10-1-2012

Probing the Role of Asn 152 in the Class C β-lactamase AmpC

Brianne Docter
Grand Valley State University

Mujahid Anwar
Grand Valley State University

David Leonard
Grand Valley State University, leonardd@gvsu.edu

Rachel Powers
Grand Valley State University, powersra@gvsu.edu

Bradley Wallar
Grand Valley State University, wallarb@gvsu.edu

Probing the role of Asn 152 in the class C β-lactamase AmpC

Brianne Docter[1], Mujahid Anwar[2], David Leonard[1], Rachel Powers[1], and Bradley Wallar[1]

[1]Departments of Chemistry and [2]Cell and Molecular Biology, Grand Valley State University,
558 Cook-DeVos Center for Health Sciences, 301 Michigan Ave NE, Grand Rapids, MI 49503

Abstract

AmpC, a class C β-lactamase, is a main cause of antibiotic resistance to cephalosporins in many species of bacteria. The current proposed mechanism of action involves an acyl-intermediate, where the enzyme becomes covalently attached to the drug at serine-64, before an activated water hydrolyzes the bond and regenerates the enzyme. Although this mechanism is generally accepted, the exact roles that the other active site residues play in recognition and breakdown of the substrate are not fully understood. Here, we investigate the role of the active site residue asparagine-152 (Asn152) in *E. coli* AmpC by mutating it to a glycine, serine, or threonine residue and examining the effect that these mutations have on kinetic and structural properties, when acting upon three different β-lactam drugs: cefotaxime, cefoxitin, and oxacillin. We found that the mutations cause 50 to 200 times higher k_{cat} values against cefotaxime and also allow the enzyme to break down oxacillin, which is not hydrolyzed at a detectable rate by wild type AmpC. We obtained the crystal structure of wild type AmpC and AmpC N152G bound to cefotaxime and found a rotation of glutamine-120 and lysine-67 to be the only significant differences in the active site residues as well as a slight conformational change in the drug itself. Uncovering the specific role of Asn152 in the function of AmpC in conjunction with work done to understand the roles of other active site residues will be useful in the development of inhibitors to these enzymes that may help combat antibiotic resistance.

Introduction

β-lactam drugs, such as penicillins and cephalosporins, have been used to treat bacterial infections since the 1950's, but resistance to these drugs is increasingly becoming a problem in both gram-positive and gram-negative species[1]. The β-lactam drugs mimic the substrates of penicillin-binding proteins (PBPs) which are involved in synthesis of the cell wall. The drugs covalently bind to the PBPs and thereby inactivate them and cause the destruction of the bacteria[2]. One of the main causes of resistance to these β-lactam drugs is the production of β-lactamases. These enzymes, found both chromosomally and on plasmids, are capable of hydrolyzing the β-lactam ring, rendering the antibiotic unable to bind PBPs and thus harmless to the bacterium[3].

One important β-lactamase that is a problem in many gram-negative pathogens is AmpC[4]. Found chromosomally in many *Enterobacteriaceae* and in some other species of bacteria, this class C β-lactamase is normally expressed at very low levels; however, different mutations can cause hyperproduction of the protein[5,6]. AmpC is able to hydrolyze extended-spectrum antibiotics, including 2nd and 3rd generation cephalosporins and cephamycins, which are usually active against a wide range of bacteria[7]. The proposed mechanism of action of AmpC, shown in figure 1, is based on a nucleophilic attack by serine-64 on the carbonyl carbon of the β-lactam ring. This nucleophilic attack breaks open the

β-lactam ring and forms an acyl-enzyme intermediate with the substrate. Deacylation occurs as an activated water acts as the nucleophile, attacking the same carbonyl carbon and breaking the acyl bond, regenerating the enzyme and releasing the harmless product[8].

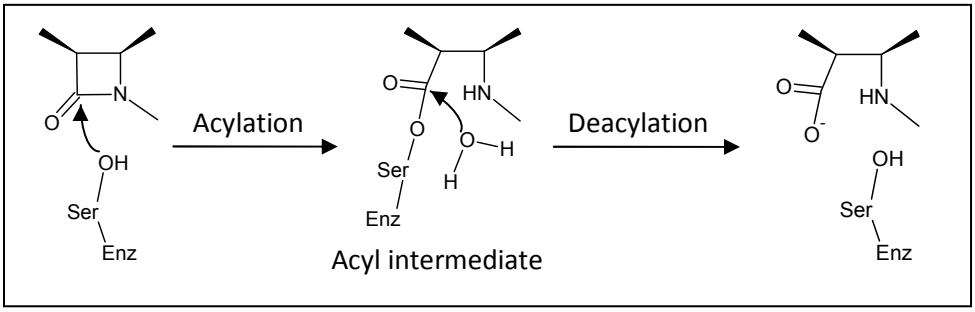

Figure 1: Proposed mechanism of β-lactam hydrolysis for AmpC

Although the general mechanism is understood, it is not known how exactly the specific residues of the active site contribute to the catalysis, nor how they are involved in recognition of the substrate. Elucidating the roles of each of the key residues would not only provide a clearer picture of the mechanism of hydrolysis of the drugs, but could also provide details of substrate recognition and binding which would be useful in the development of new drugs.

One of the key residues in the active site is asparagine-152[9]. The Asn152 residue is highly conserved in class C β-lactamases and takes part in the active site hydrogen-bonding network[10,11]. This residue appears to play a role in substrate selectivity, as a study done on P99 cephalosporinase, another class C β-lactamase, revealed that mutations to small, polar residues at this site led to a switch in substrate selectivity, causing some substrates to be hydrolyzed more quickly than the wild type enzyme but others to be hydrolyzed more slowly[10]. Understanding the exact role of this residue could help determine the reason for the substrate specificity of AmpC. This information could be used to develop inhibitors that bind very well to the active site but are not hydrolyzed, thereby inactivating the β-lactamase and helping to kill the infectious bacteria. Here, we mutate Asn152 to glycine, serine, or threonine and measure the Michaelis-Menten kinetic constants with three β-lactam drugs, structures of which are shown in figure 2. Cefotaxime is a third generation cephalosporin, cefoxitin is a semi-synthetic cephamycin, and oxacillin is a penicillinase-stable penicillin[12,13].

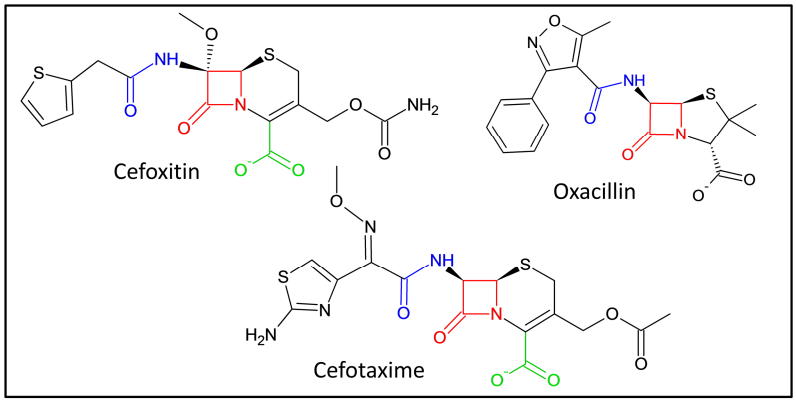

Figure 2: β-lactam drugs used in this study, with the β-lactam ring in red, C_4 carboxylate in green, and the amide of the R_1 sidechain in blue.

Results and Discussion

The Michaelis-Menten kinetic constants were determined using UV-Vis spectroscopy to follow the hydrolysis of the β-lactam amide bond. The values and standard deviations for the wild type AmpC enzyme and the N152G/S/T mutants with 3 different β-lactam drugs are shown below in table 1.

Cefotaxime

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
WT	1.32±0.22‡	0.012±0.005	0.008±0.003
N152G	455±116	1.09±0.57	0.002±0.001
N152S	47±20	0.56±0.14	0.012±0.003
N152T	799±129	1.85±0.01	0.002±0.000

Cefoxitin

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
WT	0.99±0.018‡	0.11±0.01	0.11± 0.01
N152G	56±18	0.050±0.023	0.0010±0.0005
N152S	1.16±0.22‡	0.008±0.000	0.0071±0.0000
N152T	1.12±0.08‡	0.004±0.000	0.0038±0.0001

Oxacillin

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
WT	0.053±0.034‡	ND*	ND*
N152G	0.27±0.07‡	0.41±0.17	1.52±0.64
N152S	0.068±0.012‡	0.21±0.02	3.17±0.24
N152T	1.2±0.5‡	1.38±0.09	1.15±0.08

‡Estimated as K_i *Not detected

Table 1: Michaelis-Menten kinetic constants for AmpC

With cefotaxime, we discovered that the mutants have higher k_{cat} values than the wild type enzyme, but also have higher K_m values, leading to k_{cat}/K_m values that are all within an order of magnitude of each other. With cefoxitin, however, we observed a 10 - 100 times reduction in k_{cat}/K_m values for the mutants compared to the wild type. We find that N152S and N152T mutants have similar K_m values as wild type but have the lowest k_{cat} values of all 4 enzymes. The N152G mutant, however, shows a 50 times higher K_m value than wild type, with a k_{cat} that is half the value of that of AmpC WT. And finally, with oxacillin, we demonstrated that the wild type enzyme does not have measureable activity against this drug, but all three mutants show significant activity. And although the mutants do not all bind oxacillin as well as the wild type enzyme, their relatively low K_m values and high k_{cat} values lead to k_{cat}/K_m values that are the highest of any of the three drugs. Overall, we established that the mutations created a substrate specificity switch—allowing the enzyme to now break down oxacillin, at the cost of reducing activity against cefoxitin, as well as reducing affinity for cefoxitin and cefotaxime.

Focusing at the N152G mutant, we find that it binds cefotaxime and cefoxitin worse than the wild type enzyme. However, while it is slower to break down cefoxitin than wild type, it is significantly faster than the wild type enzyme when acting upon cefotaxime. To examine the cause of this difference, the structures of AmpC N152G acylated to cefoxitin or cefotaxime were determined by X-ray crystallography. The structures of the active site in complex with cefoxitin and cefotaxime are shown in figures 3 and 4, respectively.

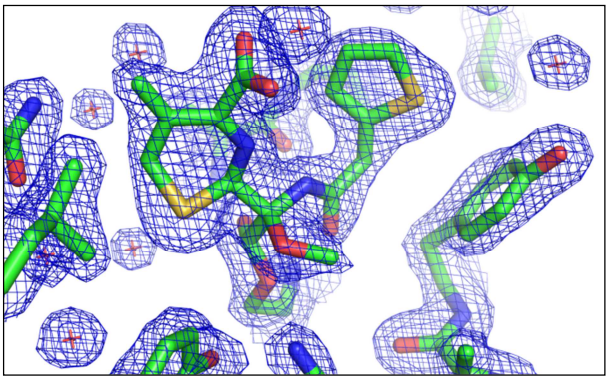

Figure 3: Active site of AmpC N152G in an acyl-intermediate complex with cefoxitin. Blue cages represent electron density contoured at 1σ. Image and subsequent images were produced with Pymol 1.3.

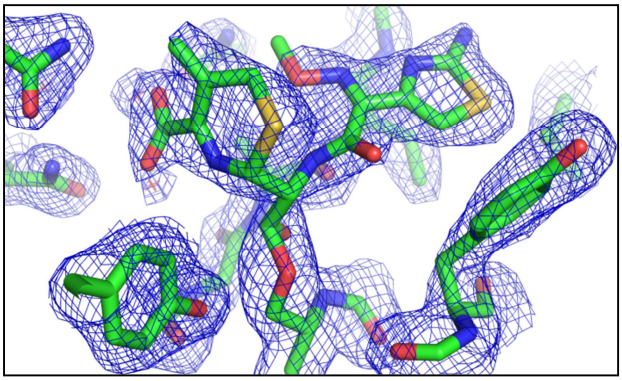

Figure 4: Active site of AmpC N152G in an acyl-intermediate complex with cefotaxime. Blue cages represent electron density contoured at 1σ.

By overlaying the two structures, we can compare the orientations of the two drugs. One obvious difference is the orientation of the C_4 carboxylate, shown in figure 5. It is evident that the C_4 carboxylate group on the dihydrothiazine ring of the cefotaxime structure hydrogen bonds to waters, while the cefoxitin structure shows a flip of the ring and the carboxylate now bonds to a water and Gln120.

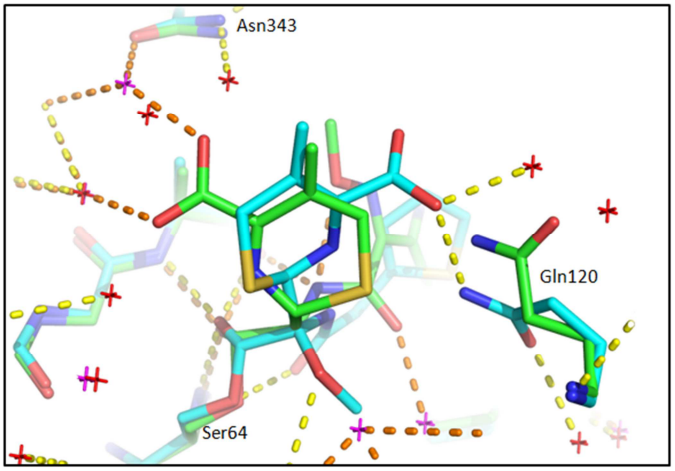

Figure 5: Overlay of AmpC N152G acylated to cefoxitin (cyan) or cefotaxime (green), focusing on C_4 carboxylate. Red waters and yellow hydrogen bonds are associated with the cefoxitin structure, while magenta waters and orange hydrogen bonds are associated with the cefotaxime structure.

One other major change between the two structures is the orientation of the amide which is on the R_2 sidechain, shown in figure 6. In the cefotaxime structure, the carbonyl of the amide points out toward residue-152 and hydrogen bonds to a water and Gln120. In the structure of AmpC bound to cephalothin, which a good substrate for AmpC, the carbonyl is seen in a similar position and it also hydrogen bonds with Asn152[14]. Additionally, we see the nitrogen of the amide hydrogen bonding with the main chain of Ala318, as is also seen in the cephalothin structure. But in the cefoxitin structure, we observe that the carbonyl has rotated away from Gln120 and towards Ser64, and it now hydrogen bonds with the main-chain nitrogen of Ser64. The nitrogen shows no hydrogen bonds. This different conformation may be the reason behind the slower activity of AmpC against cefoxitin.

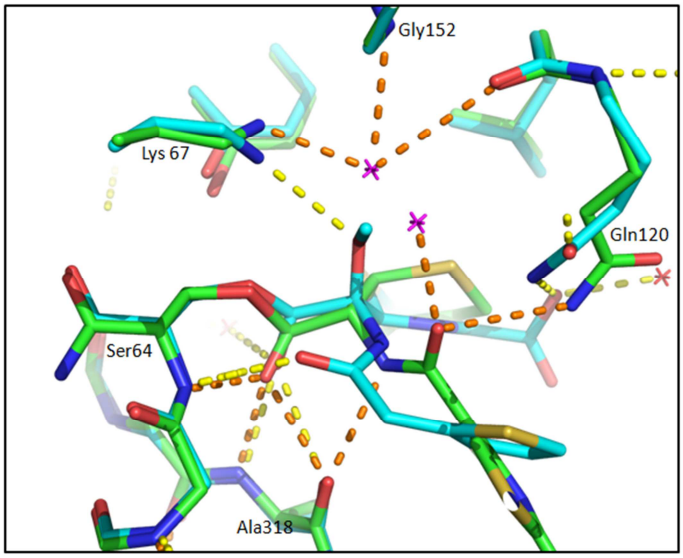

Figure 6: Overlay of AmpC N152G acylated to cefoxitin or cefotaxime, focusing on the amide of R_1 side chain. Coloring is the same as in figure 5.

In conclusion, we determined that although mutations at residue 152 in AmpC decrease the binding affinity of 3 different β-lactam drugs, they increased the catalytic activity of AmpC against cefotaxime and oxacillin and decreased activity against cefoxitin. The crystal structures of AmpC N152G acylated to cefoxitin and cefotaxime were used to infer possible reasons behind the different rates of hydrolysis of the two drugs, which include rotation of the R_2 amide and flipped dihydrothiazine ring, moving the C_4 carboxylate. Future work includes determining the structure of AmpC with oxacillin, as well as additional measurements of kinetic constants in order to obtain more precise values. I plan to present this work at the West Michigan Regional Undergraduate Science Research Conference this fall, as well as the Enzyme Mechanisms Conference in January.

Acknowledgements

We would like to thank the Office of Undergraduate Research and Scholarship at Grand Valley State University for supporting this work.

Materials and Methods

Materials:

Cefoxitin and oxacillin were purchased from Sigma-Aldrich. Cefotaxime was purchased from Calbiochem. CENTA {(6R,7S)-3-[(3-carboxy-4-nitrophenyl)sulfanylmethyl]-8-oxo-7-[(2-thiophen-2-ylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid}, a chromogenic cephalosporin, was synthesized using a published method[15].

Growth and expression:

E. coli AmpC wt and N152G/S/T mutants in the pET24a vector were expressed using BL21(DE3) E. coli cells. Cells were grown in LB medium with 30 µg/mL kanamycin at 37°C. Once reaching an OD600 of 0.6, the cells were induced with 100 µM IPTG and incubated at 17°C with shaking overnight. The cells were harvested using centrifugation at 8200 x g for 10 minutes at 4°C. The pellets were stored at -80°C until purification.

mAPB resin preparation:

Affi-gel 10 (Biorad) was washed with isopropanol and water, then incubated with 2 volumes of 1 M KHCO3, 267 mM m-aminophenyl-boronic acid hemisulfate (MAPB, Aldrich), 534 mM sorbitol for 4 hours at 4°C with mixing, maintaining the pH at 8.0 with 1 M KHCO3. It was then washed with 2 volumes of 1 M NaCl, 0.5 M sorbitol, pH 7.0, then with 2 volumes of 0.5 M borate, pH 7.0, and finally with 2 volumes of 0.5 M NaCl and 20 mM Tris-HCl, pH 7.0.

Protein purification:

Cell pellets were thawed and resuspended in 20 ml loading buffer (25 mM Tris-HCl, 500 mM NaCl, 25 mM borate, pH 7.0) with 200 µl Halt Protease Inhibitor Single-Use Cocktail, EDTA-free (Thermo Scientific) and 50 units of DNAse I. Cells were lysed with sonication, and lysates were centrifuged at 26900 x g for 20 minutes at 4°C. The supernatant was incubated with 10 ml mAPB resin for 30 minutes at 4°C with shaking before loading onto a column. The column was washed with 100 ml of wash buffer (25 mM Tris-HCl, 500 mM NaCl, 50 mM borate, pH 7.0), and then the protein was eluted with 500 mM NaCl, 500 mM borate, pH 7.0. The protein was concentrated using ultrafiltration, followed by dialysis against either 50 mM sodium phosphate, pH 7.0 (used in kinetics experiments) or 5 mM potassium chloride, 5 mM potassium phosphate, pH 7.0 (used in crystallization). The concentration of the protein was determined using the A280 with an extinction coefficient of 93850 M^{-1} cm^{-1}. The dialyzed protein was aliquoted, snap frozen in liquid nitrogen, and stored at -80°C.

Kinetic characterization:

Kinetics measurements were performed on either an Agilent Cary 60 or Cary 100, at 25°C in 50 mM sodium phosphate, pH 7.0. The concentration of enzyme varied from 1 to 9 µM depending on the substrate and enzyme. Rates of hydrolysis were determined for at least 7 different substrate concentrations and the initial velocities were fit to the Michaelis-Menten equation using non-linear

regression analysis to determine K_m and k_{cat} values. Extinction coefficients for substrates were: cefotaxime Δε=-7500 M-1cm-1 at 260 nm; cefoxitin Δε=-7700 M-1cm-1 at 260 nm; oxacillin Δε=+258 M-1cm-1 at 260 nm; CENTA Δε=+6400 M-1cm-1 at 405 nm. For all AmpC enzymes with oxacillin, wt/N152S/N152T with cefoxitin, and wt with cefotaxime, the K_m was too low to be determined by this method, so K_i was determined using a competition with CENTA. Competitions were carried out by varying inhibitor concentrations (from 25 nM to 25 μM, depending on substrate) in the presence of constant amounts of enzyme (from 10 to 150 nM, depending on enzyme) and CENTA (170 to 800 μM, depending on enzyme). The initial velocities were fit to the equation: $v_o = A\text{-}V^*[I]/(K_{dapp}+[I])$. K_i was calculated from the K_{dapp} using the equation: $K_i=K_{dapp}/(1+[CENTA]/K_m(CENTA))$. For all enzymes with oxacillin, N152S/N152T with cefoxitin, and wt with cefotaxime, k_{cat} was determined by measuring hydrolysis at saturating concentrations of substrate, typically 75 to 125 μM, at least 50 times higher than the measured K_i.

Crystal growth:

AmpC N152G crystals were grown using hanging drop diffusion with microseeding over 1.7 M potassium phosphate, pH 8.7. Initial protein concentration in the drop was 88 μM and initial buffer conditions in the drop were 1.1 M potassium phosphate, pH 8.7. Crystals appeared within 2-5 days at 25°C. For the cefotaxime structure, a crystal was soaked in 50 mM cefotaxime in crystallization buffer for two minutes, then dipped in 20% sucrose in crystallizing buffer before freezing in liquid N_2. For the cefoxitin structure, a crystal was soaked in 50 mM cefoxitin in crystallizing buffer for 1.5 minutes, then dipped in 20% sucrose in crystallizing buffer before freezing in liquid N_2.

Data collection and processing:

Data were measured from a single crystal on LS-CAT beam line (21IDD or 21IDF) of the Advanced Photon Source at Argonne National Lab at 100 K using a Mar CCD detector. Reflections were indexed, integrated, and scaled using HKL2000. The structure of the complex was determined with Phaser, using a native apo AmpC structure (PDB entry 1KE4)[16], with water molecules and ions removed, as the initial phasing model. Refinement and electron density map calculations were performed with Refmac5 in the CCP4 Program Suite. Manual rebuilding of the model was done with Coot. For the AmpC N152G-cefotaxime structure, the space group was C2, with four AmpC molecules in the asymmetric unit. Cefotaxime was modeled into each of the active sites based on initial F_o-F_c difference electron density maps and further refined with Refmac5. For the AmpC N152G-cefoxitin structure, the space group was C2, with one AmpC molecule in the asymmetric unit. Cefoxitin was modeled into each of the active sites based on initial F_o-F_c difference electron density maps and further refined with Refmac5.

References

1. Davies, J. (1994). Inactivation of antibiotics and dissemination of resistance genes. *Science*, *264*(5157), 375-382.

2. Fisher, J. F., Meroueh, S. O., & Mobashery, S. (2005). Bacterial resistance to β-lactam antibiotics: Compelling opportunism, compelling opportunity. *Chem. Rev. 105*, 395-424.

3. Neu, H. C. (1992). The crisis in antibiotic resistance. *Science*, *257*(5073), 1064-1073.

4. Choi, S., Lee, J. E., Park, S. J., Choi, S., Lee, S., Jeong, J., & Kim, M. (2008). Emergence of antibiotic resistance during therapy for infections caused by *Enterobacteriaceae* producing AmpC β-lactamase: Implications for antibiotic use. *Antimicrob. Agents Chemother. 52*(3), 995-1000.

5. Livermore, D. M. (1995). β-Lactamases in laboratory and clinical resistance. *Clin. Microbiol. Rev. 8*(4), 557-584.

6. Jacoby, G. A. (2009). AmpC β-lactamases. *Clin. Microbiol. Rev. 22*(1), 161-182.

7. Thomson, K. S. (2010). Extended-spectrum-β-lactamase, AmpC, and carbapenemase issues. *J. Clin. Microbiol. 48*(4), 1019-1025.

8. Trehan, I., Beadle, B. M., & Shoichet, B. K. (2001). Inhibition of AmpC β-lactamase through a destabilizing interaction in the active site. *Biochemistry*, *40*, 7992-7999.

9. Dubus, A., Normark, S., Kania, M., & Page, M. G. (1995). Role of asparagine 152 in catalysis of β-lactam hydrolysis by *Escherichia coli* AmpC β-lactamase studied by site-directed mutagenesis. *Biochemistry*, *34*, 7757-7764.

10. Lefurgy, S. T., De Jong, R. M., & Cornish, V. W. (2007). Saturation mutagenesis of Asn152 reveals a substrate selectivity switch in P99 cephalosporinase. *Protein Sci. 16*, 2636-2646.

11. Goldberg, S. D., Iannuccilli, W., Nguyen, T., Ju, J., & Cornish, V. W. (2003). Identification of residues critical for catalysis in a class C β-lactamase by combinatorial scanning mutagenesis. *Protein Sci. 12*, 1633-1645.

12. Page, M.G.P. (2012). B-lactam antibiotics. In Antibiotic Discovery and Development (Dougherty, T.J. & Pucci, M.J., eds), pp. 79-117, Springer, Berlin, Germany.

13. Greenwood, D. (2008). Antimicrobial drugs : chronicle of a twentieth century medical triumph, pp. 124, Oxford University Press, New York.

14. Beadle, B. M., Trehan, I., Focia, P. J. & Shoichet, B. K. (2002). Structural milestones in the reaction pathway of an amide hydrolase: substrate, acyl, and product complexes of cephalothin with AmpC β-Lactamase. *Structure*, 10, 413–424.

15. Bebrone, C., Moali, C., Mahy, F., Rival, S., Docquier, J. D., Rossolini, G. M., Fastrez, J., Pratt, R. F., Frère, J. M. and Galleni, M. (2001). CENTA as a chromogenic substrate for studying β-lactamases. *Antimicrob. Agents Chemother.* 45, 1868–1871.

16. Powers, R. A., Shoichet, B.K. (2002). Structure-based approach for binding site identification on AmpC beta-lactamase. *J. Med. Chem.* 45, 3222-3234.