

**Understanding the structure and function of proteins involved in the inducible
expression of AmpC β -lactamase**

By

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Abstract

The inducible expression of AmpC β -lactamase is a major cause of β -lactam antibiotic resistance in several clinically relevant Gram-negative bacteria, including the opportunistic pathogen *Pseudomonas aeruginosa*. AmpC induction is regulated by the transcriptional regulator AmpR, which binds to the divergent *ampR-ampC* operon and is activated by 1,6-anhydromuramoyl-peptide – an anabolite of peptidoglycan (PG) recycling that is generated by the *N*-acetyl- β -glucosaminidase NagZ. To investigate the molecular basis of *ampC* induction, the archetypal AmpR protein *Citrobacter freundii* (CfAmpR) was structurally and biophysically characterized. CfAmpR forms a homotetramer that is stabilized by binding the intergenic region of the *ampR-ampC* operon, and it interacts with up to four repressor ligands (UDP-MurNAc-pentapeptide) in an apparent stepwise manner. Moreover, protocols were developed to measure *in vitro* gene expression from the *ampR-ampC* operon by CfAmpR using either radiolabelled mRNA synthesis, or qPCR. Since NagZ generates the AmpR activator ligand, blocking its activity enhances β -lactam efficacy against bacteria with inducible AmpC systems. Crystal structures of NagZ from *Burkholderia cenocepacia* were determined in complex with the glycosidase inhibitor O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-*N*-phenylcarbamate (PUGNAc) and its NagZ-selective derivative ethylbutyryl-PUGNAc, 3-acetamido-4,5,6-trihydroxyazepane (MM-124) and its NagZ-selective derivative MM-156, showing that plasticity within the NagZ active site could be exploited to improve the design of inhibitors that selectively bind NagZ over functionally related human *N*-acetyl- β -glucosaminidases from glycoside hydrolase family 20 (GH20). An improved understanding of the catalytic mechanism of GH20 enzyme function was also explored by determining the crystal structure of a GH20 *N*-acetyl- β -hexosaminidase from *Streptomyces plicatus* in complex with inhibitors from the *N*-acetyl glycal

class, namely *N*-acetyl-D-glucal and *N*-thioacetyl-D-glucal. Furthering our understanding of the role of NagZ inhibition on β -lactam resistance in *P. aeruginosa*, it was found that the NagZ inhibitor PUGNAc could prevent the emergence of high-level AmpC-mediated β -lactam resistance, and significantly enhanced β -lactam susceptibility in synergy with the potent β -lactamase inhibitor avibactam in an *ampC* derepressed *P. aeruginosa* strain. Finally, the potency of avibactam was also structurally defined in a recently discovered class A serine carbapenemase from *Vibrio cholerae*. Collectively, this research explores a number of small molecule based strategies to potentiate β -lactam efficacy against Gram-negative bacterial pathogens.

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List of Acronyms

Å	Angstrom
Ala	Alanine
bp	Base Pair
°C	Degrees Celsius
<i>BcNagZ</i>	<i>Burkholderia cenocepacia</i> Exo-β- <i>N</i> -Acetylglucosaminidase
CAZ	Ceftazidime
CAZy	Carbohydrate-Active enZymes
CCP4	Collaborative Computational Project Number 4
<i>CfAmpR</i>	<i>Citrobacter freundii</i> AmpR Protein
CLS	Canadian Light Source
<i>m</i> -DAP	<i>meso</i> -diaminopimelic acid
DBD	DNA-binding domain
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EBD	Effector-Binding Domain
ESI	Electrospray Ionization
EtBuPUG	ethylbutyryl-PUGNAc
F	Diffracted X-ray Structure Factor Amplitude
FPLC	Fast Protein Liquid Chromatography
GdnHCl	Guanidine Hydrochloride
GH	Glycoside Hydrolases
GlcNAc	<i>N</i> -acetylglucosamine
Glu	Glutamic Acid
hrs	Hours
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
kDa	kiloDalton
K_i	Inhibition Constant (moles/litre)
LTTR	LysR Type Transcriptional Regulator
M	Molar
MALDI	Matrix-Assisted Laser Desorption/Ionization
MBq	Mega Becquerel
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
μg	microgram
mg	milligram
μL	microlitre
mL	millilitre
μM	micromolar
mM	millimolar
MR	Molecular Replacement
MS	Mass spectrometry
MurNAc	<i>N</i> -acetylmuramic acid
MW	Molecular Weight
NagZ	Exo-β- <i>N</i> -Acetylglucosaminidase

<i>N</i> -butyryl-PUGNAc	[[[(3R,4R,5S,6R)-3-(butanoylamino)-4,5-dihydroxy-6-(hydroxymethyl)oxan-2-ylidene]amino]N-phenylcarbamate
Ni-NTA	Nickel-nitrilotriacetic acid
ng	nanogram
nM	nanomolar
<i>N</i> -valeryl-PUGNAc	[[[(3R,4R,5S,6R)-4,5-dihydroxy-6-(hydroxymethyl)-3-(pentanoylamino)oxan-2-ylidene]amino] N-phenylcarbamate
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PEG	Polyethylene Glycol
PG	Peptidoglycan
PMSF	Phenylmethanesulphonylfluoride
PUGNAc	O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-phenylcarbamate
RNA	Ribonucleic Acid
RMSD	Root Mean Square Deviation
SAXS	Small Angle X-ray Scattering
σ	Standard Deviation
UDP-GlcNAc	uridinediphosphate- <i>N</i> -acetylglucosamine
UDP-MurNAc	uridinediphosphate- <i>N</i> -acetylmuramic acid
UV	Ultraviolet
<i>Vc</i> NagZ	<i>Vibrio cholerae</i> Exo- β - <i>N</i> -Acetylglucosaminidase

Chapter 1 – Introduction and literature review

A. Multidrug resistance in bacteria

Environmental microbes have evolved to protect themselves from naturally occurring antibiotics throughout evolution (1), and for the last century, they have been subjected to the hazards of human activities ranging from heavy metals, antibiotics and other toxic compounds (1, 2). Several studies suggest that the development of antibiotic resistance is an ancient, naturally occurring phenomenon (2, 3), which is now dangerously over-represented as a result of the selective pressure exerted on microbes by our use and misuse of antibiotics in agriculture, industry and medicine. With the rise of extensively drug-resistant and totally drug-resistant pathogenic bacteria, humanity is now facing a critical health care crisis that threatens a return to the pre-antibiotic era (1, 4, 5). Indeed, if left unchecked, it is estimated that by 2050, antibiotic resistant infections will kill more people annually than cancer (6).

The difficulty in treating multidrug-resistant (MDR) infections stems from the combination of defensive and offensive measures employed by bacteria. Resistance can be intrinsically encoded in the bacterial chromosome, and/or acquired by spontaneous mutations or horizontal gene transfer from the immediate environment (7, 8). The efficacy of the administered antibiotic can be impeded by: 1) obstructing access to its target, 2) modification of the drug target, and 3) inactivation of the drug.

The outer membranes of Gram-negative bacteria render them intrinsically less permeable than Gram-positive species. Exposure to antibiotics can increase membrane impermeability by the resulting down-regulation of porins, or by alterations imparting greater porin specificity (7, 8). For example, *Pseudomonas aeruginosa*, a frequently multidrug resistant Gram-negative

pathogen, has been shown to develop carbapenem-resistance by decreased expression of OprD, an outer membrane porin (9, 10). Additionally, over-expression of efflux systems such as the resistance-nodulation-division (RND) family in Gram-negative bacteria causes clinical multidrug resistance due to their wide substrate range (8, 11). Recent evidence of an RND pump encoded on a plasmid along with resistance determinants imparting resistance to β -lactam and aminoglycoside antibiotics in the Gram-negative microbe *Citrobacter freundii* is even more concerning considering its potential to be mobilized to additional human pathogens (12).

Antibiotic efficacy can be impeded by structural changes in their targets arising from mutations, such as point mutations in the topoisomerase gene *gyrA* that confer fluoroquinolone resistance (8, 13). Reduced antibiotic affinity can also result from recombination events, as observed between penicillin-binding protein (PBP) genes across some pneumococci and gonococci that result in mosaic penicillin-insensitive PBPs (14). Chemical modification of a drug target like the methylation of 16S rRNA or 23S rRNA also lends protection from antibiotics (8, 13).

Enzyme-catalyzed inactivation of drugs is a significant mode of resistance, and the expression of β -lactamases in particular is clinically pervasive, resulting in hydrolysis of β -lactam antibiotics (7, 8, 13). The over-expression of efflux pumps only serves to heighten the effect of β -lactamases, and these mechanisms frequently co-exist in MDR pathogens such as *P. aeruginosa* (15, 16). Certain functional groups on antibiotics can also be modified by bacterial enzymes, rendering them ineffective. Aminoglycosides are particularly susceptible to this mechanism as their exposed hydroxyl and amide groups can be modified by the transfer of acetyl, phosphate, and nucleotidyl groups (8).

An emerging paradigm of drug discovery is predicated on using our understanding of complex intercellular gene-protein networks to devise combinations of small molecule drugs that maximize their therapeutic effect and suppress the emergence of resistance (17, 18). Successful clinical examples of now classical combinatorial drug therapies include trimethoprim/sulfamethoxazole which synergistically impair folate metabolism, and the β -lactam amoxicillin with clavulanic acid, a β -lactamase inhibitor (17, 19).

While the primary protein targets of clinically used antibiotics are known, current understanding of the series of molecular events culminating in cell stasis and/or death is often less clear (19). A deeper understanding of molecular mechanisms that are involved in the inhibitory events has the potential to reveal novel drug targets that can enhance the lethality of antibiotics. Additionally, given our growing understanding of the importance of the gut microbiome to human health (20, 21), design of a narrow-spectrum antibiotic strategy that seeks to target genus and species-specific pathways in pathogens may ultimately prove more effective at treating infections (17).

Of particular relevance to this thesis is the MDR human pathogen *Pseudomonas aeruginosa*. In addition to colonizing surfaces in medical facilities, *P. aeruginosa* is a frequent concern in the immune-compromised, such as patients with severe burns, cystic fibrosis and those admitted in the intensive care unit (22, 23). In 2011, *P. aeruginosa* was responsible for 7.1% and 8.9% of health-care associated infections in the United States and Europe, respectively (24). Chronic *P. aeruginosa* infections persist for years in the airways of CF patients despite aggressive antibiotic therapy (25). The overall aim of this thesis is to develop a structural, biochemical and microbiological understanding of proteins involved in β -lactam antibiotic

resistance in *P. aeruginosa*, with a primary focus on understanding how metabolites of peptidoglycan (PG) recycling induce the expression of chromosomal AmpC β -lactamase, a clinically problematic mechanism of β -lactam resistance in *P. aeruginosa* and several other clinically relevant Gram-negative bacteria. Using small molecule inhibitors that block PG recycling, potentially in combination with β -lactamase inhibitors during β -lactam antibiotic therapy could provide a useful clinical strategy to suppress AmpC-mediated β -lactam resistance in numerous Gram-negative pathogens.

B. Mode of action of β -lactam antibiotics and resistance

Though it was discovered in 1927, penicillin was not manufactured on a large scale and introduced into the market until 1946 (26). This ushered in the ‘golden age’ of antibiotic drug discovery and the development of naturally-occurring and synthetic derivatives of numerous antibiotics, including a number of β -lactam antibiotic classes. β -Lactams are currently the mainstay treatment for bacterial infections, constituting over 50% of all antibiotics in clinical use (27). β -Lactam antibiotics are structural analogues of the terminal D-Ala-D-Ala which are cross-linked to adjacent peptide chains within the growing PG sacculus by the transpeptidase activity of PBPs (28). The bactericidal effect of β -lactam antibiotics stems from their ability to irreversibly bind to PBPs as mimics of the D-Ala-D-Ala dipeptide (29) and destabilize the biosynthesis of peptidoglycan.

B.1. Peptidoglycan synthesis and inhibition

PG is a heteropolymer consisting of repeating units of β -1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) that are cross-linked by short peptide chains

attached to MurNAc (Fig. 1.1). The pentapeptide stem in Gram-negative bacteria is typically comprised of L-Ala-D-Glu-*meso* diaminopimelic acid (DAP)-D-Ala-D-Ala, while most Gram-positive bacteria contain L-Ala-D-Glu-L-Lys-D-Ala-D-Ala pentapeptides (30). The core pentapeptide composition varies amongst certain bacteria, and substitution of the terminal D-Ala-D-Ala motif with a D-Ala-D-lactate, for example, confers resistance to the antibiotic vancomycin (13). Peptidoglycan synthesis occurs in three stages: first, the PG precursor molecules UDP-GlcNAc and UDP-MurNAc-pentapeptide are synthesized in the bacterial cytosol (31). The transferase MraY catalyzes the transfer of phospho-MurNAc-pentapeptide to the lipid-carrier undecaprenyl-phosphate to generate undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide (lipid I) (32). Next, MurG links the GlcNAc moiety in UDP-GlcNAc to lipid I to form the inner membrane-bound lipid II complex, which is subsequently translocated from the cytoplasmic side to the periplasmic side of the membrane (32, 33). Finally, the disaccharide-pentapeptide of the complex is released from undecaprenyl phosphate and incorporated into the PG sacculus (Fig. 1.1 A) (34). This is achieved by the combined action of glycosyltransferases which polymerize the GlcNAc-MurNAc-pentapeptide to growing glycan strands within the cell wall, and transpeptidases that cross-link the growing glycan strands together via the peptide stem (Fig. 1.1 A, B) (34, 35). A recent study has revealed that in addition to PBPs with dual glycosyltransferase and transpeptidase function, the RodA glycan polymerase from the shape, elongation, division and sporulation (SEDS) family appears to work together with the transpeptidase PBP2 and contributes to cell wall biogenesis (36).

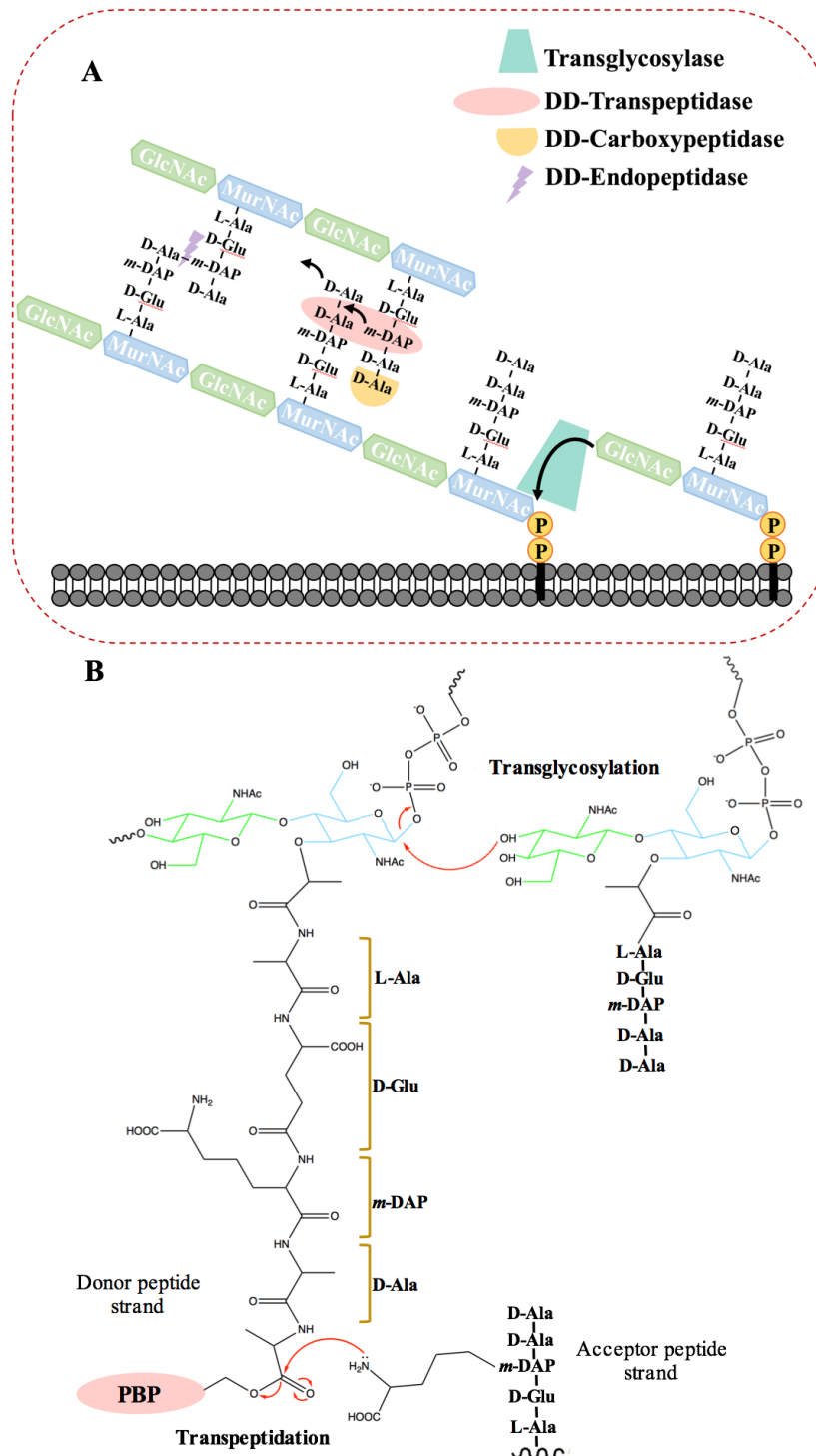


Figure 1.1 – Schematic of transglycosylation and transpeptidation during peptidoglycan (PG) synthesis in Gram-negative bacteria.

A) The PG polymer consists of alternating units of β -1,4-linked GlcNAc-MurNAc residues that are linked via transglycosylation by lytic transglycosylases and penicillin-binding proteins

(PBPs). Attached to the MurNAc residue via a lactyl group is the stem peptide L-Ala-D-Glu-*meso* diaminopimelic acid (DAP)-D-Ala-D-Ala. Transglycosylation releases GlcNAc-MurNAc-peptides from the lipid-bound undecaprenyl phosphate, which is then cross-linked to the growing PG chain by DD-transpeptidases. DD-carboxypeptidases remove the terminal D-Ala residue from the pentapeptide stem, while DD-endopeptidases cleave peptide cross-links during PG repair and recycling. **B)** A chemical schematic shows the transglycosylation and transpeptidation sites in PG subunits. During transpeptidation, the catalytic serine residue of a penicillin-binding protein (PBP) with DD-transpeptidase activity forms a covalent acyl-enzyme bond with the penultimate D-Ala in the donor peptide strand (releasing the terminal D-Ala residue), followed by covalent linkage with the *m*-DAP residue in the acceptor peptide.

B.1.1. Penicillin-binding proteins (PBPs)

Each bacterial species has a variable number of PBPs, which are categorized into high-molecular mass (HMM) or low-molecular mass (LMM) enzymes based on their mass and range of function (37). HMM PBPs include bifunctional Class A enzymes that possess both transglycosylase and transpeptidase activity (PBP1a and PBP1b in Gram-negative bacteria), and Class B D-Ala-D-Ala-dependent transpeptidases (PBP2 and PBP3 in Gram-negative bacteria) (Fig. 1.1 A) (28, 37). LMM Class C PBPs are DD-carboxypeptidases that hydrolyze the terminal D-Ala from the stem pentapeptide to prevent further transpeptidation, or DD-endopeptidases that cleave the peptide cross-links between PG strands (Fig. 1.1 A) (37). LMM PBPs are mainly involved in PG maintenance, repair and recycling. The presence of at least two HMM PBPs (one of each from Class A and Class B) is essential for cell viability (38), and these enzymes constitute the primary targets of β -lactam antibiotics (39).

All PBPs have a penicillin-binding domain responsible for DD-peptidase activity (37). Carboxypeptidation and transpeptidation are initiated by the formation of a reversible, noncovalent interaction between the PBP enzyme and the peptide stem (donor strand; Fig. 1.1 B) (37). Acylation occurs when the catalytic serine residue in the PBP active site launches a nucleophilic attack on the carbonyl carbon of the penultimate D-Ala residue in the donor strand, forming an acyl-enzyme intermediate that causes the terminal D-Ala residue to be released from the active site (Fig. 1.1 B). Deacylation in carboxypeptidases proceeds by hydrolysis and yields a trimmed peptide, whereas in transpeptidases, it is accomplished by cross-link formation with a pentapeptide stem residue (e.g. *m*-DAP) of the neighbouring acceptor strand (Fig. 1.1 B) (37). DD-Endopeptidases function in reverse to the transpeptidases, and hydrolyse the peptide cross-link, instead (34, 37).

The potency of β -lactam antibiotics against PBPs stems from 1) rapid acylation to the enzyme, and 2) slow deacylation (40). It has been proposed that β -lactam antibiotics bind to PBPs with a geometry close to the transition state conformation adopted by the D-Ala-D-Ala motif during transpeptidation (41), and coupled with their innate reactivity, this allows acylation by the catalytic serine to occur rapidly. Slow β -lactam deacylation rates occur due to higher intrinsic resistance to hydrolysis by PBPs, a feature that distinguishes these enzymes from β -lactamases, which, in contrast, rapidly turn over β -lactam substrates (see section B.3 below for details on the biochemistry and classification of β -lactamases) (40).

B.2. Classification of β -lactam antibiotics

β -Lactam antibiotics are broadly classified into 4 major classes: penams, cepheems, carbapenems, and monobactams. All β -lactam antibiotics share the eponymous four-membered β -lactam ring, and except for the monobactams, are bicyclic structures with an additional heterocyclic five or six-membered ring, and a variable side-chain (Fig. 1.2).

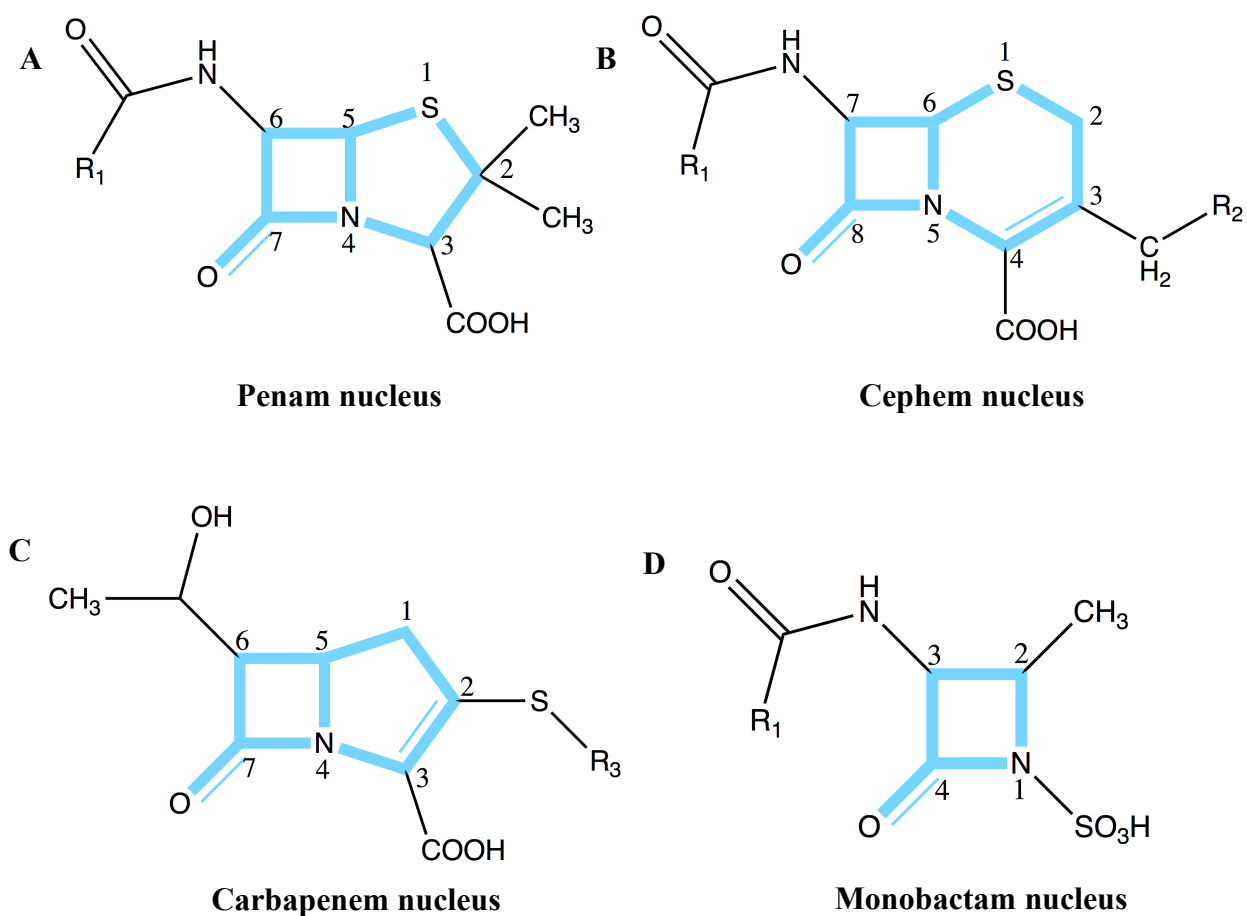


Figure 1.2 – Chemical schematic of the four classes of β -lactam antibiotics.

All β -lactam antibiotics contain a four-membered β -lactam ring. The nucleus of each class of β -lactam antibiotics is shown in blue. **A)** Penams include benzylpenicillin and contain a thiazolidine ring attached to the β -lactam ring. Synthetic derivatives modified at the C3 carboxyl group have been shown to exhibit broad-spectrum activity towards several Gram-negative pathogens. **B)** Cephems include cephalosporins and cephamycins, where the latter possess a methoxy group off C7. Modifications at R_1 and R_2 have been shown to extend target range and increase resistance to β -lactamases. **C)** Carbapenems have increased hydrolytic stability due to the C2=C3 double bond, and the broadest spectrum of inhibition among β -lactams due to their ability to bind several PBPs. **D)** A monobactam in clinical use is aztreonam, which possesses an N_1 sulfonic acid substituent.

Penams – Comprised of penicillin and its derivatives, this class includes benzylpenicillin (penicillin G), which was the first clinically used β -lactam. Subsequent derivatives were created by the addition of side chains to the penicillin nucleus off the C3 carboxyl group (Fig. 1.2 A) (42). Synthetic penicillin derivatives include methicillin, which is resistant to penicillinase (a penicillin specific β -lactamase), and used against Gram-positive infections, extended-spectrum piperacillin and ticarcillin, and the broad spectrum aminopenicillins, ampicillin and amoxicillin, whose amino groups allow them to more readily penetrate the outer membrane of Gram-negative bacteria (42). Nevertheless, the prevalence of β -lactamase-mediated resistance has limited the therapeutic efficacy of numerous penicillins as monotherapies, and it is typical to use them in combination with β -lactamase inhibitors (see B.4. for details on β -lactamase inhibitors). To reduce the likelihood of spontaneous resistance from point mutations, combination therapy can also involve the use of penicillins targeting different PBPs, such as the PBP2-inhibitor mecillinam and PBP1/PBP3-inhibitors ampicillin and piperacillin (43, 44).

Cephems – Whereas both cephalosporins and cephamycins contain the cephem nucleus (Fig. 1.2 B), the latter possesses a methoxy group at C7. Isolated from *Cephalosporium acremonium* in the 1950s (45), the progenitor of this family, Cephalosporin C was resistant to the effects of penicillinase (42). Subsequent generations of cephalosporins modified the original cephem nucleus at C7, R1 and R2 in order to widen their antibacterial target range and increase hydrolytic stability (Fig. 1.2 B). Class I cephalosporins (e.g. cefazolin) were susceptible to hydrolysis by β -lactamases, and were thus followed by class II cephalosporins such as cefuroxime, cephamycins (e.g. cefoxitin), expanded-spectrum cephalosporins of class III (e.g. ceftazidime) and class IV (e.g. cefepime), which exhibited much greater stability against β -lactamase hydrolysis (42). Interestingly, a class V siderophore-substituted cephalosporin (S-

649266) was recently found to have increased penetrability by exploiting bacterial iron-uptake pathways, as well as higher resistance to hydrolysis by carbapenemases (46). The recently developed ceftolozane (class VI) is notable for its efficacy against several Gram-negative bacilli, including multidrug resistant isolates of *P. aeruginosa*, and is now approved for use with the β -lactamase inhibitor tazobactam for the treatment of complicated intra-abdominal and urinary tract infections (47). Cephalosporins also demonstrate a range of binding affinities for PBPs. For example, the class I cephalosporin cephaloridine binds tightly to PBP1a (48), whereas the class III cephalosporin ceftazidime binds with high affinity to PBP3 (49), and the anti-methicillin resistant *S. aureus* (MRSA) cephalosporin ceftaroline (class V) binds to PBP2a (50).

Carbapenems – In carbapenems, the thiazolidine ring sulfur-atom of the penicillin nucleus is replaced with a carbon, and a double bond links carbons C2 and C3 (Fig. 1.2 C), which confers greater chemical and hydrolytic stability (51). Carbapenems (e.g. imipenem) possess broad-spectrum activity due to their ability to bind to both PBP2 and PBP3 (52). They can also bind to PBP1a and PBP1b (53), and notably, to LD-transpeptidases (54), which catalyze the formation of 3,3 PG peptide cross-links rather than the typical 4,3 peptide cross-link. The 3,3 PG cross-link is present in *Mycobacterium tuberculosis*, and the co-administration of carbapenems with the β -lactamase inhibitor clavulanic acid has been found to have a bactericidal effect (55, 56). Although considered antibiotics of last resort, the efficacy of carbapenems is being increasingly eroded by resistance mechanisms such as carbapenemase production or mutations in porins such as OprD, the primary channel of carbapenem entry in *P. aeruginosa* (57–59).

Monobactams –As the only FDA-approved monobactam, aztreonam has an N1-sulfonic acid substituent (Fig. 1.2 D), and binds to PBP3 (56). BAL30072 is a monobactam with a siderophore moiety (60), and is currently in Phase I clinical trials (as of August 2016) for treatment of Gram-negative bacterial infections (42). Highly effective against Gram-negative bacteria, aztreonam is more resistant to hydrolysis by metallo- β -lactamases as its monobactam nucleus does not make it a good substrate, and co-treatment with serine β -lactamase inhibitors may be a promising therapeutic approach in bacteria expressing multiple β -lactamases (42, 61).

B.3. β -Lactamases

β -Lactam antibiotics constitute more than half the antibiotics in clinical use, and comprise 65% of prescribed injectable antibiotics in the United States (42). However, their clinical use has selected for the presence of hundreds of different chromosomal and plasmid-encoded β -lactamases in clinically relevant Gram-negative bacteria (42, 62). Some Gram-negative clinical isolates include multidrug resistant strains of *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *P. aeruginosa*, which can express multiple β -lactamases that render them insensitive to all available β -lactam antibiotics (8).

It has been proposed that serine β -lactamases evolved from PBPs as far back as 3 billion years ago (63), and like their ancestral PBPs, facilitate substrate hydrolysis via a catalytic serine residue, unlike metallo- β -lactamases which use zinc atoms (Fig. 1.3) (62). While PBPs cannot retain both transpeptidation efficiency and the ability to effectively hydrolyze β -lactams (40, 63), β -lactamases have evolved a tighter binding pocket than their ancestral DD-transpeptidases, which allows the accommodation of β -lactams, but not the original D-Ala-D-Ala dipeptide

substrate (63). Since the original functional divergence between PBPs and β -lactamases, hundreds of β -lactamases have evolved that cover a broad-spectrum of β -lactam substrate specificities. The Ambler system classifies β -lactamases based on their primary amino acid sequence into four classes A-D (64), while Bush *et al.* classifies them primarily based on their β -lactam substrate and inhibitor profiles (65). Whereas hydrolysis by class A, C and D β -lactamases is initiated by a nucleophilic attack by the catalytic serine residue on the electrophilic β -lactam ring carbonyl carbon resulting in the formation of an acyl-enzyme intermediate (Fig. 1.3 A), class B β -lactamases are metalloenzymes that use active site zinc atoms to hydrolyze the β -lactam ring (Fig. 1.3 B) (62). This thesis focuses specifically on a class C cephalosporinase known as AmpC, and a recently discovered food-borne class A carbapenemase.

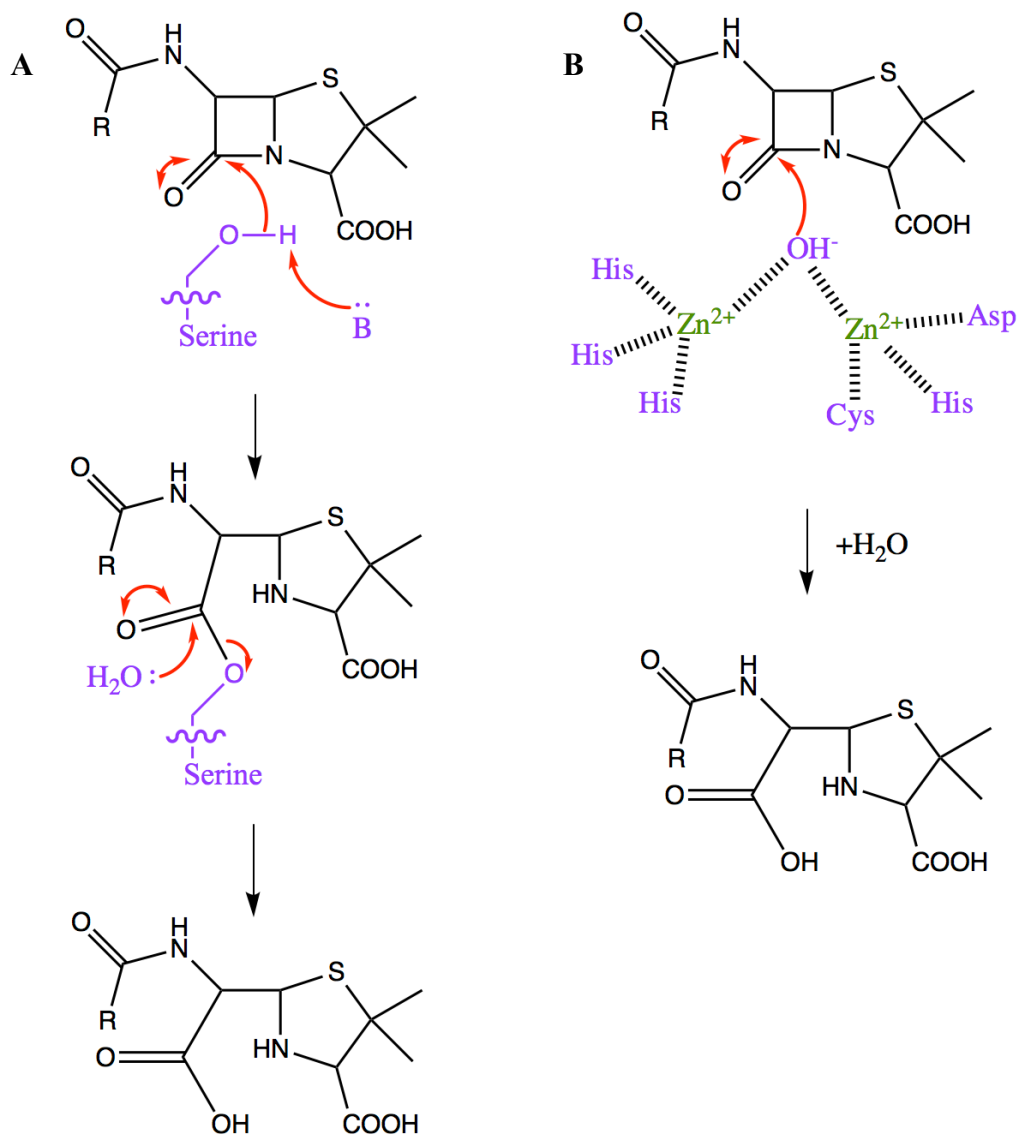


Figure 1.3 – Mechanism of β -lactam hydrolysis by β -lactamases.

A) Simplified schematic of hydrolysis by serine β -lactamases (Ambler class A, C and D). The catalytic serine residue is assisted by a general base in the active site to launch a nucleophilic attack at the site of the electrophilic carbonyl carbon of the β -lactam ring, resulting in formation of a covalent acyl-enzyme intermediate that causes β -lactam ring opening. Deacylation results from covalent bond hydrolysis by a water molecule activated by a neighbouring active site residue (75), yielding an inactive β -lactam product. **B)** Simplified schematic of hydrolysis by metallo- β -lactamases (Ambler class B). Two active-site zinc atoms facilitate hydrolysis of the β -lactam ring via a hydroxide-ion mediated nucleophilic attack of the β -lactam carbonyl carbon.

B.3.1. Class A serine carbapenemases

Serine carbapenemases are capable of inactivating most β -lactam antibiotics, including penicillins, cephalosporins, aztreonam, and of course, carbapenems (57, 62, 66). Class A serine carbapenemases can be chromosomal or plasmid-borne and are found in both Gram-positive and Gram-negative bacteria. Plasmid-encoded class A carbapenemases are far more clinically pervasive, and include KPC (*K. pneumoniae* carbapenemase), which is found on self-conjugative plasmids and can hydrolyze β -lactams of all classes (66, 67). First characterized in *K. pneumoniae*, which is notorious for its ability to disseminate resistance determinants, KPC enzymes are now also found in other enterobacteria, *P. aeruginosa* and *A. baumannii* isolates (66, 67). The global spread of transferable plasmid-encoded class A carbapenemases like KPC is of significant clinical concern, and is currently treated using β -lactams in combination with the β -lactamase inhibitors clavulanate, tazobactam or the recently FDA approved β -lactamase inhibitor avibactam (42, 66). Chromosomally-encoded carbapenemases like SME-1 (*Serratia marcescens* enzyme) and IMI (imipenem-hydrolyzing β -lactamase) are less common and have been characterized in isolates of *Serratia* and *Enterobacter* (66, 67).

B.3.2. Class C AmpC β -lactamases

AmpC β -lactamases are periplasmic proteins found in Gram-negative species that preferentially hydrolyze cephalosporins, such as ceftazidime, cefotaxime and ceftriaxone (62, 68). They can also hydrolyze extended-spectrum β -lactams such as the penam piperacillin, cepheems such as cefoxitin and cefpirome, and monobactams such as aztreonam (62, 68, 69). Interestingly, in *P. aeruginosa*, the combined upregulation of efflux pumps and downregulation of carbapenem-specific porin pumps such as OprD can severely limit the penetration of

carbapenems, thus allowing AmpC to hydrolyze remaining carbapenem molecules even though they are poor AmpC substrates (68). *P. aeruginosa* and many enterobacteria possess a chromosomally encoded *ampC* gene that is expressed only at basal levels, unless expression is induced by the presence of certain β -lactams (68). Mutational derepression of the *ampC* β -lactamase pathway is a frequent occurrence in clinical isolates of *P. aeruginosa* that have been subjected to extended β -lactam treatment and tend to constitutively overproduce AmpC (15, 68, 70). On the other hand, some Gram-negative species do not encode an inducible *ampC* gene, and only express it at low levels, as observed in *E. coli* and *A. baumannii* (68); however, these and other bacteria can also acquire plasmids that very often constitutively overexpress *ampC* (71), sometimes alongside additional genes that impart resistance to aminoglycosides, chloramphenicol, fluoroquinolones, sulfonamide, tetracycline, trimethoprim, and other β -lactamases such as carbapenemases (62, 68, 71).

B.4. β -Lactamase inhibitors

Since the FDA-approval of clavulanic acid in 1984 (72), only three additional β -lactamase inhibitors have been approved for clinical use: sulbactam, tazobactam and, most recently, avibactam (42). Like clavulanic acid, sulbactam and tazobactam are both β -lactam-based and derived from penicillanic acid (Fig. 1.4 A) (73, 74). Clavulanic acid, tazobactam and sulbactam are primarily class A enzyme inhibitors, though tazobactam and sulbactam have varying effectiveness against AmpC (68, 75). Ironically, clavulanic acid actually behaves as a weak inducer of AmpC expression (76).

In 2015, avibactam received FDA approval for use with ceftazidime and is the first clinically available non- β -lactam-based inhibitor of β -lactamases from the class of

diazabicyclooctanes (DBOs) (Fig. 1.4 B). Avibactam displays high potency, broader β -lactamase target range, and a higher resistance to inactivation by hydrolysis (77). Avibactam is a potent inhibitor of AmpC and serine carbapenemases, and has been successfully used to reverse clinical resistance (42, 78, 79). Recently however, AmpC resistance to avibactam has been shown to arise under laboratory conditions (80, 81). Furthermore, resistance to avibactam by the class A serine carbapenemase KPC-3 has been documented, both *in vitro* and clinically (82, 83). Unlike β -lactam-based inhibitors which are irreversibly inactivated by resistant β -lactamases, the DBO ring in avibactam undergoes a reversible recyclization which maintains its potency for additional rounds of inhibition (84). With the success of avibactam, two additional DBOs, relebactam and RG6080 (Fig. 1.4 B) have been developed and are currently in clinical trials for combination therapy with imipenem (85, 86). Another new β -lactamase inhibitor, RPX7009, is under phase III clinical trials, and is a boronic acid, where the boron atom is analogous to the electrophilic carbon in β -lactams (Fig. 1.4 C) (87). Effective against serine β -lactamases, RPX7009 is currently being developed as a serine carbapenemase inhibitor to use in conjunction with meropenem (88).

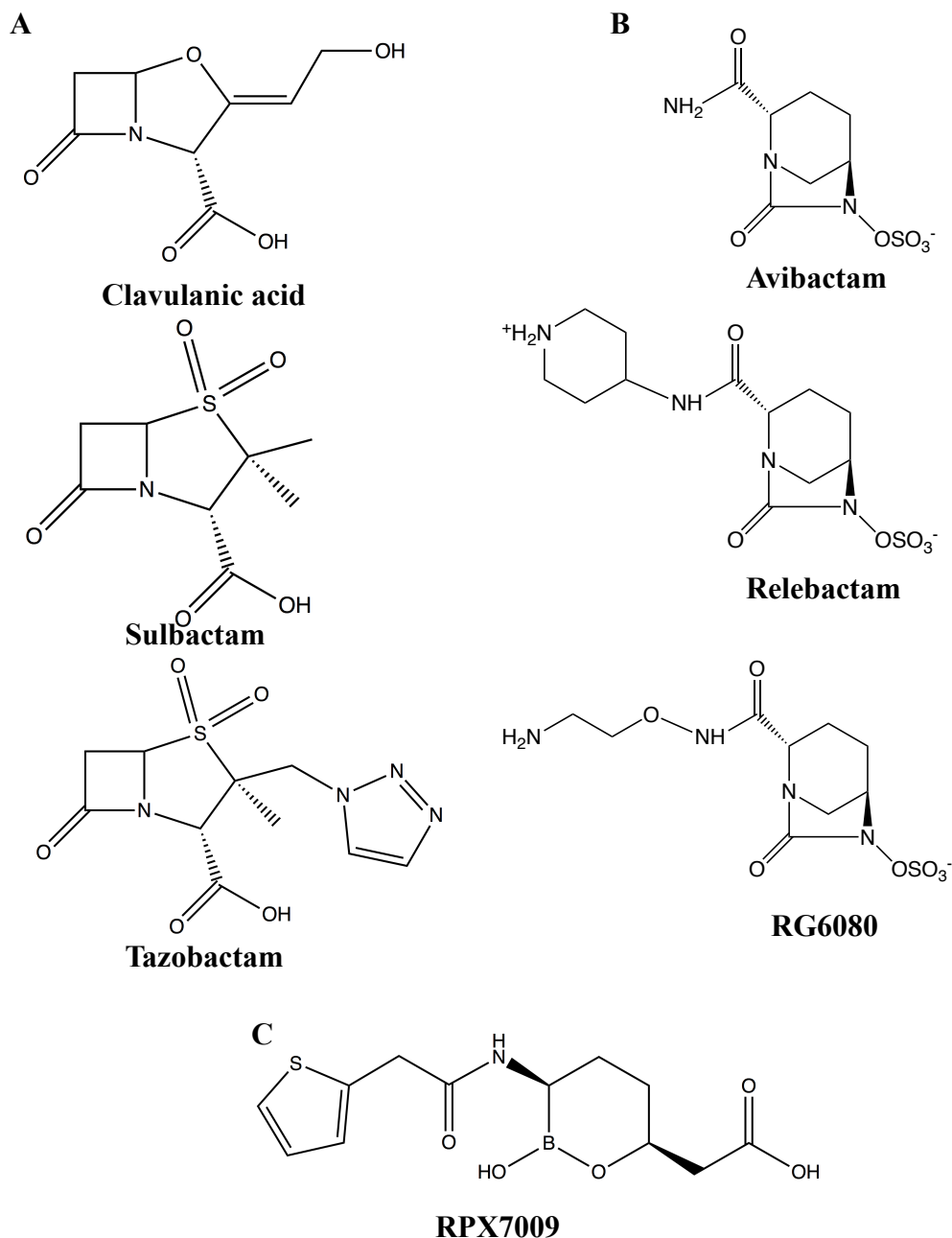


Figure 1.4 – Chemical schematic of the β -lactamase inhibitors.

A) Penicillanic acid derivatives were the first class of β -lactamase inhibitors, and include clavulanic acid, sulbactam and tazobactam. **B)** Diazobicyclooctanes (avibactam, relebactam, and RG6080) are a potent class of inhibitors with higher resistance to β -lactamase inactivation. The carbonyl carbon in the diazobicyclooctane ring is analogous to the electrophilic carbonyl carbon in β -lactams. **C)** Boronic acids, such as RPX7009, have a boron atom that is analogous to the carbonyl carbon in β -lactams.

Obtaining a deeper understanding of the pathways governing β -lactamase-mediated antibiotic resistance mechanisms could provide additional small molecule based strategies that either block β -lactamase activity directly, or block ancillary protein targets that facilitate β -lactamase gene expression. Co-administration of such small molecule inhibitors with existing β -lactams has the potential to provide new alternatives to suppress β -lactam antibiotic resistance and thus contribute towards maintaining the utility of this clinically vital class of antibiotics.

C. PG recycling and its link to inducible AmpC expression

Inducible AmpC gene expression is coupled to PG recycling in Gram-negative bacteria (89, 90). Considering that PG recycling is a conserved process in Gram-negative bacteria, blocking PG recycling using small molecule based approaches is a promising strategy to potentiate β -lactams against Gram-negative bacteria that harbor an inducible *ampC* gene (91, 92).

C.1. PG recycling

As the bacterial cell grows and divides, a host of enzymes steadily break down PG into its component sugar and peptide components (Fig. 1.5) (93–95). This PG turnover allows the PG cell wall to be remodeled as bacteria grow and also creates space for the insertion of cell wall-spanning proteins (93, 96). Given the importance of the PG layer in defining cell shape and resisting internal turgor pressure, PG degradation and recycling is stringently controlled to protect the bacterial cell from lysis (97). While peptide links within the PG layer are hydrolyzed by endopeptidases, carboxypeptidases and amidases, lytic transglycosylases cleave GlcNAc- β (1,4)-MurNAc linkages within the glycan strands (97). Together, these enzymatic activities

generate a pool of periplasmic GlcNAc-1,6-anhydroMurNAc-peptides that are transported to the cytoplasm through the membrane permease AmpG (Fig. 1.5) (98, 99). Of the GlcNAc-1,6-anhydroMurNAc-peptides generated, GlcNAc-1,6-anhydroMurNAc-tetrapeptide makes up the majority, with lesser amounts of -tripeptide and trace amounts of -pentapeptide species comprising the remainder (100). Alternatively, in a minor PG recycling pathway described for *E. coli*, stem peptides can be liberated from the GlcNAc-1,6-anhMurNAc disaccharide by amidases and subsequently enter the cytoplasm via the oligopeptide permease Opp, which is aided by the muropeptide binding protein MppA (101, 102). The free disaccharide is transported in via AmpG (99). In the cytoplasm, GlcNAc is cleaved off the imported PG fragments by the *N*-acetyl- β -glucosaminidase NagZ, generating 1,6-anhydroMurNAc-peptide and free GlcNAc (Fig. 1.5) (103, 104). AmpD, an amidase, then cleaves the stem peptide from 1,6-anhydroMurNAc (105, 106). The majority of the released peptide is the tripeptide L-Ala- γ -D-Glu-*meso*-diaminopimelic acid (DAP), since the tetrapeptide stem on the imported PG fragments is rapidly converted to tripeptide species by LdcA, a cytosolic carboxypeptidase that removes the terminal D-Ala residue from the tetrapeptide (107).

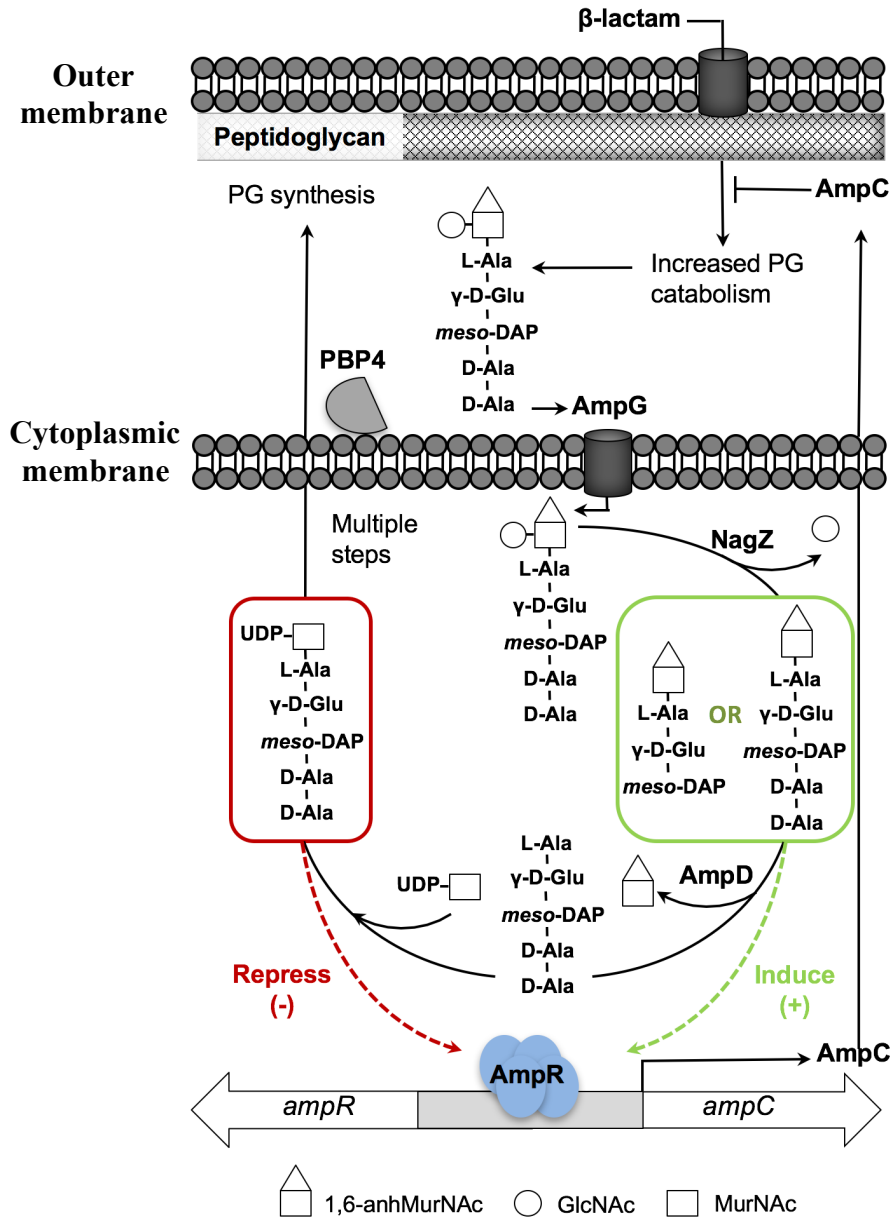


Figure 1.5 – Simplified schematic of peptidoglycan recycling-linked AmpC induction in Gram-negative bacteria. During PG recycling, autolysins break down the PG polymer, releasing GlcNAc-1,6-anhydroMurNac-tri, -tetra and -pentapeptide which enter the cytoplasm via the AmpG permease (only pentapeptide species shown for clarity). The glycoside hydrolase NagZ catalyzes removal of GlcNAc, generating 1,6-anhydroMurNac-peptide. AmpR-mediated activation of *ampC* expression from the divergent *ampR-ampC* operon is induced by either 1,6-anhydroMurNac-tripeptide or -pentapeptide. The amidase AmpD releases the stem peptides from 1,6-anhydroMurNac, and these products are recycled to synthesize UDP-MurNac-pentapeptide, the repressor of AmpR. The presence of β-lactam antibiotics causes increased PG fragmentation that overwhelms the ability of AmpD to break down the AmpR inducer, which outcompetes the repressor UDP-MurNac-pentapeptide to cause an upregulation in *ampC* expression.

The released tripeptide is subsequently transferred to UDP-MurNAc by the mucopeptide ligase Mpl, which can also ligate PG derived pentapeptides to UDP-MurNAc (108, 109). In the case of UDP-MurNAc-tripeptide, a terminal D-Ala-D-Ala peptide is attached by the murine synthase MurF (110), and the resulting UDP-MurNAc-pentapeptide product is directed into Lipid II synthesis before finally being incorporated into the PG sacculus (31, 32). These mechanisms prevent the incorporation of UDP-MurNAc-tetrapeptide into the cell wall, which has been shown to distort cell morphology since transpeptidases can only use the pentapeptide species to cross-link glycan chains within the PG sacculus (107). The released 1,6-anhydroMurNAc and GlcNAc sugars are phosphorylated by the sugar kinases AnmK (111) and NagK (112), respectively, and MurNAc-6-P is further processed into GlcNAc-6-P by the etherase MurQ (113). The resulting pool of GlcNAc-6-P is then either absorbed into PG synthesis or redirected towards glycolysis (114).

C.2. AmpC induction

To be inducible, the *ampC* gene must be correctly associated with an additional gene, *ampR*, which codes for a transcriptional regulator that controls the expression of both genes. Together, the genes form a divergent *ampR-ampC* operon (Fig. 1.5) (115). Based on a DNase I protection assay, Lindquist *et al.* identified a 38-bp binding site for AmpR in the intergenic region of the *ampR-ampC* operon from *Citrobacter freundii*, which enables the regulator to associate with the *ampC* promoter as well as the promoter of its own gene (115). By binding to this intergenic region, AmpR was found to autorepress its own expression while controlling expression of *ampC* in response to specific metabolites generated by the PG recycling pathway (Fig. 1.5).

Jacobs *et al.* discovered that upon transformation of *E. coli* with a plasmid bearing the *ampR-ampC* operon, *ampD* null mutants, which commonly arise in the presence of β -lactams, exhibit derepressed production of AmpC β -lactamase and a significant accumulation of 1,6-anhydroMurNAc-tripeptide in the cytoplasm (89). This finding led to the hypothesis that 1,6-anhydroMurNAc-tripeptide acts as the secondary messenger that binds to and converts AmpR into an activator of *ampC* expression (89, 90). *In vitro* studies subsequently showed that UDP-MurNAc-pentapeptide represses the ability of AmpR to activate *ampC* expression, whereas addition of 1,6-anhydroMurNAc-tripeptide restores *ampC* expression, implying that UDP-MurNAc-pentapeptide and 1,6-anhydroMurNAc-tripeptide act antagonistically on AmpR to either repress or induce *ampC* expression, respectively (Fig. 1.5) (90).

Though 1,6-anhydroMurNAc-tripeptide accumulation in the *ampD* mutant was confirmed by Dietz *et al.* (116), 1,6-anhydroMurNAc-pentapeptide was also found to accumulate to a lesser extent. Furthermore, in growing wild-type *E. coli* cells transformed with the *ampR-ampC* operon from *E. cloacae*, β -lactam treatment led to a 2 to 4-fold increase in cytosolic 1,6-anhydroMurNAc-tripeptide concentrations, whereas strongly inducing β -lactams such as imipenem led to a 25-fold increase in 1,6-anhydroMurNAc-pentapeptide levels relative to that induced by weak AmpC β -lactamase inducers (116). This remarkable increase was attributed to inhibition of DD-carboxypeptidases such as PBP 4,5 and 6 (which typically cleave the terminal D-Ala from stem pentapeptides in the PG layer) in addition to essential PBPs (37, 116). By preventing DD-carboxypeptidase activity, strong AmpC β -lactamase inducers like cefoxitin and imipenem allowed GlcNAc-MurNAc-pentapeptides to accumulate in the periplasm. After generation of 1,6-anhydroMurNAc-pentapeptide in the cytoplasm by NagZ, this pentapeptide species was proposed as the inducer of AmpR activity and corresponding AmpC β -lactamase

production (Fig. 1.5) (116). It has thus remained controversial which PG metabolite, the 1,6-anhydroMurNAc-tripeptide or –pentapeptide species is the bona fide activator of AmpR.

To add further uncertainty as to which molecule activates AmpR, Park and Uehara demonstrated that large quantities of free pentapeptide are produced in the cytoplasm of Gram-negative bacteria when challenged with cefoxitin, and a smaller amount of 1,6-anhydroMurNAc-pentapeptide (93). Though there is strong evidence to support UDP-MurNAc-pentapeptide as the repressor of AmpR-mediated *ampC* β -lactamase induction (90), the precise identity of the competing inducer ligand remains controversial and is a topic of considerable interest in order to fully understand the AmpC induction pathway. The present consensus is that in bacteria harboring the *ampR-ampC* system, the absence of β -lactam antibiotics allows AmpR to be repressed by UDP-MurNAc-pentapeptide, thus maintaining *ampC* expression at basal levels (91, 92, 117). Exposure to β -lactams causes increased PG degradation relative to synthesis, thereby causing PG recycling metabolites to accumulate in the cytosol, which displace UDP-MurNAc-pentapeptide from AmpR and convert it into an activator of *ampC* transcription (91, 92, 117). AmpC produced by this activation mechanism is then exported to the periplasm where it deactivates the β -lactam antibiotic in an attempt to restore PG homeostasis (Fig. 1.5) (68, 117).

C.3. Clinically relevant mutations causing *ampC* derepression in *P. aeruginosa*

Inducible *ampC* expression is the foremost cause of clinical resistance to β -lactams in *P. aeruginosa*, and is particularly concerning as this opportunistic pathogen is associated with chronic lung infections in cystic fibrosis patients and is a frequent cause of severe nosocomial infections (22, 70, 118, 119). Although β -lactams such as imipenem strongly induce *ampC* expression, imipenem is not easily hydrolyzed by AmpC and is thus effective against AmpC

producing bacteria (68). Interestingly, while susceptible to hydrolysis by AmpC, some late generation β -lactams such as ceftazidime and cefepime are weak inducers of AmpC induction and thus they too exhibit antipseudomonal activity (120). Unfortunately, clinical use of antipseudomonal β -lactams frequently selects for chromosomal mutations in *P. aeruginosa* that can dramatically boost AmpC production to levels that confer clinically relevant resistance to these β -lactams, such as mutations in the OprD porin that confer carbapenem resistance (68).

The most common mutations cause loss-of-function in the genes for the cytosolic AmpD amidase (121–125), and PBP4 (encoded by *dacB*) (126–128), which is a dual function DD-endopeptidase/DD-carboxypeptidase. Indeed, high-level β -lactam antibiotic resistance in *P. aeruginosa* is often caused by combined mutations in *ampD* and *dacB* (122, 127). Interestingly, *P. aeruginosa* expresses two *ampD* homologs, namely *ampDh2* and *ampDh3*, and it has been shown that the inactivation of each homolog leads to a stepwise derepression of *ampC* and concomitant increase in β -lactam resistance (129). However, the hyperproduction of AmpC resulting from the inactivation of all three *ampD* genes leads to reduced fitness and virulence (130), and inactivation of all these genes has not been observed clinically (131). In addition to driving *ampC* overexpression, inactivation of *dacB* gene also specifically activates the two-component CreBC (carbon-source responsive)/BlrAB (β -lactam resistance) system, which has been implicated in regulating global metabolic processes as well as high-level β -lactam resistance in Gram-negative bacteria such as *P. aeruginosa*, and appears to be linked with PG recycling and *ampC* induction by an as yet undetermined mechanism (127, 132).

Mutations in *ampR* have also been reported to cause constitutive high-level AmpC expression (122, 133), albeit at a frequency much lower than mutations in *ampD* and *dacB*,

potentially due its wider role as a global transcriptional regulator in *P. aeruginosa* (134, 135). The *ampC* gene is itself subject to polymorphisms in *P. aeruginosa*, and certain mutations have been shown to extend AmpC substrate specificity towards extended-spectrum cephalosporins, including the recently developed molecule ceftolozane (formerly CXA-101) (81, 136–138).

AmpC induction, thus, heavily depends upon proteins involved in PG recycling, and while the loss AmpD and/or PBP4 activity greatly increases β -lactam resistance in *P. aeruginosa*, the inactivation of other PG recycling proteins such as the AmpG permease and glycoside hydrolase NagZ have been shown to suppress the effects of *ampD* and/or *dacB* mutations and thus reverse β -lactam resistance in these commonly arising *P. aeruginosa* mutants. Thus, blocking PG recycling at strategic points within the pathway using small molecule based approaches presents a promising strategy to overcome AmpC-mediated β -lactam resistance in this microbe, as discussed below.

D. Targeting PG recycling to suppress AmpC-mediated β -lactam resistance

The glycoside hydrolase NagZ is directly responsible for catalyzing the formation of the AmpR inducer molecules 1,6-anhydroMurNAc-tri and –pentapeptide species by removing GlcNAc from these molecules upon entry to the cytoplasm (FIG 1.5) (103, 104). Due to their key roles in inducing AmpC β -lactamase expression, AmpG, NagZ and AmpR are all attractive targets for the development of small molecules that block their function to suppress AmpC production during β -lactam therapy. This thesis is focused on the characterization of NagZ and AmpR.

D.1. NagZ

NagZ is an *exo-N*-acetyl- β -glucosaminidase and belongs to the glycoside hydrolase family 3 (GH3) (139, 140). NagZ enzymes possess a conserved active site aspartate that serves as a catalytic nucleophile (141) and a unique consensus motif that contains a histidine general acid/base catalyst (142, 143). The two residues participate in removing GlcNAc from GlcNAc-1,6-anhydromuramoyl-peptide using an enzyme-assisted two-step double-displacement mechanism (Fig. 1.6 A) (141, 144). In the first step, the aspartate acts as a nucleophile to attack the anomeric carbon of GlcNAc, while the histidine behaves as a general acid to assist the departure of the anhydromuramoyl-peptide leaving group (Fig. 1.6 A). This results in a covalent glycosyl-enzyme intermediate that is broken down in the second step, where the catalytic histidine acts as a general base to activate an incoming water that cleaves the glycosyl-enzyme linkage and releases GlcNAc from the enzyme with retention of anomeric stereochemistry (Fig. 1.6 A). Alternatively, a recent study suggests that GH3 catalysis is facilitated by an incoming phosphate molecule that yields GlcNAc-1-P (145). Use of histidine as a general acid/base, as opposed to glutamate or aspartate (146), appears to enable GH3 *N*-acetyl- β -glucosaminidases to accommodate a negatively charged phosphate in the active site (145, 147); however, the presence of a histidine general acid/base is not always indicative of phosphorolytic activity in GH3 enzymes (148) and it remains to be determined which product GH3 NagZ enzymes generate.

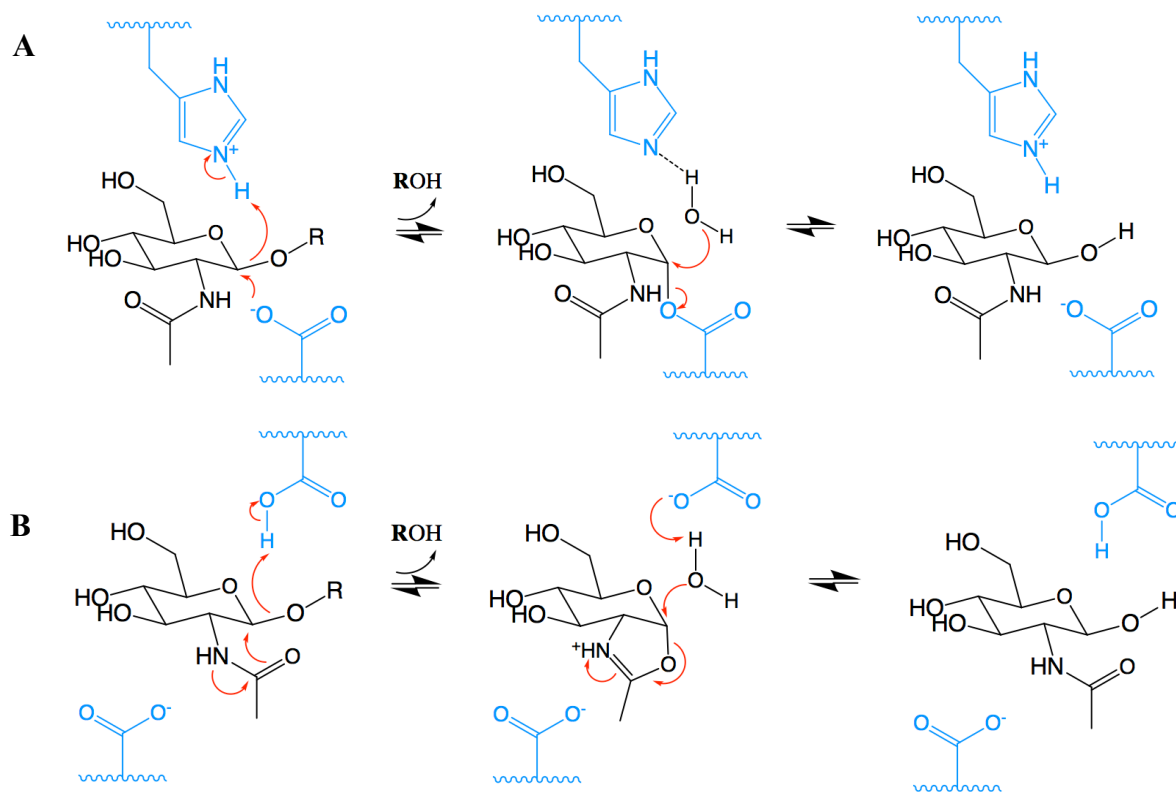


Figure 1.6: Catalytic mechanism of glycoside hydrolases.

A) GH3 enzymes like NagZ proceed via an enzyme-assisted catalytic mechanism, where an aspartate residue behaves as the catalytic nucleophile to form a glycosyl-enzyme intermediate with the substrate. This step is assisted by a histidine residue in NagZ, which is the general acid/base. This histidine residue behaves as a base in the next step, enhancing the nucleophilicity of an incoming water molecule that attacks the anomeric carbon of the covalent intermediate, releasing a hemiacetal product with retention of stereochemistry. **B)** GH20 and GH84 enzymes utilize a substrate-assisted mechanism, where the nucleophilic 2-acetamido group of the substrate attacks the anomeric carbon, generating an oxazolinium ion intermediate. Assisted by a carboxylate-containing active site residue, an incoming water molecule facilitates the release of a hemiacetal product with retained stereochemistry.

Interestingly, in contrast to GH3 NagZ enzymes, functionally related human *N*-acetyl- β -glucosaminidases from GH20 and GH84 use a substrate-assisted catalytic mechanism that does not proceed through a glycosyl-enzyme intermediate (Fig. 1.6 B) (144, 149, 150). Instead, the carbonyl oxygen of the 2-acetamido group of GlcNAc acts as the nucleophile, attacking the anomeric carbon of the sugar to yield an oxazolinium ion intermediate (Fig. 1.6 B). Departure of the leaving group is assisted by general acid catalysis from an active site glutamic acid residue, which acts as a general base in a second step to activate an incoming water that attacks the anomeric carbon of oxazolinium ion intermediate to release GlcNAc with retained anomeric stereochemistry (Fig. 1.6 B).

The mechanistic differences between GH3 NagZ and *N*-acetyl- β -glucosaminidases from GH20 and GH84 have been exploited to develop bacterial PG recycling inhibitors that selectively block NagZ over functionally related human enzymes from GH20 and GH84 (151, 152). *N*-Acetyl- β -glucosaminidases from all three families are potently inhibited by the small molecule inhibitor PUGNAc (O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate) (Fig. 1.7 A) (153, 154). However, the active site architectures of GH20 and GH84 differ sufficiently from NagZ to allow bulky substituents to be installed on the 2-acetamido group of PUGNAc to make the inhibitor selective for NagZ over GH20 and GH84 enzymes (151), such as the PUGNAc derivatives *N*-butyryl-PUGNAc and *N*-valeryl-PUGNAc (Fig. 1.7 A) (155). The active sites of GH20 and GH84 *N*-acetyl- β -glucosaminidases must envelop the 2-acetamido group to guide it toward the anomeric center of GlcNAc, which leaves minimal space around the group for functionalization (Fig. 1.7 B) (151). This is not the case for NagZ, where the 2-acetamido group projects into an open pocket in the NagZ active site, where bulky additions to the group can be accommodated (Fig. 1.7 C) (142, 151).

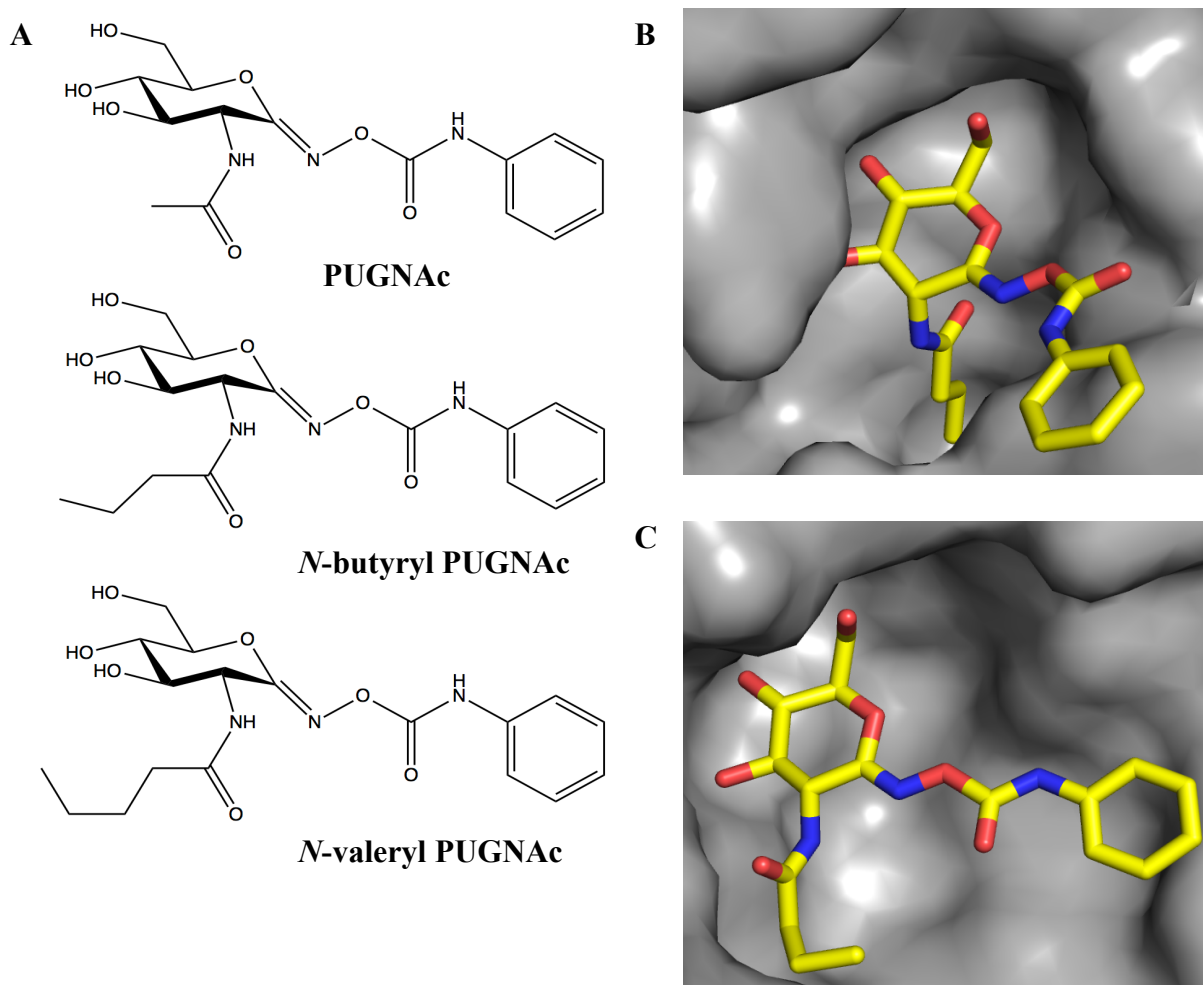


Figure 1.7. Structural basis for NagZ-selective inhibitors.

A) Schematic of the glycosidase inhibitor PUGNAc, and its 2-*N*-acyl NagZ-selective derivatives, *N*-butyryl PUGNAc and *N*-valeryl PUGNAc. **B)** A surface representation of a GH84 human *O*-GlcNAcase homologue from *Bacteriodes thetaiotamicron* in complex with *N*-butyryl PUGNAc (PDB ID 2WCA) shows that the inhibitor fits into a tight binding pocket, where additions to the 2-*N*-acyl group are not easily accomodated. **C)** In NagZ from *Vibrio cholera*, *N*-butyryl PUGNAc binds in an open solvent-exposed pocket (PDB ID 3GS6), where modifications to the 2-*N*-acyl group are easily accommodated, unlike family GH20/84 enzymes, and underscores the basis for design of NagZ-selective inhibitors.

The genetic inactivation of *nagZ* has been shown to decrease the minimum inhibitory concentration (MIC) of ceftazidime for the *ampC*-derepressed *P. aeruginosa ampD-dacB* double mutant from 96 µg/ml to 4 µg/ml (156–158). Pharmacologically blocking NagZ function using PUGNAc in conjunction with β-lactam antibiotics also discernibly attenuates β-lactam resistance in *P. aeruginosa ampD* and *dacB* null mutants. Similarly, in combination with cefoxitin or ceftazidime, ethylbutyryl-PUGNAc (EtBuPUG), the most potent NagZ-selective PUGNAc derivative, caused up to a twofold or fourfold reduction in β-lactam resistance in *P. aeruginosa ampD* null mutants, respectively (152, 156). Remarkably, population analysis of *P. aeruginosa* has demonstrated that *nagZ* inactivation also appears to prevent the emergence of spontaneous β-lactam resistant mutants at ceftazidime concentrations ≥ 8 µg/ml (susceptibility breakpoint) for the *nagZ* mutant, unlike the wild-type, for which spontaneous resistance mutants were obtained even at 16 µg/ml ceftazidime (157).

Similarly to *P. aeruginosa*, the inducible expression of *ampC* in *E. cloacae* is regulated by NagZ (159, 160), but was recently found to also proceed via a poorly understood NagZ-independent mechanism that appears to be a consequence of the type of β-lactam used (161). *Stenotrophomonas maltophilia* is a nosocomial pathogen that expresses two β-lactamases L1 and L2 in response to the AmpR-mediated sensing of PG recycling metabolites via NagZ-dependent and independent pathways (162, 163). More recently, L1/L2 β-lactamase induction has been shown to be part of a complex regulatory network including lytic transglycosylase activity and the CreBC system (164). While NagZ inactivation in *P. aeruginosa* was shown to suppress CreBC-mediated β-lactam resistance, its precise role in influencing the CreBC system requires further exploration (157).

D.2. AmpR

AmpR is a 32 kDa protein from the LysR-type transcriptional regulator (LTTR) family. LTTR-regulated gene products encompass a range of diverse functions such as those involved in amino acid biosynthesis, quorum sensing, virulence and antibiotic resistance (165). LTTR proteins typically form homotetramers in solution and constitute the largest and most ubiquitous known family of DNA-binding transcriptional regulators in bacteria, often acting as activators and/or repressors of single or divergently transcribed genes in response to co-effector molecule binding (165, 166). Co-effector molecules are frequently metabolites of biosynthesis pathways involving LTTR activity (167, 168), and can either co-induce or co-repress gene transcription by binding to an LTTR (169). Most LTTRs repress transcription in the absence of an effector molecule and only initiate transcription upon binding an activator ligand (167, 168, 170). AmpR, however, is controlled by both, a repressor ligand (UDP-MurNAc-pentapeptide) and an activator ligand (1,6-anhydroMurNAc-tri or -pentapeptides), providing an additional level of metabolite-controlled regulation over AmpC expression (90, 116).

Like other LTTRs, AmpR has a well-conserved N-terminal DNA-binding domain (DBD) containing a winged helix-turn-helix motif (usually spanning residues 20-80), and a C-terminal effector-binding domain (EBD) (171). It recognizes and binds to a 38 base-pair (bp) region within the overlapping promoters of the *ampR-ampC* operon (Fig. 1.8), shown by Lindquist *et al.* (115) to be protected by AmpR in DNase footprinting assays. This region includes a canonical LTTR high-affinity T-N₁₁-A motif (165, 166) and, according to the ‘sliding-dimer’ model of LTTR proteins (165, 166), may also include two additional low-affinity binding sites for AmpR that directly control *ampC* gene expression as described below (Fig. 1.8). When LTTR proteins tetramerize, the N-terminal DNA-binding domains of the monomers work together in pairs to

bind DNA. While one pair of DNA-binding domains binds with high-affinity to the T-N₁₁-A motif (166), the other pair is thought to be mobile and interacts with at least two low-affinity sites that are close to the RNA polymerase recognition sites (-35 site) of the regulated gene (172–174). According to the sliding dimer model, interaction of the mobile pair of DNA binding domains to these sites causes DNA bending to occur, and when the pair binds to a low-affinity site near the -35 site they shield the site from the RNA polymerase and suppress transcription. However, when bound to an appropriate effector ligand, conformational changes in the LTTR protein prompt the domains to 'slide' upstream to an alternate low-affinity site, relaxing the DNA bend and exposing the -35 site to the polymerase to permit transcription (165, 166). While some LTTRs have been shown to interact with the α C-terminal domain (CTD) subunit of RNAP (175, 176), the interactions between LTTR-RNA polymerase are still not well understood (165, 166).

Though primarily considered for its role as a key regulator of AmpC β -lactamase, AmpR has been shown to regulate genes involved in the expression of a novel class D β -lactamase, virulence and quorum sensing in *P. aeruginosa* (134, 177). With evidence suggesting that AmpR may have a broader role to play in bacterial metabolism and pathogenesis (135, 178, 179), further structural and biochemical characterization of AmpR to identify the inducer molecules as well as effector binding sites can expedite the process of small molecule inhibitor design to suppress AmpR activity, and consequently AmpC β -lactamase resistance and virulence.

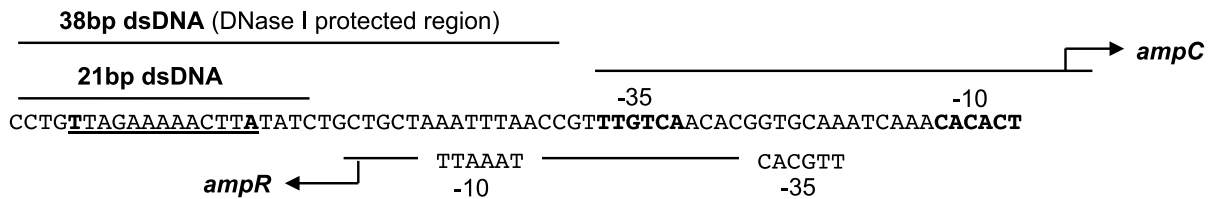


Figure 1.8: The intergenic region of the *ampR-ampC* operon of *C. freundii* containing AmpR binding sites.

This region contains the high affinity LTTR binding motif T-N₁₁-A (underlined) and the promoter regions of the divergently transcribed *ampR* and *ampC* genes (ref), which according to the sliding dimer model of LTTR proteins (165) are predicted to contain low affinity binding sites for AmpR. The sequences of the 21- and 38-bp DNA that were bound to AmpR in Chapter 2 are as indicated, with the latter region corresponding to the DNase-protected region identified by Lindquist *et al.* (115).

E. Thesis objectives

Overall, this work investigates β -lactam antibiotic resistance, with a primary focus on understanding the AmpC β -lactamase induction pathway. The objectives of this thesis are as follows:

- 1) Elucidating the regulatory mechanism of AmpR – The molecular events responsible for turning AmpR into an activator of *ampC* expression are still unknown. Accordingly, the interactions of AmpR from *C. freundii* with its repressor UDP-MurNAc-pentapeptide and operator DNA were investigated using a combination of non-denaturing mass spectrometry, X-ray crystallography, and small angle X-ray scattering (SAXS). Since the identity of the AmpR inducer molecule remains controversial, an *in vitro* transcription protocol was developed to generate mRNA from the *ampR-ampC* operon from *C. freundii* in the presence of AmpR. Methods were devised to measure relative changes in gene expression either directly by synthesis of radiolabelled mRNA, or indirectly by RT-qPCR.
- 2) Targeting the glycoside hydrolase (GH) NagZ using small molecule inhibitors – The structural basis for development of selective inhibitors of the GH3 enzyme NagZ over functionally related GH20 enzymes was investigated using crystal structures of NagZ from *Burkholderia cenocepacia* in complex with general and NagZ-selective inhibitors. Additionally, crystal structures of a model GH20 β -*N*-acetylhexosaminidase from *Streptomyces plicatus* in complex with *N*-acetyl-glycal inhibitors were used to explain their potency and provide further structural and biochemical insights into the differences between human GH20 hexosaminidases and bacterial GH3 NagZ enzymes.

Interestingly, the genetic inactivation of *nagZ* has previously been shown to prevent the rise of spontaneous β -lactam resistant mutants in *P. aeruginosa* (PAO1) (156, 157), and in this work, a population analysis was performed to investigate whether blocking NagZ pharmacologically using the glycosidase inhibitor PUGNAc could achieve similar results. Moreover, whole-genome sequencing was used to determine the source of mutations in genes that give rise to spontaneous AmpC-mediated β -lactam resistance in *P. aeruginosa* (PAO1). Additionally, the ability of PUGNAc and the new β -lactamase inhibitor avibactam to work synergistically to optimally restore β -lactam susceptibility in the *ampC* derepressed strain PA Δ *dacB* was investigated.

3) Determining the structural basis of inhibition of a novel carbapenemase – Recently, a class A serine carbapenemase was isolated from *Vibrio cholerae* in shrimp imported into Canada (180). Fortunately, we found that this new *V. cholerae* carbapenemase (VCC-1) can be effectively blocked by avibactam, and a crystal structure of VCC-1 was determined in its unliganded form and in complex with avibactam to gain insight into its catalytic mechanism and mode of inhibition by this recently approved β -lactamase inhibitor.

Chapter 2 – The β -lactamase gene regulator AmpR is a tetramer that recognizes and binds the D-Ala-D-Ala motif of its repressor UDP-N-acetylmuramic acid (MurNAc)-pentapeptide

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A. Introduction

LTTR proteins consist of a C-terminal effector-binding domain (EBD), and an N-terminal DNA-binding domain (DBD) comprised of a winged helix-turn-helix motif (166). Low solubility is a distinctive commonality shared by proteins within the LTTR family, and has been attributed to the tendency of DBDs to form extensive oligomerization contacts that result in protein aggregation (181, 182). Due to similar problems with solubility of full-length AmpR, a crystal structure of only the AmpR EBD was previously solved to gain insight into its potential mode of binding with its co-effector molecules (171). The native AmpR EBD structure was shown to form a dimer in solution (171), and while full-length AmpR was expected to associate as a dimer of dimers in solution like most LTTRs (165, 166), this had not been experimentally

confirmed. Modeling studies based on the AmpR EBD structure were used to speculate on the binding site of its co-effectors 1,6-anhydroMurNAc-peptide and UDP-MurNAc-pentapeptide, but in the absence of a crystal structure of the protein in complex with these co-effectors, the suggested binding mode awaited experimental verification (171).

While a number of crystal structures of full-length LTTRs have been determined, including one bound to its natural activator ligand (183), none have been determined in complex with a natural repressor molecule. Although a crystal structure of the LTTR BenM DBD was determined in complex with its operator DNA (184), the lack of full-length structures of LTTR proteins in complex with their DNA operator sequences means that the interactions with promoter elements and associated conformational changes that underpin the switch from gene repression to activation remain poorly understood.

This chapter provides new structural insights into the archetypal AmpR protein from *Citrobacter freundii* to further our understanding of how this LTTR protein modulates AmpC β -lactamase gene expression. Non-denaturing mass spectrometry (MS; performed in collaboration with Drs. Lynda Donald and Ken Standing) shows that AmpR is a tetramer that possesses two independent DNA binding sites that each have high affinity for the LTTR T-N₁₁-A DNA binding motif. Moreover, the non-denaturing MS analysis reveals that AmpR is able to bind up to four molecules of UDP-MurNAc-pentapeptide. To investigate how AmpR binds UDP-MurNAc-pentapeptide, I completed the determination of the crystal structure of the AmpR EBD bound to UDP-MurNAc-pentapeptide, which is the first structure of an LTTR bound to a natural repressor ligand. Interestingly, this structure revealed that the terminal D-Ala-D-Ala motif of UDP-MurNAc-pentapeptide plays a central role in the interaction of the ligand with AmpR.

Finally, small angle X-ray scattering (SAXS) studies (performed in collaboration with Dr. Trushar Patel) show that full-length AmpR tetramer bound to DNA adopts a conformation remarkably similar to AphB, an LTTR protein involved in virulence gene regulation in *Vibrio cholera*. The AmpR-DNA complex also appears to undergo a subtle conformational change upon binding UDP-MurNAc-pentapeptide. Modeling the repressor bound AmpR:DNA complex based on our SAXS data and the AphB structure, suggests that while the pentapeptide motif of the repressor and activator ligands are involved in binding AmpR, it is likely the UDP-MurNAc- and 1,6-anhydroMurNAc- moieties of these molecules are responsible for modulating AmpR function at the quaternary level and regulate its ability to repress or activate *ampC* transcription, respectively.

B. Experimental procedures

B.1. Protein expression and purification of the AmpR EBD

The AmpR EBD was expressed and purified for crystallography by Thomas Reeve (Mark lab alumnus, Dept. of Microbiology, University of Manitoba). *E. coli* BL21 (DE3) GOLD HTE cells harboring pMB400 (171) were grown to mid-log phase [optical density at 600 nm (OD_{600}) = 0.5] at 37 °C, with shaking, in 500 ml volumes of LB media supplemented with 35 µg/ml kanamycin. Expression of the AmpR EBD was induced with 1 mM IPTG for 4 h at 28 °C, with shaking. Cells were pelleted by centrifugation and stored at -80 °C. Pellets were thawed in 20 ml of ice-cold lysis buffer [1 M NaCl, 20 mM Tris-HCl (pH 8.0) and 1 µM PMSF] and lysed using a French pressure cell press (AMINCO). The lysate was clarified by centrifugation and loaded onto a Ni-NTA agarose column that had been pre-equilibrated with binding buffer [1 M NaCl and 20 mM Tris-HCl (pH 8.0)]. The column was washed with 10 column volumes of binding

buffer, followed by 2 column volumes of binding buffer supplemented with 25 mM imidazole (pH 7.4) and 50 mM imidazole (pH 7.4), respectively. The AmpR EBD protein was eluted from the column using binding buffer supplemented with 250 mM imidazole (pH 7.4). The eluate was dialyzed overnight against 300 mM NaCl and 20 mM MES (pH 6.0). Chromatographic steps were performed using AKTA FPLC. All centrifugation, chromatography and dialysis steps were conducted at 4 °C.

B.2. Protein expression, refolding and purification of full-length AmpR

The full-length *ampR* gene [Genbank ID: GU322411.3] was PCR- amplified from *Citrobacter freundii* ATCC8090 genomic DNA using oligonucleotide primers 5'-AGATATACATATGACGCGTAGCTATATCCCTC-3' and 5'-GATATAACTAGTTTTGTGCAGCACCCCGGTC-3' and ligated into a modified pET-28 vector using NdeI and SpeI restriction sites to generate plasmid pMB-AmpR (plasmid constructed by Dr. Misty Thomas, Mark lab alumna, Dept. of Microbiology, University of Manitoba). *E. coli* BL21 (DE3) GOLD HTE cells transformed with plasmid pMB-AmpR were grown in 4 x 500 ml volumes of LB media supplemented with 35 µg/ml kanamycin to an OD₆₀₀ of 1.0 at 28°C. AmpR expression was induced with 1 mM IPTG for 16 hours at 28°C, with shaking. Cells from 4 x 500 ml cultures were combined together to yield 2 x 1L volumes of culture, pelleted by centrifugation, resuspended in lysis buffer [50 mM Tris-HCl (pH 7.0) and 1 µM PMSF], and lysed using a French pressure cell press (AMINCO). The lysate was then centrifuged at 12,000 rpm for 1 hour, and the pellets from each combined 1L preparation reconstituted in 4 ml resuspension buffer (RB) [500 mM KCl, 10% glycerol, 10 mM Tris-HCl (pH 7.0) and 1 mM DTT] with 0.1% Triton X-100. Resuspended pellets were centrifuged again at 12,000 rpm for 20 minutes at 4°C, supernatant discarded, and pellets washed with RB

containing 0.5% Triton X-100. Subsequently, the centrifugation and wash steps were repeated using RB, then RB containing 10 mM guanidine hydrochloride (GdnHCl), and RB containing 50 mM GdnHCl, and finally in a final reconstitution buffer of RB only. The resuspended pellets were then sonicated (Branson Sonicator) in 2 x 15 bursts each at 50% duty cycle at a power setting of 5. The sonicated pellet resuspensions were then combined and denatured in 16 ml of denaturation buffer [2 M GdnHCl, 10% glycerol, 10 mM Tris-HCl (pH 7.0), and 1 mM DTT] at room temperature for 1 hour, with vortexing every 10 minutes.

The denatured pellet was centrifuged for 40 min at 12,000 rpm at 4°C, and the supernatant poured into a syringe fitted with a stopcock. Denatured protein was slowly dripped drop-wise into 350 ml refolding buffer [300 mM KCl, 10% glycerol, 10 mM Tris-HCl (pH 7.0) and 1 mM DTT] at 4°C with slow stirring. Refolded AmpR was centrifuged for 40 min at 3700 rpm at 4°C to pellet insoluble protein, then vacuum-filtered through a 0.22 µm cellulose membrane and concentrated to a final concentration of 0.7 mg/ml prior to being loaded on to a size-exclusion gel filtration column (Superdex 200) pre-equilibrated in gel filtration buffer [300 mM KCl, 10% glycerol, 10 mM Tris (pH 7.0) and 1 mM DTT] at 4°C. Gel filtration fractions collected at a flow rate of 1 ml/min containing purified AmpR were pooled together in the presence of 50 mM NDSB-195 to prevent protein aggregation before further concentration and biophysical analyses.

B.3. Preparation of double-stranded DNA (dsDNA)

Pairs of oligonucleotides were purchased from AlphaDNA (Montreal) or Integrated DNA Technologies (IDT) to prepare 21bp and 38 bp dsDNA corresponding to the AmpR binding site in the intergenic region of the *ampR-ampC* operon (Fig. 1.8). Oligonucleotide set 1 (21 bp) had only the T-N₁₁-A motif: sense strand 5'-CCTGTTAGAAAACTTATATC-3' (MW 6388.2 Da);

antisense strand 5'-GATATAAGTTTTCTAACAGG-3' (MW 6459.3 Da). Set 2 (38 bp) had both the T-N₁₁-A motif with the additional region shown to be protected during DNase footprinting (115): sense strand 5'-CCTGTTAGAAAACTTATATCTGCTGCTAAATTTAACC-3' (MW 11594.6 Da); antisense strand 5'-GGTTAAATTTAGCAGCAGATATAAGTTTTCTAACAGG-3' (MW 11754.7 Da). Equimolar amounts of each pair of sense and antisense DNA oligonucleotides were added to buffer containing 50mM NaCl, 10mM Tris-HCl (pH 7.9), 10mM MgCl₂, 1mM DTT (NEBuffer 2; New England Biolabs) and annealed by heating for 3 minutes at 98°C, with a slow cool for 1 hour to room temperature.

B.4. Binding dsDNA to AmpR

dsDNA was added to purified AmpR in a molar excess of 2:1 to the purified AmpR protein. Concentrated AmpR:dsDNA complex was purified by gel filtration (Superdex 200) in a column pre-equilibrated in gel filtration buffer [300 mM KCl, 10% glycerol, 10 mM Tris (pH 7.0) and 1 mM DTT] at 4°C. Purified AmpR:dsDNA fractions collected at a flow rate of 1 ml/min were pooled together and stored at 4°C.

B.5. Binding and purification of AmpR:dsDNA with UDP-MurNAc-pentapeptide

A molar excess of UDP-MurNAc-pentapeptide (BaCWAN, University of Warwick) was added to the purified AmpR:21 bp dsDNA complex, left to bind at room temperature (RT) for 30 minutes, and then reloaded on to a Superdex 200 gel filtration column pre-equilibrated in gel filtration buffer at RT. Purified AmpR:21 bp dsDNA:UDP-MurNAc-pentapeptide gel filtration fractions were pooled together and concentrated for analysis by dynamic light scattering, small-angle X-ray scattering and mass spectrometry, or stored at 4 °C for up to 5 days.

B.6. Non-denaturing Mass Spectrometry (MS)

Dr. Lynda Donald (Dr. Ken Standing's lab, Dept. of Physics, University of Manitoba) was provided with purified protein samples in order to perform MS analysis as described below. After refolding and purification of full-length AmpR, the pure protein was provided to Dr. Donald, who digested the protein with trypsin and analysed it by MALDI mass spectrometry to confirm its identification. The MS spectra were acquired on a prototype MALDI QqToF instrument (185). For analysis of full-length AmpR and the AmpR:dsDNA complexes with and without UDP-MurNAc-pentapeptide by MS, the samples were desalted into 0.3 to 0.5 M ammonium acetate buffer (Aldrich 99.99%) with 1 mM DTT (Aldrich 99%), using "waterbugs" (186, 187). Samples were then diluted to about 2 μ M and inserted into a New Objective PicoTip™ nanospray capillary attached to an electrospray time-of-flight mass spectrometer built for these kinds of experiments (188). The declustering voltage was adjusted to give the best resolution (189) but was also used to test the relative stability of large non-covalent complexes (examples in 40, 41). It was found that UDP-MurNAc-pentapeptide was lost during dialysis of full-length AmpR:dsDNA into volatile buffer. Therefore, the AmpR:dsDNA complex dialyzed into 250 mM ammonium acetate and 1 mM DTT was mixed with increasing concentrations of repressor UDP-MurNAc-pentapeptide (dissolved in water) at 0, 5, 10 and 50 μ M, keeping the final concentration of AmpR:dsDNA complex fixed at 2 μ M. The mixtures were then held on ice for 1 h before the mass spectrometry experiments. All data were analysed using TOFMA, software developed with the instruments, in the department of Physics and Astronomy at the University of Manitoba.

B.7. Crystallization of the AmpR EBD bound to UDP-MurNAc-pentapeptide and X-ray data collection

Crystallization and X-ray data collection of the AmpR EBD in complex with UDP-MurNAc-pentapeptide were performed by Thomas Reeves, while I processed and determined the crystallographic structure. Purified wild-type AmpR EBD was concentrated to 4.5 mg/ml using an Amicon Ultra Centrifuge spin cartridge (Merck Millipore) and co-crystallized with 5mM UDP-MurNAc-pentapeptide. X-ray diffraction data were obtained from a single crystal formed in a solution of 10% PEG 3350, 9% glycerol and 100 mM MES pH 6.2 via hanging drop vapour diffusion at room temperature. Crystals were flash cooled in liquid N₂ after replacing the mother liquor in the drop with a cryosolution consisting of 35% PEG 4000 and 15% glycerol.

X-ray diffraction data were collected from a single crystal (held at 100 K in an N₂ gas stream) using beamline 08ID-1 at the Canadian Light Source (Saskatoon, Canada). The X-ray data were indexed using Mosflm (190), then scaled and averaged using SCALA (191). The AmpR EBD: UDP-MurNAc-pentapeptide complex structure was determined by molecular replacement (MR) using PHASER (PHENIX package, 45) and a structure of the AmpR EBD (PDB ID: 3KOS) as the search model (from which all solvent and ligands had been removed). The MR model was rebuilt using phenix.autobuild (192). The restraint file for UDP-MurNAc-pentapeptide was generated using PHENIX eLBOW (192), and the ligand manually fit into its electron density in the complex. Further refinement and model building was carried out using phenix.refine (192) and Coot (193).

B.8. Dynamic light scattering (DLS)

The dynamic light scattering profile for a complex of full-length AmpR bound to 21 bp dsDNA containing the high affinity LTTR binding motif from the *ampR-ampC* promoter (Figure 1.8) with and without UDP-MurNAc-pentapeptide was measured using the Zetasizer Nano S system (Malvern Instruments Ltd., Malvern, UK) as described earlier (194). AmpR:21 bp dsDNA was filtered through a 0.1 μm filter via centrifugation, and diluted in 300 mM KCl, 10% glycerol, 10 mM Tris-HCl (pH 7.0) and 1 mM DTT, to final concentrations ranging from 2.9-4.1 mg/ml. Similarly, AmpR:21 bp dsDNA:UDP-MurNAc-pentapeptide complexes were diluted within 0.8- 2.3 mg/ml. For each measurement, the protein was allowed to equilibrate for 4 minutes at 20°C prior to DLS measurements, after which multiple records of the DLS profile were collected for data analysis.

B.9. Small-angle X-ray scattering (SAXS)

SAXS data collection and analysis were performed by Dr. Trushar Patel (Dr. Joerg Stetefeld's lab, Dept. of Chemistry, University of Manitoba). SAXS data for the AmpR:21 bp dsDNA complex and for AmpR:21 bp dsDNA:UDP-MurNAc-pentapeptide complex were collected at multiple concentrations (3, 4, 4.6 and 4.8 mg/mL and 0.8, 1.0, 1.4, and 1.8 mg/mL respectively) using a Rigaku 3-pinhole camera (S-MAX3000) equipped with a MicroMax+002 microfocus sealed tube and Confocal Max-Flux (CMF) optics operating at 40 W, as described earlier (194). The system was also equipped with 200 mm multi-wire 2D detector for data collection. SAXS data were collected at a wavelength of 1.54 Å (λ) with a scattering vector (q) ranging from $0.008 \leq q \leq 0.26 \text{ Å}^{-1}$ [$q = (4\pi \sin\theta)/\lambda$, where 2θ is the scattering angle] with 3 hours of exposure for each concentration. Raw data were processed and merged together using the

program PRIMUS (195) to obtain a single output file (represented graphically in the inset to Fig. 2.6 B), which was further processed using GNOM (196) to obtain the radius of gyration (R_g) and maximum particle dimension (D_{max}) for both complexes as previously described (197, 198). Multiple *ab initio* models for the AmpR:21 bp dsDNA complex and for the AmpR:21 bp dsDNA:UDP-MurNAc-pentapeptide complex were built using the program DAMMIF (199) with enforced particle symmetry of P22. These models were then rotated and averaged using the program DAMAVER (200) to obtain a representative model.

The program HYDROPRO (201) was employed to calculate hydrodynamic properties such as the R_h , R_g and D_{max} for each *ab initio* model as described earlier (194, 197). The partial specific volume of 0.738 mL/g for AmpR was calculated using SEDNTERP from its amino acid sequence whereas that for DNA was considered to be 0.57 mL/g (202). By using equation 1 from Dzananovic *et al.*, 2013 (203), and considering the M_w of the CfAmpR tetramer (130.8 kDa) and 21 bp dsDNA (25.7 kDa), the partial specific volume of 0.710 mL/g was obtained. Buffer density (1.03 g/mL) and viscosity (1.276 cP) was also calculated using the program SEDNTERP (204) and was used as the input parameters to run HYDROPRO.

B.10. Structure coordinates

The structure of the AmpR EBD in complex with UDP-MurNAc-pentapeptide was deposited in the PDB under the accession code 4WKM.

C. Results and discussion

C.1. *C. freundii* AmpR is a tetramer in solution with two active DNA-binding domains

C. freundii AmpR was over-produced as inclusion bodies in *E. coli* BL21 (DE3) GOLD HTE cells. Following denaturation of inclusion bodies in 2 M guanidine hydrochloride, the protein could be refolded and then purified using an approach significantly modified from that originally described by Bishop *et al.* (205) (see section B.2). Refolded AmpR was obtained as a single peak of ~130 kDa by gel filtration, indicating that the protein is a tetramer in solution. The purified full-length protein was stable only at low concentrations (< 1 mg/ml). The poor stability and solubility of LTTR proteins (165) is often associated with their DNA-binding domains which have been suggested to oligomerize in solution in the absence of DNA, thus contributing to their aggregation and precipitation (181, 182). However, binding AmpR to 21 bp or 38 bp dsDNA fragments of the *ampR-ampC* intergenic promoter containing the T-N₁₁-A motif only, or the complete region that is protected by AmpR during DNase footprinting, respectively (Fig. 1.8, chapter 1), greatly stabilized the protein and allowed it to be concentrated to ~ 4.8 mg/ml for biophysical studies.

Nanospray MS was used to measure the mass of the components in AmpR:dsDNA complexes to investigate the oligomeric state of the folded protein, and to determine the stoichiometry of AmpR complexed with either the 21 bp or 38 bp dsDNA. The measured mass of the AmpR monomer was 32725 Da, identical to the predicted mass of the recombinant protein (Fig. 2.1 D & E); and MS analysis also revealed transient ions consistent with a tetramer (Fig. 2.2). The AmpR tetramer was considerably more stable when bound to DNA and the spectra indicated that the difference in mass of the most abundant ions could be accounted for by the mass of two molecules of dsDNA bound to one AmpR tetramer (Fig. 2.3). For the AmpR:21 bp dsDNA complex, the observed mass of 156916 Da (Fig. 2.3 A) agreed well with that expected for a tetramer bound to two copies of dsDNA, calculated to be 156590 Da. Similarly, for the AmpR:38 bp dsDNA complex (Fig. 2.3 B), the observed mass of 177825 Da agreed with the calculated 177596 Da. Both spectra show a less abundant species that is probably from a dimeric form of AmpR.

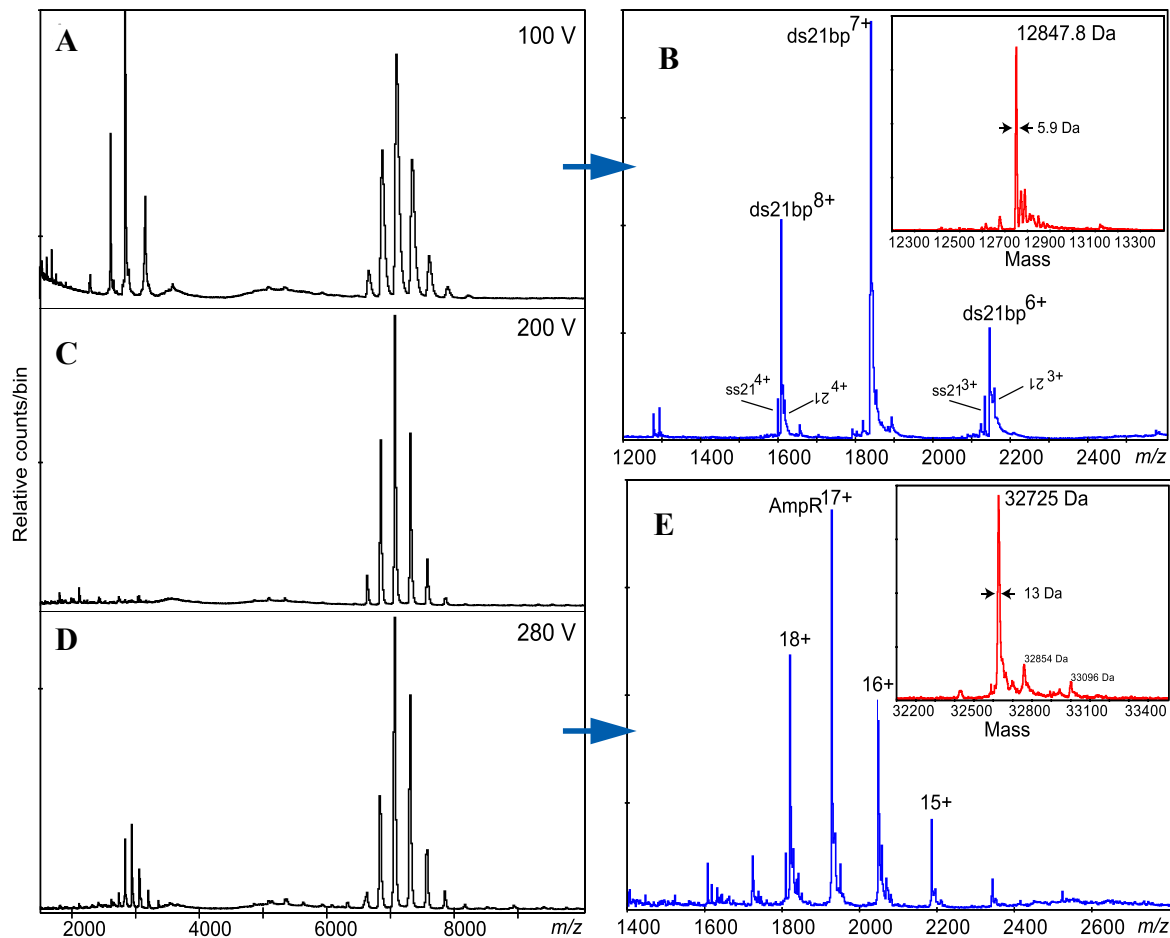


Figure 2.1 - Nanospray spectra from the AmpR: 21 bp dsDNA complex

A capillary containing AmpR: 21 bp dsDNA was subjected to changing declustering voltage, thus changing the internal energy of the ions, and revealing different components of the AmpR: 21 bp dsDNA complex. **A)** At low voltage, there are the major ions of the complex, and monoisotopic resolution of free ds and ssDNA. **B)** Expansion of the spectrum at low m/z , and, in the inset, deconvolution of the labeled ions. The mass of each oligonucleotide comprising the dsDNA was 6388.2 and 6459.3 Da, respectively, with dsDNA expected at 12847.5 Da. Ions flanking the 6+ and 8+ of the dsDNA ions are the 3+ and 4+ ions of the sense and antisense oligonucleotides, at monoisotopic resolution. A similar experiment with the AmpR: 38 bp dsDNA complex verified the mass of the 38 bp dsDNA to be 23350 Da (data not shown). **C)** Under control conditions (200V), there is only one major ion envelope (see Fig. 2.4A). **D)** At maximum voltage, a new ion envelope from the protein monomer is present at low m/z . **E)** Expansion of the low m/z region and, in the inset, deconvolution of the main ions give a protein with mass 32725 Da and a small amount at 32824 Da, indicating some retention of the initial Met residue. From the published amino acid sequence, the expected mass of AmpR is 32523 Da (Swissprot P12529). The AmpR protein in this study has four extra vector derived amino acids, TSGS, on the C-terminus. Addition of that extra mass gives an expected value of 32855 Da, or 32724 Da if the initial Met is processed off.

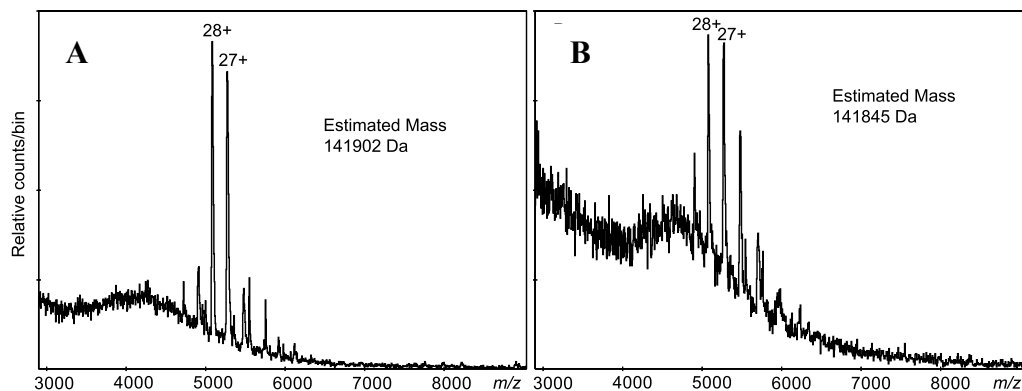


Figure 2.2 – Nanospray spectra showing transient AmpR tetramer species.

A) The beginning acquisition of the purified protein prepared without dsDNA, and buffer-exchanged into 0.3 M ammonium acetate, 1 mM DTT. Within 5 min, this ion envelope was replaced by a series best explained by aggregation; **B)** The beginning acquisition of the AmpR: 21 bp dsDNA complex showing the same ions. Within 5 min, this ion envelope was replaced by the one shown in Fig. 2.3A. Although the observation was transient, it happened frequently, always at the very beginning of the experiment when there might be residual salts in the new capillary being used for the test. Mass was manually determined from the m/z values of the labeled ions.

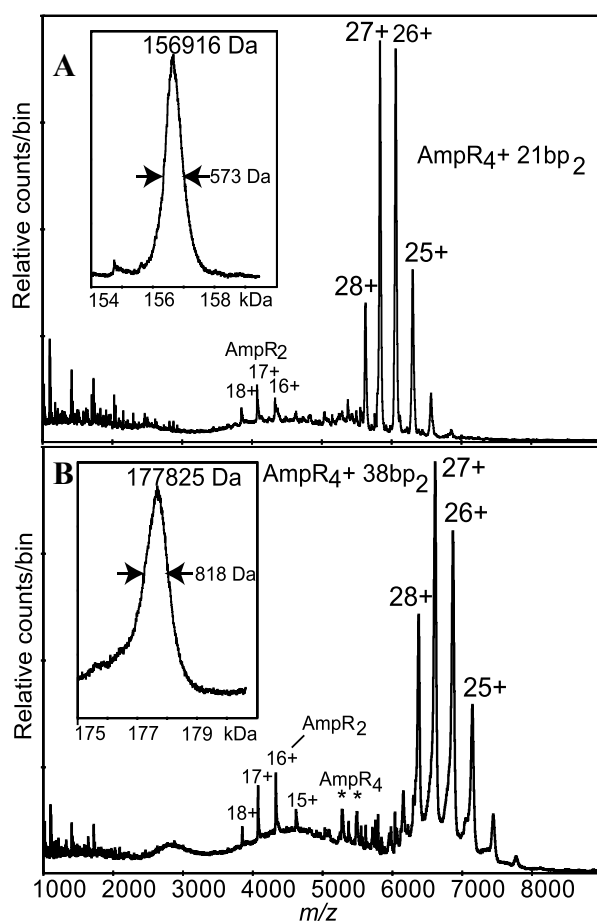


Figure 2.3 – Nanospray spectra of the complexes of AmpR with 21 and 38 bp dsDNA.

Insets are deconvolutions of the major labeled ions. The Full-Width-Half-Maximum (FWHM) indicated at the arrows is a measurement of error (see (189)). **A)** With the 21 bp dsDNA containing only the T-N₁₁-A motif there is one major charge envelope, the 24+ to 28+ ions of a species of mass 156916 Da (FWHM 573 Da). A minor series, 16+ to 18+ belongs to a species of mass 68987 Da (FWHM 22 Da). The complex was in 100 mM ammonium acetate buffer and the declustering voltage was 200 V. **B)** With the 38 bp dsDNA containing both the T-N₁₁-A motif and two putative low-affinity binding sites, as with 21 bp dsDNA, there is one major ion envelope with almost the same charge states, but shifted to higher m/z with measured mass 177825 Da (FWHM 818 Da). Also present in low abundance are ions at m/z 4000 (as in A), and unresolved ions in the m/z 5000 region, some of which, marked *, may be from the tetramer (see Fig. 2.2). The complex was in 50 mM ammonium acetate, 0.5 mM DTT and the declustering voltage was 200 V.

Also present in the sample, but only seen at low declustering voltage, were ions from the DNA (Fig. 2.1 A, B), which meant that some dissociation was occurring in the mass spectrometer. If each AmpR tetramer was able to bind both the T-N₁₁-A motif and a putative low-affinity site present in the 38 bp fragment of dsDNA at the same time, then that complex would have been smaller, only 154246 Da. However, the same 4:2 binding stoichiometry of AmpR monomer to dsDNA was observed with both the 38 bp and 21 bp dsDNA. These data suggest that each pair of DNA-binding domains of tetrameric AmpR preferentially binds the high-affinity T-N₁₁-A site on the DNA molecules in our experiments, rather than binding in tandem to the high- and low-affinity binding sites, as is assumed to occur *in vivo*.

C.2. The AmpR:dsDNA complex binds up to four UDP-MurNAc-pentapeptide repressor molecules

Non-denaturing mass spectra collected from mixtures of 2 μ M AmpR:21 bp dsDNA complex and increasing amounts of UDP-MurNAc-pentapeptide (up to 50 μ M) revealed four repressor-binding sites (Fig. 2.4). At 5 μ M repressor (Fig. 2.4 B) the most intense ions correspond to those of the unliganded AmpR: 21 bp dsDNA complex, having charge states ranging from 24⁺-28⁺. However, an additional series of ions appeared with the same charge states but with an m/z ratio equivalent to the complex plus one repressor molecule (Fig. 2.4 B). At 10 and 50 μ M concentration of UDP-MurNAc-pentapeptide (Fig. 2.4 C, D), additional series of ions appear, and increase in intensity while the abundance of the unliganded species decreases. At the 50 μ M concentration of UDP-MurNAc-pentapeptide there are five ions at each charge state, with a difference in m/z between each series corresponding to the expected m/z for the repressor molecule (mass 1,193 Da). These MS results provide a direct determination of the binding stoichiometry of UDP-MurNAc-pentapeptide to the AmpR:DNA complex. The data

reveal that the tetramer can bind four molecules of UDP-MurNAc-pentapeptide (Fig. 2.4 D), though full saturation was not possible because the high concentration of the repressor and associated sodium overloaded the detector on the mass spectrometer. The observed binding stoichiometry suggests that the effector-binding domain of each monomer in the AmpR tetramer binds one molecule of repressor. This proposed binding model is supported by our crystal structure of the AmpR EBD bound to UDP-MurNAc-pentapeptide as described below.

It is well known that AmpR is able to 'tune' *ampC* expression in response to various levels of activator ligand, such as the stepwise increase in AmpC production that occurs when the three AmpD homologues are systematically deleted from *P. aeruginosa* (129). Based on this observation, we suggest that four UDP-MurNAc-pentapeptide molecules are needed to most efficiently repress AmpR, and as the concentration of the activator ligand increases during β -lactam exposure, AmpR-mediated *ampC* transcription increases in a step-wise manner until all four UDP-MurNAc-pentapeptide molecules have been displaced by activator ligand, at which point AmpR drives *ampC* transcription at its maximal rate.

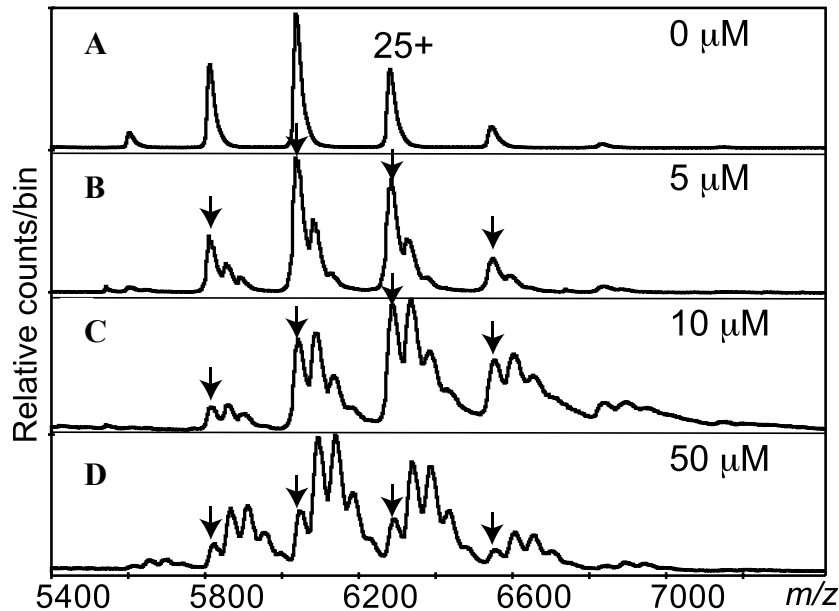


Figure 2.4 – Nanospray spectra of the AmpR:21 bp dsDNA complex mixed with increasing amounts of UDP-MurNAc-pentapeptide.

Arrows indicate the ions at the same m/z as those in the control. Spacing between the adducts can be explained by sequential additions of the repressor molecule, with up to four present. **A)** Control, no repressor; **B)** 5 μM repressor; **C)** 10 μM repressor; **D)** 50 μM repressor. High ion counts at low m/z from the free repressor molecules and fragments thereof caused serious interference at higher concentrations of repressor. All samples were in 125 mM ammonium acetate, 0.5 mM DTT, and all spectra were at 200 V declustering voltage.

C.3. AmpR recognizes and binds the peptide motif of UDP-MurNAc-pentapeptide

Though we were unable to crystallize full-length AmpR, its effector-binding domain (EBD) (residues 94-289) could be crystallized in complex with UDP-MurNAc-pentapeptide. The structure of the complex was determined by molecular replacement using the unliganded AmpR EBD (PDB ID: 3KOS) as a search model (171) and refined to 2.15 Å (see Table 2.1 for crystallographic and refinement statistics).

The AmpR EBD is known to dimerize in solution (171) and it crystallized in space group P2₁2₁2 when bound to UDP-MurNAc-pentapeptide, with four homodimers present within the asymmetric unit. Each monomer of the AmpR EBD dimer is comprised of two subdomains that adopt α/β -Rossmann-like folds and are connected to each other by a hinge region (Fig. 2.5 A) (171). The effector-binding site is located between the two subdomains of each monomer and electron density maps clearly revealed that the effector-binding site of each monomer was occupied by the pentapeptide motif of one UDP-MurNAc-pentapeptide molecule (Fig. 2.5 A, B, C). The UDP-MurNAc portion of each ligand (Fig. 2.5 B) was disordered in all copies of the AmpR EBD monomer with the exception of four monomers where the MurNAc sugar could be modeled into density.

It was striking to observe that the terminal D-Ala-D-Ala motif of the pentapeptide plays a primary role in interacting with the effector-binding site of AmpR (Fig. 2.5 C). Buried deep within the binding pocket, the carboxylate on the terminal D-Ala group acts as an acceptor of three hydrogen bonds from the side-chain hydroxyl groups of Thr103 (2.6 Å), Ser221 (2.7 Å) and Tyr264 (2.5 Å), which together form the base of the effector binding pocket. The backbone oxygen of the penultimate D-Ala residue forms a 3.0 Å hydrogen bond with the amide backbone

of Tyr192. Three additional hydrogen-bonding contacts are made between the pentapeptide and AmpR residues at the entrance of the effector-binding pocket: the indole-ring nitrogen of Trp152 participates in a 2.8 Å hydrogen bond with the side-chain carboxylate group of the D-Glu within the pentapeptide. The carbonyl group of the *meso*-DAP side chain forms a hydrogen bond within 2.8 Å and 3.0 Å of the side-chain groups of Arg193 and Asn132. Six water molecules are also present within the effector-binding pocket and form hydrogen-bonding interactions with either the bound repressor or residues of the pocket (Fig. 2.5 C).

Previously, in an attempt to predict how both 1,6-anhydroMurNAc-tri and pentapeptide species appear to behave as AmpR inducers, it was speculated that the sugar moiety, 1,6-anhydroMurNAc, played a key role in converting AmpR into an activator of *ampC* transcription by binding within the effector binding pocket (171), since the 1,6-anhydroMurNAc sugar is common to both the 1,6-anhydroMurNAc-tri and pentapeptide species (90, 116). However, our crystal structure of the AmpR EBD bound to UDP-MurNAc-pentapeptide repressor now shows that it is the pentapeptide stem of the repressor that is solely responsible for binding to the AmpR EBD. This binding mode implies that 1,6-anhydroMurNAc-pentapeptide (116) or possibly free pentapeptide (as suggested by Park and Uehara, 7) bind in an identical fashion to UDP-MurNAc-pentapeptide to outcompete the repressor in a concentration dependent manner and convert AmpR into an activator of *ampC* transcription.

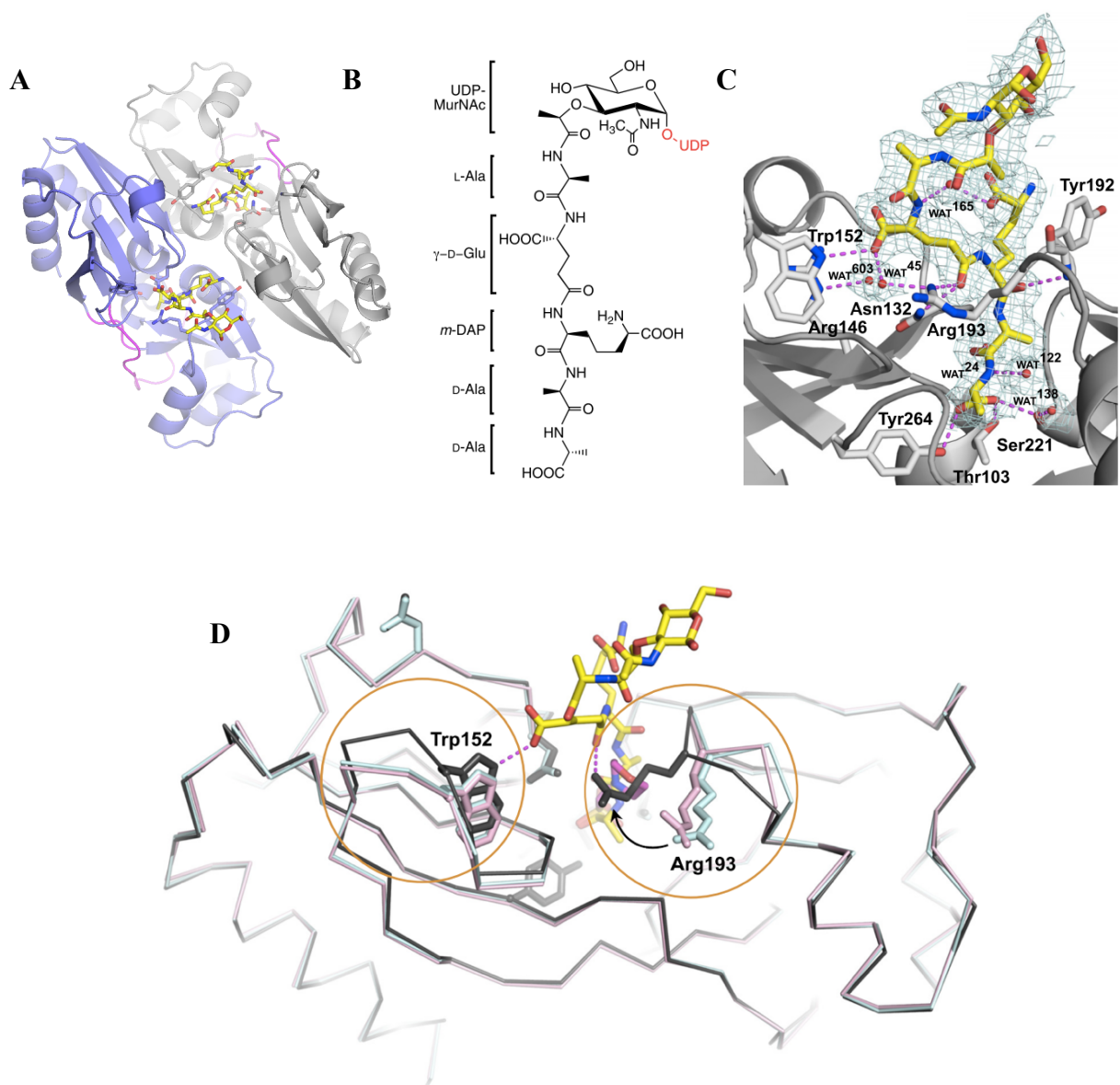


Figure 2.5 – Investigation of AmpR EBD - UDP-MurNAc-pentapeptide binding interactions

A) The crystal structure of the AmpR effector-binding domain (EBD) dimer (monomers indicated by purple and grey cartoons) bound to UDP-MurNAc-pentapeptide (stick format; oxygen atoms are indicated in red, nitrogen atoms in blue and carbon atoms in yellow) refined to 2.15Å resolution. The hinge regions connecting each subunit within a monomer are coloured magenta. The UDP moiety of the repressor was disordered and not visible in electron density maps, as were some of the MurNAc sugars. **B)** Chemical schematic of UDP-MurNAc-pentapeptide (the UDP moiety not visible in the electron density maps is coloured red). **C)** Sigma-A-weighted $2F_o - F_c$ density map contoured at 1.0 σ showing the MurNAc-pentapeptide portion of the repressor bound within the AmpR effector-binding pocket. Hydrogen-bonding

interactions between residues forming the binding site and the pentapeptide stem of the repressor are shown by dashed magenta lines. Waters are shown as red spheres. **D)** Superposition of AmpR EBD monomers comprising the asymmetric unit of the AmpR EBD: UDP-MurNAc-pentapeptide crystal structure (grey) with the AmpR EBD bound to 2-(N-morpholino)ethanesulfonic acid (MES) (PDB ID: 3KOS; shown in cyan) and the inactivated variant AmpR EBD (T103V/ S221A/Y264F) (PDB ID: 3KOT; shown in pink). UDP-MurNAc-pentapeptide and MES molecules are represented by sticks, with carbon atoms indicated in yellow and pink, respectively, and oxygen and nitrogen atoms in red and blue. Regions with relative shifts in loop/residue position are circled in orange.

Table 2.1 – Crystallographic and refinement statistics for the AmpR EBD from *C. freundii* bound to UDP-MurNAc-pentapeptide

Data collection	AmpR EBD: UDP-MurNAc-pentapeptide
X-ray source	CLS 081D-1
Space group	P 2 ₁ 2 ₁ 2
Unit cell dimensions	$a=62.7 \text{ \AA}$, $b=183.6 \text{ \AA}$, $c=197.81 \text{ \AA}$ $\alpha=\beta=\gamma=90^\circ$
Wavelength (Å)	0.9795
Resolution (Å)	59.3 – 2.15 (2.27 - 2.15)*
R _{merge}	0.096 (0.497)
CC(1/2)	0.995 (0.667)
I / σ I	8.3 (3.0)
Completeness (%)	97.7 (98.0)
Redundancy	4.1 (3.6)
Refinement	
R _{work} / R _{free}	0.20 (0.25)
B-factor (Å ²)	36.29
Protein	36.03
Ligands	37.34
Solvent	39.87
<i>RMSDs</i>	
Bond lengths (Å)	0.013
Bond angles (°)	1.46
Ramachandran favoured/allowed (%)	96.9/ 3.1

* Values in parentheses refer to the high-resolution shell.

Although the terminal D-Ala-D-Ala motif contributes the majority of the interactions of the ligand to the AmpR EBD, additional interactions between the D-Glu and *meso*-DAP of the repressor and Arg193, Asn132 and Trp152 on the surface of the AmpR EBD ligand binding pocket suggest that 1,6-anhydroMurNAc-tripeptide could also displace UDP-MurNAc-pentapeptide from AmpR as observed by Jacobs *et al.* (90). However, these binding interactions of the tripeptide species would likely be weaker than those of 1,6-anhydroMurNAc-pentapeptide or free pentapeptide that contain the D-Ala-D-Ala motif. Moreover, involvement of the D-Ala-D-Ala motif in AmpR regulation is entirely consistent with the observed AmpC hyperproduction that occurs upon loss of PBP4 (127), as this protein has a DD-peptidase activity that removes the terminal D-Ala residue from PG catabolites (206). Thus, our findings support a scenario where loss of PBP4 causes 1,6-anhydro-MurNAc-pentapeptide (or free pentapeptide) to accumulate and drive AmpR to hyper-produce AmpC. Finally, as previously pointed out by Park and Uehara (93) it is interesting to note that the strongest inducers of AmpR-mediated *ampC* transcription, such as cefoxitin and imipenem, are those that generate pentapeptide-containing PG fragments (93, 116).

A general examination of the repressor-bound AmpR EBD monomers revealed little change in the relative orientation of secondary structural elements between each monomer within the asymmetric unit. However, superposition of a crystal structure of the AmpR EBD monomer bound to the buffer molecule 2-(*N*-morpholino)ethanesulfonic acid (MES) (PDB ID: 3KOS), or a variant of the AmpR EBD (T103V, S221A and Y264F) that is unable to bind MES (PDB ID: 3KOT), with the AmpR:UDP-MurNAc-pentapeptide complex revealed that the hydrogen bonds with γ -D-Glu and *meso*-DAP residues in the pentapeptide tether Trp152 and Arg193 such that they point towards the center of the interdomain cleft (Fig. 2.5 D). Similarly to the terminal D-

Ala stem of the repressor, MES forms H-bonding interactions at the base of the binding pocket with Thr103, Ser221 and Tyr264. However, in the absence of H-bonding interactions at the opening of the binding cleft, Trp152 and Arg193 and the loop regions they reside on exhibit more flexibility in the wild-type AmpR EBD:MES complex and the unliganded AmpR variant relative to the UDP-MurNAc-pentapeptide-bound structure (Fig. 2.5 D). This change might be involved in modulating AmpR function, as it has been suggested that conformational changes in the EBD upon effector molecule binding may trigger larger reorganization of the LTTR homotetramer (207).

C.4. The AmpR:21 bp dsDNA complex adopts a flat conformation that undergoes only a slight conformational change upon binding of UDP-MurNAc-pentapeptide

Given that we identified a stable AmpR: 21 bp dsDNA quaternary complex by non-denaturing MS, we decided to examine the effects of repressor ligand binding on the conformation of this complex using small angle X-ray scattering (SAXS) measurements. Prior to SAXS data collection, dynamic light scattering (DLS) was used to confirm that the complex remained monodisperse in the presence and absence of UDP-MurNAc-pentapeptide over a protein concentration range of 0.8 to 4.1 mg/ml (Fig. 2.6 A, Table 2.2). Analysis of the SAXS data collected from the complex with and without UDP-MurNAc-pentapeptide using GNOM (196) yielded a radius of gyration (R_g) of 4.40 ± 0.32 nm for the AmpR:DNA complex, and R_g of 4.63 ± 0.10 nm for the AmpR:DNA:repressor complex (Table 2.2).

The maximal particle dimension ($D_{\max}$) of the repressor-free AmpR:21 bp dsDNA complex was calculated from the pairwise distribution $[p(r)]$ function as 12.5 nm, which only increased slightly to 13.8 nm upon binding UDP-MurNAc-pentapeptide (Fig. 2.6 B). Using the program DAMMIF (199), *ab initio* models of the AmpR:DNA complex alone and bound to UDP-MurNAc-pentapeptide were calculated and were in excellent agreement with the experimental SAXS data (Table 2.2). Interestingly, a representative *ab initio* model for the AmpR:21 bp dsDNA complex possesses a large central cavity, which is flanked at opposite ends by two smaller cavities present along a diagonal of the tetramer, which are absent in the *ab initio* model of the complex bound to UDP-MurNAc-pentapeptide, suggesting that the surface indents may become occupied by repressor ligands (Fig. 2.6 C, D).

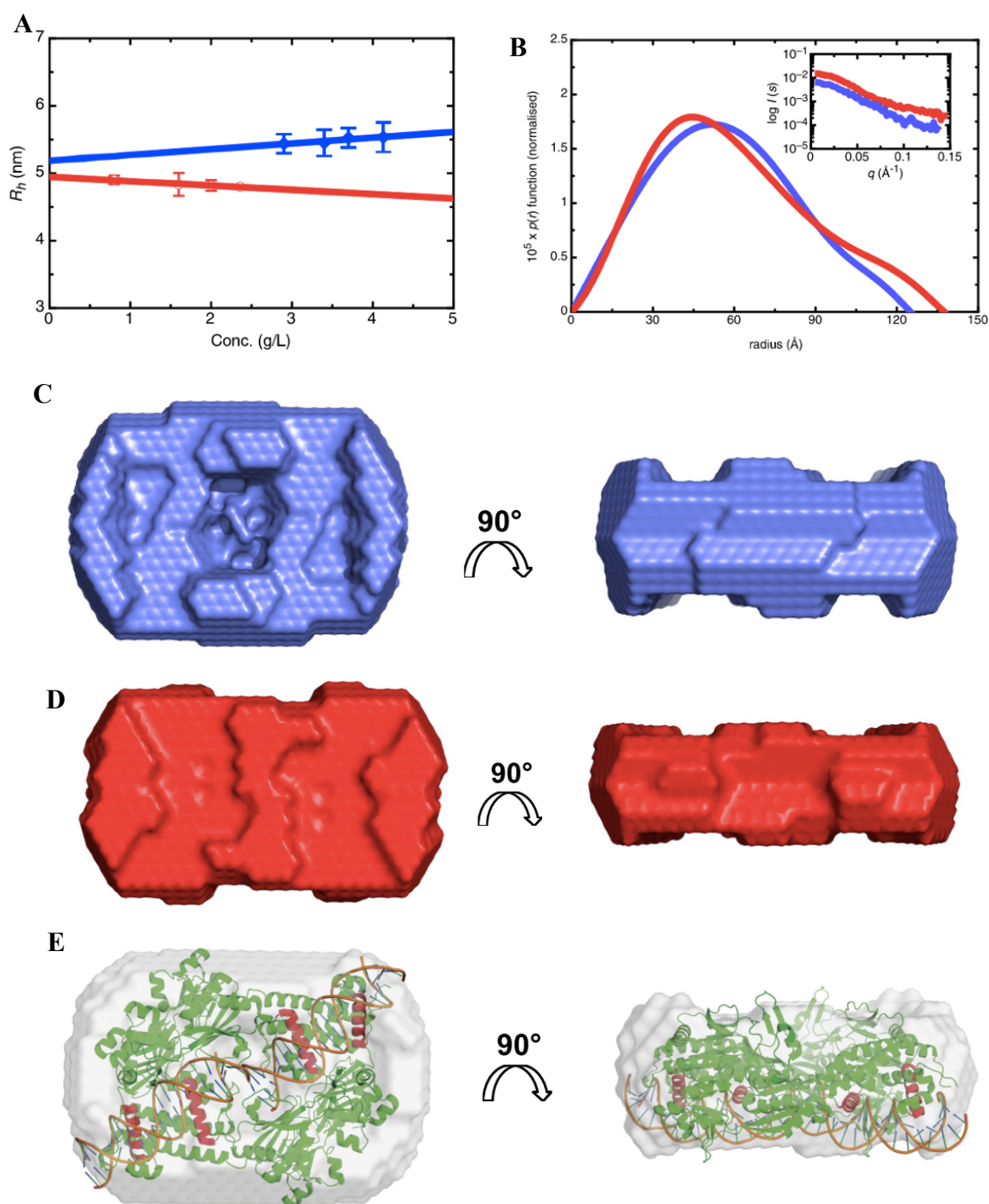


Figure 2.6 – SAXS analysis of the AmpR:21 bp dsDNA complex

A) Hydrodynamic radii (R_h) determined for the AmpR:21 bp dsDNA complex (blue) and AmpR:21 bp dsDNA:UDP-MurNAc-pentapeptide complex (red) at various protein concentrations. SAXS Data were collected using an in-house instrument (Rigaku) at multiple

concentrations, from which **B**) Pair distribution function ($p(r)$) for the AmpR:21 bp dsDNA complex (blue) and AmpR:21 bp dsDNA:UDP-MurNAc-pentapeptide complex (red; scaled data to AmpR:DNA $p(r)$ data) were calculated using GNOM (196) from merged data revealing that AmpR undergoes only a slight conformational change upon binding the repressor. Guinier plots for AmpR complexes are shown in **B** (inset). *Ab initio* modeling of the unliganded AmpR:21 bp dsDNA complex (**C**) and UDP-MurNAc-pentapeptide-bound complex (**D**) using DAMMIF (199) with a particle symmetry of P22 supports that repressor binding causes a slight conformational change in AmpR, most notably demonstrating an absence of the central surface pocket observed in the unliganded AmpR:DNA complex. **E**) Fitting the structure of the AphB (N100E) variant on to the *ab initio* SAXS model of the AmpR:DNA complex indicates that the central cavity in the AphB tetramer is consistent with the indent in the SAXS model. 21 bp dsDNA fragments are placed within the *ab initio* model such that the $\alpha 3$ -recognition helices of AphB (N100E) (coloured in red) fit into the major groove of the dsDNA.

Table 2.2 – Measurements and statistics from SAXS and DLS analysis of the AmpR:21 bp dsDNA complex with and without repressor UDP-MurNAc-pentapeptide

	AmpR:DNA complex		AmpR:DNA:UDP-MurNAc-pentapeptide	
	Experimental value	HYDROPRO – DAMMIF	Experimental value	HYDROPRO - DAMMIF
R_h (nm)	4.95 ± 0.01^b	5.10 ± 0.03^a	5.18 ± 0.01^b	5.05 ± 0.02^a
R_g (nm)	4.20 ± 0.10^c	-	4.30 ± 0.20^c	-
R_g (nm)	4.40 ± 0.32^d	4.45 ± 0.05^a	4.63 ± 0.10^d	4.72 ± 0.08^a
D_{max} (nm)	12.5^d	13.4 ± 0.30^a	13.8^d	15.0 ± 0.28^a
Chi		0.85		0.72
NSD		0.53 ± 0.18		0.68 ± 0.11

* R_h , R_g , D_{max} , and NSD represent hydrodynamic radius, radius of gyration, maximum particle dimension, and normalized spatial discrepancy, respectively.

^a Data calculated from *ab initio* models using HYDROPRO

^b Experimentally determined using Dynamic light scattering

^c Experimentally determined from SAXS data using Guinier analysis

^d Experimentally determined from SAXS data from $p(r)$ analysis by GNOM

Only slight conformational changes appear to occur in AmpR upon binding UDP-MurNAc-pentapeptide according to our SAXS data. The prevailing model of LTTR activation proposes that small conformational changes in the EBD of LTTR proteins translate to larger conformational changes in their linker-helix (LH) and DNA binding domains to trigger transcription (208). TsaR, the only full-length LTTR crystallized with its native inducer ligand did not demonstrate any significant conformational changes relative to the unliganded protein (183). In the case of the DntR EBD, however, it was observed that binding of its inducer (salicylate) triggered a noticeable conformational change within the protein relative to other LTTRs bound to their respective ligands (207). We did observe a small conformational change within the crystal structure of the AmpR EBD when bound to UDP-MurNAc-pentapeptide (Fig. 7D), and while our SAXS data do not suggest that this translates into large changes at the quaternary level, it is possible that the full scope of conformational changes within full-length AmpR may only become apparent when it is bound to one contiguous strand of dsDNA, rather than to separate fragments. This would be technically challenging to achieve however, due to preferential binding of the DNA-binding domains to the high-affinity T-N₁₁-A motif when presented with a molar excess of promoter DNA *in vitro*, a scenario that may be common to other tetrameric LTTR proteins.

C.5. The SAXS-based solution structure of the AmpR:21 bp dsDNA complex closely resembles the structure of *V. cholerae* virulence regulator AphB

By individually fitting all known crystal structures of full-length LTTR proteins into the SAXS-based model of the AmpR:21 bp dsDNA complex, it became clear that the shape of the AmpR:21 bp dsDNA complex most closely resembles that of the tetrameric LTTR AphB, a protein that regulates virulence factors in *V. cholerae* (209). A transcriptional activator ligand for

AphB has not been identified, however the protein is known to respond to intracellular pH and oxygen concentrations (209). Unlike other full-length tetrameric LTTRs that have been structurally characterized (183, 210, 211), AphB adopts a distinctively flat shape, with a linear arrangement of its DNA-binding domains (209). Whereas AphB and a variant of the protein (N100E) both bind DNA in solution, AphB (N100E) crystallized in a conformation where the relative orientations of the α -helices in its DNA-binding domains suggests that this variant can bind a linear stretch of DNA, unlike in the wild-type crystal structure where the DNA binding domains are not appropriately aligned (209). Thus, AphB (N100E) was used as a basis for modeling the AmpR:21 bp dsDNA complex into the *ab initio* SAXS envelope. The AphB (N100E) tetramer fit comfortably into the SAXS envelope of the AmpR:21 bp dsDNA complex (Fig. 2.6 E), and interestingly, AphB (N100E) has a somewhat hollow core that is consistent with the central cavity we observed in our SAXS model of the AmpR:21 bp dsDNA complex (Fig. 2.6 C, E).

After fitting AphB (N100E) into the SAXS envelope, room remained available within the envelope to also fit two 21 bp dsDNA oligomers onto the DNA-binding domain of AphB (Fig. 2.6 E). The dsDNA was oriented such that the α 3-recognition helices of each DNA-binding domain (coloured in red, Fig. 2.6 E) fit into the major groove of the dsDNA as observed in a crystal structure of dsDNA bound to the isolated DNA-binding domain from the LTTR protein BenM (184). Based on the fitting of AphB (N100E) and dsDNA into the SAXS envelope, we built a model of AmpR bound to the dsDNA molecules by superposing our crystal structure of the AmpR EBD homodimer bound to UDP-MurNAc-pentapeptide onto the equivalent EBD dimers of the modeled AphB:dsDNA complex. Interestingly, the superposition revealed that the effector binding site of each of the four AmpR EBD domains were fully accessible to the solvent

since they reside on the side of the tetramer opposite from the bound length of dsDNA (Fig. 2.7). Moreover, while the pentapeptide motif of each repressor is bound deep within the effector-binding pocket of each EBD monomer, the superposition suggests that the UDP-MurNAc portion of each bound repressor may project into the central cavity of AmpR, which is consistent with the filling of the central cavity in the *ab initio* SAXS model of the AmpR:21 bp dsDNA complex that occurred after binding UDP-MurNAc-pentapeptide (Fig. 2.6 C, 2.6 D, 2.7).

C.6. Proposed model for the regulation of AmpR by PG recycling intermediates

Ruangprasert *et al.* recently proposed two structural schemes, I and II that describe the inactive and active conformations of tetrameric LTTR proteins, respectively (181). In Scheme I (as adopted by the LTTR CbnR), the tetrameric interface is comprised of contacts between the DNA-binding domains and linker helices that connect the EBDs to the DBDs, as well as direct interactions between adjacent effector-binding domains, which together result in a ‘closed’ conformation of the protein (181, 210). Scheme II, on the other hand (as observed for the unliganded TsaR, ArgP, AphB LTTR proteins, and a putative transcriptional regulator from *P. aeruginosa* (PDB ID: 2ESN)), presents a more ‘open’ conformation, with an expanded hollow core within the tetramer, and associating solely through the linker helices and DNA-binding domains.

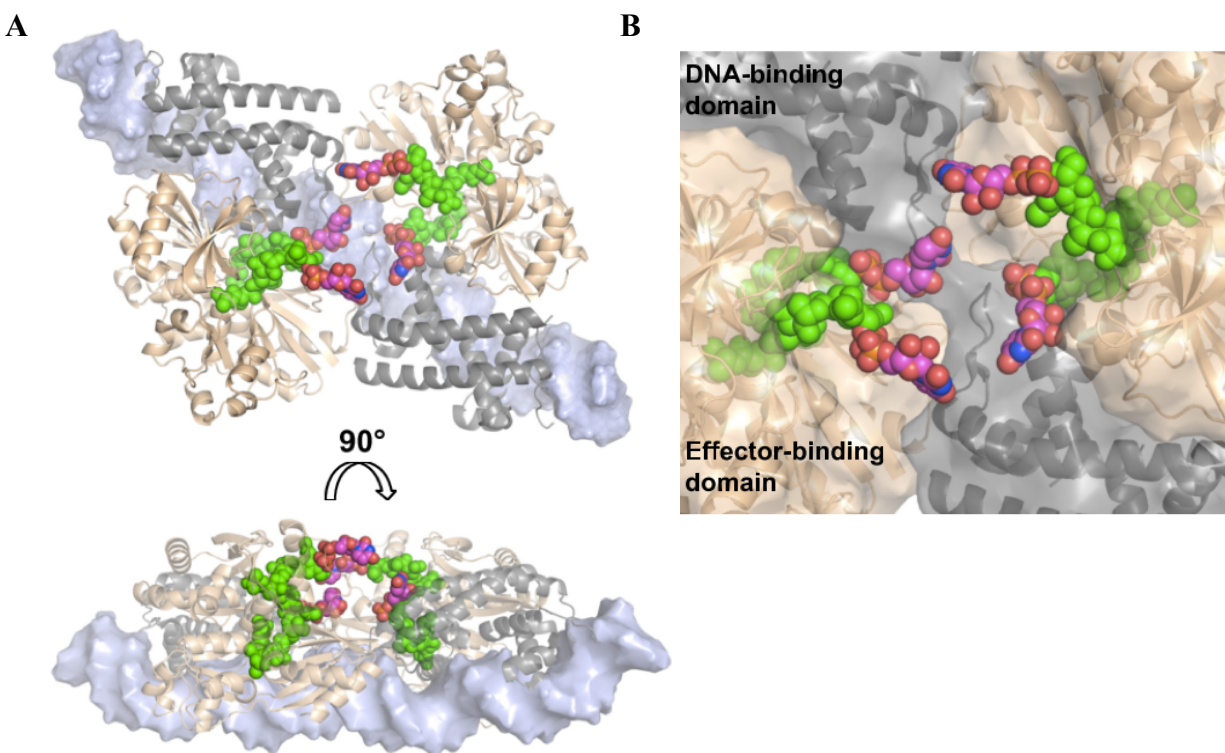


Figure 2.7 – Modeling of the AmpR:DNA complex bound to UDP-MurNAc-pentapeptide.

A) Based on the fitting of AphB (N100E) and dsDNA into our SAXS envelopes of the unliganded and UDP-MurNAc-pentapeptide bound AmpR:DNA complexes, we built a molecular model of AmpR bound to the dsDNA molecules by superposing our crystal structure of the AmpR EBD homodimer bound to UDP-MurNAc-pentapeptide (shown in gold) onto the equivalent EBD dimers of the modeled AphB:DNA complex (shown in grey). A short, continuous fragment of double-stranded DNA (light blue) is shown docked onto the DNA-binding domains of AphB. UDP moieties (carbon atoms are shown in magenta, nitrogen in blue, oxygen in red and phosphorus in yellow) were manually modeled on MurNAc-pentapeptide portions of the repressor that were visible in the electron density maps (green). **B)** A close-up of the central cavity predicted to exist within the AmpR tetramer showing how the UDP-MurNAc moieties of the bound repressor ligands may become positioned to interact with each other or with residues of the DNA-binding domains and/or EBDs to trigger conformational rearrangements in the quaternary structure of AmpR that cause it to bind the operator DNA in a manner that represses gene transcription.

Activation of a tetrameric LTTR by inducer-binding is thought to trigger a conformational rearrangement of the tetramer, converting it from Scheme I to Scheme II (181, 209). Since Jacobs *et al.* found that AmpR is fully active in the absence of PG metabolites (90), our SAXS envelope for the unliganded, activated AmpR:21 bp dsDNA complex likely mimics the inducer-bound form with a central cavity that corresponds to the ‘open’ tetramer described by Scheme II. In comparison, the SAXS model for the repressed AmpR:DNA:UDP-MurNAc-pentapeptide complex does not contain any surface indents, unlike the unliganded, active protein complex. It is possible that when AmpR is repressed by UDP-MurNAc-pentapeptide, the ligand occupies the space within the cavity and thus fills in the indents observed in the unliganded complex. However, binding of the repressor ligand may also trigger conformational changes within the monomers of the tetramer that result in closing of the central core via contacts between the EBDs, thus converting the protein into a form described by Scheme I.

Finally, our model of the AmpR:dsDNA complex bound to UDP-MurNAc-pentapeptide (Fig. 2.7 A) predicts that the UDP-MurNAc-moiety of the repressor molecules are oriented such that they could interact with each other, as well as with residues comprising the DNA-binding domains and areas of the EBDs that flank the pentapeptide binding pocket (Fig. 2.7 B). Given that the UDP-MurNAc moiety is unique to the repressor UDP-MurNAc-pentapeptide, compared to the putative activator ligands, it is tempting to speculate that the UDP-MurNAc moiety of the repressor may effect a conformational change in the quaternary structure of AmpR that causes the protein to repress *ampC* transcription (Fig. 2.7). Competitive binding of 1,6-anhydroMurNAc-pentapeptide, and perhaps the tripeptide species would effectively remove the UDP-MurNAc moiety and convert the protein into an activator of *ampC* transcription. The

molecular details underlying these ligand-induced changes await high-resolution crystallographic analyses.

D. Conclusions

The work presented in this chapter is currently the only structure-based model of AmpR regulation, and adds to our understanding of the regulatory mechanism of LTTR proteins. While UDP-MurNAc-pentapeptide is generally accepted as the PG metabolite responsible for repressing AmpR-mediated gene transcription, the identity of the activator ligand(s) remains controversial (90, 93, 116). Our finding that the terminal D-Ala-D-Ala motif of UDP-MurNAc-pentapeptide forms the primary interactions that bind the repressor to AmpR, support 1,6-anhydroMurNAc-pentapeptide and free-pentapeptide as being more potent activators of AmpR over the –tripeptide species. Identification of the activator ligands of AmpR has important implications in understanding how AmpR modulates *ampC* gene expression and why some β -lactams induce AmpC production more potently than others, which could be taken into account when developing next generation β -lactam antibiotics.

Chapter 3 – Development of an *in vitro* assay to measure AmpR-mediated transcription from the *ampR-ampC* operon

A. Introduction

The study published by Jacobs *et al.* remains the only direct evidence demonstrating that AmpR can be activated to express *ampC* *in vitro* using the PG metabolite 1,6-anhydroMurNAc-tripeptide (90). In contrast, support for 1,6-anhydroMurNAc-pentapeptide as the preferred AmpR inducer ligand was based on indirect observations published by Dietz *et al.* (116) that the strongest AmpC inducing β -lactam antibiotics lead to an accumulation of 1,6-anhydroMurNAc-pentapeptide (and free pentapeptide (93)) in the cytosol, rather than 1,6-anhydroMurNAc-tripeptide. Indeed, the crystal structure of the AmpR EBD (Chapter 2) revealed a key role of the D-Ala-D-Ala motif in binding of UDP-MurNAc-pentapeptide to AmpR, which strongly suggests that AmpR would preferentially bind 1,6-anhydroMurNAc-pentapeptide over the -tripeptide species (212). While 1,6-anhydroMurNAc-tripeptide may lead to increased *ampC* expression as observed by Jacobs *et al.* (90), 1,6-anhydroMurNAc-pentapeptide may be a more competitive AmpR activator due to the presence of its terminal D-Ala-D-Ala motif. However, whether these additional binding interactions allow the pentapeptide species to cause greater *ampC* expression relative to the tripeptide species has not been directly investigated.

Jacobs *et al.* initiated *in vitro* transcription from pNU305, a plasmid DNA template containing the inducible *C. freundii ampR-ampC* operon (213) using an *E. coli* RNA polymerase holoenzyme, which is comprised of the core enzyme complex and a σ_{70} specificity subunit (90). The σ_{70} factor associates with the core RNA polymerase complex (consisting of $\alpha_2\beta\beta'\omega$ subunits) and is involved in the recognition of the -10 and -35 promoter regions (214). The

ability of AmpR to effect changes in gene expression was established by the addition of refolded *CfAmpR* or soluble *CfAmpR* extracts in the presence of the PG recycling metabolites UDP-MurNAc-pentapeptide and 1,6-anhydroMurNAc-tripeptide (90). Interestingly, it was observed that both refolded AmpR and soluble AmpR extracts were capable of inducing *ampC* expression in their native conformations without the additional requirement of 1,6-anhydrMurNAc-tripeptide (90). Coupled with the observation that UDP-MurNAc-pentapeptide inhibition of AmpR activity can be reversed by titrating in 1,6-anhydroMurNAc-tripeptide, the results led the authors to suggest that AmpR must be maintained in an inactive state by UDP-MurNAc-pentapeptide *in vivo*, unless activated by 1,6-anhydroMurNAc-tripeptide (90).

This chapter details my attempt to reproduce the original *in vitro* transcription assay performed by Jacobs *et al.*, using purified *CfAmpR*, which is expected to actively induce *ampC* expression in the absence of its binding ligands (90). Additionally, I have developed highly sensitive procedures to directly measure changes in gene expression from the *ampR-ampC* operon via radiolabeled mRNA synthesis *in vitro*, and indirectly by generating cDNA via reverse transcription (RT) for relative quantification by qPCR.

B. Experimental procedures

B.1. Bacterial strains

E. coli DH5 α (F⁻ Φ 80*lacZ* Δ M15 Δ (*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (rK⁻, mK⁺) *phoA supE44* λ -*thi-1 gyrA96 relA1*), *E. coli* BL21 (DE3) GOLD (F- *ompT hsdS*(r_B⁻m_B⁻) *dcm*⁺ Tet^r *gal* λ (DE3) *endA* Hte), and *E. coli* MG1655 (F⁻ λ ⁻ *ilvG*⁻ *rfb-50 rph-1*) were obtained from NEB, Stratagene and the Coli Genetic Stock Center (<http://cgsc.biology.yale.edu>), respectively.

B.2. Construction of a full-length *ampR* expression plasmid to generate recombinant AmpR for *in vitro* transcription assays

The pET-based full-length *C. freundii ampR* expression plasmid pMB-AmpR characterized in Chapter 2 (Table 3.1) contained a vector-derived cloning artefact that generated the amino acids TSGS fused to the C-terminus of AmpR (*CfAmpR1*) (212). To remove the cloning artifact, the entire plasmid pMB-AmpR (expressing *CfAmpR1*) was amplified by PCR to exclude the bases corresponding to the cloning artefact (using primers P1 and P2 listed in Table 3.1), then ligated using T4 DNA ligase, and transformed into chemically competent *E. coli* DH5 α . The resulting plasmid pGV1 (*CfAmpR*-WT) was isolated from a single kanamycin-resistant colony (selected from LB supplemented with 35 μ g/ml kanamycin) and sequence-verified at The Center for Applied Genomics (TCAG), SickKids hospital, Toronto, ON. Following sequence verification, chemically competent *E. coli* BL21 (DE3) GOLD cells were transformed with pGV1 for the purpose of *CfAmpR*-WT overexpression and purification (described in B.4.)

B.3. Construction of plasmids harbouring the *C. freundii ampR-ampC* operon

In vitro transcription studies by Jacobs *et al.* utilized template DNA in which the *ampR-ampC* operon was inserted into a pBR322-derived vector (90). To maintain consistency, the following procedure describes the construction of a pBR322-based vector containing the full-length *ampR-ampC* operon from *C. freundii*. The full-length *C. freundii ampR-ampC* operon was PCR amplified from plasmid pMB300 (pACYC184/*ampR-ampC*, (171)) using Phusion DNA polymerase (NEB) and primers P3 and P4 (listed in Table 3.1). Following digestion of the PCR amplicon and the plasmid pBR322 (NEB) with restriction enzymes AhdI and AatII, the

fragments were ligated using T4 DNA ligase (NEB) and used to transform competent *E. coli* DH5 α cells with pGV2. The restriction sites AhdI/AatII were chosen in pBR322 in order to remove the resistance genes for ampicillin (*amp*^R). Single colony transformants were selected using tetracycline (selected from LB supplemented with 10 μ g/ml tetracycline) from which plasmid pGV2 was isolated and verified by DNA sequencing at The Center for Applied Genomics (TCAG), SickKids hospital, Toronto, ON.

B.4. Protein expression and purification

CfAmpR1 and *CfAmpR*-WT were expressed in *E. coli* BL21 (DE3) GOLD cells transformed with pMB-AmpR and pGV1, respectively, and purified as described in chapter 2 (section B.2), in the presence of gel filtration buffer (300 mM KCl, 10% glycerol, 10 mM Tris pH 7, and 1 mM DTT).

Table 3.1 – Plasmids and primers

Plasmid	Genotype	Cloning primers
pMB-AmpR	Modified pET28b (Kan ^R) containing the full-length <i>ampR</i> gene from <i>C. freundii</i> and a cloning artefact encoding residues TS GS	Listed in Chapter 2, section B.2.
pGV1	Modified pET28b (Kan ^R) containing the full-length <i>ampR</i> gene from <i>C. freundii</i> minus the cloning artefact	P1: 5'phos-CTC GAG CAC CAC CAC CAC CAC C-3' P2: 5'phos-TTA TTT GTG CAG CAC CCC GGT CAA CC-3'
pGV2	pBR322 (tet ^R)/ full-length <i>ampR-ampC</i> operon from <i>C. freundii</i>	P3: 5'-GTC AGG ACT CCC CGT CGT ACA TGC GTT-3' P4: 5'-CTG ACG ACG TCG ATT TAC CGG ACC CC-3'

* In pGV2, AhdI and AatII recognition sites in forward and reverse cloning primers, respectively, are bolded.

B.5. In vitro transcription

In vitro transcription reactions were performed in transcription buffer (40 mM Tris pH 7.9, 150 mM KCl, 1 mM DTT, 8 mM MgCl₂, 5% glycerol) to a final volume of 50 µl and contained ~3 nM of purified pGV2 (500 ng/reaction), 1 U RiboLock RNase inhibitor (ThermoFisher Scientific), and 0.5-6 µM of purified homotetrameric *CfAmpR1* or *CfAmpR-WT* (negative control reactions contained an equivalent volume of gel filtration buffer in place of *AmpR* protein). Reactions were incubated at 37°C for 10 min, and after the addition of 1 U σ_{70} -saturated *E.coli* RNA polymerase holoenzyme (NEB), they were incubated for an additional 10 min at 37°C, followed by the addition of rNTPs (NEB) to a final concentration of 1 mM, to allow mRNA synthesis to proceed at 37°C either for 20 min or up to 2 hrs. Transcription reactions were subsequently diluted with the addition of 1X TURBO DNase buffer (ThermoFisher Scientific) to a final volume of 100 µl, to which 2 x 1.5 µl TURBO DNase (1 U/µl, ThermoFisher Scientific) was added, with a 30 minute incubation at 37°C between each addition of enzyme. DNA-digested mRNA was purified on-column using the RNeasy MinElute Cleanup kit (Qiagen), and eluted in 15 µl nuclease-free water. Following this RNA transcription and purification method, RNA yields were typically 15-75 ng, as measured by UV-absorbance using NanoDropTM (Thermo Fisher Scientific) or fluometric quantitation using QubitTM (Thermo Fisher Scientific). mRNA samples were stored at -80°C, and held on ice when in use.

B.6. Radiolabeled cDNA synthesis

Primers meant for annealing to *tet^R*, *ampC*, and *ampR* (listed in Table 3.2) were designed to yield cDNA products of 201, 178 and 363 nucleotides in length, respectively. They are identical to those used by Jacobs *et al.* (90) and are listed in Table 3.2. Primers were labelled

with γ - ^{32}P (PerkinElmer) at the 5'-ends using an AMV reverse transcriptase kit (Promega), which also provided control *kan^R* RNA, its associated oligonucleotide primer for cDNA extension, and a ϕX174 DNA marker. Briefly, 10 μl reactions containing 10 pmol primer/ ϕX174 DNA marker, 1X T4 PNK buffer, 1 U T4 PNK, and (γ - ^{32}P)-ATP (375 MBq/ml) were incubated for 15 min at 37°C, and denatured for 2 min at 90°C. Radiolabeled primers were diluted in nuclease-free water, and purified on-column using the QIAquick Nucleotide Removal kit (Qiagen) as suggested by the manufacturer. Purified radioalabeled samples were eluted in elution buffer (Qiagen) and stored at -20°C.

For cDNA synthesis, approximately 100 ng of RNA generated *in vivo* was used per reaction, however, RNA quantities generated *in vitro* were too low to be accurately detected by NanoDropTM (Thermo Fisher Scientific), and were likely less than 5 ng per reaction. Purified RNA samples (or nuclease-free water for negative controls) were mixed with an equal volume of 1X AMV Primer Extension buffer (Promega), and aliquoted into 3 X 10 μl reactions per sample, which were each annealed with 2 μl radiolabeled primers for *tet^R*, *amp^R* and *amp^C* at 58°C for 20 minutes. After cooling to room temperature, primer extension was initiated by the addition of 9 μl master mix (0.5X AMV primer extension buffer, 2.5 mM Na pyrophosphate, 1 unit AMV reverse transcriptase), and reactions were incubated for 30 min at 42°C. 1X Loading dye (Promega) was added to each 21 μl sample, which were denatured at 90°C for 10 min. Samples were loaded onto a precast 7M urea-6% polyacrylamide gel (Invitrogen) pre-run for 30 min at 100V, resolved at 150V for 50 min in 1X Tris-borate-EDTA (TBE) buffer (89 mM Tris pH 7.6, 89 mM boric acid, 2 mM EDTA), and visualized using a phosphorimager (Bio-Rad).

Table 3.2 – Primers used for radiolabeled cDNA synthesis and non-radiolabeled qPCR.

Primers for radiolabelled cDNA synthesis	
<i>tet^R</i>	P5: 5'-CGG ACAGTGCTCCGAGAACGGGTGC-3'
<i>amp^R</i>	P6: 5'-CAGCGGGAAAAGAC AGCCGA-3'
<i>amp^C</i>	P7: 5'-GCCTGCTCCTGCATCAGTGGTGTG-3'
Primers for qpcr	
<i>tet^R</i>	P10: 5'-CCT GGA TGC TGT AGG CAT AG-3' P11: 5'-CGG GTG CGC ATA GAA ATT G-3'
<i>amp^R</i>	P12: 5'-CGG TTC TCG TGG ATT AAT GTT G-3' P13: 5'-TTC CTG GGT CTG TTT AGT GG-3'
<i>amp^C</i>	P14: 5'-CAA ACG CTG TTT GAG CTA GG-3' P15: 5'-AGT ATT TCG TGA CCG GAT CG-3'

B.7. Quantification of AmpC β -lactamase activity

Competent *E. coli* MG1655 and *E. coli* DH5 α cells transformed with pGV2 were grown to an OD₆₀₀ ~ 0.5 at 37°C in M9CA minimal media supplemented with 0.2% glucose, 5 μ g/ml thiamine, 50 μ g/ml uracil and 10 μ g/ml tetracycline. AmpC β -lactamase activity was determined by spectrophotometrically measuring the hydrolysis of nitrocefin, a chromogenic cephalosporin substrate for β -lactamase enzymes. Specifically, nanomoles of nitrocefin (Calbiochem) hydrolyzed per minute per milligram of protein were determined after pGV2-bearing cells were grown in the presence or absence of 16 μ g/ml cefoxitin, a strong AmpC-inducer, for 1 hour at 37°C. Crude lysates from un-induced and FOX-induced cells resuspended in PBS buffer were sonicated (3x8 bursts at 50% duty cycle), centrifuged, and protein concentration in lysate supernatants was measured via a Bradford protein assay (Bio-Rad). The linear rate of liberation of 2,4-dinitrophenolate from nitrocefin (100 μ M/reaction) by AmpC β -lactamase in lysate samples was measured by the addition of 10 μ l nitrocefin to 90 μ l lysate diluted in 1X PBS buffer (~3 ng protein/reaction), and monitoring changes in absorbance at 486 nm every minute for 5 minutes.

B.8. Whole cell mRNA extraction

Outgrowths of pGV2-bearing *E. coli* MG1655 were grown at 37°C in M9CA supplemented with 0.5% glycerol, 5 μ g/ml thiamine, 50 μ g/ml uracil and 10 μ g/ml tetracycline to an OD₆₀₀ ~ 0.3, followed by another hour of incubation at 37°C in the presence or absence of 16 μ g/ml FOX. Cells were centrifuged and resuspended in 500 μ l of the same media, to which 1 ml of RNAlater (Qiagen) was added followed by a 5 min incubation at RT to stabilize RNA.

Cells were once again pelleted and stored at -80°C until further use. Whole cell RNA was extracted from un-induced and FOX-induced cell pellets using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. Purified RNA was eluted in 30 µl of nuclease-free water. DNase digestion was performed by diluting 25 µl RNA in 45 µl 1X DNase buffer (NEB) with the sequential addition of 2 x 2.5 µl DNase I (NEB) 30 min apart, over a 1 hour period at 37°C. Digested samples were subjected to another on-column purification using the RNeasy Mini kit. Integrity of the purified RNA was verified by running 5 µl purified nucleic acid on an agarose gel, and these RNA samples were used as templates for cDNA synthesis as described previously.

B.9. Electrophoretic mobility shift assay (EMSA)

Binding reactions (20 µl) of *CfAmpR*-WT with a 1613 bp PCR fragment of the *C. freundii ampR-ampC* operon (containing the AmpR binding site) were set up in AmpR gel filtration buffer using 2 nM DNA (20 ng per reaction) and increasing concentrations of *CfAmpR*-WT ranging from 3.5-35 µM homotetramer (0.45-4.5 µg per reaction), and incubated for 40 min at 37°C. The DNA template was generated by PCR using pGV2 template and primers P8 (5'-GTC AGG ACT CCC CGT CCA GCG GGA AAA G-3') and P9 (5'-CTG ACG ACG TCG ATT TAC CGG ACC CC-3'). After the addition of 1X loading buffer (3% glycerol and 0.1 g bromophenol blue in 5X TBE), samples were resolved by native-PAGE at 120V for 110 min. The gel was stained in SYBR green (Bio-Rad) diluted 1:10,000 in 0.5X TBE for 10 min and visualized using the GelDoc system (Bio-Rad).

B.10. Radiolabeled mRNA synthesis

Transcription reactions were performed similarly to as described above in B.5, except 40 ng pGV2 DNA template was incubated for 20 min at 37°C with varying concentrations of *CfAmpR*-WT (details in Fig. 3.2) in transcription buffer. Additionally, to ascertain the effect of salt on transcription, template DNA was incubated with equivalent volumes of the gel filtration buffer that AmpR was purified in. To 23.5 µl reactions, 0.5 µl 1 U/µl of σ_{70} -saturated *E.coli* RNA polymerase holoenzyme (NEB) was added and incubated for 15 minutes at 37°C. Finally, 1.5 µl of rNTP master mix (500 µM rATP/rGTP/rCTP, 10 µM rUTP, 5µCi α -³²P-UTP) was added and incubated at 37°C for 15 min. Reactions were stopped by the addition of 20 µl loading dye (95% formamide, 20 mM EDTA pH 8, 0.05% bromophenol blue), and denatured at 95°C for 3 min. Urea-polyacrylamide gels (Invitrogen) were pre-run at 100 V for 30 minutes prior to loading 10 µl mRNA samples per well. Bands were resolved over 1.5 hours at 180V in 1X TBE, and visualized by phosphorimager.

B.11. RT-qPCR

mRNA generated *in vitro* was reverse transcribed using the iScriptTM Reverse Transcription Supermix for RT-qPCR (Bio-Rad) as follows: 3 µl mRNA (1-5 ng/µl) was incubated with either 4 µl RT or no-RT control supermix, and diluted with nuclease-free water to a final volume of 20 µl. Reaction constituents were primed at 25°C for 15 min, reverse transcribed at 46°C for 20 min, and the reverse transcriptase inactivated by incubation at 95°C for 1 min.

The following reactions were assembled on ice. Ten-fold dilution series of cDNA generated from RT and no-RT controls were prepared in nuclease-free water for use in a standard curve, and ranged from 10 pg-0.01 pg per qPCR reaction. Primers for qPCR were designed using NCBI PrimerBlast and IDT PrimerQuest (listed in Table 3.2). qPCR master mixes were prepared for each primer set for a 300nM final primer concentration in 1X SsoAdvancedTM Universal SYBR® Green Supermix (Bio-Rad). In a 96-well PCR plate (Bio-Rad), 9 µl of qPCR master mix for each primer pair was added to 1 µl cDNA (or no-RT and no template controls), such that amplification reactions per gene per sample were performed in duplicate. Amplification reactions were performed using the CFX ConnectTM Real-Time PCR Detection System (Bio-Rad) as recommended for cDNA amplification by the manufacturer, followed by a melt-curve analysis.

The purity of each amplicon was confirmed by melt-curve analysis, and by restriction fragment length polymorphism (RFLP) analysis. Amplicons from multiple qPCR reactions for each gene were pooled and subjected to restriction enzyme digest using BfaI (NEB), TatI (ThermoFisher Scientific), and EcoRV (NEB) to generate digested fragments of *ampC*, *ampR* and *tet^R*, respectively. Following the addition of 1X loading buffer (used for EMSAs in above section), samples of digested and un-digested amplicons were run on a 10% polyacrylamide-TBE gel in 1X TBE buffer for 50 min at 180V.

C. Results and discussion

C.1. Optimization of AmpR-regulated *in vitro* transcription

The DNA template (pNU305) used by Jacobs *et al.* for *in vitro* transcription studies consisted of the *ampR-ampC* operon inserted into a pBR322-based vector, and the vector-derived

tet^R gene as a positive control (90, 213). In addition to the *ampR-ampC* operon, the plasmid pNU305 also contained a partial fumarate reductase (*frd*) operon immediately upstream of the *ampR* gene (213). In the previously generated pMB-300 plasmid (171), the *C. freundii ampR-ampC* operon was inserted into a pACYC184 vector with a chloramphenicol resistance marker. In order to reproduce the assay conditions employed by Jacobs *et al.* (90) as closely as possible, the *C. freundii ampR-ampC* operon from pMB-300 was PCR-amplified and inserted into pBR322 to generate the plasmid pGV2 that included a *tet^R* resistance marker. The pBR322 plasmid also contains an innate ampicillin resistance gene, which was removed and replaced with the *ampR-ampC* insert during plasmid construction to prevent competing expression of another β -lactamase enzyme. Additionally, since the aim was to observe AmpR-mediated expression from the *ampR-ampC* operon only, the partial *frd* operon was not included.

The *in vitro* transcription assay described by Jacobs *et al.* was performed using 3 nM DNA template, and 40-400 nM refolded *CfAmpR* to observe an induction in *ampC* expression. To reproduce this original procedure, 3 nM DNA template was used, however, prior to establishing the optimized *in vitro* transcription protocol described in B.5, it was proving difficult to completely eradicate the template by DNase digestion following mRNA synthesis. Consequently, transcription reactions were initially performed with lower starting concentrations of DNA template (0.2 nM pGV2) in the presence or absence of 125 nM *CfAmpR1* tetramer (initially expressed from pMB-AmpR and containing the C-terminal TSGS cloning artefact (171, 212)), and allowed to proceed for up to 2 hrs to maximize mRNA yields. The molar ratio of AmpR to template DNA was higher than that used in the original protocol (90) in an attempt to ensure that final mRNA yields were sufficient to be detected by radiolabeled-cDNA synthesis following a rigorous RNA purification procedure. The DNase-digestion and mRNA purification

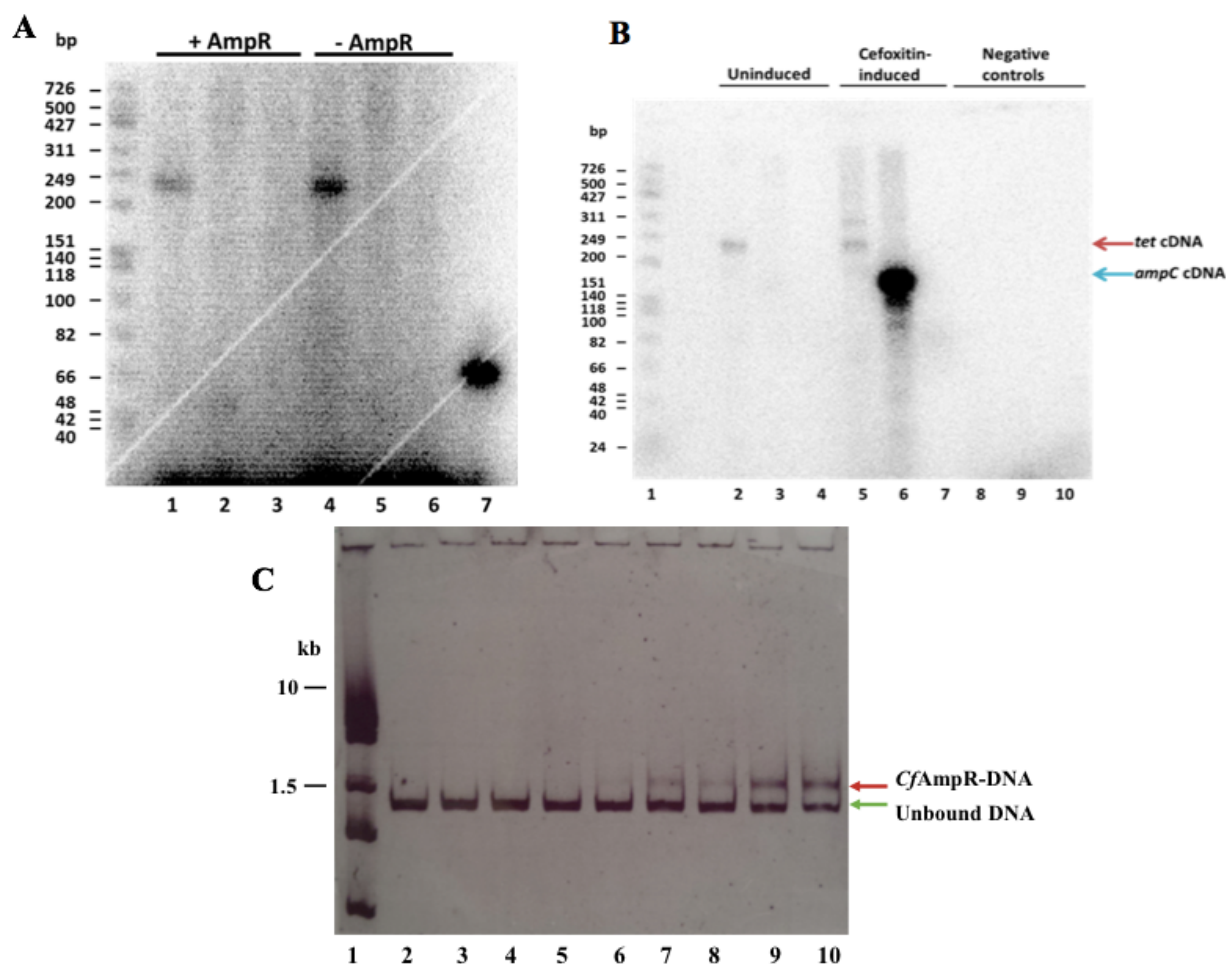
methods were still less than optimal at this stage and yielded mRNA amounts that could not be accurately detected by UV-absorbance, and for the sake of clarity, only the optimized procedure described in B.5. was ultimately adopted for experiments described in section C.2 onwards.

Despite low initial mRNA yields, the *tet^R* gene was constitutively transcribed in the presence and absence of *CfAmpR1*, indicating that the RNA polymerase holoenzyme was functional (Fig. 3.1 A). Failure to detect *ampC* and *ampR* transcripts could have been due to a malfunctioning *ampR-ampC* operon, lack of primer hybridization during cDNA synthesis, or malfunctioning AmpR.

Cefoxitin (FOX)-induction of *E. coli* MG1655 cells was found to result in a β -lactamase activity of 7.23 $\mu\text{mol} / \text{min.mg}$ relative to 0.47 $\mu\text{mol} / \text{min.mg}$ in the uninduced control, and validated the functionality of the *ampR-ampC* operon in pGV2 in live cells. To address the possibility that the radiolabeled primers failed to anneal to *ampR* and *ampC* transcripts, cDNA synthesis was performed using mRNA generated *in vivo* from pGV2 in *E. coli* MG1655 cells that had been treated with the strong AmpC-inducer cefoxitin (FOX). As expected, *tet^R* cDNA was observed with and without FOX-induction, as indicated by the presence of a 201 base nucleotide (nt) band of similar intensity under both conditions (Fig. 3.1 B). More significantly, based on the intense band observed for *ampC* cDNA (178 nt, Fig. 3.1 B), FOX-induction appeared to have resulted in a substantial increase in the production of *ampC* mRNA. The presence of *ampR* transcripts could not be detected either *in vitro* or *in vivo*, possibly due to low levels of expression combined with loss of total RNA yields following the extensive purification. These results confirmed that not only did the γ -³²P-labelled primers anneal to *tet^R* and *ampC* transcripts,

they provided confirmation that the pGV2 plasmid contains a fully functional inducible *ampC* β -lactamase system from which transcripts can be detected by generating radiolabeled cDNA.

To address the possibility that the four-amino acid (TSGS) cloning artefact at the C-terminal end of *CfAmpR1* may have been interfering with AmpR function *in vitro*, *CfAmpR*-WT was generated to exclude the extraneous sequence. Increasing concentrations of *CfAmpR*-WT were then incubated with the linear truncated *ampR-ampC* DNA template (1613 bp) containing the intergenic region, the entire *ampC* gene, and the first 336 bp of the *ampR* gene. Using a truncated *ampR-ampC* operon (instead of full-length) prevented impediments to the migratory ability of the large AmpR-DNA complex through the native gel, allowing the observation of clearly perceptible shifts between unbound and AmpR-bound DNA. The EMSA demonstrated that while *CfAmpR*-WT did appear to bind the *ampR-ampC* operon, it occurred when AmpR was in an approximately 1000-fold molar excess to the template DNA (Fig. 3.1 C). The extremely high concentration of AmpR relative to DNA template required to cause a binding shift may be indicative of non-specific binding, or that only a small portion of the total AmpR protein was functional. Although refolded *CfAmpR*-WT was capable of binding DNA, it may have possessed a conformation incapable of initiating *ampC* mRNA production under *in vitro* settings. Its functional capability was thus explored in more detail as described in the following section.



3.1 – *In vitro* and *in vivo* analysis of transcription products of genes *tet^R*, *ampC* and *ampR*.

A) Urea-PAGE showing 5'- γ^{32} P-end labelled cDNA generated by reverse transcription of mRNA synthesized *in vitro* for the genes *tet^R* (lanes 1,4), *ampC* (lanes 2,5) and *ampR* (lanes 3,6) in the presence (lanes 1-3) or absence (lanes 4-6) of CfAmpR1. cDNA products corresponding to *tet^R* and the kanamycin mRNA (positive control, lane 7) were 201 and 87 nucleotides (nt) in length, respectively. **B)** Urea-PAGE showing 5'- γ^{32} P-end labelled cDNA generated from mRNA synthesized *in vivo* from uninduced and cefoxitin-induced cells for genes *tet^R* (lanes 2,5,8), *ampC* (lanes 3,6,9) and *ampR* (lanes 4,7,10). Negative controls (lanes 8-10) contain water in place of RNA. cDNA products for *tet^R* and *ampC* are 201 nt and 178 nt, respectively. **C)** EMSA performed using a 1613 bp fragment of AmpR operator DNA with increasing concentrations of CfAmpR-WT (lanes 2-10, 0-4.5 μ g). Lane 10 contains AmpR at a 1750X molar excess to DNA, and clearly indicates a gel shift.

C.2. *CfAmpR*-WT may be causing an increase in *ampC* mRNA synthesis and a corresponding decrease in *ampR* mRNA synthesis

An advantage of generating radiolabeled mRNA products *in vitro* is that their relative abundance can be immediately analyzed by gel electrophoresis, and bypasses the requirement for purification procedures that risk diminishing mRNA yields. If purified AmpR was present in a functionally active conformation capable of inducing *ampC* mRNA expression as demonstrated by Jacobs *et al.* (90), then it was expected that performing *in vitro* transcription analysis using *CfAmpR*-WT and the pGV2 DNA template should directly generate large quantities of radiolabeled *ampC* transcript, and correspondingly, should result in a decrease in *ampR* transcripts due to AmpR auto-repression. Accordingly, radiolabeled mRNA products incorporating α -³²P-UTP were synthesized *in vitro* from DNA template pGV2 in the presence or absence of *CfAmpR*-WT. The *tet^R* coding region (1191 bp) is very close in size to *ampC* (1146 bp), and it was not expected that mRNA expressed from these two genes would resolve separately on a gel, unlike the *ampR* coding region (876 bp). However, in the event of *ampC* induction, an accumulation of *ampC* transcript was expected, and in the absence of a radiolabeled size marker, this was hoped to distinguish relative increases in *ampC* mRNA production from basal *tet^R* mRNA expression.

Transcription reactions were carried out over 15 min and for the most part, several independent reactions confirmed that although mRNA products were generated, no distinct variation was observed in their relative abundance irrespective of the presence of *CfAmpR*-WT, barring an unexpected instance: when reactions were set up as described in section B.9, *CfAmpR*-WT appeared to cause a significant increase in expression of an mRNA transcript relative to the *tet^R* control. To confirm the reproducibility of this observation, *in vitro*

transcription reactions were performed containing 0.47 nM pGV2 as a template and increasing concentrations of *CfAmpR*-WT ranging from 0.28-1.4 μ M of homotetrameric protein (Fig. 3.2).

In the absence of *CfAmpR*-WT, the single transcript generated *in vitro* from an empty pBR322 vector was presumably from the *tet^R* gene (Fig. 3.2 – lanes 1 and 2). Interestingly, in addition to what may be *tet^R*, expression from pGV2 shows an abundance of a smaller product in the absence of *CfAmpR*, and it is possible that this may be *ampR* mRNA (Fig. 3.2 – lane 4). Indeed, increasing concentrations of *CfAmpR*-WT caused a gradual decrease in the abundance of the smaller transcript, which may be a reflection of auto-repression by AmpR (Fig. 3.2 – lanes 5-9). This corresponded with a gradual accumulation of a larger transcript closer in size to the predicted *tet^R* mRNA product, that could be *ampC* mRNA (Fig. 3.2 – lanes 6-9). This observation seemed promising in terms of demonstrating the ability of *CfAmpR*-WT to actively regulate changes in expression from the *ampR-ampC* operon, although one cannot speculate further considering that the identities of the mRNA products have yet to be verified experimentally.

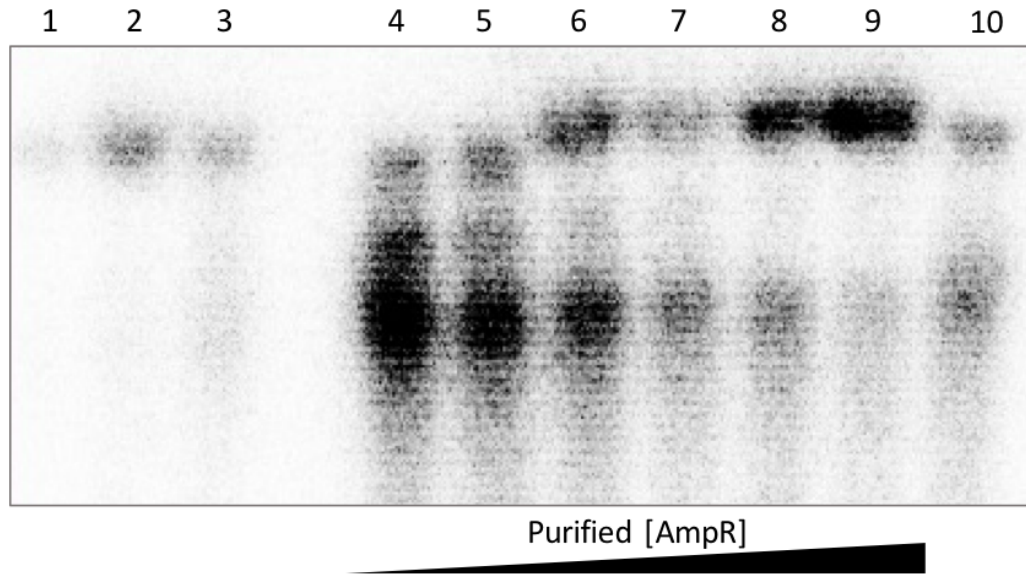


Figure 3.2 – *In vitro* analysis of radiolabeled *tet^R* and *ampC* and *ampR* mRNA.

Urea-PAGE showing $\alpha^{32}\text{P}$ -UTP-incorporated mRNA generated *in vitro* by incubating pBR322 (lane 1) or pGV2 (lane 4) with RNA polymerase holoenzyme (NEB) only. Additionally, to ascertain the effect of salt on transcription, pBR322 and pGV2 were incubated with 5 μl AmpR gel filtration buffer (lanes 2 and 10, respectively). Lanes 5-9 represent changes in transcription from pGV2 when incubated with increasing amounts of purified *CfAmpR* ranging from 1-5 μl (0.9-4.5 μg total OR 0.28-1.4 μM). Lane 3 represents transcription from pBR322 when supplemented with 5 μl AmpR (4.5 μg OR 1.4 μM). The coding regions for *tet^R*, *ampC* and *ampR* are 1191, 1146 and 876 bp, respectively. It appears that increasing amounts of AmpR cause a simultaneous upregulation in expression of a large transcript (lanes 6-9) and downregulation of a smaller transcript that may correspond to *ampC* and *ampR*, respectively. It is difficult to distinguish predicted *tet^R* and *ampC* products since they are similar in size, however, based on the empty vector controls (lanes 1-3), it appears that differential expression from the *ampR-ampC* operon is mediated by AmpR.

It must be pointed out that at its most concentrated, *CfAmpR*-WT is present at a 3500-fold molar excess relative to DNA template (Fig. 3.2 - lane 5), which is exceedingly high to effect a change in gene expression, considering that *ampC* induction has been previously documented for a 13 to 130-fold molar excess of AmpR relative to template (90). Perhaps the reason why the assay may have worked in the above instance is due to degradation or unfolding of the AmpR sample over the course of two weeks since it had been initially purified, such that only a small fraction of the protein was actually functional. The results in the previous chapter demonstrate that binding to double-stranded DNA stabilizes AmpR significantly, potentially by minimizing oligomerization resulting from protein-protein contacts. Thus, addition of such a large quantity of AmpR protein may actively interfere with transcription rather than facilitate it by promoting the formation of inactive protein oligomers that are incapable of binding DNA *in vitro*. Future attempts at synthesizing mRNA *in vitro* from the *ampR-ampC* operon using a more reasonable molar ratio of AmpR to template DNA may allow AmpR to remain in a soluble, functionally active form capable of consistently mediating gene transcription.

C.3. RT-qPCR based mRNA detection

As a convenient alternative to radiolabelling as a means of quantifying relative changes in gene transcription, an RT-qPCR protocol was developed since it is also capable of sensitively detecting gene expression from minute starting concentrations of mRNA. qPCR primer efficiencies for primer pairs amplifying *ampC*, *ampR*, and *tetR* cDNA (primers listed in Table 3.2) were determined by performing a standard curve using a 10-fold dilution series of the plasmid pGV2, and were 106%, 104.6% and 102%, respectively, with slope values of approximately -3.2. Although the % efficiencies were higher than desirable, melt curve and

agarose gel analyses for each amplicon indicated the presence a single major PCR product, and the primer pairs were deemed suitable to amplify cDNA templates.

mRNA was generated *in vitro* as described in section B.5, using only σ_{70} -saturated *E.coli* RNA polymerase holoenzyme and pGV2 as a template. While transcription products generated in the presence of purified *CfAmpR* were also subjected to qPCR, the expected changes in expression of *ampC* and *ampR* transcripts were not observed, possibly due to reasons discussed in section C.2., and consequently, are not described in the following analysis. Purified mRNA template (3-15 ng) generated by the RNA polymerase holoenzyme was reverse transcribed to generate cDNA, which was diluted to quantities as low as 0.1-10 pg, and subjected to qPCR amplification. mRNA samples were also used to set up negative control reactions, which lacked reverse transcriptase (RT) enzyme, and would not have generated cDNA. Dilutions of these no-RT control reactions lacking RT enzyme were also included in the qPCR assay. Any amplification from these control reactions would be wholly attributable to carryover of plasmid DNA template from the DNA digestion and mRNA purification process.

Interestingly, when *in vitro* transcription is performed in the absence of recombinant AmpR protein, detectable cDNA was generated for *ampC*, *ampR* and *tet^R*. This indicated that even in the presence of RNA polymerase only, basal levels of transcription occur from the *ampC* and *ampR* genes. Comparison of amplification from all three genes to that from no-RT controls reveals a substantially higher cycle threshold (C_T) in the latter instance, which indicates that contaminating plasmid DNA must undergo several additional cycles of amplification prior to being detected over background fluorescence, and thus has a limited influence on observed basal transcription levels. Primer efficiencies for the amplification of *ampC*, *ampR* and *tet^R* cDNA were either nearly or within an acceptable range (88.6%-97.7%, Fig. 3.3 A). In case primer

efficiencies for selected primer pairs (e.g. *ampC*) are not improved by further optimization of reaction conditions, new primer sets may have to be tested to ensure that amplification is occurring with similar efficiencies for all templates. RFLP analysis was performed to support qPCR melt curve data, and confirmed the purity of amplicons generated from *ampC*, *ampR* and *tetR* cDNA (Fig. 3.3 B). Collectively, these results clearly highlight the feasibility of an RT-qPCR approach towards defining relative changes in gene expression even with very low starting concentrations of mRNA template synthesized *in vitro*.

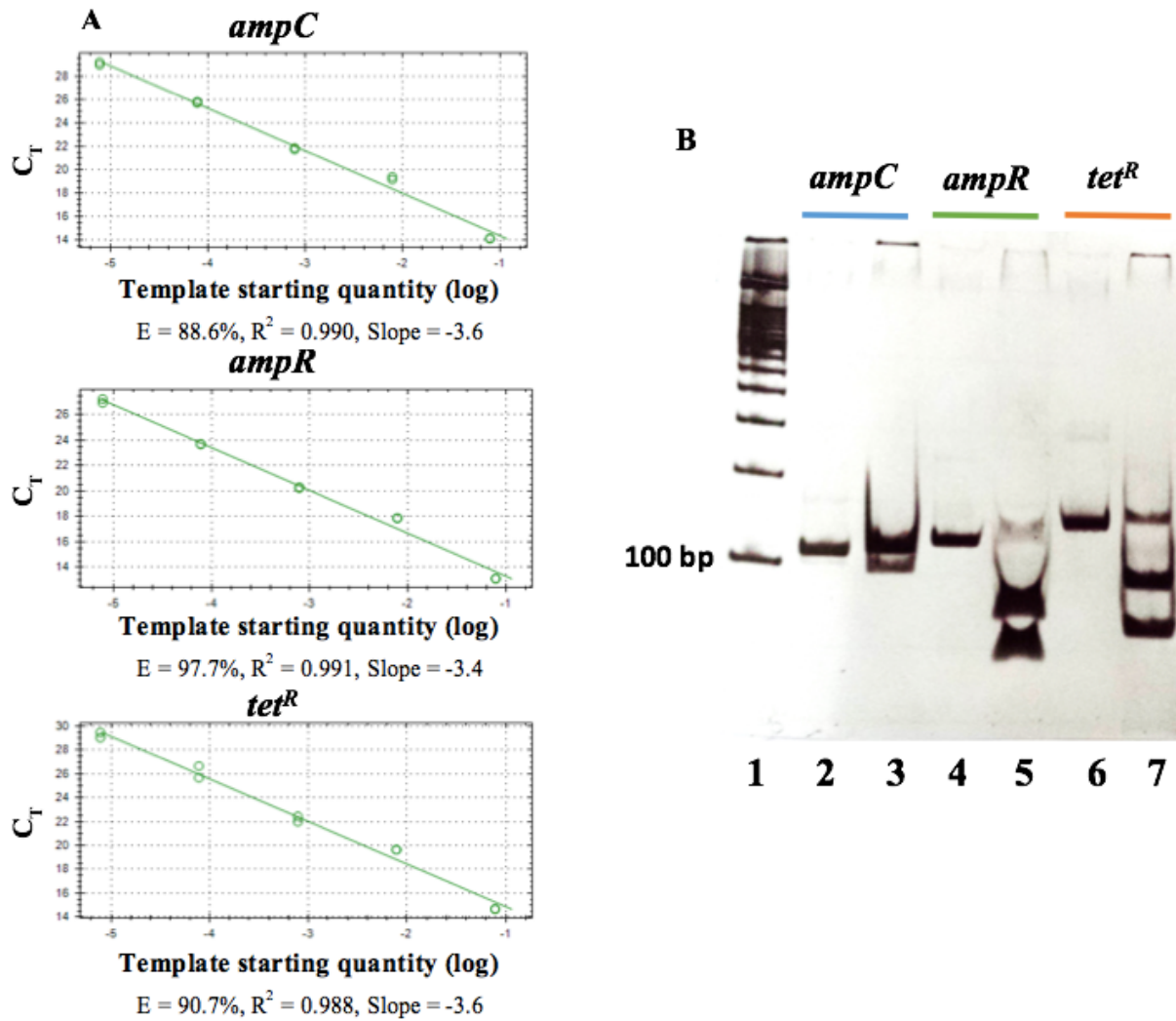


Figure 3.3 – qPCR primer efficiencies and amplicon purity

A) Standard curves (in duplicate) were generated using *ampC*, *ampR* and *tet^R* cDNA and indicate qPCR primer efficiencies for each primer pair. **B)** Amplicons generated during qPCR for *ampC* (109 bp), *ampR* (118 bp), and *tet^R* (140 bp) are shown in lanes 2, 4, 6. Restriction enzyme digested amplicons for *ampC* (17 and 92 bp; smaller fragment ran off the gel), *ampR* (51 and 67 bp), and *tet^R* (55 and 85 bp) are shown in lanes 3, 5, and 7. The amplicons were confirmed to correspond to *ampC*, *ampR*, and *tet^R* based on the observation that undigested and digested fragments for each gene were of expected sizes.

D. Conclusions

Although the crystal structure of the AmpR EBD bound to UDP-MurNAc-pentapeptide suggests that 1,6-anhydroMurNAc-pentapeptide may bind to AmpR more competitively than the tripeptide species, the methodology presented in this chapter can be developed to provide direct confirmation of this preference *in vitro*. Limiting the concentration of AmpR to a 10 to 100-fold molar excess relative to DNA template during *in vitro* transcription may facilitate the expected upregulation of *ampC* mRNA and a corresponding downregulation of *ampR*. To confirm that AmpR is indeed in a transcriptionally active conformation in the absence of its effector molecules, an *in vitro* transcription assay can be performed comparing its expression profile to that mediated by the constitutively active Gly102Gln AmpR mutant (215), which causes constitutive *ampC* overexpression in its native conformation. The work presented in this chapter offers two approaches towards quantifying relative changes in AmpR-mediated gene expression. First, generating radiolabelled mRNA transcripts was found to directly and sensitively detect relative changes in gene expression from the *ampR-ampC* operon *in vitro*. Reproducing this assay can be followed by Northern blot analysis in the future to verify the identities of the transcripts generated *in vitro*. Secondly, as a promising alternative to radiolabelling, rapid and highly sensitive RT-qPCR could also be used to accurately measure relative changes in *ampC* expression in response to binding of 1,6-anhydroMurNAc-tripeptide and –pentapeptide to AmpR *in vitro*, and thus resolve a long-standing controversy about the mechanism of AmpR activation.

Chapter 4 – Conformational flexibility of the glycosidase NagZ allows it to bind structurally diverse inhibitors to suppress β -lactam antibiotic resistance

This research was originally published in *Protein Science*, 2017, and copyright permission has been obtained to reproduce it in this thesis: Vadlamani, G., Stubbs, K.A., Désiré, J., Blériot, Y., Vocadlo, D.J., and Mark, B.L. (2017) Conformational flexibility of the glycosidase NagZ allows it to bind structurally diverse inhibitors to suppress β -lactam antibiotic resistance. *Protein Sci.* doi: 10.1002/pro.3166. The structural characterization of *BcNagZ* in complex with MM-124 (discussed in this chapter) was published separately in *Chem. Commun. (Camb)*, 2013: Mondon, M., Hur, S., Vadlamani, G., Rodrigues, P., Tsybina, P., Oliver, A., Mark, B. L., Vocadlo, D. J., and Blériot, Y. (2013) Selective trihydroxyazepane NagZ inhibitors increase sensitivity of *Pseudomonas aeruginosa* to β -lactams. *Chem. Commun. (Camb)*. **49**, 10983–10985. I carried out all of the protein purification and structural biology research presented in this chapter.

A. Introduction

The exo- β -*N*-acetylglucosaminidase NagZ is a member of the glycoside hydrolase (GH3) family and catalyzes the breakdown of GlcNAc-1,6-anhydromuramoyl-peptide into GlcNAc and 1,6-anhydroMurNAc-peptide (Fig. 4.1 A) (104), which activates AmpR-mediated *ampC* expression. Due to its key role in PG recycling and *ampC* induction, several studies have validated that small molecule inhibition of NagZ in conjunction with β -lactam therapy is a viable strategy for overcoming β -lactam antibiotic resistance (152, 156, 157). β -glucosaminidase inhibitors that have been tested specifically against NagZ include: *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino *N*-phenylcarbamate (PUGNAc) (151, 152), iminosugars (e.g. deoxynojirimycin) (216, 217), and trihydroxyazepanes (discussed in this chapter, (218)).

PUGNAc is a potent, yet indiscriminate inhibitor of several glycosidases (153, 154), including family GH20 and GH84 human β -N-acetylglucosaminidases, which are functionally related to GH3 NagZ. Fortunately, differences in the catalytic mechanisms followed by NagZ and its functionally related homologs (described in Chapter 1, section D.1) provide an avenue for the design of NagZ-selective inhibitors. A crystallographic structure of *V. cholerae* NagZ in complex with PUGNAc had revealed that the 2-acetamido group of PUGNAc (Fig. 1.7 A, C) extends into a relatively spacious pocket in NagZ, in contrast to GH20 and GH84 glycosidases, where the 2-acetamido group fits into a snug pocket so as to facilitate its participation in substrate-assisted catalysis (Fig. 1.7 B) (152). Based on this structure, NagZ-selective inhibitors were synthesized by the addition of 2-acetamido substituents on PUGNAc, including ethylbutyryl-PUGNAc (EtBuPUG) (Fig. 4.1 B), which is the most selective inhibitor known for NagZ (152). Although the structural basis for the NagZ-selectivity of EtBuPUG was not determined at the time, structural analysis of a GH84 homolog in complex with *N*-butyryl-PUGNAc confirmed that its NagZ-selectivity stemmed from steric constraints in accommodating substituents at the site of the 2-acetamido group in functionally-related human enzymes (151). This strategy can be extended towards the class of the trihydroxylated azepane inhibitors (Fig. 4.1 B), where the seven-membered heterocyclic ring possesses greater conformational plasticity compared to six-membered iminosugars (219). Accordingly, this chapter includes an exploration of the structural interactions of the 3-acetamido-4,5,6-trihydroxyazepane MM-124, and its NagZ-selective derivative MM-156 containing a 3-acetamido substituent (Fig. 4.1 B), and contrasts them with the binding interactions of PUGNAc-derived inhibitors.

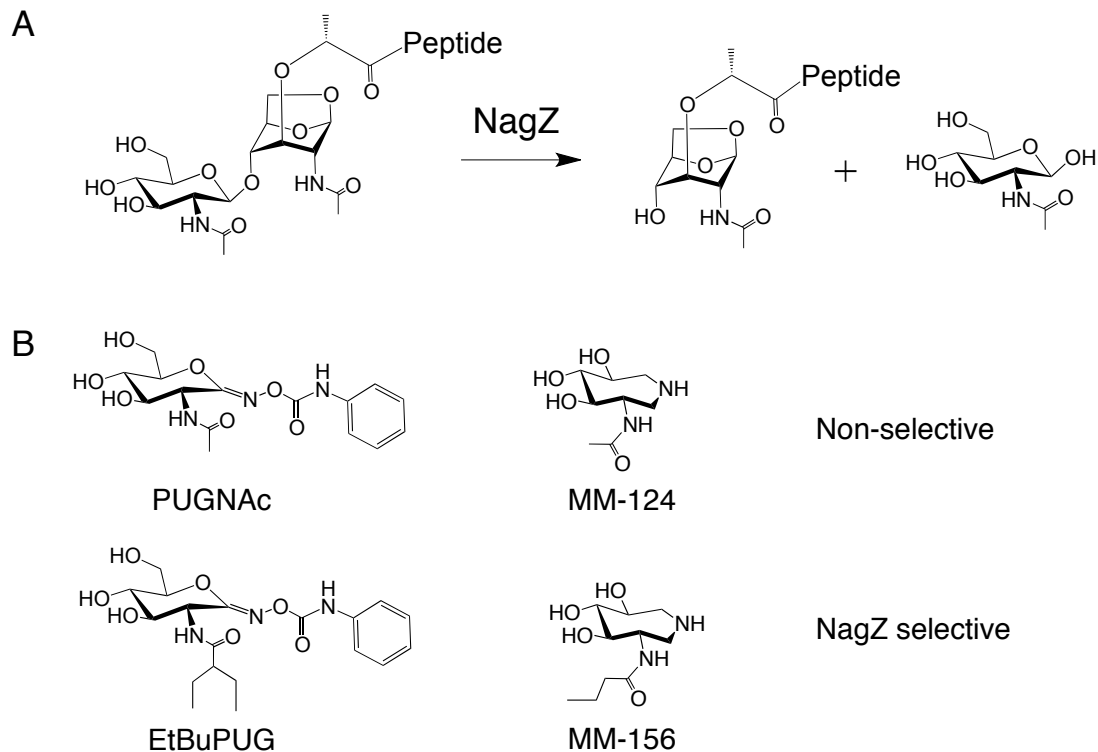


Figure 4.1 – Chemical schematic of NagZ substrate and inhibitors

The above structures depict **A**) NagZ substrate (GlcNAc-1,6-anhydromuramoyl-peptide) and products (GlcNAc and 1,6-anhydromuramoyl-peptide), **B**) non-selective PUGNAc and trihydroxyazepane (MM-124) NagZ inhibitors (top), and their NagZ-selective *N*-acetamido derivatives, EtBuPUG and MM-156 (bottom).

Compared to functionally related human glycosidases that have rigid active site architectures, GH3 NagZ enzymes position their histidine general acid/base residue on a highly mobile loop that moves in and out of the active site during catalysis in order to coordinate substrate distortions that facilitate glycosidic bond cleavage (143). Remarkably, the catalytic histidine can move as much as ~20 Å away from the position it must adopt to participate in acid/base catalysis (143). In this chapter, the structural investigations of *Bc*NagZ in complex with MM-156 and EtBuPUG reveal that their *N*-acetamido substitutions designed to impart selectivity toward NagZ are easily accommodated within the NagZ active site. However, unlike MM-124 and MM-156, PUGNAc and EtBuPUG possess a phenylcarbamate group which appears to prevent the mobile catalytic loop in NagZ from adopting a catalytically competent orientation, thus highlighting a feature of NagZ enzymes that can potentially be exploited to improve inhibitor specificity toward NagZ in addition to modifications at the *N*-acetamido site.

B. Experimental procedures

B.1. *Bc*NagZ expression and purification

Construction and sequence verification of the pBCNagZ expression plasmid was performed by Prerana Rodrigues (Mark lab alumna, Dept. of Microbiology, University of Manitoba). The open reading frame for *Bc*NagZ (UniProtKB B4EA43) was amplified by PCR from *Burkholderia cenocepacia* J2315 genomic DNA (provided by Dr. Silvia Cardona, Dept. of Microbiology, University of Manitoba) as template and the following oligonucleotide primers: 5'-GATATACATATGGCGCATCACCATCACCATCACATGAAAACGACTCCCGGCCCC-3' and 5'-GATATAGGATCCTTACGCCAGCGCGCTGCTCAACAGCGC-3'. The resulting PCR amplicon was restricted with NdeI and BamHI and ligated into a version of a pET28 plasmid

(Novagen) containing a reduced multicloning site (Nde1/Spe1/BamH1). The resulting plasmid (pBCNagZ) was used to transform *E. coli* BL21 (DE3) GOLD cells. Transformants were selected on Luria-Bertani (LB) agar supplemented with 50 µg/ml kanamycin. The resulting expression plasmid pBCNagZ was isolated from a single kanamycin-resistant transformant and confirmed by DNA sequencing at The Center for Applied Genomics (TCAG), SickKids hospital, Toronto, ON.

E. coli BL21 (DE3) GOLD cells containing the expression plasmid pBCNagZ (218) were grown to OD₆₀₀ of 0.5 at 37°C in 500-ml volumes of LB media supplemented with 35 µg/ml kanamycin, then induced with 1 mM IPTG for 3 hours at 28°C. Cells were pelleted by centrifugation and stored at -80°C. Thawed pellets were resuspended in 20 ml lysis buffer (0.5 M NaCl, 5% glycerol, 25 mM HEPES pH 7, 1 µM PMSF), and the cells lysed using a French pressure cell press (AMINCO). Following centrifugation, the soluble protein fraction of the lysate containing His-tagged *BcNagZ* was incubated with nickel-nitrilotriacetic acid (Ni-NTA) resin at 4°C for 1 hour, prior to loading on a gravity column. Resin-bound protein was subjected to washes with binding buffer (25 mM HEPES pH 7, 0.5 M NaCl and 5% glycerol) supplemented with 0, 10 and 20 mM imidazole, and eluted using wash buffer containing 250 mM imidazole. The eluted protein was dialyzed overnight against 2 L binding buffer, and concentrated to 13-20 mg/ml.

B.2. Crystallization and structure determination of *BcNagZ* with inhibitors

BcNagZ crystals were grown at 20°C using the hanging drop vapour-diffusion method by mixing equal volumes of reservoir buffer (30-32% PEG8000, 0.1M MES pH 6.2-6.8) with protein solution (13-20 mg/ml). The inhibitors MM-124 and MM-156 were synthesized by Dr.

Blériot's lab (Université de Poitiers, France) (218), and PUGNAc and EtBuPUG were prepared by Dr. Stubbs (Dr. Vocadlo's lab, Simon Fraser University, BC, Canada) as described elsewhere (155). A single droplet containing several *BcNagZ* crystals in reservoir buffer was soaked for 24 hours in MM-124, MM-156, PUGNAc or EtBuPUGNAc at final concentrations of 1 mM to obtain the protein-inhibitor complex. Prior to screening, crystals were cryo-protected in 30% PEG3500, 15% PEG8000 and 0.1M MES pH 6.6, and flash-cooled in liquid nitrogen. X-ray data for the MM-124, EtBuPUG and PUGNAc-bound complexes were collected using a Rigaku R-Axis IV++ detector and 007HF MicroMax X-ray generator at the University of Manitoba. Data for the *BcNagZ*-MM-156 complex were collected using beamline 08B1-1 at the Canadian Light Source (Saskatoon, Canada). The X-ray data were indexed using iMosfilm (220), then scaled and averaged using SCALA (CCP4 package) (191). The *BcNagZ*:inhibitor complex structures were determined by molecular replacement using PHASER (from within the PHENIX package) (192) and a structure of *BcNagZ* (PDB ID: 4G6C) from which solvent had been removed prior to use as the search model. The MR model was subsequently rebuilt using PHENIX.AUTOBUILD (192). The ligand restraint files for MM-124, MM-156, PUGNAc and EtBuPUG were generated using PHENIX eLBOW (192) and a model of each inhibitor was manually fit into its electron density. Subsequent refinement of the complex and addition of solvent was carried out using PHENIX.REFINE (192) and COOT (193). Crystallographic and refinement statistics are presented in Table 4.1.

B.3. Multiple sequence alignments

The amino acid sequences of all GH3 family NagZ enzymes from Gram-positive bacteria, and selected Gram-negative species were obtained from the CAZy database (<http://www.cazy.org/>). Multiple sequence alignments were performed comparing Gram-

negative species, and Gram-positive species, respectively, using the Clustal Omega program from EMBL-EBI bioinformatics services (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).

B.4. Structure coordinates

The crystallographic structures of *BcNagZ* in complex with PUGNAc, EtBuPUG, MM-124, and MM-156 were deposited in the Protein Data Bank under the coordinates 5UTQ, 5UTP, 4MSS, and 5UTR, respectively.

C. Results and discussion

C.1. The phenylcarbamate extension on PUGNAc and EtBuPUG displaces the catalytic loop from the *BcNagZ* active site

To investigate the effects of inhibitor binding on the orientation of the catalytic loop in *BcNagZ*, crystals of the enzyme were soaked with 1 mM PUGNAc, EtBuPUG, MM-124 or MM-156 for 24 hours and structures of the complexes determined by molecular replacement using an unliganded model of *BcNagZ* (PDB 4G6C) from which solvent had been removed. *BcNagZ* is a single domain enzyme that adopts a characteristic TIM-barrel (β/α)₈ (Fig. 4.2). Interestingly, it was found that MM-156 allows the catalytic loop to enter the active site and correctly position the general acid/base residue His183 within ~6 Å of the active site nucleophile (Asp 253) (Fig. 4.2 A), which is consistent with the distance between the analogous residues of other configuration retaining glycosidases (221).

The position of the loop is also consistent with the superposed structures of *BcNagZ* bound to MM-124 (PDB ID: 4MSS; data not shown for clarity) (218) and the product GlcNAc (PDB ID: 4GNV), indicating that the propyl extension off the 3-acetamido group in MM-156

does not affect positioning of the catalytic loop and His183 within the enzyme active site (Fig. 4.3 A, B). Moreover, the catalytic loop in these complexes adopts the same position as found in the GH3 NagZ from the Gram-positive bacteria *Bacillus subtilis* (*BsNagZ*) bound to GlcNAc-MurNAc substrate (PDB ID: 4GYJ), where the histidine (His234) of the catalytic loop of *BsNagZ* interacts directly with the glycosidic oxygen of the disaccharide through a 2.8 Å hydrogen bond (Fig. 4.3 B) (143). Unlike the single domain structure of NagZ enzymes of Gram-negative bacteria, *BsNagZ* is a two-domain enzyme, as are most NagZ enzymes from Gram-positives. Nevertheless, the flexibility of the catalytic loop and its ability to place the catalytic histidine within hydrogen bonding distance of the oxygen of the scissile glycosidic bond appears conserved in GH3 NagZ enzymes from Gram negative and positive bacteria (142, 143).

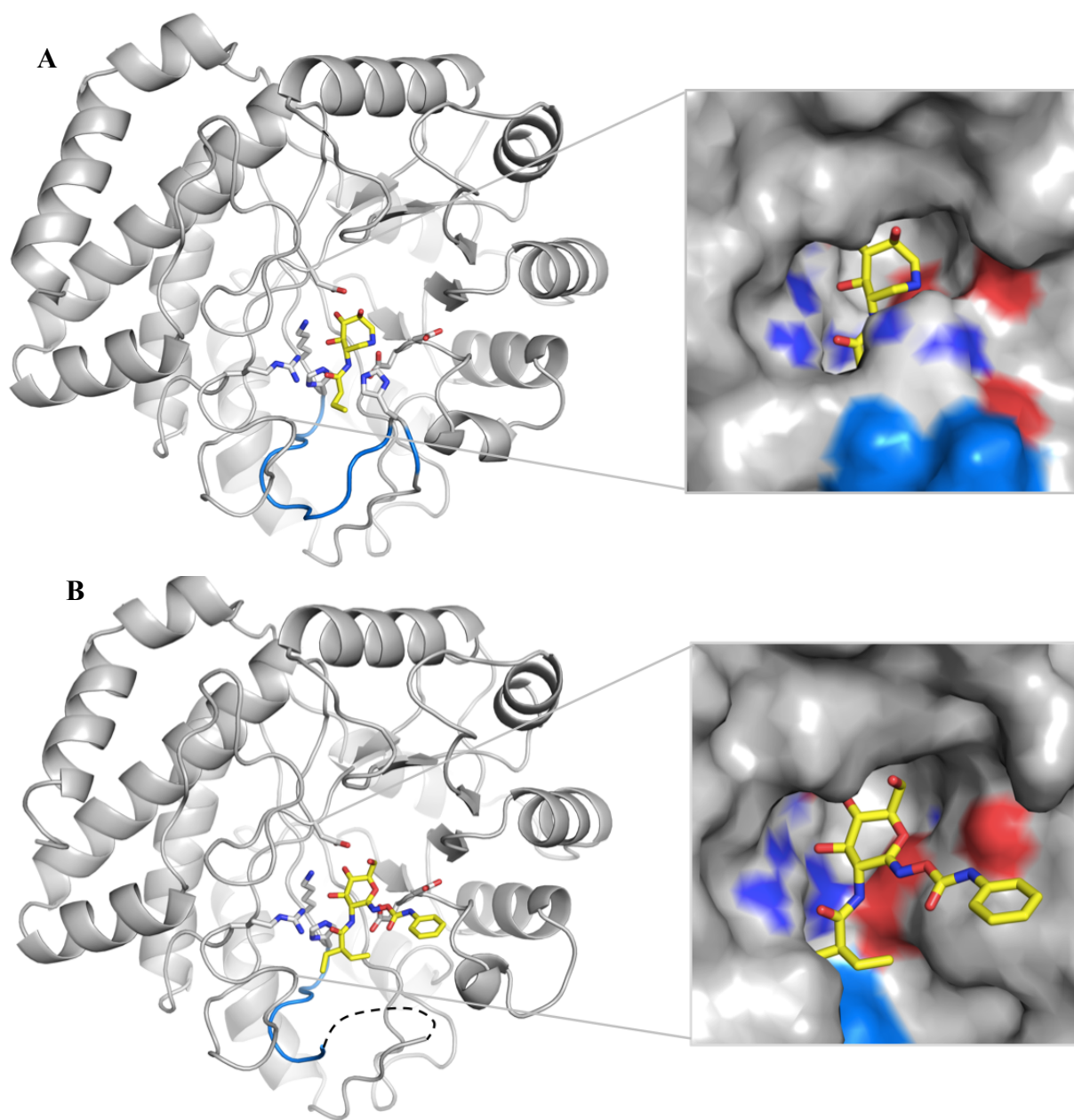


Figure 4.2 – *BcNagZ* in complex with MM-156 and EtBuPUG.

The general acid/base histidine-bearing catalytic loop is highlighted in blue in the *BcNagZ*: MM-156 complex (A), and was disordered in both the EtBuPUG and PUGNAc-bound complexes (B) (PUGNAc not shown for clarity), pictured very clearly in surface representations of the active site (inset). MM-156 and EtBuPUG are shown as yellow sticks, and nitrogen and oxygen atoms are coloured dark blue and red, respectively.

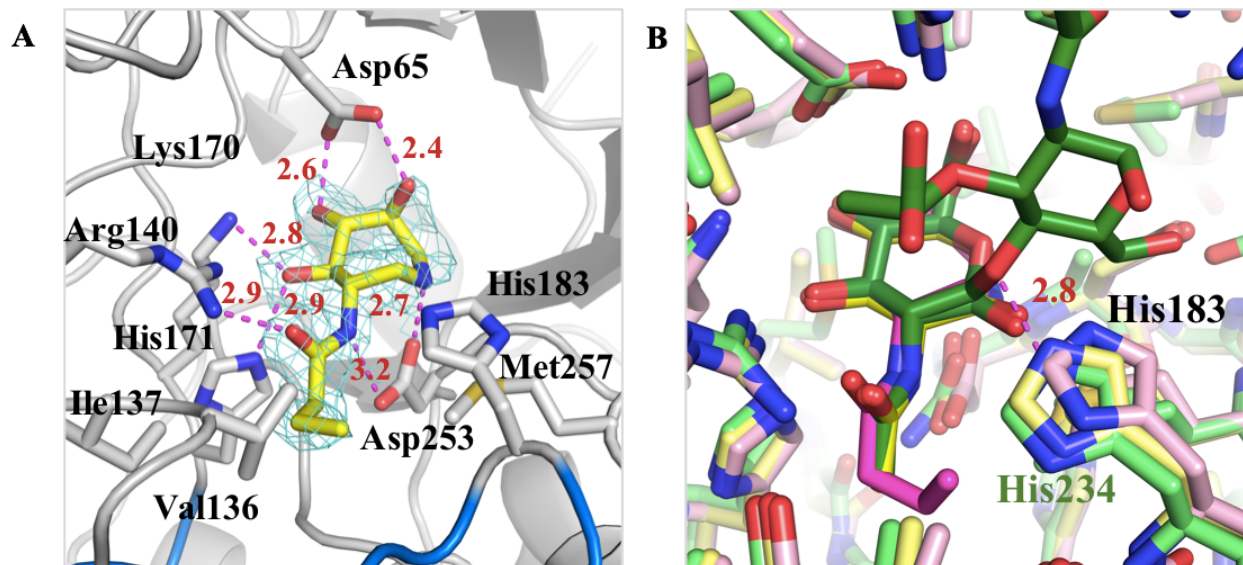


Figure 4.3 – Active site interactions of *BcNagZ* with MM-156

A) Maximum-likelihood weighted $F_{\text{obs}} - F_{\text{calc}}$ omit map contoured at 3.5σ for MM-156 (carbon atoms shown as yellow sticks) bound to *BcNagZ*, with dashed lines indicating hydrogen-bonding interactions with active site residues. **B)** Superposition of *BcNagZ*-MM-156 (pink), *BcNagZ*-GlcNAc (yellow, PDB 4GNV), and *BsNagZ*-GlcNAc-MurNAc (green, PDB 4GYJ). Hydrogen bond between the disaccharide glycosidic oxygen and catalytic His234 in the *BsNagZ* structure is shown as a dashed magenta line. MM-124 is not shown for clarity, but binds very similarly to MM-156. Nitrogen and oxygen atoms are coloured blue and red, respectively.

Table 4.1: Crystallographic statistics for structures of *BcNagZ* bound to MM-156, PUGNAc, and EtBuPUG.

Data collection	<i>BcNagZ</i>: MM-124	<i>BcNagZ</i>: MM-156	<i>BcNagZ</i>: PUGNAc	<i>BcNagZ</i>: EtBuPUG
X-ray source	Rigaku MicroMax 007HF	CLS 08B1-1	Rigaku MicroMax 007HF	Rigaku MicroMax 007HF
Space group	P 1 2 ₁ 1	P 1 2 ₁ 1	P 1 2 ₁ 1	P 1 2 ₁ 1
Unit cell dimensions	$a = 48.76 \text{ \AA}$, $b = 87.58 \text{ \AA}$, $c = 67.21 \text{ \AA}$; $\alpha = \gamma = 90^\circ$, $\beta = 91.89^\circ$	$a = 48.66 \text{ \AA}$, $b = 87.82 \text{ \AA}$, $c = 66.97 \text{ \AA}$; $\alpha = \gamma = 90^\circ$, $\beta = 91.92^\circ$	$a = 48.67 \text{ \AA}$, $b = 87.67 \text{ \AA}$, $c = 66.70 \text{ \AA}$; $\alpha = \gamma = 90^\circ$, $\beta = 91.75^\circ$	$a = 51.21 \text{ \AA}$, $b = 85.89 \text{ \AA}$, $c = 65.61 \text{ \AA}$; $\alpha = \gamma = 90^\circ$, $\beta = 102.79^\circ$
Wavelength (Å)	1.54	1.03	1.54	1.54
Resolution (Å)	29.59 – 1.80 (1.864 - 1.80)	66.93 - 2.15 (2.21 - 2.15)	43.83 - 2.20 (2.27 - 2.20)	49.94 - 2.20 (2.27 - 2.20)
R _{merge}	0.090 (0.55)	0.091 (0.264)	0.124 (0.458)	0.137 (0.249)
CC(1/2)	0.997 (0.655)	0.993 (0.889)	0.966 (0.647)	0.950 (0.908)
I / σ I	9.3 (2.0)	9.7 (4.5)	6.4 (2.0)	5.1 (3.1)
Completeness (%)	98.0 (96.2)	100 (100)	99.4 (99.2)	98.2 (98.0)
Redundancy	3.2 (3.1)	3.4 (3.3)	2.2 (2.2)	2.9 (2.8)
Refinement				
R _{work} / R _{free}	0.14/ 0.19	0.14/ 0.19	0.16/ 0.21	0.17/ 0.23
B-factor (Å ²)	21.00	17.80	22.58	17.03
Protein	19.40	17.13	22.34	16.90
Ligands	16.60	16.57	30.71	18.60
Solvent	31.0	23.15	24.65	18.51
<i>RMSDs</i>				
Bond lengths (Å)	0.01	0.002	0.003	0.002
Bond angles (°)	1.34	0.62	0.64	0.63
Ramachandran favoured/allowed (%)	97/ 0	96/ 4.0	95/ 5.3	96/ 4.2

* Values in parentheses refer to the high-resolution shell.

In contrast to the lack of influence of MM-124 or MM-156 on the positioning of the catalytic loop within the active site of *BcNagZ*, we found that both PUGNAc and EtBuPUG completely displace the catalytic loop from the enzyme active site (Fig. 4.2 B). Superposition of *BcNagZ* structures bound to NagZ-selective inhibitors MM-156 and EtBuPUG reveal that the position of the phenylcarbamate extension off C1 of EtBuPUG (or PUGNAc) would result in a direct steric clash with His183 of the catalytic loop, which is likely responsible for displacing the loop from the active site (Fig. 4.4). Unlike the natural substrates of NagZ, which undergo considerable distortion during catalysis that allows the catalytic loop to enter and position the catalytic histidine in the active site (Fig 4.3B) (143), the sp²-like hybridization state of C1 of PUGNAc and its 2-acetamido group derivatives does not allow a comparable distortion, thus permitting only an orientation that leads to displacement of the mobile loop.

On the other hand, the ethylbutyryl extension off the 2-acetamido group of EtBuPUG, which occupies the same region in the active site as the propyl extension off the 3-acetamido group of MM156, was found to have no appreciable effect on the conformation of nearby residues in the enzyme, suggesting that even though it is slightly larger than the *N*-butyryl group on MM156, it does not participate in loop displacement. Interestingly, MM-156 and EtBuPUG both have low micromolar binding affinities against NagZ enzymes from Gram negative bacteria ($K_i = \sim 7 \mu\text{M}$ and $\sim 3 \mu\text{M}$, respectively) (152, 218) and PUGNAc remains the most potent NagZ inhibitor known ($K_i = \sim 0.05 \mu\text{M}$) (152); thus, displacement of the catalytic loop does not appear to incur a binding penalty. The additional space in the active site that is opened up by the displacement of the catalytic loop by the phenylcarbamate group on PUGNAc and its derivatives (Fig 4.2B) could therefore be exploited to improve selectivity of these inhibitors for bacterial

NagZ enzyme, such as the generation of a locked cyclic derivative where extensions off the 2-acetamido group could be linked to a phenylcarbamate group.

C.2. Molecular interactions not involving the catalytic loop of *Bc*NagZ underlie the binding potency of the NagZ-selective inhibitors MM156 and EtBuPUG

The azepane of MM-156 and GlcNAc component of PUGNAc and EtBuPUG all bind similarly within the active site of *Bc*NagZ active site (Fig. 4.4). The azepane hydroxyl groups of MM-156 form several hydrogen-bonding interactions with the enzyme that mimic the interaction of the enzyme with hydroxyl groups of the terminal GlcNAc residue of PG recycling intermediates and the GlcNAc moieties of PUGNAc and EtBuPUG. However, an additional 2.8 Å hydrogen bond occurs between the endocyclic nitrogen of MM-156 and the catalytic nucleophile of *Bc*NagZ (Asp253) (Fig. 4.3 A, 4.5 A, B), that likely contributes to the binding potency of azepane-based inhibitors to NagZ enzymes (218). The propyl extension to the 3-acetamido group of MM-156 provides excellent selectivity for GH3 NagZ over GH20 *N*-acetyl- β -glucosaminidase (> 1000-fold) and modest selectivity over GH84 *N*-acetyl- β -glucosaminidases (~ 7-fold) due to the steric constraints of the GH20 and GH84 enzyme active sites (kinetic analysis performed by Dr. Bleriot's lab, (218)). The extension also forms favorable hydrophobic interactions with residues that help form the pocket within which the extension binds (Val136, Ile137 and Met256) (Fig. 4.3 A). Moreover, the pocket is able to accommodate even larger substituents as described below for EtBuPUG.

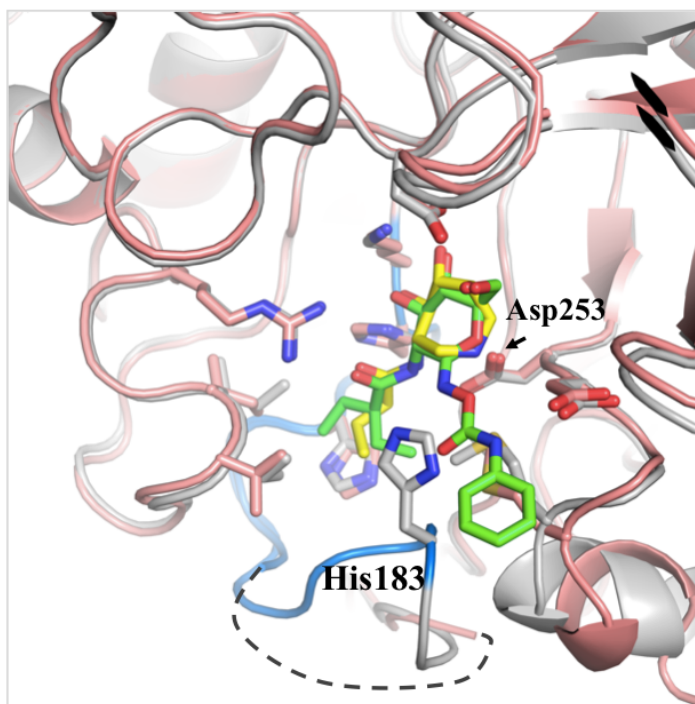


Figure 4.4 – Superposition of *BcNagZ* structures bound to MM-156 and EtBuPUG

The catalytic loop is highlighted in blue in the *BcNagZ*: MM-156 complex (grey), and depicted as a dashed line in the EtBuPUG-bound complex (salmon; PUGNAc not shown for clarity). The phenylcarbamate arm of EtBuPUG is oriented such that it would sterically clash with His183 from the MM-156-bound complex. MM-156 and EtBuPUG are shown as yellow and green sticks, respectively; nitrogen and oxygen atoms are coloured dark blue and red.

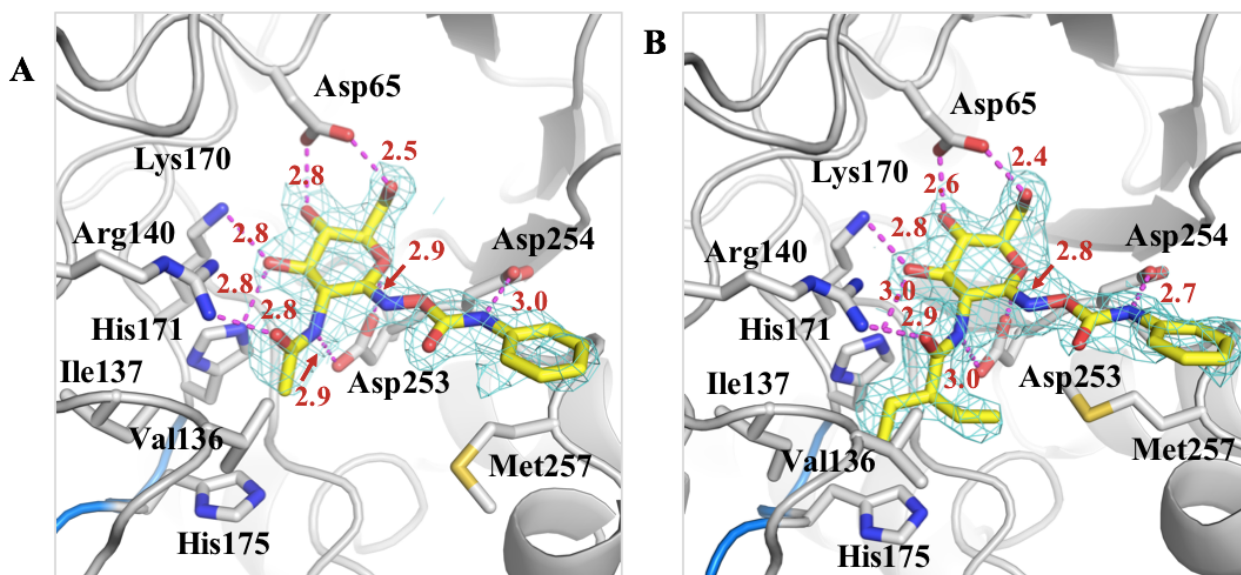


Figure 4.5 – Active site interactions of *BcNagZ* with PUGNAc and EtBuPUG

A) Maximum-likelihood weighted $F_{\text{obs}} - F_{\text{calc}}$ omit map contoured at 3.5σ for PUGNAc and **B)** EtBuPUG bound to *BcNagZ*, with dashed lines indicating hydrogen-bonding interactions with active site residues. The ethylbutyryl gr appears to be easily accommodated within the active site. No electron density was observed for the catalytic His183 residue-bearing loop. Carbon, nitrogen and oxygen atoms are coloured grey/yellow, blue and red, respectively.

EtBuPUG has excellent selectivity for NagZ over both GH20 (~1000-fold) and GH84 (~100-fold) *N*-acetyl- β -glucosaminidases (152), which arises from its large *N*-ethylbutyryl group. Similar to the propyl extension on MM-156, the ethylbutyryl group of EtBuPUG extends into a pocket where it forms hydrophobic interactions with Val136, Ile137, Met256 and His175 (Fig. 4.5 B). Notably, even though the ethylbutyryl extension is slightly larger than the propyl group on MM156, it does not significantly alter the conformation of the residues comprising the pocket into which it binds when compared to unliganded *Bc*NagZ. The major conformational difference in *Bc*NagZ that does arise from binding EtBuPUG or PUGNAc is the displacement of the catalytic loop by the phenylcarbamate extension, which forms a 2.7 Å hydrogen bonding interaction with Asp254 of the enzyme (Fig. 4.5 B). The presence of this interaction with Asp254 appears to favour this positioning over an alternate conformation that might otherwise allow the catalytic loop to enter the active site, as observed for *Bs*NagZ bound to PUGNAc (142) (see below). Additional interactions of PUGNAc and EtBuPUG with *Bc*NagZ include a hydrogen-bonding interaction between the 2-acylamido group nitrogen and the active site nucleophile Asp253 as well as hydrogen bonding interactions between the enzyme and hydroxyl groups of the GluNAc moiety of the inhibitor. Finally, the geometric resemblance of PUGNAc and its 2-acetamido group derivatives to the proposed oxocarbenium ion-like transition states that arise along the reaction coordinate is also believed to contribute significantly to binding potency of these inhibitors to NagZ enzymes (152).

C.3. The phenylcarbamate extension of PUGNAc adopts different conformations depending on the active site architecture of NagZ

Crystal structures of NagZ enzymes from both Gram-negative and –positive bacteria have now been determined in complex with PUGNAc (142, 152). While NagZ from Gram-negative bacteria are typically single-domain enzymes that adopt a TIM-barrel (β/α)₈, NagZ enzymes from Gram positive bacteria have a two-domain architecture; however, the N-terminal domain adopts a TIM-barrel fold that is conserved with NagZ enzymes from Gram-negative bacteria (142). Although Gram-positive bacteria lack the *ampR-ampC* system that is blocked by NagZ inhibitors, they do appear to have a PG recycling pathway in which NagZ participates (114).

Interestingly, superposing PUGNAc-bound complexes of *BsNagZ* and *BcNagZ* reveals that in the latter complex, the conformation of the phenylcarbamate extension of PUGNAc differs significantly from the conformation it adopts when bound to *BcNagZ* (Fig. 4.6 A). This difference arises due to the presence of Ala319 in *BsNagZ*, which replaces the aspartate in this position (Asp254) in *BcNagZ*. The alanine creates sufficient space for the phenylcarbamate of PUGNAc to adopt a conformation that permits accommodation of the catalytic loop of *BsNagZ* within the active site and correctly places His234 for catalysis (Fig. 4.6 A). Conversely, in *BcNagZ*, Asp254 blocks the phenylcarbamate of PUGNAc from adopting such a conformation and instead forms a hydrogen bonding interaction with the extension, which holds it in a conformation that does not allow the catalytic loop to enter the active site (Fig. 4.6 A).

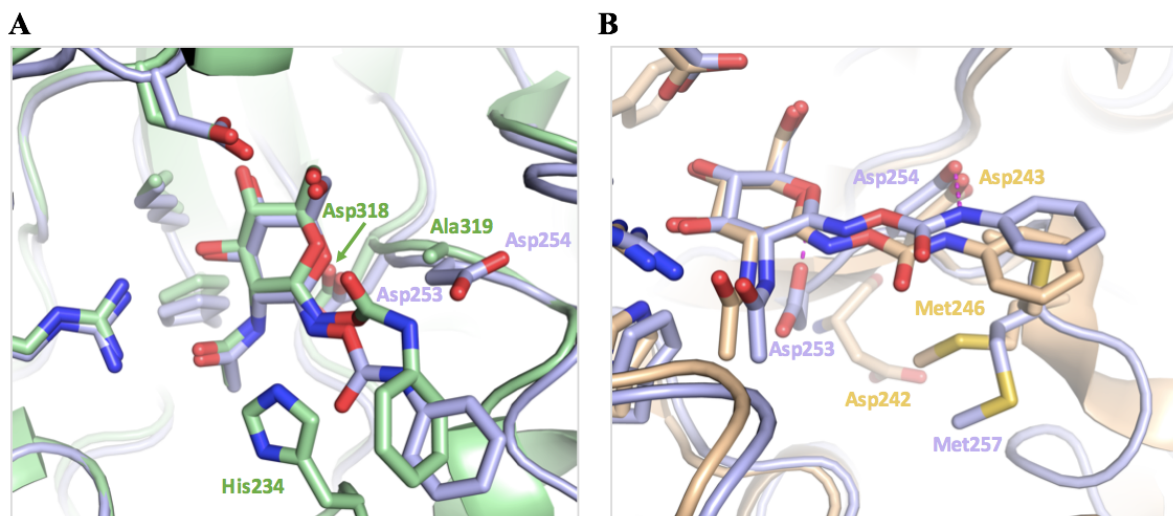


Figure 4.6 – Active site superposition of PUGNAc-bound NagZ enzymes

A) Superposition of the NagZ: PUGNAc complex from *B. cenocepacia* (light blue) and *B. subtilis* (green, PDB 3NVD) reveals that the catalytic aspartate nucleophiles for each structure are present in a physiologically relevant conformation. The PUGNAc phenylcarbamate group distortion when bound to *Bs*NagZ appears to be accommodated due to the presence of Ala319. Instead, in *Bc*NagZ, there is Asp254 at this site which is within hydrogen-bonding distance of the nitrogen bonded to the phenyl group. **B)** Superposition of PUGNAc-bound structures of *B. cenocepacia* (light blue) and *V. cholera* (light brown, PDB 2OXN). Unlike the *Bc*NagZ complex, the catalytic nucleophile Asp242 is flipped away from the active site in *Vc*NagZ, while the PUGNAc phenyl group competes for occupancy with Met246. Key active site residues are labelled in corresponding colours. Hydrogen-bonding interactions between *Bc*NagZ and PUGNAc are represented by dashed magenta lines. Nitrogen and oxygen atoms are indicated in dark blue and red, respectively.

The phenylcarbamate extension adopts a nearly identical conformation when PUGNAc is bound to another single domain GH3 NagZ from the Gram negative bacteria *V. cholerae* (*VcNagZ*) and is within 3.6 Å of Asp243, the analogous residue of Asp254 in *BcNagZ* (Fig. 4.6 B). Multiple sequence alignments of GH3 NagZ enzymes from Gram-negative and –positive bacteria reveal Asp254 of *BcNagZ* (Asp243 *VcNagZ*) is highly conserved in Gram-negative bacteria whereas this position in NagZ enzymes from Gram-positive bacteria, while predominantly alanine, exhibits a higher degree variability, including the presence of aspartate or cysteine (Fig. 4.7).

It is interesting to observe that the phenylcarbamate extension of PUGNAc can adopt different conformations depending on differences in the active site architectures of NagZ enzymes from Gram-negative and –positive bacteria. Given the Asp254 of *BcNagZ* is highly conserved among NagZ enzymes from other Gram-negative bacteria, we predict the catalytic loop will be displaced by PUGNAc in these enzymes whereas, in NagZ from Gram-positive bacteria that have an alanine in this position, it is expected that the phenylcarbamate extension of PUGNAc will adopt a conformation that could allow the catalytic loop to enter the active site as observed for *BsNagZ* (Fig. 4.6 A).



Figure 4.7 – Multiple sequence alignments of NagZ enzyme active site residues

Multiple sequence alignments are shown for a conserved active site region within GH3 family NagZ proteins from Gram-negative species (top) and Gram-positive bacteria (bottom). Active site residues corresponding to Asp254 in *BcNagZ* are highly conserved in Gram-negative bacteria, while residues homologous to Ala319 in *BsNagZ* exhibit greater variability (positions shown by arrow). Sequence alignments were performed using the Clustal Omega web server (<http://www.ebi.ac.uk/Tools/msa/clustalo/>)

D. Conclusions

Inhibiting NagZ activity with small molecule inhibitors has been shown to suppress AmpC-mediated β -lactam antibiotic in Gram-negative bacteria by blocking PG recycling and the formation of PG recycling intermediates that induce *ampC* gene expression. Introducing 2-acylamido substituents on PUGNAc has been used to favour NagZ-selectivity in the past, and the work presented in this chapter demonstrates that this strategy can be successfully applied to generate NagZ-selective 3-acetamido trihydroxyazepanes. Furthermore, it is apparent that the remarkable plasticity of NagZ enables it to adopt different conformations in response to various inhibitor designs. In the case of PUGNAc and its 2-acetamido group derivative EtBuPUG, the displacement of the catalytic loop by this inhibitor class opens up the entrance to the active site considerably (Fig. 4.2), which could be exploited to develop new PUGNAc derivatives with substituents that occupy the open space to significantly enhance selectivity for bacterial NagZ enzymes over functional related GH20 and GH84 human *N*-acetyl- β -glucosaminidases.

Chapter 5 – *N*-Acetyl glycals are tight-binding and environmentally insensitive inhibitors of GH20 hexosaminidases

The work presented in this chapter was published in *Chem. Commun.*, 2016: González, A., Vadlamani, G., Mark, B. L., and Withers, S. G. (2016) *N*-Acetyl glycals are tight-binding and environmentally insensitive inhibitors of hexosaminidases. *Chem. Commun.* **52**, 7943–7946. I carried out all of the structural biology research described in the above publication, which has been rewritten here with a focus on the structural data. A.G. carried out all enzyme kinetics analyses on *SpHex*.

A. Introduction

N-Acetyl- β -hexosaminides are ubiquitous in nature and are components of glycoproteins, glycolipids, bacterial peptidoglycan, and glycosaminoglycans contained within the extracellular matrix (222, 223). Glycosidases such as *N*-acetyl- β -hexosaminidases of the GH20 family play important biological roles in the degradation of glycolipids and glycoproteins, which make them attractive targets for inhibitor design (224). *O*-GlcNAcase is a family GH20 hexosaminidase that is involved in the *O*-GlcNAc biosynthesis pathway, and its inhibition by thiamet-G has been shown to reduce the aggregation of tau fibrils, thus impeding neurodegeneration that causes Alzheimer's disease in human and mouse models (224, 225).

Interestingly, unlike NagZ (described in Chapter 4) and other GH3 glycosidases, family 20 enzymes use a substrate-assisted catalytic mechanism whereby the carbonyl oxygen of the 2-acetamido group of the terminal GlcNAc to be removed acts as the catalytic nucleophile instead of an acidic residue on the enzyme (see Fig. 1.6 for mechanisms) (144, 149, 150). A previous crystal structure of a β -*N*-acetylhexosaminidase from the Gram-positive bacteria *Streptomyces*

plicatus bound to the reaction intermediate analogue NAG-thiazoline (Fig. 5.1 A) provided structural support that this class of GH20 enzymes proceeds via a substrate-assisted catalytic mechanism, and revealed that the inhibitory activity of NAG-thiazoline was due to its similarity to the oxazolinium-ion like transition state (150). Several additional classes of *N*-acetylhexosaminidase inhibitors have been studied to block this catalytic mechanism as well, including aldonolactones, 5-amino-5-deoxy-D-hexopyranoses, isofagomines, and glycals (226, 227). A previous study showed that the glycal inhibitor *N*-acetyl-D-glucal (NA-glucal; Fig. 5.1 B) bound tightly to β -*N*-acetylhexosaminidases from human placenta, jack bean and bovine kidney, with K_i values in the micromolar range (226). The 2-acetamido group on NA-glucal was suggested to stabilize the transition state-like binding interactions, and contribute to its potency against β -*N*-acetylhexosaminidases (226).

This chapter investigates the structural basis for the tight-binding inhibition of NA-glucal against a β -*N*-acetylhexosaminidase from *S. plicatus* (*SpHex*), which is a model enzyme for the structural and functional characterization of inhibitors of GH family 20 enzymes (150, 222, 223, 227). As described previously, catalysis by GH20 enzymes is initiated when the general acid residue (Glu314 in *SpHex*) assists the 2-acetamido group of the substrate in launching a nucleophilic attack on the anomeric carbon, which generates a cyclic oxazoline intermediate (Fig. 5.2, left) (150, 224). Glu314 then behaves as a general base, and facilitates hydrolysis of the oxazoline intermediate to yield a product with retention of stereochemistry (150, 224). In *SpHex*, Asp313 plays a vital role in stabilizing the transition states as catalysis proceeds, and moreover, correctly orients the 2-acetamido group of the substrate for catalysis (222). Lai *et al.* also observed that the ring double bond in NA-glucal undergoes slow enzyme-catalyzed hydration (226). Kinetic analysis performed by my collaborator, Dr. Andres Gonzalez (Dept. of Chemistry,

University of British Columbia) validated the occurrence of NA-glucal hydration in *SpHex*, and a likely hydration mechanism beginning with Glu314 acid-catalysed formation of the oxazoline intermediate was proposed (Fig 5.2, right). A thioamide version of NA-glucal (NTA-glucal, Fig. 5.1 C) was expected to be resistant to this hydration mechanism (224), and was synthesized to generate an inhibitor that would be more metabolically stable. However, kinetic analysis described below indicates that although capable of inhibiting *SpHex* activity, NTA-glucal bound considerably more weakly than NA-glucal. Additionally, modification of key active site residues did not appear to affect the binding affinities of NA-glucal or NTA-glucal towards *SpHex*. To elucidate the structural basis for their invariant binding affinities, NTA-glucal was crystallized bound to *SpHex*. Overall, the kinetic and structural data presented in this chapter indicate that as charge-neutral compounds, *N*-acetyl glycals are a promising class of hexosaminidase inhibitors with an imperviousness to mutations in active site residues that could render them immune to the development of resistance from point mutations.

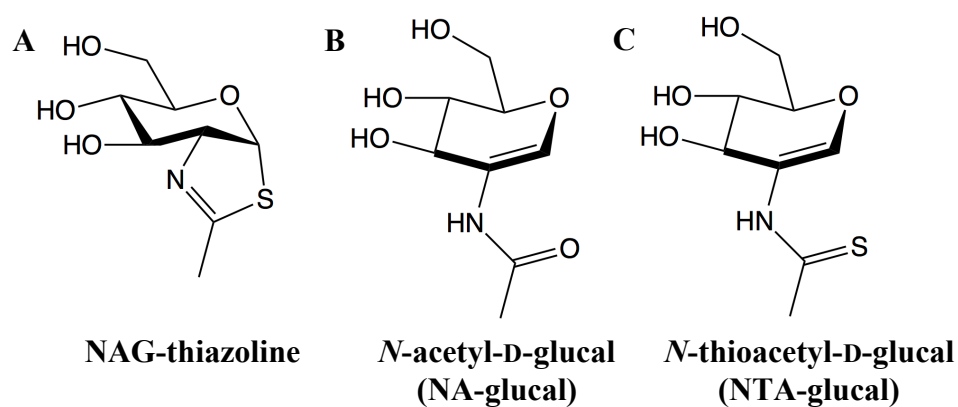


Figure 5.1 –Chemical schematic of *N*-acetylhexosaminidase inhibitors.

A) NAG-thiazoline is a transition state analogue of *N*-acetylhexosaminidase substrate. Inhibitors from the *N*-acetyl glycal class characterized in this chapter include **B)** *N*-acetyl-D-glucal (NA-glucal), and **C)** *N*-thioacetyl-D-glucal, which is a thioamide version of NA-glucal.

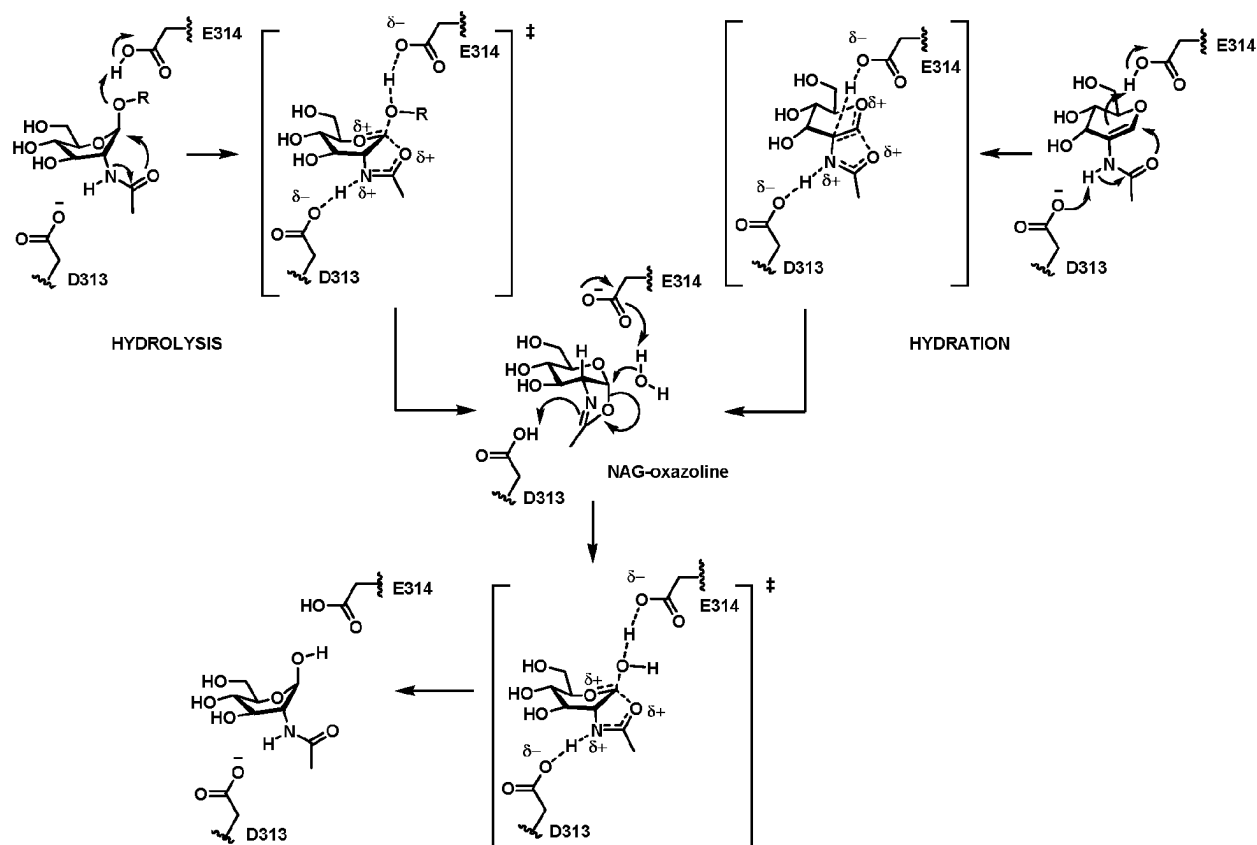


Figure 5.2 – Proposed mechanism for NA-glucal hydration by *SpHex*.

Both GlcNAc hydrolysis (left) and NA-glucal hydration (right) proceed via a NAG-oxazoline transition intermediate. During hydration, however, formation of the reaction intermediate is proposed to be preceded by hydration of the double-bond ring carbon (C2) that converts NA-glucal into GlcNAc. This figure was reproduced from Ref. 224 (Gonzalez *et al.*, 2016) with permission from the Royal Society of Chemistry.

B. Experimental procedures

B.1. SpHex expression and purification

E. coli BL21 (DE3) cells containing the expression plasmid p3AHEX-1.8 (previously constructed by Dr. Brian Mark, (150)) were grown to OD₆₀₀ of 0.5 at 37°C in 500-ml volumes of LB media supplemented with 150 µg/ml ampicillin, then induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside for 3 hours at 25°C. Cells were pelleted by centrifugation and stored at -80°C. Thawed pellets were resuspended in 20 ml lysis buffer (20 mM Tris pH 8, 300 mM NaCl, 10 mM imidazole pH 8, and 10 mM β-mercaptoethanol), and the cells lysed using a French pressure cell press (Aminco). Following centrifugation, the soluble protein fraction of the lysate containing His₇-tagged *SpHex* was incubated with nickel-nitrilotriacetic acid (Ni-NTA) resin at 4°C for 1 hour, prior to loading on a gravity column. Resin-bound protein was washed with lysis buffer supplemented with 80 mM imidazole pH 8, and eluted using lysis buffer containing 250 mM imidazole pH 8. The eluted protein was dialyzed overnight against 2 L dialysis buffer (50 mM trisodium citrate pH 6, 300 mM NaCl, and 0.5 mM DTT), and concentrated to 3-12 mg/ml.

B.2. SpHex crystallization and structure determination

The *SpHex* crystallization procedure was adapted from Mark *et al.*, 2001 and optimized within a range of AmSO₄ concentrations (1.6-2.6 M), with reduced glycerol (5-20%) (150). Crystals were grown at room temperature by the hanging drop vapour diffusion method using equal volumes of reservoir buffer (1.8 M AmSO₄, 0.1 M trisodium citrate pH 6) and protein (3.6 mg/ml), and maintained as stocks for streak-seeding in subsequent co-crystallization of *SpHex* with inhibitors. *SpHex* was co-crystallized with NA-glucal or NTA-glucal at final concentrations

of 4 mM to obtain protein-inhibitor complexes. The crystals were cryoprotected in 1.8 M AmSO_4 , 0.1 M trisodium citrate pH 6, and 25% glycerol prior to flash cooling to 100K for X-ray data collection. X-ray data were collected using a Rigaku R-Axis IV++ detector and 007HF Microfocus X-ray generator at the University of Manitoba. The X-ray data were indexed using Mosflm (220), then scaled and averaged using SCALA (191). Structures of the SpHEX complex with either NA-glucal or NTA-glucal were determined by molecular replacement using PHASER (from within the PHENIX package (192)) and a structure of SpHEX (PDB ID: 1HP4) (from which solvent molecules had been removed) as a search model. 3D models of NA-glucal and NTA-glucal were constructed using Jligand (228) and restraint files generated using PHENIX eLBOW (192). The inhibitor models were manually fit into their ascribed electron densities using Coot (193). Refinement of the modeled complexes and addition of solvent was carried out using PHENIX.REFINE (192) and COOT (193). Crystallographic and refinement statistics are presented in Table 5.1.

B.3. Inhibition Kinetics

Kinetic studies were performed by Dr. Andres Gonzalez (Dept. of Chemistry, University of British Columbia) at 25 °C in 25mM sodium citrate, 25mM sodium phosphate, 100mM sodium chloride buffer in the presence of 1% BSA either at pH 5 or pH 7, using a known concentration of enzyme ($SpHex_{WT} = 2.1 \times 10^{-7}$ mM; $SpHex_{D313A} = 2.85 \times 10^{-5}$ mM; $SpHex_{E314Q} = 5.35 \times 10^{-5}$ mM). The enzyme was incubated with different concentrations of the inhibitors for 2-5 minutes before initiating the reaction by the addition of 4-nitrophenyl *N*-acetyl- β -D-glucosaminide (NAG-PNP) substrate and the initial rate was measured by monitoring the increase in absorbance at 400 nm (pH 7, $\epsilon = 10587 \text{ M}^{-1} \text{ cm}^{-1}$) or at 348 nm (PNP isosbestic point,

pH 5-6, $\epsilon = 6700 \text{ M}^{-1}\text{cm}^{-1}$) for up to ten minutes. K_i determinations were performed using two to four different substrate concentrations. For each inhibitor, a range of four to six inhibitor concentrations bracketing the K_i value was used for each substrate concentration. Dixon plots ($1/v$ vs $[I]$) were constructed to validate the use of competitive inhibition model and to assess the fit of the rest of the data. A competitive inhibition model was fit to the data by using non-linear regression analysis with Grafit 7.0 (229).

B.4. Structure coordinates

Crystallographic structures of *SpHex* in complex with NA-glucal and NTA-glucal were deposited in the Protein Data Bank under the coordinates 5FD0 and 5FCZ, respectively.

C. Results and discussion

C.1. Structure of *SpHex* in complex with NA-glucal and NTA-glucal

Although NA-glucal has previously been shown to potently inhibit β -*N*-acetylhexosaminidase activity, the structural basis for this tight binding was unclear (226). To complement the kinetic analysis investigating the binding affinities of NA-glucal and the hydration-resistant NTA-glucal towards *SpHex*, structures of *SpHex* co-crystallized with NA-glucal and NTA-glucal were determined to 2.0 Å and 2.45 Å, respectively (Table 5.1). *SpHex* is a two domain protein, with domain I consisting of residues 1-151, and domain II containing residues 152-506 (Fig. 3). Domain I is comprised of seven antiparallel β -sheets and two near-parallel α -helices, and is well-conserved within the GH20 family (150). Domain II has a solvent-exposed active site situated within a $(\beta/\alpha)_8$ barrel, in which helix $\alpha 5$ is truncated into a single turn of a 3/10 helix, and helix $\alpha 7$ is replaced by an extended loop. Following $\alpha 8$, another α -helix

located at the C-terminal end extends out between domains I and II, and appears to stabilize the two domains with respect to each other (150).

NA-glucal and NTA-glucal bind very similarly within the *SpHex* active site (only NTA-glucal shown in Fig. 5.3 for clarity), however, kinetic analysis (Table 5.2) indicates that NA-glucal and NTA-glucal have K_i values of 2.4 μM and 68.0 μM at pH 5, the optimum pH for *SpHex*. The fact that the K_i value of NTA-glucal is nearly 30-fold higher than that of NA-glucal suggests that the sulfur atom in NTA-glucal is not as well accommodated by *SpHex*. NAG-thiazoline also contains a sulfur atom, and in order to determine if this adversely affected its binding potency to *SpHex* compared to that of NAG-oxazoline, further kinetic analysis was performed on both inhibitors at neutral pH to account for the acid lability of the oxazoline. Interestingly, NAG-thiazoline had nearly 12-fold higher affinity to *SpHex* ($K_i = 6.9 \mu\text{M}$) than NAG-oxazoline ($K_i = 78.5 \mu\text{M}$), thus indicating that in the case of the cyclic NAG-thiazoline, substituting an oxygen with a sulfur tightens inhibitor binding, possibly because the longer C-S bond more closely mimics transition state configuration (224, 230).

Table 5.1 – Crystallographic and refinement statistics for *Sp*HEX bound to NTA-glucal and NA-glucal.

Data collection	<i>Sp</i>HEX NTA-glucal	<i>Sp</i>HEX NA-glucal
X-ray source	Rigaku MicroMax 007HF	Rigaku MicroMax 007HF
Space group	P 6 ₁ 2 2	P 6 ₁ 2 2
Unit cell dimensions	$a = b = 133.87 \text{ \AA}$, $c = 174.91 \text{ \AA}$ $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$	$a = b = 133.17 \text{ \AA}$, $c = 176.52 \text{ \AA}$ $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$
Wavelength (Å)	1.54	1.54
Resolution (Å)	62.51 - 2.45 (2.55 - 2.45)*	54.81 - 2.00 (2.05 - 2.00)
R _{merge}	0.135 (0.544)	0.123 (0.704)
CC(1/2)	0.990 (0.867)	0.995 (0.772)
I / σ I	12.4 (3.4)	11.1 (2.3)
Completeness (%)	96.7 (96.0)	98.2 (94.1)
Redundancy	9.0 (8.7)	7.2 (6.6)
Refinement		
R _{work} / R _{free}	0.15 / 0.21	0.15 / 0.18
B-factor (Å ²)	29.54	25.39
Protein	29.20	23.28
Ligands	39.21	35.70
Solvent	32.11	36.96
<i>RMSDs</i>		
Bond lengths (Å)	0.012	0.011
Bond angles (°)	1.15	1.06
Ramachandran favoured/allowed (%)	98/ 1.8	99/ 1.4

* Values in parentheses refer to the high-resolution shell.

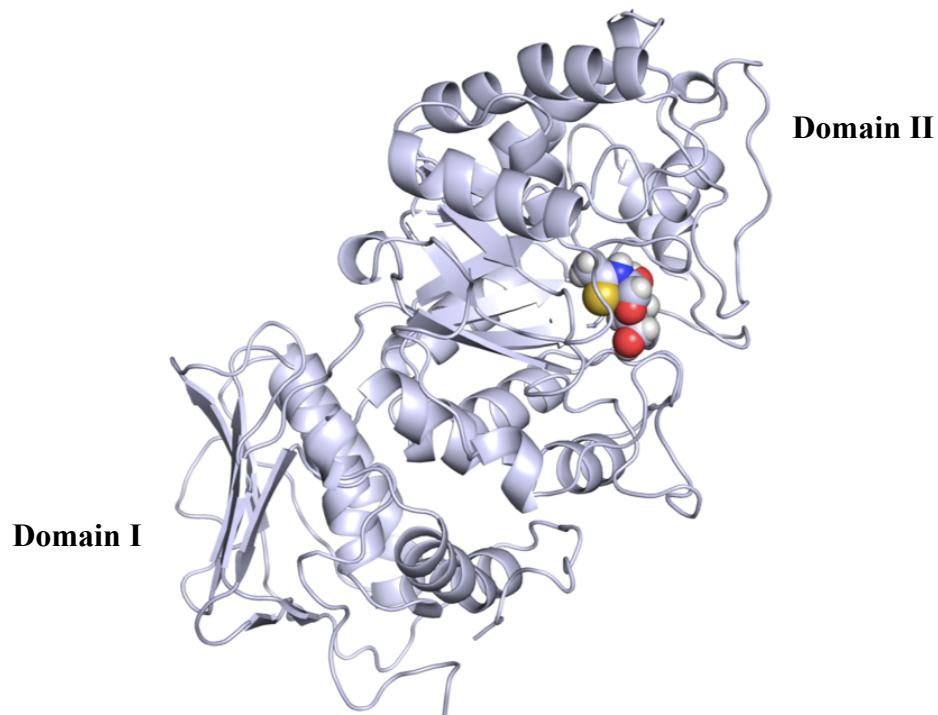


Figure 5.3 – Full-length structure of *SpHex* bound to NTA-glucal.

SpHex is a two-domain enzyme, where NTA-glucal binds to domain II, and is represented by spheres where carbon, oxygen, nitrogen and sulfur atoms are pale blue, red, dark blue and gold, respectively.

Table 5.2 – Kinetic analysis of *SpHex* inhibition

		pH 5			pH 7		
		<i>SpHex</i> WT	<i>SpHex</i> D313A	<i>SpHex</i> E314Q	<i>SpHex</i> WT	<i>SpHex</i> D313A	<i>SpHex</i> E314Q
K_i	NA-glucal	2.4	5.3	3.1	2.1	5.6	1.4
	NTA-glucal	68.0	12.9	154	63.0	178	32.0
	NAG-oxazoline	ND	ND	ND	78.5	197	34.2
	NAG-thiazoline	2.8	175	254	6.9	350	490
NAG-PNP	K_m	23.4	76.7	23.0	9.3	142.0	9.9
	k_{cat}	308.0	0.9	2.0	98.5	2.7	1.3
	k_{cat}/K_m	13.2	0.012	0.087	10.6	0.019	0.131

* K_m and K_i values in μM and k_{cat} in s^{-1} . The above data were reproduced from Ref. 224 (Gonzalez *et al.*, 2016) with permission from the Royal Society of Chemistry.

C.2. Active site interactions between *SpHex* and inhibitors NA-glucal and NTA-glucal

A closer look at the *SpHex* active site reveals that NAG-thiazoline binds similarly to NTA-glucal (Fig. 5.4 A, B), with the exception of its ring C1, which is flattened in order to accommodate the constraints of its double bond geometry. NTA-glucal and NA-glucal bind in the active site in a ${}^4E/{}^1,4B$ conformation, thus mimicking ${}^4H_3$ transition-state geometry, and form a network of seven hydrogen-bonding interactions with surrounding residues (Fig. 5.4 A, C). As expected based on the substrate-assisted catalytic mechanism, Asp313 is within hydrogen-bonding distance of the 2-acetamido group (Fig. 5.4 A). The general acid/base residue Glu314 does not participate in hydrogen-binding interactions, but is poised over the site of the anomeric carbon. To assess the impact of the key residues D313 and E314 on inhibitor binding, Dr. Gonzalez measured binding affinities of NA-glucal, NTA-glucal and NAG-thiazoline on the *SpHex* mutants D313A and E314Q, which still displayed some catalytic function (Table 5.2). The loss of hydrogen-bonding and electrostatic interactions in the D313A mutant resulted in a 50-70 fold reduction in NAG-thiazoline binding affinity, and a 70-90 fold reduction in the non-acidic E314Q (Table 5.2). In contrast, NA-glucal was barely affected by these mutations, and although K_i values for NTA-glucal exhibited slightly more variation in the mutants, they were not as significant as observed for NAG-thiazoline. Since NAG-thiazoline carries a diffuse positive charge within its thiazoline ring substituent, it relies more heavily on interactions with charged catalytic residues, unlike NA-glucal and NTA-glucal which are more chemically inert, and thus less sensitive to these changes in the active site.

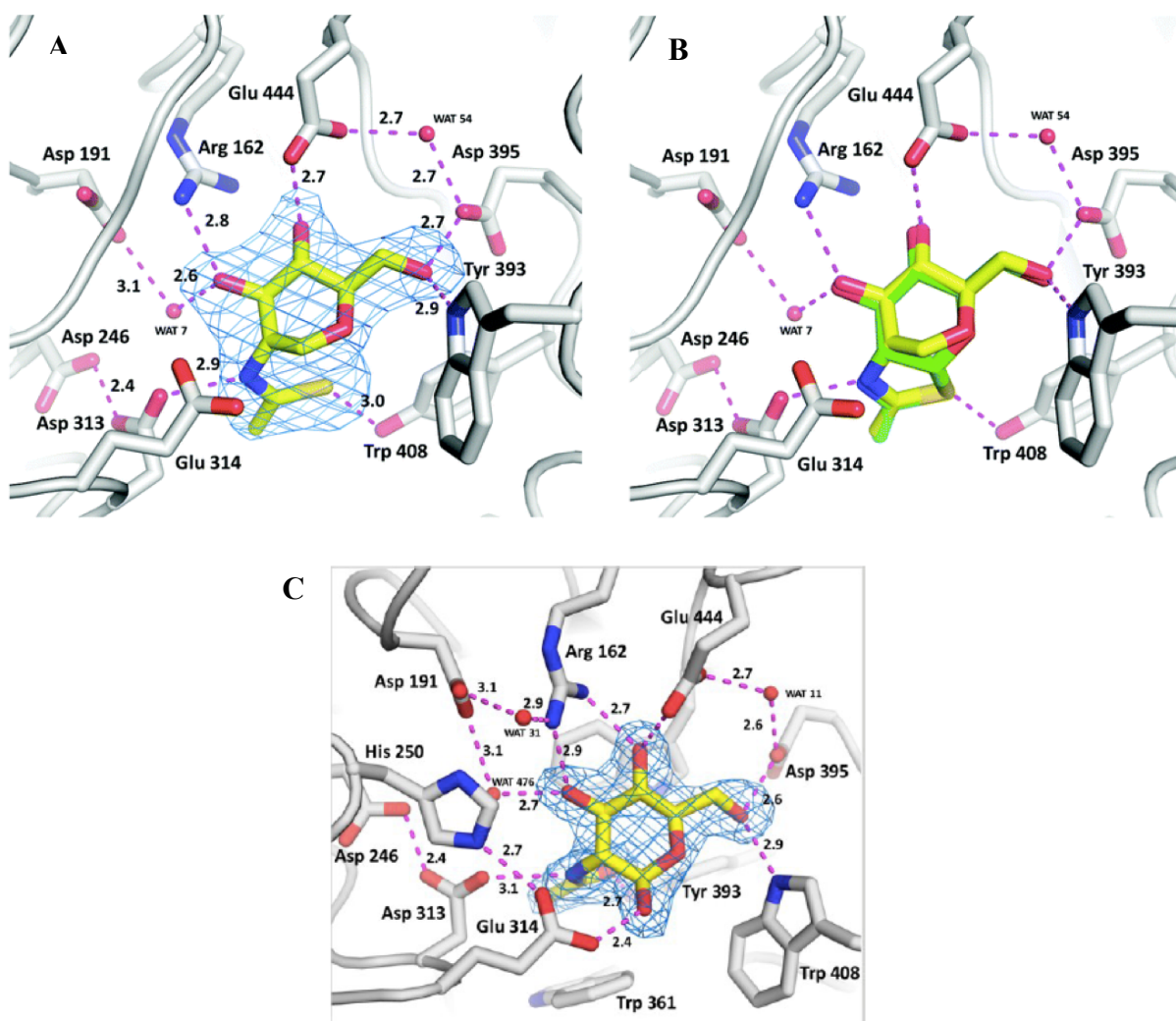


Figure 5.4 – Active site interactions of *SpHex* bound to NTA-glucal and NA-glucal.

A) NTA-glucal (yellow sticks) forms several hydrogen-bonding interactions with *SpHex* active site residues (grey). Oxygen, nitrogen and sulfur atoms are red, blue and gold, respectively. Red spheres represent waters. Hydrogen-bonds are shown as dashed lines. Electron density is an $F_{\text{obs}} - F_{\text{calc}}$ omit map contoured at 3.0σ . **B)** Superposition of *SpHex* structures bound to NTA-glucal and NAG-thiazoline (green carbons) (PDB: 1HP5), indicates that NTA-glucal mimics the binding mode of the transition intermediate. The co-crystallized structure of *SpHex* bound to NA-glucal revealed a GlcNAc molecule within the active site, indicating that the NA-glucal was converted to GlcNAc by hydration. These figures were reproduced from Ref. 224 (Gonzalez *et al.*, 2016) with permission from the Royal Society of Chemistry.

Structural support for kinetic analysis demonstrating the slow hydration of NA-glucal was provided by the observation that instead of NA-glucal, electron density maps clearly revealed GlcNAc in the *SpHex* active site (Fig. 5.4 C). It is possible that the inhibitory activity of NA-glucal is due to rapid formation of a slowly hydrolyzing oxazoline species that blocks the enzyme active site. Like NA-glucal, turnover of NAG-PNP also involves passing through an oxazoline intermediate, where opening of the oxazoline ring is the rate limiting step. However, NA-glucal had a k_{cat} value approximately 2500-fold lower than NAG-PNP. If NA-glucal turnover was limited during hydrolysis of the oxazoline intermediate like NAG-PNP, both would have similar turnover rates. The fact that NA-glycal is catalyzed much slower than NAG-PNP is then because its turnover is limited at a step preceding ring-opening of the oxazoline intermediate, i.e. during protonation of the double bond C2 (Fig. 5.2, right). This is confirmed by kinetic and structural data that show that unlike NA-glucal, NTA-glucal is resistant to hydration, due to the presence of a thioamide group (Fig. 5.4 A).

D. Conclusions

The above results structurally validate kinetic studies demonstrating that NA-glucal is hydrated by β -*N*-acetylhexosaminidases, as evidenced by the presence of a hydroxylated NA-glucal (GlcNAc) in the *SpHex* active site. A superposition of the transition-state analog NAG-thiazoline in the *SpHex* active site with NTA-glucal also suggests that the affinity of *N*-acetyl glycols is potentially due to their structural similarity to the transition-state intermediate. The crystallographic structures of NA-glucal and NTA-glucal reveal that the inhibitors form hydrogen bonding interactions with several residues within the enzyme active site, including the key residue Asp313. This explains why loss of electrostatic interactions with Asp313 and Glu314 resulting from mutation of these residues causes the binding affinity of NAG-thiazoline to suffer,

since it carries a diffuse positive charge, while the binding affinities of the charge-neutral NA-glucal and NTA-glucal remain largely unaffected. The chemical neutrality of NA-glucal and NTA-glucal thus renders them relatively insensitive to mutation of key catalytic residues, suggesting that they have therapeutic potential as they can be more resistant to adaptive mutations acquired within the enzyme active site.

Chapter 6 – Investigations into the effect of PUGNAc and the AmpC-inhibitor avibactam on suppressing high-level AmpC-mediated resistance against ceftazidime in *P. aeruginosa*

A. Introduction

P. aeruginosa is an opportunistic pathogen often associated with severe nosocomial infections. The combination of innate and acquired resistance pathways endows *P. aeruginosa* with a formidable arsenal of multi-drug resistance mechanisms and has necessitated the use of a range of combination therapies during treatment. Resistance to antipseudomonal β -lactam/ β -lactamase inhibitor combinations such as piperacillin-tazobactam and ticarcillin-clavulanate predominantly results from selection of mutations in proteins regulating inducible *ampC* expression (*dacB*, *ampD*, *ampR*) causing hyperproduction of AmpC. The clinically available β -lactamase inhibitors tazobactam and clavulanate (Fig. 1.4 A) have very limited efficacy against AmpC. Recently, however, avibactam, a non β -lactam β -lactamase inhibitor (Fig. 1.4 B) has entered the marketplace and is currently approved for use with ceftazidime in the US and Europe (231, 232), and is being evaluated for use with ceftaroline and the monobactam aztreonam (42, 81).

β -Lactamase inhibitors like tazobactam and clavulanic acid inhibit serine β -lactamases like AmpC by covalently linking to the active site serine via an ester bond, which is more susceptible to hydrolysis than the carbamoyl linkage formed by avibactam (84). Coupled with this increased resistance to hydrolysis, another advantage of avibactam is that it does not result in AmpC induction like clavulanic acid (233, 234). Sequence analysis of over 500 *P. aeruginosa* clinical isolates revealed that AmpC proteins retained a high degree of conservation within active site residues directly involved in avibactam binding, which also play an important role in β -

lactam substrate binding, although some variation within key residues was observed (81). It has been suggested that the ability of avibactam to mimic β -lactam substrate binding and its rigid mode of binding to AmpC implies that active site mutations hindering avibactam binding while maintaining turnover of native substrate may be less prone to develop (81). Indeed, *in vitro* studies assessed the development of spontaneous avibactam resistance in ceftazidime-resistant *P. aeruginosa* strains to a frequency ranging from 2.5×10^{-8} – 5.8×10^{-10} (80). The mutations were all sourced to the chromosomal *ampC* gene, specifically, within the Ω -loop region that forms a part of the avibactam-binding pocket (80). Nevertheless, while the frequency is low, resistance to avibactam can occur, which reaffirms the importance of continuing the development of strategies to combat AmpC-mediated resistance.

Genetic inactivation of *ampG* and *nagZ* has been shown to completely suppress the emergence of high-level ceftazidime resistance in *P. aeruginosa* (157, 158), and a number of studies have demonstrated NagZ-inhibition as a viable strategy to restore β -lactam susceptibility in *ampC* derepressed strains of *P. aeruginosa* with mutations in *ampD* and *dacB* (152, 156, 157), the two most commonly occurring mutations that cause clinically relevant AmpC hyperproduction (121, 122, 127, 129). Previously, Zamorano *et al.* (2010) demonstrated that genetic inactivation of *nagZ* abolishes the rise of high-level ceftazidime resistance in *P. aeruginosa*, and based on MIC assays on these mutants, attributed the cause to chromosomal mutations in the *ampD* and *dacB* genes that cause AmpC hyperproduction (157). This chapter reproduces the trends observed by Zamorano *et al.* and using whole-genome sequencing of high-level ceftazidime-resistant *P. aeruginosa* mutants, confirms the presence of mutations in the *dacB* gene. Further, the *N*-acetyl- β -glucosaminidase inhibitor PUGNAc has previously been shown to potentiate β -lactam efficacy against *P. aeruginosa* by blocking NagZ activity (156,

157), and the work presented in this chapter demonstrates that blocking NagZ using PUGNAc also suppresses the emergence of high-level ceftazidime resistant mutants in *P. aeruginosa*.

We hypothesized that simultaneously blocking NagZ and AmpC would augment β -lactam antibiotic susceptibility in AmpC-expressing *P. aeruginosa*. To test this hypothesis, the lowest effective combination of avibactam and PUGNAc capable of enhancing ceftazidime susceptibility in the AmpC derepressed strain *P. aeruginosa* PA Δ *dacB* was identified, against which their ability to work in synergy to enhance β -lactam antibiotic susceptibility was clearly demonstrated using minimum inhibitory concentration (MIC) assays. As a proof-of-concept study, these results successfully highlight the potential in development of selective NagZ-inhibitors as a supplemental therapeutic to be used in combination with avibactam to further potentiate β -lactam antibiotics, and to suppress the rise of high-level β -lactam resistant mutants.

B. Experimental procedures

B.1. Population analysis

A population analysis of wild-type PAO1 was performed to quantify the sub-population of high-level ceftazidime-resistant spontaneous mutants, using a procedure adapted from Zamorano *et al.* 2010, and performed in triplicate (157). Serial dilutions of 3 ml overnight cultures of PAO1 in MHB++ were plated on MH agar plates containing 0, 2, 4, 8, and 16 μ g/ml of CAZ, whereas a PAO1 overnight culture grown in the presence of 5 mM PUGNAc was plated on MH agar plates containing 0, 2, 4, 8, and 16 μ g/ml of CAZ supplemented with 5 mM PUGNAc. The concentration of PUGNAc was relatively high at 5 mM, and was used to account for limited penetration of the inhibitor into cells and ensure the inhibition of high-level

ceftazidime-resistant mutants. CFU/ml at each CAZ concentration was determined from colony counts after 24 h of incubation at 37°C. The population analysis experiment described in Zamorano *et al.* 2010 was also reproduced using PAO1 and PA Δ nagZ in triplicate, similarly as described above, except with 0, 2, 4, 8, and 16 μ g/ml of CAZ only. Glycerol stocks were made using PAO1 colonies isolated from MH agar plates containing 0, 8 and 16 μ g/ml CAZ, and were stored at -80°C for further analysis by whole-genome sequencing. For each CAZ concentration, two colonies were picked to ensure sufficient sequence redundancy of each representative concentration for whole-genome analysis (described next).

B.2. DNA extraction and purification for whole-genome sequencing

DNA extraction and purification procedure was provided by Dr. Jacik Switala (Dr. Loewen's lab, Dept. of Microbiology, University of Manitoba), and was adapted from Martin-Platero *et al.*, 2007 (235). Overnight cultures of abovementioned stocks from 0, 8 and 16 μ g/ml CAZ were grown in LB broth, and 1-1.5 ml of overnight LB broth cultures were pelleted and resuspended in 100 μ l TES buffer (10% w/v sucrose, 25 mM Tris pH 8, 10 mM EDTA, 10 mg/ml lysozyme, and 40 μ g/ml RNase). Following the addition of 600 μ l lysis buffer (100 mM Tris pH 8, 100 mM EDTA, 10 mM NaCl, and 1% SDS), samples were inverted to mix and incubated for 10-15 minutes at room temperature. Samples were incubated for another 15 minutes at 37°C with 10 mg/ml Proteinase K, and then for 5 minutes at 80°C. After addition of 200 μ l of 3M sodium acetate pH 5.2, samples were vortexed for 15 seconds, chilled on ice for 10-15 minutes, and centrifuged to precipitate proteins. Supernatant was transferred to a fresh tube, and DNA was precipitated using an equal volume of isopropanol. DNA was pelleted, washed with 1 ml of 70% ethanol, and air-dried at room temperature. Final pellets were resuspended in 100 μ l 1X TE buffer (10 mM Tris pH 8, 1mM EDTA). Purified DNA samples

were analyzed on an agarose gel to verify their integrity, and sent to the Children's Hospital Research Institute of Manitoba for analysis on the MiSeq paired-end sequencing system (Illumina) with 150X coverage. Whole genome sequencing results were aligned and analyzed using Geneious software (MAUVE alignment) with an annotated *P. aeruginosa* PAO1 genome for reference (NCBI Reference Sequence: NC_002516.2).

B.3. Checkerboard analysis

The checkerboard dilution assay is typically used in microbiology to determine the synergistic effect of drugs against bacterial cells. In a simple assay performed in a 96-well plate, each of two drugs is diluted column-wise and row-wise, respectively, in an array of twofold dilution series, giving the appearance of a 'checkerboard'. The synergistic interaction of the drugs is calculated using a fractional inhibitor concentration (FIC) index, and assumes that each drug is capable of suppressing bacterial growth on its own (236). Avibactam and PUGNAc do not inhibit *P. aeruginosa* growth on their own, and strictly according to conventional nomenclature, their synergistic interaction cannot be defined in terms of the FIC index. However, if they are capable of enhancing ceftazidime-susceptibility to a greater extent in combination than individually, they could be loosely said to be working in synergy, and following discussions of this interaction assume this definition of synergy.

To set up a checkerboard assay, twofold serial dilutions of ceftazidime (CAZ) at a concentration range of 96-0.75 µg/ml were performed in 100 µl of cation-adjusted Mueller-Hinton broth (MHB++) in a row-wise layout in a 96-well plate (beginning from the bottommost row and ending at the topmost). An additional twofold dilution series was then performed in a column-wise layout (beginning from leftmost column and ending at rightmost) using either

avibactam at a concentration range of 64-0.125 µg/ml or PUGNAc at a concentration range of 1.7-0.003 mg/ml diluted in MHB++. These checkerboard assays established the lowest effective concentrations at which avibactam and PUGNAc individually inhibit growth in combination with ceftazidime, and provided a narrower concentration range at which to test the synergistic ability of avibactam and PUGNAc in the subsequent analysis. A checkerboard assay was performed using a fixed concentration of ceftazidime (1.5 µg/ml) diluted in MHB++. Twofold dilutions of avibactam (1-0 µg/ml) were performed column-wise from left to right in a 96-well plate, against row-wise (bottom to top) twofold dilutions of PUGNAc (355-0 µg/ml), similarly to as described above (results and plate layout shown in Fig. 5.2). Positive controls for each assay contained 100 µl MHB++. Following inoculation with 100 µl of $\sim 10^5$ cells of *P. aeruginosa* $\Delta dacB$ (PA $\Delta dacB$) grown to an optical density (OD₆₀₀) of ~ 0.5 , plates were incubated for 18 h at 37°C in a shaker incubator.

B.4. Minimum inhibitory concentration (MIC) assays

MICs were performed in duplicate or triplicate using the broth microdilution method (Clinical and Laboratory Standards Institute) in a 96-well plate. The results of the checkerboard analysis indicated that the lowest effective concentrations of avibactam and PUGNAc with the synergistic potential to enhance ceftazidime susceptibility were 0.5 µg/ml and 177.5 µg/ml. Accordingly, for the MIC assay, avibactam or PUGNAc were diluted to a final concentration of 0.5 µg/ml and 177.5 µg/ml, respectively, in 100 µl of MHB++. In this inhibitor-broth, twofold serial dilutions of CAZ were performed within the range 96-0.18 µg/ml, followed by inoculation with 100 µl of $\sim 10^5$ cells of PA $\Delta dacB$ grown to an optical density (OD₆₀₀) of ~ 0.5 . As a control, 100 µl of MHB++ was used in place of inhibitors in the above assay. MICs were determined after incubation of the cells for 18 h at 37°C in a shaker incubator.

C. Results and discussion

C.1. Blocking NagZ using PUGNac suppresses the emergence of high-level ceftazidime resistance in *P. aeruginosa*

The population analysis carried out by Zamorano *et al.* was reproduced using PAO1 and PA Δ nagZ, and similar trends in growth were observed: 1) no difference between growth was observed in the absence of ceftazidime (CAZ), 2) similar growth was observed at 2 and 4 μ g/ml CAZ, and 3) complete inhibition of growth for PA Δ nagZ at ≥ 8 μ g/ml CAZ, while resistant PAO1 colonies were still observed (Fig. 6.1 A).

To investigate if the high-level ceftazidime resistance observed for PAO1 was due to the occurrence of spontaneous mutations in genes contributing to *ampC* hyper-expression, genomic DNA was extracted from selected PAO1 colonies isolated from media containing 0, 8 and 16 μ g/ml CAZ. As expected, colonies grown in the absence of CAZ had wild-type sequences for the *dacB*, *ampD*, *ampR*, and *ampC* genes. However, all isolates that grew at 8 and 16 μ g/ml CAZ (which is approximately 5 and 10X the MIC of CAZ against wild-type PAO1) displayed mutations in the *dacB* gene. Indeed, *dacB* null mutations are the most frequently occurring clinical mutations that give rise to clinically relevant, high-level *ampC*-mediated β -lactam resistance (127). Two strains contained frameshift deletions and premature stop codons interrupting the *dacB* gene, while one had a single point mutation (Gly366Ser). These mutations are distinct from those previously documented in clinical *P. aeruginosa* AmpC overproducers with *dacB* mutations (127). Other frequently occurring mutations such as those in *ampD*, *ampR*, and *ampC* genes were not observed.

The hyperinduction of *ampC* resulting from *dacB* inactivation depends on the ability of NagZ to generate 1,6-anhydroMurNAc-peptide, which activates AmpR. Interestingly, Zamorano *et al.* found that genetic inactivation of *nagZ* dramatically suppresses the emergence of spontaneous CAZ resistance in PAO1(157), as evidenced by the complete absence of *P. aeruginosa* growth beyond 8 µg/ml (Fig. 6.1 A). Notably, blocking NagZ function pharmacologically using PUGNAc also reduced the development of resistance at 8 and 16 µg/ml CAZ, although not to the extent achieved by genetic inactivation (Fig. 6.1 B). The inability of PUGNAc to suppress CAZ resistance as efficiently as genetic deletion of *nagZ* is likely due to limited uptake of PUGNAc into the microbe.

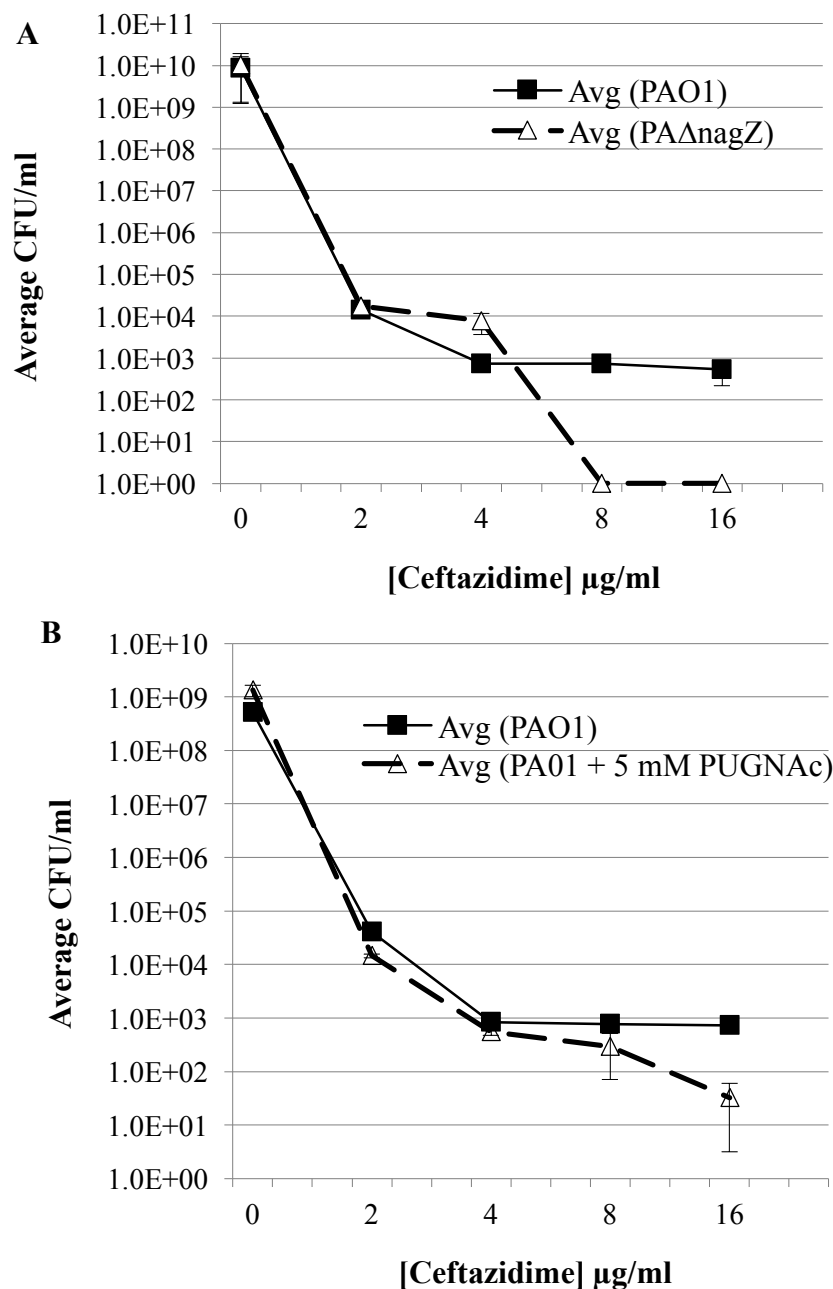


Figure 6.1 – Population analysis of *P. aeruginosa* ceftazidime susceptibility

Population analysis of **A)** *P. aeruginosa* PAO1 and PA Δ nagZ ceftazidime susceptibility, and **B)** PAO1 ceftazidime susceptibility +/- 5mM PUGNAC, demonstrating that NagZ inactivation leads to a remarkable loss in the ability of *P. aeruginosa* to develop high-level resistance towards ceftazidime, thus providing a promising means for increasing β -lactam sensitivity by blocking NagZ function.

C.2. Co-inhibition of AmpC and NagZ enhances ceftazidime efficacy in the *ampC* derepressed PA Δ *dacB* mutant

P. aeruginosa PA Δ *dacB* has an MIC value of 24 μ g/ml towards ceftazidime, which is 16-fold higher than that observed for PAO1, which has an MIC value of 1.5 μ g/ml. Initial checkerboard analysis revealed that avibactam is very effective at suppressing PA Δ *dacB* growth, with as little as 1.5 μ g/ml avibactam required to restore wild-type PAO1 levels of susceptibility (MIC = 1.5 μ g/ml). On the other hand, even in the presence of 355 μ g/ml PUGNAc (5 mM), PA Δ *dacB* has an MIC value of 6 μ g/ml ceftazidime, and indicated that PUGNAc is not nearly as potent an inhibitor as avibactam, likely due to limited uptake into the cytosol of the microbe. Nonetheless, despite the high concentration of PUGNAc required to suppress growth of PA Δ *dacB*, it still serves as a suitable investigation compound for the proof-of-concept study described in this chapter. In a subsequent checkerboard analysis of avibactam versus PUGNAc (Fig. 6.2), the concentration of ceftazidime was fixed at 1.5 μ g/ml, on the basis that since PUGNAc is unable to restore ceftazidime susceptibility of PA Δ *dacB* to wild-type PAO1-levels on its own, synergistic effects with avibactam will be more readily apparent at this concentration of ceftazidime. The results of this analysis (Fig. 6.2) reveal that while 0.5 μ g/ml avibactam and 177.5 μ g/ml PUGNAc are individually unable to inhibit PA Δ *dacB* growth in the presence of 1.5 μ g/ml ceftazidime, they achieve complete inhibition of growth in combination, thus displaying a synergistic ability.

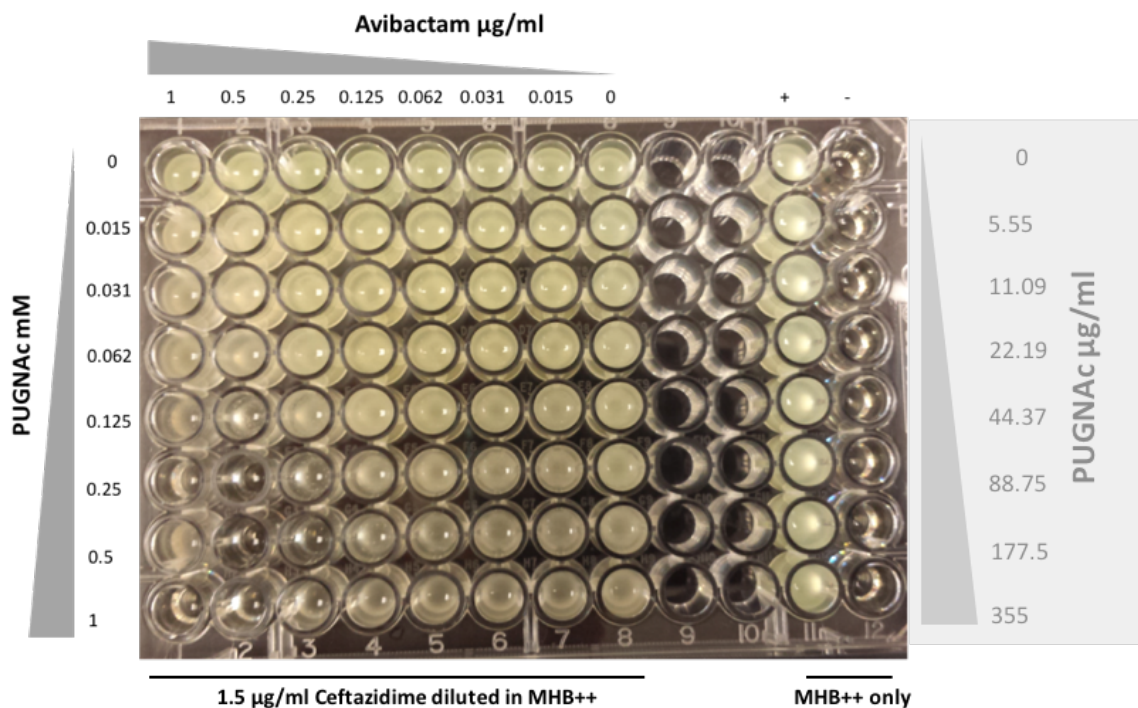


Figure 6.2 – Checkerboard analysis of PUGNAc vs. avibactam against *PA Δ dacB*

Checkerboard analysis testing *PA Δ dacB* susceptibility towards ceftazidime (CAZ) were performed by varying twofold serial dilutions of 0-355 $\mu\text{g/ml}$ PUGNAc against 0-1 $\mu\text{g/ml}$ avibactam in the presence of a fixed concentration of 1.5 $\mu\text{g/ml}$ ceftazidime.

To verify this result, standard MIC assays were performed in duplicate using *PA Δ dacB* in the presence of 96-0.18 μ g/ml ceftazidime. Avibactam (0.5 μ g/ml) and PUGNAc (177.5 μ g/ml) were added to the broth individually, and in combination. Relative to the control where *PA Δ dacB* was only treated with ceftazidime (MIC of 24 μ g/ml), avibactam and PUGNAc cause a two-fold (12 μ g/ml) and three-fold (6 μ g/ml) reduction in the ceftazidime MIC of *PA Δ dacB*, respectively (Table 6.1). However, in combination, they cause a remarkable 16-fold decrease in the MIC value of *PA Δ dacB* to 1.5 μ g/ml ceftazidime (Table 6.1), which is normally expected of the more susceptible PAO1 wild-type strain. This result clearly indicates that avibactam and PUGNAc are capable of a significant degree of synergy in facilitating ceftazidime to suppress growth of AmpC-overproducing *P. aeruginosa*.

Table 6.1 – Minimum inhibitory concentration (MIC) values of ceftazidime towards PA Δ *dacB* in the presence of avibactam and/or PUGNAc.

+ Inhibitor (s)	CAZ-MIC (μg/ml)
None	24
Avibactam (0.5 μ g/ml)	12
PUGNAc (177.5 μ g/ml)	6
Avibactam (0.5 μ g/ml) + PUGNAc (177.5 μ g/ml)	1.5

D. Conclusions

This chapter reproduces a population analysis study demonstrating that a small subpopulation of wild-type *P. aeruginosa* PAO1 possesses spontaneous mutations that confer high-level ceftazidime resistance. Whole-genome sequencing of these mutants indicates that all of them possess mutations in the *dacB* gene, one of the most commonly occurring clinical mutations giving rise to β -lactam resistance. Blocking NagZ function in PAO1 using the glycosidase inhibitor PUGNAc causes a significant reduction in the emergence of high-level ceftazidime resistant mutants, and it is possible that the resistance exhibited by these mutants is eroded by blocking PG recycling, and consequently, *ampC* induction. Additionally, checkerboard and MIC assays clearly demonstrate the ability of PUGNAc and avibactam to work synergistically in enhancing ceftazidime susceptibility in the *ampC* derepressed strain PA Δ *dacB*. Although avibactam alone is effective at blocking AmpC-mediated resistance in PA Δ *dacB*, its potency is potentiated by PUGNAc, since blocking NagZ reduces the amount of AmpC that needs to be inhibited. Moreover, a possible advantage of targeting two disparate targets in the AmpC expression pathway is that the likelihood of simultaneously developing resistance mutations in both *nagZ* and *ampC* to overcome the effect of both inhibitors is much lower than a resistance mutation occurring in a single gene. This possibility could be explored in the future by assessing mutational frequencies of *P. aeruginosa* in the presence of PUGNAc and avibactam. The work presented here provides proof-of-concept that the growing clinical threat of β -lactam antibiotic resistance in *P. aeruginosa* and other Gram-negative pathogens with inducible *ampC* systems can be effectively suppressed by combining inhibitors of PG recycling with AmpC inhibitors such as avibactam.

Chapter 7 – Structural determination of a novel class A carbapenemase from *Vibrio cholerae* in complex with the potent β -lactamase inhibitor avibactam

Protein chemistry and structural biology studies of VCC-1 were carried out by G.V. Enzymology studies of VCC-1 were carried out by Dr. Chand Mangat, Public Health Agency of Canada.

A. Introduction

Among β -lactam antibiotics, carbapenems possess the broadest spectrum of activity and potency against both Gram-negative and Gram-positive bacteria, and are frequently administered as a drug of last resort during the treatment of resistant infections (57). Unfortunately, the efficacy of this powerful class of antibiotics, particularly against multidrug resistant Gram-negative pathogens, is being eroded by the increasing clinical prevalence of Class A and D serine carbapenemases (57, 62). While surveillance data of antimicrobial resistance is predominantly based on human pathogens isolated from clinical samples, carbapenemase resistance has been reported in environmental microbes, some of which have been associated with human foodstuffs (237–239).

Recently, a novel class A carbapenemase was characterized in a nontoxigenic *Vibrio cholerae* strain isolated from retail shrimp imported into Canada for human consumption, and was named *V. cholerae* carbapenemase-1 (VCC-1) (180). Two identical copies of the gene encoding the β -lactamase VCC-1 (named *bla*_{VCC-1}) were identified in the *V. cholerae* genome, and found to be associating with insertion elements in an 8.5-kb region on the chromosome that may have been acquired by transposition (180). The *bla*_{VCC-1} gene was inserted into the 3'-end of the *vrgG* gene (180), which encodes for an effector protein involved in type VI secretion (240). Importantly, VCC-1 was found to effectively hydrolyze penicillins, first-generation

cephalosporins, aztreonam, and carbapenems, and was only moderately inhibited by the β -lactam-based inhibitors clavulanic acid and tazobactam (180). However, class A carbapenemases are known to be effectively inhibited by avibactam (241). Based on the sequence homology of VCC-1 with other class A carbapenemases such as KPC-2 (*Klebsiella pneumoniae* carbapenemase-2), it was hypothesized that avibactam may act as an effective inhibitor against the newly discovered VCC-1 enzyme, which would be welcome news for those at the frontlines in our growing battle against antimicrobial resistance.

Avibactam inhibits class A, C and D β -lactamases, with an inhibitory profile that includes extended spectrum β -lactamases and β -lactamases resistant to β -lactam-based inhibitors (79). Although class A β -lactamases have sequences of variable lengths, key catalytic residues participating in β -lactam hydrolysis are highly conserved, and led to the Ambler classification system which proposed that all catalytically relevant residues within class A β -lactamases be referred to by the same residue number (242). Accordingly, the main catalytic serine residue in all class A β -lactamases (regardless of actual sequence length) is assigned as Ser70, a convention that will be adhered to throughout this chapter. When Ser70 is deprotonated, it is hypothesized to launch a nucleophilic attack on avibactam at the site of the electrophilic C7 carbonyl group (Fig. 7.1 A), similarly to the way Ser70 reacts with the carbonyl carbon of β -lactam rings (see Fig. 1.6 A). This results in the formation of a stable covalent carbamoyl-enzyme linkage between avibactam and the catalytic Ser70 residue, by a mechanism that remains contentious, and is discussed in the Results and Discussion (79). In the case of a typical β -lactam-based substrate, formation of the covalent bond with Ser70 causes opening of the β -lactam ring; whereas in avibactam it is the urea ring of this bicyclic inhibitor that opens (Fig. 7.1 A).

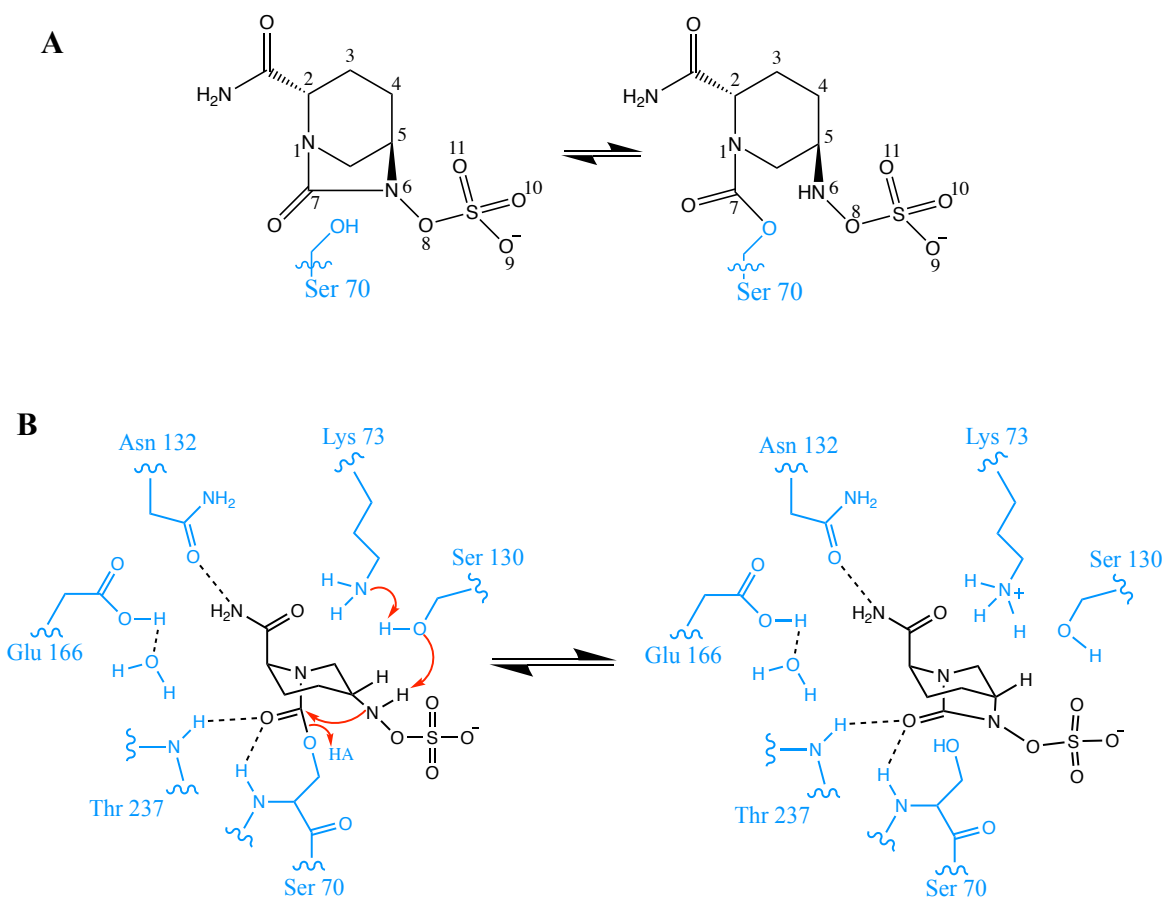


Figure 7.1 – Class A β -lactamase inhibition by avibactam

A) Acylation of avibactam to the enzyme active site results in formation of a covalent carbamoyl linkage between avibactam and the catalytic serine residue (Ser70). **B)** Deacylation is proposed to occur via recyclization and regeneration of active avibactam in class A enzymes. Figure B was adapted from research originally published in the *Journal of Biological Chemistry* by Ehmann, D. E., Jahic, H., Ross, P. L., Gu, R.-F., Hu, J., Durand-Réville, T. F., Lahiri, S., Thresher, J., Livchak, S., Gao, N., Palmer, T., Walkup, G. K., and Fisher, S. L. Kinetics of avibactam inhibition against Class A, C, and D β -lactamases. *J. Biol. Chem.* 2013; 288: 27960–71 © the American Society for Biochemistry and Molecular Biology.

The release of β -lactam-based substrates from the acyl-enzyme complex proceeds via a water molecule that is activated by Glu166 in class A β -lactamases (243). Conversely, avibactam deacylation is proposed to occur by N6 deprotonation mediated by Ser130 and Lys73 (Fig. 7.1 B, left) (241, 244, 245). The conformational similarity of the ring-opened avibactam to its closed-ring structure within the enzyme active site favours recyclization of its five-membered urea ring over hydrolysis (Fig. 7.1 B, right) (79). In contrast, the relatively constrained β -lactam ring may not be capable of recyclization, and so in the case of β -lactamase inhibitors like clavulanic acid, the inhibitor is hydrolyzed via multiple alternate pathways (79, 246). Furthermore, the regeneration of intact avibactam has the potential to perpetuate additional rounds of inhibition in a recurrent cycle of acylation/deacylation, which appears to enhance its potency (79, 84).

The efficacy of avibactam inhibition against serine β -lactamases stems from its rapid acylation to the enzyme, and a relatively slower rate of deacylation (79, 241). Another feature of avibactam that distinguishes it from β -lactam-based inhibitors, is its higher resistance to enzyme-assisted hydrolytic inactivation, resulting from the more stable carbamoyl linkage (Fig. 7.1 B) as compared to an ester linkage (Fig. 1.6 A) (84, 247). Although avibactam hydrolysis into inactive byproducts has been documented in a class A β -lactamase from *Mycobacterium tuberculosis*, and the carbapenemase KPC-2 (where hydrolysis has been shown to involve desulfation), inhibitory potency towards these enzymes is maintained due to the slow rates at which hydrolysis occurs to yield chemically inactive inhibitor fragments (241, 248, 249).

Presently, crystal structures of only four class A β -lactamases in complex with avibactam have been determined (244, 245, 248, 249). This chapter describes the crystallographic structure of a recently discovered novel carbapenemase in Canada, VCC-1, and a structure of the enzyme

covalently bound to avibactam, which furthers our understanding of the inhibition mechanism of this clinically potent inhibitor and provides an example of how renewed efforts into discovering antibiotic potentiators can have a major positive impact on overcoming antimicrobial drug resistance mechanisms.

B. Experimental procedures

B.1. VCC-1 expression and purification

VCC-1 expression and purification using pET-28b-VCC-1-transformed *E. coli* BL21 (DE3) cells (provided by Dr. Chand Mangat from Dr. Michael Mulvey's lab, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg) was previously described by Mangat *et al.* (180). Briefly, VCC-1 expressing cells were grown to OD₆₀₀ of 0.6 at 37°C in 500-ml of Terrific Broth supplemented with 35 µg/ml kanamycin, then induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside for 18-20 hours at 22°C. Cells were pelleted by centrifugation at 3000 rpm for 10 minutes, and gently resuspended in 20 ml TSE buffer (0.2 M Tris pH 8, 0.5 M sucrose, 1 mM EDTA, and 1 tablet cOmplete™ Protease inhibitor (Roche)) to release the periplasmic protein fraction. The resuspension was incubated on ice for 10 min, centrifuged at 12,000 rpm for 30 min, and the supernatant concentrated prior to a five-fold dilution in 50 mM sodium acetate (pH 5) buffer. The diluted protein fraction was loaded on to 1-ml HiTrap SP XL cation-exchange column (GE Healthcare) preequilibrated with 50 mM morpholineethanesulfonic acid (MES) pH 6, and eluted with a linear gradient of 0 to 300 mM NaCl. The presence of eluted VCC-1 was confirmed by SDS-PAGE analysis, and selected fractions were concentrated in a 10 kDa molecular weight cut-off spin concentrator (Millipore) before loading on to a Superdex 75 (GE Healthcare) size exclusion column. VCC-1 protein was

purified in 50 mM Tris pH 7.2 buffer, and concentrated to 12-18 mg/ml (determined by absorption at 280 nm, and an extinction coefficient (ϵ) value of 30,035 M⁻¹ cm⁻¹).

B.2. VCC-1 crystallization and structure determination

Purified VCC-1 (12-18 mg/ml) was subjected to a Classics II suite crystallization screen (Qiagen), and crystals were obtained in 0.2 M NaCl, 0.1 M Bis-Tris pH 5.5, and 25% (w/v) PEG 3350. Crystal growth was further optimized within a range of varying concentrations of NaCl, Bis-Tris and PEG3350, although pH was maintained at 5.5 (150). Optimized crystallization buffer consisted of 0.7 M NaCl, 0.2 M Bis-Tris pH 5.5 and 22% PEG 3350. Crystals were grown at room temperature by the hanging drop vapour diffusion method using equal volumes of crystallization buffer and protein. VCC-1 crystals were soaked with avibactam at final concentrations of 5 mM to obtain protein-inhibitor complexes. The crystals were cryoprotected in crystallization buffer supplemented with 25% glycerol prior to flash cooling to 100K for X-ray data collection. X-ray data for native VCC-1 was collected from a single crystal (held at 100 K in an N₂ gas stream) using beamline 08ID-1 at the Canadian Light Source (Saskatoon, Canada). Data for the VCC-1:avibactam complex were collected using a Rigaku R-Axis IV++ detector and 007HF Microfocus X-ray generator at the University of Manitoba. The X-ray data were indexed using Mosflm (220), then scaled and averaged using Aimless (CCP4 suite) (250). Structures of the native and avibactam-bound VCC-1 complex were determined by molecular replacement using PHASER (from within the PHENIX package (192)) and a reference structure of the class A carbapenemase NMC-A from *E. cloacae* (PDB ID: 1BUE) from which solvent molecules had been removed. The protein sequence of VCC-1 was accessed from the NCBI database (Genbank ID ALU63998.1). Models of avibactam and its desulfated hydroxylamine byproduct were created using Jligand (CCP4 suite) (228) and restraint files generated using

PHENIX eLBOW (192). Avibactam was manually fit into its ascribed electron densities using Coot (193). The covalent carbamoyl linkage between avibactam and the catalytic serine residue, and associated geometrical restraints were generated using Phenix (192). Refinement of the modeled complexes and addition of solvent was carried out using PHENIX.REFINE (192) and COOT (193). Crystallographic and refinement statistics are presented in Table 7.1. The amino acid residues in figures of the VCC-1 structure are numbered to be consistent with the Ambler system for class A β -lactamases (242). Determination of root-mean square deviations (RMSD) between structurally conserved C-alpha carbon-atoms was done using the DaliLite v.3 server (http://ekhidna.biocenter.helsinki.fi/dali_server/)

B.3. VCC-1 protein sequence homology analysis

NCBI Protein BLAST was used to determine the percentage homology between the class A β -lactamases VCC-1, KPC-2, CTX-M15, SHV-1 and BlaC. The amino acid sequences of KPC-2, CTX-M15, BlaC, and SHV-1 were downloaded from the UniProt database deposited under the IDs Q93LQ9, Q2PUH3, A5U493, and P0AD63, respectively. A multiple protein sequence alignment was performed using the Clustal Omega program from EMBL-EBI bioinformatics services (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).

C. Results and discussion

C.1. VCC-1 sequence homology analysis

Sequence comparison of VCC-1 with all class A β -lactamases that have been structurally characterized in complex with avibactam reveal that VCC-1 shares highest sequence identity with KPC-2 (57%), followed by CTX-M15 (43%), BlaC (41%), and SHV-1 (39%). A multiple

sequence alignment shows four conserved motifs (Fig. 7.2) that include key catalytic residues (pointed out in Fig. 7.1 B): a) $^{70}\text{S}(\text{S/T})\text{FK}^{73}$, where Ser70 is the catalytic serine residue involved in forming a covalent intermediate with substrate, and Lys73 is proposed to play a role in avibactam deacylation (Fig. 7.1 B), b) $^{130}\text{SDN}^{132}$, where Ser130 is typically involved in protonation of the β -lactam nitrogen during acylation, and potentially deprotonates the N6 nitrogen of avibactam that facilitates urea-ring recyclization during deacylation (Fig. 7.1 B), c) $^{166}\text{EXXXN}^{170}$, containing the general base Glu166 as part of the Ω -loop, which plays a key role in β -lactam substrate acylation/ deacylation, and d) $^{234}\text{KTG}^{236}$, constituting the oxyanion hole that stabilizes the tetrahedral substrate transition states that arise during acylation and deacylation of class A β -lactamases (251–255). Among *Mycobacterium* species, BlaC enzymes have been shown to fall into two lineages, displaying either the canonical $^{130}\text{SDN}^{132}$ motif, or the variant $^{130}\text{SDG}^{132}$, where interestingly, the G¹³²N substitution caused an increase in the efficiency of clavulanate hydrolysis in *M. abscessus* (256).

SHV-1	-----MRYI-----RLCIISLLATLPLA--VHASQPQLEQIKLSQSLSGRVG	41
BlaC	MRNRGFGRRRELLVAMAMLVSVTGRCARHSGARPASTTLPAGADLADRFAELERRYDARLG	60
CTX-M-15	MVKKSLRQFTLMA-----TATVTLLLG---SVPLYAQTAADVQQKLAELERQSGGRLG	49
VCC-1	-----MKRIAMYV-----ALSI-----STSTAFADENHNKNMADIEAAFEGRVG	38
KPC-2	--MSLYRRLVLLS-----CLSWPLA---GFSATALTNLVAEPFAKLEQDFGGSIG	45
	. : * . : *	
SHV-1	MIEMDLASGRTLTAWRADERFPMMSTFKVVLGCAVLARVDAGDEQLERKIHYRQQDLVDY	101
BlaC	VYVPATGTAA-IEYRADERFAFCSTFKAPLVA AVLHQN--PLTHLDKLITYTSDDIRSI	117
CTX-M-15	VALINTADNSQ-ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQVEIKKSDLVNY	108
VCC-1	VYAIN TGSGKA-YSYRANERFPLCSSFKAF LAAAVLKMDQDSPGVLLKVNHNRTMEPH	97
KPC-2	VYAMDTGSGAT-VSYRAEERFPLCSSFKGFLAAAVLARSQQQAGLLDTPIRYGNALVPW	104
	: . :*:*** : * : * .*** * : . :	
SHV-1	SPVSEKHLADGMTVGELCAAAITMSDN SAANLLLA-TVGGPAG----LTAFLRQIGDNVT	156
BlaC	SPVAQQHVQTGMTIGQLCDAAIRYSDGTAANLLLA-DLGGPGGGTAAFTGYLRS LGDTVS	176
CTX-M-15	NPIAEKHVNGTMSLAELSAAALQYSDNVAMNKLIA-HVGGPAS----VTAFARQLGDETTF	163
VCC-1	SPITEKFQSQGMAVGELAAATLQYSDNGAANLLMEKYIKGPEG---MTQFMNSIGDTKF	153
KPC-2	SPISEKYLTTGMTVAELSAAAVQYSDNAAAANLLLK-ELGGPAG----LTAFMRSIGDTTF	159
	.*:***. *:***. **: * * *: : ** . .*: :.***	
SHV-1	RLDRWETELNEALPGDARDTTTPASMAATLRKLLTSQRLSARSQRQLLQWMVDDR VAGPL	216
BlaC	RLDAEEPELNRDPPGDERDTTTPHAIALVLQQLVLGNALPPDKRALLTDWMARNTTGAKR	236
CTX-M-15	RLDRTEPTLNTAIPGDPDRTTSPRAMAQTLRNLT LGKALGDSQRAQLVTWMKGNTTGAAS	223
VCC-1	RLDRWELDLNSAIPGDERDTSTPKAVAESLNKLISNTVLDNYHQEIFKKWMIGNTTGDNR	213
KPC-2	RLDRWLELNSAIPGDARDTSSPRAVTESLQKLT LGSALAAPQRQFVDWLKGNTTGNHR	219
	*** * ** *** ***: * :*: * . * : : * : : ..	
SHV-1	IRSVLPAGWFIADKTGA-GERGARGIVALLG-PNNKAERIVVIYLRDTPA--SMAERNQQ	272
BlaC	IRAGFPADWKVIDKTGT-GDYGRANDIAVVSPTG-VPYVVAVMSDRAGGGYDAEPREAL	294
CTX-M-15	IQAGLPASWVVGDKTGS-GGYGTTNDIAVIWP-KDRAPLILVYFTQPQ--PKAESRRDV	279
VCC-1	IRAAVPDGWVVGDKTGTGCKGYGTANDHAFILQGNNAAPLILSIYTRKG--EHMKHDDDEV	271
KPC-2	IRAAVPADWAVGDKTGTGCVYGTANDYAVVWP-TGRAPIVLAVYTRAPN--KDDKHSEAV	276
	*:: . * . * : ***: * * . *.: . . . ::	
SHV-1	IAGIGAALIEHWQR--- 286	
BlaC	LAEAAATCVAGVLA---- 307	
CTX-M-15	LASAAKIVTDGL----- 291	
VCC-1	IAKAARIAIENVK---- 284	
KPC-2	IAAAARLALGLGVNGQ 293	
	:* .	

Figure 7.2 –Multiple sequence alignment of VCC-1 and selected class A β -lactamases.

Multiple sequence alignments show conserved catalytically relevant motifs in indicated class A β -lactamases that are involved in catalytic turnover of substrate, and contain residues i) ⁷⁰S(S/T)FK⁷³ (red), ii) ¹³⁰SDN¹³² (cyan), iii) ¹⁶⁶EXXXN¹⁷⁰ (green), and iii) ²³⁴KTG²³⁶ (purple). Although actual residue numbers for conserved residues are different in each protein, they are numbered according to the Ambler classification scheme (242). Sequence alignments were performed using the Clustal Omega web server (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).

C.2. Structures of native VCC-1 and in complex with avibactam

The crystallographic structures of native VCC-1 and the avibactam-bound complex were obtained at resolutions of 1.65 Å and 2.2 Å, respectively (X-ray diffraction data and refinement statistics are shown in Table 7.1), using as a search model the structure of NMC-A, a class A β -lactamase that shares 59% sequence identity with VCC-1. The crystallographic data for the VCC-1: avibactam complex was processed at a resolution cut-off of 2.2 Å; however, given the high signal to noise ratio ($I / \sigma I$) in the high-resolution shell (2.2 – 2.27 Å) of 9.7, the crystal was diffracting to a higher resolution than was collected. Nevertheless, density maps calculated using these data clearly defined avibactam bound within the active sites of the four VCC-1 molecules that comprised the asymmetric unit of the crystal. Overall, VCC-1 was found to adopt an $\alpha\beta\alpha$ -sandwich fold typical of class A β -lactamases (66, 257), and Fig. 7.3 A highlights each of the four important sequence motifs within the VCC-1 structure. Consistent with previous structural studies of β -lactamases which found that avibactam binding does not significantly alter β -lactamase conformation (79, 244, 245, 249), the structure of VCC-1 bound to avibactam aligned well with its unliganded structure (RMSD 0.7). The only notable difference in the active site was a slight rotation in the orientation of the Leu167 side chain (Fig. 7.3 B).

Table 7.1 – Crystallographic and refinement statistics for native VCC-1 and VCC-1 bound to avibactam.

Data collection	VCC-1	VCC-1 – avibactam
X-ray source	CLS 08B1-1	Rigaku MicroMax 007HF
Space group	C 1 2 1	C 1 2 1
Unit cell dimensions	$a = 209.82 \text{ \AA}$, $b = 46.57 \text{ \AA}$, $c = 113.45 \text{ \AA}$, $\alpha = \gamma = 90^\circ$, $\beta = 99.22^\circ$	$a = 209.47 \text{ \AA}$, $b = 46.32 \text{ \AA}$, $c = 113.07 \text{ \AA}$, $\alpha = \gamma = 90^\circ$, $\beta = 98.82^\circ$
Wavelength ($\text{\AA}$)	1.03	1.54
Resolution ($\text{\AA}$)	37.55 – 1.65 (1.71 – 1.65)*	70.71 – 2.2 (2.27 - 2.2)
R_{merge}	0.017 (0.280)	0.04 (0.076)
CC(1/2)	1.0 (0.910)	0.997 (0.987)
$I / \sigma I$	10.8 (1.6)	16.3 (9.7)
Completeness (%)	99.0 (99.0)	92.7 (82.7)
Redundancy	2.0 (2.0)	2.8 (2.8)
Refinement		
$R_{\text{work}} / R_{\text{free}}$	0.18 / 0.20	0.17 / 0.23
B-factor ($\text{\AA}^2$)	29.31	17.25
Protein	28.14	16.71
Ligands	N/A	19.66
Solvent	37.95	23.05
<i>RMSDs</i>		
Bond lengths ($\text{\AA}$)	0.007	0.008
Bond angles ($^\circ$)	0.87	0.99
Ramachandran favoured/allowed (%)	97/ 2.7	95/ 3.7

* Values in parentheses refer to the high-resolution shell.

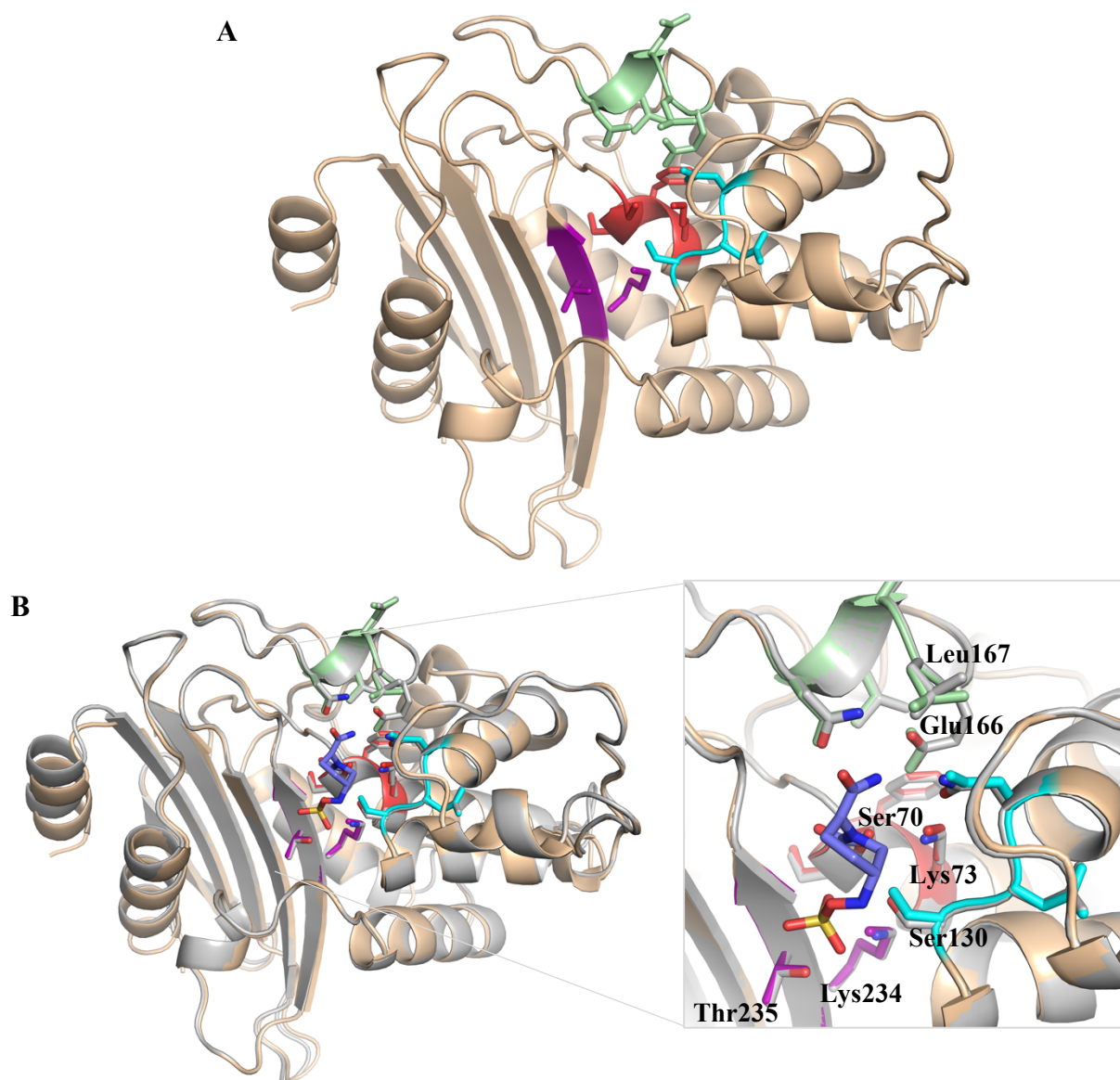


Figure 7.3 – Full-length crystal structures of VCC-1 and VCC-1 bound to avibactam

Structures of **A**) VCC-1 in its native conformation (bronze), and **B**) superposed with the avibactam-bound complex (grey; avibactam shown as dark blue sticks) show that avibactam binding does not significantly affect VCC-1 conformation. Catalytically relevant motifs containing i) ⁷⁰SSFK⁷³ (red) ii) ¹³⁰SDN¹³² (cyan) iii) ¹⁶⁶ELDLN¹⁷⁰ (green), and iv) ²³⁴KTG²³⁶ (purple) are highlighted. Oxygen, sulfur and nitrogen atoms in avibactam are coloured red, gold, and dark blue, respectively.

Despite low completeness (92.7% overall and 82.7% in the outer shell) of these initial VCC-1: avibactam structural data (Table 7.1), clear electron density for avibactam covalently attached to the deprotonated hydroxyl oxygen of Ser70 via atom C7 can be observed (Fig. 7.4 A). The six-membered ring of avibactam (Fig 7.1 A) is present in a chair conformation, while the urea ring is absent due to cleavage of the C7-N6 bond by the enzyme. The carbamoyl-carbonyl group of the covalently linked avibactam interacts with a region of the oxyanion hole of the enzyme that is comprised of the peptide backbone nitrogen atoms of residues Ser70 and Thr237, where a 3.1 Å hydrogen bond forms between the carbonyl oxygen atom of avibactam and the peptide nitrogen of Thr237 (Fig. 7.4 A). The sulfate moiety of avibactam forms the largest number of hydrogen-bonding interactions with the enzyme. It is situated in a pocket that typically accommodates carboxylate moieties of β -lactams and it forms hydrogen bonds with Ser130 (2.7 Å), Thr235 (2.6 Å), and Thr237 (2.5 Å), while the avibactam amide group forms a hydrogen bond with Asn132 (3.0 Å). Interestingly, the 2.9 Å H-bond with Arg220 also appears to orient Thr237 within range of the sulfate moiety. Replacement of Arg220 in KPC-2 has been shown to play a role in avibactam resistance, potentially in part due to loss of its interaction with Thr237 and the sulfate moiety (249, 258). Avibactam also forms hydrophobic interactions with Leu167 (Fig. 7.4 A), Phe72 and Leu169 (not shown for clarity).

The above data shows that avibactam binding within the VCC-1 active site is stabilized by numerous interactions, suggesting that it is a potent inhibitor of VCC-1. Indeed, this potency was confirmed by a determination of its inhibitory constant (K_i) against VCC-1, with a K_i value of 0.016 ± 0.002 μ M (data provided by Dr. Mangat, personal communication). The inhibitory potency of avibactam is more remarkable when compared to that of the primarily class A β -lactamase inhibitors tazobactam and clavulanic acid, which were previously shown to inhibit

VCC-1 with K_i values of $2.0 \pm 0.4 \mu\text{M}$ and $13 \pm 3 \mu\text{M}$, respectively (180). Considered in context of the reversible nature of avibactam acylation to β -lactamases, these results reinforce the potential of avibactam as a potent inhibitor of VCC-1.

C.2.1. A closer look at VCC-1 active site residues potentially involved in avibactam acylation/deacylation

Aside from interactions with avibactam, the active site residues in VCC-1 are also involved in an extensive hydrogen-bonding network with each other (Fig. 7.4 B), which is notable in terms of their predicted roles during avibactam acylation/deacylation. Whereas Lys73 of VCC-1 forms several hydrogen bonding contacts with other residues in the active site, particularly noteworthy is its interaction with Ser130 (2.6 Å with Ser130 backbone and 3.2 Å with Ser130 hydroxyl), which is proposed to be its partner in avibactam deacylation (see Fig. 7.1 B for mechanism). (241, 244, 245). Additionally, Lys234 also forms a 2.9 Å hydrogen bond with Ser130. The catalytic water molecule (W1) that is proposed to be activated by the general base Glu166 in class A β -lactamases to initiate β -lactam deacylation (252, 254) is consistently observed within the active sites of all four monomers of the asymmetric unit in the native VCC-1 and VCC-1: avibactam complex crystals, and it indeed forms a hydrogen bond with Glu166 (2.6 Å) of VCC-1 (Fig. 7.4 B), but it is also within hydrogen-bonding distance of Asn170 (2.7 Å). Deacylation of avibactam from β -lactamases has been suggested to be a consequence of recyclization rather than hydrolysis by W1, partly due to the proximity of the electron dense amide and sulfate moieties of avibactam, which makes W1 the weaker nucleophile for attack on the inherently stable carbamoyl-carbonyl link (84, 244).

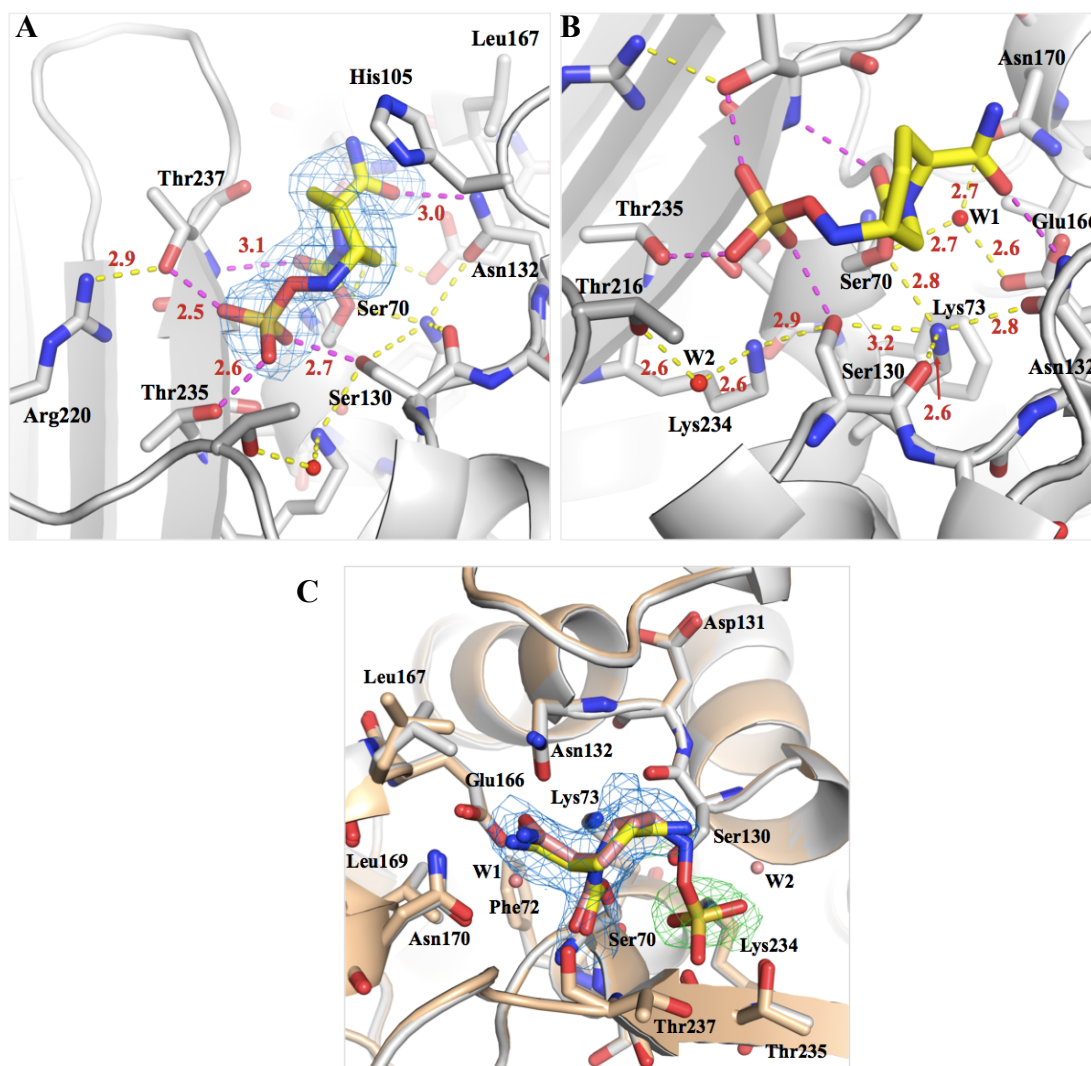


Figure 7.4 – Active site interactions of VCC-1 bound to avibactam

The structure of avibactam (yellow sticks) covalently linked to monomer D of VCC-1 (grey) is shown with a focus on interactions between **A)** avibactam and VCC-1 active site residues, and **B)** active site residues only. Hydrogen-bond interactions between protein-avibactam and protein-protein are shown as dashed magenta and yellow lines, respectively. **C)** VCC-1 monomers C (bronze) and D (grey) are covalently linked to desulfated (yellow sticks) and intact avibactam (salmon sticks) species, respectively. Superposition of these structures shows that the planar N6 imine nitrogen of the desulfated avibactam species is positioned slightly higher than the tetrahedral N6 atom in intact avibactam. The positive electron density in the VCC-1 monomer C complex superposes with the sulfate moiety of avibactam, suggesting partial occupancy of the active site by avibactam and its desulfated byproduct. Electron density is an $F_{\text{obs}} - F_{\text{calc}}$ omit map contoured at 3.0σ . Oxygen, nitrogen and sulfur atoms are red, blue and gold, respectively. Red spheres represent waters.

Although the current consensus is that Lys73 and Ser130 promote avibactam deacylation via recyclization, the identity of the residues involved in acylation remains debatable (244, 245, 259). Based on their crystal structure of CTX-M15 bound to avibactam, Lahiri *et al.* proposed that Glu166 activated the deacylating water for Ser70 deprotonation, thus allowing it to launch a nucleophilic attack on C7 of avibactam (245). However, King *et al.* showed by kinetic analysis that the substitution E166Q in CTX-M15 allowed retention of wild-type avibactam acylation efficiency; whereas, substitutions of K73A and S130A drastically reduced the ability of avibactam to acylate the enzyme (244). A moderate decrease in acylation rates by K234A replacement was also observed, and was proposed to be due to its interaction with Ser130. Similarly, in KPC-2, an S130G variant displayed severely reduced acylation efficiency, as did K234R, albeit to a lesser extent (249). Taken together, these studies suggest that Lys73 may deprotonate Ser70 rather than a Glu166-activated water molecule, and implicate Lys73 and Ser130 in avibactam acylation, assisted by Lys234. In both the native and avibactam-bound VCC-1 structures, the hydroxyl oxygen of the catalytic Ser70 (carbamoyl linked oxygen in the avibactam-bound structure) is consistently within hydrogen-bonding distance of both Lys73 and W1 (within 2.8 Å of Lys73 and 2.7 Å from W1 in Fig. 7.4 B), suggesting that it may be deprotonated by either Lys73 or W1, allowing it to launch a nucleophilic attack on the C7 carbon of avibactam (Fig. 7.1 A). Comprehensive mutational analysis of residues Lys73, Ser130 and Glu166 on acylation efficiency of avibactam in VCC-1, and structural interactions of these substituted residues with avibactam could shed more insight on the acylation mechanism of avibactam in VCC-1.

C.3. VCC-1 desulfates avibactam in the crystalline state

Of the four monomers comprising the asymmetric unit, clearly defined electron density for the intact avibactam molecule acylated to Ser70 could be observed within two monomers (monomers B and D). For the remaining two monomers (monomers A and C), while avibactam was observed to be acylated to Ser70, electron density for the sulfate group of avibactam was interrupted at the site of the N6-O8 bond (Fig 7.4 C A). Considering how potently avibactam inhibits VCC-1 activity through its numerous contacts within the enzyme active site, this loss of electron density is likely indicative of avibactam desulfation. Among class A β -lactamases that have been characterized enzymatically and structurally, VCC-1 bears closest homology to KPC-2, which is known to slowly desulfate avibactam (241). KPC-2 was previously found to slowly hydrolyze avibactam over the course of 24 hours, the fragments consisting predominantly of a desulfated imine species of avibactam, and to a lesser extent, a hydroxylamine intermediate of the molecule (Fig. 7.5) (241, 249).

It has been hypothesized that desulfation occurs either in a two step process, with the sequential loss of sulfate and water (Fig. 7.5 A), or in one step that results in direct formation of an imine species (Fig. 7.5 B). This latter step could be facilitated by Lys73 and Ser130, where Lys73 accepts a proton from Ser130, prompting Ser130 to deprotonate the N6 atom of avibactam, thus causing imine formation to occur via loss of sulfate (Fig. 7.5 B) (241).

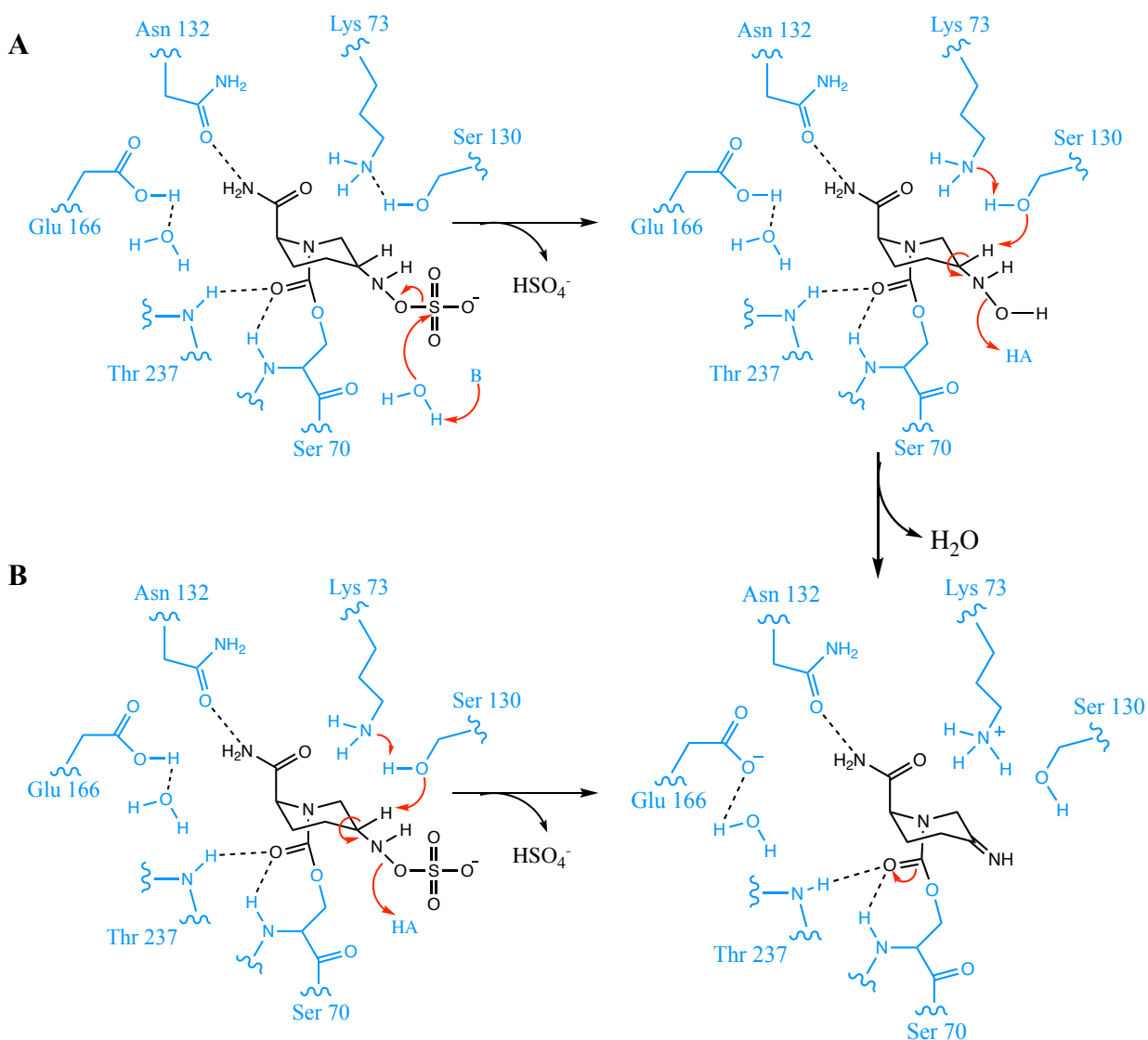


Figure 7.5 – Possible mechanisms of avibactam desulfation

Desulfation of avibactam is predicted to proceed either by **A**) two-step hydrolysis that involves passing through a hydroxylamine intermediate species, or by **B**) one-step hydrolysis that directly results in imine formation. In both models, Ser130 and Lys73 are proposed to key roles in assisting the process of desulfation. Figures were adapted from research originally published in the Journal of Biological Chemistry by Ehmann, D. E., Jahic, H., Ross, P. L., Gu, R.-F., Hu, J., Durand-Réville, T. F., Lahiri, S., Thresher, J., Livchak, S., Gao, N., Palmer, T., Walkup, G. K., and Fisher, S. L. Kinetics of avibactam inhibition against Class A, C, and D β -lactamases. *J. Biol. Chem.* 2013; 288: 27960–71 © the American Society for Biochemistry and Molecular Biology.

While deacylation of β -lactam containing substrates is believed to occur with assistance from Glu166 and the deacylating water in class A β -lactamases (W1 in VCC-1, Fig. 7.4 A), deacylation of avibactam is proposed to be facilitated by Lys73 and Ser130, instead, because the proximity of Glu166 and W1 to the charge-dense amide and sulfate groups is predicted to weaken the ability of the activated water W1 to launch a nucleophilic attack on the C7 atom of avibactam (84, 244). In contrast, the desulfated imine byproduct of avibactam lacks the negatively-charged sulfate group, and could thus permit the water molecule W1 to nucleophilically attack the C7 carbon in avibactam, triggering deacylation from VCC-1 with assistance from Glu166 (241).

Interestingly, when the VCC-1 structure was refined with intact acylated avibactam within monomers A and C of the asymmetric unit where electron density for the bond between N6-OSO₃ in avibactam was interrupted, positive electron density was present beside the N6 nitrogen, which may be reflective of a planar imine N6 nitrogen (Fig. 7.4 C). When the acylated imine species was modeled in the active site and refined with 100% occupancy, the positive electron density peak was no longer present (Fig. 7.4 C), suggesting that VCC-1 monomers A and C were bound to the desulfated imine species of avibactam (Fig. 7.5 B). A positive peak possibly corresponding to electron density from the sulfate group was still present, and may be indicative of partial occupancy of monomer C by both sulfated and desulfated avibactam species.

When sulfated avibactam (acylated to Ser70 of VCC-1 in monomer D) was superposed over the imine species in monomer C, the active site residues in both VCC-1 subunits were positioned near identically (Fig. 7.4 C), except for a slight rotation in the Leu167 sidechain (Fig. 7.4 C). The distance between the N6 atom in the imine species and the nearest sulfate oxygen in

the superposed intact avibactam molecule is ~ 1.8 Å (Fig. 7.4 C), which suggests that when an avibactam molecule that is acylated to VCC-1 is desulfated to an imine species, a free sulfate ion is unlikely to be tolerated in such close proximity and therefore displaced from the active site. Additionally, the deacylating W1 was present within 2.5 Å of Glu166, and 3.0 Å of the carbamoyl-carbonyl C7 atom of avibactam in the active site, and well positioned to possibly participate in deacylating the imine species from the enzyme. Thus, as observed for KPC-2, the X-ray structure of avibactam-bound VCC-1 suggests that the enzyme may be causing the slow desulfation of avibactam, which could then lead to deacylation via W1-mediated hydrolysis. Further biochemical investigation into the products generated by the hydrolytic inactivation of avibactam by VCC-1 is required to support the structural observation that suggests a desulfated imine species is acylated to VCC-1.

C.4. Active site comparisons of avibactam-bound VCC-1 with KPC-2 and CTX-M15

The sulfate group in avibactam forms a majority of the hydrogen-bonding interactions with VCC-1 (Fig. 7.4 A), and fits into an electropositive pocket that is predicted to stabilize avibactam as a precatalytic Michaelis complex (244). Consequently, loss of this sulfate group would compromise avibactam potency against VCC-1, due to an increased vulnerability to hydrolysis by W1. To gain more insight into potential VCC-1 mediated desulfation of avibactam, its structure was superposed with the avibactam-bound complexes of KPC-2 (Fig. 7.6 A, PDB ID: 4ZBE) and CTX-M15 (Fig. 7.6 B, PDB ID: 4HBU). VCC-1 and KPC-2 adopt very similar conformations (RMSD 1.0) as does the orientation of the acylated avibactam (Fig. 7.6 A). A notable difference is that the N6 atom of avibactam in the CTX-M15 structure is situated much closer to the carbamoyl-carbonyl C7 (3.0 Å), as compared to KPC-2 and VCC-1, where in the latter structure, the N6 atom faces away from C7 and is 3.9 Å away (Fig. 7.6 B). Additionally, in

CTX-M15, N6 also makes a 2.9 Å hydrogen bond with Ser130, whereas this distance is 3.4 Å in VCC-1. With its proximity to C7 and Ser130, the N6 nitrogen appears poised to recyclize the urea ring in CTX-M15 (245). This has been suggested as a potential reason for the slower recyclization/deacylation rate of avibactam by KPC-2, where $t_{1/2}$ is 82 min as opposed to 40 min for CTX-M15 (249). However, enzyme kinetics measurements of the inhibition of VCC-1 by avibactam that were performed by Dr. Mangat (National Microbiology Laboratory, Winnipeg; *personal communication, data not shown*) revealed that VCC-1 deacylates avibactam with a $t_{1/2}$ of 27 min, which is unexpected considering the structural similarities between the avibactam-bound structures of VCC-1 and KPC-2. Additionally, Dr. Mangat found the avibactam acylation rate of VCC-1 to be $1.7 \pm 0.1 \times 10^3 \text{ (M}^{-1}\text{s}^{-1}\text{)}$ (*personal communication, data not shown*), which is about an order of magnitude slower than that of KPC-2, which has a rate of $1.3 \pm 0.1 \times 10^4 \text{ (M}^{-1}\text{s}^{-1}\text{)}$ (241, 249). Currently, the structural analysis of VCC-1 bound to avibactam does not immediately reveal the basis for the kinetic differences between KPC-2 and VCC-1, and further investigations will be needed.

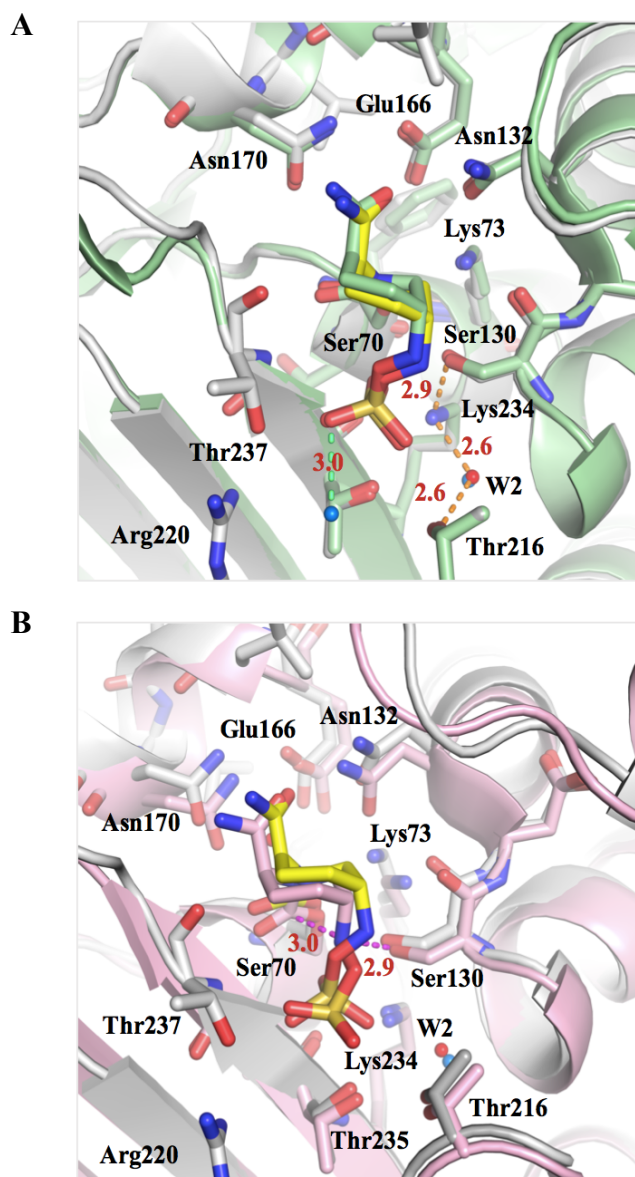


Figure 7.6 – Active site superposition of avibactam-bound VCC-1, KPC-2 and CTX-M15.

VCC-1 (grey) bound to avibactam (yellow sticks) was superposed with **A**) KPC-2-avibactam complex (green, PDB ID: 4ZBE), and **B**) CTX-M15-avibactam complex (pink, PDB ID: 4HBU). The superposed structures show that the binding mode of avibactam in VCC-1 is more similar to that observed in KPC-2. Waters in VCC-1 are shown as red spheres, and in KPC-2/CTX-M15 as blue spheres. Hydrogen-bonds are shown as dashed lines coloured orange (VCC-1), green (KPC-2) and magenta (CTX-M15), respectively. Oxygen, nitrogen and sulfur atoms are red, blue and gold, respectively.

The avibactam acylation rate of VCC-1 is closer to values exhibited by the class C *P. aeruginosa* AmpC and the class D OXA-48 (241). While these may be a feature of innate differences in catalytic mechanisms between enzyme classes, it is interesting to note that *P. aeruginosa* AmpC also potentially participates in avibactam desulfation to a lesser degree than KPC-2 (249). Based on structural comparisons of KPC-2 and *P. aeruginosa* AmpC, Krishnan *et al.* found that these enzymes lack hydrogen bonding interactions with nitrogen N6 of avibactam, and have a water molecule interacting with the sulfate oxygen of the inhibitor within the active site (Fig. 7.6 A), which could potentially desulfate avibactam by the mechanism shown in Fig. 7.5 A (249). However, there is no analogous water present at this site in the VCC-1: avibactam structure. Instead, water 2 (W2) is present within hydrogen-bonding distance of Lys234 (2.6 Å), which further hydrogen-bonds with Ser130 (2.6 Å) (Fig. 7.6). A similar arrangement is present in the avibactam bound complexes of KPC-2 and CTX-M15 (Fig. 7.6). As outlined in the mechanism in Fig. 7.5 B, Ser130 deprotonation by Lys73 may trigger one-step desulfation in VCC-1, however, it is possible that Lys234 could also deprotonate Ser130 and achieve the same, and warrants further investigation.

D. Conclusions

Inhibition kinetics demonstrate that avibactam inhibits the novel class A carbapenemase VCC-1 much more potently than class A β -lactamase inhibitors tazobactam and clavulanic acid. Due to the 82% completeness of the VCC-1: avibactam X-ray data, further data sets of higher completeness will need to be acquired in order to publish these results. Nonetheless, the X-ray structure of VCC-1 in complex with avibactam provides the structural basis for its potency, showing extensive hydrogen-bonding and hydrophobic interactions between avibactam and residues within the VCC-1 active site. The arrangement of VCC-1 active site residues Lys73 and

Ser130 within hydrogen-bonding distance of the N6 nitrogen attached to the sulfate group in avibactam support the current view that these residues are involved in avibactam deacylation. Among two of the four monomers within the crystallographic asymmetric unit, structural evidence supports the presence of a desulfated imine species of avibactam acylated to VCC-1. Comparing the avibactam-bound structures of the class A carbapenemases VCC-1, KPC-2, and CTX-M15, reveal that VCC-1 binds avibactam similarly to KPC-2, where unlike in CTX-M15, avibactam does not appear to be poised to favour recyclization following deacylation. Based on its sequence identity with KPC-2, it is probable that avibactam desulfation also occurs slowly in VCC-1, and may not significantly mitigate the potency of this β -lactamase inhibitor towards this carbapenemase.

Chapter 8 – Summary and future directions

β -Lactam antibiotics constitute over half of the clinically used antibiotics used to treat bacterial infections (27). Especially concerning is the dissemination of genes expressing β -lactamases that can erode the efficacy of most β -lactam drugs (62). Inducible AmpC β -lactamase production is a significant cause of clinical resistance in several Gram-negative bacteria, including *P. aeruginosa* (68). While the use of β -lactamase inhibitors has met with some success, particularly with the recently approved drug avibactam, they too are frequently prone to resistance mechanisms (42, 80, 81, 246). AmpC induction is linked to the peptidoglycan (PG) recycling pathway in Gram-negative bacteria, and controlled by the transcriptional regulator AmpR via the divergent *ampR-ampC* operon. AmpR function is activated by 1,6-anhydroMurNAc-peptide, a catabolite of the PG recycling pathway generated by the glycoside hydrolase NagZ. Due to their critical roles in inducing *ampC* expression, AmpR and NagZ are attractive targets for the design of small molecule inhibitors aimed at mitigating AmpC-mediated β -lactam resistance. With this aim in mind, my research primarily focused on understanding the structure and function of these two proteins.

A. Characterization of AmpR regulatory function using structural, biophysical and functional methods

In collaboration with Dr. L. Donald (University of Manitoba), we used non-denaturing mass spectrometry to establish that AmpR from *C. freundii* is a homotetramer in solution, and that it binds two molecules of double-stranded DNA corresponding to its high-affinity T-N₁₁-A recognition motif. Binding to operator DNA was found to significantly stabilize the AmpR tetramer, causing an increase in protein solubility. Non-denaturing MS also indicated that the

AmpR-DNA complex is able to bind four molecules of its repressor UDP-MurNAc-pentapeptide in a stepwise fashion. Using crystallographic data collected by T. Reeves, a previous graduate student, I determined the 2.15 Å crystal structure of the AmpR effector-binding domain (EBD) bound to UDP-MurNAc-pentapeptide, which is the first crystal structure of an LTTR protein bound to its native repressor ligand. UDP-MurNAc-pentapeptide was found to bind within a deep pocket in the AmpR EBD, where the terminal D-Ala-D-Ala residue formed extensive hydrogen-bonding interactions with AmpR. On this basis, we suggested that 1,6-anhydroMurNAc-pentapeptide may be the preferred inducer of AmpR, rather than the –tripeptide species. Finally, in collaboration with Dr. T. Patel (University of Manitoba), small angle X-ray scattering (SAXS) data was used to show small conformational changes were incurred by the DNA-bound AmpR upon binding its UDP-MurNAc-pentapeptide. Collectively, these data let us to propose a model for AmpR regulation, whereby the UDP-anhydroMurNAc group of the repressor may interact with quaternary protein structure to maintain AmpR in a conformation incapable of inducing *ampC* transcription. These results were published in the *Journal of Biological Chemistry* (212) and add to our understanding of the regulatory function of AmpR as a part of the *ampC* induction pathway.

To further investigate the identity of the preferred inducer of AmpR, I developed a protocol to generate mRNA *in vitro* from an *ampR-ampC* operon-bearing plasmid in the presence of *CfAmpR*. Preliminary data indicated that *CfAmpR* causes a simultaneous upregulation and downregulation of two different transcripts *in vitro*, that may correspond to *ampC* and *ampR*. I have outlined two methods to sensitively detect and measure relative yields of *ampR* and *ampC* mRNA: 1) *in vitro* synthesis of $\alpha^{32}\text{-P}$ -labeled mRNA (direct approach) and 2)

RT-qPCR (indirect approach). These methods can be developed further to confirm which of 1,6-anhydroMurNAc-tripeptide or -pentapeptide is the preferred inducer of AmpR.

B. Small molecule inhibition of NagZ activity to suppress AmpC β -lactamase resistance

In collaboration with our synthetic chemistry colleagues (Dr. K. Stubbs, University of Western Australia; Dr. D. Voadlo, Simon Fraser University; Dr. Y. Blériot, Université de Poitiers) I characterized the structural interactions of the GH3 enzyme NagZ from *B. cenocepacia* in complex with four of its inhibitors: PUGNAc (2.2 Å), its NagZ-selective derivative EtBuPUG (2.2 Å), the trihydroxyazepane MM-124 (1.8 Å), and its NagZ-selective derivative MM-156 (2.15 Å). The crystal structure *BcNagZ* bound to MM-124 was published in *Chemical communications* (218). These structures revealed that *N*-acetamido substitutions do not appear to affect the mobility of the flexible catalytic loop in NagZ. Particularly, the structures of PUGNAc and EtBuPUG showed that the phenylcarbamate moiety displaces the catalytic loop from the NagZ active site – a feature that can be exploited to design potent and selective inhibitors of NagZ to suppress AmpC mediated β -lactamase resistance.

The genetic inactivation of *nagZ* has been shown to suppress high-level ceftazidime resistance in *P. aeruginosa* (157), and I have demonstrated by a population analysis experiment that pharmacological inhibition of NagZ using PUGNAc can also significantly suppress the emergence of high-level ceftazidime resistant mutants in wild-type PAO1. Additionally, I used whole-genome sequencing to show that resistant PAO1 mutants isolated from 8-16 μ g/ml ceftazidime have mutations in the *dacB* gene, one of the most frequent causes of clinical *ampC* derepression (126–128). Additionally, I have demonstrated using minimum inhibitory concentration (MIC) assays that co-inhibition of NagZ and AmpC in the *ampC* derepressed

PA Δ *dacB* using low concentrations of PUGNAc and avibactam augments ceftazidime susceptibility beyond the extent of their individual capabilities. This strategy of simultaneously blocking AmpC induction and function has the potential to reduce the frequency of AmpC β -lactamase resistance, and is a promising therapeutic strategy.

C. Structural analysis of GH20 enzyme inhibition

Unlike GH3 enzymes, GH20 enzymes proceed via substrate-assisted catalysis, and play important biological roles that make them promising targets for inhibitor development. In collaboration with Drs. A. Gonzalez and S. Withers (University of British Columbia), I crystallized the model family GH20 β -*N*-acetylhexosaminidase from *Streptomonas plicatus* in complex with two inhibitors from the family of *N*-acetyl glycals, to provide a structural rationale for their inhibitory potency. The structures of SpHex bound to NA-glucal (2.0 Å) and NTA-glucal (2.45 Å) also revealed why these inhibitors appear to retain potency despite substitutions of key active site residues. The structural data were published along with kinetic analyses by our collaborators in *Chemical communications* (224), and can aid the development of potent inhibitors of this important class of glycoside hydrolases.

D. Structural characterization of VCC-1, a novel food-borne carbapenemase

In collaboration with Drs. C. Mangat and M. Mulvey (National Microbiology Laboratory, Public Health Agency of Canada), I crystallized a 1.65 Å crystallographic structure of a novel class A serine carbapenemase from *V. cholerae* (VCC-1) originally isolated from shrimp imported into Canada (180). I also solved a 2.2 Å structure of VCC-1 in complex with avibactam, which reveals that avibactam forms numerous interactions with enzyme active site residues that contribute to its stability, thus supporting kinetic data that shows avibactam is a

potent inhibitor of VCC-1. My structural data also suggests that VCC-1 may be causing desulfation of avibactam. However, current studies characterizing class A β -lactamase-mediated hydrolysis of avibactam indicate that this is a slow process (241). Overall, this work supports avibactam as a potent inhibitor of this newly discovered carbapenemase.

E. Future directions

E.1. Determining the mechanism of AmpR regulatory function

While the work presented in Chapter 2 proposes a structural model for AmpR regulation, a full-length crystal structure of AmpR in complex with its repressor UDP-MurNAc-pentapeptide and inducer 1,6-anhydroMurNAc-pentapeptide remains to be determined in order to validate the model. Since full-length AmpR bound to 21 bp dsDNA has increased solubility in solution, this complex could be subjected to commercial crystallization screens to obtain crystals for X-ray structure determination. Having a full-length structure of AmpR would be particularly insightful in interpreting the previously discussed SAXS data.

The *in vitro* transcription methods discussed in Chapter 3 could be further optimized using lower concentrations of AmpR in assays to prevent potential protein oligomerization effects that may have interfered with *ampR-ampC* gene regulation. Once a functional assay is established, relative changes in gene expression can be assessed using competing amounts of 1,6-anhydro-MurNAc-tri and –pentapeptide to determine whether the latter species causes increased *ampC* expression using methods discussed in Chapter 3. The binding affinity of each inducer species for AmpR could also be defined using isothermal microcalorimetry (ITC). Given the success of MS in characterizing the binding of the repressor UDP-MurNAc-pentapeptide to AmpR, electrospray ionization (ESI) MS could also be used to titrate in AmpR repressor and

inducers to characterize their binding stoichiometry. Additionally, a comparison of relative intensities of competing AmpR:ligand complexes on the mass spectrum could reveal the identity of the predominant species, thus identifying which of 1,6-anhydro-MurNAc-tri or –pentapeptide has greater affinity for AmpR.

E.2. Further development of therapeutic strategies based on NagZ inhibition

To overcome the membrane impermeability of *P. aeruginosa*, selective NagZ inhibitors could be synthesized to include catechol moieties at the site of the phenylcarbamate and 2-acetamido groups in PUGNAc to potentially facilitate their uptake into the cell via iron acquisition pathways, a strategy that has been successfully applied to improving β -lactam antibiotic uptake into the periplasmic space (260). Additionally, given the flexibility of the catalytic loop in NagZ, inhibitors could be developed with locked cycling rings that link the phenylcarbamate group in PUGNAc to the 2-acetamido group such that they selectively bind NagZ over related family GH20 and GH84 glycosidases that have more rigid active site architectures. Finally, an assessment of the mutational frequencies of high-level β -lactam resistant mutants in *ampC* derepressed strains of *P. aeruginosa* that might arise during co-administration of PUGNAc and avibactam with ceftazidime could be used to reveal if simultaneously blocking AmpC induction and function may provide a strategy to tightly suppress the emergence of AmpC-mediated resistance.

E.3. Investigating VCC-1-mediated hydrolysis of avibactam

While kinetic and structural data highlight the remarkable potency of avibactam against VCC-1, its efficacy against *V. cholerae* expressing VCC-1 should also be determined by an MIC assay. To gain further insight into the mechanisms of VCC-1 inhibition and catalysis, the crystal

structures of VCC-1 in complex with the β -lactamase inhibitors clavulanic acid and tazobactam could also be determined. These structures could potentially reveal the basis for the greater relative potency of avibactam against VCC-1, and also highlight features of active site architecture responsible for avibactam desulfation. The ability of active site residues (particularly Lys73, Ser130, Glu166 and Lys234) to influence avibactam desulfation could also be assessed by generating VCC-1 mutants for each residue, which can then be crystallized with avibactam and subjected to kinetic analysis. Subjecting the VCC-1-avibactam complex to a time-course ESI-MS experiment to identify various acyl-enzyme species generated as avibactam is slowly hydrolyzed should also be considered. Together, these experiments would add to our understanding of inhibitory and potential resistance mechanisms against this important new class of β -lactamase inhibitors, and potentially inform future work aimed at improving their efficacy against serine β -lactamases.

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