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

Alen, I. van


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General discussion and outlook

... words are where most change begins.

- Brandon Sanderson, Words of Radiance

Overview and perspectives

Antibiotic resistance is a global health problem that severely complicates the treatment of infectious diseases. One mechanism that allows bacteria to become resistant is the evolutionary adaptability of β-lactamases, such as *M. tuberculosis* BlaC. In order to hydrolyze β-lactam antibiotics, BlaC needs to be correctly folded, stable, and catalytically active. The function of β-lactamases also requires adaptability, and it has been suggested that evolvability is a selection pressure in itself.[230] These constraints make it difficult to predict possible future evolutionary changes, as attempts to adapt or expand the function, e.g. to increase catalytic activity or decrease sensitivity to inhibitors, often have great consequences for the other enzymatic properties. In addition, the frequency of mutations displaying epistatic interactions increases along the evolutionary pathway,[231] further complicating the adaptive process.

The work presented in this thesis aimed to map several evolutionary paths that BlaC can take to evade the action of inhibitors. Previous work in the Ubbink group resulted in a method for laboratory evolution of BlaC and yielded the mutations G132N and K234R, both conferring resistance against clavulanic acid.[118] At the start of the research project described here, we set out to find mutations that make the enzyme less sensitive to sulbactam and to elucidate mechanisms of inhibitor resistance. One of the mutations that was discovered is G132S. The introduction of the Ser sidechain affects the conformation of the loop between residues 102 and 108, resulting in a partial blockage of the entrance to the active site and therefore lowering affinity for sulbactam (chapter 2).[172] Interestingly, lower affinity was not limited to the inhibitor, as the apparent K_M for the substrates ampicillin and nitrocefin was found to be higher for BlaC G132S than for the wild-type enzyme, negatively affecting catalytic activity for these substrates. In addition, we found that BlaC G132S is more sensitive to another inhibitor, avibactam. These observations illustrate the evolutionary tradeoff between the acquisition of a new function and keeping the other enzymatic properties.

Other mutations found using laboratory evolution, A55E, D172N, and G269S also showed a decrease in sensitivity to sulbactam in cells, but not *in vitro*, demonstrating that a change in catalytic properties for sulbactam hydrolysis is not the only possible pathway to resistance. An increase in mRNA or protein stability, or changes in folding or translocation can increase the amount of active protein in cells and result in a decrease in the sensitivity of the bacterial cells to sulbactam. *In vitro* kinetic data show a 2-fold higher k_{cat} for BlaC D172N with ampicillin. This provides another explanation for the increase in growth of cells expressing a mutant compared to wild-type BlaC, as ampicillin was used as a substrate during screenings. If the conversion rate of the substrate is higher, the cells can withstand a higher amount of inhibitor.

The mutation D179N was identified during previous research into the role of non-catalytic highly conserved residues, as Asp179 is present in 99.8% of class A β-lactamases.[127] Whereas most mutations of conserved residues decrease the overall fitness of BlaC, this is not the case

for D179N, which inspired further characterization as described in chapter 3. It was found that BlaC D179N is slightly more stable than the wild-type enzyme, as evidenced by a 1.5 °C increase in melting temperature. Furthermore, cells producing BlaC D179N grow slightly better in the presence of ampicillin or avibactam than cells expressing the wild-type enzyme. As with BlaC A55E, D172N, and G269S, the resistance could not be demonstrated *in vitro* and likely stems from an increase in stability.

The effect of individual amino acid substitutions can differ depending on the genetic background of an enzyme. It was questioned whether these epistatic effects would be present if mutations would be combined in BlaC, or whether the phenotype of a double mutant would be the sum of the effects of individual mutations. More specifically, we wondered whether a decrease in sensitivity to sulbactam caused by G132S would be additive to decreased sensitivity to avibactam caused by D179N. Mutation I105F was also included in this study, as it had previously been identified to increase the catalytic activity for ampicillin in BlaC.[125] It was found that combining mutations G132S and D179N results in a slightly positive epistatic effect as it decreases the sensitivity of *E. coli* cells to sulbactam more than for BlaC G132S alone and it results in a more stable protein (chapter 4). Adding I105F to BlaC G132S yields an even greater reduction in sulbactam sensitivity of the cells than for G132S+D179N. Combining all three mutations results in an additive effect of stability caused by I105F and D179N, and the sensitivity observed for I105F+G132S. In addition, analysis of the chemical shift perturbations of the backbone amides of the BlaC variants shows additive effects of the mutations. Interestingly, no combination can compensate for the increased sensitivity of BlaC G132S to avibactam, and negative epistasis was observed for BlaC I105F+G132S, BlaC G132S+D179N, and the triple mutant. Attempts to perform laboratory evolution with BlaC G132S and screen for avibactam resistance were unsuccessful and generally resulted in a reversal of the enzyme back to the wild type (data not shown), suggesting that resistance against sulbactam is not compatible with resistance against avibactam and making them interesting candidates for combinational therapies with two or more inhibitors.

It is debatable whether these findings of BlaC variants translate to other class A β-lactamases. The effects of mutations in many residues are similar between the most studied β-lactamases as observed for Cys/Met69, Ser130, Lys234, and Arg220/244.[54,94,118,232,233] However, the results of chapter 3 point out crucial differences between the orthologs as residue Asp179 can be mutated to Asn in BlaC without loss of function or stability, but is essential in other class A β-lactamases. When studying the class A β-lactamases CTX-M-14, KPC-2, NMC-A, and TEM-1, it was found that cells producing the D179N variants were more sensitive to penicillins and meropenem than those producing the wild-type enzyme. Moreover, cells producing either KPC-2 D179N or NMC-A D179N are less sensitive to ceftazidime than those producing the wild-type enzymes, showing a shift in substrate specificity, while the opposite is true for BlaC and CTX-M-14. Comparison of the whole cell lysates and soluble fractions showed that for CTX-M-14, KPC-2, NMC-A, and TEM-1, the D179N variant is less soluble than the wild-type protein. The D179N mutation causes the loss of the salt bridge with Arg164 in the Ω-loop of these four β-

lactamases, likely destabilizing the enzymes. A decrease in sensitivity for cephalosporins, like ceftazidime, has previously been associated with mutations in residues Arg164 and Asp179,[163,166,167,174–176] and has been attributed to destabilization of the Ω-loop.[161] As BlaC has an Ala in position 164, it does not have this salt bridge and therefore does not suffer the loss, as the Ω-loop is stabilized by a hydrogen bond between Asp172 and Asn179.

Asn179 may be a next step in the evolution of BlaC, although it should be considered that observed effects might be specific to *E. coli* cells. In the Ubbink group, BlaC is used as a model for protein evolution and *E. coli* is employed as an expression system because of its fast growth rate and low biosafety level. However, one could question whether results obtained for BlaC variants expressed in *E. coli* translate to more physiological conditions. To test this, the native *blaC* gene of *M. marinum* was knocked-out using a recently developed protocol,[207] and *M. tuberculosis blaC* variants were inserted into the genome. The effect of mutations I105F, G132S/N, D172N, D179N, and K234R on *M. marinum* cell growth was checked in the presence of sulbactam and avibactam (chapter 5). Reassuringly, the results for most mutations were like those observed for *E. coli*. However, the mutations D172N and D179N did not seem to have any influence on growth. Interestingly, while K234R was originally included as a clavulanic acid-resistant mutant,[54,118] we observed that it also confers resistance against avibactam, a property that was known for KPC-2 K234R,[208] but not in BlaC. As a next step, we aimed to test the effect of BlaC variants in zebrafish, based on infection assays developed by the Meijer group.[234] Zebrafish can be used as a model system for tuberculosis, as the initial immune response to infection with *M. marinum* and the formation of granulomas is very similar to that in human patients infected with *M. tuberculosis*.[198,200–202] After infecting the embryos with *M. marinum* expressing *M. tuberculosis blaC,* and treating them with ampicillin, avibactam, or a combination of the two, it was discovered that embryos treated with both ampicillin and avibactam significantly lowered the infection levels 4 dpi. Additionally, zebrafish infected with *M. marinum* producing BlaC K234R show higher infection levels than larvae infected with wild-type BlaC when treated with the same combination. These results demonstrate that the effects of mutations observed in *E. coli* translate to more physiological conditions and that the difference in morphology does not limit the uptake of ampicillin or avibactam for *M. marinum*. It also shows that ampicillin/avibactam does not just influence bacterial growth on plate, but also when the bacteria are phagocytosed by macrophages and present in granulomas inside a eukaryotic system, suppressing the development of the disease. Lastly, we can observe relatively subtle effects, 8-10x differences in inhibitor susceptibility between BlaC variants in zebrafish.[208]

Outlook

The results described in chapter 5 offer new research opportunities. Apart from testing other BlaC variants or other substrate/inhibitor combinations in zebrafish, it could be interesting to expand this research to other model systems. Monocyte cell lines could be used to test the effect of treatment with ampicillin and avibactam on human cells infected with *M. tuberculosis* producing wild-type BlaC, as previously done for ceftazidime/avibactam,[235] and BlaC mutants. If successful, it could be tested if ampicillin/avibactam can be used to treat TB in a mammalian model organism, and whether this treatment is safe for potential use in clinics. It should be noted that caution is required when deciding to clone genes coding for inhibitor-resistant BlaC variants in *M. marinum or M. tuberculosis* strains, even when using an attenuated biosafety level 2 strain of the latter,[236,237] as infection of humans is still a possibility.

The results obtained for *M. marinum* also instill more confidence in the use of *E. coli* for the selection and characterization of BlaC variants. The main focus of this thesis was on evolution against the inhibitors sulbactam and avibactam, but there are many potential evolutionary paths still to be studied, both for substrates and inhibitors, as new promising inhibitors are in constant development.[238,239] One project in the Ubbink group uses laboratory evolution to study the fitness effects of BlaC variants with mutations in specific residues by analyzing libraries of *blaC* variants using deep sequencing (M. Radojkovic, personal communication). This technique yields mutations that confer resistance against inhibitors but also allows for studying of mutations that have a lower fitness effect than the wild type and teaches us more about the role of specific residues in BlaC. Laboratory evolution experiments could be expanded to include other class A β-lactamases. The work in this thesis shows that there are important differences between them, and evolution experiments could teach us more about the different evolutionary paths of enzymes with similar substrate profiles. Comparing the mechanisms these β-lactamases can employ to evade inhibitors or increase activity will give information on protein adaptation in different genetic backgrounds, as the β-lactamases are optimized for different organisms and environments. Further research into BlaC and β-lactamases will undoubtedly find more evolutionary pathways to increased antibiotic resistance and it would be interesting to see this knowledge being used to develop new therapeutic strategies.

While in-cell experiments are useful for the selection and initial characterization of BlaC variants, *in vitro* experiments are invaluable for in-depth characterization. The techniques used for the research described in this thesis, e.g. thermal shift assays, x-ray crystallography, and kinetic assays, gave some important insights into mechanisms of resistance, but there are questions still left to answer. For instance, the reasons behind the increased catalytic activity of BlaC I105F or BlaC D172N for ampicillin are unknown, making these variants good candidates for further *in vitro* studies. One possible reason for the difference in activity compared to wild-type BlaC could be a change in protein dynamics, as is the case for BlaC G132N.[118] Two ways to study protein dynamics are NMR spectroscopy and molecular dynamics (MD) simulations. NMR dynamics studies can provide information about the motions of backbone amides on various time scales, while simulations can be used to visualize these movements. Combining MD simulations with experimental data will allow for a comprehensive

understanding of the dynamics of BlaC variants that show higher activity or a decrease in sensitivity to inhibitors.

In conclusion, this thesis provides a modest contribution to the understanding of the evolutionary adaptability of *M. tuberculosis* BlaC. It describes the effects of mutations in BlaC and other class A β-lactamases, shows possible evolutionary steps to decrease sensitivity to inhibitors, as well as provides evidence that results obtained in *E. coli* translate to more physiological conditions.