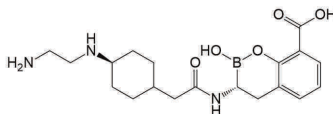


nacubactam

Cyclic boronate inhibitors



vaborbactam



taniborbactam

Figure 1.3. β -Lactamase substrates and inhibitors discussed in this thesis.

Clavulanic acid, sulbactam and tazobactam

The first β -lactamase inhibitor, clavulanic acid, was discovered in the 1970s and is a naturally occurring β -lactam compound produced by *Streptomyces clavuligerus*.^{88,89} It is most commonly available as a potassium salt and combined with the antibiotics amoxicillin (brand name Augmentin®) and ticarcillin (Timentin®). Clavulanic acid can inhibit the enzyme both reversibly and irreversibly and various intermediates and reaction products have been observed in literature (Figure 1.4).^{81,87,90,91} Sulbactam and tazobactam (formerly known as CP45899

and YTR830, respectively) are sulfones that were developed by the pharmaceutical industry in the early 1980s.^{92,93} Sulbactam is combined with either ampicillin (Unasyn[®]) or cefoperazone (Sulperazon[®]), while tazobactam is used in combination with either piperacillin (Zosyn[®]) or ceftolozane (Zerbaxa[®]). There are many similarities between these inhibitors; all three contain a β -lactam ring (Figure 1.3) and inhibit the enzyme covalently and reversibly. Just like in substrates, the nucleophilic attack on the β -lactam carbonyl results in a covalent interaction with residue Ser70 of BlaC and the opening of the β -lactam ring. However, a major difference between penicillins and β -lactam inhibitors is that the inhibitors have good leaving groups, which cause the opening of the 5-membered ring (Figures 1.4 and 1.5).⁹⁴ These leaving groups also cause differences between different inhibitors. BlaC can recover from inhibition with sulbactam and tazobactam in 30 and 45 minutes, respectively, but recovery from clavulanic acid takes much longer.^{81,95} This is caused by the enol ether oxygen of clavulanic acid, which is a better leaving group when opening the ring than the sulfone present in tazobactam and sulbactam.⁹⁴

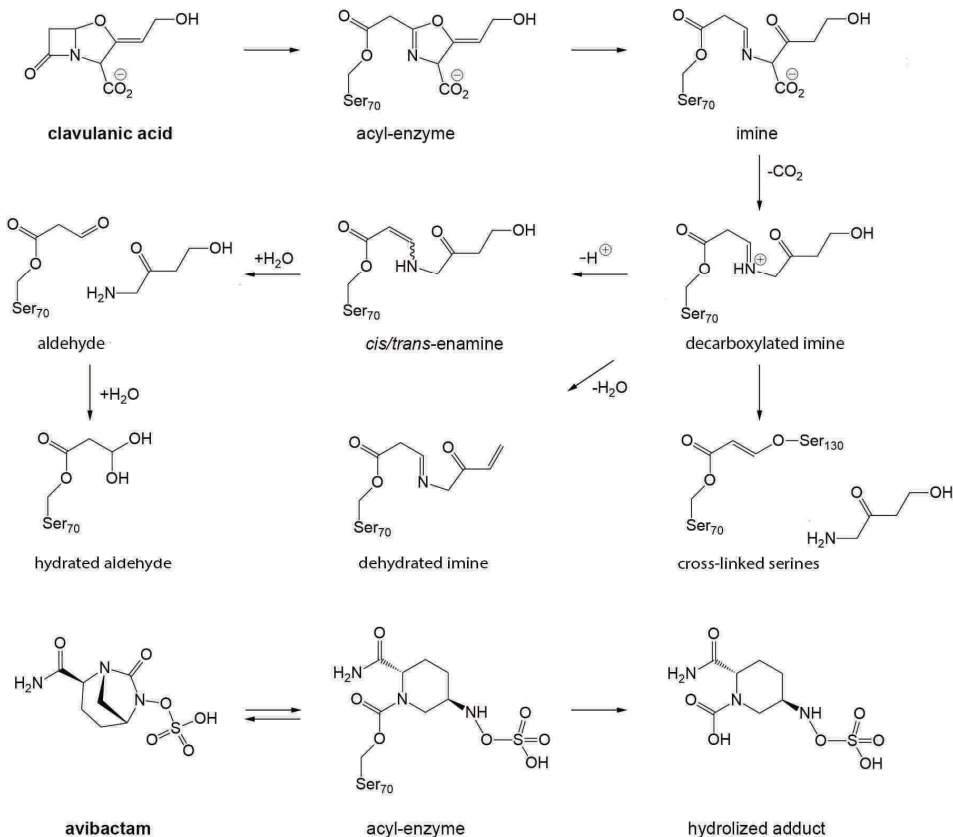


Figure 1.4. (Top) Mechanism for β -lactamase inhibition by clavulanic acid based on mass spectrometry data in literature.^{81,87,90,91} Reaction mechanisms for sulbactam and tazobactam are similar.^{96–101} (Bottom) Reversible inhibition and hydrolysis of the avibactam adduct by BlaC.^{102,103}

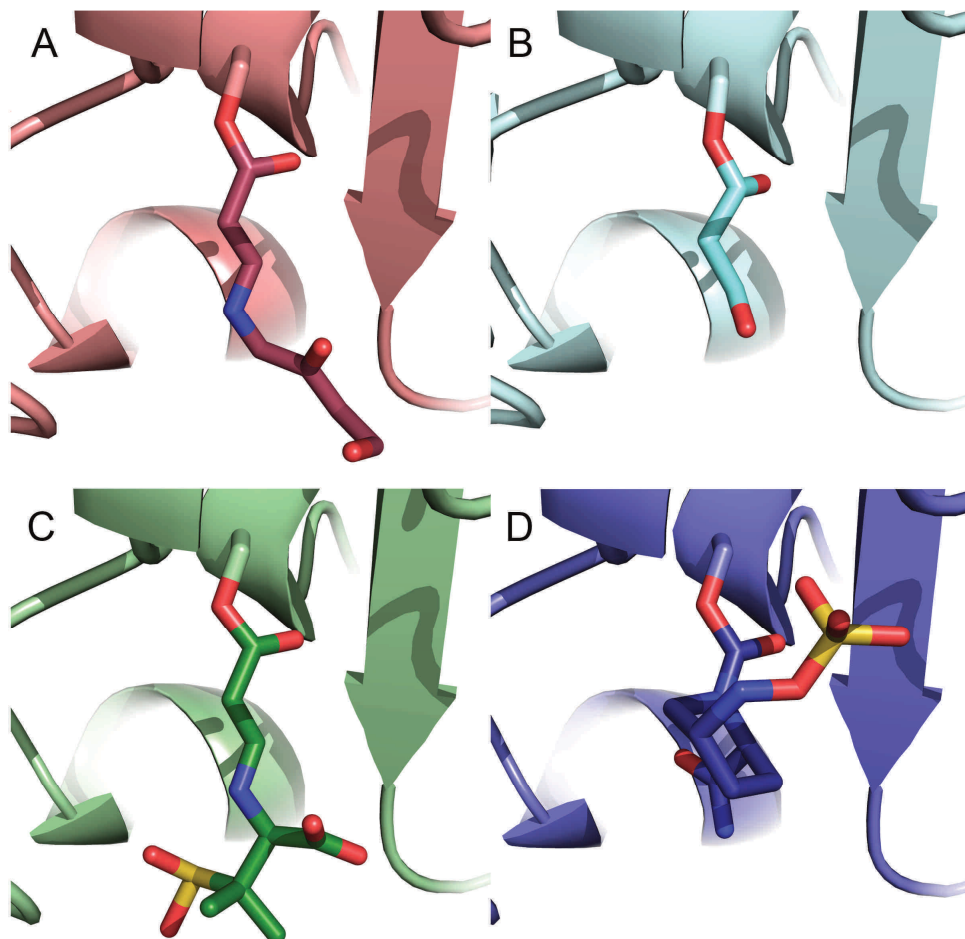


Figure 1.5. Crystal structures of *M. tuberculosis* BlaC in complex with (A) the trans-enamine adduct of clavulanic acid (PDB entry 6H2C), (B) the aldehyde adduct of clavulanic acid (PDB entry 6H2K), (C) the trans-enamine adduct of sulbactam (PDB entry 6H2K), and (D) the adduct of avibactam (PDB entry 6H2H). All structures are described in ref.¹⁰⁴

Non- β -lactam inhibitors

The search for lactamase inhibitors is not limited to β -lactam compounds. Inhibitors that were approved more recently include avibactam and vaborbactam, compounds that are structurally different from penicillins (Figure 1.4).

Avibactam (formerly known as AVE1330A and NXL104) is a DBO and is FDA-approved for the treatment of infections caused by multi-drug resistant pathogens when combined with ceftazidime (Avycaz[®]).¹⁰⁵ As with the β -lactam inhibitors, the DBO inhibitors form a covalent bond with Ser70, resulting in reversible inhibition. Recovery from avibactam was shown to take 48 hours for BlaC, longer than for any of the β -lactam inhibitors mentioned.¹⁰² In contrast to those inhibitors, intact avibactam can be generated after release via deacylation and

recyclization of the five-membered urea ring.^{103,106} This is the case for β -lactamases TEM-1 and OXA-10, making it a very potent inhibitor, but hydrolysis by BlaC results in an inactive inhibitor (Figure 1.4).^{102,106} Desulfation of the adduct has been observed for KPC-2.^{106,107}

The success of avibactam against different classes of β -lactamases resulted in the development of more DBO inhibitors, like relebactam (MK-7655), which differs from avibactam only in the addition of a piperidine ring, and nacubactam (OP0595), which contains an oxyethylamine group.^{108,109} The cyclic boronates make up the third generation of inhibitors. As the name suggests, these inhibitors contain a boron atom that can form a covalent bond with Ser70. Examples of boronic acid inhibitors are vaborbactam (RPX7009), the first inhibitor of its generation, and taniborbactam (VNRX-5133).^{110,111} Several cyclic boronates, including taniborbactam, can inhibit enzymes from all classes of β -lactamases by interchanging between different hybridization states.^{112,113} This allows them to mimic tetrahedral transition states in β -lactam hydrolysis.^{113–115}

BlaC mutants that are less susceptible to inhibitors

Several mutations in BlaC have been discovered that show reduced susceptibility to inhibitors. One of these mutations is G132N, which restores the SDN motif that is present in most class A β -lactamases. The presence of an Asn in position 132 results in enhanced hydrolysis of clavulanate.^{116–118} However, this mutant is also more sensitive to avibactam than wild-type BlaC.¹¹⁷ Other mutations that confer resistance against clavulanate are C69L, S130G, R220A, R220S, K234R, T237A, and T237S, but these also cause a loss in substrate affinity or a lower hydrolysis rate (Figure 1.6a).^{54,119} Cys69 is situated next to Ser70, the residue that binds the substrate. Mutations of residue 69 are found quite frequently in class A β -lactamases, and often result in either a hydrophobic amino acid (e.g. Leu) or one that produces steric hindrance in that position, changing interactions with substrates and inhibitors in the oxyanion hole.^{54,120} Ser130 is a conserved amino acid residue that is involved in substrate binding and proton transfer (Figure 1.2). The mutation to Gly has not only been found in BlaC but also in the class A β -lactamases TEM and SHV.^{121,122} The mutation to Gly prevents cross-linking between Ser70 and Ser130 and therefore irreversible inhibition.¹²³ Arg220 and Lys234 contribute to the binding of the carboxyl group found in all substrates. In TEM-1, Arg244 has the same function as Arg220 in BlaC, which has an Ala at position 244 (Figure 1.6b). On their own, mutations R220A and R220S confer resistance against clavulanic acid, but at the cost of catalytic activity. Introducing both R220A and A244R in BlaC results in partial restoration of catalytic activity and inhibitor susceptibility.¹¹⁹ When combining mutations R220S and K234R or S130G and K234R, an additive effect was observed.⁵⁴ Residue Thr237 forms the oxyanion hole together with Ser70 and is important in binding the carbonyl group of β -lactams.¹²⁴ Mutating Thr273 to a Ser results in a slight reduction in catalytic function and an increase in clavulanic and sulbactam resistance. Larger differences were observed when introducing an Ala in this position, resulting in resistance against clavulanic acid, sulbactam, and tazobactam.¹¹⁹ Another notable mutation is I105F. I105F was discovered during directed evolution experiments and

enhances the ability of BlaC to hydrolyze ampicillin, even in the presence of clavulanic acid.¹²⁵ Ile105 is present in a loop that covers the active site and has been named gatekeeper residue.¹²⁵

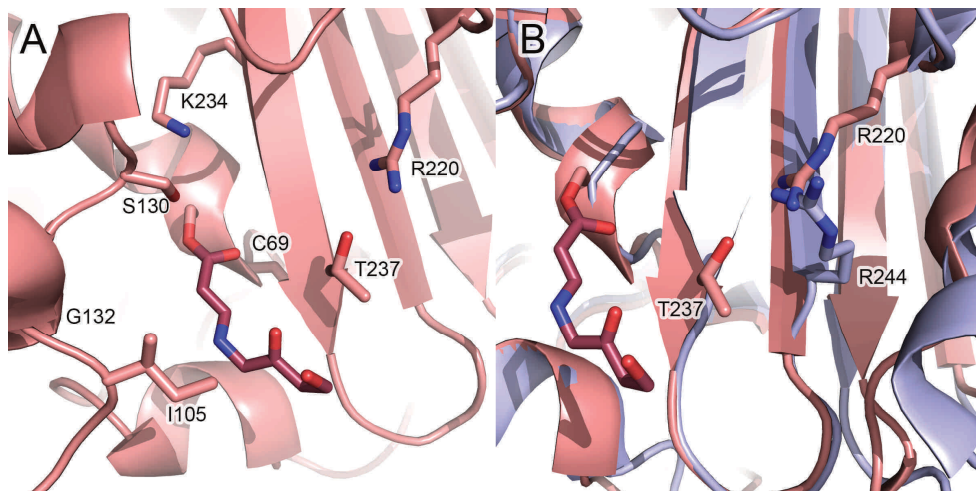


Figure 1.6. Positions of known inhibitor-resistant mutations in BlaC. (A) BlaC bound to the trans-enamine adduct of clavulanic acid. Residues for which mutations can confer inhibitor resistance are shown as sticks (PDB entry 6H2C¹⁰⁴). (B) Overlay of BlaC bound to the trans-enamine adduct of clavulanic acid (salmon) and TEM-1 (pale blue), showing that the positive charge provided by the side chain of BlaC Arg220 is provided by Arg244 in TEM-1 (PDB entries 6H2C¹⁰⁴ and 1ZG4¹²⁶).

Previous inhibitor-related work in the Ubbink group focused on interactions of BlaC with clavulanic acid and avibactam. It was established that clavulanic acid inhibition is reversible and that recovery times are influenced by the environment.⁸¹ The presence of phosphate in the buffer enhanced recovery rates and both a crystal structure and NMR data showed the presence of phosphate in the active site. It was also shown that, upon binding of clavulanic acid or avibactam, dynamics in the active site of BlaC increase.⁷⁰ Two mutations that confer resistance against clavulanic acid, G132N, and K234R, the latter found in laboratory evolution experiments, were characterized using x-ray crystallography and NMR dynamics studies. While K234R decreases the dynamics in the active site on the millisecond time scale, the opposite is true for G132N, which exists in two states as evident from NMR data.¹¹⁸ These results are important for our understanding of inhibitor evasion and show that there are different evolutionary paths to resistance.

Other recent work focused on the role of conserved amino acids. It was found that residues surrounding the active site are important for the stability of the protein and fine-tuning of the active site. Changing the interactions of the side chain with the active site residues was more detrimental than introducing a smaller side chain and removing those interactions altogether. Conserved residues far away from the active site are often involved in the folding of the protein.¹²⁷

Research objectives

The research described in this thesis aims to explore the evolutionary adaptability of *M. tuberculosis* β -lactamase BlaC. The rise of antibiotic-resistant *M. tuberculosis* increases the need for new treatments against tuberculosis. Widely used, and thus safe, β -lactam antibiotics would be good candidates for new treatments, when combined with β -lactamase inhibitors. However, as it is expected that this enzyme and other β -lactamases will continue to evolve in order to evade these inhibitors, we aimed to map the evolutionary paths that the enzyme might take. We hypothesized that while it could be possible for the enzyme to gain resistance against one inhibitor, it will be difficult to evade multiple inhibitors while maintaining both stability and catalytic activity. The first objective was to identify mutations that confer resistance to a β -lactam inhibitor and to determine whether combining mutations that individually provide resistance against different inhibitors, would result in resistance against multiple inhibitors. Subsequently, we aimed to characterize the mutant proteins and identify the changes on a molecular level, and to test inhibitor-resistance in more physiological conditions, including a zebrafish model for tuberculosis.

Outline of this thesis

Chapter 1 provides an introduction into the topics of protein evolution, β -lactamase BlaC, and β -lactamase inhibitors, and describes the research objectives. In **chapter 2**, laboratory evolution and selective screening experiments are described yielding several amino acid substitutions that resulted in increased resistance against the inhibitor sulbactam. The single mutants were characterized both in cells and *in vitro*, and analysis with NMR spectroscopy and x-ray diffraction of crystals gave insight into the structural changes in the BlaC G132S variant that help to explain the reduced sensitivity for sulbactam. In **chapter 3** an explanation is sought for the observation that the highly conserved residue Asp179 can be mutated to Asn and several other residues without loss of function of BlaC. It is demonstrated that this residue is essential in other class A β -lactamases but interestingly not in BlaC, showing that conservation does not necessarily equate with essentiality. The structural explanation for this observation is provided with the crystal structure of the D179N variant. To investigate whether combining mutations in BlaC leads to additive effects or epistatic interactions, mutations that individually result in increased catalytic activity or reduced sensitivity to inhibitors were combined. BlaC variants harboring mutations I105F, G132S, and/or D179N were characterized in cells and *in vitro* to get a better understanding of the effects of the individual mutations. These results are discussed in **chapter 4**. **Chapter 5** describes a model to test BlaC variants in more physiological conditions, using *M. marinum*. Like *M. tuberculosis* in human patients, *M. marinum* infection results in the formation of granulomas in zebrafish. Zebrafish embryos were infected with *M. marinum* that produce BlaC variants and treated with a combination of antibiotic and inhibitor. It is demonstrated that the effects of mutations observed in *E. coli* can also be observed in this model. The final chapter, **chapter 6**, provides a general discussion and an outlook on further research.