Inactivation of the Glycoside Hydrolase NagZ Attenuates Antipseudomonal β-Lactam Resistance in *Pseudomonas aeruginosa*

By

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Chapter 1 INTRODUCTION

Chapter 2 MATERIALS AND METHODS

Chapter 3 RESULTS

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ABSTRACT

Pseudomonas aeruginosa is a versatile Gram-negative opportunistic pathogen notorious for its ability to chronically colonize and deteriorate the pulmonary function of the cystic fibrosis lung. It exhibits high resistance to β-lactam antibiotics, including cephalosporins and monobactams, via induction of their chromosomally encoded AmpC β-lactamase. Regulation of *ampC* expression is coupled to the bacterial cell wall recycling pathway by the activity of NagZ, a glycosidase that produces 1,6-anhydroMurNAc-tri-(or penta-) peptides from internalized peptidoglycan metabolites. During β-lactam therapy, this tripeptide rapidly concentrates in the bacterial cytosol to levels sufficient for it to bind and activate AmpR, the transcriptional activator of *ampC*. *P. aeruginosa* also encodes three *ampD* genes, each expressing an *N*-acetylmuramyl-L-amidase that cleaves the peptide stems from 1,6-anhydroMurNAc or GlcNAc 1,6-anhydroMurNAc. AmpD thus suppresses 1,6-anhydroMurNAc-peptide accumulation and moderates *ampC* induction. Selection of AmpD null mutants during therapy thus causes chronic hyperproduction of β-lactamase, presumably from an increase in NagZ product, and have been identified in *P. aeruginosa* strains isolated from chronically infected CF patients. Mutants harboring an inactivated *nagZ* gene in a wild-type *P. aeruginosa* background were isolated and were found to have increased antibiotic susceptibility to antipseudomonal β-lactams. Inactivating *nagZ* in a triple *ampD* mutant substantially decreased the expression of *ampC* and rendered these high-level resistant strains susceptible to antipseudomonal β-lactams at wild-type strain levels. This brings the susceptibility of the *P. aeruginosa* strains down to the β-lactam therapy range accepted by CLSI for use in cystic fibrosis patients suffering from chronic *Pseudomonas*

aeruginosa infections.

To assess whether *P. aeruginosa* expresses more than one *N*-acetyl-β-glucosaminidase that could contribute to the production of the activating tripeptide, residual activity assays were conducted on *nagZ* deficient mutants. Mutants were devoid of activity so it was concluded that *P. aeruginosa* expresses only the one *N*-acetyl-β-glucosaminidase in study, NagZ.

Complementation studies using the wild type *nagZ* gene restored the wild type phenotypes, particularly evident in the triple *ampD* null mutants. These findings suggest that NagZ activity is required for *ampC* induction, and that an intricate balance exists between NagZ and AmpD activity to regulate the concentration of the inducer molecule 1,6-anhydroMurNAc-tripeptide.

DEDICATION

To Jeni (1984 – 2008)

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ABBREVIATIONS

A	- Optical absorbance
A_2pm	- Diaminopimelate
A_{486} or A_{525} or A_{600}	- Absorbance at 486 nm or 525 nm or 600 nm respectively
Ala	- Alanine
Ap^R	- Ampicillin resistant
ATM	- Aztreonam
β	- Beta
BGG	- Bovine gamma globulin
bp	- Base pair(s)
BSA	- Bovine serum albumin
˚C	- Degrees Celsius
C-terminus	- Carboxyl terminus
CAZ	- Ceftazidime
CIP (enzyme)	- Calf intestinal phosphatase
CIP	- Ciprofloxacin
CF	- Cystic fibrosis
CFTR	- Cystic fibrosis transmembrane conductance regulator gene
CLSI	- Clinical Laboratory and Institute Standards
cm	- Centimeter
ddH_2O	- Double distilled water
DNA	- Deoxyribonucleic acid
dNTP	- Deoxynucleotide 5'-triphosphate
E. coli	- *Escherichia coli*
EDTA	- Ethylenediamine tetra acetic acid
FEP	- Cefepime
FOX	- Cefoxitin
γ	- Gamma
g	- Gram
G (G needle)	- Gauge
GlcNAc	- *N*-acetyl-glucosamine
Gm^R	- Gentamicin resistant
Glu	- Glutamate
HCl	- Hydrochloric acid
ICU	- Intensive care unit
IMP	- Imipenem
IR	- Induction ratio
kb	- Kilobase
KCl	- Potassium chloride
kV	- Kilo volts
L	- Liter
LB	- Luria-Bertani medium

m	- Meso
µg	- Micrograms
µL	- Microlitres
M	- Molar
mM	- Millimolar
mm	- Millimeter
µM	- Micromolar
mL	- Milliliter
MCS	- Multiple cloning site
MH	- Mueller Hinton medium
MIC	- Minimum inhibitory concentration
min	- Minute
MRM	- Merodiploid-resolving media
mRNA	- Messenger ribonucleic acid
MurNAc	- *N*-acetyl-muramic acid
4-MU	- 4-methylumbelliferone
4-MUGlcNAc	- 4-methylumbelliferyl-β-D-*N*-acetylglucosamine
NEB	- New England Biolabs
N-terminus	- Amino-terminus
NagZ	- *N*-acetyl-β-glucosaminidase
ng	- Nanograms
NHAc	- Acetamido group
nm	- Nanometer
nM	- Nanomoles
O	- Oxygen
OH	- Hydroxyl group
OD	- Optical density
ORF	- Open reading frame
ori	- Origin of replication
P. aeruginosa	- *Pseudomonas aeruginosa*
PBS	- Phosphate buffered saline
PCR	- Polymerase chain reaction
PIA	- *Pseudomonas* Isolation Agar
PIP	- Piperacillin
PIP/TZ	- Piperacillin/tazobactam
Pol	- Polymerase
PTC	- Peltier Thermal Cycler
PUGNAc	- *O*-(2-Acetamido-2-deoxy-D-glucopyranosylidene)amino *N*-phenylcarbamate
rbs	- Ribosome binding site
rcf	- Relative centrifugal force
spp.	- Species
SOB	- Super Optimal Broth
SOC	- Super Optimal broth + catabolite repression
TAE	- Tris-acetate EDTA buffer
Tc^R	- Tetracycline resistant

Tc^S	- Tetracycline sensitive
TRIS	- Tris (hydroxymethyl) aminomethane
UDP	- Undecaprenyl-PP
v	- Volume
VAP	- Ventilation-associated pneumonia
x	- Times
xg	- Centrifugal force

Chapter 1 - Introduction

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative bacterium that is ubiquitous in the environment and is notorious for its high intrinsic resistance to β-lactam antibiotics (1). β-lactams are front line defense antimicrobial agents against bacterial, often pseudomonal, infections in hospitals (2). As a significant opportunistic pathogen, *P. aeruginosa* frequently capitalizes on the weakened immunity of its human host and, as a result, accounts for over 10% of all nosocomial infections (1, 3, 4). *P. aeruginosa* is often responsible for ventilation-associated pneumonia (VAP) and secondary bacteremia in intensive care units (ICU), targets burn-wound victims, and chronically colonizes the weakened lungs of cystic fibrosis (CF) patients (5-8). Pseudomonal infections are prevalent in cystic fibrosis patients at a young age and by early adulthood, almost 80% of CF patients have been infected with the bacteria (9). The microbe commonly establishes chronic infections by overcoming clinical antibiotic therapy, contributing to the extensive morbidity and mortality of CF patients (4).

1.1.1 Clinical Relevance: Cystic Fibrosis

Cystic fibrosis is an autosomal recessive disorder caused by a mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. Those who are afflicted suffer debilitating physical effects and a shortened lifespan (10-13).

The primary, incurable problem of CF is the secretion of viscous, dehydrated mucous in the airways of the patients which can be explained by abnormalities in the transport and regulation of chloride ions; the airway cells are impermeable to Cl^- (11, 14). The physiologically defective electrolyte transport is characterized by the clinical manifestations of CF, that is, the thickened and obstructive secretions that results in organ failure (11). The mechanisms of defense in the lungs, both to remain healthy and to avoid colonization and infection, involves mucociliary clearance and phagocytosis of antigens, both of which become virtually ineffective because of the thick mucous and dysfunctional osmolarity regulation in the CF lung (14). Thus, cystic fibrosis patients are highly susceptible to secondary infections (11).

Amongst CF patients, it has been found that the appearance of *P. aeruginosa* isolates occur at a much higher frequency than with other common airway pathogens such as *Haemophilus influenzae,* and *Streptococcus pneumoniae* (9). Chronic infections with *P. aeruginosa* are a major barrier to the survival of CF patients, resulting in a shortened life expectancy and general demise in health. Respiratory disease associated with chronic infections by resistant strains in CF patients is the leading cause of death; 95% of CF patients die of respiratory failure (11, 15, 16).

1.2 *Pseudomonas aeruginosa* Intrinsic Antibiotic Resistance

The ability to challenge and defeat β-lactam antibiotic therapy in the clinical setting is frequently mediated by the microorganism's considerable intrinsic resistance to antibiotics and more extensively through the selection of mutated, highly resistant strains

(17). The intrinsic resistance of *P. aeruginosa* involves components of the bacterial membrane that serve to protect the microbe. These include a pump system that was the reason for their resistance (18). This pump system, MexAB-OprM, most likely had natural roles that involved the removal of molecules that could disrupt the bacterial membrane but also served to remove a variety of antimicrobials including fluoroquinolones, macrolides, sulfonamides, novobiocin, as well as dyes, disinfectants and detergents (17, 19-21). Previous studies on pseudomonal resistance through efflux systems have confirmed the role of the membrane's limited permeability, though other systems confer a more narrow range of resistance to certain antimicrobials; MeXCD-OprJ, MexEF-OprN and MexXY-OprM (22-24).

Another mechanism by which *P. aeruginosa* exhibits resistance to β-lactam antibiotics is via the induction of AmpC β-lactamase. AmpC is an enzyme that deactivates β-lactams through hydrolysis and expression is induced by the degradation components of the Gram-negative cell-wall recycling pathway.

1.3 The Bacterial Cell Wall

P. aeruginosa, like other Gram-negative bacteria, utilizes a recycling pathway involving the degradation and reintegration of the cell wall turnover products. By reusing turnover material that is extracted from the cell wall, which is composed of a highly cross-linked heteropolymer of sugars and amino acids known as peptidoglycan, the bacteria conserve energy and have the additional benefit of reusing precursor molecules, a

feature that is particularly beneficial when the organism is in conditions with low nutrients or is in competition with other microorganisms (25, 26).

Peptidoglycan structure is crucial to the maintenance of microbe shape, rigidity and protection from osmolytic lysis (25, 27, 28). The basic subunit of peptidoglycan is made up of alternating linear glycan chains of *N*-acetyl-glucosamine (GlcNAc) and *N*-acetyl-muramic acid (MurNAc) sugar residues, connected by a β-(1,4) glycosidic linkage (28, 29). Attached to the carboxyl side chain of the MurNAc residue by an amide bond is a short peptide of three to five amino acids; L-alanyl-γ-D-glutamyl-mesodiaminopimelyl-D-alanine (L-Ala- D-Glu-m-A_2pm-D-Ala), which is the peptide sequence found in Gram-negative bacteria (Figure 1.1) (27).

The first major catalytic step in synthesis of the cell wall is organization of the membrane-bound disaccharide peptide monomer unit in which transglycosylases catalyze formation of the GlcNAc-MurNAc glycan chains. The second major type of membrane-bound activity is that of the membrane-anchored cell-wall transpeptidases, or serine-type D-alanyl-D-alanine carboxypeptidases (DD-carboxypeptidases) that catalyze formation of the peptide cross-bridges that link the alternating glycan strands of peptidoglycan (30, 31). DD-carboxypeptidases are specific to the C-terminus of the donor peptide containing two alanine residues, such as D-alanyl-D-alanine, as on the MurNAc short peptides (32). The activities of the transpeptidases employ an active site serine by forming a serine ester-linked acyl-enzyme complex upon interaction with an amide carbonyl-donor (31, 33). Specific to Gram-negative cells, the head-to-tail cross-linking involves the peptide bond formed between the free amino end of a diamino pimelic acid on one glycan chain and a penultimate D-alanine on the other glycan chain (31).

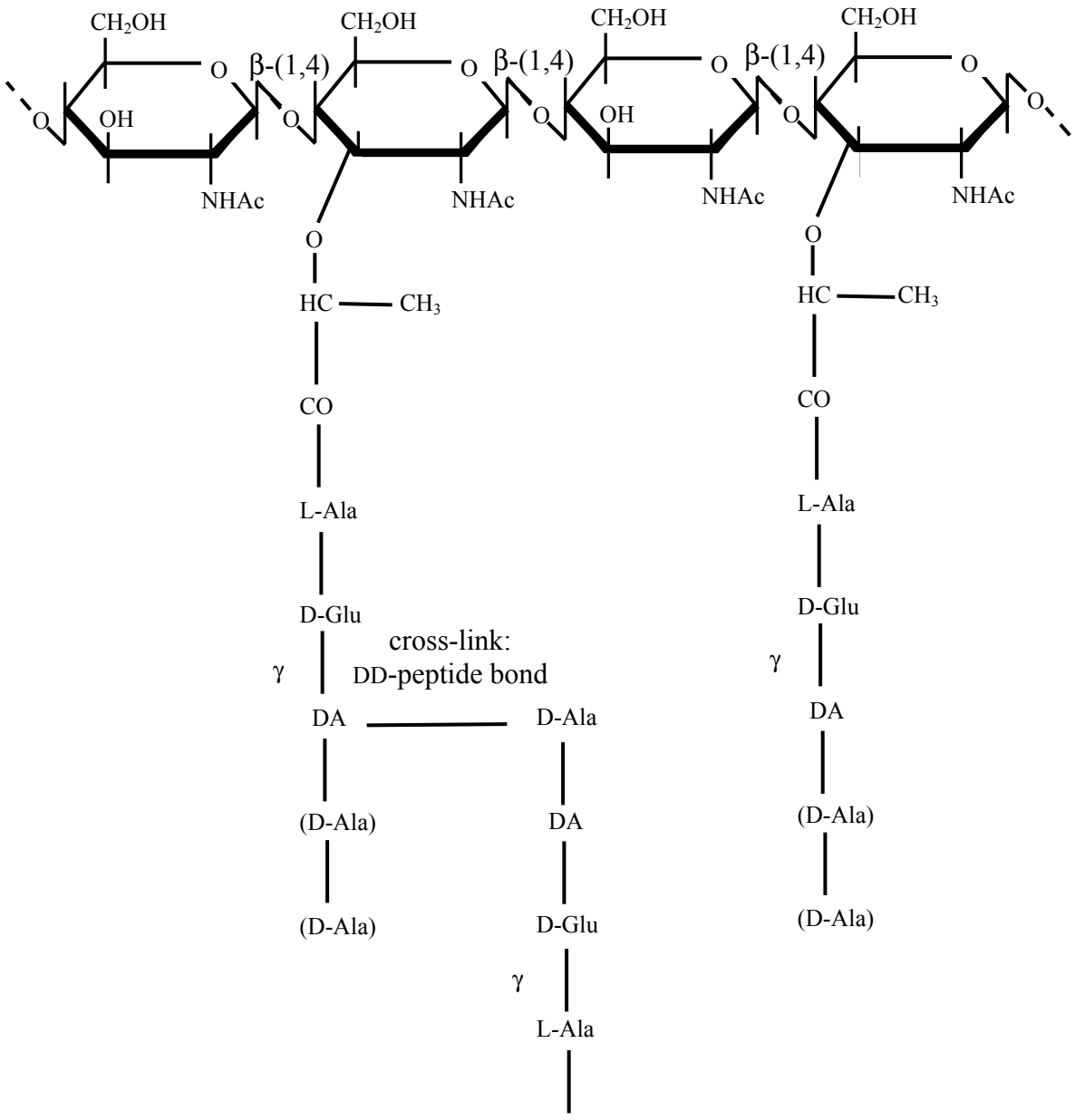


Figure 1.1. The basic subunit of the heteropolymer peptidoglycan of Gram-negative bacteria. Alternating GlcNAc and MurNAc sugar residues, connected by a β-(1,4) glycosidic linkage, form repeating linear glycan chains. A short peptide L-alanyl-γ-D-glutamyl-mesodiaminopimelyl-D-alanine (L-Ala-D-Glu-m-A_2pm-D-Ala) is attached to the MurNAc residue by an amide bond to the carboxyl side chain. The short peptide forms a bridge with the opposing peptide, giving rise to an oligopeptide that forms an interconnected mesh of peptidoglycan that defines the cell wall.

The continuous cross-linking reactions of glycans and peptidoglycan remodeling involves the insertion of new glycan-peptide strands into the existing cell wall, enabling the bacteria to expand its cell wall as it grows and divides (26, 27).

1.4 Peptidoglycan Turnover

P. aeruginosa carefully monitors the integrity of the cell wall and responds by induction of AmpC β-lactamase when b-lactams are present (34). Recycling peptidoglycan is an endogenous pathway and is balanced by tracking the precursors in the cytosol as the accumulation of certain molecules will trigger the bacteria's defenses.

The cell wall recycling pathway is a catalytic cycle beginning at the excision of the peptidoglycan molecules and leading to the activity of the transpeptidase enzymes through means of an acylation/deacylation procedure.

Synthesis of cell wall precursor molecules is energy-demanding, thus bacteria use a recycling pathway to degrade existing peptidoglycan and use the degradation products to efficiently synthesize new peptidoglycan which can be integrated into the existing cell wall, reusing approximately 50% of the cell wall components per generation (35). Degraded sugar-peptide fragments of peptidoglycan are excised from the cell wall and brought into the cytoplasm by a transmembrane permease encoded by AmpG. The fragments are cleaved by enzymes in the cytoplasm to give rise to murein oligopeptides that undergo a series of metabolic steps to be reintegrated into the existing cell wall.

The recycling of peptidoglycan is not only an advantageous metabolic shortcut, it also plays an important role in bacterial pathogenicity, as the peptidoglycan precursor molecules have been found to regulate intrinsic β-lactam resistance by modulating *ampC* gene expression (25, 28). Transcription of AmpC, from the very low basal level it is normally expressed at, can be expressed at quantities high enough to hydrolyze and inactivate most β-lactams (36).

1.5 Inducible β-Lactam Resistance

1.5.1 AmpC β-Lactamase Pathway

β-lactamases encoded by *ampC* are found in many Gram-negative bacteria such as *P. aeruginosa*, *Citrobacter freundii* and other enterobacteria (34, 37). *P. aeruginosa* produces low basal levels of β-lactamase when uninduced although the presence of β-lactam agents that alter the tightly-regulated AmpC pathway precipitates increased expression (36). Based on the Ambler system of classification, there are four classes of β-lactamases produced; classes A, B, C and D, categorized based on their catalytic mechanisms and sequence homology (38).

The β-lactamase produced by *P. aeruginosa* is classified as a Group I Class C chromosomally encoded cephalosporinase. β-lactamases evolved with a role that likely intended to defend the bacteria from external agents and its expression is coupled to the cell wall recycling pathway. β-lactam compounds inhibit peptidoglycan biosynthesis and stimulate overproduction of lytic enzymes that are naturally produced by bacteria at carefully controlled levels involved in remodeling of peptidoglycan (39). Thus β-lactam

resistance mediated by β-lactamases was likely a mechanism evolved as part of normal cell wall maintenance and protection from self lytic enzymes (40).

In a normal healthy cell, a delicate balance exists between the degradation of peptidoglycan and biosynthesis of new molecules that will eventually be integrated into the growing cell wall. During bacterial growth cycles, peptidoglycan fragments are constantly excised from the cell wall and are recycled intracellularly (41).

Disruption in the equilibrium alerts the bacteria to changes in its environment, and triggers its defense mechanisms. In the presence of β-lactam antibiotics, the equilibrium of the peptidoglycan-recycling pathway is shifted and the ratio of peptidoglycan degradation and biosynthesis becomes unbalanced (34).

The AmpC-mediated β-lactamase pathway of *P. aeruginosa* is closely coupled to the bacteria's cell wall recycling pathway (Figure 1.2). Peptidoglycan is a unique molecule to the bacterial kingdom and the entire pathway to synthesize peptidoglycan is highly specific in bacteria themselves (42). β-lactams inhibit the proper synthesis of the peptidoglycan cell wall, leading to microbial death. However, Gram-negative bacteria have evolved a number of mechanisms, including the AmpC-mediated β-lactamase pathway to defend themselves against β-lactams.

1.5.2 NagZ: *N*-acetyl-β-glucosaminidase

NagZ is a family 3 β-glucosaminidases located in the cytoplasm of bacteria. NagZ cleaves GlcNAc from the incoming the GlcNAc-1,6-anhdroMurNAc-peptides to

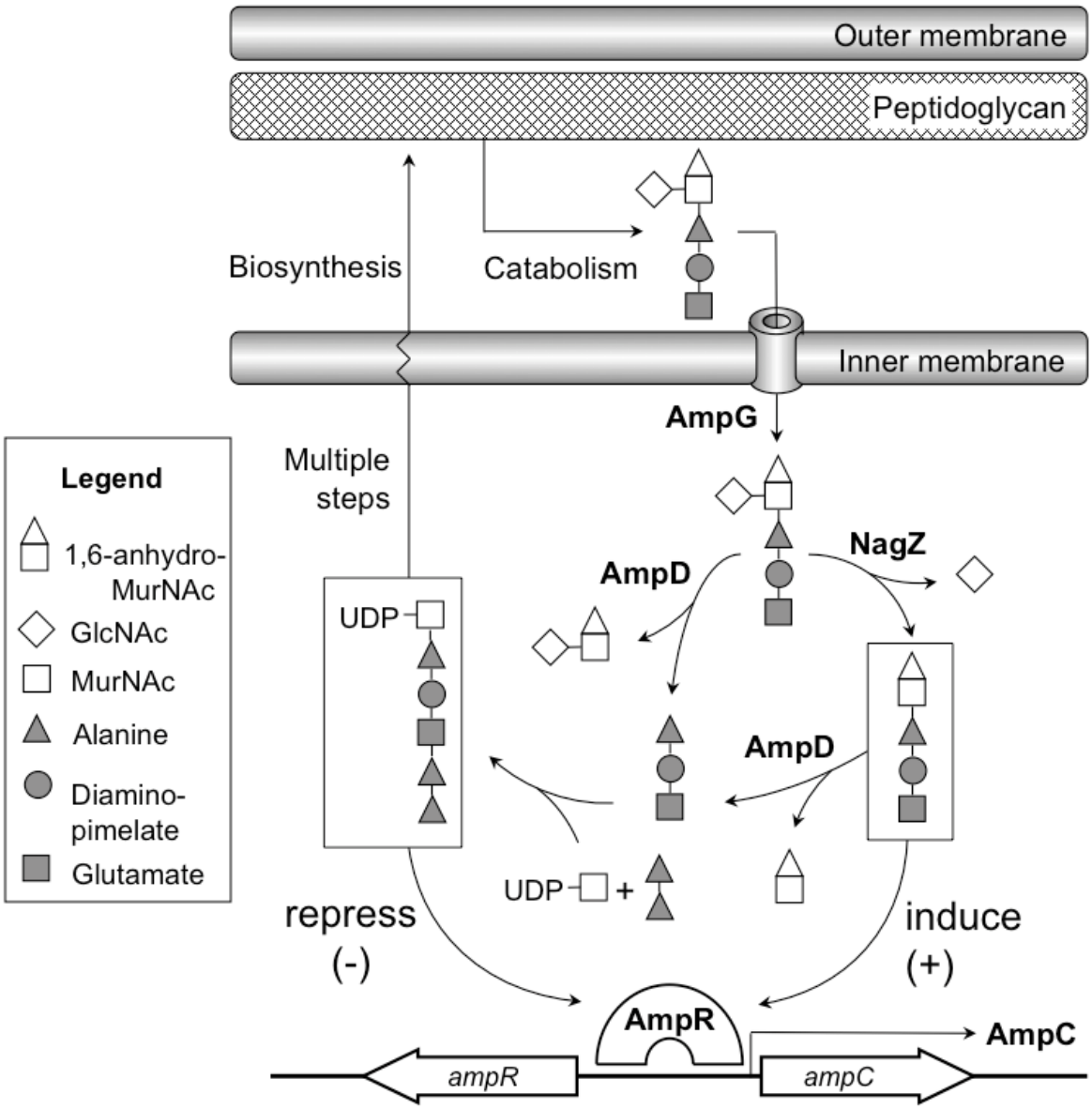


Figure 1.2. Schematic representation of the cell wall recycling pathway. As bacteria grow, the cell wall must enlarge to accommodate. GlcNAc-1,6-anhydroMurNAc tri-, tetra- and pentapeptides (tripeptides shown in figure) are excised from the cell wall and imported into the cytoplasm by AmpG permease. NagZ cleaves GlcNAc, generating 1,6-anhydroMurNAc peptide (boxed at right), and either the tri- or pentapeptide species is believed responsible for activating AmpR to express *ampC* from the *ampC-ampR* operon. AmpD clears the cytoplasm of peptides by removing the stem peptides from both GlcNAc-1,6-anhydroMurNAc and 1,6-anhydroMurNAc. These degradation products are eventually recycled into UDP-MurNAc pentapeptide (boxed at left), a precursor molecule of peptidoglycan synthesis and a repressor of AmpR. Exposure to β-lactams causes an increased cytosolic concentration of 1,6-anhydro-MurNAc peptides that is sufficient to displace UDP-MurNAc pentapeptides to convert AmpR into an activator of *ampC* transcription.

generate GlcNAc and 1,6-anhydroMurNAc-tri-, tetra- and pentapeptides (Figure 1.2) (43). In healthy cells, there is very little cytosolic accumulation of free 1,6-anhydroMurNAc-oligopeptides (43). The concentration of the 1,6-anhydroMurNAc-tripeptide (or pentapeptide species) is the inducer molecule that up regulates transcription of the *ampC* β-lactamase, thus their concentration in the cytoplasm is carefully monitored.

Under normal cellular conditions, the AmpC inducer molecule produced by NagZ, 1,6-anhydroMurNAc-tripeptide (or pentapeptide), is degraded by AmpD amidases which removed the stem peptide from 1,6-anhydroMurNAc to keep cytosolic levels at a basal level to prevent the unnecessary or excessive induction of AmpC. In the presence of β-lactam antibiotics, an increased influx of GlcNAc-1,6-anhydroMurNAc-peptides flood the cell and bacteria accumulate NagZ product, presumably because AmpD activity becomes saturated. The inducer up regulates transcription of *ampC* from the very low basal level it is normally expressed at to quantities high enough to hydrolyze and inactivate most β-lactams to clear the antibiotic that induced its expression (36).

1.5.3 AmpD: Anhydro-*N*-acetylmuramyl-L-Ala Amidases

There are three homologous *ampD* genes present in the genome of *P. aeruginosa,* all of which actively contribute to regulating the amount of inducer molecule present in the cell and are thus clearly involved in AmpC β-lactamase induction (25, 44). Each AmpD enzyme is located in the cytoplasm and acts to degrade incoming peptidoglycan components. The *ampD* genes encode *N*-anydromuramyl-L-alanine amidases, which are negative regulators of β-lactamase expression (25). Their activity prevents the accumulation of the β-lactamase expression inducer molecules 1,6-anhydroMurNAc tri-

(or penta-) peptides by removing the stem peptide from 1,6-anhydroMurNAc, the NagZ product, as well as from GlcNAc-1,6-anhydroMurNAc-tripeptide (Figure 1.2)

In deducing the specific substrate of AmpD, it has been shown that AmpD exhibits activity at a rate 10,000 times faster on the 1,6-anhydroMurNAc-tripeptide species versus the tetra- and pentapeptide species, providing evidence that, although all the oligopeptide species may play the role of *ampC* inducer, the tripeptide species may bind with higher affinity to AmpR (44). Furthermore, in *ampD* null mutants of *E. coli*, a greater accumulation of the AmpG intake product GlcNAc-anhydroMurNAc-tripeptide was identified in the cytoplasm, suggesting this was the substrate for AmpD activity (44).

AmpD displays strict specificity to substrates with an anhydro function on the muramic acid residue, even though this is not its site of cleavage. This necessary 1,6-anhydro bond only forms during intercellular transglycosylation of peptidoglycan fragments brought in from the cell wall and this allows AmpD amidases to recognize recycled precursors from those synthesized *de novo* (44, 45). This allows the bacteria more accurate detection and monitoring of its environment as AmpD targets the fragments from the existing cell wall. As β-lactams bind the DD-peptidases and disrupt the balance of the recycling pathway, peptidoglycan fragments (GlcNAc-anhydroMurNAc-oligopeptides) will flood the cell and saturate AmpD activity, alerting the bacteria to the presence of threatening compounds.

AmpD activity in the balance of peptidoglycan degradation and biosynthesis is regulated in the cell by the dual regulator AmpR, both repressor and activator of the AmpC β-lactamase expression.

1.5.4 Transcriptional Regulation of AmpC

The AmpC β-lactamase is an inducible cephalosporinase under the control of the transcriptional regulator AmpR. Control is closely associated with cell wall turnover products and the presence of β-lactams; thus, regulation of the *ampC-ampR* genetic operon closely involves the cell wall recycling pathway. Transcription of *ampC* is modulated by AmpR, the constitutively transcribed LysR-type transcriptional regulator, part of the LysR family of bacterial regulators (46). The AmpR operon is divergent from and located immediately upstream of AmpC on the *ampC-ampR* operon. AmpR positively and negatively regulates transcription of the *ampC* gene depending on the cytosolic concentrations of the AmpR inducer or repressor molecules (34). The inducer molecules, considering their intimate link to peptidoglycan recycling, are precursors from cell-wall turnover that have over accumulated in the cytoplasm, prompted by the result of β-lactam activity (37).

A byproduct of NagZ activity, the released murein peptides, are promptly bound to UDP-MurNAc (pentapeptide) and recycled for synthesis of peptidoglycan (43). However UDP-MurNAc pentapeptide, as a precursor of peptidoglycan, also binds the AmpR regulator (34). The 1,6-anhydroMurNAc-oligopeptides can also undergo several turnover steps to yield UDP-MurNAc-pentapeptide. In turn, the bound AmpR conformation prevents transcription of AmpC thereby acting as a negative regulator (34, 36, 37).

In the presence of β-lactams that have disrupted peptidoglycan synthesis, if the inducer molecule 1,6-anhydroMurNAc-tri-(or penta-) peptide is present in concentrations

high enough to compete for AmpR binding , it can displace UDP-MurNAc-pentapeptide in which AmpR will be shifted into a positive regulator and transcription of *ampC* will occur (Figure 1.3) (28, 46). Essentially, AmpR acts as the repressor of *ampC* expression under normal conditions, but once bound to the 1,6-anhydroMurNAc-tri- (or penta-) peptide, it induces β-lactamase transcription (47).

1.6 β-Lactam Antibiotics

β-lactam antibiotics specifically target the serine-type D-alanyl-D-alanine carboxypeptidases whose enzymatic activity gives rise to the DD-peptide bonds that cross-link the peptide chains of neighboring MurNAc residues (Figure 1.4) (29). These carboxypeptidases serve essential functions in the recycling and expansion of the cell wall of *P. aeruginosa*. β-lactams are active against bacteria because they mimic the structure of a muramyl pentapeptide with a C-terminal D-alanyl-D-alanine (48).

1.6.1 Mode of Action

The last step of peptidoglycan synthesis occurs on the outermost side of the cytoplasmic membrane, in the extracellular space, providing easy access for β-lactams, whose target enzymes are located in the same region. β-lactam activity specifically inhibits the transpeptidase reactions (31). The serine-type D-alanyl-D-alanine carboxypeptidases, or DD-carboxypeptidases, are enzymes which act to catalyze the cross-linking between the free amino end of a diamino pimelic acid on one chain and a penultimate D-alanine on the other chain (Figure 1.1) (31). β-lactams are stereochemical

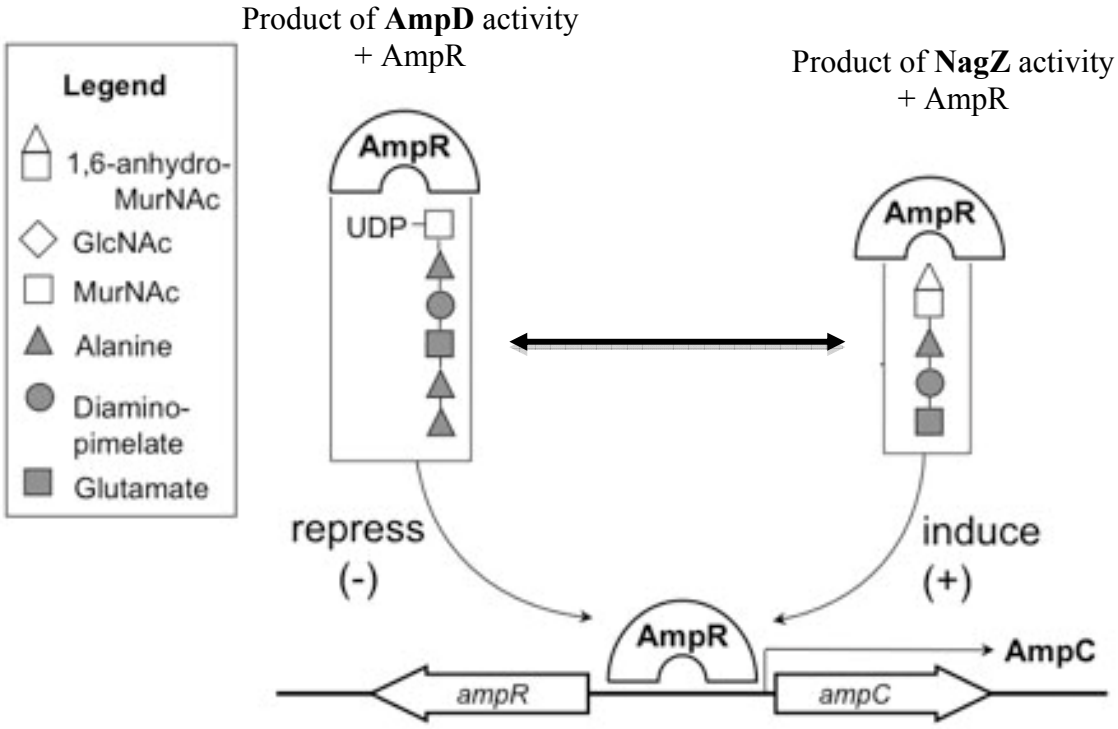


Figure 1.3. The AmpR transcriptional regulator both negatively and positively regulates the *ampC* gene. In cells without β-lactam (at left) UDP-MurNAc-pentapeptide dominates and represses *ampC* transcription. Cells in the presence of ß-lactams exhibit disrupted balance and 1,6-anhydro-MurNAc-oligopeptides accumulate and displace repressor molecule, shifting AmpR and inducing *ampC* transcription.

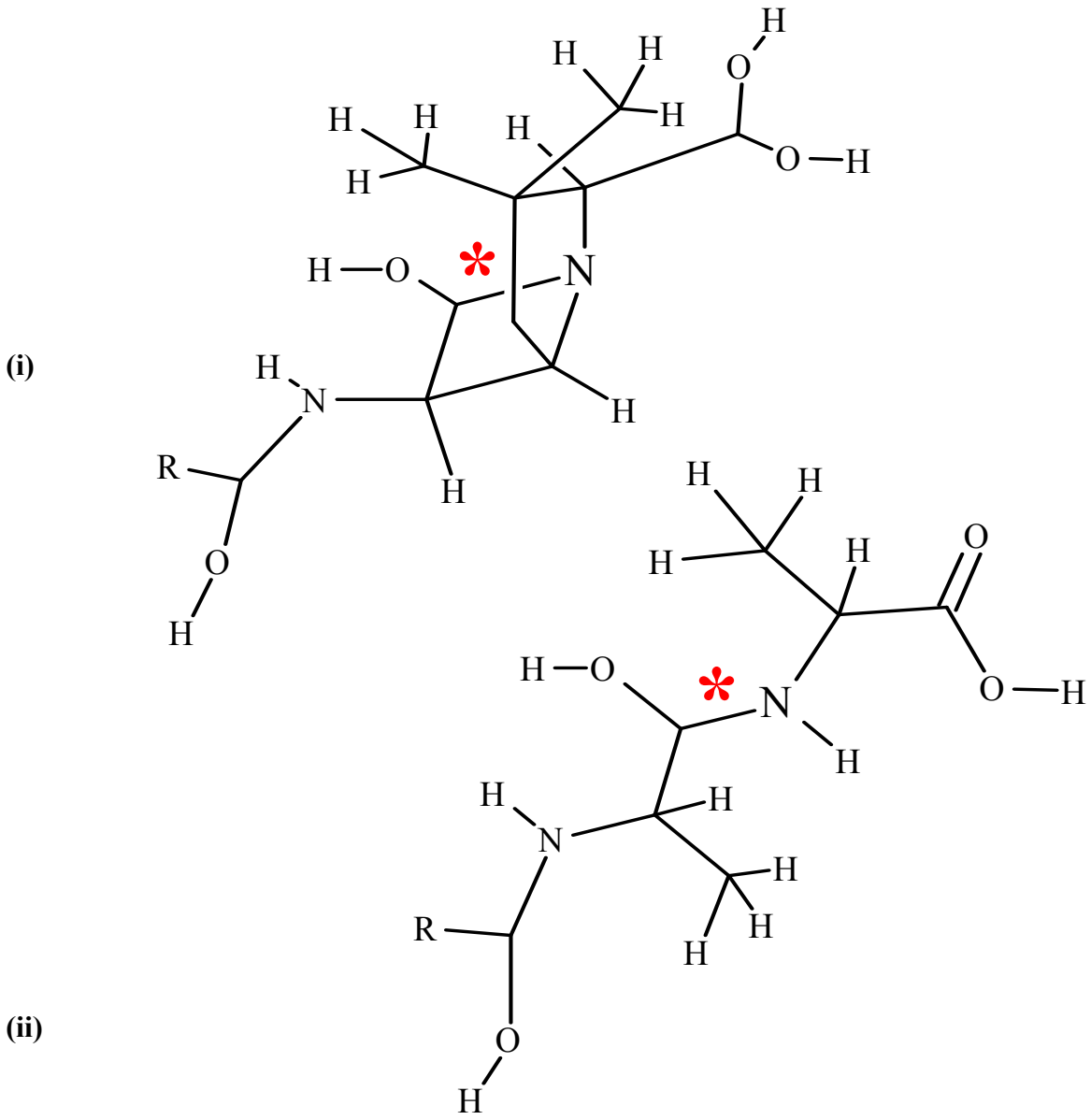


Figure 1.4. Stereochemical structures of (i) penicillin and (ii) the D-alanyl-D-alanine terminus of the pentapeptide of peptidoglycan. The CO-N bond indicated by the asterisk is the target bond of bacterial transpeptidases that result in cross-linking. Transpeptidases also bind the CO-N bond of penicillin, leading to their immobilization. Figure adapted from Rice, L.B., 2004 (38).

carbonyl-donor analogues of the D-alanyl-D-alanine substrate and thus bind with high affinity to the transpeptidases (31). Upon binding, the β-lactam ring opens and the carbon atom forms an irreversible covalent bond with the enzyme, acylating the active-site serine of the DD-peptidases and inhibiting a normal transpeptidase reaction and thus, cross-linking of the glycan strands (Figure 1.5, 1.6) (33, 49, 50). The end result is that the bacteria are inhibited from normal growth because the product of synthesis is soluble uncross-linked peptidoglycan, which gives rise to a weak, unstable peptidoglycan mesh (46).

1.7 β-Lactamase: Mode of Action

As discussed earlier the existing peptidoglycan from the cell wall is excised using lysins produced by the bacteria. Gram-negative bacteria can then rebuild the cell wall by use of an active-site-serine residue found on the dissaccharide oligopeptides. Accordingly, class C β-lactamases produced by *P. aeruginosa* are also known as the serine type β-lactamases. These β-lactamases utilize a conserved serine as the catalytic nucleophile required for hydrolysis of the ester on the opened β-lactam ring (Figure 1.7) (31). Upon hydrolysis of the ester, the enzyme is released in an active state however the β-lactam lactam-amide bond has been inactivated and can no longer bind the target transpeptidases (51). The enzyme can then go on to locate and inactivate another β-lactam molecule, resulting in a rapid turnover of inoperative β-lactams.

β-lactams locate to the periplasmic space of Gram-negative bacteria, carrying out their immobilization of transpeptidases, where the last step of peptidoglycan synthesis

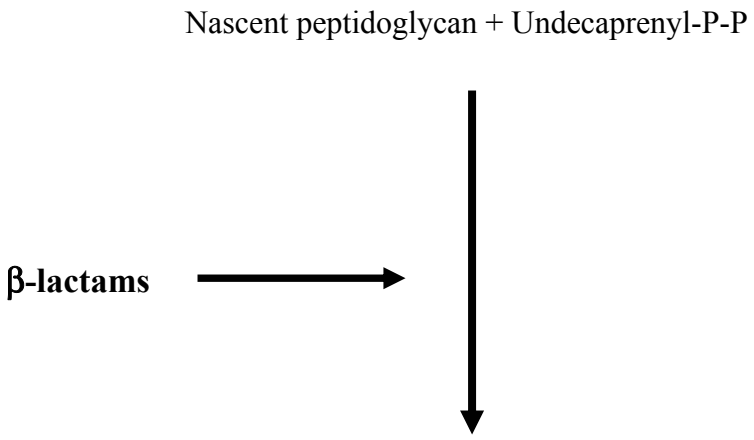


Figure 1.5. Target of β-lactam antibiotics in peptidoglycan synthesis.

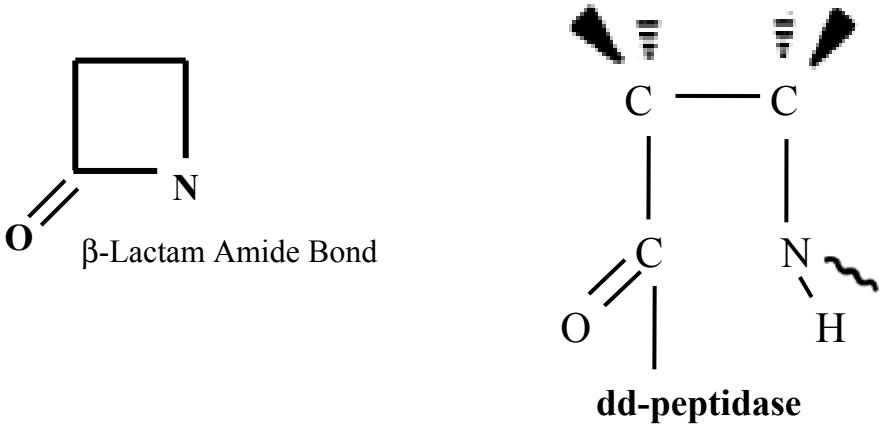


Figure 1.6. Acyl-enzyme complex formed by β-lactam ring acting as an amide carbonyl-donor and covalently binding transpeptidase. Active-site-serine DD-carboxypeptidase becomes immobilized in a long-lived acyl-enzyme complex with the β-lactam compound.

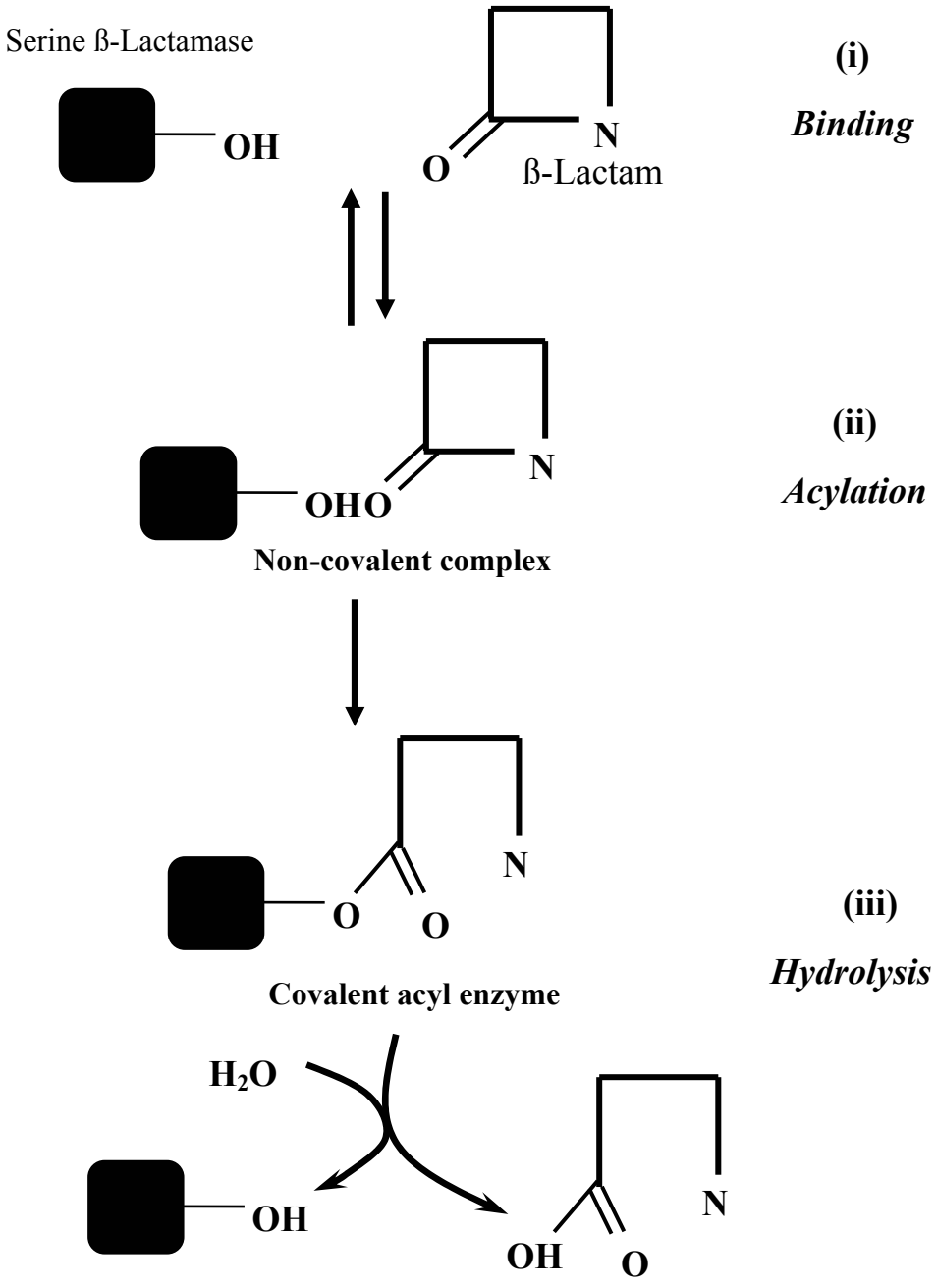


Figure 1.7. Mode of action of Class C β-lactamase: serine β-lactamase through a Michaelis complex. (i) Binding is the first step of the β-lactamase activity in which a Michaelis non-covalent complex forms between the β-lactam rings upon attack by the free hydroxyl on the side chain of a serine residue located at the active site of the enzyme. (ii) A covalent acyl ester is yielded. (iii) Final step of β-lactam breakdown is hydrolysis of the ester, producing an active β-lactamase enzyme and the inactivated β-lactam. Figure adapted from Livermore, 1995 (40).

occurs. It is for this reason that β-lactamase activity peaks in the periplasmic space (40, 51).

As stated, the cross-linking DD-carboxypeptidases also form a complex with β-lactams but, unlike the β-lactamases, they cannot partake in the hydrolysis of the antibiotic to inactivate it, which renders the transpeptidases immobilized from further enzymatic activity (40, 51). Thus the protective mechanism of β-lactamase production is hydrolyzing and clearing the bacterial cytosol of antibiotic before the antibiotic can bind and inactivate the transpeptidases. In this way, the production of β-lactamases results in the most common mechanism of bacterial resistance to β-lactams in clinical settings. The quantity of β-lactamase expression induced by an individual β-lactam antibiotic presence in the cell differs depending on the induction potential of the antibiotic (40).

1.8 Induction Potentials of β-Lactam Antibiotics

The mechanism in which the presence of a given β-lactam antibiotic results in the induction of AmpC β-lactamase transcription is through induction of the AmpC pathway. Normally, AmpC β-lactamase is expressed at very low levels but is inducible by the activity of β-lactam antibiotics (37, 52, 53). Different β-lactams vary greatly in their ability to induce β-lactamase expression (54).

Induction Ratio of β-Lactam Antibiotics

$$IR = E\ (induced)/E\ (uninduced)$$

The induction ratio (IR) is equal to the quantity of β-lactamase produced by cells grown with inducer (β-lactam) divided by the quantity of enzyme produced by cells grown without inducer (β-lactam) (37).

1.8.1 Antipseudomonal β-Lactams

Antipseudomonal β-lactams are indeed antipseudomonal because, although they contain the characteristic lactam ring that all β-lactam antibiotics have and can still immobilize transpeptidases, they do not cause a large amount of induction of AmpC β-lactamase. β-lactams with high binding affinities for transpeptidases of a bacterium would cause the bacterium to be more susceptible to the antibiotic than those antibacterial agents with low affinity of the transpeptidases (55). Only recently have studies begun to investigate the mechanism of *ampC* induction by a β-lactam and the means by which a noninducing β-lactam, frequently the antipseudomonals, alter peptidoglycan recycling in a different manner than strong *ampC* inducers, such as cefoxitin (56, 57). The degree of induction is associated with the rate and amount of peptidoglycan degradation by the inducer β-lactam (58).

When β-lactams enter the cell, the peptidoglycan-recycling pathway gets disrupted and alters the equilibrium between murein degradation and biosynthesis. The increase in incoming muropeptides saturates the activity of the *ampD* amidases, resulting in an accumulation of *ampC* inducer molecule. The increased concentration of 1,6-anhydroMurNAc oligopeptides displace the UDP-MurNAc-pentapeptides inactivating the AmpR regulator and leads to transcription of β-lactamase. Thus, resistance to β-lactams is associated with an increase in the anhydroMurNAc oligopeptide pool and in β-

lactamase expression, triggered by the presence of β-lactams themselves. Indeed it has been shown that in wild-type cells, upon addition of the β-lactamase inducer cefoxitin, the intracellular concentration of recycled muropeptides increases, and there is an even more dramatic increase in *ampD* null mutants (59). Another effect of the presence of cefoxitin is a decreased pool of UDP-MurNAc-pentapeptide, the negative gene modulator of the AmpR repressor; the presence UDP-MurNAc-pentapeptide results in the repression of *ampC* expression. Thus, although there are less anhydroMurNAc oligopeptides to induce AmpC expression through displacement of AmpR binding, there are less UDP-MurNAc-pentapeptides present that must be outcompeted by the anti-repressor 1,6-anhydro-MurNAc-tripeptides.

The altered equilibrium between peptidoglycan recycling and biosynthesis is characterized by increased breakdown of peptidoglycan and thus, increased generation of GlcNAc-anhydroMurNAc-tetrapeptide and GlcNAc-anhydroMurNAc-tripeptide (57). Rapid entry of GlcNAc-anhydroMurNAc-tripeptide components into the cytoplasm results in accumulation of the 1,6-anhydroMurNAc tripeptides that bind AmpR, converting it into an upregulater for *ampC* transcription (60).

The dual regulatory role of AmpR monitors cytoplasmic levels of peptidoglycan components, which is greater in the presence of β-lactam antibiotics, consequently β-lactamase continues to be expressed in the presence of β-lactams until the entire antibiotic concentration has been hydrolyzed and cleared from the cytoplasm (57). Antipseudomonals are categorized as such because they exhibit activity against *Pseudomonas aeruginosa*; strong β-lactamase inducers at high concentrations, weak β-lactamase inducers at lower concentrations (60).

The concept of antibiotic induction potentials can be applied in a clinical setting as well and can help explain why some antipseudomonals are more active than others. β-lactams such as the penicillins, cefoxitin, and narrow-spectrum cephalosporins have low β-lactamase stability and so destroy their own activity as a result of their strong AmpC induction potential (40, 61).

Piperacillin and extended spectrum (third generation) cephalosporins are weak inducers of AmpC thus, although they are susceptible to β-lactamase activity, they weakly induce AmpC transcription; thus, they can sustain concentrations sufficient to exhibit antimicrobial activity even in strains that have inducible AmpC (54). However, the selection of loss of AmpD activity during beta-lactam therapy can lead to hyper expression of the *ampC* gene because this mutation causes a build up of the 1,6-anhydroMurNAc oligopeptides that hyper-activate the AmpR regulator; thus, even weak inducers of AmpC, such as the antipseudomonals, become susceptible to hydrolysis by these mutants (37).

Imipenem, a carbapenem, is a strong inducer of β-lactamase; however it is not susceptible to hydrolysis by AmpC β-lactamase. To deduce the induction potential of imipenem, β-lactamase specific activity has been assessed when induced with imipenem and has been shown to produce copious amounts of β-lactamase, significantly higher than when strains are grown uninduced (53, 62).

Imipenem has been shown to have high activity against *P. aeruginosa* while exhibiting enough stability to β-lactamases such that the bacterium has been, for the most part, incapable of establishing resistance through AmpC (53, 63). The chemical structure

of imipenem influences its ability to act as an inhibitor of hydrolytic activity when bound to AmpC β-lactamase. Modifications on the acyl side chain, commonly of an acid function, confer resistance to cephalosporinases. Imipenem forms a complex with the enzyme, causing a conformational change to take place in which the bulky side chain forces rotation away from its acyl center, and steric strain results in complete inhibition of a hydrolytic attack (63). Imipenem thus remains active and it is for this reason it is commonly known as an effective antipseudomonal.

True to its notoriety however, *P. aeruginosa* can acquire resistance to imipenem through mutations in its active efflux systems resulting in loss of the D2 porin that allows carbapenems to gain cytoplasmic access (7, 23, 62, 64). As *P. aeruginosa* evolves and mutates, acquiring resistance to the β-lactams currently used in antibiotic therapy, new antibiotics must be continually enhanced and designed with greater efficiency. β-lactams are generally categorized based on their antipseudomonal activity – high, moderate and low, as well as their stability in the presence of β-lactamase – whether they are susceptible or stable. The stability of the antibiotic is related to its antipseudomonal activity; the more stable an antibiotic it is, the more likely it is that it will retain antipseudomonal activity (Table 1.1).

1.9 Derepressed AmpC Cephalosporinase-Producing Mutants

Frequent AmpR-regulated derepressed cephalosporinase-producing mutants that are found in clinical isolates of CF patients are strains of *P. aeruginosa* lacking functional AmpD activity (14). The *ampD* gene product exhibits activity that contributes directly to

Table 1.1. Categorization and Examples of Antipseudomonal β-Lactams

Group	Antipseudomonal Activity	β-Lactamase Stability	Examples of β-Lactams
Group I.	Moderate	High	• Ceftriaxone • Ceftizioxime • Cefotaxime • Moxalactam • Cefmenoxime • Nocardicin-A
Group II.	Moderate	Susceptible	• Ticarcillin • Cefoperazone • Mezlocillin
Group III.	High	High	• Ceftazidime • Cefuslodin • Thienamycin
Group IV.	High	Susceptible	• Piperacillin • Azlocillin • Furazlocillin • Apalicillin

Categorized based on biological activity and β-lactamase stability in *P. aeruginosa*.

the quantity of AmpC inducer molecule by breaking down the NagZ product (8, 36, 57). In the best interest of the cell to conserve energy, AmpD activity serves to maintain a concentration of inducer molecule below the levels required for induction of β-lactamase transcription (57). Mutations in *ampD* are the main mechanism by which the hyperproduction of AmpC is achieved (8, 36, 57, 65, 66).

The second mechanism by which β-lactamase can be constitutively expressed is through the selection of mutants lacking negative regulation (61). During antipseudomonal therapy with these antimicrobial agents, derepressed mutants are selected for under the distinct conditions of CF lungs (37, 67). Higher rates of derepressed mutants are seen in cystic fibrosis patient (40).

Antipseudomonal β-lactam antibiotics such as ceftazidime and piperacillin selects for isolates that produce the AmpC cephalosporinase beyond wild-type levels. A series of chromosomal mutations can give rise to strains that can constitutively produce β-lactamase (8). Increased and constitutive β-lactamase expression occurring from mutations in the genome often arises from excessive antibiotic usage, leading to the proliferation of antibiotic resistant strains (16, 40, 68). These mutants pose a great challenge to antimicrobial chemotherapy and high rates of resistance in *P. aeruginosa* is found amidst cystic fibrosis patients, where antibiotics are used repeatedly throughout the chronic illness in attempts to eliminate infections (9, 40).

1.10 NagZ: *N*-acetyl-β-glucosaminidase

1.10.1 Deletion Mutants in a Model *E. coli* System

The β-*N*-acetylglucosaminidase NagZ cleaves GlcNAc from the incoming disaccharide peptides, producing the inducer molecules 1,6-anhydroMurNAc tri-(or penta-) peptides that binds AmpR and promotes the transcription of AmpC β-lactamase (26, 69).

Previous studies of *nagZ* deletion mutants in *E. coli* showed large quantities of GlcNAc-anhydroMurNAc collected in the cytoplasm, and mutants showed no NagZ β-*N*-acetylglucosaminidase activity whatsoever (69). These deletion mutants were also incapable of establishing AmpC-mediated antibiotic resistance to β-lactams; they displayed increased susceptibility to cefoxitin relative to wild-type *E. coli* harboring an artificially introduced *ampC-ampR* operon cloned from *Enterobacter cloacae*, carried on a plasmid (26). The NagZ-deficient mutants exhibited sensitivity comparable to bacteria lacking *ampC* β-lactamase genes (26).

This study confirmed NagZ is needed to establish β-lactam resistance, and suggested that inhibiting NagZ activity could be a possible method for ceasing expression of inducible β-lactamases, such as the AmpR-regulated (inducible) *ampC-ampR* operon within *P. aeruginosa*. These strains should then be locked in a sensitive state, incapable of establishing resistance.

1.10.2 Inactivation of NagZ in a Model *E. coli* System

Previous studies have shown the use of a PUGNAc-derived β-lactamase inhibitor selective for NagZ in an *E. coli* system harboring the *ampC-ampR* operon from *E. cloacae* capable of mitigating resistance to several clinically applicable β-lactams (34). The inhibitor binds the active site of NagZ, essentially immobilizing the enzymatic activity of the *nagZ* gene such that the inducer molecule 1,6-anhydroMurNAc-tripeptides, intermediates of the cell wall recycling pathway, are no longer generated at the same levels as in the uninhibited state. By attenuating formation of the AmpR inducer molecule, the *ampC* β-lactamase gene is no longer induced (34).

1.11 Genetic Inactivation in *Pseudomonas aeruginosa*

Schweizer et al. have carried out research in knocking out genes in *P. aeruginosa* using a positive selection gene replacement method, employing a suicide vector delivery system. These vectors, such as the pEX18Tc employed in this thesis, contain a greater variety of antibiotic resistance markers compatible with *P. aeruginosa*, in addition to the *sacB* gene which encodes a levan sucrase cloned from *Bacillus subtilis* for counter selection (70). Levan sucrase is toxic to *P. aeruginosa* and other Gram-negative bacteria in the presence of sucrose. The addition of sucrose to growth media allows for positive selection for those recombinants that have undergone a second crossover event to eliminate the plasmid from their genome (70).

Studies of inactivation of *ampD* through homologous recombination yielded *P. aeruginosa* with a derepressed AmpC phenotype (71). As there are multiple *ampD* genes, the stepwise inactivation of these genes results in, in addition to the low-level and inducible state of the wild-type *P. aeruginosa* PAO1, three hyper inducible states, with the triple AmpD null mutant strain exhibiting the highest level of hyper induced expression of β-lactamase (36). In a clinical setting, these AmpD null mutants are frequently selected for in CF patients, often leading to failure of antibiotic therapy, and leaving patients even more vulnerable to secondary infection (36, 72, 73).

1.12 Thesis Objectives

It is apparent that current therapeutics available for *P. aeruginosa* infections is limited and inevitably has influenced the emergence of multi-drug resistance strains of *P. aeruginosa*. The focus of this thesis was to investigate the extent of antibiotic susceptibility in *nagZ* deficient mutants of *P. aeruginosa* PAO1 and a triple null mutant of AmpD by way of the inducible AmpC-mediated β-lactamase pathway, and to study the role of *nagZ* in intrinsic and acquired resistance to antipseudomonal β-lactams. Recall that NagZ activity results in formation of 1,6-anhydroMurNAc-tripeptides by hydrolyzing the β-(1-4) glycosidic bond between *N*-acetyl-glucosamine and anhydro-*N*-acetyl-muramic acid cell wall degradation products. The molecule produced by NagZ is believed to convert AmpR into an activator of *ampC* gene expression. AmpD is considered to be the negative regulator of *ampC*, by preventing accumulation of 1,6-anhydroMurNAc-tripeptide, the inducer molecule of AmpC β-lactamase. The specific objectives were to (i) to genetically inactivate the *nagZ* gene in *P. aeruginosa* PAO1 and in an isogenic triple AmpD null mutant, and (ii) to characterize these *nagZ* null mutants with respect to their antibiotic susceptibility and NagZ and AmpC enzymatic activities; thereby determining if blocking NagZ activity in AmpD null mutants can counteract the hyperproduction of AmpC β-lactamase.

Chapter 2 – Materials and Methods

2.1 Bacterial Strains, Plasmids, Media, Growth Conditions and Reagents

Pseudomonas aeruginosa PAO1 (1) was used as the wild-type strain for this work. *P. aeruginosa* strain PAΔ*DDh2Dh3* (kindly provided by Dr. Antonio Oliver; Servicio de Microbiología, Hospital Son Dureta, Palma de Mallorca, Spain) was also employed. All mutant derivatives of PAO1 (PAΔ*nagZ,* PAΔ*DDh2Dh3nagZ*), plasmid constructs and *E. coli* strains are described in Table 1. Growth media used for culturing and plating were from Becton Dickinson Canada (Oakville, ON) as follows, Luria-Bertani (LB) broth, LB agar, Pseudomonas isolation agar (PIA), cation-adjusted Mueller-Hinton (MH) agar and cation-adjusted Mueller-Hinton broth. MH agar and MH broth were supplemented with 25 mg/L of Ca^{++} and 12.5 mg/L of Mg^{++} to contain the correct concentrations of the divalent cations, according to Clinical and Laboratory Standards Institute's (CLSI) accepted method for minimum inhibitory concentration assays (MICs)(74). Recipes for Merodiploid-resolving agar supplemented with 5% sucrose (MRM + 5% sucrose), Super Optimal broth (SOB) and Super Optimal broth with catabolite repression (SOC) are found in Appendix 1.

Bovine gamma globulin (BGG) was acquired from Sigma-Aldrich, Oakville, ON. Bovine-serum albumin (BSA), calf intestinal phosphatase (CIP) and all restriction endonucleases and enzyme buffers were acquired from New England Biolabs

Table 2.1. Plasmids and bacterial strains

Strain or plasmid	Genotype or description	Source
Plasmids		
pEX18Tc	Tc^R; *ori*T^+ *sacB*$^+$, gene replacement vector with MCS from pUC18	(70)
pUCP27	Tc^R pUC-19-derived broad-host range vector	(75)
pUCP*NagZ*	Tc^R; pUCP27 containing wild-type PAO1 *nagZ* gene (PA3005)	This work
pUCGM	Ap^R Gm^R; source of Gm^R cassette (*aacC1* gene)	(70)
pEXΔ*nagZ*	pEX18Tc containing 5' and 3' flanking sequence of Δ*nagz*	This work
pEXΔ*nagZGm*	pEX18Tc containing 5' and 3' flanking sequence of Δ*nagz::*Gm	This work
E. coli		
S17-1	RecA *pro* (RP4-2Tet::Mu Kan::Tn*7*)	(76)
P. aeruginosa		
PAO1	Reference strain completely sequenced	(1)
PAΔ*DDh2Dh3*	PAO1 Δ*ampD*::*lox* Δ*ampDh2*::*lox* Δ*ampDh3*::*lox*	(36)
PAΔ*nagZ*	PAO1 Δ*nagZ::Gm*	This work
PAΔ*DDh2Dh3nagZ*	PAO1 Δ*ampD*::*lox* Δ*ampDh2*::*lox* Δ*ampDh3*::*lox* Δ*nagZ::Gm*	This work

(Ipswich, Massachusetts). Gibco ligase buffer (5X) was acquired from Invitrogen (Carlsbad, CA). Protein quantification was carried out using the BioRad Bradford protein assay according to the manufacturer. All additional chemicals were of laboratory reagent grade and obtained through Thermo Fischer Scientific (Ottawa, ON) or VWR Scientific (Westchester, PA).

2.2 Standard DNA Manipulations and Procedures

2.2.1 Plasmid and Genomic DNA Purification

Plasmid preparations and DNA purifications were performed with the QIAprep Spin Miniprep plasmid preparation kit (Qiagen Inc., Hilden, Germany). By modifying the QIAprep Spin Miniprep mini plasmid preparation kit, genomic preparations of *P. aeruginosa* PAO1, PAΔ*DDh2Dh3* and the *nagZ* deficient mutants PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ* were carried out as follows: bacterial DNA was sheared using a 22 gauge (G) needle after addition of lysis buffer (Qiagen buffer P2) and prior to addition of alkylating agent (Qiagen buffer N3). The remainder of the procedure was identical to the Qiagen protocol and yielded a preparation of genomic DNA suitable for Polymerase chain reaction (PCR) template. PCR and restriction endonuclease reactions were purified using the Qiagen PCR Purification Kit (Qiagen Inc.) to remove excess deoxynucleotides, primers, and non-specific PCR products; or excess enzymes, buffers, salts and fragmented DNA, respectively.

2.2.2 Agarose Gel Electrophoresis

Purified DNA fragments were mixed with an equal part of DNA Loading Buffer

(Appendix 1) and run with 1 kilobase (kb) DNA ladder (Invitrogen, Carlsbad, CA) in Tris-acetate buffer (77) on a 1 % agarose gel. DNA cut from agarose gels was purified using the QIAquick Gel Extraction Kit (Qiagen Inc.) according to the manufacturer.

2.2.3 Oligonucleotide Primer Preparation

Oligonucleotide primers (synthesized by AlphaDNA, Montreal, Quebec) were diluted to a stock concentration of 100 mM in 10 mM Tris pH 8.0. Working stocks of 5 µM were generated by appropriately diluting 100 mM stocks of primers.

2.2.4 Restriction Enzyme Digests

Single digests to linearize vectors were prepared using 50 µL circular plasmid, 7.2 µL 10X *Eco*RI NEB buffer, 3.6 µL 20X BSA, 3.6 µL of enzyme, and if digesting to linearize plasmid for ligation, plasmid was dephosphorylated with 0.5 µL CIP. Double digests were prepared as above however 3.6 µL of second enzyme was added to the mixture. Restriction digests of PCR product for ligation into plasmid were prepared as follows; 28 µL purified PCR product, 1.8 µL 20X BSA, 3.6 µL *Eco*RI NEB buffer and 1.75 µL of enzyme. Single digests containing *Pst*I used NEB buffer 3. Digests were incubated at 37°C for 4 hours. Digests were run on 1.0 % agarose gels for verification.

2.2.5 Standard Polymerase Chain Reaction

Standard PCR reactions were prepared to final volumes of 50 µL. For general template amplification with *Pfu* polymerase (Stratagene), reactions were prepared as follows, 1 µL of DNA template (25-65 ng), 27.5 µL ddH_2O, 5.1 µL10X *Pfu* buffer, 8.1 µL 2.5mM dNTPs, 2 µL $MgCl_2$, 1 µL *Pfu* enzyme as well as 2 µL of each forward and

reverse primer at working stocks of 5 µM. Cycling conditions were as follows: template was initially denatured at 97°C for 3 minutes, followed by 35 cycles of denaturation at 96°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 2 minutes. Final extension was prescribed at 72°C for 10 minutes prior to cooling at 3°C. According to the manufacturer (Stratagene), the extension time for *Pfu* polymerase is 2 minutes per kilobase thus extension time was adjusted according to the size of the amplicon to be generated.

For reactions with *Vent* polymerase (NEB), reactions were prepared as follows, 1 µL of DNA template (25-65 ng), 33.5 µL ddH_2O, 5 µL thermopol buffer, 6 µL 2.5mM dNTPs, 0.44 µL $MgSO_4$, 0.67 µL *Vent* enzyme as well as 2 µL of forward and reverse primer (5 µM). Cycling conditions were as follows: template was initially denatured at 97°C for 5 minutes, followed by 30 cycles of denaturation at 96°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 2 minutes 30 seconds. Final extension was prescribed at 72°C for 10 minutes prior to being held cooling at 3°C. Extension time for *Vent* polymerase is 1 minute per kilobase, thus extension time was adjusted according to the size of the amplicon to be generated.

PCR reactions were performed in a PTC-200 Peltier Thermal Cycler (MJ Research, South San Francisco, CA). For accuracy, large reactions were prepared in one master mix and appropriate volumes were diluted to individual tubes containing appropriate primers or template DNA. Any samples requiring addition of a volume of 1 µL or less was either diluted in ddH_2O and reaction volume was adjusted accordingly, or a master mix was made and then aliquoted.

Table 2.2. Oligonucleotide primers

Primer Name	Primer Sequences (5' – 3')[a]	Restriction Enzyme	Use/Target
NagZ-WTF	GATATACATATGCAAGGCTCTCTGATGCTC	*Nde*I	*NagZ* amplification
NagZ-WTR	GATATAGGATCCATCAATCAGTTGCGCAGC	*Bam*HI	
NagZ-FH1	CATATCAAGCTTCCAGTCGGAAACCGTCGAACGC	*Hin*dIII	*nagZ* inactivation
NagZ-RP1	GATATACTGCAGCGATGTCGAGCATCAGAGAGCC	*Pst*I	
NagZ-FP2	GATATACTGCAGGCCCATGTGGTCGGCGAC	*Pst*I	
NagZ-RE2	GATATAGAATTCTGGCCGCCTAGCCGGCCAGG	*Eco*RI	
sacB-F	TACACGCCATGATATGCTGC		*sacB* verification
sacB-R	AGCTGTCTGATGCTGATACG		
NagZ-FpUC	GATATACTGCAG*AAGAAGGAGATATACATATGCAAGGCTCTCTGATGCTC*	*Pst*I	*nagZ* complement PA3005
NagZ-RpUC	GATATAGGATCCTCA**GTGATGGTGATGGTGATG**ATCAATCAGTTGCGCAGC	*Bam*HI	
pEX-F	GGGATGTGCTGCAAGGCG		*aacC1* cassette (orientation)
pEX-R	TCCGGCTCGTATGTTGTG		
Gm-F	CATACTTGAGCCACCTAAC		
Gm-R	TGACGATCCCGCAGTGGC		
Genome-F	GCGTCACGATGAAGTTGGCC		Genome crossover verification
Genome-R	CGCACCTCCTGCGCAATAGC		
NagZ-XF	GCAGGTGATGCACCTGATGG		*nagZ* crossover verification
NagZ-XR	CGCTCAGCGTCAGCCCTTCC		
pUCP-F	CGATTAAGTTGGGTAACGCC		pUCP MCS
pUCP-R	AGTTAGCTCACTCATTAGGC		

[a] Primer sequences were obtained from the published PAO1 genome (1). Sites for restriction endonucleases are underlined. Ribosomal binding site derived from pET vector T7 shown italicized. The His_6 fusion tag added to the C-terminus of the *nagZ* open reading frame of the complementation plasmid pUCP*nagZ* is shown in bold.

For amplifications involving bacterial colonies or genomic DNA, reactions were held at 98°C for 8 minutes prior to cycling as an additional denaturation step to ensure cells were lysed and to completely melt genomic DNA to allow primers and polymerase full access to the template. This step was added to reactions in which primers sacB-F, sacB-R, Genome-X and Genome-F were used (Table 2.2). Annealing temperatures were increased to a maximum of 68°C to reduce the amount of non-specific PCR products generated. Samples were purified using QIAquick PCR Purification Kit (Qiagen Inc.). Reactions were routinely run on 1 % agarose gels for verification.

2.2.6 Ligation Reactions

Ligation reactions containing one insert were prepared using 3.8 µL (50 – 70 ng) gel extracted insert (or ddH_2O as negative control), 1.5 µL (30 – 50 ng) linearized and dephosphorylated vector plasmid, 2.0 µL 5X Gibco ligase buffer and 0.6 µL T4 DNA ligase. Reactions containing two inserts were prepared as prior however 3.8 µL of second insert was included to mixture. Due to low efficiency of ligation, reaction of *aacC1* and pEXΔ*nagZ* were set up as follows, 7.2 µL *aacC1* gel extracted insert, 1.5 µL linearized and dephosphorylated vector plasmid, 2 µL 5X Gibco ligase buffer and 0.6 µL T4 DNA ligase. Ligation reactions were incubated a 16°C for 16 hours. Chemically competent *E. coli* cells were then transformed with ligations (see section 2.3)

2.3 Preparation of Chemically Competent Cells and *E. coli* Transformations

E. coli NM522 chemically competent cells were prepared using the Z-Competent™ *E. coli* Transformation Kit & Buffer Set (Cedarlane Laboratories, Ltd.,

Burlington, ON) as per manufacturer's instructions. 65 µL of chilled competent cells were mixed gently with 3 µL of ligation reaction, or ddH_2O as a negative control, and incubated at 4°C for 20 minutes, then heat-shocked at 42°C for 45 seconds. The transformation was rescued in 1 mL pre-warmed SOC and incubated at 37°C for one hour with aeration. To isolate single transformants, 10, 50, 100 µL and remainder of rescues were plated onto pre-warmed LB agar plates, supplemented with appropriate antibiotic. Transformants were plated on gentamicin (Gm; 10 µg/mL) (Sigma-Aldrich, Oakville, ON) for maintenance and selection of suicide delivery vectors pEXΔ*nagZ* and pEXΔ*nagZGm*, or vector pUCGm (see section 2.5 for vector construction) (Table 2.1). Transformants of pEXΔ*nagZGm* were also plated on LB agar plates supplemented with tetracycline 100 µg/mL, the antibiotic selectable marker of the plasmid backbone. For maintenance and selection of constitutive expression vector pUCP*nagZ* and vector pEX18Tc (Table 2.1), LB agar plates were supplemented with tetracycline (Tc; 10 µg/mL) (Sigma-Aldrich, Oakville, ON). Individual colonies were allowed to grow up overnight at 34°C.

2.4 Sequence Analysis

DNA sequencing, using appropriate primers as indicated by Table 2.2, was used to verify all plasmid constructs and genome mutants. The Centre for Applied Genomics DNA Sequencing and Synthesis Facility (Toronto, ON) performed all sequencing. Sequences were analyzed using the program BioEdit (Ibis Biosciences, Carlsbad, CA, USA).

2.5 Inactivation of *Pseudomonas aeruginosa* PAO1 *nagZ*

Using purified *P. aeruginosa* PAO1 genomic DNA as template, a 950-bp region upstream of and including the first 31-bp of *nagZ* (PA3005) (Entrez GeneID: 880216) was PCR amplified using primers NagZFH1 and NagZRP1 (Table 2.2) and digested with *Hin*dIII and *Pst*I. A second 1015-bp region containing the last 246-bp of *nagZ* and adjacent downstream DNA was amplified using primers NagZFP2 and NagZRE2 (Table 2.2) and digested with *Pst*1 and *Eco*RI (Figure 2.1). The restricted amplicons were ligated together via a three-way reaction into pEX18Tc (70) that had been linearized with *Hin*dIII/*Eco*RI and dephosphorylated with CIP. The ligation reaction was used to transform chemically competent *E. coli* NM522 and transformants were selected on LB agar supplemented with 5 μg/mL tetracycline. Recombinant plasmid, pEXΔ*nagZ*, was isolated from a single transformant and verified by restriction analysis and DNA sequencing.

To generate the mobilizable suicide plasmid pEXΔ*nagZGm*, the gentamicin resistance cassette (*aacC1*) was excised from plasmid pUCGm (70) by *Pst*I restriction and ligated into the unique *Pst*I sites that had been introduced by PCR into the truncated *nagZ* gene of pEXΔ*nagZ* (Figure 2.2). Recombinant pEXΔ*nagZGm* was isolated from a single transformant of *E. coli* NM522 that had been selected on LB agar supplemented with 20 μg/mL gentamicin and was verified by restriction analysis. Due to the fact that there was one *Pst*I restriction site for the insertion of the *aacC1* cassette, the marker could

Figure 2.1. Schematic representation of the wild-type *nagZ* gene in the *P. aeruginosa* PAO1 genome. Boxed sequence (i) indicates 950-bp flanking sequence upstream of and including the first 31-bp of the *nagZ* gene. Boxed sequence (ii) indicates 1015-bp flanking sequence downstream of and including the last 246-bp of the *nagZ* gene.

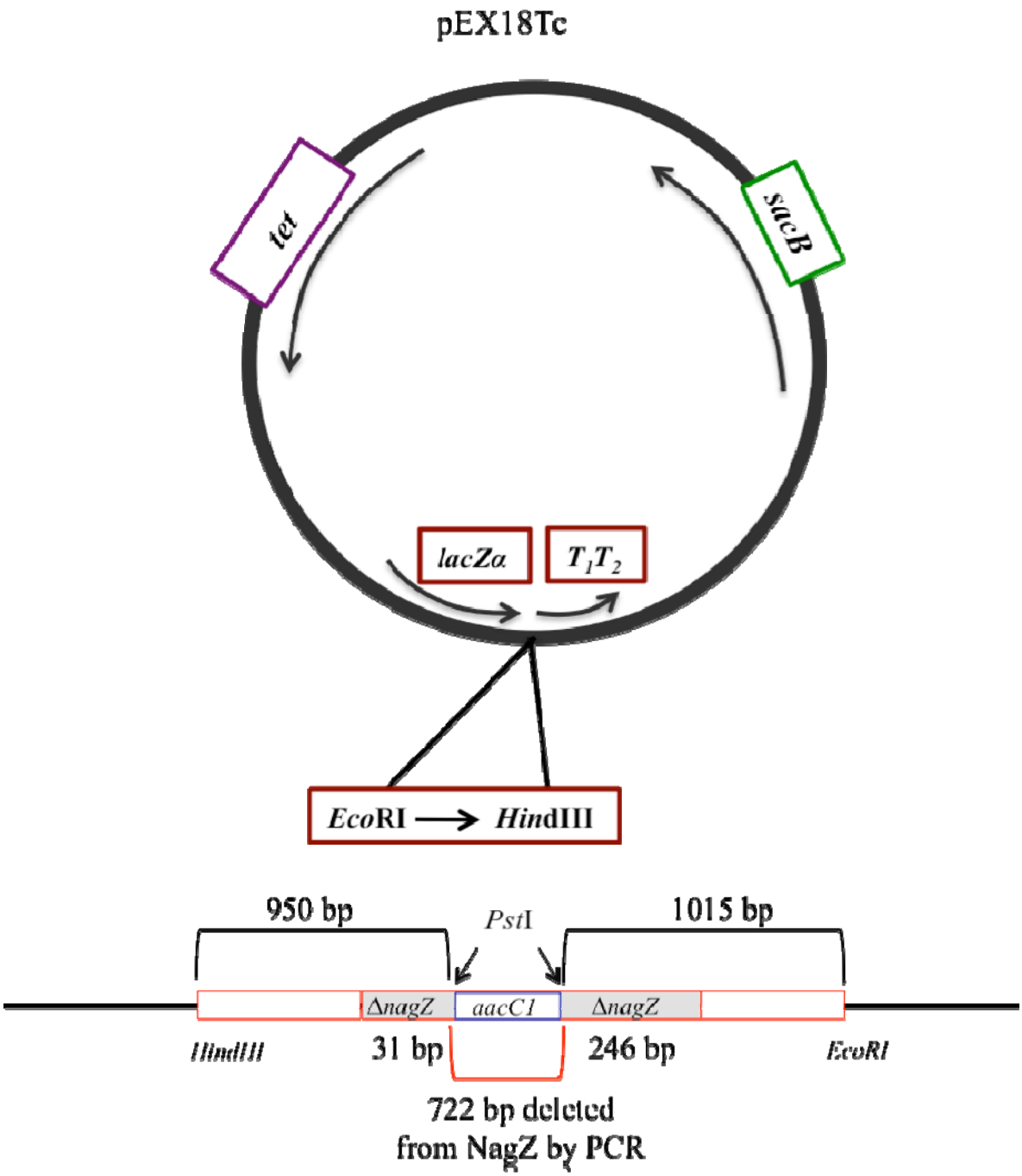


Figure 2.2. Schematic representation of the inactivated Δ*nagZ* construct. Gentamicin resistance cassette; *aacC1*. Restriction sites as indicated. Note: *Hin*dIII and *Eco*RI sites were added by PCR and are not present in the *P. aeruginosa* PAO1 *nagZ* at the indicated positions. *Pst*I sites were also added by PCR and are not present in the *P. aeruginosa* PAO1 *nagZ* gene. Plasmid pEXΔ*nagZGm* contains inactivated Δ*nagZ*.

be inserted into the center in either direction. The correct orientation of the *aacC1* cassette in selected transformant was ensured using primers pEX-F, pEX-R, Gm-F and Gm-R (Table 2.2). The selected transformant was also verified for tetracycline resistance (Tc; 5 μg/mL), the selectable marker found on the plasmid backbone.

2.6 Insertional Inactivation of *nagZ* by Biparental Mating

Cultures of *P. aeruginosa* PAO1 and the triple *ampD* null mutant PAΔ*DDh2Dh3* (Table 2.1) and donor *E. coli* S17-1 (76), previously transformed with pEXΔ*nagZGm*, was grown to an $OD_{600} \approx 0.5$. Cultures were pelleted (Eppendorf 5415D, rcf = 15,600 xg), washed with fresh LB and repelleted. Pellets were resuspended in 1.5 mL LB and donor to recipient cultures were mixed at a ratio of 2:1 to a total volume of 1.0 mL. Mixed culture was pelleted and gently resuspended in 50 μL LB. Entire mixture was spotted on pre-warmed LB agar plates and allowed to dry prior to incubation for 18 hours at 28°C. Cell spot was resuspended in 1 mL LB prior to centrifugation for 10 minutes. Supernatant was aspirated and the pellet was resuspended in 1.5 mL LB. To isolate single colonies, 10 μL aliquots of 1/10 and 1/100 dilutions were plated on PIA supplemented with 50 μg/ml gentamicin and incubated overnight for 18 hours at 28°C. Selection for gentamicin resistance gives rise to individual colonies that have integrated plasmid pEXΔ*nagZGm*, identified as merodiploids (78) and these colonies were patched to PIA supplemented with 50 μg/ml gentamicin. To promote homologous recombination and excision of plasmid, patching was followed by selection on MRM + 5% sucrose. Presence of the *Bacillus subtilis sacB* gene in the suicide delivery vectors like pEX18Tc

provides a counter selectable marker; all plasmid-integrated mutants will be sensitive to sucrose (78). Though growth is not exclusive to colonies that have lost the plasmid, differentiation is relatively simple based on the proficient growth of bacteria that have fully resolved the plasmid from their genome.

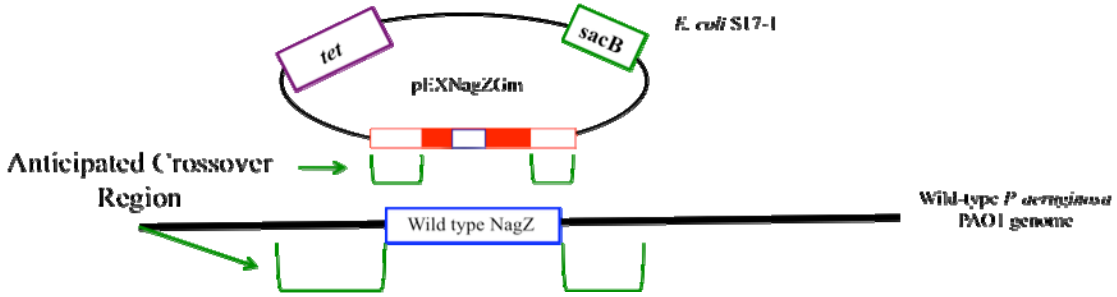
Putative PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ* mutants, were verified by assaying for resistance to gentamicin and sucrose, and susceptibility to tetracycline by replica plating. The presence of the Gm^R cassette was confirmed by restriction analysis and sequencing of genomic PCR products (Figure 2.3). These PCR products were generated using primers FwdFlank and RevFlank, which target sites on the *P. aeruginosa* genome, flanking the sequence that was used to construct pEXΔ*nagZGm*. The purpose of using primers designed to target regions beyond the area of anticipated homologous crossover was a precautionary measure to ensure amplicons generated from PCR were not priming to the plasmid, should it have still been present in the mutants.

2.7 Cloning of Wild-Type *nagZ* for Complementation Studies

Full-length *nagZ* (PA3005) was PCR amplified from purified *P. aeruginosa* PAO1 genomic DNA using *Pfu* polymerase and oligonucleotide primers NagZFpUC and NagZRpUC (Table 2.2). Primers were designed to fuse a His_6 tag to the C-terminus of NagZ in the event that NagZ activity was not restored with the inclusion of the complement plasmid and could be used to verify expression of the complement *nagZ* gene.

The PCR amplicon was restricted with *Pst*I and *Bam*HI, as these sites were

(i)



CCAGTCGGAAACCGTCGAACGCATTCTCGATGCGGCGGAACAGCTG
TTCGCGGAAAAAGGCTTCGCCGAAACCTCGTTGCGTCTGATCACCA
GCAAGGCCGGGGTCAACCTGGCGGCGGTGAATTATCACTTCGGTTC
GAAGAAGGCGCTGATCCAGGCGGTGTTCTCGCGCTTCCTCGGGCCA
TTCTGCGCCAGCCTGGAAAAGGAGCTGGATCGTCGCCAGGCCAAGC
CCGAGGCCCAGCACGCCACCCTGGAGGACCTCCTGCACCTGCTGGT
GTCCCAGGCGATGGCGGTGAAGCCGCGCAGCGGCAACGACCTGTCG
ATCTTCATGCGCTTGCTCGGCCTGGCCTTCAGCCAGAGCCAGGGGCA
CCTGCGCAAGTACCTGGAGGAGGTCTACGGCAAGGTCTTCCGGCGC
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AGCTGTTCTGGCGCGTGCACTTCATGCTCGGCGCGGCCGCCTTCAGC
ATGTCGGGGATCAAGGCCCTGCGGGCGATGGCGGAAACCGATTTCG
GCGTGAACACTTCCACCGAGCAGGTGATGCACCTGATGGTGCCGTT
CTTCGCTGCCGGCATGCGCGCCGAGAGCGGCATCGACGATCCGCTG
CTGGCCGGGGCGCAACTGCGCCCGCGGAACAAGACGCCCGCCAAG
GCCTGATCGCAGACGGCGCCCCAGGGCGCCGTTTTCCTTTCTCCTTT
CTCCTTTCTCCTTTCTCTGTGCTCCGGGCGGAACTCCATGCGCCGGG
CCGGCTCCATTGGCAGGCGCGCGGTACCGCCCCGGGACTTAAGTCT
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GGGAGCTGTCGATGAGTGTCTACGCGATTATCGGAGGAACCGGCCT
GACCCAGCTGGAAGGGCTGACGCTGAGCGAGTCCCTGCCGATCGAA
ACGCCGTATGGCGCGCCGTCCGCGCCGCTCCAGCGTGGCCGCTACG
CCGGGCGCGAGGTGCTGTTCCTGGCACGTCACGGTCATCCGCACCG
CTTCCCGCCGCACCAGGTGAACTACCGGGCCAATCTCTGGGCGCTG
AAGCAGGCGGGTGCCGAGGCGGTGATCGCGGTCAACGCGGTGGGT
GGCATCCATGCGGCGATGGGAACGGGGCACCTGTGCGTGCCGCACC
AGTTGATCGACTACACCTCGGGACGCGAGCACACCTATTTCGCCGG
CGATATCGAGCATGTCACCCATATCGACTTCAGCCACCCCTATGACG
AGCCGTTGCGCCAGCGCCTGATCGAGGCGTTGCGTGCGTTGGGGCT
GGCGCACAGCAGCCATGGGGTCTACGCCTGCACCCAAGGGCCGCGC
CTGGAAACGGTGGCGGAAATCGCCCGGTTGGAGCGGGACGGCAAC
GATATCGTCGGCATGACCGGCATGCCCGAGGCGGCACTGGCCCGCG
AGCTGGACCTGCCCTACGCCTGCCTGGCGCTGGTGGTCAATCCCGCG
GCGGGGAAGTCGGCGGGGATCATCACCATGGCCGAAATCGAACAG
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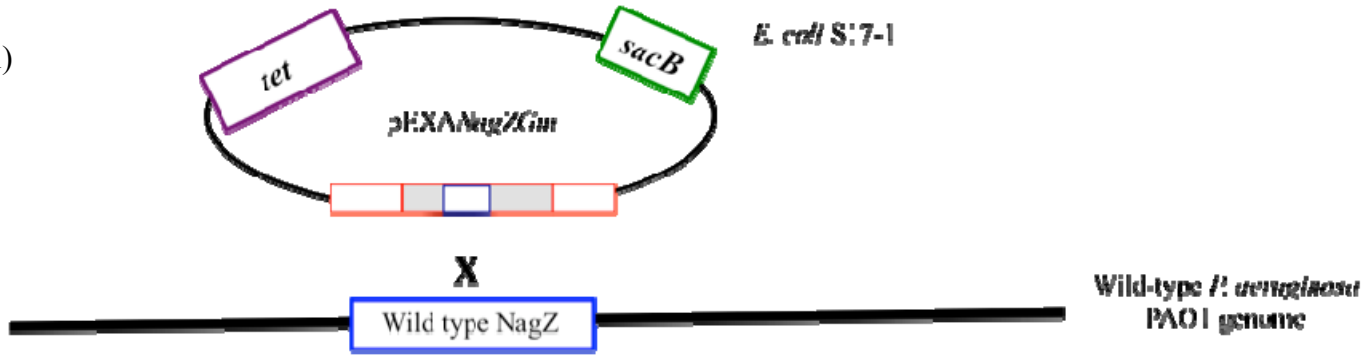
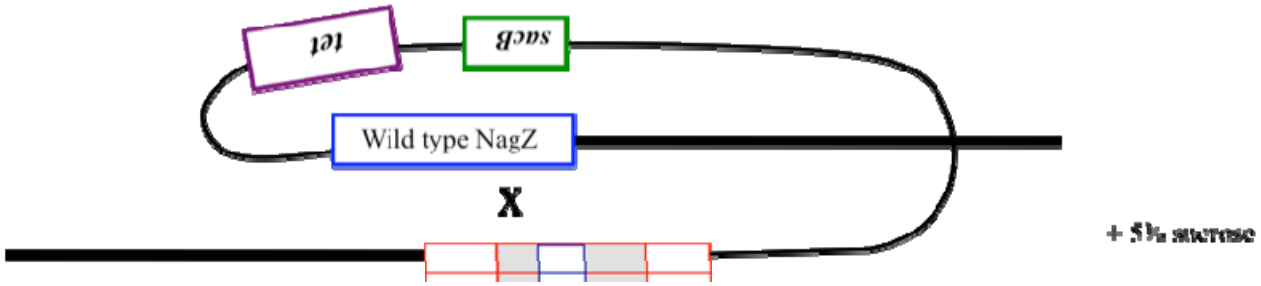
TCCTGGCCGGCTAGGCGGCCA

(ii)



(iii)



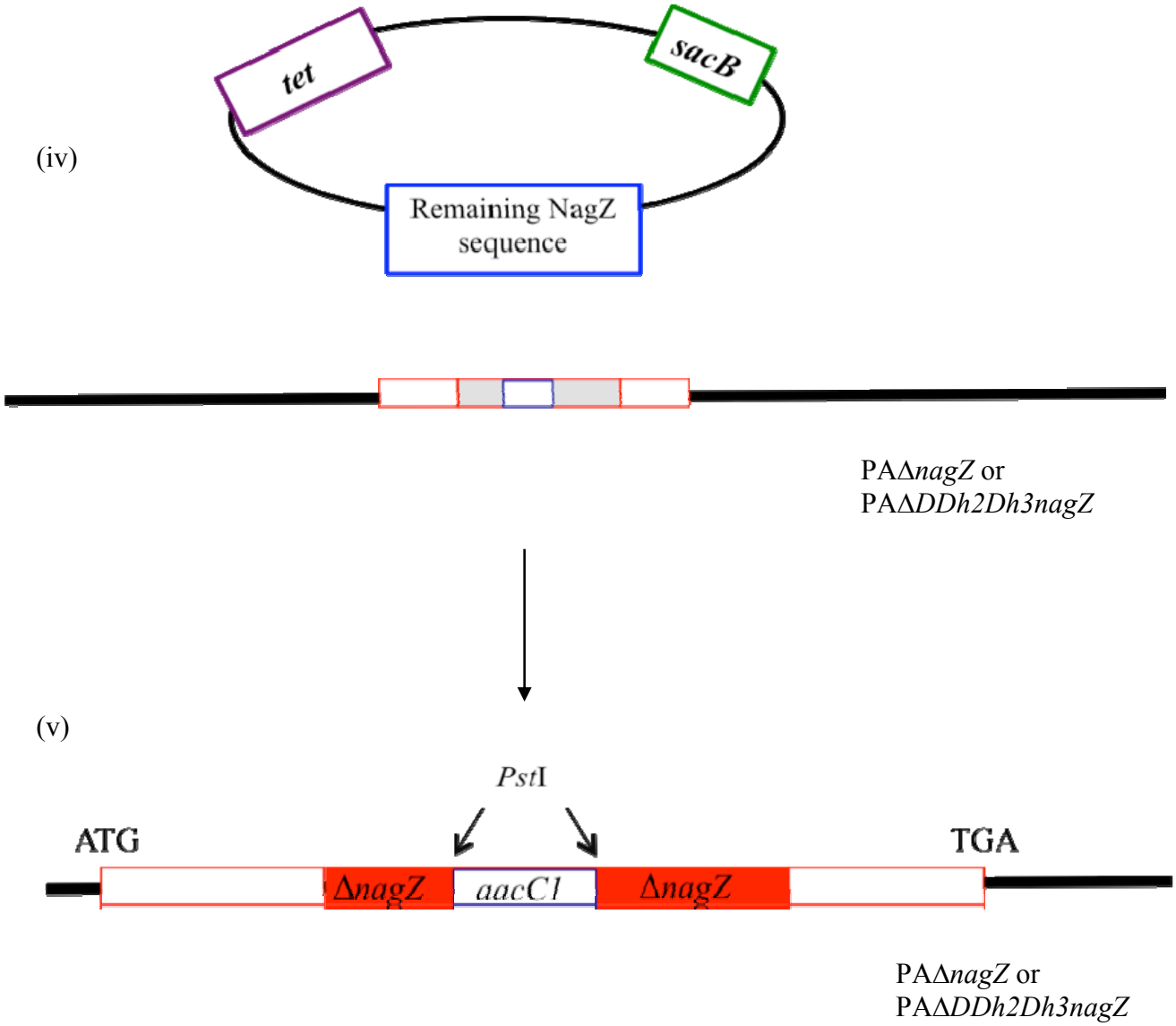


Figure 2.3. Schematic representation of the insertional inactivation of *nagZ* by biparental mating. (i) Schematic representation of *E. coli* S17-1 donor cell harboring pEXΔ*NagZGm*, and wild-type *P. aeruginosa* PAO1 genome, with flanking portions of DNA where expected region of homologous recombination will occur as indicated. (ii) Donor and recipient are incubated together to initiate the primary homologous crossover, initiated in the section flanking *nagZ*. (iii) To promote resolution of the plasmid through a second homologous recombination event, merodiploids are selected against with the addition of 5% sucrose. (iv) Excision of the plasmid removes the wild-type *nagZ* gene from the genome and leaves Δ*nagZ* integrated in the genome, giving rise to the *nagZ*-deficient mutant strains of PAO1 and PAΔ*DDh2Dh3nagZ*. (v) In detail, Δ*nagZ* in the genome.

engineered into the PCR primers and are not restriction sites present in the wild-type PAO1 *nagZ* gene, and ligated into *Pst*I/*Bam*HI-linearized pUCP27 (75). Vector pUCP27 is pUCP19-derived, an *E. coli* shuttle vector that can replicate in *P. aeruginosa* strains using a pMB1 origin of replication, (*ori*), and $ori_{1600,}$ the broad-host range *ori* from strain pRO1600 (75, 79).

The ligation reaction was used to transform chemically competent *E. coli* NM522 and transformants were selected on LB agar supplemented with 5 μg/ml tetracycline. Recombinant plasmid was isolated from a single transformant and verified by restriction analysis and DNA sequencing. The resulting *nagZ* expression plasmid pUCP*NagZ* (Table 1) was electroporated into the *nagZ* deficient *P. aeruginosa* PAO1 mutants PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ* (Table 2.1). Transformants were selected on LB agar supplemented with 100 μg/mL tetracycline to generate strains PAΔ*nagZ* (pUCP27), PAΔ*nagZ* (pUCP*NagZ*), PAΔ*DDh2Dh3nagZ* (pUCP27) and PAΔ*DDh2Dh3nagZ* (pUCP*NagZ*), respectively (Table 2.1).

2.8 Electroporation of *Pseudomonas aeruginosa* Cells

Preparation of electrocompetent *P. aeruginosa* cells were prepared by slightly modifying the protocol of Smith and Iglewski (80). Overnight cultures were sub-cultured into 30 mL fresh LB and grown to approximately $OD_{600} \approx 0.5$. Cells were harvested by centrifugation (Sorvall Legend RT, rcf = 1300 xg) at 4°C for 15 minutes. Pellets were washed in 6 mL 0.3M glucose twice and were then resuspended in the same volume 0.3M glucose. Resuspensions were transferred to new tubes and repelleted to remove all of the supernatant. To each pellet, 112.5 μL of chilled 0.3M glucose and 30 μL chilled

nuclease-free DNA water was added. Fifty microlitres was used for each electroporation harboring either 3 μL of sample plasmid (55 ng) or elution buffer as a control. Samples were electroporated at 2.5 kV and rescued in SOC for 1 hour with aeration at 37°C. Dilutions of 10-, 100- and 1,000-fold were plated as well as the remainder of the cells on LB agar plates supplemented with 100 μg/mL tetracycline. Plates were incubated overnight at 37°C to obtain individual colonies.

2.9 Minimum Inhibitory Concentration Assays (MICs)

MIC assays were performed by creating a suspension of the appropriate culture to an OD_{525} of 1.0 in a 0.85% NaCl solution. A lawn of bacteria was swabbed onto MH agar plates. For complemented strains, agar plates were supplemented with 75 μg/mL tetracycline. An Etest strip (ABiodisk, Sweden) was then applied to the surface. Plates were incubated for 18 hours at 34°C. MICs were read to be the lowest value at which bacterial growth was completely inhibited, including individual colonies, as described by the manufacturer. Etest strips are a CLSI approved method of determining MIC breakpoint values for *P. aeruginosa* (81). Etest strips contain a predefined gradient of antibiotic that diffuses into agar upon application. The concentration of the given antibiotic produces an MIC value that inhibits growth of strains under these particular experimental conditions. All determinations were performed in triplicate.

Broth microdilution MIC assays were performed testing antibiotic susceptibility where MIC values exceeded the range of Etest strips, or when the resolution of the Etest strips was insufficient to reveal differences in MIC values. The assays were performed in

200 µL per well cation-adjusted MH broth as recommended by the CLSI (81), supplemented with 50 µg/mL for strains complemented with pUCP27 and pUC*PNagZ* inoculated with 10^6 cells per ml. Antibiotic sensitivity for wild type *P. aeruginosa* PAO1 and PAΔ*nagZ* to ceftazidime and aztreonam were tested at the following concentrations: 32, 16, 18, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.0625 µg/mL. Antibiotic sensitivity of cefoxitin was tested at the following concentrations: (i) for wild type *P. aeruginosa* PAO1 and PAΔ*nagZ*; 3200, 2400, 1600, 1200, 800, 600, 400, 300, 200 and 150 µg/mL; and (ii) PAΔ*nagZ* (pUCP27/pUCP*nagZ*); 2400, 1600, 1200, 800, 600, 400, 300, 200, 150 and 100 µg/mL; and (iii) PAΔ*DDh2Dh3*; 9600, 6400, 4800, 3200, 2400, 1600, 1200, 800, 600 and 400 µg/mL; and (iv) PAΔ*DDh2Dh3nagZ*; 4800, 3200, 2400, 1600, 1200, 800, 600, 400, 300 and 200 µg/mL. Antibiotic sensitivity of ciprofloxacin was tested at the following concentrations for *P. aeruginosa* PAO1, PAΔ*nagZ*, PAΔ*DDh2Dh3* and PAΔ*DDh2Dh3nagZ*; 0.8, 0.3, 0.4, 0.15, 0.2, 0.075, 0.1, 0.0375, 0.05 and 0.01875 µg/mL. MIC was determined to be the lowest concentration of antibiotic in which no growth was exhibited after a 20-hour incubation period at 35°C, as recommended by the CLSI (81). All determinations were performed in triplicate.

2.10 Agar Diffusion Tests

The appropriate bacterial culture was prepared by inoculating 3 mL of MH broth with the appropriate glycerol stock and the culture was allowed to grow at 37°C until the OD_{600} reached ≈ 0.5. The cells were harvested by centrifugation then resuspended in 2 mL of MH broth and a lawn swabbed onto MH agar plates. Antibiotic discs (6 mm

diameter) previously loaded with antibiotic (Becton Dickinson Canada, Oakville, ON) were placed on the agar plates. After incubation for 18 hours at 35°C, the diameter of the inhibition zone was measured. All determinations were performed in triplicate.

2.11 Residual *N*-acetyl-β-glucosaminidase Activity Assay

Cell lysates of *P. aeruginosa* null mutants PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ*, PAΔ*DDh2Dh3* and wild type *P. aeruginosa* (PAO1) (Table 1) were assayed for *N*-acetyl-β-glucosaminidase activity using 4-methylumbelliferyl-β-D-*N*-acetylglucosamine (4-MUGlcNAc) as substrate (Sigma-Aldrich, Oakville, ON). For each strain, 3 mL of MH broth was inoculated with a few milligrams of glycerol stock and allowed to grow at 37°C to an OD_{600} of approximately 0.5, at which time each culture was diluted to an OD_{600} of 0.25 with fresh MH broth and allowed to grow for an additional 1.5 hours at 37°C then harvested by centrifugation. Pellets were washed by resuspending twice in phosphate-buffered saline (PBS) buffer (Appendix 1), followed by centrifugation. Supernatants were discarded and the washed pellets were stored at -80°C. Cell pellets were lysed by sonication in 200 µL chilled PBS buffer and 15 µg of protein from each lysate was assayed for *N*-acetyl-β-glucosaminidase activity at 37°C in a total volume of 100 µL PBS supplemented with 2 mM 4-MUGlcNAc. Reactions were allowed to proceed for 1 hour, 2 hours, 4 hours and 8 hours then quenched by addition of 0.9 mL of 0.1 M glycine/NaOH buffer (pH 10.7). Liberated 4-methylumbelliferone (4-MU) was detected by fluorescence measurement using an excitation wavelength of 360 nm and monitoring emission at 450 nm using a Synergy 2 Multi-Mode Plate Reader (Biotek). Assays were carried out in triplicate and controls included thermally denatured lysates (heated to

100°C for 20 minutes), native lysates in PBS lacking 4-MUGlcNAc, 2 mM 4-MUGlcNAc alone in PBS and blanks containing PBS only.

2.12 Quantification of β-Lactamase Specific Activity Assay

For each strain; *P. aeruginosa* null mutants PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ*, PAΔ*DDh2Dh3* and wild type *P. aeruginosa* (PAO1) (Table 2.1), 3 mL of MH broth was inoculated with a few milligrams of glycerol stock and the resultant culture allowed to grow at 37°C overnight. A 300 µL aliquot of overnight cells were used to inoculate 20 mL of MH broth, which were then grown at 37°C to an $OD_{600} \approx 0.5$. To determine β-lactamase specific activity (nanomoles of nitrocefin hydrolyzed per minute per milligram of protein), strains were induced to express *ampC* by supplementing cultures with 50 µg/mL cefoxitin for 3 hours and compared to strains cultured under the same conditions without cefoxitin. Cefoxitin is a β-lactam antibiotic known to be a strong inducer of *ampC* induction (82). Cells were harvested by centrifugation. Using a Branson Sonifer 450 and 102 Converter (Branson Ultrasonics Corporation), cells were lysed in 200 µL chilled PBS buffer using 6 bursts at 50% duty cycle and pelleting between each sonication, 3 times. Cell debris was removed by centrifugation (rcf = 15,600 xg) for 60 minutes at 4°C. Reactions of 500 µL were carried out by adding 5 µL of diluted supernatant (PAO1 and PAΔ*nagZ*; 1/5, PAΔ*DDh2Dh3*; 1/500, PAΔ*DDh2Dh3nagZ*; 1/50) and monitored every minute, over a 5 minute continuous assay procedure, which followed the linear rate of liberation of 2,4 dinitrophenolate from an initial concentration of 100 µM of nitrocefin (Calbiochem, LaJolla, CA) as substrate, as determined at 486 nm using a Spectronic 3000 Array (Milton Roy, Ivyland, PA). Mutant PAΔ*DDh2Dh3* hyperexpresses

ampC β-lactamase, resulting in quantities of enzyme so high that the nitrocefin concentration would limit the production of 2,4-dinitrophenolate unless cell extract was adequately diluted. Dilutions varied between strains in order to generate a sufficient trace where a linear measurement could be obtained for at least 3 minutes. Protein concentrations of supernatants were quantified by the BioRad Bradford assay using BGG as standard, as per manufacturer's instructions. All nitrocefin assays were carried out in triplicate at 37°C.

Nanomoles of nitrocefin hydrolyzed per minute per milligram of protein. Calculated from the following formula (derived from Beer's Law):

$$c = A\,(\varepsilon\lambda)^{-1}$$

A = optical absorbance of sample at 486 nm; $\varepsilon = 20{,}500\ M^{-1}\ cm^{-1}$, the molar extinction coefficient of hydrolyzed Nitrocefin at 486 nm; λ = path length of cuvette as 1 cm. A graph of nitrocefin concentration (M) over time (t = 0 – 5 minutes) was used to calculate the rate of product formation. The slope of the linear portion of each time plot was multiplied by the reaction volume (liters) to calculate the nanomoles of nitrocefin hydrolyzed per minute for each sample.

The rate (nanomoles of nitrocefin hydrolyzed/minute) was divided by the total milligrams of protein added to the reaction. This gave rise to the final quantity of β-lactamase specific activity in nanomoles of nitrocefin hydrolyzed per minute per milligram of protein.

Chapter 3 – Results

3.1 Construction of Suicide Delivery Vector pEXΔ*nagZGm*

To investigate the role of a gene, the gene of interest must be mutated in the chromosome to get a measure of its purpose and to study the effects of the mutation on the microbe. In order to demonstrate the role of NagZ in antipseudomonal β-lactam resistance of *P. aeruginosa*, *nagZ* (PA3005) was inactivated by allelic exchange.

The inactivated *nagZ* gene, truncated in the centre by deletion of 722 base pairs and with a *PstI* restriction site engineered in the middle, was ligated into the pEX18Tc suicide delivery vector, to give pEXΔ*nagZ* (Table 2.1). A diagnostic restriction digest of pEXΔ*nagZ* to excise the insert verified the Δ*nagZ* gene was present in the multi cloning site of pEX18Tc (Figure 3.1). Vector pEXΔ*nagZ* was then linearized with *PstI* and the *aacC1* cassette was ligated into the centre of the Δ*nagZ* to give pEXΔ*nagZGm*. A diagnostic restriction digest of pEXΔ*nagZGm* with *PstI* excised the gentamicin resistance marker, resulting in a band of approximately 900 bp, as well as linearized vector pEXΔ*nagZ* (Figure 3.2).

The inactivated *nagZ* was introduced into *P. aeruginosa* reference strain PAO1 (1) and in the hyperinduced, highly antipseudomonal β-lactam resistant triple *ampD* null mutant PAΔ*DDh2Dh3* (36).

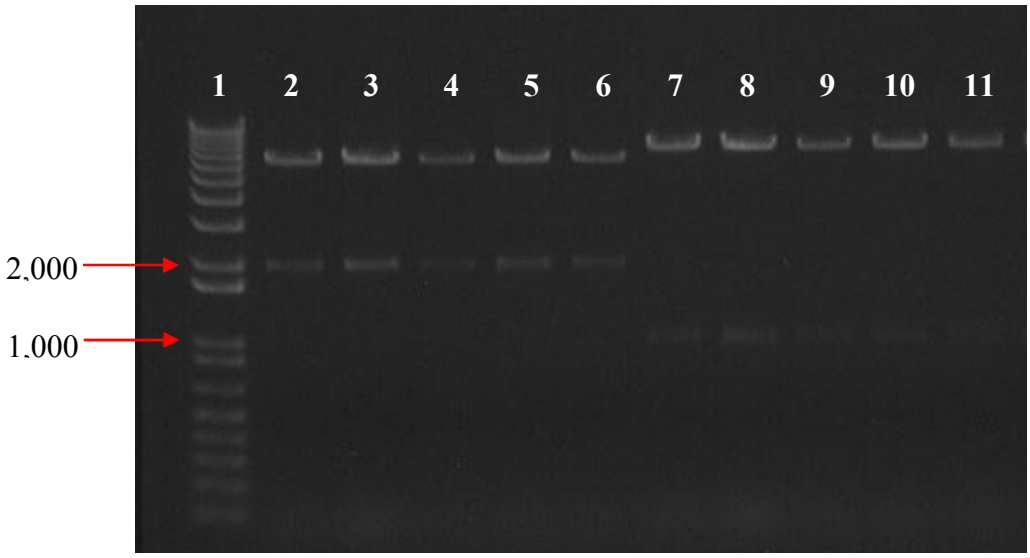


Figure 3.1. DNA agarose gel containing diagnostic restriction digests of pEXΔ*NagZ* clones. Lanes 2-6 contain plasmid DNA digested with *Eco*RI/*Hin*dIII which excises the inactivated NagZ construct (~270 bp) as well as flanking DNA (~2,000 bp). Lanes 7-11 contain plasmid DNA digested with *Eco*RI/*Pst*I which excises half the inactivated *nagZ* construct as the *Pst*I sites are located at the center of the gene (~1,000 bp).

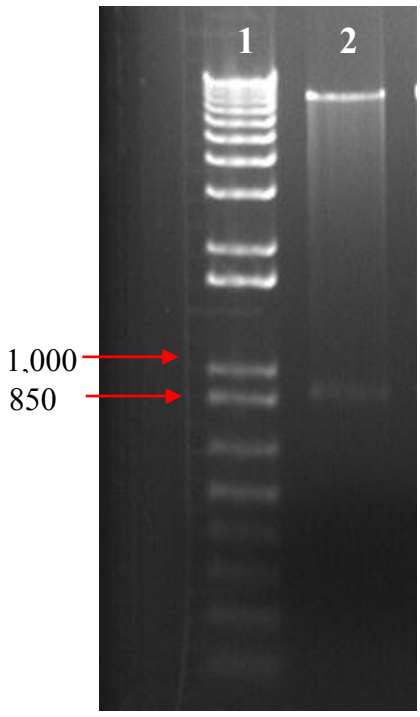


Figure 3.2. DNA agarose gel containing diagnostic digest with restriction endonuclease *Pst*I of pEXΔ*nagZGm*. The excised *aacC1* cassette runs at ~900 bp.

3.2 Insertional Inactivation of *nagZ* PA3005

3.2.1 Isolation and Phenotypic Characterization of *nagZ* Deficient Double Crossover Mutants PAΔ*nagZ and* PAΔ*DDh2Dh3nagZ* (PAΔ*nagZ::Gm and* PAΔ*ampD*::*lox* Δ*ampDh2*::*lox* Δ*ampDh3*::*lox* Δ*nagZ::Gm*)

To inactivate *nagZ* in *P. aeruginosa* strain PAO1 and PAΔ*DDh2Dh3*, the suicide delivery vector pEXΔ*nagZGm* (Tc^R, Gm^R, *sacB* sensitive) was first used to transform the helper strain *E. coli* S17-1 (Table 2.1). Plasmid pEXΔ*nagZGm* contained the truncated *nagZ* gene disrupted by the *aacC1* cassette flanked by sufficient genomic DNA (~1000 bp) on each end of the gene for homologous recombination to occur.

To perform a biparental mating, *E. coli* S17-1 (pEXΔ*nagZGm*) acted as the donor strain and the recipient strains were *P. aeruginosa* PAO1 and PAΔ*DDh2Dh3*. Cultures were prepared as in Section 2.6.

The first crossover event leads to integration of the plasmid into the genome and incorporates the vector with inactivated *nagZ,* resulting in the vector-borne *nagZ* sequence and genomic *nagZ* sequence aligned one after another. The resulting phenotype of these transconjugants was Tc^RGm^R and sucrose sensitive from the *sacB* counter selectable marker on the plasmid backbone. The transconjugants from the mating mixture were selected for gentamicin resistance located in the inactivated *nagZ* construct with 50 μg/mL gentamicin on Pseudomonas isolation agar. PIA contains Irgasan, a potent broad-spectrum antimicrobial that does not exhibit activity against *Pseudomonas* spp., thus eliminating the growth of any other bacteria, including the donor *E. coli*.

Transconjugants of interest were tested to verify resistance to tetracycline, attributed to the presence of plasmid in the genome.

The transconjugants that grew overnight on PIA supplemented with gentamicin were merodiploids; those isolates where single crossovers had occurred. Merodiploids were plated overnight on media lacking NaCl but containing 5% sucrose. In allelic exchange, the efficiency of plasmid excision tends to be quite low under normal conditions thus having a positive selection method for plasmid loss is beneficial to facilitate screening (83). The presence of sucrose selected for clones that had undergone subsequent recombination between the repeat sequences, losing the vector through a second crossover event. Double recombination events gave rise to *nagZ* gene exchange and growth of true recombinants. The phenotype exhibited by true recombinants was $Tc^{S}Gm^{R}$ and sucrose resistant. True recombinants were replica plated on 5% sucrose to ensure resistance, LB agar supplemented with gentamicin (50 µg/mL) to ensure resistance as well as LB agar supplemented with tetracycline (100 µg/mL) to ensure sensitivity, confirming excision of the plasmid (Table 3.1).

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Table 3.1. Phenotypic characterization of *P. aeruginosa* PAO1 and PAΔ*DDh2Dh3* and their Δ*nagZ* mutants PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ.*

Strains	*nagZ*	*Marker Phenotype*		
		Sucrose	Tetracycline	Gentamicin
P. aeruginosa PAO1	$nagZ^+$	R	S	S
PAΔ*nagZ*	$nagZ^-$	R	S	R
PAΔ*DDh2Dh3*	$nagZ^+$	R	S	S
PAΔ*DDh2Dh3nagZ*	$nagZ^-$	R	S	R

Excision of the plasmid would result in either exchange of Δ*nagZ*, replacing the wild-type gene with the inactivated version, or restoring the genome to wild type. A negative selection using the *sacB* gene was used to identify mutant strains (PAΔ*nagZ*) that had undergone complete double crossover events to resolve the *sacB* containing suicide plasmid pEXΔ*nagZGm*. When screening for the PAΔ*nagZ* strain, 100% of randomly selected clones (9/9) were found to be *sacB* negative (not sensitive to sucrose) and thus had resolved the suicide plasmid, whereas when screening for PAΔ*DDh2Dh3nagZ*, 90% of randomly selected clones (8/9) revealed double crossovers had taken place. The incidence of double crossovers in both cases was higher than the normally observed frequency of ~25%.

3.2.2 Growth and Morphology

The resulting mutants created by insertional inactivation of *nagZ*, PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ*, were monitored for negative effects in growth rate and morphology compared to their parent strains PAO1 and PAΔ*DDh2Dh3*. Because NagZ is involved in the cell-wall recycling pathway, there was a possibility that the mutation could adversely affect the ability of *P. aeruginosa* to grow normally.

Within the time frames of experimentation, up to 48 hours in broth cultures or on agar plates, there was no apparent effect on the growth rate or morphology of mutants PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ* compared to their parent strains *P. aeruginosa* PAO1 and PAΔ*DDh2Dh3*.

3.3. Genotypic Characterization of *nagZ* Deficient Double Crossover Mutants

Primary screening of PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ* true recombinants was through determination of their mutant phenotypes; $Tc^{S}Gm^{R}$ and sucrose resistant. To then verify that the recombination mutants contained the Δ*nagZ* construct integrated in their genome, polymerase chain reaction amplicons of the region were generated using genomic DNA of selected recombinants. For each potential clone, DNA was amplified using primers NagZ-WTF and NagZ-WTR, specific to the *nagZ* sequence. The amplicons were compared to a positive control of the parental *P. aeruginosa* PAO1 genomic DNA amplicon generated using the same primers. By comparing the generated amplicons by DNA gel electrophoresis, it was determined which clones contained the Δ*nagZGm* insertion in their genomes. The positive control amplicon was 1,000 base pairs, the size of the wild-type *nagZ* and the amplicons from clones were slightly larger at 1,200 base pairs. This shift in size indicated incorporation of the inactivated *nagZ* in the genome.

The *nagZ* mutant amplicons were purified and restricted with *PstI* to target and verify the engineered restriction site in the inactivated construct. The excised *aacC1* cassette, as when excised from the pEXΔ*nagZGm* vector (Figure 3.2) was approximately 900 bp. The remaining fragment from the digestion of Δ*nagZGm*, because of its small size of ~300 bp, was difficult to see.

The *P. aeruginosa* PAO1 wild-type *nagZ* gene does not contain a *PstI* restriction site in its sequence. To verify this and to demonstrate that the band excised from the mutant-generated amplicons after digestion with *PstI* was indeed the *aacC1* cassette, the

control amplified genomic DNA from *P. aeruginosa* PAO1 was also digested with *PstI*. Both undigested and digested control amplicons were verified by gel electrophoresis and were uncut bands at 1,000 base pairs, the size of the wild-type NagZ.

Final genotypic verification that double recombination events had taken place in the chosen recombination mutants and to rule out the presence of merodiploids, PCR amplification of the *sacB* marker was attempted using genomic DNA isolated from each recombination mutant selected using primers sacB-F and sacB-R (Table 2.2). Positive amplification from control pEX18Tc was compared to the resultant PCR of selected recombinants. The lack of amplification of the *sacB* target from the chosen recombination mutants verified that double crossover events and the suicide plasmid had been fully resolved from the genome, leaving behind the inactivated *nagZ* gene as shown in Figure 2.3.

3.3.1. Mutant PAΔ*nagZ* (PAΔ*nagZ::Gm*)

Nine PAΔ*nagZ* clones were randomly selected for screening from colonies growing on MRM supplemented with 5% sucrose. Amplification of *nagZ* from the genome showed that all the amplicons were ~1200 base pairs, the increased size of the gene attributed to allelic exchange from vector pEXΔ*nagZGm* (Figure 3.3). All of the selected clones were true recombinants harboring the inactivated *nagZ* gene. Subsequent digestion of purified amplicons with *PstI* resulted in excision of the *aacC1* cassette for all PAΔ*nagZ* mutants (Figure 3.4). Attempted amplification of *sacB* was negative for the nine clones selected, confirming loss of the plasmid (Figure 3.5).

3.3.2. Mutant PAΔ*DDh2Dh3nagZ* (PAΔ*ampD*::*lox* Δ*ampDh2*::*lox* Δ*ampDh3*::*lox* Δ*nagZ::Gm*)

Nine PAΔ*DDh2Dh3nagZ* clones were randomly selected for screening from colonies growing on MRM supplemented with 5% sucrose. Amplification of *nagZ* from the genome showed that all the amplicons were ~1200 base pairs, the increased size of the gene attributed to allelic exchange from vector pEXΔ*nagZGm* (Figure 3.6). Eight of the nine selected clones were true recombinants harboring the inactivated *nagZ* gene. Subsequent digestion of purified amplicons with *PstI* resulted in excision of the *aacC1* cassette for all PAΔ*DDh2Dh3nagZ* mutants (Figure 3.7). Attempted amplification of *sacB* was negative for the nine clones selected, confirming loss of the plasmid (Figure 3.8).

3.3.3. Crossover Sequence Verification of PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ*

Sequence verification of PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ* mutants ascertained there were no mutations in the allelic exchanges. Amplification using genomic DNA as templates from PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ* were performed with primers Genome-F and Genome-R. Primers were designed to target a 20-bp region flanking the external primers used in the PCR amplification to inactivate NagZ, thus external to the region in which crossovers will have occurred. These primers were also beyond the region where the plasmid pEXΔ*nagZGm* would have integrated into the parental genomes. The resulting amplicons were purified and then used as the template for sequencing using primers NagZ-XF and NagZ-XR. The amplicons generated using these primers and the template of the crossover region harboring the *nagZ* mutation gave the

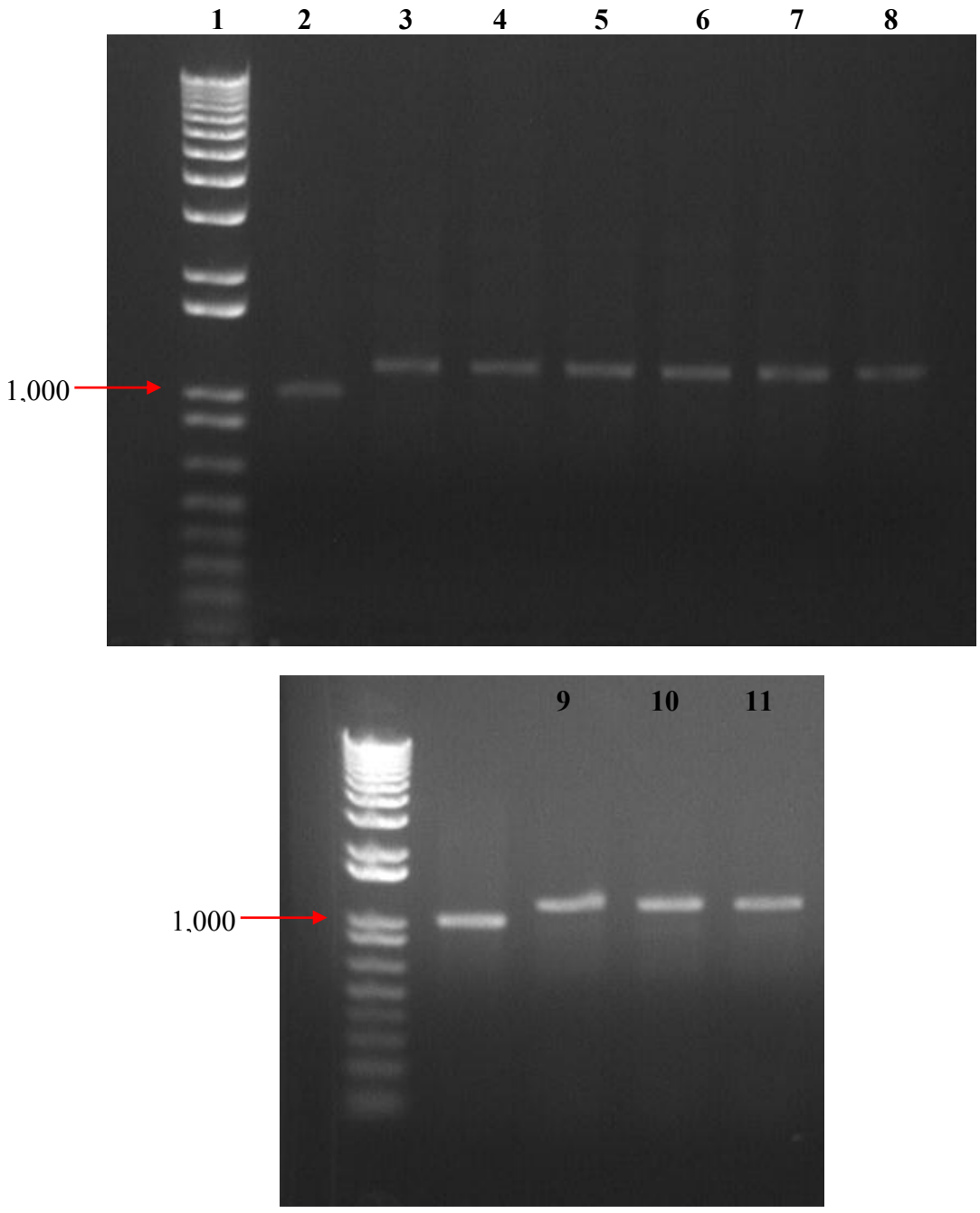


Figure 3.3. DNA agarose gel of PCR amplification of genomic DNA of nine randomly picked potential homologous crossover clones of PAΔ*nagZ*. Primers NagZ-WTF and NagZ-WTR were used for the reaction. PCR amplicons in lanes 2 is the wild-type *nagZ* and runs at ~1,000 bp. Lanes 3-11 of both gels are the potential clones which contain Δ*NagZGm* and are slightly larger than the wild-type *nagZ* with the amplicons running at ~1,200 bp.

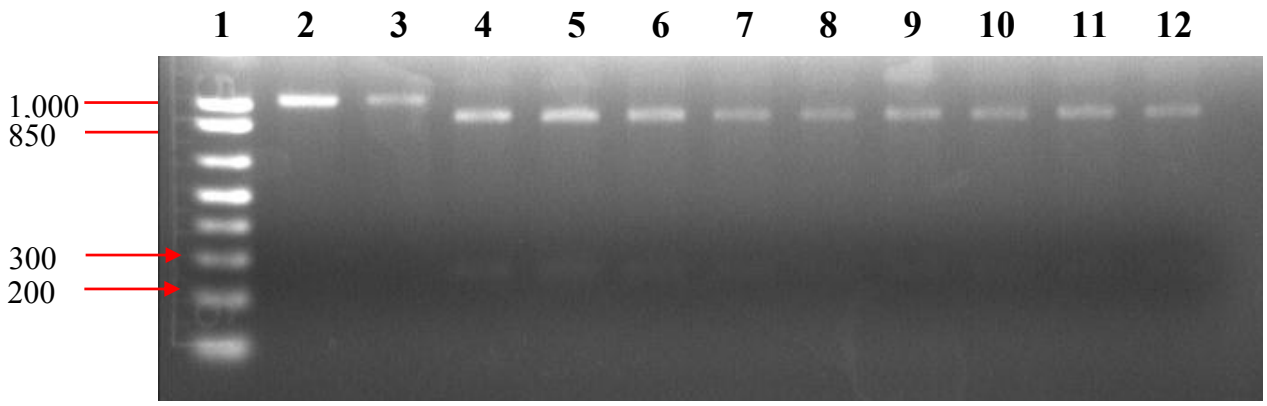


Figure 3.4. DNA agarose gel of *Pst*I digested PCR products from potential PAΔ*nagZ* clones. Lanes 4-12. *Pst*I double digest excises *aacC1* cassette which runs at ~ 900 bp. The second fragment of inactivated *nagZ* from clones runs at ~300 bp (lanes 4-12). The remaining fragment is too small to see on the gel. Lane 2 displays the PCR amplicon from primers NagZ-WTF and NagZ-WTR from genomic DNA of wild-type *P. aeruginosa* PAO1, and the amplicon digested with *Pst*I (lane 3). Both fragments run at ~1,000 bp, the size of the wild-type *nagZ*.

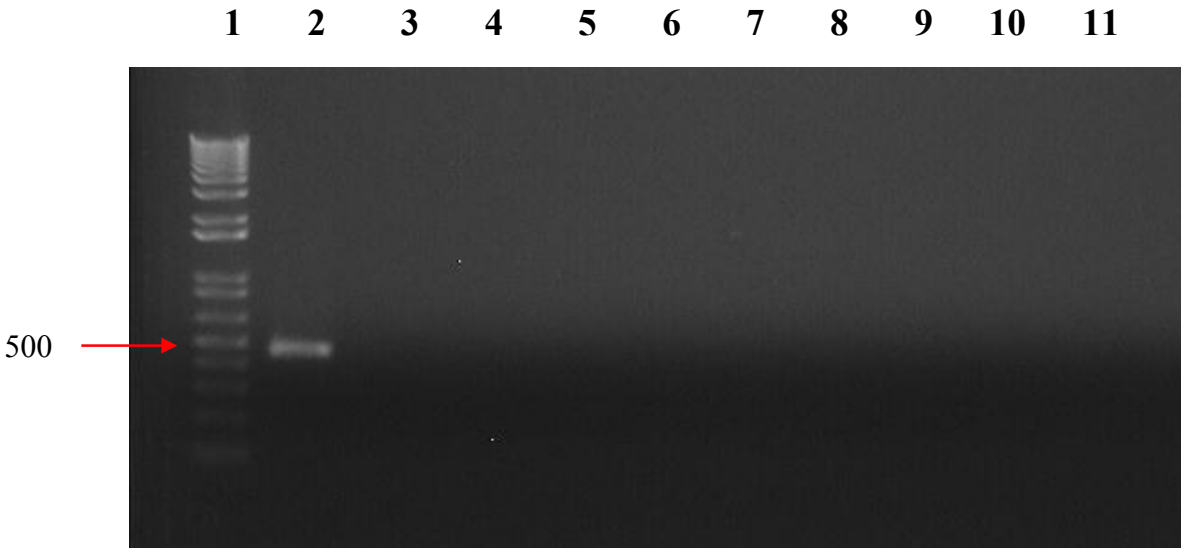


Figure 3.5. DNA agarose gel on attempt at amplifying *sacB* from genomic DNA of putative PAΔ*nagZ* double recombinants (lanes 3-11) with sacB-F and sacB-R primers which are designed to amplify approximately half of the *sacB* gene. Lane 2 is a positive control from *E. coli* harboring pEX18Tc.

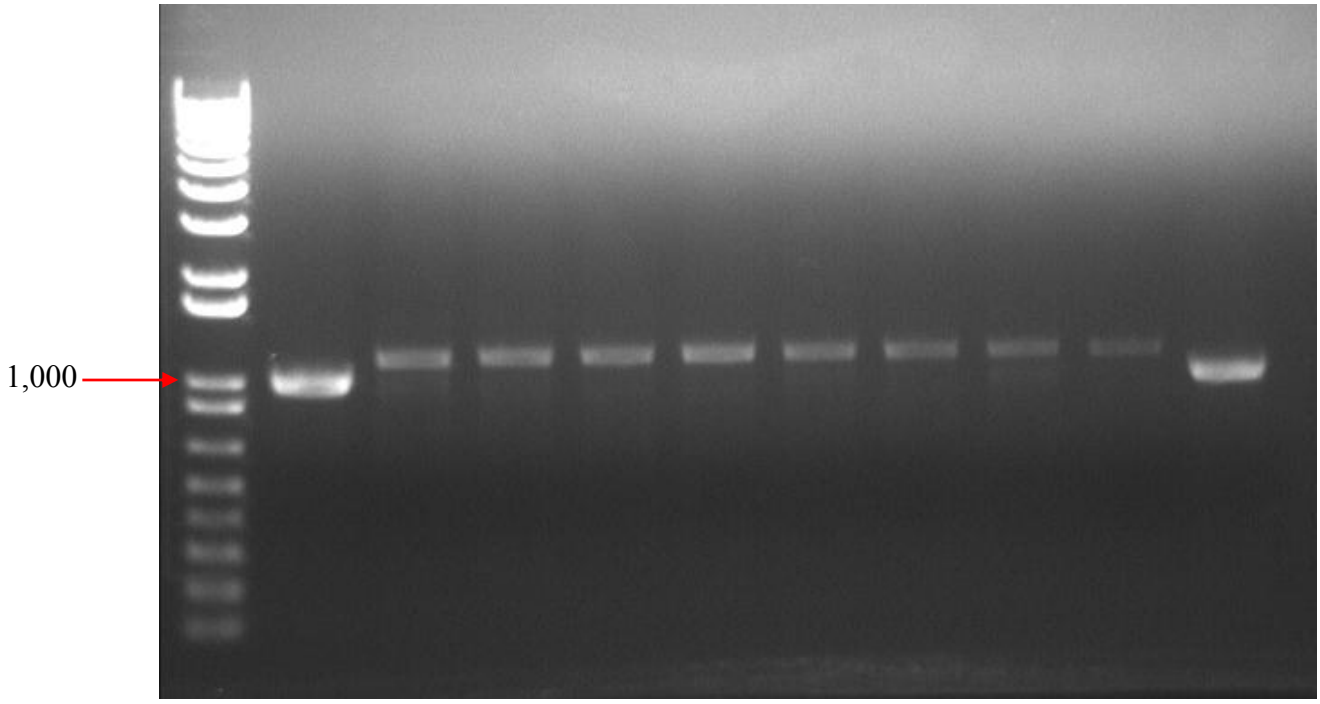


Figure 3.6. DNA agarose gel of PCR amplified genomic DNA of nine putative homologous crossover clones of PAΔ*DDh2Dh3nagZ, nagZ*-deficient triple *ampD* null mutants, using primers NagZ-WTF and NagZ-WTR. The PCR amplicon in lanes 2 is the wild-type *nagZ* and runs at ~1,000 bp. Lanes 3-10 are the potential clones which contain Δ*NagZGm* and are slightly larger than the wild-type *nagZ*, with the amplicons running at ~1,200 bp. Lane 11 contains a clone that passed phenotypic screening but does not appear to contain Δ*NagZGm*.

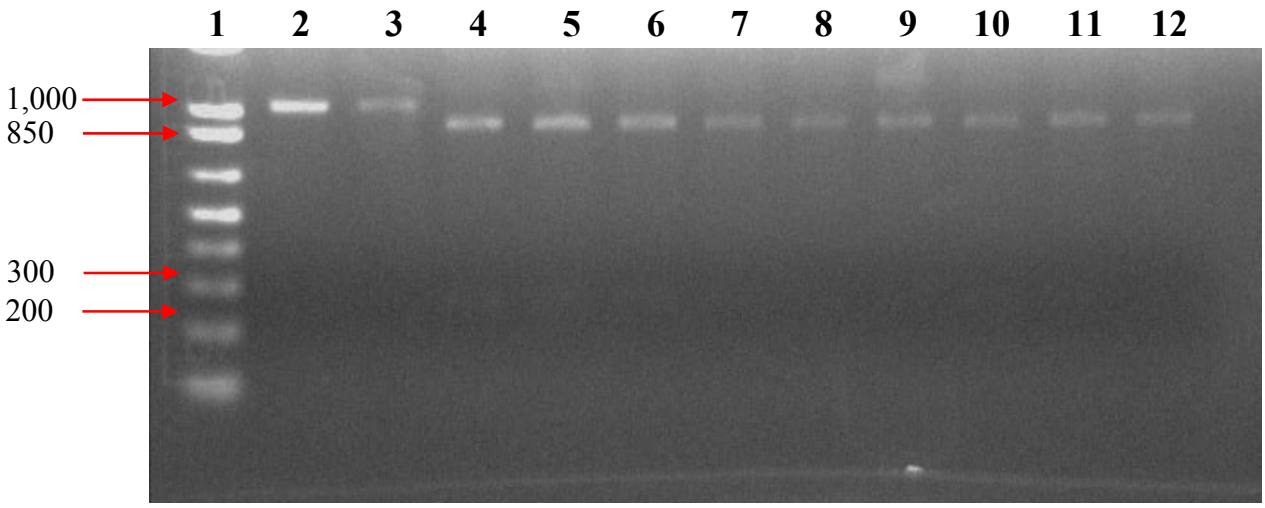


Figure 3.7. DNA agarose gel of *Pst*I digested potential PAΔ*DDh2Dh3nagZ* clones. *Pst*I double digest excises the *aacC1* cassette which runs at ~ 900 bp. The second fragment of inactivated *nagZ* from clones runs at ~300 bp (lanes 4-12). The remaining fragment is too small to see on gel. Lane 2 displays the PCR amplicon from primers NagZ-WTF and NagZ-WTR from genomic DNA of wild-type *P. aeruginosa* PAO1, then digested with PstI (lane 3). Both fragments run at ~1,000 bp, the size of the wild-type NagZ.

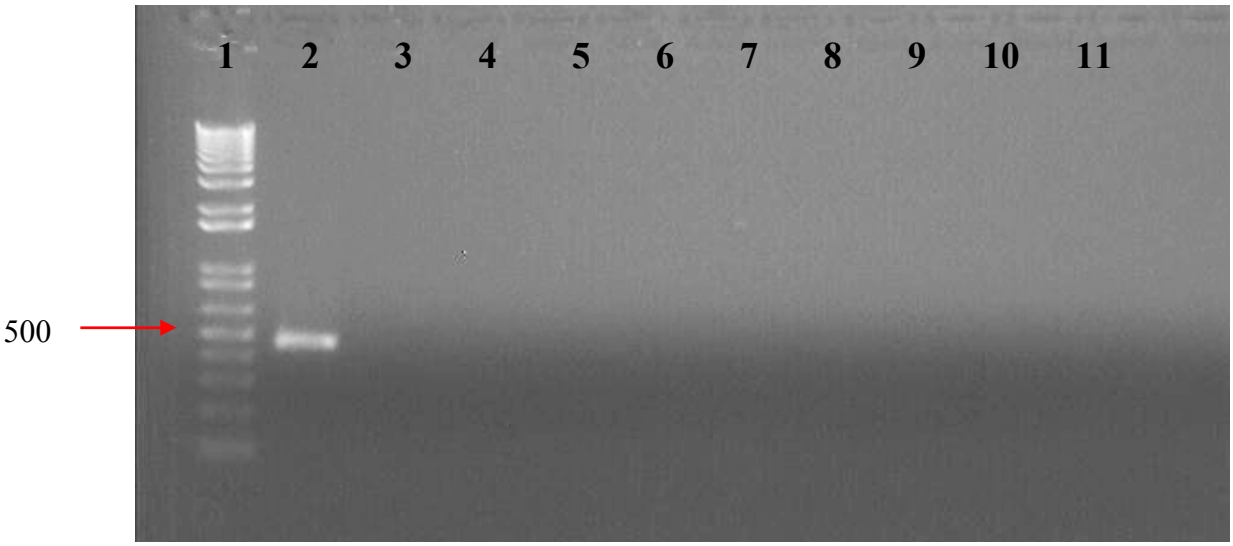


Figure 3.8. DNA agarose gel of an attempt at amplifying the *sacB* from genomic DNA of nine putative colonies of PAΔ*DDh2Dh3nagZ* double recombinants (lanes 3-11) with sacB-F and sacB-R primers, which are designed to amplify approximately half of the *sacB* gene. Lane 2 is a positive control from *E. coli* harboring pEX18Tc.

sequence of the entire mutation that was created, and sequence verification showed no homology with the plasmid pEXΔ*nagZGm*. Sequencing the crossover region of the *P. aeruginosa* PAO1 background as well as in the triple *ampD* null mutant showed there were no additional mutations acquired from genetic crossover. This also confirmed that PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ* had successfully excised the delivery vector.

3.4 Assaying Enzymatic Activities Involving *nagZ*

3.4.1 Residual *N*-acetyl-β-glucosaminidase Assay

In *P. aeruginosa*, there are three *ampD* homologues, *ampD*, *ampDh2* and *ampDh3* that encode for *N*-acetyl-anhydromuramyl-L-alanine amidases involved in the cell-wall recycling pathway and thus, indirectly control *ampC* β-lactamase expression. Knowledge of multiple *ampD* homologues working in concert to regulate *ampC* induction led to speculation that there may be *nagZ* homologues with functional activity in the cell-wall recycling pathway. To explore whether more than one *nagZ* gene encoded a *N*-acetyl-β-glucosaminidase, a residual activity assay was performed on lysates of PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ*.

For this assay, the initial concentration of 2 mM 4-methylumbelliferyl-β-D-*N*-acetylglucosamine (4-MUGlcNAc) was used as substrate to detect residual *nagZ* activity. Substrate 4-MUG in buffer PBS by itself degrades over time in solution, liberating fluorescent 4-methylumbeliferone (4-MU) product, which can be measured spectrophotometrically at a monitoring emission of 450 nm. This background fluorescence was not due to NagZ activity and was subtracted at each time point for all

samples. Any *N*-acetyl-β-glucosaminidase activity would liberate additional 4-MU. Each reaction was quenched from further activity before measurement with the addition of 0.1 M glycine/NaOH buffer pH 10.7. Controls included thermally denatured PAO1, PAΔ*nagZ*, PAΔ*DDh2Dh3* and PAΔ*DDh2Dh3nagZ* and blanks containing buffer only and buffer supplemented with 4-MUGlcNAc. Results showed that cellular lysates of PAO1 and PAΔ*DDh2Dh3* exhibited NagZ activity; however both mutants PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ* were devoid of *N*-acetyl-β-glucosaminidase activity as indicated by liberation of 4-MU similar to thermally denatured controls (Figure 3.9).

These residual activity assays thus demonstrated that *P. aeruginosa* expresses only one NagZ, encoded by PA3005, which had been successfully inactivated in strains PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ*. This finding concurred with that of the *E. coli* system where only one *nagZ* gene was found present (84). Having confirmed there is no other enzyme with *nagZ* activity, the findings of the assay also ascertained that *nagZ* was completely inactivated in the *nagZ* deficient mutant strains.

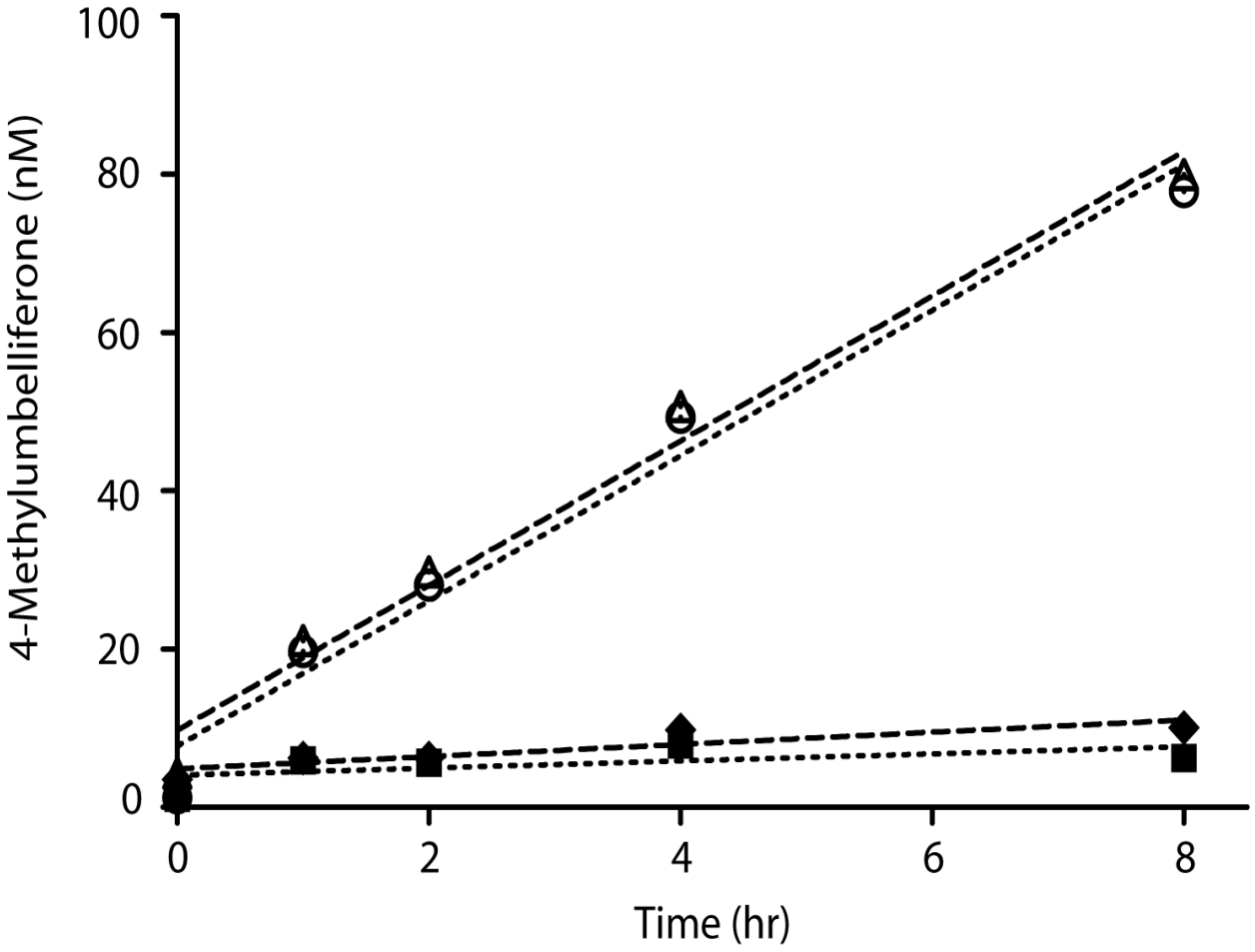


Figure 3.9. Residual *N*-acetyl- β-glucosaminidase assay of wild-type *P. aeruginosa* and deletion mutants. PAO1 (--O--), PAΔ*nagZ* (--■--), PAΔ*DDh2Dh3* (--Δ--) and PAΔ*DDh2Dh3nagZ* (--◆--). Thermally denatured controls of PAO1, PAΔ*nagZ*, PAΔ*DDh2Dh3* and PAΔ*DDh2Dh3nagZ* and blanks containing buffer only and buffer supplemented with 4-MUGlcNAc, showed liberation of 4-MU at levels similar to *nagZ*-deficient knock outs (data not shown).

3.5 Resistance Profiles of *P. aeruginosa* PAO1 and PAΔ*DDh2Dh3* and Their Δ*nagZ* Mutants PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ*

P. aeruginosa is notorious for its ability to overcome antibiotic therapy in clinical settings by rapidly developing mutations in components of the cell-recycling pathway to successfully evade the capacity of β-lactams. Derepressed, high-level β–lactamase expression is exhibited through null mutations in one, two or all of the *ampD* homologues (40, 52, 85); however, only loss of AmpD activity has been found clinically. In this way, *P. aeruginosa* has the ability to achieve increased *ampC* expression via loss of function mutations in AmpD; however, can still maintain sufficient cell wall recycling by maintaining active *ampDh2* and *ampDh3* (71).

3.5.1 Analysis of Antibiotic Susceptibility of *P. aeruginosa* Strains to Antipseudomonal β-Lactams

Some β-lactams exhibit antipseudomonal properties against wild-type *P. aeruginosa* (PAO1), apparently because these beta-lactams do not cause a sufficient level of peptidoglycan fragmentation to produce enough 1,6-anhydroMurNAc tri- (or penta-) peptide to saturate the endogenous AmpD activity. Thus, AmpD breaks down these molecules and removes them from the cytoplasm before they reach a concentration sufficient to convert AmpR into an activator of *ampC* expression. Perhaps for this reason, PAΔ*nagZ* did not display large increases in susceptibility to antipseudomonal β-lactams compared to PAO1 when using Etest strips. This was evidenced by the fact that Etest strips were unable to provide the resolution necessary to detect a change in MIC for PAΔ*nagZ* compared to PAO1. To probe if PAΔ*nagZ* did exhibit an increased susceptibility to antipseudomonal β-lactams, MIC measurements were carried out using

the broth microdilution method over a narrow serial dilution range of 32 – 0.0625 μg/mL. This approach offered significantly more precise MIC measurements in this antibiotic concentration range compared to that achievable using Etest strips. The broth microdilution method indeed confirmed that, compared to PAO1, PAΔ*nagZ* exhibits a 2-fold and 4-fold reduction in MIC values for ceftazidime and aztreonam respectively (Table 3.2). Both strains were also assayed for susceptibility against cefoxitin, a β-lactam that is known to be a strong inducer of AmpC β-lactamase expression. Although the MIC values are very high relative to antipseudomonal β-lactams (due to the large production of AmpC β-lactamase), PAΔ*nagZ* was also found to be at least 2-fold more susceptible to cefoxitin compared to PAO1.

Previous studies (24) have shown that the complete removal of AmpD activity from *P. aeruginosa* yields a mutant (PAΔ*DDh2Dh3*) that exhibits complete derepression of *ampC*. AmpD activity is required to suppress *ampC* induction by catalyzing the breakdown of the 1,6-anhydroMurNAc tri- (or penta-) peptide inducer molecule. In accordance with the complete derepression of *ampC*, PAΔ*DDh2Dh3* exhibits high, clinical-level resistance to antipseudomonal β-lactams (36). Interestingly, inactivation of *nagZ* in this strain yielded a mutant (PAΔ*DDh2Dh3nagZ* (Table 2.1)) that was profoundly more susceptible to all antipseudomonal β-lactams that were tested compared to parent strain PAΔ*DDh2Dh3*. Compared to PAΔ*DDh2Dh3,* the *nagZ* deficiency in PAΔ*DDh2Dh3nagZ* resulted in a 10- to 16- fold increase in susceptibility to piperacillin and piperacillin/tazobactam, and a 4- to 6- fold increase in susceptibility to aztreonam, cefepime and ceftazidime. Importantly, the observed increase in susceptibility from the

Table 3.2. MICs (μg/mL) of β-lactams and non β-lactam control for strains PAO1 and PAΔ*DDh2Dh3,* and *nagZ* null mutants of *P. aeruginosa*.

Strains	β-lactams							Control
	ATM[b]	**CAZ**[b]	**PIP**	**PIP/TZ**	**FEP**	**IMP**	**FOX**[b]	**CIP**
	(≤8 ->16)	(≤8 ->16)	(≤64 ->64)	(≤64 - >64)	(≤8 ->16)	(≤4 ->8)	n/a	(≤1 ->4)
PAO1 (wild type reference strain (47))	1	1	1	1	0.5	3	1600	0.2
PAΔ*nagZ*	0.25	0.5	1	1.5	0.38	2	800	0.2
PAΔ*DDh2Dh3*	24	48	>256	≥256	12	1.5	3200	0.2
PAΔ*DDh2Dh3nagZ*	4	12	24	16	3	1.5	2400	0.2
Complementation with wild-type *nagZ*								
PAΔ*nagZ* (pUCP27)							600	
PAΔ*nagZ* (pUCP*nagZ*)							1200	
PAΔ*DDh2Dh3nagZ* (pUCP27)	6	12	16					
PAΔ*DDh2Dh3nagZ* (pUCP*nagZ*)	24	48	256					

[a] ATM; aztreonam, CAZ; ceftazidime, FEP; cefepime, PIP; piperacillin, PIP/TZ; piperacillin/tazobactam, IMP; imipenem, FOX; cefoxitin, CIP; ciprofloxacin. MICs were determined using E-test strips unless otherwise indicated. CLSI resistance breakpoints are shown below each antibiotic. Measurements were performed in triplicate.

[b] MICs of aztreonam, ceftazidime and cefoxitin for PAO1 and PAΔ*nagZ* were determined via broth microdilution as recommended by the CLSI (see results). Measurements were performed in triplicate.

loss of functional NagZ brought the MIC values of all antipseudomonal β-lactams to well below their pharmacological resistance breakpoint concentrations (Table 3.2).

3.5.2 Analysis of Antibiotic Susceptibility of *P. aeruginosa* Strains to Control Antibiotics

Ciprofloxacin is a fluoroquinilone, a non-β-lactam antibiotic that targets the bacterial DNA gyrase and thus its mode of action is independent of the AmpC β-lactamase. For the purpose of emphasizing the role of *nagZ* and its specificity its role in *ampC* induction and β-lactam antibiotic resistance, the MIC values of the strains were measured using ciprofloxacin. Although the parent strains and *nagZ*-deficient mutant strains show a large variety of resistance to the β-lactam antibiotics tested, there was no difference in resistance to ciprofloxacin between any of the strains, they were all equally susceptible to the antibiotic. This confirmed that the changes seen in the resistance profiles of the strains to the β-lactams are attributed to the inactivation of *nagZ* and not due to non-specific activity or other NagZ effects.

The expression of AmpC has been supported thus far by MIC determinations of β-lactam antibiotics, all of which are substrates for β-lactamase activity. To demonstrate that the observed increases in susceptibility were to do with the specific effects of NagZ activity in the induction pathway of AmpC, all strains were challenged with imipenem, a non-AmpC-substrate. Imipenem is a carbapenem that is resistant to hydrolysis by AmpC. Changes in β-lactamase levels due to *ampD* mutations or *nagZ* deficiencies did not affect MICs of imipenem.

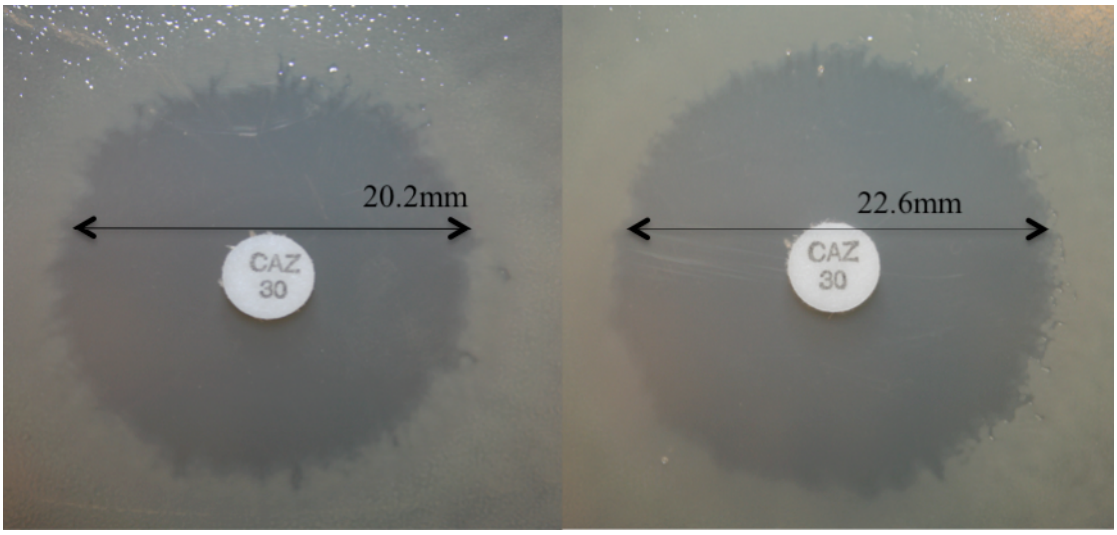
All strains were found to be fully susceptible to both imipenem and ciprofloxacin as compared to the wild-type strain PAO1, thereby demonstrating the specific effect of NagZ activity within the AmpC induction pathway (Table 3.2) (10).

3.5.3. Phenotypic Analysis of Antibiotic Susceptibility of *P. aeruginosa* PAO1 and PAΔ*DDh2Dh3* and their Δ*nagZ* Mutants

The difference in the resistance profiles of PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ* to front-line defense antibiotics aztreonam and ceftazidime and the markedly visible reductions in resistance in the triple AmpD null mutant strain where NagZ had been inactivated was also confirmed visually using a disc diffusion assay, as described in Figures 3.10 through 3.13.

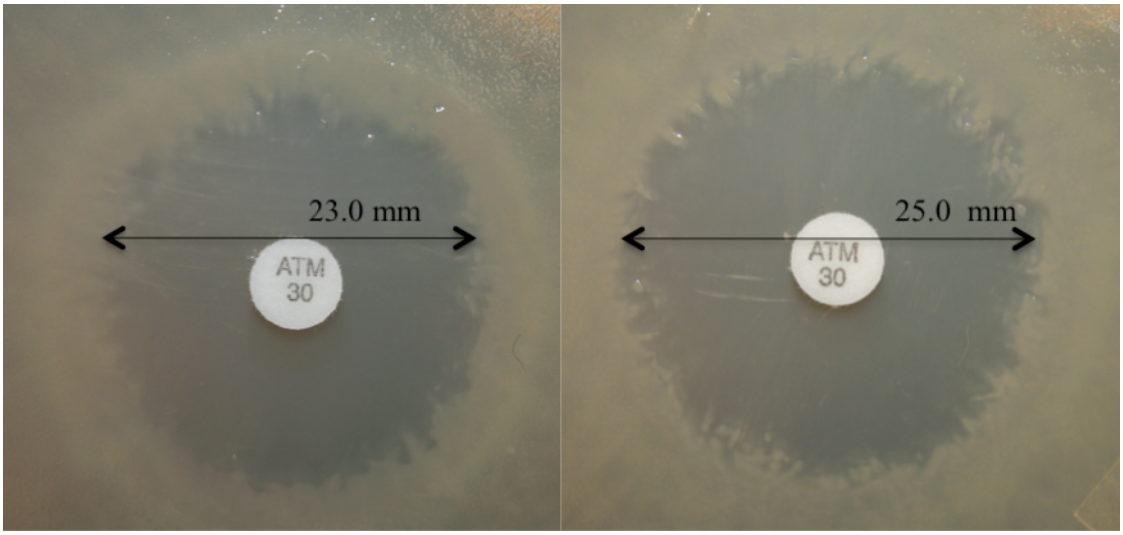
AmpC β-lactamase expression and inducibility correlates with observed changes in β-lactam resistance as evidenced in observed changes in MIC values (Table 3.2) and quantified β-lactamase specific activity assay (see section 3.7).

Table 3.3 lists results of strains based on diameters of zones of inhibition of antibiotic-infused discs. Measurements that defined resistance, inhibition and susceptibility were according to CLSI performance standards (74).



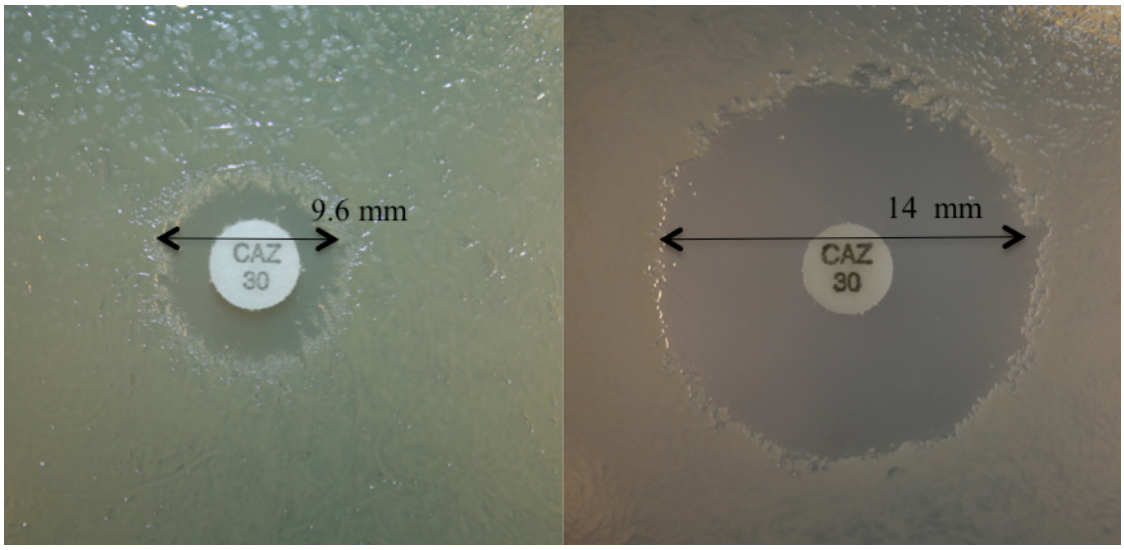
(a) (b)

Figure 3.10. Zones of inhibition: (a) Strain PAO1, susceptible to ceftazidime; and (b) Strain PAΔ*nagZ,* susceptible to ceftazidime.



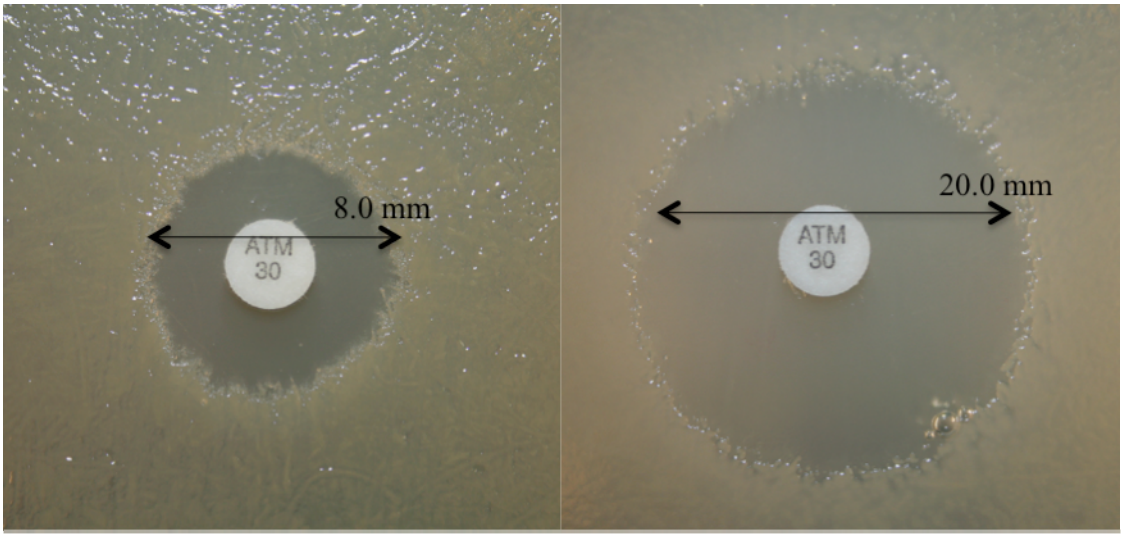
(a) (b)

Figure 3.11. Zones of inhibition: (a) Strain PAO1, susceptible to aztreonam; and (b) Strain PAΔ*nagZ,* susceptible to aztreonam.



(a) (b)

Figure 3.12. Zones of inhibition: (a) Strain PAΔ*DDh2Dh3,* hyperinduced AmpC β-lactamase expression; and (b) Strain PAΔ*DDh2Dh3nagZ.*



(a) (b)

Figure 3.13. Zones of inhibition: (a) Strain PAΔ*DDh2Dh3*, hyperinduced AmpC β-lactamase expression; and (b) Strain PAΔ*DDh2Dh3nagZ.*

Table 3.3. Zone diameter measurements (mm) of β-lactams aztreonam and ceftazidime for strains PAO1 and PAΔ*DDh2Dh3,* and *nagZ* null mutants PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ.*

Strains	**Antibiotic Strain Discs + Zone Diameter (mm)**	
	Ceftazidime	**Aztreonam**
	(≤8 ->16)*	(≤8 ->16)*
PAO1	20.2 Susceptible**	23.0 Susceptible
PAΔ*nagZ*	22.6 Susceptible	25.0 Susceptible
PAΔ*DDh2Dh3*	9.6 Resistant	8.6 Resistant
PAΔ*DDh2Dh3nagZ*	14.0 Intermediate	20.0 Intermediate

* Zone diameter measured to nearest whole (mm); ranges as defined by CLSI standards (74).
**Interpretive criteria; susceptible, intermediate, resistant, as defined by CLSI standards.

3.6 Complementation of *nagZ* in *P. aeruginosa* PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ*

Plasmid-based complementation using full-length *nagZ*, carried out with the *nagZ* expression plasmid, pUCP*NagZ* (Table 2.1) was used to verify that the measured effects on MIC were solely due to inactivation of NagZ. As expected, complementation of PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ* with the full-length *nagZ* gene via electroporation of these strains with pUCP*NagZ* was found to restore antibiotic resistance profiles to near wild-type levels (Table 3.2).

3.7 Quantification of β-lactamase Specific Activity

Given that NagZ increases the susceptibility of *P. aeruginosa* PAO1 and PAΔ*DDh2Dh3* to β-lactams and the proposed role of NagZ in peptidoglycan recycling, inactivation of *nagZ* was appearing to block the induction of AmpC β-lactamase expression by reducing the levels of inducer molecule in the cytoplasm. This allowed AmpD activity to accommodate the concentrations and sufficiently break down NagZ product, clearing the cytoplasm of inducer. Collectively, this should result in increased susceptibility to β-lactam antibiotics.

A method to quantify AmpC β-lactamase expression under basal conditions is by measuring β-lactamase specific activity, a value quantified as nanomoles of nitrocefin hydrolyzed per minute per milligram of protein. However, to compare basal levels of AmpC expression to β-lactam induced conditions and to correlate β-lactamase specific activity to *nagZ* activity, cells were induced with cefoxitin, a potent inducer of β-lactamase production. Using this AmpC activity assay, the effect of NagZ activity on the

levels of AmpC induction was measured by testing PAO1, PAΔ*nagZ* and PAΔ*DDh2Dh3* and PAΔ*DDh2Dh3nagZ* in the presence of absence of cefoxitin.

The results showed b-lactamase expression is correlated to NagZ activity both in PAO1 and the triple *ampD* null mutant and PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ.* Under cefoxitin-induced conditions, compared to PAO1, strain PAΔ*nagZ* displayed decreased b-lactamase specific activity 2-fold. In the triple *ampD* null mutant PAΔ*DDh2Dh3nagZ*, b-lactamase specific activity was reduced 4-fold (Table 3.4). These reductions in b-lactamase specific activity were consistent with the observed increases in susceptibility to antibiotics (Table 3.2). These results confirmed that the changes in resistance profiles were due to a decline in the production of the AmpC b-lactamase.

Table 3.4. Basal and cefoxitin-induced β-lactamase specific activities of *P. aeruginosa* PAO1, PAΔ*nagZ,* triple mutant PAΔ*DDh2Dh3* and PAΔ*DDh2Dh3nagZ*

Strain	Average β-lactamase specific activity ± SD	
	Basal	Induced[a]
PAO1	2.8 ± 0.4	230 ± 20
PAΔ*nagZ*	3.9 ± 0.8	95 ± 7.8
PAΔ*DDh2Dh3*	3792 ± 121	3935 ± 11
PAΔ*DDh2Dh3nagZ*	288 ± 32	1019 ± 64

[a] Induction was carried out by culturing strains in the presence of 50 μg/ml cefoxitin for 3 hours 37 °C.

Chapter 4 – Discussion and Conclusion

4.1 Inactivation of *nagZ* in *Pseudomonas aeruginosa* PAO1 and PAΔ*DDh2Dh3*

Schweizer et al. developed an efficient, accurate method for genetic replacement in *Pseudomonas aeruginosa.* This method focused on the design of suicide delivery vectors, such as pEX18Tc, which was used in this thesis for the genetic inactivation studies (70). The inactivated *nagZ* construct that was created, pEXΔ*nagZGm* (Table 2.1) harbored a substantial deletion in the center of the gene. This was to ensure the gene was truncated sufficiently, so that there would be no translation of nonsense transcripts that could potentially affect the growth of the bacteria. The *aacC1* gentamicin resistance marker was also inserted in the center of the truncated *nagZ* gene as a selection and verification marker that could be used after allelic exchange into the recipient strains. As the studies focused on the change in susceptibility of the knock out mutants to β-lactam antibiotics, gentamicin was an ideal resistance marker as its mode of action differs from that of β-lactams. β-lactams inhibit peptidoglycan synthesis and disrupt the cell wall. Gentamicin is a broad-spectrum aminoglycoside that inhibits protein synthesis by binding the 30S ribosomal subunit and preventing the formation of an initiation complex with messenger RNA (mRNA) (86). This activity would not affect the studies of peptidoglycan recycling and the AmpC β-lactamase pathway and thus would not falsely modify the resistance profile results.

Approximately 1,000 bp of *P. aeruginosa* genomic DNA flanking the inactivated NagZ products was added to increase the likelihood of homologous sequence recognition

during the biparental mating procedure. The intent of this was to increase the frequency of recombination events to result in more clones containing the knock out construct as opposed to the more frequently occurring merodiploids.

The suicide delivery vector pEXΔ*nagZGm* was first introduced into the mobilizable strain of *E. coli*, S17-1, which carries the transfer genes that enable conjugative DNA transfer in Gram-negative bacteria dissimilar to *E. coli*. The biparental mating was carried out with recipient strains *P. aeruginosa* PAO1 and the isogenic triple *ampD* null mutant PAΔ*DDh2Dh3* (Table 2.1).

Attempts to isolate clones in which allelic exchange had occurred was facilitated by the addition of sucrose as a selection step. Supplementing growth media with sucrose when plating merodiploids allows for relatively simple differentiation of recombination mutants at highly efficient frequencies (87). Replication of this frequency was achieved in selection of double crossover mutants by replica plating gentamicin resistant colonies onto medium containing 5% sucrose, therefore those that had the plasmid integrated into their genomes. Those colonies that grew successfully in the presence of sucrose must have excised the plasmid from their genomes, thereby having removed the toxic levan sucrase encoded by *sacB*. This selection step was intended to facilitate selection of the knock out mutants sought, by aid of the *sacB* selection marker contained on the plasmid. Therefore, plating clones with sucrose promotes easier and more accurate selection of transconjugants that have undergone gene replacement via double homologous recombination, excising unwanted plasmid DNA sequence, and exchanging the disruptive Gm resistance cassette into the targeted gene, *nagZ*.

Furthermore, the donor to recipient strains were plated at ratios of 2:1 as it was found that at this ratio, as opposed to the 1:1 and 1:2 ratio that were also attempted, resulted in an increased frequency of transconjugants.

Upon isolation of recombination mutants through phenotypic screens, and identification of recombination mutants via PCR screening, further genotypic analysis of a *PstI* restriction digest would reveal patterns of the wild-type *P. aeruginosa* PAO1 strain that would be different to the clones in which allelic exchange occurred, removing the wild-type *nagZ* gene. The *aacC1* cassette was ligated in the center of the inactivated NagZ construct by introduction of two *PstI* restriction sites. Digestion of wild-type *nagZ* gives rise to an intact fragment of 1000 base pairs as there is no restriction site present in the wild-type gene. The digestion pattern of a mutant would result in a 900 bp fragment of the *aacC1* cassette, and a band of approximately 300 bp, the remaining fragment of *nagZ*. These patterns were different enough from each other that it could confidently be used to identify recombination mutants from merodiploids and the parent strains. In addition, as stated in results, this was verified using primers targeting the genome of each strain (PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ*), external to the potential crossover regions used in the construction of pEXΔ*nagZGm*, followed by sequence verification.

4.2 Attenuation of β-Lactam Resistance in Hyperexpressing AmpC Mutants

Although recurring infections with *P. aeruginosa* are treated in the clinical setting with β-lactams, the success of antibiotic therapy is declining with the selection of strains harboring chromosomal mutations related to the expression of these inducible β-

lactamases (61). The prevalence of hyperexpressible β-lactamases poses a challenge for the antipseudomonal β-lactams, which are usually active against *P. aeruginosa* infections. They are effective against *P. aeruginosa*, despite the presence of its inducible chromosomal cephalosporinase because they are weak inducers of AmpC and thus are not hydrolyzed before reaching the targeted transpeptidases (40). Mutants that hyperexpress AmpC can immobilize even the most effective antipseudomonal drugs (36). Wild-type strains, though they exhibit high intrinsic resistance to many antibiotics, are largely threatening to immunocompromised patients but remain susceptible to the majority of the clinical front line of defense antipseudomonal β-lactams such as aztreonam (a monobactam). The development of AmpC hyperexpressing mutants, commonly through AmpD loss of function mutations, which have been clinically isolated from cystic fibrosis patients suffering from chronic infections, highlights the need for alternative approaches to β-lactam therapy (14).

Previous studies have shown the crucial role NagZ plays in cell wall recycling systems of Gram-negative bacteria and its relation to the inducible AmpC β-lactamase (29). Given the highly conserved nature of the NagZ gene (70, 88), and publishing of the NagZ crystal structure (34), NagZ is a well-characterized choice for further investigation. Given this, research into the role of a chromosomally encoded NagZ is carried out in *P. aeruginosa* and these *nagZ*-deficient mutants; PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ*.

To begin understanding the role of the NagZ *N*-acetyl-β-glucosaminidase, the *nagZ* gene was inactivated in two different strains; PAO1 and the triple AmpD null mutant PAΔ*DDh2Dh3* (34). Previously, Vötsch and Templin performed research on NagZ activity in *E. coli* carrying the *ampC-ampR* operon from *Enterobacter cloacae* on a

plasmid. They provided evidence that *nagZ*-deficient mutants could not establish sustained AmpC-mediated β-lactamase resistance when compared to its parental strain, wild-type PAO1 (26). Sensitivity to β-lactams in *E. coli* containing a *nagZ* mutation and carrying the plasmid-mediated *ampC-ampR* operon is closely similar to *E. coli* in which there is no *ampC* gene present at all. It has been suggested that inhibiting *nagZ* gene activity could be a possible means of ceasing expression of inducible β-lactamases (such as with those associated with the *ampC-ampR* operon); therefore, bacteria capable of producing AmpC β-lactamase would be locked in a "sensitive state". This offers the possibility that strains would be either unable to establish resistance, or in the case of strains that are already β-lactam resistant, could be rendered sensitive to antipseudomonals again.

From the collection of previously conducted research, it was concluded that inactivating NagZ in *P. aeruginosa* might result in increased β-lactam susceptibility in this organism. Given that this was effective in systems that contain an AmpR-regulated (inducible) *ampC* β-lactamase gene, *P. aeruginosa* was an ideal candidate for knocking out *nagZ* using the Schweizer et al. suicide delivery vectors. Blocking *nagZ* activity in *P. aeruginosa* may provide a novel strategy to enhance the efficacy of antipseudomonal β-lactams against bacteria encoding inducible *ampC*. The consequence of its inhibition would be the suppression of intrinsic *ampC* induction and the hyperinduction that occurs from the selection of *ampD* null mutants.

In a study complementary to the work described here, Stubbs et al. (2007) recently showed that NagZ activity could be inhibited with the use of selective small molecules to give rise to a 2-fold increase in the susceptibility of PAO1, PAΔ*D*, PAΔ*DDh2* and

PAΔ*DDh2Dh3* to Aztreonam when compared to these strains exposed to Aztreonam alone (60). However to test the true extent of the role of NagZ in AmpC mediated β-lactam resistance, inactivating *nagZ* in PAO1 and PAΔ*DDh2Dh3* gave a more accurate scope of susceptibility as opposed to using these inhibitors, which could be prevented from reaching the cytoplasm by membrane exclusion or degradation. Chromosomal mutations would not be dependent on membrane permeability or specificity and would demonstrate the maximum level of inhibition possible.

4.2.1 PAΔ*nagZ*

It has been shown that functional *nagZ* activity results in the production of 1,6-anhydroMurNAc-tripeptides, the up regulating molecule of the *ampC-ampR* operon (26, 69). Impaired *nagZ* function would correlate with a decline in *ampC* gene transcription and thus increased susceptibility to β-lactams (Figure 4.1).

Although the plasmid-based studies carried out by Vötsch and Templin showed a visibly detectable decline in resistance to cefoxitin, as measured by disc assay zones of inhibition, *E. coli* does not exhibit an intrinsic resistance to cefoxitin as high as *P. aeruginosa*. Susceptibility to the β-lactams piperacillin, piperacillin/tazobactam and cefepime that were assayed remained, for the most part, statistically unchanged in PAΔ*nagZ* compared to PAO1. However, PAO1 demonstrated resistance to the strong β-lactamase inducer cefoxitin at high concentrations, but PAΔ*nagZ* was found to be 2-fold more susceptible than PAO1. Furthermore, PAΔ*nagZ* was 2- to 4-fold more susceptible to the favored clinical β-lactams, aztreonam and ceftazidime, compared to PAO1, a significant difference at very low and thus clinically applicable concentrations (89).

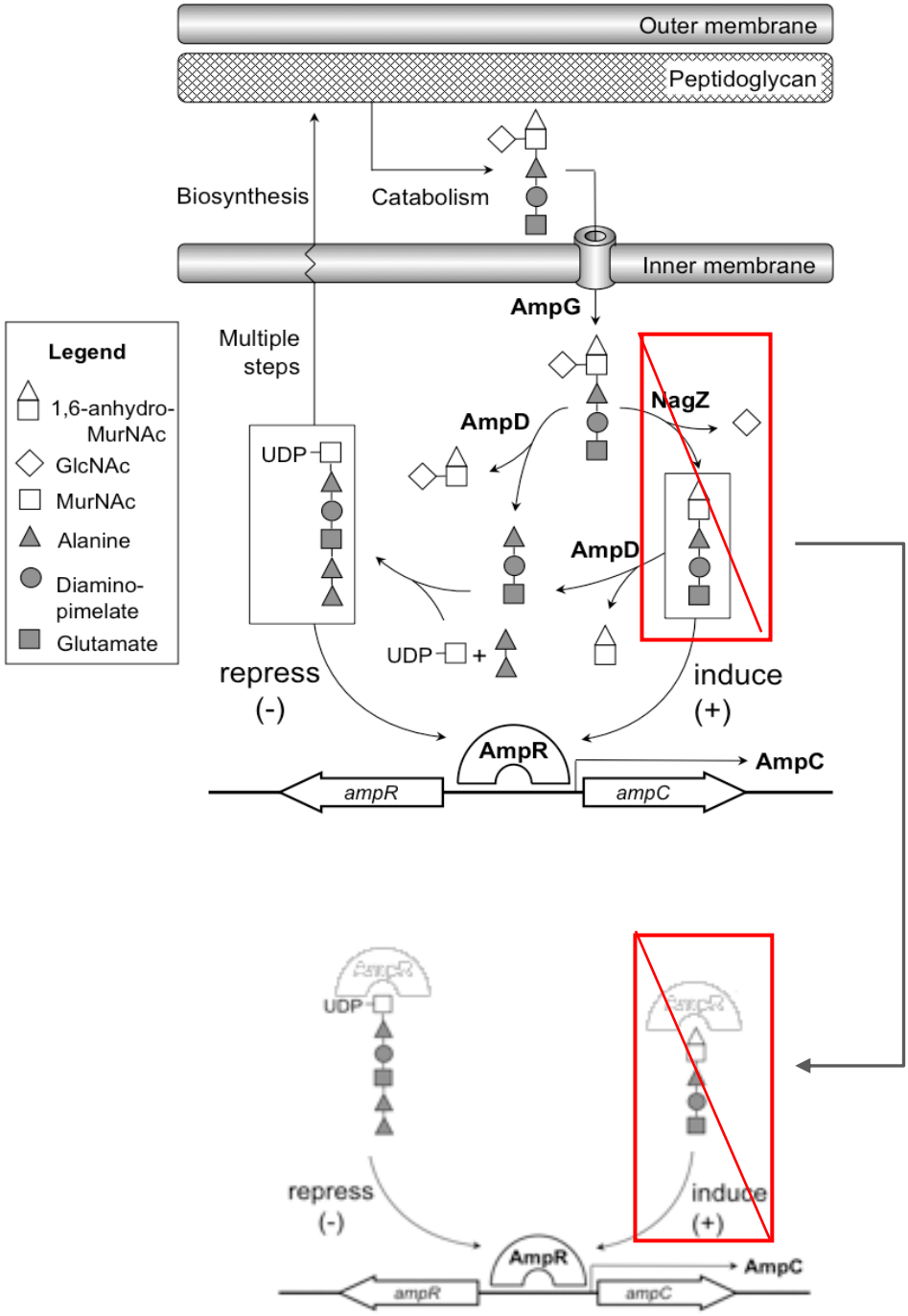


Figure 4.1. Schematic representation of the proposed effect to the AmpC pathway due to insertional inactivation of the *nagZ* gene. NagZ removes terminal *N*-acetylglucosamine residues from internalized cell wall degradation intermediates, producing a pool of 1,6-anhydro-MurNAc-oligopeptides (tri-, tetra- and pentapeptides). At right: removing this activity should promote a decline in *ampC* transcription by preventing formation of the AmpR-inducer molecule complex that up regulates *ampC* transcription. Figure modified from Asgarali et al. (2009).

Inactivating NagZ in the wild-type background showed the same effect as seen in the studies performed with *E. coli* carrying a plasmid-borne *ampC-ampR* operon for antipseudomonals. The recent publication of the research performed by Stubbs et al. supported the results of Vötsch and Templin, where the use of the selective small molecule inhibitors to attenuate β-lactam resistance in strains of *E. coli* transformed with the plasmid-borne *ampC-ampR* operon cloned from *Citrobacter freundii* is also effective (34).

NagZ inactivation results in a 2-fold increase in susceptibility in PAΔ*nagZ,* with high concentrations of cefoxitin needed to detect this change, compared to the strains in prior studies can possibly be attributed to the fact that *P. aeruginosa* can probably pump cefoxitin out of the cell, hence the high background resistance. In addition, as previously mentioned, *P. aeruginosa* is intrinsically more resistant to antibiotics (β-lactams) than *E. coli.*

However, the resistance profiles obtained in this thesis are supported by a similar series of studies carried out by Juan et al. (2006). They showed minimum inhibitory concentration (MIC) values to a variety of antipseudomonal β-lactams that matched the wild-type PAO1 MIC values of this thesis. At a very high concentration of cefoxitin, PAΔ*nagZ* did exhibit a nearly 2-fold reduction in resistance compared to PAO1. Recall that cefoxitin is a very strong inducer of AmpC, thus contributing to the high intrinsic resistance of *P. aeruginosa* to this antibiotic. A high concentration of a strong inducer is required to flood the immobilizing activity of β-lactamase. Antibiotics that strongly induce AmpC β-lactamase do so by triggering increased concentrations of 1,6-anhydroMurNAc-oligopeptides that induce the AmpR regulator of AmpC, and this results in the catalyzing their own hydrolysis (90).

4.2.2 PAΔ*DDh2Dh3nagZ*

The three homologous *ampD* genes encode *N*-acetyl-anhydromuramyl-L-alanine amidases that are responsible for removing the stem peptide on the recycled muropeptides to be reused in biosynthesis of peptidoglycan. These *ampD* genes coordinate to contribute to and repress β-lactamase expression via the AmpR regulator (Figure 1.5, 1.6). In wild-type PAO1, where the *ampD* genes are fully functional, the *ampC* cephalosporinase remains inducible through AmpR binding with the inducer molecule, resulting in a conformation that leads to transcription of *ampC*.

Generation of *AmpD* null mutants, and possibly *AmpDh2* mutants, are naturally selected for in chronically infected cystic fibrosis patients (72, 91). These mutants do not efficiently breakdown the 1,6-anhydroMurNAc oligopeptides produced by NagZ and thus NagZ product levels accumulate in the cytoplasm, inducing *ampC* transcription chronically even without the presence of β-lactams (Figure 4.2) (52). This is advantageous to the pathogen as it contributes to its ability to defeat clinical antibiotic therapy. PAΔ*DDh2Dh3* mutants exhibit very high resistance to antipseudomonals by having β-lactamase present at all times, at levels that are difficult to combat with antibiotics (40, 71, 92). The relentless production of β-lactamase by hyper inducing mutants is not only expensive, it is more likely that treatment will be longer, have greater risks and be at concentrations that is toxic to patients (92).

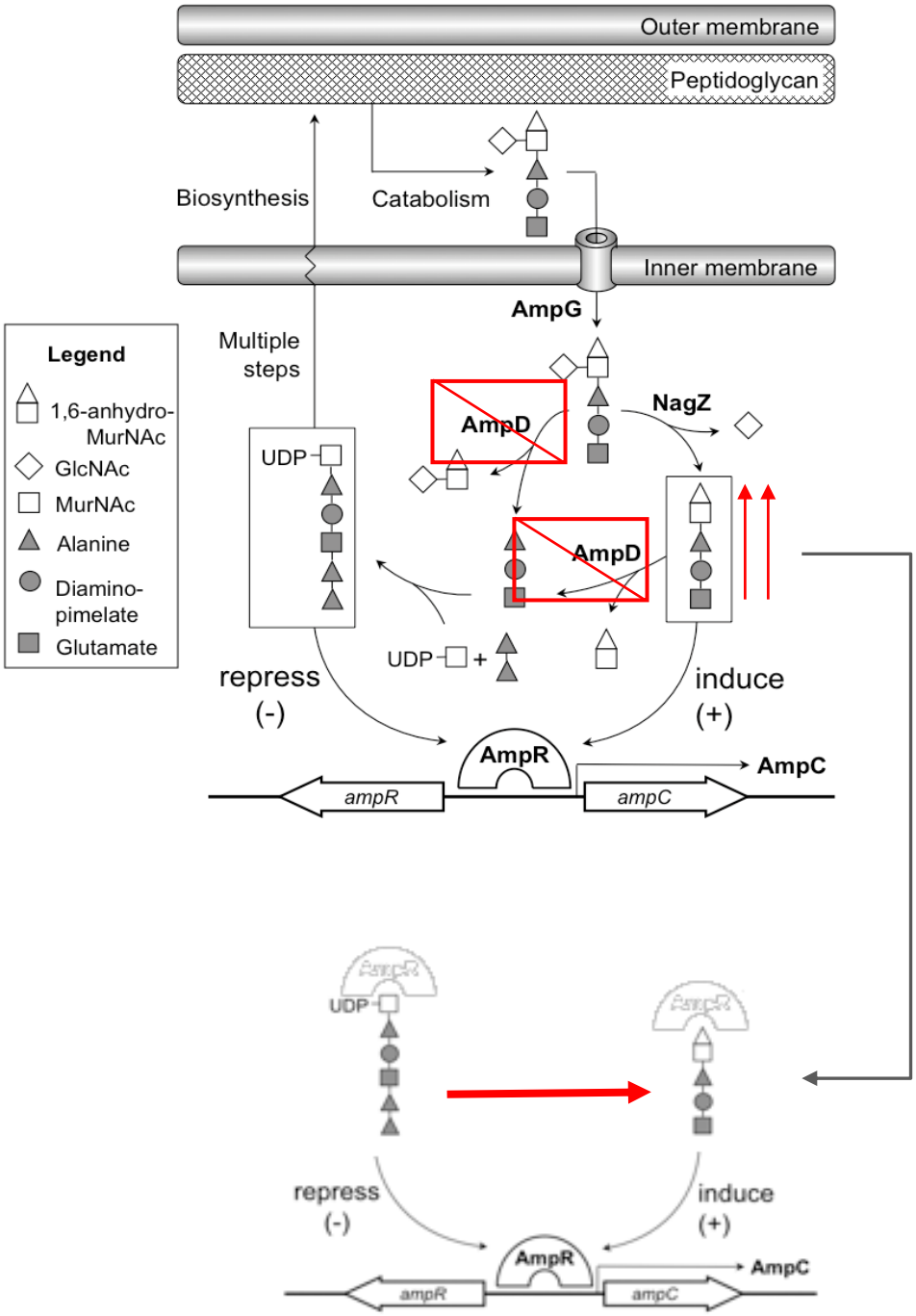


Figure 4.2. Schematic representation of the proposed effect of AmpD and AmpD homologue(s) inactivation. Inactivation of AmpD results in an accumulation of NagZ product, an increasing pool of 1,6-anhydro-MurNAc-oligopeptides (tri-, tetra- and pentapeptides). At right: the balance between AmpR repressor and AmpR inducer complexes is disturbed and an increase of AmpC transcription will occur with the increased binding of NagZ product to AmpR. Figure modified from Asgarali et al. (2009).

Given that NagZ produces the substrate for AmpD activity, it should follow that by impairing the generation of the AmpD substrate by inactivating *nagZ,* the pool of inducer molecules would decline (although they may not be completely eliminated). As is the case amongst mutants lacking *ampD* activity, the cytoplasm does not get cleared of the sugar-peptide inducer molecules. However, by deleting *nagZ* from the triple null *ampD* mutant, PAΔ*DDh2Dh3*, the cell wall recycling pathway becomes uncoupled to the inducibility of *ampC* transcription (36).

Although AmpD is no longer active, the less 1,6-anhydro-MurNAC oligopeptides that are present in the cytoplasm as a result of loss of function *nagZ* gene activity, the less inducer molecule there is to be competitively displaced from binding AmpR. UDP-MurNAc pentapeptide, the negative regulator molecule that binds AmpR and prevents transcription of *ampC*, can then outcompete 1,6-anhydroMurNAc-tripeptides to prevent induction via AmpR (Figure 4.3). This uncoupling of the two pathways had a greater effect on antibiotic susceptibility of PAΔ*DDh2Dh3* and PAΔ*DDh2Dh3nagZ* compared to PAO1 and PAΔ*nagZ*.

PAΔ*DDh2Dh3nagZ* exhibited a significant drop in resistance to all antipseudomonal β-lactams that were assayed, which can only be attributed to the deleterious effects of NagZ. When compared to the parent strain PAΔ*DDh2Dh3* that exhibits a clinically relevant highly resistant phenotype to antipseudomonal β-lactams, but also harbors a fully functional *nagZ* gene, the only genotypic difference between the two strains is the presence of *nagZ.* The overall increase in susceptibility, as anticipated, was indeed greater in PAΔ*DDh2Dh3nagZ* than PAΔ*nagZ* compared to their parent strains.

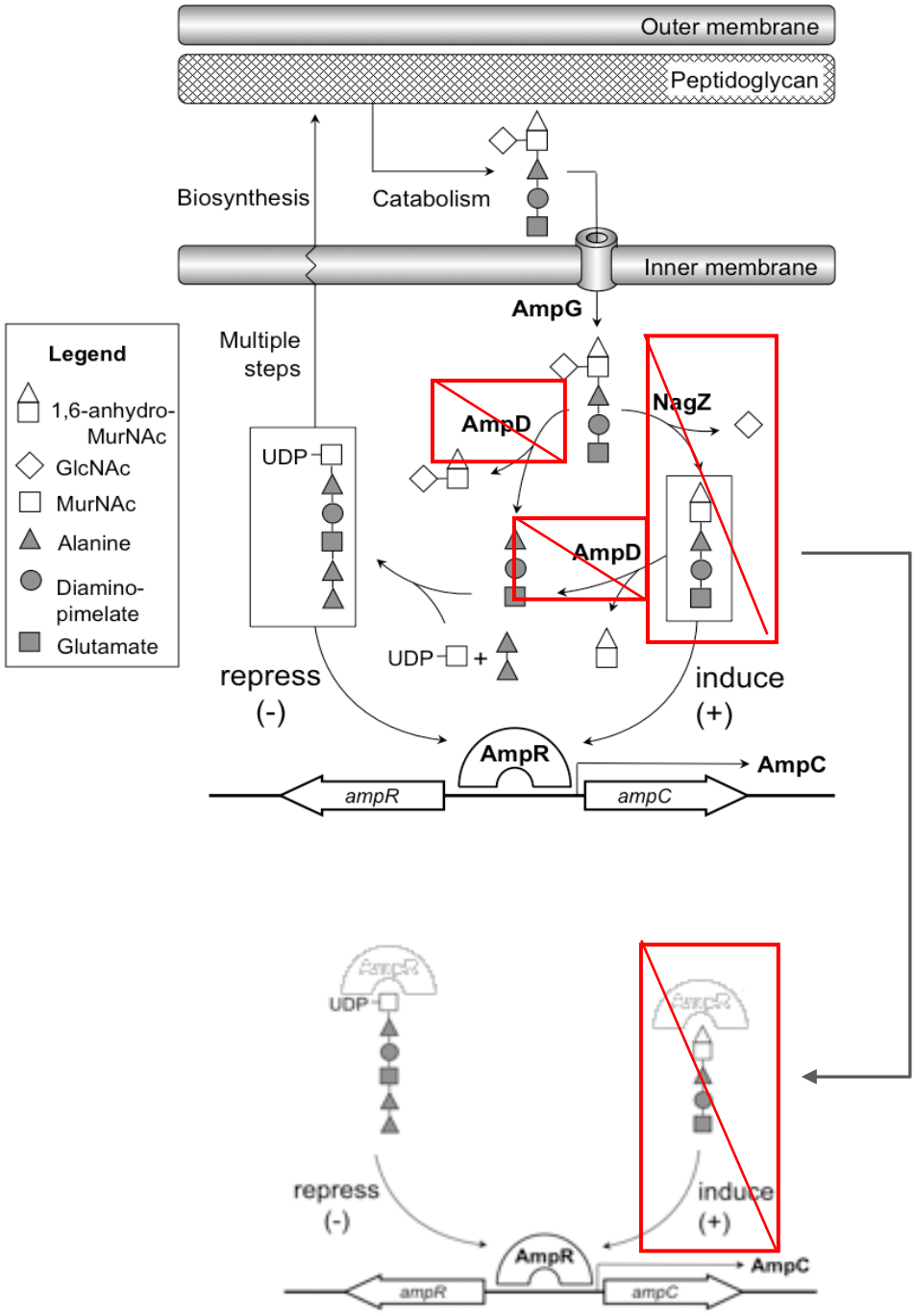


Figure 4.3. Schematic representation of the proposed effect of AmpD and NagZ inactivation. Inactivation of AmpD as well as NagZ results in a decline in production of 1,6-anhydro-MurNAc-oligopeptides (tri-, tetra- and pentapeptides). At right: the balance between AmpR repressor and AmpR inducer complexes is disturbed and an increase of AmpR-repressor complexes will down regulate *ampC*. Figure modified from Asgarali et al. (2009).

4.2.3 Gene Complementation Analysis

To confirm the change in susceptibility to antipseudomonal β-lactam antibiotics exhibited by PAΔ*DDh2Dh3nagZ* and PAΔ*nagZ* compared to their parent strains was indeed due to inactivation of *nagZ* PA3005, vector pUCP27 was selected to carry the wild-type *nagZ* gene for complementation. This vector was selected as it exhibits several features essential to gene complementation. It displays a constitutive copy number of ten to 25 copies per cell that was designed to be stable through modification of the 1.9-kb *Pst*I fragment found on pRO1614 (79, 93), which was cloned into the single *Nar*I site of pUC19 by Schweizer et al. (93). The complement gene is expressed under the control of the *lac* promoter. Primers used to clone full-length *nagZ* included a ribosome-binding site (rbs) derived from pET vector T7 (94) to initiate protein synthesis of the NagZ complement gene located on pUCP*NagZ* (Table 2.1).

4.3 Investigating Residual β-Lactam Resistance

4.3.1 NagZ Homologues

Strains that exhibit constitutive, hyperinduced β-lactamase expression such as the triple *ampD* null mutant PAΔ*DDh2Dh3* have large accumulations of 1,6-anhydroMurNAc-tri- (or penta-) peptides in the cytoplasm (Table 2.1) (36). Strain PAΔ*DDh2Dh3* has nonfunctional *ampD* genes and therefore is incapable of clearing the cytoplasm of muropeptides and consequently, *ampC* inducer molecule. Inactivating *nagZ*

in strain PAΔ*DDh2Dh3* should increase susceptibility to β-lactams, despite the hyperinduced β-lactamase expression, by reducing the levels of NagZ product. This might result in increased susceptibility as β-lactamase expression could be reduced to levels similar to that of wild-type *P. aeruginosa* PAO1 or PAΔ*nagZ.*

The MIC data agrees with this hypothesis as shown in Table 3.2. PAΔ*nagZ* shows increased susceptibility to ceftazidime, aztreonam and cefoxitin compared to parent strain PAO1. Compared to PAΔ*DDh2Dh3*, which shows high, clinical-levels of resistance to several β-lactams such as aztreonam, ceftazidime, piperacillin and cefepime, the *nagZ*-deficient PAΔ*DDh2Dh3nagZ* displays a reduction in resistance up to 16-fold (Table 3.2). The reason as to why there PAΔ*nagZ* displays little change in susceptibility to cefepime, piperacillin and piperacillin/tazobactam is due to the fact that they are weak inducers of *ampC* expression; it is only within the AmpD deficient strains that the difference becomes clear. The presence of three AmpD homologues involved in the AmpC pathway suggests *P. aeruginosa* could potentially have NagZ homologues, especially as this is a critical enzymatic component of cell wall recycling and *ampC* expression. It was investigated whether or not there were additional *nagZ* gene homologues present that could contribute to residual resistance. However, several of the experiments conducted here confirmed that the *nagZ* gene was completely disrupted and non functional. This evidence, including β-lactamase specific activity assays as well as residual activity assays, are in accordance with recently published data identifying only one *nagZ* gene encoding an *N*-acetyl-β-glucosaminidase present in PAO1 using an activity based proteomics probe selective for this enzymatic activity (60, 95). Both *nagZ*-deficient mutants PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ* were devoid of the glucosaminidase activity that is required to

catalyze the formation of the 1,6-anhydroMurNAc tripeptide inducer molecule. This confirmed an absence of alternate *nagZ*-like activity or a NagZ homologue.

A β-lactamase specific activity assay demonstrated the decline in NagZ activity and, accordingly, generation of 2,4-dinitrophenolate product from the reference substrate nitrocefin of both *nagZ*-deficient mutants. This assay provided evidence that *P. aeruginosa* does not have any NagZ homologues and confirmed that the observed increases in susceptibility to β-lactams were due to decreased β-lactamase expression in mutants PAΔ*nagZ* and PAΔ*DDh2Dh3nagZ*. Given the absence of *nagZ* gene homologues, it must be speculated that *P. aeruginosa* employed other means to induce *ampC* transcription, despite having lost *nagZ* activity, to exhibit residual resistance.

Jacobs et al. observed constitutive expression of *ampC* β-lactamase *in vitro* by way of an apo form of AmpR. The unmodified conformation of AmpR thus activates transcription of *ampC* until the addition of UDP-MurNAc pentapeptide; only then will AmpR bind the repressor molecule and inhibit *ampC* transcription (96).

Undoubtedly, the cell wall recycling pathway is advantageous to *P. aeruginosa* and other bacteria as it allows them to conserve energy and reuse building blocks under nutrient-deprived conditions. Nevertheless, uncoupling the cell wall recycling pathway to the AmpC pathway by genetic inactivation of the *nagZ* gene and the three *ampD* homologues obviously did not completely debilitate the mutants. Bacteria are still capable of *de novo* synthesis of peptidoglycan and thus, UDP-MurNAc pentapeptide continues to be produced and recycled. Due to the fact that they are likely producing the AmpR repressor molecule *de novo*, the quantities of UDP-MurNAc pentapeptide may not

be sufficient to completely bind and repress AmpR, leading to a modest level of transcription of *ampC*.

4.4 Relevant Mutations

P. aeruginosa is notorious for its ability to mutate, giving rise to select mutants that can overcome clinical antibiotic therapy. It must therefore be considered that the using novel antibiotic therapies will likely lead to the development of new multi-drug resistant strains of *P. aeruginosa*.

The use of antibiotics that target different defenses of the bacteria, that is, that have a different mode of action than β-lactams, may lead to mutations in the genome that give rise to resistant isolates. As with imipenem, the carbapenem that is very stable against hydrolysis by β-lactamases, *P. aeruginosa* has developed resistance to the antibiotic during the course of imipenem therapy that involves loss of the D2 porin (53). Any mutation, such as those related to membrane permeability, can also affect the transport of other molecules that may utilize the D2 porin to gain access to the cytoplasm, such as selective inhibitors.

Although mutations in *ampD* have been identified in clinical isolates, and these mutants have developed resistance to antipseudomonal β-lactams, the development of novel methods to target NagZ activity may simply be circumvented. Other mutations have been identified which often are a result of loss of genes, such as *ampR,* that do not result in constitutive production of a gene. Circumvention of a pathway appears to be

favored as mutations resulting in hyperproduction of a gene have been linked to reduced fitness over time (97).

On the other hand, the design and use of small molecule inhibitors, such as the NagZ inhibitor PUGNAc used by Stubbs et al., could be hindered by selection of mutations in the *nagZ* active site that may reduce the binding specificity of the enzyme to inhibitors (34). However, a less efficient NagZ may lead to declined formation of the 1,6-anhydro-MurNAc oligopeptides that are required to bind and induce AmpR to transcribe *ampC*. Overall, the mutation could mean the microbe may not produce concentrations of β-lactamase sufficient enough to overcome antibiotic therapy. A mutation of this nature may be clinically advantageous in defeating an infection, or it could have more detrimental effects by giving rise to new mutants, resistant to selective small molecule inhibitors.

4.5 Future Work

Studies performed by Park (1996) noted that although AmpC β-lactamase is found in *E. coli*, this microbe does not have an AmpR transcriptional regulator nor does it have a DNA-binding site for AmpR (98); hence, *ampC* is only expressed at basal levels even in the presence of β-lactams. Consequently, its *ampC* is not inducible through control of AmpR as that found in *P. aeruginosa*. Park states in his research that *E. coli* can monitor the cell wall status by its recycling pathway but this raises the question, to what purpose does the connection between the cell wall recycling pathway and AmpC production serve, if its peptidoglycan degradation components cannot induce transcription of *ampC*? In this

case, *P. aeruginosa*, as previously mentioned, can monitor environmental conditions both internally and externally through the cell wall degradation components inducing transcription of AmpC. Therefore it may be beneficial to further research into the cell wall recycling pathway by genetically inactivating the *ampG* gene. This gene encodes the transmembrane permease that is responsible for capturing and transport of peptidoglycan fragments that are excised from the cell wall during recycling. AmpG has been identified as necessary and relevant to the induction process (66). Genetic inactivation and small molecule inhibitor studies of *ampG* could result in the development of clinically therapeutic selective inhibitors for *ampG* in particular. Studies of inactive *ampG* in *E. cloacae* demonstrate that AmpG is a required component in order to induce *ampC* transcription through AmpR. Without functional AmpG, inducibility and high-level expression of AmpC appears stunted. Investigating this in *P. aeruginosa* may provide an understanding as to the pathways in study.

Just as with the use of β-lactams in clinically therapy, a combination of antibiotics and inhibitors to, at this stage of knowledge, *nagZ* (but with

further studies *ampG* may be as likely a target), may be necessary to eradicate infections and reduce the chances of spontaneous or selective mutations. The work in this thesis shows that genetic inactivation of *nagZ* effectively reduces resistance to antipseudomonal β-lactams. Genetic inactivation of the gene of interest demonstrated the full extent to which inhibition of NagZ activity could reduce resistance to antipseudomonal β-lactams in PAO1 and the *ampD* deficient mutant PAΔ*DDh2Dh3*. It has also been shown that use of a selective small molecule inhibitor can also reduce resistance to antipseudomonals, providing a benchmark to which the potency of small molecule inhibitors targeting *nagZ*

can be compared (60). Attenuation of β-lactam resistance by genetic inactivation provides strong evidence that strategically-designed drugs targeting *nagZ* could potentially reduce resistance levels to concentrations accepted for clinical therapy use.

Ultimately, targeting both *nagZ* and *ampG* genes to inhibit their activity could suppress the cell wall recycling pathway and its direct connection to the inducible AmpC β-lactamase. A cocktail of selective small molecule inhibitors holds the potential to offer a therapeutic strategy that may lengthen the life span and enhance the quality of life of cystic fibrosis patients suffering from chronic *P. aeruginosa* infections.

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Appendix 1

Growth Media

Merodiploid-Resolving Agar + 5% Sucrose (MRM + 5% sucrose)
6.0 g tryptone
2.5 g yeast extract
0.5 g dextrose
7.5 g agar
Add ddh_2O to final volume of 500 mL.
Autoclave at 121°C for 15 minutes.
Add 50 mL 50% filter-sterilized sucrose.

Super Optimal Broth (SOB)
20 g tryptone
5.0 g yeast extract
2 mL 5M NaCl
0.5 mL 1M KCl
10 mL $MgCl_2$
10 mL $MgSO_4$
Add ddh_2O to final volume of 1 liter.
pH adjusted to 6.0 – 7.0 with NaOH.
Autoclave at 121°C for 15 minutes.

Super Optimal Broth with Catabolite Repression (SOC)
To 1 mL of SOB medium, add 10 µL 1M filter-sterilized glucose.

Electrophoresis Buffer

Tris-Acetate EDTA Buffer (TAE)
242 g Tris base
57.1 mL glacial acetic acid
100 mL 0.5 M EDTA pH 8.0
Add ddh_2O to final volume of 1 liter.
Dilute 50X prior to use.

DNA Loading Buffer
0.06 g - (0.25%) bromophenol blue
0.25% xylene cyanol FF
7.5 mL (30%) – 100 % glycerol in ddH_2O

ddH_2O volume to 25 mL.
Filter sterilize 0.22 µ
Dilute ¼ with ddH_2O prior to use.

Solutions for DNA Analysis

Phosphate-Buffered Saline (PBS)
50 mM NaPi, pH 7.4
100 mM NaCl
Add ddh_2O to final volume of 500 ml.
Autoclave at 121°C for 15 minutes.