

3-20-2015


Characterization of the poxAB Operon Encoding a Class D Carbapenemase in *Pseudomonas aeruginosa*,

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DOI: 10.25148/etd.FI15050210

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

CHARACTERIZATION OF THE *POXAB* OPERON ENCODING A CLASS D
CARBAPENEMASE IN *PSEUDOMONAS AERUGINOSA*

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Diansy Zincke

2015

To: Dean Michael R. Heithaus
College of Arts and Sciences

This dissertation, written by Diansy Zincke, and entitled Characterization of the *poxAB* Operon Encoding a Class D Carbapenemase in *Pseudomonas aeruginosa*, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Florida International University, 2015

DEDICATION

This work is dedicated to my grandmother Marina Diaz Linares and to my parents Maria R. Quintero and Armando Zincke for their constant guidance, support and unconditional love.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my PhD advisor Dr. Kalai Mathee for her continued guidance and support throughout my journey with her. I have learned a lot from her and I'm sure her influence and impact on my life will persist for a long time. I would also like to thank my committee members, Alejandro Barbieri, Ruben Gonzalez, John Makemson and Lynn Silver, for their insightful criticism and guidance throughout the years. I have met many wonderful people throughout my time in the Mathee lab and have derived a lot of support from these colleagues. The Mathee crew has become my family and I'm very grateful to have shared this journey with such a diverse and determined group of people. I'm also very grateful to the financial support provided by the FIU MBRS-RISE (NIH/NIGMS R25 GM61347), the FIU Biology TAsip, the Student Summer Research Award (RISE Biomedical Research Initiative, 08/09) and the Cystic Fibrosis Foundation (ZINCKE08H0). Last but not least, I would like to thank my family and friends for being supportive and patient.

ABSTRACT OF THE DISSERTATION

CHARACTERIZATION OF THE *POXAB* OPERON ENCODING A CLASS D
CARBAPENEMASE IN *PSEUDOMONAS AERUGINOSA*

by

Diansy Zincke

Florida International University, 2015

Miami, Florida

Professor Kalai Mathee, Major Professor

Pseudomonas aeruginosa is a dreaded opportunistic pathogen that causes severe and often intractable infections in immunocompromised and critically ill patients. This bacterium is also the primary cause of fatal lung infections in patients with cystic fibrosis and a leading nosocomial pathogen responsible for nearly 10% of all hospital-acquired infections. *P. aeruginosa* is intrinsically recalcitrant to most classes of antibiotics and has the ability to acquire additional resistance during treatment. In particular, resistance to the widely used β -lactam antibiotics is frequently mediated by the expression of AmpC, a chromosomally encoded β -lactamase that is ubiquitously found in *P. aeruginosa* strains. This dissertation delved into the role of a recently reported chromosomal β -lactamase in *P. aeruginosa* called PoxB. To date, no detailed studies have addressed the regulation of *poxB* expression and its contribution to β -lactam resistance in *P. aeruginosa*. In an effort to better understand the role of this β -lactamase, *poxB* was deleted from the chromosome and expressed *in trans* from an IPTG-inducible promoter. The loss of *poxB* did not affect susceptibility. However, expression *in trans* in the absence of *ampC* rendered strains

more resistant to the carbapenem β -lactams. The carbapenem-hydrolyzing phenotype was enhanced, reaching intermediate and resistant clinical breakpoints, in the absence of the carbapenem-specific outer membrane porin OprD. As observed for most class D β -lactamases, PoxB was only weakly inhibited by the currently available β -lactamase inhibitors. Moreover, *poxB* was shown to form an operon with the upstream located *poxA*, whose expression *in trans* decreased *pox* promoter (P_{pox}) activity suggesting autoregulation. The transcriptional regulator AmpR negatively controlled P_{pox} activity, however no direct interaction could be demonstrated. A mariner transposon library identified genes involved in the transport of polyamines as potential regulators of *pox* expression. Unexpectedly, polyamines themselves were able induce resistance to carbapenems. In summary, *P. aeruginosa* carries a chromosomal-encoded β -lactamase PoxB that can provide resistance against the clinically relevant carbapenems despite its narrow spectrum of hydrolysis and whose activity *in vivo* may be regulated by polyamines.

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LIST OF SYMBOLS AND ABBREVIATIONS

Term/Unit of Measurement	Symbol/Abbreviation
5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside	X-gal
6-aminopenicillanic acid	6-APA
7-aminocephalosporanic acid	7-ACA
Airway surface liquid	ASL
Carbapenem-hydrolyzing class D β -lactamase	CHDL
Congo Red	CR
Coomassie brilliant blue	CB
Cystic fibrosis	CF
Cystic fibrosis transmembrane conductance regulator	CFTR
Degrees Celsius	$^{\circ}\text{C}$
Diaminopimelic acid	DAP
Electrophoretic mobility shift assay	EMSA
<i>et alia</i>	<i>et al.</i>
Epsilometer test	E-test
Extended-spectrum β -lactamases	ESBL
Extended-spectrum oxacillinase	ES-OXA
Gram	g
High molecular mass	HMM
High pressure liquid chromatography	HPLC
Hour	h

Intensive care unit	ICU
Isopropyl β -D-1-thiogalactopyranoside	IPTG
<i>Klebsiella pneumoniae</i> carbapenemase	KPC
Lipopolysaccharide	LPS
Liter	L
Low molecular mas	LMM
Luria-Bertani	LB
LysR-type transcriptional regulator	LTTR
Metallo- β -lactamase	MLB
Microgram	μ g
Microliter	μ l
Micromolar	μ M
Milligram	mg
Milliliter	ml
Millimolar	mM
Minimum inhibitory concentration	MIC
Minutes	min
Mucociliary clearance	MCC
Multicloning site	MCS
Multidrug resistant/resistance	MDR
Open reading frame	ORF
Optical density	OD

Ortho-nitrophenyl- β -galactoside	ONPG
Outer membrane	OM
Overnight	O/N
Penicillin-binding protein	PBP
Percent	%
Periciliary liquid	PCL
Peptidoglycan	PG
<i>pox</i> promoter	P _{<i>pox</i>}
Resistance-Nodulation-Division	RND
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis	SDS-PAGE
Transposon	Tn
Two-component system	TCS
Urinary tract infection	UTI
Ventilator-associated pneumonia	VAP

Chapter 1

Introduction

1.1 Preface

Pseudomonas aeruginosa is one of the most dreaded pathogens in the clinical setting. It is one of the leading causes of nosocomial infections and one of the most troublesome and intractable organisms colonizing the lungs of patients with cystic fibrosis (CF), a deadly genetic disease. Although there are antibiotics and treatment strategies to combat such infections, the organism's inherent and acquired resistance renders many such antipseudomonal treatments ineffective. One important mechanism of resistance, the expression of β -lactamases, is the leading cause of β -lactam resistance in the clinical setting and is the subject of this dissertation. The following introduction is a review of the relevant literature on this important organism and its mechanisms of resistance, with special attention to β -lactam antibiotics and the expression of β -lactamases.

1.2 *Pseudomonas aeruginosa*

P. aeruginosa is an aerobic, non-spore forming, monoflagellated, Gram-negative rod ubiquitously found in the environment (Figure 1.1) (1). It is positive for catalase and oxidase, and produces a fruity or grape-like odor due to the production of 2-aminoacetophenone (2). Although it is unable to ferment lactose and its metabolism is mostly respiratory, it can also grow under anaerobic conditions by using nitrate as a terminal electron acceptor or by fermenting arginine (3, 4).

Like other pseudomonads, renowned for their nutritional versatility, *P. aeruginosa* has very simple nutritional needs and can use a multitude of organic compounds for growth (3). As a chemoorganotroph, it can metabolize a variety of simple

and complex organic substrates as a sole source of carbon and energy. It is also a very versatile, adaptable microbe that is able to tolerate a variety of physical conditions. It has an optimum growth temperature of 37°C, but it is also able to grow at 42°C, unlike some other species of the same genera (3). Most notably, *P. aeruginosa* is inherently resistant to many commonly used antibiotics and chemotherapeutic agents (1, 5).

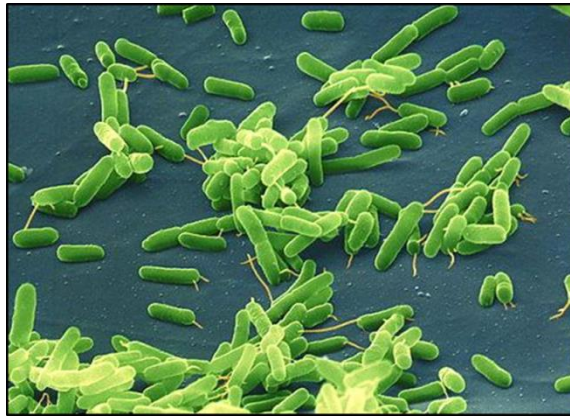


Figure 1.1. Colored scanning electron micrograph of *P. aeruginosa*.
(http://www.visualphotos.com/image/1x6040105/coloured_sem_of_pseudomonas_aeruginosa_bacteria)

Most strains of *P. aeruginosa* produce pyocyanin, a blue-green, water-soluble, non-fluorescent phenazine pigment (3). Phenazine compounds are commonly found in nature and are produced by bacteria such as the *Streptomyces* and *Pseudomonas* spp. Pyocyanin (1-hydroxy-5-methyl-phenazine) is derived from chorismate, an intermediate in the biosynthesis of aromatic amino acids in plants and microorganisms (3). Pyocyanin and other phenazine compounds appear to contribute to the virulence and competitive fitness of the producing organisms (3, 6). For instance, in *P. aeruginosa* pyocyanin contributes to its ability to colonize the lungs of patients with CF as it has been shown to

disrupt the beating of human cilia and inhibit mammalian cell respiration (7, 8). Additionally, this pigment inhibits lymphocyte proliferation and epidermal cell growth (9, 10). Pyocyanin can also act as an antibiotic agent against other bacterial cells by the generation of reactive oxygen intermediates (6). This antimicrobial activity is oxygen-dependent and involves the oxidation of NADH to produce superoxide and hydrogen peroxide (6).

Some strains also produce pyoverdine, a water-soluble, fluorescent pigment that gives *P. aeruginosa* a yellowish-green appearance (1). Pyoverdine functions as a siderophore or iron-scavenging compound, and as such it is known to increase the rate of bacterial iron transport. Siderophores, in general, are extracellular compounds that have a high affinity for iron; they are produced under iron-deficient conditions, and have been known to stimulate and increase bacterial growth rate (1, 11). Production of siderophores during infection has been implicated with bacterial virulence as a consequence of increased growth rate; it also appears to be a factor that can limit the growth of other bacteria as *P. aeruginosa* can effectively compete for iron with other microorganisms (1, 11).

Besides pyocyanin and pyoverdine, *P. aeruginosa* can produce and secrete a myriad of other virulence factors that contribute to its success as a pathogen and allow it to compete against other microorganisms. These include elastase, a zinc protease that destroys immunoglobulins and eukaryotic proteins responsible for maintaining cell structure; exotoxin A, a very toxic protein that inhibits the host cell protein synthesis; exoenzymes S and T, proteins with cytotoxic activity secreted by the type III secretion

system; LasA protease, a staphylolytic enzyme that can destroy β -casein, and rhamnolipids (1, 12). Rhamnolipids, in particular, are very powerful virulence factors capable of destroying polymorphonuclear leukocytes, inhibiting phagocytosis by macrophages, and causing disruption of the mucociliary clearance and of epithelial tight junctions leading to paracellular infiltration of *P. aeruginosa* (13-16). Rhamnolipids have also been shown to have antibacterial activity against a number of microbes including *Staphylococcus aureus*, *Enterobacter aerogenes*, *Streptococcus faecalis*, *Serratia marcescens*, *Klebsiella pneumoniae* and *Proteus vulgaris* to name a few (17, 18).

1.2.1 Habitat

P. aeruginosa is a ubiquitous microorganism commonly found in water, soil, and sewage as well as on the surfaces of plants, animals, insects, fruits and vegetables (1, 19). Natural bodies of water such as lakes and rivers, are also natural sources of the bacterium, especially near sites of urban drainage. It is sometimes part of the human microbial flora infrequently colonizing the skin especially in areas that are moist, and the intestine after ingestion with contaminated foods or fluids (20-23). Its prevalence and colonization in healthy individuals is often low (2.6-25%) with intestinal loads being reported at 3% (24-26).

Its ability to thrive in aqueous environments has made it a problem in the hospital setting where it is often isolated from soaps, ointments, disinfectants, irrigation fluids, and eye drops (27). Its very simple nutritional needs allow it to grow even on the negligible impurities contained in aqueous solutions found in hospitals. Specifically, a study found *P. aeruginosa* was able to not only grow but thrive in the distilled water of

several mist therapy units (28). Tap water is also a major source of nosocomial infections when it is inadvertently used for the preparation of solutions to be used in that setting (29-33). The prevalence of *P. aeruginosa* in tap water likely results from the colonization of showerheads, faucets, and sinks from which it has also been detected (32, 34-38). Additionally, *P. aeruginosa* has also been isolated from holy water, aerators, baby baths, hot tubs, swimming pools, contact lens solutions, cosmetics and even the innersole of sneakers (39-46). In short, aqueous and moist environments seem to be the natural and preferred reservoirs for *P. aeruginosa* and such environments, when contaminated, can be potential sources of infection.

1.2.2 *P. aeruginosa* infections in healthy individuals

P. aeruginosa is rarely a cause for concern in healthy individuals with such infections often resulting from contact with contaminated water or solutions or after sustaining some form of external trauma like a puncture wound (19). Folliculitis, for example, is an infection of the hair follicles caused by bacteria such as *P. aeruginosa*, and can occur after bathing in swimming pools, hot tubs, and whirlpools that are not adequately treated with chlorine (43, 44, 47, 48). Individuals involved in aquatic sports as well as those swimming and bathing in contaminated waters can also develop superficial infections of the ear canal known as external otitis (49-52).

Minor injury to the eye or cornea, often related to the use of contacts lens, especially extended wear lenses, can predispose an individual to eye infections, or keratitis, with *P. aeruginosa* (53-57). Contact lens solutions that are contaminated, or even tap water used for the handling of contact lens, can all serve as potential sources of

infection (19). Osteomyelitis, or infection of the bone, has also been reported, especially in children after incurring puncture wounds in the feet, with the source of the infection often being the sole or inner pad of the sneaker that was worn at the time of the injury (46, 58). Osteomyelitis can also occur in intravenous drug users (59, 60).

One of the most severe *P. aeruginosa* infections that can affect an otherwise healthy person is endocarditis, or inflammation of the inner lining of the heart, often requiring replacement of the affected valve (19). The majority of *P. aeruginosa* endocarditis occurs in intravenous drug users, as the drugs are often mixed with contaminated water leading to bacteremia and endocarditis (61-63). *P. aeruginosa*-related endocarditis, however, can also occur in burn and open heart surgery patients (64-66).

1.2.3 *P. aeruginosa* in nosocomial or healthcare-associated infections

P. aeruginosa is one of the leading causes of hospital-acquired infections accounting for as much as 10-13% of all nosocomial infections with incidences as high as 22% reported in intensive care units (ICUs) (67-71). *P. aeruginosa* was the sixth most frequently isolated pathogen accounting for 7.1% of all healthcare-associated infections in a recent survey of 183 US hospitals from 10 different states (72). Similarly, the latest hospital reporting to the CDC ranks *P. aeruginosa* as the fifth most commonly reported pathogen in nosocomial infections accounting for 7.5% of all healthcare-associated infections (73).

Pneumonia accounts for the majority of nosocomial diseases caused by *P. aeruginosa* (72, 73). *P. aeruginosa* can easily colonize endotracheal tubes and mechanical ventilators and as such has been reported as one of the leading causes of

ventilator-associated pneumonia (VAP), second only to *S. aureus* (73-75). VAP has been associated with high mortality rates that exceed those of other types of pneumonia such as community-acquired, healthcare-associated or hospital-acquired pneumonia (75, 76). Mortality rates have been reported as high as or higher than 70% when *P. aeruginosa* or *Acinetobacter* spp. are the causative agents (74, 76-78). About 11% of nosocomial urinary tract infections (UTIs) are caused by *P. aeruginosa* (72, 73). A higher incidence of infection is associated with the use of urinary catheters, *P. aeruginosa* being the second most commonly identified pathogen in catheter-associated UTIs (73, 79).

P. aeruginosa is also a leading cause of nosocomial infections in burn units primarily colonizing burn wounds but also responsible for pneumonia, bacteremia and UTIs in that setting (80-84). On admission, generally *S. aureus* and coagulase-negative staphylococci predominate the wounds of burn patients but the incidence of *P. aeruginosa* quickly increases during the first week of admission and continues to rise with time, often surpassing the incidence of other microorganisms (82, 85, 86). Burn wound infections with *P. aeruginosa* are especially worrisome since they are correlated with bacteremia, a high rate of sepsis and mortality (81, 82, 87, 88). Other nosocomial infections caused by *P. aeruginosa* include endocarditis, meningitis, bacteremia, gastrointestinal and surgical site infections (72, 73, 89).

1.2.4 *P. aeruginosa* in immunocompromised patients

P. aeruginosa is an opportunistic human pathogen that readily exploits any deficiency in the host immune system to mount an infection. Since it is often intractable and resistant to a wide range of antibiotics, it represents a very serious problem not only

for critically ill individuals, such as those in the hospital setting and ICUs, but also for immunocompromised patients. *P. aeruginosa* has often been isolated as one of the most common pathogens causing septicemia in patients with primary immunodeficiencies (90, 91). A number of other conditions can predispose the host to *P. aeruginosa* bacteremia including taking broad spectrum antibiotics, receiving chemotherapy, as well as being an HIV, leukemia, cancer, diabetes, bone marrow or organ transplant patient (92-99).

Although *P. aeruginosa* bacteremia has been reported in patients with AIDS (100, 101), a number of studies agree that pathogens other than *P. aeruginosa* are the most common cause of bacteremia in that population (95, 102). *P. aeruginosa* however, is one of the leading causes of pneumonia in HIV patients (103-106) and a common respiratory pathogen in patients with AIDS, where pulmonary and respiratory disease infections are often chronic and intractable (107-111).

Before 1968, *P. aeruginosa* bacteremia in cancer patients resulted in ~80-90% fatalities (112-115). The development of more potent antipseudomonal drugs has greatly improved outcomes and survival rates in these patients, provided that the bacteremia is treated rapidly, with studies from the 1980s and 2000s showing a 60 and 80% cure rate, respectively (92, 98, 116, 117). Although prognosis has improved, the incidence of *P. aeruginosa* infections in cancer patients for the decade of the '90s was found to range from 5-12% (118), while in a report from 2008, carbapenem-resistant *P. aeruginosa* infections were documented in 20% of the cancer patients (119). A recent study found that *P. aeruginosa* was the leading causative agent of bloodstream infections (42%) and

pneumonia in patients with solid tumors, with 22% incidence of bloodstream infections in patients with haematological malignancies (120).

1.2.5 *P. aeruginosa* and cystic fibrosis

P. aeruginosa is the most common and clinically relevant pathogen found in patients with CF (121-123). It is estimated that over 80% of CF patients will be infected with this bacterium by the time they reach adulthood (122). Infection of the airways with *P. aeruginosa*, and the inflammation that follows, represent a major problem for CF patients as the lungs steadily deteriorate auguring very poor overall prognosis and high mortality rates (124-126). As of 2012, the life expectancy for a patient with CF in the United States was around 37.8 years (122).

CF is an autosomal, recessive genetic disease affecting about 30,000 people in the US and about 70,000 worldwide (122). This disease mainly affects Caucasian populations of European descent and is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene located in the long arm of chromosome VII (127-129). The CFTR protein is a cAMP-regulated chloride channel found on the apical surface of various epithelial cells and responsible for the transport of chloride ions into and out of the cell (130-136). Additionally, the CFTR protein is known to regulate other membrane conductance channels including the outwardly rectifying chloride channel (137-139) and the epithelial sodium channel, ENaC (140-143).

More than 1800 *CFTR* mutations have been reported so far, the majority being rare and not yet well understood (122, 144). Most mutations can be grouped into one of six classes according to the level of disruption or impairment of CFTR function (Figure

1.2). Classes I, II and III are associated with a more severe form of the disease including pancreatic insufficiency and defective pulmonary function (145-147). Patients with class IV, V and VI mutations, on the other hand, generally exhibit a milder presentation and have a functional pancreas (146, 148, 149). The most common mutation, a class II mutation present in 86.7% of the CF patients worldwide, is a deletion in phenylalanine at position 508 ($\Delta F508$) resulting in misfolding of the protein and subsequent degradation by proteasomes (122, 147).

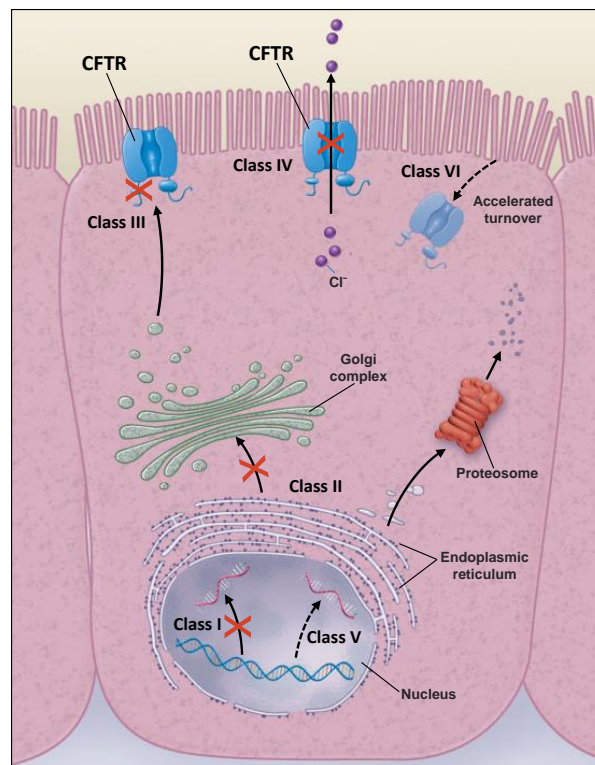


Figure 1.2. Classes of *CFTR* mutations. *CFTR* mutations can result in no CFTR protein being expressed (class I), misfolded protein that is degraded by proteasome (class II), a CFTR protein that does not respond to cAMP (class III), defective chloride conductance or channel gating (class IV), a reduced number of CFTR transcripts due to a promoter or splicing abnormality (class V) and accelerated turnover from the cell surface (class VI) (148, 150-153). Reproduced with permission from (153), Copyright Massachusetts Medical Society.

There is an ongoing debate as to how *CFTR* mutations cause lung disease in patients with CF but most proposed models converge on one point: the imbalance of water and ions created by a malfunctioning *CFTR* protein in the apical surfaces of airway epithelium ultimately leads to abnormal or defective mucociliary clearance (MCC) (154-159). MCC is an important defense mechanism against inhaled particles, allergens, bacteria and viruses (160-162). Normal airway epithelium is ciliated and covered with a layer of liquid known as the airway surface liquid (ASL) (163-165). The ASL itself is divided into a fluid layer of low viscosity known as the periciliary liquid (PCL) or sol layer that keeps the cilia hydrated and facilitates its beating, and a more viscous upper gel or mucus layer that is responsible for trapping foreign particles (Figure 1.3A) (164-167). The beating of the cilia continuously propels the mucus layer with its trapped particles upwards towards the nasopharynx and the mouth where foreign substances can be then swallowed or expectorated (161, 166, 168, 169).

The most strongly supported and generally accepted hypothesis of pathobiology in CF is that abnormal transport of chloride ions across epithelial membranes coupled with the increased absorption of sodium and water through the epithelial sodium channel, ENaC, leads to dehydration of the ASL layer (Figure 1.3B) (170-174). This hypothesis, known as the low-volume hypothesis, states that the depletion of the airway surface liquid reduces the volume or height of the PCL layer and increases the viscosity of the mucus layer leading to compression and impaction of the cilia (170, 174-176). Impaction of the cilia is thought to compromise its proper functioning and to diminish its capacity to work in concert with the mucus layer to remove foreign particles (154, 156). The end

result is impairment of the MCC apparatus setting up the stage for the persistent and chronic bacterial infections that are a hallmark of airway disease in CF patients (Figure 1.4).

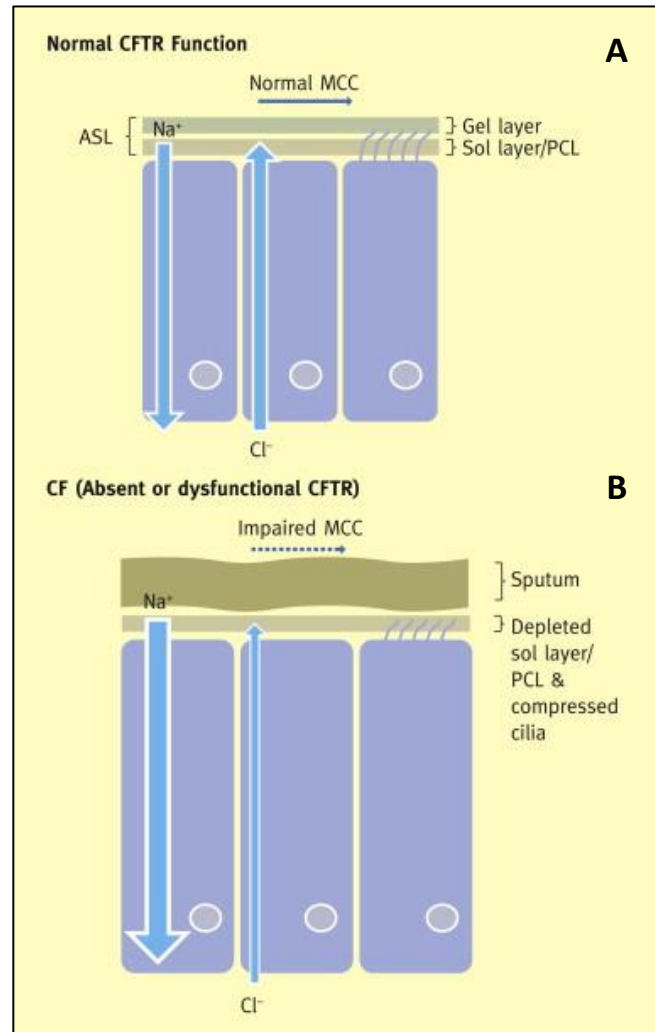


Figure 1.3. ASL layer and MCC in the normal and CF airways. **A.** Normal CFTR function. Under normal conditions, the PCL layer is hydrated and the cilia beat normally allowing MCC from the mucus layer. **B.** Abnormal or nonfunctioning CFTR leads to dehydration and reduction in volume of the PCL layer resulting in impacted and improperly beating cilia that hinders MCC (177). Reproduced from (177) with permission from Elsevier.

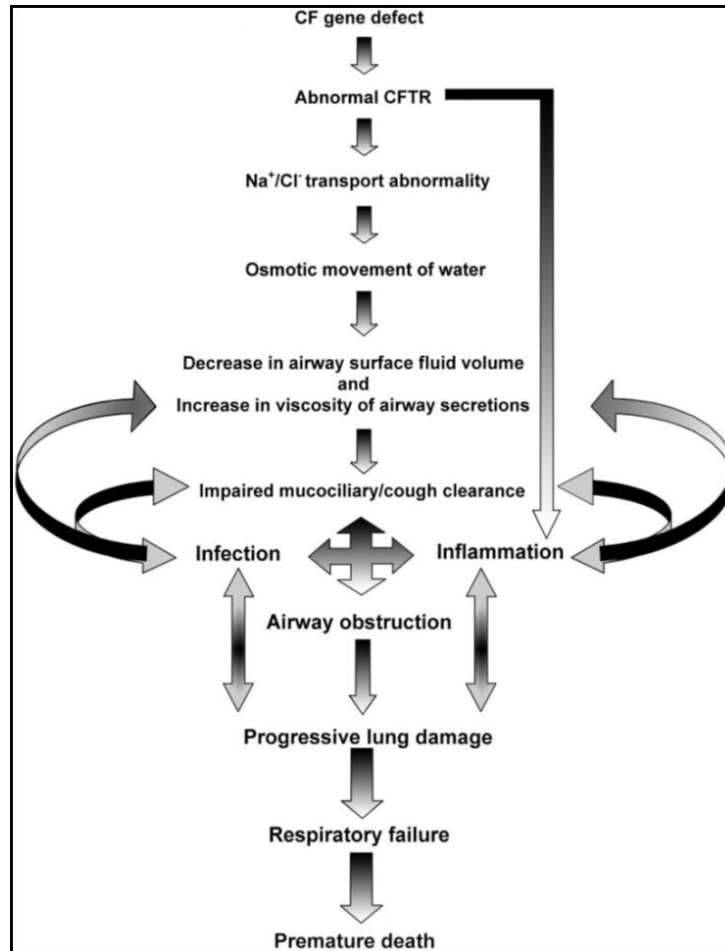


Figure 1.4. Pathobiology of CF lung disease. In the most widely accepted hypothesis of pathobiology of CF lung disease, reduced airway surface liquid results in impairment of MCC leading to successive cycles of infection and inflammation eventually resulting in obstruction of the airways, chronic deterioration of the lungs and death. Reproduced from (154) with permission from John Wiley and Sons.

CF, however, is a multi-organ disease not just limited to the lungs, as the CFTR protein is expressed in the epithelium of several other organs including the pancreas, sweat ducts, intestine, biliary tree and the vas deferens (178-184). For example, in the intestine it can lead to meconium illium, a condition where the first stool of the newborn

is impacted in the intestine (182, 185). Additionally, more than 95% of men with CF are usually infertile (186-188). Lung disease, however, remains one of the main concerns for patients with CF as continuous cycles of infection and inflammation herald gradual lung deterioration and eventual pulmonary failure (Figure 1.4).

The conditions present in CF airways, namely dehydrated, thick mucus coupled with impaired mucociliary clearance, provide the ideal environment that is conducive to colonization by a number of pathogens (123, 189). *S. aureus*, for example, often the first to colonize the respiratory tract of CF patients, is common in children less than 10 years old, and responsible for infant morbidity and mortality in the preantibiotic era (Figure 1.5) (122, 123). *Haemophilus influenzae* is another common pathogen and is predominantly found in young children (123, 189). Other less common pathogens such as *Stenotrophomonas maltophilia* (previously *Xanthomonas maltophilia*), *Alcaligenes xylosoxidans* and *Burkholderia cepacia complex*, have also been isolated from the respiratory tract of CF patients, the latter having the poorest prognosis, although found in less than 10% of patients (122). A recent study examined the microbiome of sputum from CF patients and found that although the patients were not being treated for fungal infections, many (18/19) did carry pathogenic fungal species of *Aspergillus*, *Candida*, *Cryptococcus* and *Exophiala*, among others (190). Fungal infections are in fact not uncommon in CF patients and can occur in association with other microorganisms (190-193). *P. aeruginosa*, however, remains the main pathogen associated with morbidity and mortality in CF patients and is more frequently found in adults (Figure 1.5) (122, 124, 126, 194).

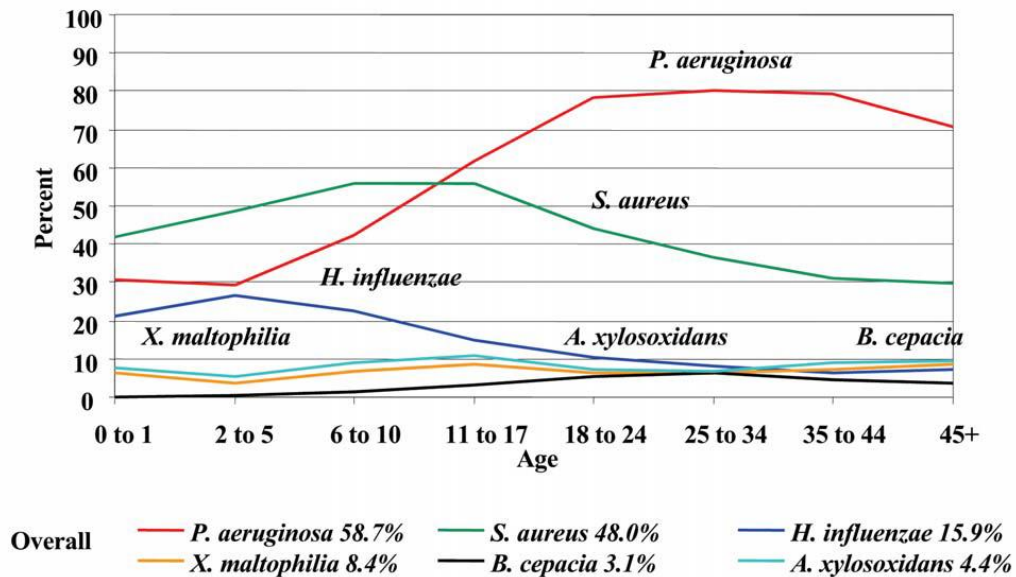


Figure 1.5. Pathogens found on the CF lungs by age. From an early age, CF patients are infected with a number of different organisms. By the time they reach adulthood, *P. aeruginosa* is the predominant pathogen (122).

An important feature of *P. aeruginosa* infections, is the tendency of this bacterium to convert to a mucoid phenotype in the lungs of CF patients, an event which signals the beginning of chronic stage of the infection leading to a progressive decline in lung function (195-197). This mucoid phenotype is characterized by the production of large quantities of alginate, a polysaccharide also known as mucoid exopolysaccharide, which plays a very important role in helping the bacterium evade the host immune system (123, 198, 199). Alginate promotes a biofilm mode of growth, which in turn not only protects the bacteria from antimicrobial agents, but also helps the microorganisms escape phagocytosis by macrophages (200). Alginate overproduction exacerbates the already detrimental conditions of the CF lungs leading to further blocking of the airways and inexorable death.

1.2.6 Treatment

Treatment of *P. aeruginosa* infections varies greatly given severity of infection and resistance profile of the organism. Current strategies include treatment with a single drug, usually a β -lactam or an aminoglycoside, or combination therapy with at least two different antimicrobial agents (189, 201, 202). β -lactams, include a broad class of bactericidal agents, widely used in the clinical setting, and with minimal side effects to eukaryotes (Figure 1.6) (203-205). Several β -lactams have shown reliable results against *P. aeruginosa*, including the extended-spectrum penicillins ticarcillin and piperacillin that can be combined with the β -lactamase inhibitors clavulanic acid and tazobactam, respectively, to enhance the antibacterial action of the β -lactam (201, 202). The cephalosporin ceftazidime is stable to most β -lactamases and is the drug of choice if the isolate is determined to be susceptible (205, 206).

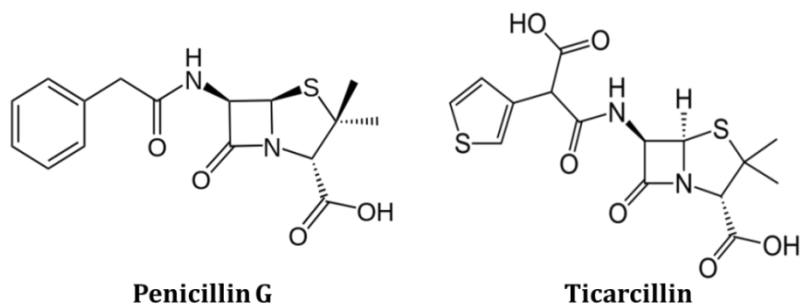


Figure 1.6. Molecular structures of two β -lactams of the penicillin class.

The carbapenem β -lactams can be used if the organism is resistant to extended-spectrum penicillins and cephalosporins (207, 208). Carbapenems have a wide spectrum of activity but can select for multidrug resistant (MDR) strains of *P. aeruginosa*, especially imipenem. The monobactam aztreonam, is the drug of choice if the patient is

allergic to penicillins and other β -lactams (209, 210). It can be helpful in treating isolates that produce metallo- β -lactamases as it is resistant to hydrolysis by class B enzymes.

Combination therapy is usually the first line of defense, particularly for MDR pseudomonal infections (189, 201). The most commonly used combination includes the administration of a β -lactam with an aminoglycoside. The aminoglycosides are powerful antimicrobial agents that inhibit bacterial protein synthesis being predominantly active against aerobic, Gram-negative bacteria (211, 212). The discovery of gentamicin in 1963 was a breakthrough in the treatment against *P. aeruginosa* infections, and subsequently other more powerful aminoglycosides, such as tobramycin and amikacin, were developed (213). Tobramycin has shown greater activity *in vitro* than most other aminoglycosides, and thus it is often the drug of choice to treat *P. aeruginosa* infections (211). Amikacin, however, is very effective against bacteria that are resistant to other aminoglycosides such as gentamicin and tobramycin. Its chemical structure makes it less susceptible to the inactivation by the enzymes that normally inactivate gentamicin and tobramycin (Figure 1.7) (211).

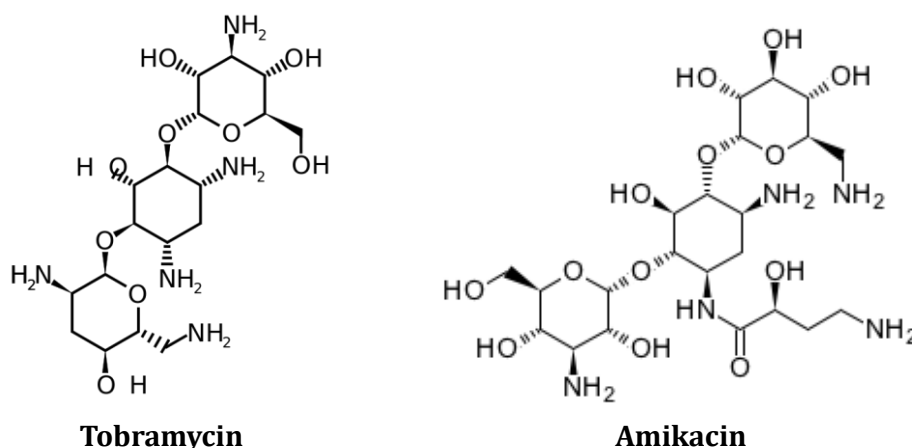


Figure 1.7. Molecular structures of amikacin and tobramycin.

Aminoglycosides, however, can often have nephrotoxic and ototoxic side effects (214). Nephrotoxicity is usually reversible but ototoxicity, or the toxic effect on the inner ear, is generally irreversible and can lead to hearing loss (215). Thus, aminoglycosides should only be administered when no other antibiotic is deemed as effective, for instance in the treatment of MDR organisms and of critically ill patients, and should be replaced with less toxic agents as soon as the sensitivity profile of the organism is ascertained (211). Combination therapy with aminoglycosides often includes amikacin or tobramycin with either piperacillin, ticarcillin, ceftazidime, imipenem, meropenem or aztreonam (189, 201).

Another option when considering therapy for *P. aeruginosa* infections, is the combination of either an aminoglycoside or a β -lactam with ciprofloxacin (216, 217). Ciprofloxacin belongs to a class of antibiotics known as fluoroquinolones (218). Fluoroquinolones have a broad-spectrum of activity and act by preventing DNA replication through the inhibition of the enzyme DNA gyrase (218). Ciprofloxacin has great activity against *P. aeruginosa* and Gram-negatives and unlike the rest of above antibiotics, the majority of which are for parenteral use or aerosolized, it is available for oral administration (219, 220). It has been particularly good at treating respiratory infections in CF patients but prolonged monotherapy can lead to resistance (219, 221-224). Additionally, serious side effects, such as damage of young cartilage, tendonitis and tendon rupture, hypoglycemia and arrhythmias, have been reported (225, 226). It is often used in conjunction with inhaled antibiotics like the aminoglycoside tobramycin or the polymyxin colistin (123, 227).

Colistin was one of the first drugs to show good *in vitro* activity against *P. aeruginosa* (228-230). It works by disrupting the structure of the outer membrane and increasing cell permeability leading to cell lysis (231). Earlier studies however, reported serious nephrotoxic and neurotoxic effects and thus its use was relegated to the external treatment of eye and skin infections (232, 233). The emergence of MDR strains, unresponsive to broad spectrum antibiotics but remaining susceptible to the drug, has brought colistin back into the antimicrobial limelight (234, 235). Despite its earlier reported toxicity, recent studies show its usefulness outweighs potential risks, which have also been shown to be lower than originally reported, perhaps as a result of better dosing strategies (236, 237). Although reported nephrotoxicity rates for intravenous use are up to 14%, in many cases a lack of better alternatives has redefined colistin as the last line of treatment or salvage therapy (238, 239). For the treatment of respiratory infections, in both CF and non-CF patients, colistin can be administered as an aerosol, which has the advantage of a more direct higher dose delivery to the site of infection while limiting toxicity (240-242). Colistin has also been shown to be effective against the inner structure (core and stalk) of the *P. aeruginosa* biofilm suggesting this antimicrobial peptide can penetrate this complex structure (243).

1.3 Mechanisms of intrinsic antibiotic resistance

P. aeruginosa can employ a number of mechanisms to evade, prevent or inhibit the actions of antibiotics, the most common of which include restricted membrane permeability, active efflux of antibiotics, alteration of drug targets, the formation of

biofilm, and the expression of β -lactamases (Figure 1.8) (244-247). The following sections will review some of these important mechanisms.

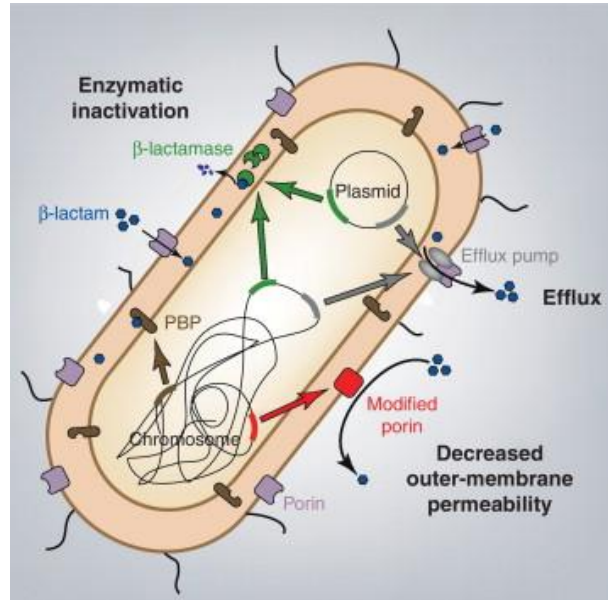


Figure 1.8. Mechanisms of antibiotic resistance in *P. aeruginosa*. *P. aeruginosa* employs multiple mechanisms to inhibit the action of antibiotics including an intrinsically low membrane permeability, expression of efflux pumps, modification of antimicrobial targets and production of β -lactam-hydrolyzing enzymes. Reproduced from (248) with permission from Elsevier.

1.3.1 Extrapermeability barrier

The cell wall of Gram-negative bacteria is composed of an inner membrane, a thin peptidoglycan layer and outer membrane (OM) that serves as a selective permeation barrier by restricting the passage of solutes across the membrane (Figure 1.9) (249, 250). The OM is generally an asymmetric lipid bilayer with an inner leaflet of phospholipids and a surface layer rich in lipopolysaccharide (LPS) (251, 252).

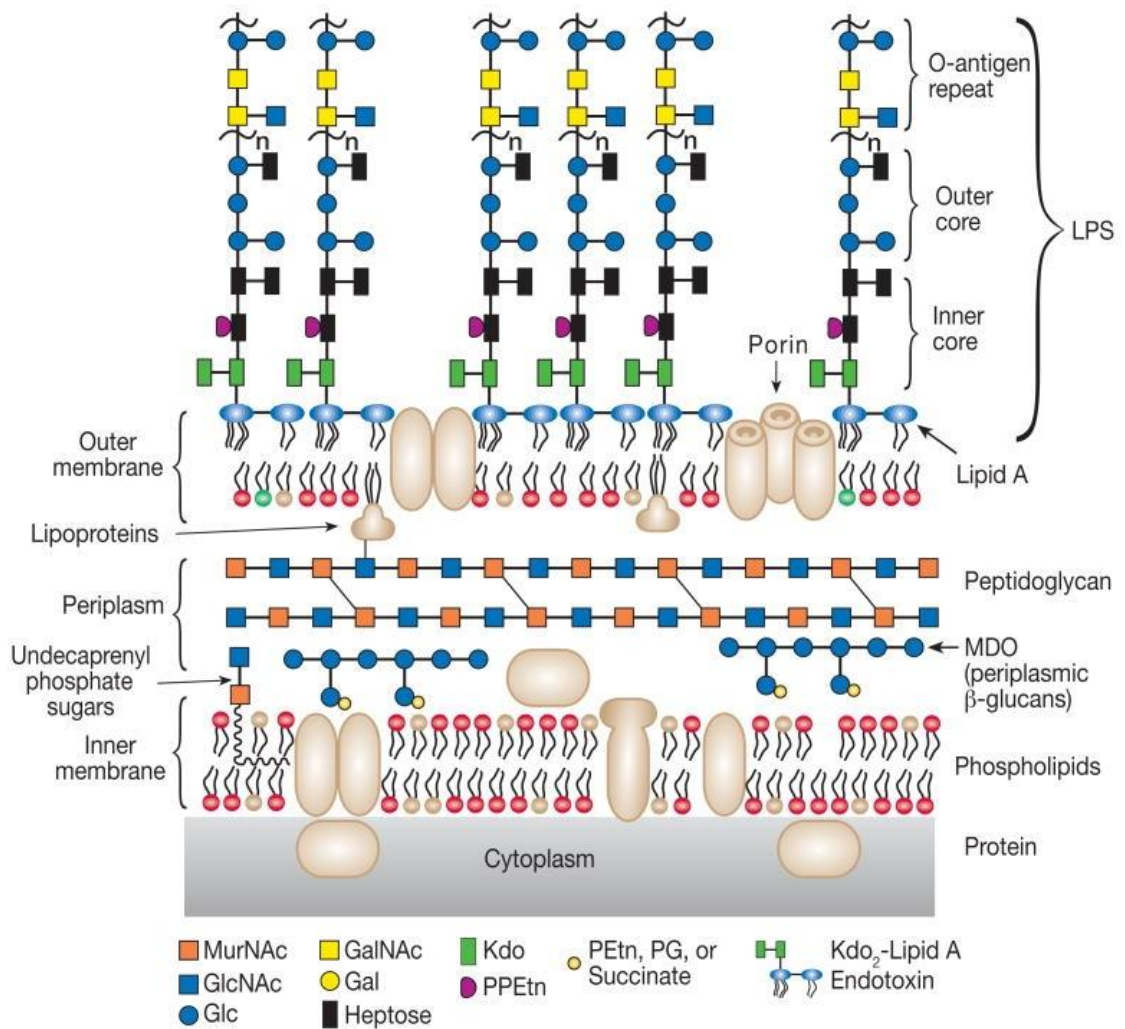


Figure 1.9. Cell wall of Gram-negative bacteria. An inner phospholipid bilayer containing mostly phosphatidylethanolamine (red lipids) with some phosphatidylglycerol (light brown lipids) surrounds the cytoplasm of the bacterial cell. The periplasm, or the space sandwiched between the inner and outer membranes, contains a thin peptidoglycan layer; membrane-derived oligosaccharides (MDO) can also be present. The outer layer of the OM is rich in LPS. Kdo, 3-deoxy-D-manno-octulosonic acid; (n) variable number of O-antigen repeats; (PPEtn), pyrophosphoethanolamine (253).

(<https://amit1b.wordpress.com/the-molecules-of-life/10-the-living-cell-gallery/>)

In order to reach their targets inside the cell, antibiotics must first cross the outer membrane. Permeability of the OM is largely governed by the low fluidity structure of the LPS layer and by the presence of channels or pores in the membrane known as porins (250, 254). Generally, small, hydrophilic compounds, like β -lactams, are taken up by porins, while lipid-mediated pathways are thought to be responsible for the uptake of hydrophobic antibiotics like aminoglycosides and polymyxin (255-257).

1.3.1.1 LPS as a barrier and lipid-mediated uptake

LPS is one of the first barriers that solutes encounter when traversing the cell. It is usually comprised of lipid A, the core sugars and the O antigen, and it is a very strongly negatively charged structure (258, 259). Divalent cations such as calcium (Ca^{2+}) and magnesium (Mg^{2+}) have a high affinity for LPS and help to neutralize some of these negative charges while at the same time bridging neighboring LPS molecules (260-265). Bridging of adjacent LPS molecules through divalent cations, as well as through hydrogen bonding, promotes strong lateral interactions, stabilizes the structure and creates a gel-like core of low fluidity in the lipid interior restricting the passage of solutes and antibiotics across the membrane (266-268).

The low fluidity of the LPS structure is thought to restrict partitioning by hydrophobic compounds and antibiotics (255, 269). Hydrophobic probes have been shown to be excluded from the membranes of *P. aeruginosa* and *Escherichia coli* (270, 271). Similarly, *P. aeruginosa* and other Gram-negative bacteria intrinsically display high MICs (minimum inhibitory concentration) against hydrophobic antibiotics such as macrolides, novobicin, clindamycin and fusidic acid. (255, 272). Defects in efflux,

however, have been shown to reduce the MICs of hydrophobic antibiotics. The intrinsic resistance to hydrophobic antibiotics thus, is determined not only by the normal structure of LPS but also by the ability of some of these compounds to be effluxed. LPS is generally considered effective at slowing down the passage of hydrophobic antibiotics and permeation rates through the outer membrane have been found to be 50-100 times slower than through a simple lipid bilayer (273).

The structure of LPS, however, can be compromised by the presence of polycationic compounds (274-276). Polycations, having sometimes a higher affinity for LPS than divalent cations, can readily displace them from their regular binding sites in the LPS, leading to disruptions in the membrane barrier and increasing permeability of the outer membrane (271, 275). Specifically, the bulkiness of the polycations is thought to create transient cracks in the structure of the LPS, thus allowing uptake of other compounds such as hydrophobic antibiotics and of the polycations themselves in a process known as self-promoted uptake. Examples of antibiotics that promote their own entry into the cell by disrupting the normal structure of LPS via their cationic nature include aminoglycosides, polymyxins, cationic detergents and polycationic peptides, among others (256, 276-281). Permeabilization of the outer membrane with such compounds can additionally sensitize the cells to permeation by other compounds and antibiotics including lysozyme, β -lactams, and a number of hydrophobic antibiotics (erythromycin, clindamycin, novobiocin, fusidic acid and cloxacillin) (274, 282-284). Membrane permeabilizers, such as the metal chelator EDTA, have a similar effect and

can enhance the uptake of a number of compounds and antibiotics that are not themselves permeabilizers (285).

1.3.1.2 Porin-mediated transport

A major determinant of permeability is the presence of water-filled channels in the outer membrane called porins. OprF is the major general porin in *P. aeruginosa* (286, 287). It is a non-specific channel for the passage of large substrates and has an exclusion limit of about 3000 daltons in contrast to 600 daltons for OmpF, the general porin in *E. coli* (287-289). Notwithstanding the large pore size and its abundance in the outer membrane, OprF is considered a slow porin because of its slow rate of diffusion and low permeability (289, 290). A study showed that the rate of arabinose influx was 50 times slower through the *P. aeruginosa* OprF than through the *E. coli* OmpF channel (291). In general, the rate of solute diffusion across the outer membrane of *P. aeruginosa* has been at least two orders of magnitude lower than that of *E. coli* (287, 292).

The slow rate diffusion has been attributed to the conformation of the OprF channel in *P. aeruginosa*. Various studies suggest that OprF can exist in two different conformations, open or closed, and that only a minority of the channels are ever open, thus accounting for the decreased permeability as compared to *E. coli* (291, 293-295). While having the majority of channels closed may seem arbitrary, the periplasmic C-terminus of the closed conformer is predicted to interact with the peptidoglycan layer as a way to stabilize the structure of the cell envelope (296-299). Thus, the closed conformer of OprF appears to play a major structural role in *P. aeruginosa* besides that of permeability, its absence leading to abnormal cell morphology and destabilization of the

outer membrane (300, 301). However, even with only about 5% of the channels open at any one time, OprF is still a major determinant of permeability in *P. aeruginosa* (293, 302).

Another porin affecting permeability and β -lactam resistance is the substrate-specific porin OprD. OprD is specific for basic amino acids, dipeptides and carbapenems (imipenem, meropenem, doripenem) (20, 21, 23, 34). In the clinical setting, exposure to imipenem often leads to mutations, deletions or insertions in *oprD* and loss of the protein resulting in impermeability and clinical resistance to imipenem (29-33, 38). Although loss of OprD also increases the MIC against meropenem, it does not by itself usually lead to clinical resistance (303-305), presumably because meropenem can also get inside the cells via other routes (306-308). Additionally, both meropenem and doripenem are subject to efflux by the MexAB system while imipenem is not (309-312). Thus, clinical resistance to imipenem can happen with just the loss of OprD (313-315), while both absence of OprD and upregulation of the MexAB pump are needed for meropenem and doripenem resistance to occur (304, 309-311, 316).

As previously mentioned, OprD also mediates the uptake of basic amino acids and peptides that structurally resemble carbapenems (Figure 1.10) (21, 23). The positively charged residues at C2 of imipenem closely resemble a number of basic amino acids such as histidine, lysine, arginine and ornithine that not surprisingly have been shown inhibit imipenem transport through OprD (317). Small peptides containing lysine have also been shown to inhibit transport, while addition of basic amino acids to different media increased the resistance of different *P. aeruginosa* strains against carbapenems

(22, 23, 36). Such finding could be of concern in the clinical setting as the empirically demonstrated MIC might not reflect the real MIC in the blood or serum where the presence of basic amino acids may reduce the efficacy of carbapenem treatment.

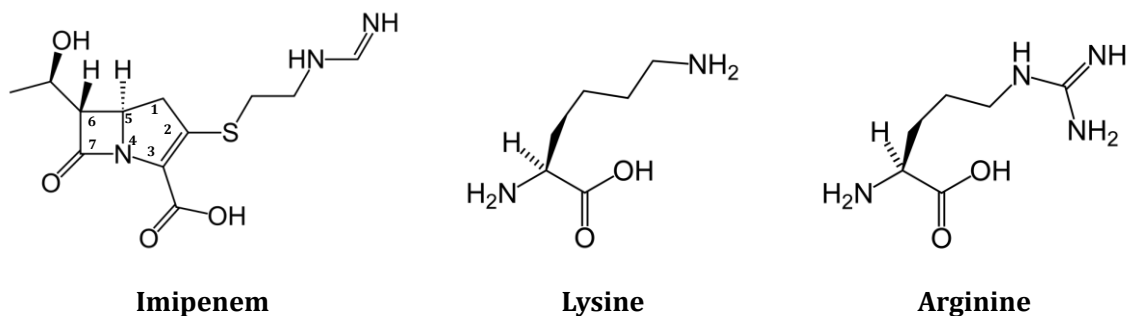


Figure 1.10. Molecular structures of the β -lactam imipenem and of the amino acids L-lysine and L-arginine.

Many other specific and gated porins, for uptake of a myriad of substrates including iron and carbohydrates, exist in the outer membrane of *P. aeruginosa* (318). As they have not been associated with resistance, they will not be further discussed here. The porins involved in efflux are examined below.

1.3.2 Efflux pumps

Efflux pumps are ubiquitous in nature and have been recognized in both prokaryotic and eukaryotic cells (319). They are membrane-bound pumps that transport a variety of solutes such as dyes, antibiotics and toxic compounds to the outside of the cell by using the energy of ATP hydrolysis or the energy derived from transmembrane electrochemical gradients, such as the proton motive force (320, 321). Efflux pumps can be substrate specific or they can transport a variety of structurally different compounds,

including different classes of antibiotics (319). Pumps that are able to export a wide range of antibiotics are often associated with multidrug resistance (319). Efflux-determined resistance can be caused by an increase in expression of efflux proteins, often as a result of mutations in efflux regulatory genes, or by mutations that enhance the export capabilities of the pump itself (320).

Five different classes of efflux pumps have been described in bacteria: the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the multidrug and toxic compound extrusion (MATE) family, the ATP-binding cassette (ABC) family, and the resistance nodulation-division (RND) family (Figure 1.11). RND efflux pumps are of particular interest in Gram-negative bacteria where they play a crucial role in intrinsic and acquired resistance in synergy with the outer membrane barrier (246, 322, 323). They are responsible for the export of a variety of substrates such as toxic substances, dyes, detergents, antibiotics, as well as substances produced by the host such as bile, hormones and antimicrobial peptides (324-328). RND pumps typically have a tripartite arrangement comprised of an inner membrane transporter, a periplasmic adaptor protein and an outer membrane channel that driven by the proton motive force, work in concert to transport solutes across two membranes towards the extracellular space (329, 330). Capture of solutes from the periplasm has also been reported in both *E. coli* and *P. aeruginosa* (331-333).

Twelve RND efflux pumps have now been described in *P. aeruginosa*: MexAB-OprM (317, 334, 335), MexCD-OprJ (336, 337), MexEF-OprN (338), MexGHI-OpmD (339, 340), MexJK (341), MexMN (342), MexPQ-OpmE (342), MexVW (343), MexXY

(344, 345), TriABC-OpmH (346), MuxABC-OpmB (347) and CzcAB-OpmN (348). Together they form a family of multidrug efflux pumps with an extensive substrate range that includes aminoglycosides, fluoroquinolones, β -lactams, tetracycline, chloramphenicol and macrolides, among others (Table 1.1).

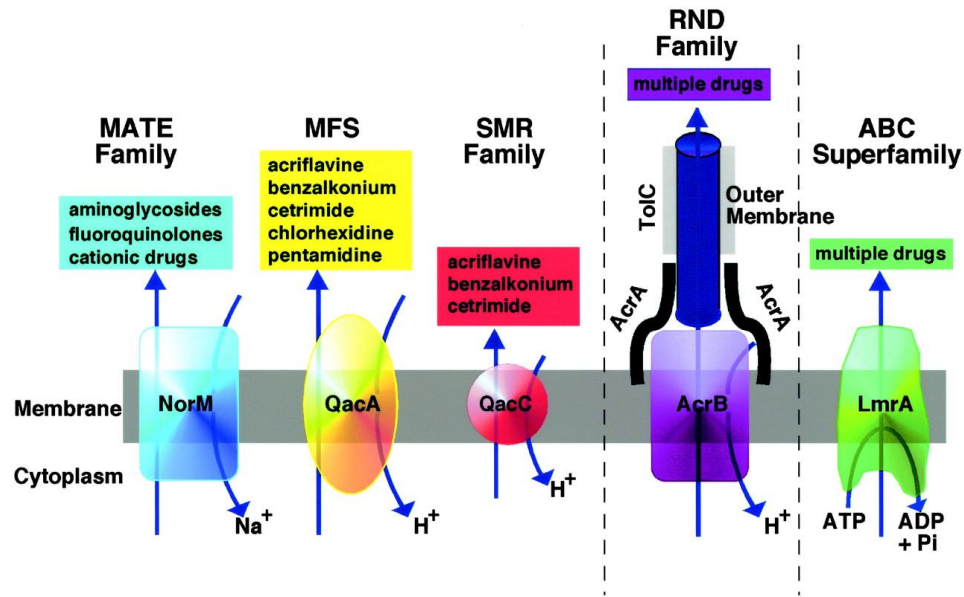


Figure 1.11. Family of efflux pumps found in bacteria and their substrates. Reproduced from (319) with permission by the American Society for Microbiology.

RND efflux-encoding genes are organized into operons frequently in association with an immediately upstream located transcriptional regulatory gene (320, 322, 349). The first gene of the RND operon (*mexA*, *mexC*, *mexE*, and *mexX*) generally encodes a membrane fusion protein connected to the cytoplasmic membrane and expanding through the periplasm. The second gene (*mexB*, *mexD*, *mexF*, and *mexY*) encodes an inner membrane transporter that moves the substrate across the cytoplasmic membrane into the periplasm. An additional gene may be present in some operons (*oprM*, *oprJ*, and *oprN*). This third gene encodes an OM protein that allows passage of substrates across the outer

membrane into the extracellular space. Operons that do not code for the outer membrane protein themselves, may instead use the OM protein from another pump. For instance, the *mexXY*, *mexJK*, *mexMN*, and *mexVW* operons, lacking an OM-encoding gene, use the OprM protein of the MexAB system as an outer membrane channel, MexJK can additionally use OpmH (349).

Table 1.1: Substrate profile of RND efflux pumps described in *P. aeruginosa* (312, 323).

Efflux pump	Substrates
MexAB-OprM	Fluoroquinolones, tetracycline, chloramphenicol, β -lactams (except imipenem), β -lactamase inhibitors, novobiocin, macrolides, trimethoprim, tigecycline, triclosan, sulfonamides, ethidium bromide, SDS, aromatic hydrocarbons, thiolactomycin, cerulenin, acylated homoserine lactones
MexCD-OprJ	Fluoroquinolones, tetracycline, chloramphenicol, β -lactams (except imipenem), novobiocin, macrolides, trimethoprim, triclosan, ethidium bromide, SDS, aromatic hydrocarbons, crystal violet
MexEF-OprN	Fluoroquinolones, chloramphenicol, trimethoprim, aromatic hydrocarbons, triclosan
MexGHI-OpmD	Norfloxacin, vanadium
MexJK	Tetracycline, erythromycin, triclosan
MexMN	Chloramphenicol, thiamphenicol
MexPQ-OpmE	Fluoroquinolones, tetracycline, chloramphenicol, macrolides
MexVW	Fluoroquinolones, tetracycline, chloramphenicol, erythromycin, ethidium bromide, acriflavine
MexXY	Fluoroquinolones, aminoglycosides, tetracycline, erythromycin macrolides, chloramphenicol several β -lactams, not imipenem,
TriABC-OpmH	Triclosan
MuxABC-OpmB	Aztreonam, macrolides, novobiocin, tetracycline
CzrAB-OpmN	Cadmium, zinc

MexAB-OprM is the major contributor of efflux-mediated intrinsic resistance in *P. aeruginosa* (350). Although constitutively expressed, the MexAB-OprM pump mediates resistance to varied antimicrobial agents having the widest substrate range of all known RND *P. aeruginosa* pumps (317, 335). Recently, Yang *et al.* studied the promoter activity of all 12 RND pumps found in *P. aeruginosa* (351). In agreement with previous reports, *mexAB-oprM* had the highest activity in all conditions tested. *mexJK*, *mexVW*, *mexXY* and *mexABC-opmB* were also constitutively expressed, but at much lower levels than *mexAB*. Still lower, but detectable activity was observed for *mexGHI-opmD*, *mexPQ-opmE* and *mexMN*, with no activity detected for the rest of the pumps.

These promoter expression studies are in agreement with previous reports that show most pumps are either silent or constitutively expressed and that their contribution to innate resistance is null (322, 350). In particular, disruptions of *mexCD-oprJ* and *mexEF-oprN* were not previously shown to affect susceptibility, suggesting these two pumps do not contribute to intrinsic resistance (338, 352). The MexXY system, on the other hand, is thought to contribute to the intrinsic resistance of *P. aeruginosa* against tetracycline, erythromycin, and gentamicin, as deletion of *mexXY* increases susceptibility against these antibiotics (353). Not surprisingly, these compounds, but not other MexXY substrates, were shown to induce expression of *mexXY*.

Although most pumps are silent or only weakly expressed, and likely not playing a role in innate resistance, their contribution should not be discounted as it is often mutations in their regulatory genes or regions (known and unknown), that lead to overexpression and multidrug resistance. For instance, MexCD-OprJ is silent in wild-type

but hyperexpression and variable levels of resistance have been attributed to *in vitro* and *in vivo* mutations in its repressor-encoding gene, *nfxB* (336, 354). Such mutations have also been reported in the regulatory genes for *mexEF-OprN* and *mexJK*, as well as for the operons already providing intrinsic resistance, *mexAB-oprM* and *mexXY* (322, 349). Thus collectively, the RND efflux pumps are a major determinant of efflux-mediated resistance in *P. aeruginosa*.

1.3.3 Alteration and modification of penicillin-binding proteins

The peptidoglycan layer of Gram-negative bacteria consists of alternating subunits of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), where MurNAc bears a stem peptide often consisting of L-alanine, D-glutamic acid, L-lysine or meso-diaminopimelic acid (meso-DAP), and D-alanyl-D-alanine (Figure 1.12) (355-358). Cross-linking of the glycan chains, or transpeptidation, is carried out by essential proteins known as penicillin-binding proteins, PBPs, and generally occurs between the third amino acid (DAP) of a stem peptide and the fourth (D-Ala) of the next peptide chain (Figure 1.12) (359). PBPs additionally catalyze other reactions in the later stages of peptidoglycan synthesis and remodeling including polymerization of the nascent glycan strand (transglycosylation), removal of the last D-alanine of the stem peptide (carboxypeptidation), and hydrolysis of the peptide bond that connects the two strands (endopeptidation) (360).

PBPs are generally classified as high molecular mass (HMM) and low molecular mass (LMM) enzymes and are further subdivided into classes A and B (360-362). A class C also exists for LMM PBPs. HMM class A PBPs are bifunctional with an N-terminal

glycosyltransferase domain and a C-terminal transpeptidase domain, while class B enzymes are only transpeptidases (Table 1.2). LMM class A and B PBPs are carboxypeptidases, and class C can be carboxypeptidases and/or endopeptidases.

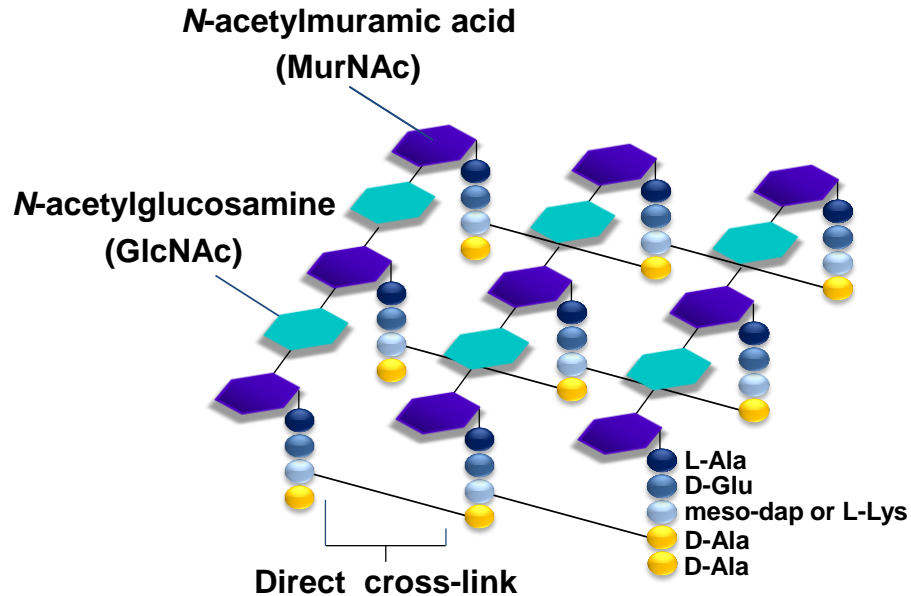


Figure 1.12. Structure of peptidoglycan. In Gram-negative bacteria the peptidoglycan layer is made up of alternating subunits of MurNAc and GlcNAc. A 4-5 amino acid stem peptide is attached to the GlcNAc subunits. Direct cross-linking can occur between the fourth D-Ala of one PG molecule and the third meso-DAP residue of the next.

Table 1.2: Classification of penicillin-binding proteins

Category	Subclasses	<i>In vivo</i> function
High Molecular Mass	A	glycosyltransferase and transpeptidase
	B	transpeptidase
Low Molecular Mass	A	carboxypeptidase
	B	carboxypeptidase
	C	carboxypeptidase/endopeptidase

Adapted from references (362) and (363).

HMM PBPs are essential for survival whilst LMM enzymes are not. All PBPs, however, have D-alanyl-D-alanine-peptidase (DD-peptidase) activity in their respective DD-transpeptidase, DD-carboxypeptidase or DD-endopeptidase domains (360, 364). Not surprisingly, this DD-peptidase domain is subject to inhibition by β -lactam antibiotics as β -lactams resemble the natural substrates of PBPs, namely D-alanyl-D-alanine (Figure 1.13). This is in fact the role of β -lactam antibiotics, to target and inhibit PBPs.

β -lactams covalently bind the active site of PBPs and form long-lived acyl-enzyme complexes effectively acting as suicide inhibitors of the enzymes (362, 365). Binding and inhibition of PBPs by β -lactams prevents the final crosslinking step of the new peptidoglycan layer, thus disrupting cell wall synthesis and leading to cell death. To successfully inhibit the action of PBPs, β -lactams rely on the specific binding to their PBP target. PBP mutations that reduce the specific binding and lower the affinity with which the β -lactam and its target bind, can render the drug ineffective and lead to resistance.

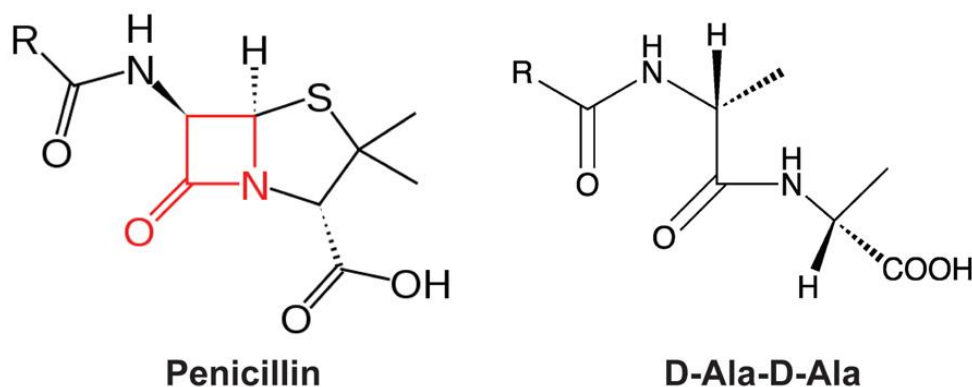


Figure 1.13. Molecular structures of penicillin and D-alanyl-D-alanine (366).

β -lactam resistance has been reported in *P. aeruginosa* following treatment of a CF patient with high dosage of tobramycin and piperacillin in association with loss of/or decreased binding to PBP3 (367). Similarly, β -lactam-resistant strains, selected *in vitro* following growth on cefsulodin, did not express β -lactamase but did show reduced affinity of PBP3 towards the two β -lactams tested, namely cefsulodin and carbenicillin (368). Recently, a new mechanism of PBP-mediated resistance has been elucidated. Specifically, *in vitro* and *in vivo* *ampC* overexpression and β -lactam resistance were correlated to inactivation of a nonessential PBP4 in an *ampD*-independent manner (369, 370). It is not known how prevalent this mechanism of resistance is in the clinical setting, but the phenotype was found to be reproducible upon deletion of PBP4.

Although modification of PBPs has also been previously reported in other Gram-negative bacteria, it is not currently considered a major cause of β -lactam resistance in the clinical setting (365, 371). PBP-mediated resistance however, is a prevalent mechanism in Gram-positive bacteria, particularly in *Streptococcus pneumoniae*, methicillin-resistant *S. aureus* and the enterococci (371, 372).

1.3.4 Expression of β -lactamases

Expression of β -lactamases is one of the most efficient and common ways of neutralizing the action of the commonly used β -lactam antibiotics (discussed in section 1.4) (373, 374). β -lactam-hydrolyzing enzymes can be chromosomal- or plasmid-encoded and expressed constitutively or induced upon β -lactam challenge. Constitutive β -lactamases are present at basal levels under normal conditions while inducible β -lactamases are derepressed in the presence of antibiotics but maintained at low levels in

their absence (375, 376). Synthesis of chromosomally encoded β -lactamases has been found to be inducible in bacterial species having a specific transcriptional regulatory system (375, 377, 378). In contrast, synthesis of chromosomally encoded β -lactamases is constitutive in bacterial species lacking such regulatory systems. Derepression of the chromosomally encoded β -lactamase AmpC is considered the most common mechanism of β -lactam resistance in *P. aeruginosa* (379-382). The classes, mode of action and genetics of β -lactamases will be discussed in section 1.5.

1.4 β -lactams

β -lactams are the most widely used class of antibiotics (204, 245, 383). They contain a signature β -lactam ring composed of one nitrogen and three carbons that is highly susceptible to hydrolysis by β -lactamases (Figure 1.14) (206). The major classes of β -lactam antibiotics include penicillins, cephalosporins, carbapenems, monobactams, and β -lactamase inhibitors (Figure 1.15).

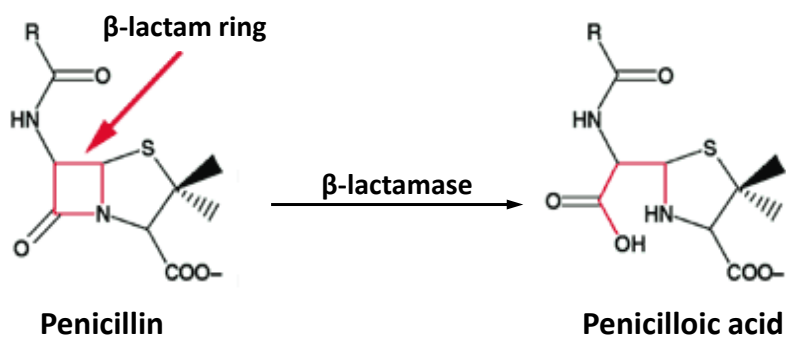


Figure 1.14. Action of β -lactamases on the β -lactam ring. β -lactamases inactivate β -lactam antibiotics by hydrolyzing the C-N bond present in the signature β -lactam ring.

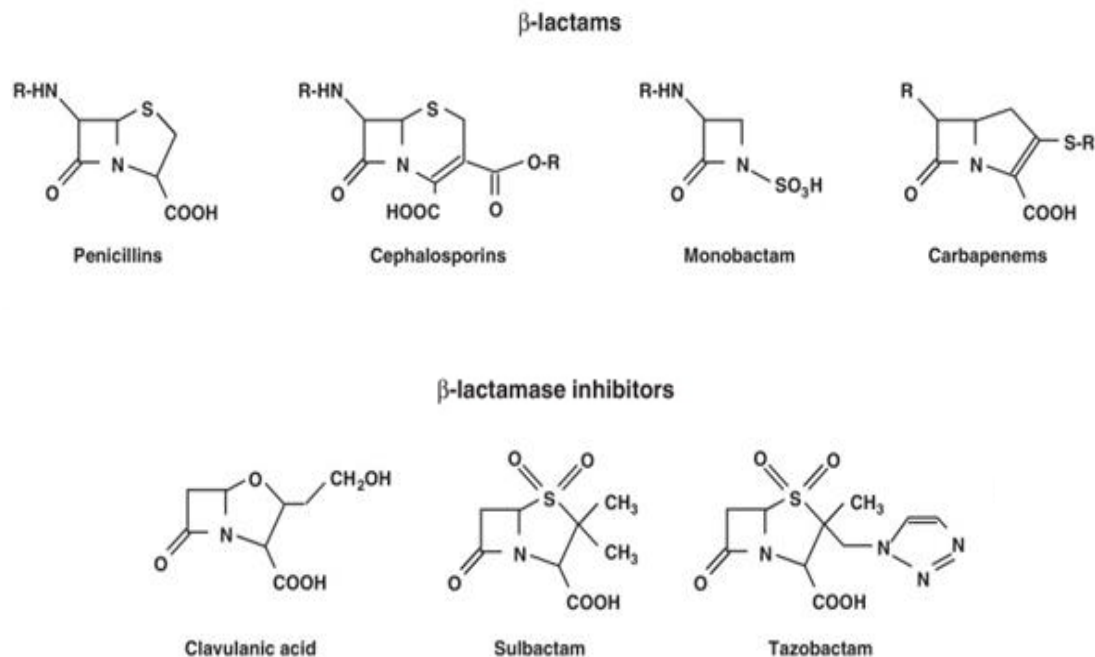


Figure 1.15. β -lactam classes. The major classes of β -lactams, penicillins, cephalosporins, carbapenems, monobactams, and β -lactamase inhibitors, differ in the ring structure fused to the four-membered signature β -lactam ring. Derivatives of each class differ in the substituent at the R groups. Adapted from (248).

Penicillin has the distinction of being the first antibiotic discovered. In 1928 the bacteriologist Alexander Fleming noticed that growth of the mold *Penicillium notatum* lysed nearby growing *Staphylococcus* cells (384). Growth of a number of other microorganisms including *Pneumococcus*, *Gonococcus*, *Streptococcus pyogenes* and *Streptococcus viridans* were also inhibited by what Fleming concluded was an antibacterial agent that was being produced by the fungus. He aptly named this compound penicillin. Subsequent attempts to purify it were relatively unsuccessful, but in 1940 Chain *et al.* succeeded in purifying penicillin and in using it as a therapeutic agent to treat mice infected with streptococci and staphylococci (385). In 1941, results from the

first small clinical trial showing low toxicity and high efficacy were published (386). The chemical structure of penicillin was finally elucidated by X-ray crystallography in 1945, although the work was not published until 1949 (387).

A naturally occurring penicillin-like compound, termed cephalosporin C, was later reported by Newton and Abraham (388-390). This compound was isolated from the fungus *Cephalosporium*, now named *Acremonium*, and also possessed antibacterial properties but contained a six-membered ring fused to the β -lactam structure, instead of the five-atom structure normally seen for penicillins (Figure 1.15). The real breakthrough came, however, with the discovery and synthesis of compounds containing only the β -lactam nucleus without a side chain, namely 6-aminopenicillanic acid (6-APA) and 7-aminocephalosporanic acid (7-ACA) (Figure 1.16) (391-393). It was possible to chemically modify these compounds to produce new and improved antibiotics with unusual side chains (394-399). Chemical additions or modifications of the side chains allowed the development of a vast number of semi-synthetic penicillins and cephalosporins with broad and extended-spectrum activity (Tables 1.3 and 1.4) (400-403).

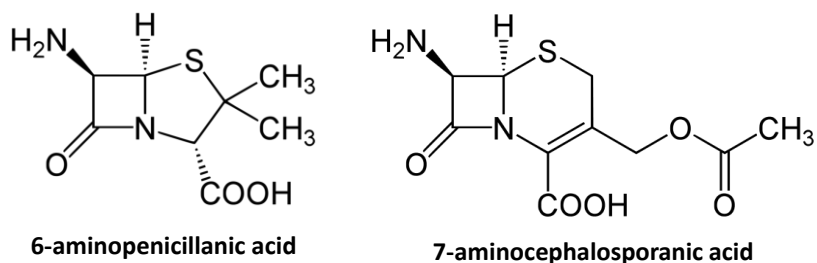


Figure 1.16. Molecular structures of 6-aminopenicillanic acid and 7-aminocephalosporanic acid. The 6-APA and 7-ACA compounds are useful structures containing only the β -lactam nucleus without a side chain. The current semi-synthetic penicillins and cephalosporins are derivatives of 6-APA and 7-ACA, where side chains have been added and modified.

Table 1.3: β -lactam antibiotics. I. Penicillins

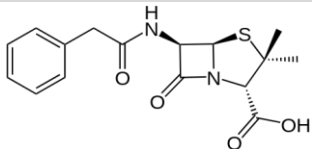
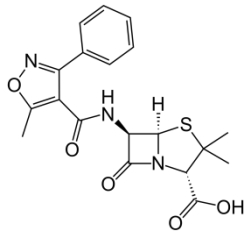
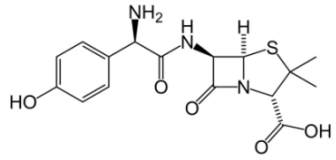
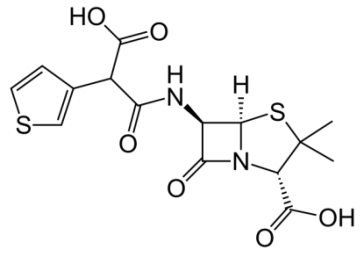
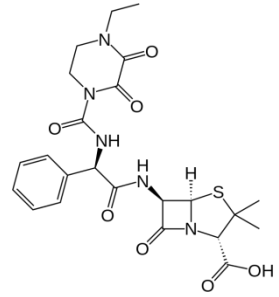
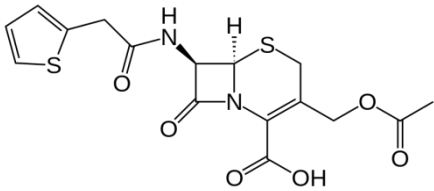
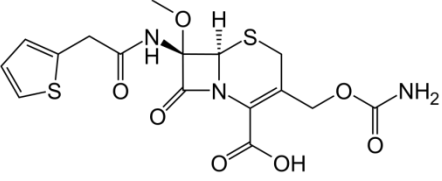
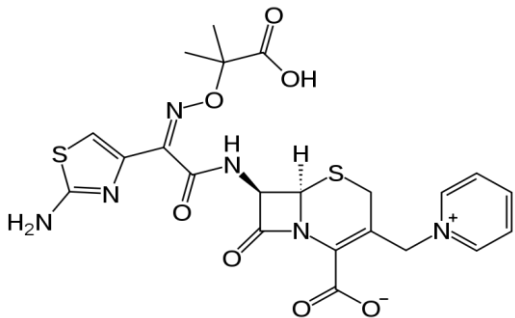
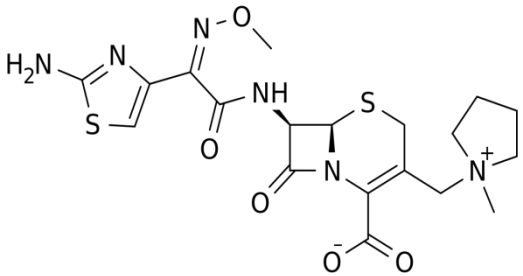
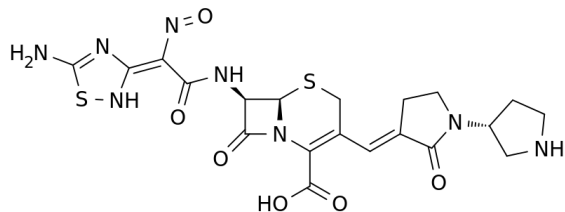
	Subclasses	Representatives	Representative Structure
Narrow Spectrum	Natural Penicillins	Penicillin G Penicillin V	 <p>Penicillin G</p>
	Penicillinase-resistant penicillins	Methicillin Isoxazolyl penicillins (oxacillin, cloxacillin, dicloxacillin)	 <p>Oxacillin</p>
Extended Spectrum	Aminopenicillins	Ampicillin Amoxicillin	 <p>Amoxicillin</p>
	Carboxypenicillins	Carbenicillin Ticarcillin	 <p>Ticarcillin</p>
	Ureidopenicillins	Piperacillin Azlocillin Mezlocillin	 <p>Piperacillin</p>

Table 1.4: β -lactam antibiotics. II. Cephalosporins

Generations	Representatives	Representative Structure
1 st	Cefazolin Cefadroxil Cefaloridine Cefalotin	 <p>Cefalotin</p>
2 nd	Cefaclor Cephamecins (Cefoxitin Cefotetan)	 <p>Cefoxitin</p>
3 rd	Cefotaxime Cefsulodin Ceftriaxone Ceftazidime Cefoperazone	 <p>Ceftazidime</p>
4 th	Cefepime Cefpirome	 <p>Cefepime</p>
5 th	Ceftobiprole Ceftaroline	 <p>Ceftobiprole</p>

A third class of β -lactams, termed carbapenems, were first discovered in species of the genus *Streptomyces* (404). This genus is the source of a plethora of other natural antibacterial compounds including tetracycline, chloramphenicol, aminoglycosides, and cephalosporin C derivatives, among others (405-409). Specifically, thienamycin, the first carbapenem, was discovered in the species *Streptomyces cattleya* which also produces cephamycin C and penicillin N (404). Thienamycin was shown to be a very potent, broad spectrum antimicrobial, generally resistant to degradation by β -lactamases. However, it was also very unstable. Semisynthetic, stable alternatives of thienamycin have been developed and include those approved for use in the US (imipenem, meropenem, ertapenem, doripenem) (Figure 1.17), and in Japan (panipenem, biapenem, and tebipenem) (207).

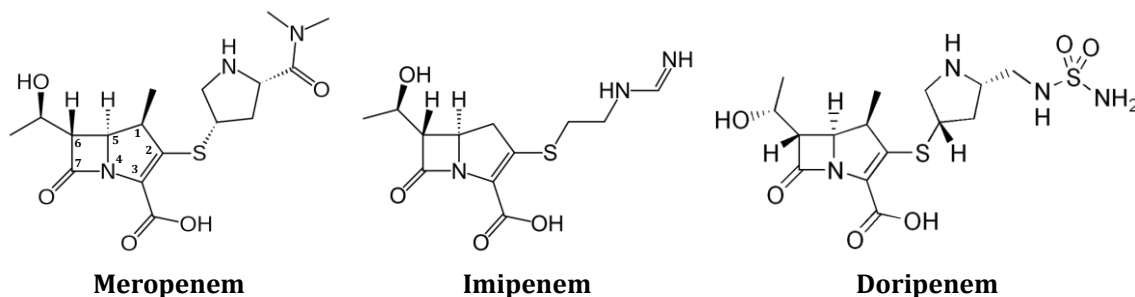


Figure 1.17. Structure of antipseudomonal carbapenems approved for used in the US. Although very similar in structure to penicillins, position 1 of carbapenems has a carbon atom instead of sulfur; the structure is also unsaturated at carbons 2 and 3.

Monobactam antibiotics are also known as monocyclic β -lactams since they do not possess a ring fused to the β -lactam nucleus as seen for penicillins, cephalosporins and carbapenems (Figure 1.15). The first monobactam discovered was nocardicin A, produced by the bacterium *Nocardia uniformis* subsp. *tsuyamanensis* (410). Several other natural monocyclic β -lactams have been identified in various other bacterial species

including *Pseudomonas acidophila*, *Agrobacterium radiobacter*, *Gluconobacter* and *Acetobacter* (411-414), the simplest of structures being produced by *Chromobacterium violaceum* (413). Development of semi-synthetic alternatives was undertaken as naturally occurring monobactams were shown to be very poor antimicrobials (415, 416). Currently, aztreonam, a synthetic derivative based on the monobactam nucleus, is the only monocyclic β -lactam approved for clinical use (Figure 1.18) (209, 210).

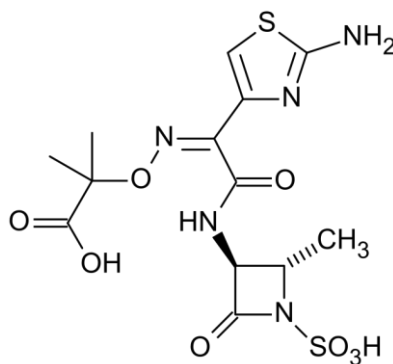


Figure 1.18. Structure of aztreonam, a monocyclic monobactam.

Aztreonam can be used to treat patients with allergies to penicillins and cephalosporins and is considered a safer alternative to aminoglycosides as this β -lactam does not cause ototoxic or nephrotoxic side effects (210). It is active against aerobic Gram-negative bacteria including *P. aeruginosa* and most of the Enterobacteriaceae, but it does not possess activity against Gram-positives or anaerobic Gram-negatives (417, 418). It is stable to most plasmid- and chromosomal-mediated β -lactamases of classes A and D, and to all class B enzymes. Aztreonam, however, can be hydrolyzed by the class A *Klebsiella pneumoniae* carbapenemases (KPCs) (419, 420). Additionally, it does not induce expression of the chromosomal cephalosporinases from *Citrobacter freundii*,

Enterobacter cloacae, *Serratia* spp. and *P. aeruginosa* but can serve as a powerful competitive inhibitor of these enzymes as it is only slowly hydrolyzed by them (421-424). Not surprisingly, it has been shown to enhance the antibacterial action of the β -lactam cefepime by protecting it from degradation by the *P. aeruginosa* cephalosporinase (425).

A final, important category of β -lactams are the β -lactamase inhibitors. Three are currently commercially available: clavulanic acid from *Streptomyces clavuligerus*; and sulbactam and tazobactam, semi-synthetic penicillin derivatives carrying sulfur dioxide at position 1 (Figure 1.15) (426-428). Structurally they are very similar to conventional β -lactams, having also a five-membered ring fused to the core β -lactam nucleus. Unlike regular β -lactams, however, β -lactamase inhibitors have only weak antibacterial action (423, 428). Nonetheless, they are good inhibitors of class A β -lactamases for which they display high affinity, but generally they are poor inhibitors of the class B, C or D enzymes (429-432).

Clavulanic acid, sulbactam and tazobactam are suicide inhibitors that irreversibly inactivate the β -lactamases (423, 428). Since their main role is inhibitory and not bactericidal, β -lactamase inhibitors must be co-administered with β -lactams to achieve the desired antibacterial effect. A good inhibitor thus is one that can competitively bind the enzyme, having a higher affinity for it than that of the co-administered β -lactam, so as to provide protection to the antibiotic against destruction by the β -lactamase. The currently approved β -lactam-inhibitor combinations are amoxicillin-clavulanic acid, ticarcillin-clavulanate, ampicillin-sulbactam and piperacillin-tazobactam (428). Studies

have shown however, that none of these inhibitors adequately inhibit the chromosomal cephalosporinase of *P. aeruginosa* and are thus deemed ineffective in the treatment of AmpC-expressing isolates (433-435).

1.4.1 Mechanism of action of β -lactams

As described in section 1.3.4, β -lactams act by inhibiting the synthesis of the peptidoglycan layer, a structure that is important for maintaining cell wall integrity and stability (245). Early studies showed that radio-labeled penicillin covalently binds enzymes that are involved in cell wall synthesis; hence these enzymes were named penicillin-binding proteins (PBPs) (371). PBPs are found in almost all bacteria, but they usually vary amongst species in their affinity for β -lactam antibiotics, number and size (361).

There are about two to four essential PBPs in any given organism and their inhibition can lead to cell lysis, death or growth arrest (361, 371). One of the most important enzymatic activities associated with PBPs is the final transpeptidation step in the synthesis of the peptidoglycan layer. The binding and inhibition of the PBPs by the β -lactam antibiotics prevents the final crosslinking step of the new peptidoglycan layer, thus disrupting cell wall synthesis (361, 371). It has been proposed that the structural and conformational similarities between penicillins and the D-alanyl-D-alanine part of the peptidoglycan (Figure 1.13) facilitates the binding of the β -lactam antibiotics to the active site of the PBPs. Seven PBPs have been reported in the bacterium *P. aeruginosa* using the techniques of polyacrylamide gel electrophoresis, but the number could be higher as

evidenced by the sequencing of the parent strain, PA01, which yielded nine PBPs (361, 436).

1.5 β -lactamases

β -lactamases (EC 3.5.2.6), as defined by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, are a diverse group of enzymes that specifically target and hydrolyze the C—N bond of the four-atom ring found in β -lactam antibiotics (Figures 1.14 and 1.15). The first enzyme with penicillinase activity was detected in *E. coli* by Abraham and Chain in 1940, before the actual introduction of penicillin in the clinical setting (437). The widespread use of penicillin in the 1940s saw the emergence of penicillin-resistant staphylococci and *S. aureus* strains carrying penicillinase (438-441). Staphylococcal penicillinase-mediated resistance became prevalent and of great concern through the 1950s (439, 442-445) until the advent of semi-synthetic and more effective β -lactams in the 1960s led to the emergence and spread of β -lactamases, especially of the plasmid-encoded types, in Gram-negatives (446-449). Although β -lactamases can be found in a number of Gram-positive pathogens, namely the staphylococci and *Bacillus* spp., these enzymes are more prevalent and widespread in Gram-negative organisms where they remain a major determinant of β -lactam resistance (373, 374, 450, 451). More than 850 β -lactamases have now been identified. A comprehensive list can be found at the Lahey Clinic (<http://www.lahey.org/Studies/>). Four main classes, A, B, C and D, are commonly recognized based on amino acid homology (452, 453).

1.5.1 Mechanism of action

Class A, C and D β -lactamases use a serine ester mechanism to disrupt the β -lactam ring. Specifically, the β -lactam ring is attacked by the free hydroxyl of the serine at the active site of the β -lactamase (Figure 1.19) (454, 455). The attack opens up the ring producing a covalent acyl enzyme complex. Hydrolysis of the complex regenerates the free, active enzyme and produces a hydrolyzed and inactive β -lactam. Class B enzymes, on the other hand, usually have one or two zinc ions in the active site, which are absolutely required for enzyme activity, hence they are known as metallo- β -lactamases (MLBs) (456, 457). A zinc ion can recruit and activate a hydroxide to attack the carbonyl carbon of the β -lactam ring leading to cleavage of the C-N bond and sometimes protonation of the nitrogen (456).

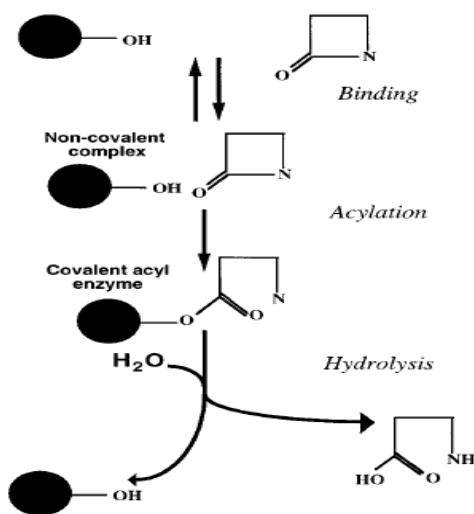


Figure 1.19. Mechanism of action of serine β -lactamases. The β -lactamase first associates with the β -lactam to form a non-covalent complex. A free hydroxyl from the side chain of the active serine residue then attacks the β -lactam ring forming a covalent acyl ester. The acyl-enzyme complex then undergoes hydrolysis to produce an inactive β -lactam. Reproduced from (373) with permission by the American Society for Microbiology.

1.5.2 Classification

One of the first classification schemes for β -lactamases was proposed by Jack and Richmond in 1970 with further updating in 1973 by Richmond and Sykes (458, 459). This classification grouped β -lactamases according to the spectrum of activity, inhibitor susceptibility, and the location of the gene coding for the enzyme (459). A major revision was proposed by Bush in 1989 (460) that was later expanded by Bush and colleagues in 1995 (461). This scheme was based on functional similarities and took into account inhibitor and substrate profiles (461).

The classification proposed by Bush recognized four major groups of β -lactamases with multiple subgroups (460, 461). Group 1 are the cephalosporinases that are poorly inhibited by clavulanic acid (462). Group 2 include the penicillinases, cephalosporinases and broad-spectrum β -lactamases that are generally inhibited by β -lactamase inhibitors (462, 463). Group 3 comprises the MLBs that can hydrolyze carbapenems, penicillins and cephalosporins (463). Group 4 contains penicillinases not inhibited by clavulanic acid (463).

Phenotypic classifications such as the one proposed by Bush and colleagues can be faulty since point mutations can change the substrate and inhibitor profile for a given enzyme (464). A classification scheme was thus proposed by Ambler based on amino acid similarities rather than phenotypic characteristics (452). The Ambler scheme is widely used today and, as previously mentioned, distinguishes four main classes of β -lactamases: A, B, C, and D.

1.5.2.1 Molecular class A

Class A enzymes are among the most common β -lactamases encountered in the clinical setting and in the Enterobacteriaceae (373, 465). They are known as penicillinases since they are mostly active against penicillin and penicillin-derived β -lactams, but extended-spectrum class A β -lactamases with activity against cephalosporins and carbapenems have also been reported (449, 461, 466). Most are well inhibited by the commercially available β -lactamase inhibitors clavulanic acid, tazobactam, and sulbactam (461, 467). The Class A β -lactamase-encoding genes are widely distributed in bacteria and are generally plasmid-borne but have also been reported in the chromosomes and integrons of Gram-negative bacteria (448, 468-472).

Common representatives include the TEM, SHV, PER and the TEM-, SHV-, and PER-derived β -lactamases (Table 1.5). TEM-1, the first plasmid-encoded β -lactamase, was reported in Greece in 1965 (473). It was originally identified in an *E. coli* isolate from a patient named Temoneira (473). TEM-1 is currently one of the most commonly reported β -lactamases in Gram-negatives being predominantly found in *E. coli* and in *K. pneumoniae* but also identified in *H. influenza* and *Neisseria gonorrhoeae* (373, 446, 474). TEM-2, a less frequently reported point variant of TEM-1, has a more active promoter than its progenitor, a different isoelectric point but the same hydrolytic profile (475, 476).

A common group of class A enzymes are the SHV-type β -lactamases, named after sulfhydryl variable, since it was thought that the inhibition of this enzyme by p-chloromercuribenzoate varied according to the substrate used (477). The earliest

representative of this group, SHV-1 is predominately found in *K. pneumonia* but plasmid dissemination has occurred to other enterobacterial species including *E. coli* and *Proteus mirabilis* (477-480). SHV enzymes are very structurally and functionally similar to TEM enzymes, SHV-1 sharing as high as 68% of its amino acid sequence with TEM-1 (478, 481). TEM and SHV enzymes provide resistance against penicillins such as amoxicillin, ampicillin, ticarcillin and carbenicillin and the narrow-spectrum cephalosporins (478). Most class A extended-spectrum β -lactamases (ESBLs) are TEM-1, TEM-2- and SHV-1-derived enzymes that differ from their precursors in as few as 1-4 amino acids (466, 482, 483). These point mutations increase the hydrolysis spectrum of the enzymes to include extended-spectrum cephalosporins and monobactams (478).

Table 1.5: β -lactamase Ambler classification

Molecular class	Representatives
A	TEM, SHV, CTX-M, KPC, IMI, SME, GES, PER, VEB
B	IMP, VIM, SPM, GIM, SIM, NDM
C	AmpC, CMY, MIR, MOX, LAT, FOX, DHA, CFE, ACT
D	OXAs (OXA-10, OXA-23, OXA-40, OXA-48, OXA-50)

Another important group of class A β -lactamases are the CTX-M-types ESBLs. These enzymes were first identified as non-TEM and non-SHV type ESBLs, exhibiting only about 40% similarity with TEM- and SHV-types, and having distinct hydrolytic activity against cefotaxime (484-487). They can occur in association with other TEM, SHV and OXA enzymes and are frequently carried on transmissible plasmids that can also carry *bla*_{TEM-1} (488-491). CTX-M-type enzymes are widely distributed among

members of the Enterobacteriaceae family including *E. coli*, *P. mirabilis*, *C. freundii*, *S. marcescens*, *Salmonella*, *Klebsiella* and *Enterobacter* spp., but have also been reported in other non-enterobacterial species such as *Vibrio* spp. and *Aeromonas hydrophila* (485, 492-503).

Unlike the TEM- and SHV-derived ESBLs that arose by point mutations of their precursors, the CTX-M enzymes appear to be the result of plasmid transfer from naturally occurring chromosomal enzymes in the *Kluyvera* genus (504-506). Many CTX-M enzymes have been reported to be identical or near identical to the CTX-M-like enzymes KLUA and KLUC of the *Kluyvera* spp. (504, 505, 507), with others like KLUG-1 sharing 85-86% similarity with the CTX-M-1 enzymes (506). Additionally, the genetic regions surrounding the acquired CTX-M enzymes have been shown to be 80-100% identical to regions surrounding the corresponding β -lactamase-encoding gene in the *Kluyvera* chromosome (504, 507-509). *ISEcpI* or *ISEcpI*-like insertion sequences have been detected upstream of many CTX-M-plasmid-encoded enzymes (490, 500, 504, 510, 511), while some CTX-M enzyme are encoded in the class I integrons InS21, In35 and In60 (508-510, 512) further strengthening the case for horizontal gene transfer from the *Kluyvera* chromosome.

A fourth relevant group of class A β -lactamases are the non-metallo-carbapenemases. The chromosomally encoded representatives of this group include the imipenem-hydrolyzing β -lactamase (IMI) and the non-metallo-carbapenemase (NMC-A) described in *E. cloacae* (513-515), as well as the SME-types isolated from *S. marcescens* (516-518). The spectrum of hydrolysis of these enzymes includes not only the

carbapenems, but also penicillins, narrow-spectrum cephalosporins and aztreonam (519, 520). These chromosomally encoded non-metallo-carbapenemases however, do not generally pose as much a threat as the TEM, SHV and CTX-M ESBLs, as they are only infrequently reported.

Plasmid-borne representatives of class A non-metallo-carbapenemases include the *Klebsiella pneumoniae* carbapenemase (KPC) and the Guiana extended spectrum (GES) family of enzymes. The first KPC was detected in a *K. pneumoniae* isolate from North Carolina in 1996 (521). KPCs remained relatively rare until the early 2000s when they reemerged in the East Coast of the United States (522-525) causing multiple nosocomial outbreaks in the New York area, sometimes through clonal spread (526-529). Subsequently KPCs have been isolated in several European and South American countries (530-538) as well as in Israel, China and Taiwan (539-541). The *bla*_{KPC} genes are usually carried on the plasmid-borne transposon, Tn4401, which undoubtedly contributes to the ease of dissemination observed (521, 524, 532, 542, 543). Thus, although most frequently observed in *K. pneumoniae* (544, 545), KPCs have also been isolated from *P. aeruginosa*, *E. coli*, *C. freundii*, *S. marcescens*, as well as from *Salmonella* and *Enterobacter* spp. (520, 522, 546, 547).

Similar to the TEM, SHV and CTX-M ESBLs, the KPC enzymes can hydrolyze penicillins and cephalosporins (520). Unlike the ESBLs, however, KPCs can also hydrolyze carbapenems to varying degrees (548). As carbapenems are often the first line of defense against microorganisms that express ESBLs, the emergence of carbapenem-resistant organisms, that are already resistant to penicillins and cephalosporins, severely

limits treatment options. The dissemination of KPCs is thus of great concern. Hydrolysis of carbapenems by KPCs, however, does not always lead to resistance, in some cases it can mean a decrease in susceptibility, with intermediate to high level resistance also being observed (523, 525).

GSE-1 was first reported in a *K. pneumonia* isolate from French Guiana (470). Currently 15 GSE enzymes are recognized, five of which display carbapenemase activity, albeit at low levels (520, 549). In every other sense they are typical ESBLs able to hydrolyze penicillins and extended-spectrum cephalosporins (519, 520). The GSE enzymes have been mostly identified in *P. aeruginosa* but have also been reported in some enterobacterial species (469, 550-552). Although GSE β -lactamases are relatively rare, nosocomial outbreaks have been reported in Korea, Portugal and South Africa (550, 553, 554).

Most of the class A enzymes, including the extended-spectrum PER- and VEB-types, have been reported in *P. aeruginosa* (482, 549). Class B and D enzymes (discussed below), however, are more prevalent in *P. aeruginosa*, whereas the class A enzymes are widely distributed in the Enterobacteriaceae (448, 477, 555, 556).

1.5.2.2 Molecular class B

Class B or metallo- β -lactamases (MLBs) are often described as being a class apart since unlike their serine counterparts they require Zn^{+2} in their active site in order to hydrolyze β -lactams (456, 457). They have a broad hydrolysis spectrum that includes the carbapenems and most often also penicillins and cephalosporins (456, 461, 463). MLBs are resistant to inhibition by the commercially available serine β -lactamase inhibitors and

are unable to hydrolyze the monobactam aztreonam (456, 557). The requirement for zinc means they are sensitive to inhibition by EDTA, a known metal chelator (457, 461). They are often carried on plasmid-borne transposons and integrons, but can also be chromosomally encoded.

Chromosomally encoded MLBs are mostly found on environmental and sometimes opportunistic pathogenic bacteria that are rarely a cause for concern, with the notable exceptions of *Bacillus anthracis* and *Stenotrophomonas maltophilia* (457). Chromosomal MLBs include CphA from *A. hydrophila* (558), BCII from *B. cereus* (559), Bla2 from *B. anthracis* (560), SFH-1 from *Serratia fonticola* (561), FEZ-1 from *Legionella gormanii* (562), TUS-1 and MUS-1 from *Myroides* spp. (563), and L1 from *S. maltophilia* (564), among others. Frequently the MLBs are coproduced with penicillinases and cephalosporinases and are induced upon β -lactam challenge. For example, *A. hydrophila* coordinately expresses two β -lactamases, CepH (cephalosporinase) and AmpH (oxacillinase) in addition to the MLB CphA, also named ImiH (565, 566). Coproduction of various β -lactamases is also observed in several other *Aeromonas* species such as *A. veronii*, *A. jandaei*, *A. caviae*, and *A. salmonicida* (566-568). Similarly, *S. maltophilia* produces two chromosomally encoded and inducible enzymes, L1, a metallo- β -lactamase, and L2, a class A cephalosporinase (569-572).

The first occurrence of transferable imipenem resistance was reported in a *P. aeruginosa* isolate from Japan (573). The identified enzyme, IMP-1, was encoded in a conjugative plasmid that could be mobilized into other *Pseudomonas* strains. IMP-1 was later identified in several *S. marcescens* strains from general hospitals in the nearby cities

of Anjyo and Okazaki from the Aichi prefecture in Japan (574-576). An IMP-type enzyme was also detected by DNA hybridization in fifteen *P. aeruginosa* clinical isolates from three different geographical areas in Japan (577). DNA fingerprinting showed that, for the most part, the strains were heterogeneous, while the *bla*_{IMP} gene displayed little to no variation, thus illustrating a plasmid-mediated proliferation that was not clonal in nature (577). Further studies have shown that the IMP-1 enzyme and variants have proliferated in Japan in the Enterobacteriaceae, including *S. marcescens*, *E. coli*, *C. freundii*, *K. pneumoniae*, *Enterobacter* spp., as well as in non-enterobacterial species such as *P. aeruginosa*, *Pseudomonas putida*, *Achromobacter xylosoxidans* and *Alcaligenes* spp. (578-583).

PCR analysis of 42 strains carrying the *bla*_{IMP} gene detected class 3 integron sequences in 79% of them (579). A larger Japanese study involving 357 *bla*_{IMP-1}-positive strains that included *P. aeruginosa*, identified class I integron sequences in 99% of them (580). Association of *bla*_{IMP} with mobile genetic elements has likely facilitated the dissemination of the enzyme in Japan. The IMP family of enzymes has also been reported worldwide in places like Portugal (584), Italy (585), Canada (586), China (587, 588), Australia (589, 590), England (591) and Korea (592, 593), among others. The current data however, suggest that these are local occurrences rather than dissemination from the alleles in Japan. Indeed, phylogenetic trees place IMP-1 in one of the later evolving branches of the dendrogram (457, 594).

A second and also prevalent class of MLBs is the integron-borne VIM-type. The first reported representative of this group, VIM-1 (Verona integron-encoded metallo- β -

lactamase) was detected in Italy in 1997 in a *P. aeruginosa* β -lactam-resistant isolate (595). Expression of the enzyme in a heterologous background conferred high level resistance against penicillins (ampicillin, carbenicillin, piperacillin and mezlocillin) and cephalosporins (cefoxitin, ceftazidime, cefoperazone and cefepime), while decreasing susceptibility to the carbapenems (595). VIM-1 was found to be only distantly related to other MLBs, sharing only 31% identity with IMP-1 (595). The *bla*_{VIM-1} gene was located in the chromosome and encoded as part of a gene cassette carried in a class 1 integron, which itself carried another gene cassette (*aacA4*) for aminoglycoside resistance (595). Not surprisingly, a *bla*_{VIM-1} gene was subsequently reported in a plasmid from an *A. xylosoxidans* isolate from the same hospital (596). A VIM-type enzyme, exhibiting a 90% amino acid similarity with VIM-1, was reported in France in a *P. aeruginosa* isolate in a nonconjugative plasmid (597). The variant, termed VIM-2, has since been found throughout Europe and the rest of the world, and is often reported as the most detected of acquired MLBs (598-604). A number of other VIM variants have also been reported throughout the world and in a number of bacterial species including *E. coli*, *K. pneumoniae*, *E. cloacae*, *S. marcescens*, *C. freundii*, *Acinetobacter baumannii*, *P. putida* and *P. aeruginosa*; the latter being the most common host for these enzymes (457, 549).

Other noteworthy MLBs include SPM-1 (for Sao Paulo MLB), GIM-1 (for German imipenemase) and SIM-1 (for Seoul imipenemase). SPM-1 was first detected in 1997 in a *P. aeruginosa* clinical isolate from Brazil (605). It was encoded in a plasmid, but unlike the IMP and VIM enzymes that are regularly associated with class 1 integrons, SPM-1 was associated with a novel transposable-like element with probable recombinase

activity (606). Thereafter, *P. aeruginosa* SPM-1-producing strains have become prevalent in Brazil and isolated from both clinical and environmental sources (607-610). To date, SPM-1 has only been reported outside of Brazil in *A. baumannii* isolates from various hospitals in Iran (611), and in a *P. aeruginosa* isolate from a Swiss man who had been previously treated in a Brazilian hospital (612).

GIM-1 was first reported in five multidrug-resistant *P. aeruginosa* isolates from a hospital in Dusseldorf, Germany (613). Subsequent reports identified more GIM-1-producing *P. aeruginosa* isolates in hospitals within a 40 km radius from the location of the original report (614). Although so far GIM-1 has not been reported outside of Germany, it has however, spread to a number of enterobacterial species including *Acinetobacter pittii*, *E. cloacae*, *Klebsiella oxytoca*, *S. marcescens*, *E. coli*, and *C. freundii* (615-618).

SIM-1 was first detected in the chromosome of *Acinetobacter* clinical isolates from Korea (619). Later, *bla*_{SIM-1} was also detected in two *Acinetobacter* (genomospecies 10) isolates that additionally carried *bla*_{IMP-1} or *bla*_{VIM-2} (620). To date, SIM-1 has not been detected outside of Korea, except for an *Acinetobacter baylyi* clinical isolate reported in China that additionally harbored OXA-23 in a large 360-kb plasmid (621). Similar to the IMP and VIM enzymes, both GIM-1 and SIM-1, are carried in class I integrons (613, 619). Although both SPM-1 and GIM-1 have been detected in *P. aeruginosa*, the IMP- and VIM-types are the most commonly detected MLBs in this pathogen and in the *Acinetobacter* spp. (549, 622).

1.5.2.3 Molecular class C

Class C enzymes or AmpC β -lactamases are generally chromosomally encoded but have also been reported in plasmids (623, 624). They can hydrolyze penicillins and cephalosporins including the cephamycins (cefoxitin and cefotetan) and the oxyimino-cephalosporins (cefotaxime, ceftriaxone, and ceftazidime), as well as the monobactam aztreonam (625, 626). The increased rate of hydrolysis observed against some cephalosporins as compared to penicillins, particularly the first generation cephalosporins cefazolin, cephaloridine, cephalothin and cephalexin, has given rise to their common designation of cephalosporinases (625). Although able to slowly hydrolyze some of the later generation cephalosporins, such as cefotaxime and cefoxitin, these enzymes often display high affinity for these substrates as evidenced by low K_m values (625, 627). The penicillins cloxacillin, oxacillin and carbenicillin act as transient inhibitors able to efficiently form acyl-enzyme complexes but usually being very poorly hydrolyzed by the class C enzymes (626). Additionally, the class A inhibitors are generally not good inhibitors of the cephalosporinases, although tazobactam and sulbactam have been shown to inhibit some class C enzymes (429, 430, 432).

Chromosomal class C enzymes are ubiquitous in Gram-negative bacteria and very frequently encountered in the Proteobacteria including the Aeromonadales (*Aeromonas* spp.) and the Pseudomonadales (*A. baumannii*, *P. fluorescens* and *P. aeruginosa*) (623). They are particularly well represented in members of the Enterobacteriales, including some commonly encountered species and pathogens such as *C. freundii*, *E. aerogenes*, *E. cloacae*, *E. coli*, *Morganella morganii*, *Providencia stuartii*, *S. marcescens*, *Shigella* spp.

and *Yersinia enterocolitica*, among others (555, 623). Some well-known members of the Enterobacteria group such as *K. pneumoniae*, *K. oxytoca*, *P. mirabilis*, *Salmonella* spp. and *Yersinia pestis* (623), however, do not carry a chromosomal class C enzyme, although this does not preclude the possibility of carrying plasmid-borne AmpC.

In many of the species carrying a chromosomal *ampC*, including *P. aeruginosa*, *C. freundii* and *E. cloacae*, *ampC* expression is constitutively low but induced upon β -lactam challenge (377, 628). Induction is tightly controlled by the transcriptional regulator AmpR and intricately linked to the recycling of the peptidoglycan layer (375). On the other hand, in *E. coli* and *Shigella* spp. *ampC* does not respond to induction by β -lactams and is only produced at very low levels (629, 630). Consequently the natural AmpC expression in such backgrounds does not lead to resistance. The marginal levels of AmpC β -lactamase observed in such species stem from the genetic environment surrounding the structural β -lactamase gene. In species with an inducible system (e.g., AmpC of *P. aeruginosa*), *ampR* is frequently located upstream of *ampC* and divergently transcribed; while species with a non-inducible system lack *ampR* and have a very weak promoter (378, 631, 632). Additionally, an attenuator is located between the promoter and the β -lactamase-encoding gene leading to the premature termination of the majority of transcripts (633). Derepression and hyperproduction in species having a non-inducible system, i.e., lacking *ampR*, can occur but often require mutations in the promoter and attenuator regions and/or the combined effect of different mutation types in order to provide any meaningful β -lactam resistance (634-638). Although such mutations have been reported in both *E. coli* and in *Shigella* spp. (634, 639, 640), they are rarer than

those leading to constitutive hyperexpression in species with an inducible system (376, 630). Mutational derepression or stable derepression of *ampC*, as it is also known, however, has been reported as one of the most common mechanisms of β -lactam resistance in species that have an inducible AmpC system such as *P. aeruginosa*, *C. freundii*, *E. cloacae*, *M. morganii*, *P. stuartii*, *Enterobacter* and *Serratia* spp. (380, 446, 465, 641-645). The genetics of inducible class C β -lactamases will be covered in section 1.5.3.

Although chromosomal *ampC*-types are the predominant class C enzymes in Gram-negative bacteria, dissemination into plasmids began to emerge in the late 1980s and continued to spread worldwide (646-649). They are predominantly found in species not usually known to possess chromosomal *ampC*, such as *K. pneumoniae*, *K. oxytoca* and *Salmonella* spp., but have also been reported in *E. coli* (624, 649). Representatives include MIR-1, MOX-1, LAT-1, FOX-1, ACT-1 and the CMY-types first reported in *K. pneumoniae*, as well as CFE-1 and DHA-1 first identified in *E. coli* and *Salmonella enteritidis*, respectively (Table 1.5) (624, 649, 650). The original chromosomal enzymes of Proteobacteria described above are the likely progenitors of these plasmid-borne enzymes (649). For instance, CMY-2 and the closely related types LAT-1 and CFE-1, appeared to have originated from the AmpC of *C. freundii* sharing 96, 95 and 99% amino acid sequence with that enzyme, respectively (647, 651, 652). CMY-1-types, including MOX-1, were originally thought to have descended from *P. aeruginosa*, but have since been shown to be more closely related to the chromosomal enzymes of the *Aeromonas* (646, 650, 653). The genetic support is usually an integron-based plasmid that often

carries other resistance genes such as those coding for aminoglycoside and quinolone resistance, or even other β -lactamase-encoding genes such as *bla*_{TEM-1}, *bla*_{CTX-M-3} and *bla*_{VIM-1} (649, 654-658). The hydrolysis spectrum remains the same as that of their chromosomal-encoded counterparts (623, 649).

Although plasmid-encoded AmpC enzymes have been reported worldwide, they are not as prevalent in the clinical setting as the ESBLs. They are most commonly reported in *K. pneumoniae* isolates from patients that have had long hospital stays or prolonged care at the ICU, where urinary indwelling catheters and an immunocompromised system are predisposing factors (650, 659-662). Plasmid-encoded AmpC enzymes have not so far been described in *P. aeruginosa* (549).

1.5.2.4 Molecular class D

Transferable drug resistance among Gram-negative bacteria started being reported and characterized in the early 1960s (663-665). The agents mediating resistance were dubbed resistance or *R* factors and later shown to be extrachromosomal pieces of DNA, termed plasmids (666). Datta and Kontomichalou recognized two distinct penicillinase-encoding *R*-factors, *R*_{TEM} and R1818, on the basis of substrate profile (473). The enzyme from R1818, later renamed R46 (667), was able to hydrolyze methicillin and cloxacillin more efficiently than the penicillinase from *R*_{TEM}, which displayed only slight activity against these substrates (473). Additionally the R1818 penicillinase was able to hydrolyze cloxacillin almost as fast as it could hydrolyze penicillin (473). Later work showed that the penicillinase from R1818 also hydrolyzed oxacillin faster than benzylpenicillin (668-672). Other studies characterized several more *R* factors mediating expression of β -

lactamases that resemble that of R1818 in the rapid hydrolysis of oxacillin and cloxacillin (670, 671, 673).

Two different classes of oxacillin-hydrolyzing enzymes were then distinguished on the basis of molecular weight and substrate profile (674). One group was characterized by having low molecular weight (~24,000) and high hydrolysis rate against methicillin, while the other had high molecular weight (~40,000) but relatively low hydrolysis of the same compound. The lowest rate of hydrolysis, however, was around 40-fold higher than that previously observed for the original TEM enzyme against methicillin (473). Isoelectric focusing further divided the oxacillinases into three main subclasses that were designated as OXA-1, OXA-2 and OXA-3 to reflect their preference for the isoxazolyl penicillins such as oxacillin and cloxacillin (475).

The first oxacillinases described, particularly OXA-1 (675, 676), OXA-2 (447, 669, 677) and OXA-3 (671, 678, 679) from plasmids R_{GN238}, R46, and R57b, respectively, were characterized by their ability to degrade isoxazolyl penicillins and by their sensitivity to inhibition by NaCl (674). As more OXAs were reported and characterized, it became apparent that these were a diverse group of enzymes with a heterogeneous substrate profile that in some instances did not include the oxacillin-type β -lactams (680). More than 350 oxacillinases are now recognized (<http://www.lahey.org/studies/webt.asp>) and they can be broadly divided into the narrow-spectrum, extended-spectrum and the carbapenem-hydrolyzing types (680).

The plasmid-mediated narrow-spectrum OXA enzymes include the originally characterized oxacillinases in *E. coli* (OXA-1), *S. typhimurium* (OXA-2), and *K.*

pneumoniae (OXA-3), as well as a few others also first described in *E. coli* (OXA-4, OXA-7), *K. pneumoniae* (OXA-9, OXA-47), *A. baumannii* (OXA-37) and *P. aeruginosa* (OXA-5, OXA-6, OXA-10, OXA-20, LCR-1) (680). Generally they can hydrolyze penicillins and sometimes early generation cephalosporins but not extended-spectrum cephalosporins. The hydrolysis spectrum however, can vary greatly from enzyme to enzyme. For instance, OXA-1 can generally hydrolyze amino- and ureidopenicillins, narrow-spectrum cephalosporins (681), as well as the broad-spectrum cephalosporins (675, 681), albeit weakly; whereas the *P. aeruginosa* OXA-5 can only hydrolyze penicillins and cephalothin (682). Similarly the *P. aeruginosa* OXA-10, originally categorized as a *P. aeruginosa* specific enzyme (PSE-2), hydrolyses carbenicillin and oxacillin, as well as aztreonam and some of later generation cephalosporins like cefotaxime and ceftriaxone, albeit slowly (683). The *P. aeruginosa* LCR-1 only hydrolyzes penicillins including oxacillin (684).

The extended-spectrum class D enzymes are mostly point mutation variants of the narrow-spectrum OXA-2 and OXA-10 enzymes (680, 685). These mutations extend the hydrolysis spectrum to include the extended-spectrum cephalosporins. OXA-11, the first reported OXA-10 variant, was identified in a *P. aeruginosa* isolate taken from the bloodstream of a Turkish patient (686). It was carried on a plasmid and had two amino acid substitutions (Asn146Ser and Gly167Asp). Several other OXA-10 variants, including OXA-14 (687), OXA-16 (688), OXA-17 (689), and OXA-19 (690) were also identified in *P. aeruginosa* isolates from both Turkey and France. Up to nine substitutions were observed per variant (OXA-19, OXA-28, OXA-145, OXA-147), with

OXA-14 having only one point mutation (<http://www.lahey.org/Studies/>). The Gly167Asp substitution appears to be the common thread among the variants that are very resistant to ceftazidime.

Unlike the OXA-10 ESBLs, the majority of which have more than one point mutation, the seven currently known OXA-2 variants have each only one substitution. OXA-15 (691) and OXA-36 have both a substitution at aspartate 149, to glycine and tyrosine respectively (685, 691). Both OXA-10 and OXA-2 ESBLs are carried in plasmid-borne class 1 integrons in association with aminoglycoside resistance cassettes (680). ES-OXAs are not widely distributed and have been mostly reported in *P. aeruginosa* (549).

A very important class of oxacillinases is comprised of the carbapenem-hydrolyzing β -lactamases (CHDLs). The majority has been identified in plasmids from the nosocomial and opportunistic pathogen *A. baumannii*, not usually in association with integrons (622, 692). CHDLs can significantly hydrolyze carbapenems often leading to decreased susceptibility or resistance towards meropenem and imipenem (685, 693). They are also able to hydrolyze most penicillins and provide high-level resistance to the aminopenicillins (ampicillin, amoxicillin) and the carboxypenicillins (ticarcillin) with variable hydrolysis against the ureidopenicillins (piperacillin). CHDLs, however, do not generally significantly hydrolyze cephalosporins or provide any meaningful resistance against these compounds (519, 693). The notable exception is OXA-146 which hydrolyses ceftazidime (694).

OXA-23, the first OXA carbapenem-hydrolyzing β -lactamase identified, was recovered from an *A. baumannii* isolate taken from a patient in Scotland (695). It was a plasmid-borne enzyme that showed very weak relation to other β -lactamases. Nineteen different variants have so far been reported for the OXA-23 group, the OXA-23 allele, however, remains the most common of this group with a widespread prevalence (685, 693). The OXA-51 group, currently consisting of 95 variants, is naturally present in the chromosome of *A. baumannii* (696, 697). These enzymes are not thought to play a major role in the intrinsic resistance as their expression is unregulated and uninducible. The *bla*_{OXA-51} gene however, has sometimes been reported in association with the upstream-located *ISAbal* or *ISAb9* (698-700). The promoters carried within these insertion sequences have been shown to drive expression of *bla*_{OXA-51} in carbapenem-resistant isolates (698-700).

Other important CHDLs include the OXA-40, OXA-48, OXA-58 and OXA-143 groups (701-703). Although most display low level of carbapenem hydrolysis, they have often been reported in carbapenem- or multidrug-resistant strains, whereby their transformation into *E. coli* or susceptible *A. baumannii* confers decreased susceptibility or carbapenem resistance (703-707). CHDLs are predominant and widespread in *A. baumannii* but relatively rare in *P. aeruginosa* (549, 685, 692, 693, 708, 709).

1.5.3 Genetics of inducible class C β -lactamases

The inducible expression of AmpC β -lactamase in the Enterobacteriaceae and in *P. aeruginosa* is regulated by AmpR, a LysR-type transcriptional regulator (710-712). The induction of *ampC*, however, also involves the interplay of several other *amp* genes

and is coordinately linked to the recycling of the peptidoglycan layer (375, 713, 714). In the Enterobacteriaceae model, *ampG* encodes a transmembrane protein that serves as a permease for passage of the inducer (1,6-anhydromurapeptides) that results from remodeling of the peptidoglycan layer during normal cell growth (715, 716). AmpG transports 1,6-anhydromurapeptides into the cytoplasm where the amidase AmpD hydrolyses them for murein recycling (717). It has been proposed that during normal physiological growth, the muramyl peptides processed by AmpD maintain AmpR in an inactive conformation that is unable to induce the expression of *ampC* (716, 718, 719).

In the presence of β -lactams, however, there is an excessive breakdown of murein leading to an accumulation of 1,6-anhydromurapeptides in the periplasm and in the cytoplasm (718, 720). Under these conditions, AmpD cannot keep up with the increased concentration of 1,6-anhydromurapeptides in the cytoplasm. The high number of muramyl peptides can then induce a conformational change in AmpR to promote the high level expression of *ampC* (716, 721). AmpD can thus function as a negative regulator of *ampC* expression in the absence of β -lactams. In fact, mutational inactivation of *ampD*, in both Enterobacteriaceae and *P. aeruginosa*, leads to constitutive over-expression of AmpC and consequently increased β -lactam resistance (722, 723). Recent work by Juan and colleagues reveals that three *ampD* homologs in *P. aeruginosa* are responsible for a stepwise *ampC* up-regulation mechanism that ultimately leads to constitutive hyperexpression of this β -lactamase (643). Details of this intricate mechanism are further discussed in Chapter 4.

1.5.4 Genetics of class D β -lactamases

Oxacillinase-encoding genes have been reported in a multitude of bacterial species including *P. aeruginosa*. Most of these and other β -lactamase-encoding genes appear to be unregulated, with insertion sequences often providing strong promoters for high-level expression of such genes (471, 698, 700, 724-728). To date, the only known regulatory systems of chromosomal-encoded class D β -lactamases are that of *Ralstonia pickettii* and *Aeromonas* spp. (567, 729, 730).

R. pickettii carries two inducible chromosomally encoded β -lactamases, OXA-22 and OXA-60, exhibiting a narrow-spectrum of hydrolysis that includes the penicillins (731, 732). Induction requires the presence of the divergently transcribed ORF *rp3* found 190 bp upstream of the OXA-60-encoding gene (730, 733). Imipenem was found to increase the mRNA levels of *bla*_{OXA-22} and *bla*_{OXA-60} as well as that of *rp3*, suggesting regulation by Rp3 occurs at the transcriptional level (733). Rp3 is thought to function as an activator as a recombinant Rp3 was shown to bind tandem repeats upstream of both OXA-encoding genes and to protect regions of ~30 bps there (733). RP3 was not similar to any known regulatory proteins. No significant similarities were found with *P. aeruginosa* proteins either.

Aeromonas spp. can produce two–three inducible and unlinked β -lactamases (552, 696-698). For instance, *Aeromonas jandaei* AER 14, formerly known as *Aeromonas sobria* AER 14, produces three chromosomally encoded β -lactamases, AsbA1 a class C cephalosporinase, AsbB1 (OXA-12) an oxacillinase, and AsbM1, a metallo- β -lactamase (734-736). *Aeromonas veronii* bv. *sobria* similarly produces a penicillinase (AmpS), a

cephalosporinase (CepS) and metallo- β -lactamase (ImiS) (737, 738), with the homologs, AmpH, CepH, and ImiH, also identified in *A. hydrophila* (565, 739).

The expression of all β -lactamases in *A. hydrophila* is coordinately regulated by a two-component system (TCS), encoded by *blrA* and *blrB*, and located immediately upstream of *ampH*, the oxacillinase-encoding gene (729). PCR analysis confirmed the presence of the *blrAB* operon in other species of the *Aeromonas* genera, including *A. jandaei* and *A. veronii* bv. *sobria*, where the TCS-encoding genes are also linked to the *bla_{OXA}* gene (729). Regulation by a TCS thus appears to be a common and effective mechanism of coordinate β -lactamase expression in *Aeromonas* spp.

1.5.5 The *pox* operon

Previous work showed that *ampC* mutations do not completely abolish β -lactamase activity in the *P. aeruginosa* PA01 strain (740). Furthermore, the susceptibility profile of an *ampC*-deficient strain did not show an increase in sensitivity towards the three β -lactam representatives tested, benzyl-penicillin, meropenem and cefotaxime, as compared to the wild-type PA01 (740). In view of these results, the presence of a second chromosomally encoded β -lactamase in *P. aeruginosa* was postulated. *In silico* analysis revealed that ORF *PA5514* encoded a putative β -lactamase with a signal peptide sequence at the N-terminus. The presence of a signal peptide is consistent with what is known about β -lactamases since they are localized to the periplasm and require a signal peptide for translocation.

Analysis of the sequences around *PA5514*, termed *poxB*, revealed the presence of an upstream ORF annotated *PA5513*, named *poxA*, 49 bp upstream. In the absence of

promoter-like sequences in the intergenic region between *PA5513* and *PA5514*, it was proposed that *poxA* and *poxB* form a two-gene operon (Figure 1.20). PoxA displayed high sequence similarity to hypothetical hydrolases and acyltransferases of yet unknown function. The presence of transmembrane spanning domains, as suggested by its hydrophobicity plot, and the absence of any signal sequence, indicate PoxA likely localizes to the inner membrane.

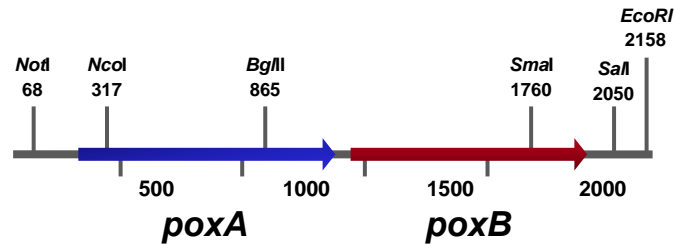


Figure 1.20. Restriction map of the *pox* operon.

PoxB, on the other hand, shows high sequence identity to class D β -lactamases from *A. baumannii* and *Fusobacterium nucleatum* (740). Alignment of the PoxB sequence with 26 conserved residues in class D β -lactamases revealed that 21 of the 26 amino acids were identical. Differences in four of the remaining five amino acids place PoxB in a new branch of a dendrogram (740). PoxB, thus appears to be a new kind of oxacillinase, markedly different from other OXA enzymes (740).

Expression studies conducted in *E. coli*, where there is a naturally low basal level of β -lactamase expression, confirmed the role of PoxB as a β -lactamase. Specifically, Girlich *et al.* showed that introduction of *poxB* in an *E. coli* background yields β -lactamase activity (741). Previous work in our lab also showed that both the *poxB* gene and the *poxAB* operon are capable of producing high basal levels of β -lactamase activity

in *E. coli* (740). The goal of this dissertation was to characterize PoxB and the *poxAB* operon and determine their contribution to β -lactam resistance in the nosocomial and intractable pathogen *P. aeruginosa*.

Chapter 2

Characterization of a carbapenem-hydrolyzing enzyme, PoxB, in

Pseudomonas aeruginosa

2.1 Abstract

Pseudomonas aeruginosa is an opportunistic pathogen often associated with severe and life-threatening infections that are highly impervious to treatment. This microbe readily exhibits intrinsic and acquired resistance to varied antimicrobial drugs. Resistance to penicillin-like compounds is commonplace and provided by the chromosomal AmpC β -lactamase. In the present work the contribution of a second, recently discovered, chromosomally-encoded β -lactamase, PoxB, and its relation to AmpC, were investigated. A single deletion of *ampC* surprisingly did not alter susceptibility against most of the penicillins or cephalosporins tested, however its overexpression provided resistance and/or decreased susceptibility against these β -lactams. Carbapenem susceptibilities, as evidenced by minimum inhibitory concentrations (MICs), were mostly unaffected by the lack of AmpC. PoxB expression decreased susceptibility against the carbapenems but did not affect susceptibility against the penicillins or cephalosporins. Additionally, PoxB was only poorly inhibited by class A inhibitors, but a novel non- β -lactam inhibitor, termed avibactam, was a slightly better inhibitor of PoxB activity. Clinical concentrations of avibactam, however, failed to produce a change in MIC against the carbapenems by E-test. In addition, *poxB* was found to be cotranscribed with the upstream open reading frame, *poxA*, which itself was shown to encode a 32-kDalton protein of yet unknown function.

Key Words: PoxB, OXA-50, AmpC, Oxacillinase, Class D β -lactamase

Abbreviations: Congo red, CR; Coomassie brilliant blue, CB; Isopropyl β -D-1-thiogalactopyranoside, IPTG; Minimum inhibitory concentration, MIC; Open reading frame, ORF; Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE

2.2. Introduction

Pseudomonas aeruginosa is a ubiquitous and versatile opportunistic pathogen commonly affecting immunocompromised individuals such as those with severe burns, AIDS and cancer (89, 92, 96, 99, 109, 202). In addition, it is a significant source of nosocomial infections and the second most common cause of ventilator-associated pneumonia in the hospital setting (72, 73). Most notably, *P. aeruginosa* is the primary pathogen associated with lung deterioration and mortality in patients with cystic fibrosis (CF), a deadly, autosomal, recessive genetic disorder affecting about 70,000 individuals worldwide (122, 742). Treatment often proves challenging and ineffective as the bacterium exhibits innate and acquired resistance to a broad range of antibiotics (246, 349, 743). In particular, resistance to the frequently used β -lactam-type antibiotics is common and mediated by the expression and derepression of the chromosomally encoded AmpC β -lactamase (379-381, 722).

The Ambler classification scheme distinguishes four different classes of β -lactamases (classes A, B, C and D) on the basis of amino acid sequences (452). All four classes have been reported in *P. aeruginosa* and are often transposon or plasmid-borne (601, 744-747). In addition to the acquired β -lactamases, *P. aeruginosa* strains frequently carry the chromosomal class C β -lactamase AmpC (711, 748). Derepression of *ampC* has been identified as a common cause of moderate to high-level β -lactam resistance in *P. aeruginosa* clinical isolates (379, 380, 643-645, 749).

A second chromosomally-encoded β -lactamase, termed PoxB (*PA5514*) or OXA-50, has been reported in *P. aeruginosa* (740, 741). PoxB belongs to the class D β -

lactamases, also termed oxacillinases, for the ability of some members of this class, particularly, the earlier reported ones, to degrade isoxazolyl penicillins such as oxacillin, methicillin, and cloxacillin (671, 674, 682, 750-752). The DBL numbering system is used to correlate homologous residues and signature sequences across class D β -lactamases that may otherwise be located at different amino acid positions (753). Differences in these amino acid signature sequences place PoxB in a new branch of the oxacillinase phylogenetic tree, suggesting it is only weakly related to other oxacillinases (740, 741). For instance, class A and D enzymes commonly have a serine-threonine-phenylalanine-lysine (STFK) motif at position 70 in the DBL numbering system, where serine, the active site residue, and lysine are conserved in serine β -lactamases and in penicillin-binding proteins (753, 754). To our knowledge, however, PoxB is only one of two oxacillinases where the traditional STFK motif of class D β -lactamases is replaced by STYK (serine-threonine-tyrosine-lysine) (740, 741), the other being OXA-62 from *Pandoraea phoenicis* (755-757). Thus, PoxB appears to be a new kind of oxacillinase, markedly different from other known OXA enzymes. In addition, previous *in silico* analysis suggested *poxB* may form a two-gene operon with the upstream open reading frame (ORF), *PA5513*, termed *poxA* (740), which is predicted to encode a putative hydrolase or acyltransferase of yet unknown function. The role of *poxA* and its relation to *poxB* is yet to be determined.

In the present work we examine the role of PoxB and its contribution to β -lactam resistance by generating single in-frame deletions of both chromosomal β -lactamase-encoding genes, namely *ampC* and *poxB*. We also study the efficacy of class A enzyme

inhibitors and of a novel non- β -lactam inhibitor termed avibactam, against PoxB. Additionally, the hypothesis that *poxA* and *poxB* form an operon and the role of PoxA were investigated.

2.3 Materials and Methods

2.3.1 Bacterial strains, plasmids and media. Bacterial strains, plasmids and primers used in this study are shown in Table 2.1. *Escherichia coli* and *P. aeruginosa* were cultured routinely in Luria-Bertani medium (LB; 10 g tryptone, 5 g yeast extract, and 5 g NaCl, per liter). Cation-adjusted Mueller Hinton broth (CAMHB) and agar (BBL, BD) were used for susceptibility testing using the broth microdilution method and E-test, respectively. T-broth (10 g Bacto tryptone and 5 g NaCl, per liter) was used for pellicle formation and Congo red (CR) binding assays. Antibiotics were used at the following concentrations: ampicillin (Ap) at 100 μ g/ml, and gentamycin (Gm) at 15 μ g/ml for *E. coli*; Gm at 75 μ g/ml for *P. aeruginosa*.

2.3.2 Construction of *poxA*, *poxB*, and *oprD* deletion mutants. A single in-frame deletion of *poxA* (PA5513) was constructed using overlap extension PCR and homologous recombination as previously described (758). Briefly, sequences upstream (816 bp) and downstream (773 bp) of the target deletion were amplified using primer pairs CApoxAUF1-CApoxAUR1 and CApoxADF2-CApoxADR2, respectively (Table 2.1). The PCR products were then ligated through another round of PCR, cloned into the suicide vector pEXG2 (759) and moved into *P. aeruginosa* PA01 for homologous

recombination with the genome. Double-cross-over recombinants were selected for by screening for Gm sensitivity and sucrose resistance. The deletion was confirmed by PCR amplification of deletion product from the genome and sequencing. The strain is referred to as PA0 Δ *poxA* henceforth. In-frame deletions of *poxB* (PA5514) and *oprD* (PA0958) were generated in a similar manner using primer pairs DB*pox*BUF1-DB*pox*BUR1 and DB*pox*BDF2-DB*pox*BDR2; and DZ*opr*DUF1-DZ*opr*DUR1 and DZ*opr*DDF2-DZ*opr*DDR2, respectively (Table 2.1). The *poxB* and *oprD* deletions were also introduced into a previously generated *ampC* deletion strain (PA0 Δ *ampC*) (760) to create PA0 Δ *ampC* Δ *poxB*, PA0 Δ *ampC* Δ *oprD* and PA0 Δ *ampC* Δ *poxB* Δ *oprD*.

Table 2.1: Bacterial strains and plasmids used in this study

Strains/Plasmids	Relevant phenotype and genotype	Reference/ Source
<i>Escherichia coli</i>		
DH5 α	F ⁺ Φ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR</i>	New England
	<i>recA1 endA1 hsdR17</i> ($r_k^- m_k^+$) <i>phoA supE44</i> λ^-	Biolabs
	<i>thi-1 gyrA96 relA1</i>	
TOP10	F ⁺ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80 <i>lacZ</i> Δ M15	Life
	Δ <i>lacX74 recA1 araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU</i>	Technologies
	<i>galK rpsL</i> (Str ^R) <i>endA1 nupG</i>	
BL21(DE3)	F ⁺ <i>ompT hsdS_B</i> ($r_B-m_B^-$) <i>gal dcm</i> (DE3)	(761)
<i>Pseudomonas aeruginosa</i>		
PA01	Wild-type	(762)

PKM119	PA0Δ <i>poxA</i> , PA01 containing in-frame deletion of <i>poxA</i>	This study
PKM120	PA0Δ <i>poxB</i> , PA01 containing in-frame deletion of <i>poxB</i>	This study
PKM201	PA0Δ <i>ampC</i> , PA01 containing in-frame deletion of <i>ampC</i>	(760)
PKM202	PA0Δ <i>ampC</i> Δ <i>poxB</i> , PA01 containing in-frame deletions of <i>ampC</i> and <i>poxB</i>	This study
PKM121	PA0Δ <i>oprD</i> , PA01 containing in-frame deletion of <i>oprD</i>	This study
PKM203	PA0Δ <i>ampC</i> Δ <i>oprD</i> , PA01 containing in-frame deletions of <i>ampC</i> and <i>oprD</i>	This study
PKM204	PA0Δ <i>ampC</i> Δ <i>poxB</i> Δ <i>oprD</i> , PA01 containing in-frame deletions of <i>ampC</i> , <i>poxB</i> and <i>oprD</i>	This study
Plasmids		
pEXG2	Gm ^R ; pMB1 <i>ori sacB</i>	(759)
pGEM-T Easy	Ap ^R ; TA cloning vector for PCR amplicons, pUC origin, f1 <i>ori</i>	Promega
pCR2.1-TOPO	Ap ^R , Km ^R ; TA cloning vector for PCR amplicons, pUC origin, f1 <i>ori lacZα</i>	Life Technologies
pET15bVP	Ap ^R ; pBR322 origin, <i>oriV</i> , expression vector, T7 promoter, His tag coding sequence	(756)
pMMB67EH-Gm	Gm ^R ; IncQ, RSF1010, <i>lacI^q</i> P _{<i>lac</i>} expression vector with <i>ampR::aacCI</i> insertion at <i>DraI</i>	(763)
pDZ427	Gm ^R ; pET15bVP carrying an 891-bp <i>XhoI</i> and <i>BamHI poxA</i> fragment	This study

pAmpC	Gm ^R ; pMMB67EH-Gm carrying a 1297-bp fragment containing the <i>ampC</i> ORF	This study
pPoxA	Gm ^R ; pMMB67EH-Gm carrying a 946-bp fragment containing the <i>poxA</i> ORF	This study
pPoxB	Gm ^R ; pMMB67EH-Gm carrying an 835-bp fragment containing the <i>poxB</i> ORF	This study
Primers		
DZ <i>ampC</i> F	5'-GGAATTCAACGACAAAGGACGCCAA-TCCTC-3'	This study
DZ <i>ampC</i> R	5'-CGGGATCCATGGCACCATCATAGCCA-GGACCGG-3'	This study
DZ <i>poxA</i> F	5'-GGAATTCTTACTGCGCGCCGTACG-AGGA-3'	This study
DZ <i>poxA</i> R	5'-CGGGATCCGATTCCCCACGGAAGGAG-CGTT-3'	This study
RT <i>poxA</i> Rev1	5'-AGGAGAAGGGCACTGAAGAGGAGA-3'	This study
RT <i>poxA</i> Ford2	5'-GACTTTCTCCTGCCCAATCCATGA-3'	This study
RT <i>poxA</i> Rev2	5'-CACCAACCAGAAGTTATCCACA-3'	This study
DZ <i>poxA</i> Ford4	5'-CTCGAGATGTCGCTATCCCCCTGGT-CC-3'	This study
DZ <i>poxA</i> Rev4	5'-GGATCCTCATGGATTGGGCAGGAGAA-AGTC-3'	This study
DZ <i>poxB</i> F	5'-GGAATTCAGGATGACCCACAGGACC-GAGCCAT-3'	This study
DZ <i>poxB</i> R	5'-CGGGATCCCAGAGCGTCAGGGCAGT-ATCC-3'	This study

<i>CApoxAUF1</i>	5'-GGAATTCGATCGTATTGCCGGGTCTC-3'	This study
<i>CApoxAUR1</i>	5'-GAGCATTCTAGCTAGCTAGAACCAGG-GGATAGCGACAT-3'	This study
<i>CApoxADF2</i>	5'-CTAGCTAGCTAGAATGCTCCCAATCC-ATGAACGCTCCTT-3'	This study
<i>CApoxADR2</i>	5'-CGGGATCCAAGCCGTAGAGCCGCTCG-TT-3'	This study
<i>DBpoxBUF1</i>	5'-GAATTCGGATTTTCGACCTCTGGCTGT-G-3'	This study
<i>DBpoxBUR1</i>	5'-GAGCATTCTAGCTAGCTAGCAGGAGA-AGGGCACTGAAGAG-3'	This study
<i>DBpoxBDF2</i>	5'-CTAGCTAGCTAGAATGCTCTCAAGGC-TCTC-GGGATACTG-3'	This study
<i>DBpoxBDR2</i>	5'-CGGGATCCACTGGGTGTGGTTGGAGA-AT-3'	This study
<i>DZoprDUF1</i>	5'-GGAATTCCAAAGCGAACATACTGACC-TCTC-3'	This study
<i>DZoprDUR1</i>	5'-GAGCATTCTAGCTAGCTAGACTTATA-GCACGCAGTGTTTCTTG-3'	This study
<i>DZoprDDF2</i>	5'-CTAGCTAGCTAGAATGCTCCCTATAA-AGGAAGGGCGTAGGTA-3'	This study
<i>DZoprDDR2</i>	5'-CGGGATCCCGGTGGAAAGCAGATACT-GACTGGTA-3'	This study

2.3.3 PCR amplification and cloning of *poxA*, *poxB* and *ampC*. The *P. aeruginosa* PA01 genome was used as the template for PCR amplification of the *poxA* (PA5513), *poxB* (PA5514) and *ampC* (PA4110) ORFs with primer pairs DZpoxAF-DZpoxAR,

DZ*poxBF*-DZ*poxBR*, and DZ*ampCF*-DZ*ampCR*, respectively (Table 2.1). The amplicons were independently cloned into pCR2.1-TOPO and sequenced. Each insert was then moved into the *EcoRI*-*Bam*HI sites of the broad-host range expression vector pMMB67EH-Gm (763) and propagated in *E. coli* TOP10 or DH5 α . The plasmid carrying *ampC*, (pAmpC), was subsequently introduced by electroporation into PA0 Δ *ampC* Δ *poxB* while pPoxA was introduced into PA0 Δ *ampC*, PA0 Δ *poxA*, and PA0 Δ *poxB*. The pPoxB plasmid was introduced into PA0 Δ *poxA*, PA0 Δ *poxB*, PA0 Δ *ampC*, PA0 Δ *ampC* Δ *poxB* and PA0 Δ *ampC* Δ *poxB* Δ *oprD*.

For protein expression in *E. coli* BL21(DE3), the *poxA* ORF was amplified using primers DZ*poxAFord4* and DZ*poxARev4*, cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced. The 891-bp fragment was then subcloned into the *Xho*I and *Bam*HI sites of the broad host range shuttle vector pET15bVP (756) and propagated in *E. coli* TOP10 (Life Technologies, Grand Island, NY). The resultant plasmid was then introduced into *E. coli* BL21(DE3) (761) for expression studies.

2.3.4 Protein expression and one-dimensional gel electrophoresis. To determine if *poxA* is translated, the ORF was cloned into the expression vector pET15bVP and introduced into *E. coli* BL21(DE3) as described in section 2.3.3. Stationary-phase cultures were diluted to an OD₆₀₀ of 0.02 in 25 ml of LB broth and incubated with shaking at 37°C until the culture density reached an OD₆₀₀ of 0.5. Cells were then induced with 1 mM of isopropyl β -D-1-thiogalactopyranoside (IPTG) and reincubated at 37°C. One-ml samples were taken at the time of induction and every hour for a total of

four hours for both IPTG-induced and non-induced samples. The cells were recovered by centrifugation, resuspended in 100 μ l of 4X SDS protein sample buffer and boiled for 10 minutes. Proteins were separated on a 12% SDS-PAGE gel and stained with Coomassie brilliant blue (CB).

2.3.5 Analysis of PA01 mRNA. To determine if *poxA* and *poxB* are cotranscribed, RNA was extracted from wild-type *P. aeruginosa* PA01, using the RNeasy mini kit (Qiagen). cDNA was synthesized with Superscript III (Invitrogen) and an (NS)₅ random primer using standard methods (764). Two sets of primers were used to amplify the intergenic between *poxA* and *poxB* plus 24 bp corresponding to the 3' end of *poxA* and 53 or 486 bp from the 5' end of *poxB*. RT*poxA*Ford2 and RT*poxA*Rev1 were designed to amplify 126-bp product, while amplification with RT*poxA*Ford2 and with RT*poxA*Rev2 would yield a 559-bp product. RNA samples not treated with Superscript were also tested to ensure no carryover DNA contamination resulted from the RNA isolation procedure.

2.3.6 β -lactamase assay. The β -lactamase assay was modified from a previously published protocol (765). Briefly, stationary-phase cultures were diluted in 25 ml of LB broth to an OD₆₀₀ of 0.02 and incubated with shaking at 37°C. At an OD₆₀₀ of 0.6, cells were induced with 50 μ g/ml of benzylpenicillin, while cells containing the expression plasmid (pPoxB) were induced with 1 mM IPTG. In both cases induction was carried out for an hour. Ten milliliter of cells were harvested by centrifugation and resuspended in 1000 μ l of 1x BugBuster solution (Novagen, EMD Millipore, Billerica, MA) containing 1

μl of benzonase (Novagen) and 1 KU of rLysozyme (Novagen). Lysate was rotated for 20 min at room temperature before centrifuging at 4°C for 15 minutes to collect the β-lactamase-containing supernatant. A 2-μl aliquot of cell lysate was added to nitrocefin (final concentration, 100 μM) in 250 μl of assay buffer. The reaction was incubated at 37°C for 20 minutes and the hydrolysis of nitrocefin was measured spectrophotometrically at 482 nm. Total protein concentration was determined with Bradford on the same supernatant. The activity was expressed as nmol of nitrocefin degraded per minute per microgram of total protein.

2.3.7 Minimum inhibitory concentration (MIC). MICs were determined by the E-test system according to the manufacturer's instructions (bioMérieux, Marcy l'Etoile) or by the broth microdilution method according to standard protocols (766, 767). E-test values were often reported as a range or as two independent values to illustrate variation seen in biological and technical replicates.

2.3.8 Colony morphology on Tryptone Agar containing Congo red. Plates containing 1% agar, 1% tryptone, 40 μg/ml of CR and 20 μg/ml of CB were spotted with 10 μl of 0.025 OD₆₀₀ cells. Plates were also supplemented with 1 mM IPTG and 10 or 20 mM NaNO₃, when appropriate. Plates were incubated at room temperature or in an anoxic chamber for 5-7 days and pictures were taken with the Epson Perfection V550 scanner (Long Beach, CA).

2.3.9 Pellicle formation and cell-surface interactions. For pellicle formation, the strains were grown in 6 ml of T-broth at room temperature without shaking for 7 days as previously described (768). The tubes were inspected visually for formation of mats at the liquid-air interphase. To assess cell-cell and cell-surface interactions, cells were grown in borosilicate glass tubes overnight (O/N) with shaking at 37°C in T-broth in the presence and absence of 1 mM IPTG and inspected visually the next day for aggregates on the walls of the tubes (769).

2.3.10 Congo red binding assay. The CR-binding assay was performed as previously described with minor modifications (770). Briefly, strains were grown in 2 ml of T-broth at 37°C without shaking for 48 hours. Cells were harvested by centrifugation, and washed with T-broth. The pellet was resuspended in 1 ml of T-broth containing 40 µg/ml of CR and incubated with shaking at 37°C. After 90 minutes, the cells and the bound CR were pelleted by centrifugation. The optical density of the supernatant was determined at 495 nm and the percentage of CR left in the supernatant was calculated.

2.4 Results and Discussion

P. aeruginosa infections are commonly treated with a combination of β -lactams and aminoglycosides. Resistance to the β -lactam antibiotics is frequently observed in the clinical setting and is commonly due to the expression of enzymes that can hydrolyze these compounds, namely β -lactamases (645, 743, 771-773). *P. aeruginosa* clinical isolates often harbor acquired β -lactamases particularly of the oxacillinase- (class D) and

metallo- β -lactamase IMP- and VIM-types (class B) (549, 608, 610, 614, 747, 748, 774, 775). Additionally, *P. aeruginosa* expresses a chromosomal cephalosporinase, AmpC, that provides intrinsic resistance and that can be expressed at high levels in the presence of β -lactams (711, 712, 776). A second chromosomally encoded oxacillinase-type β -lactamase, PoxB, has also been reported (740, 741). The aim of this study was to examine the role of the class D β -lactamase PoxB in *P. aeruginosa* PA01.

2.4.1 *poxA* and *poxB* form an operon. Previous *in silico* analysis revealed the oxacillinase-encoding gene (*PA5514*) to be located 49-bp downstream of a putative ORF annotated *PA5513* (Figure 2.1A). Given the close proximity between *PA5513* and *PA5514*, the absence of promoter like sequences in the intergenic region, and the presence of a potential ρ -independent terminator downstream of *PA5514*, we previously hypothesized that *poxA* and *poxB* form a two-gene operon (740). To determine if in fact these two genes are cotranscribed, RNA was extracted from the prototypic *P. aeruginosa* PA01, cDNA was synthesized and two sets of primers (RTpoxAFord2-RTpoxARev1 and RTpoxAFord2-RTpoxARev2) were used to amplify the intergenic region plus small portions of the 3' of *poxA* and the 5' of *poxB* (Figure 2.1A). As expected, the first set of primers yielded a 126-bp amplicon in both genomic DNA and cDNA templates (Figure 2.1B, lanes 1 and 2), with the second set yielding a 559-bp product in the same templates (Lanes 5 and 6). In the absence of template DNA or reverse transcriptase, no amplification was detected for both primer sets (Lanes 3, 4, 7 and 8). This work confirms that these two genes are cotranscribed and that they form an operon.

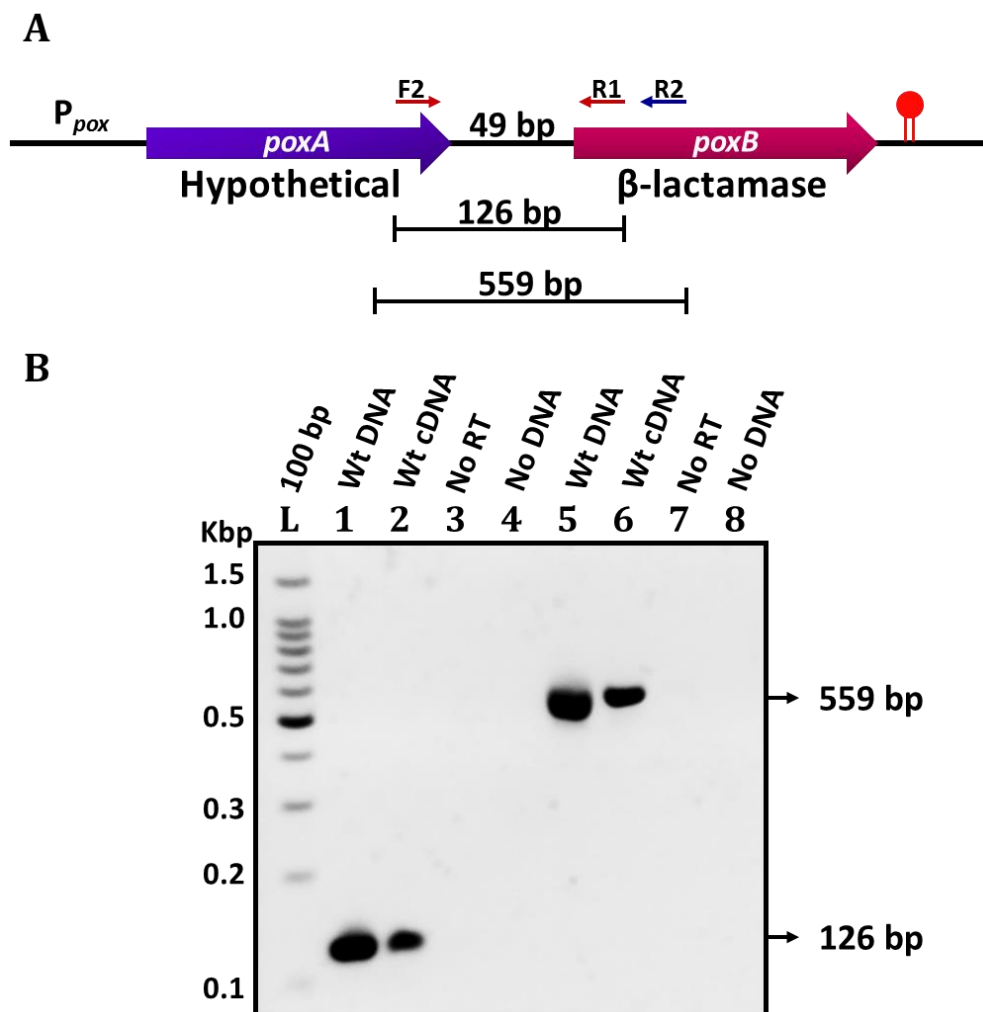


Figure 2.1. The *poxAB* operon. **(A)** The *pox* genes and the approximate location of primers used to show *PA5513* and *PA5514* form an operon. Two sets of primers were used to amplify the intergenic region as well as the 3' end of *poxA* and the 5' start of *poxB* from wild-type cDNA. Primer pair RTpoxAFord2 and RTpoxAREv1, denoted as F2 and R1 respectively, were used for amplification of a 126-bp product, while RTpoxAFord2 and RTpoxAREv2, denoted F2 and R2, were used to amplify a 559-bp region. **(B)** A 126-bp amplicon was detected in wild-type genomic and cDNA samples (Lanes 1 and 2) upon amplification with F2 and R1. Similarly, a 559-bp product was observed upon amplification with F2 and R2 in wild-type genomic and cDNA samples (Lanes 5 and 6). No amplification could be detected with either primer set in the absence of reverse transcriptase or template DNA (Lanes 3, 4, 7 and 8).

2.4.2 *poxA* is translated into a 32.4-kDalton protein. Since our present work indicates *poxA* and *poxB* are cotranscribed, it is also expected that *poxA* mRNA is translated into a protein with a function potentially related to that of PoxB. To confirm our prediction, the *poxA* ORF was amplified and cloned into the expression vector pET15bVP (756) and expressed in *E. coli* BL21(DE3) (761). Whole cell extracts were obtained from cells carrying the *poxA*-overexpressing plasmid in the presence and absence of IPTG. Proteins were visualized in an SDS-PAGE gel. A protein band corresponding to PoxA was detected at around 32 kDalton an hour after induction with IPTG and thereafter (Figure 2.2, Lanes 3-6). The same band was also present but reduced in whole cell extracts from uninduced cells that had been grown for 4 hours after induction (Lane 7). PoxA was absent from the uninduced and induced conditions at the 0 time point (Lanes 1 and 2), as well as from cells containing only empty vector (Figure 2.2, lanes 8-9). Thus the *poxA* ORF codes for a protein.

Since both *poxA* and *poxB* are part of a single operon, they could potentially have related functions. *In silico* analysis reveals that the nucleotide sequences composing this operon are not found anywhere else but in *P. aeruginosa*. Sequences showing high similarity to both the *poxA* ORF (73-82%) and the PoxA protein (66-78%) were also identified in other *Pseudomonas* species such as *P. denitrificans*, *P. entomophila*, *P. monteilii*, and *P. putida*, but were unlinked to any β -lactamase-encoding gene. Similar to PoxA, these hypothetical proteins are classified as putative hydrolases or acyltransferases of the α/β hydrolase superfamily. The α/β hydrolase fold is present in a varied number of proteins that share little in terms of function or sequence homology but show structural

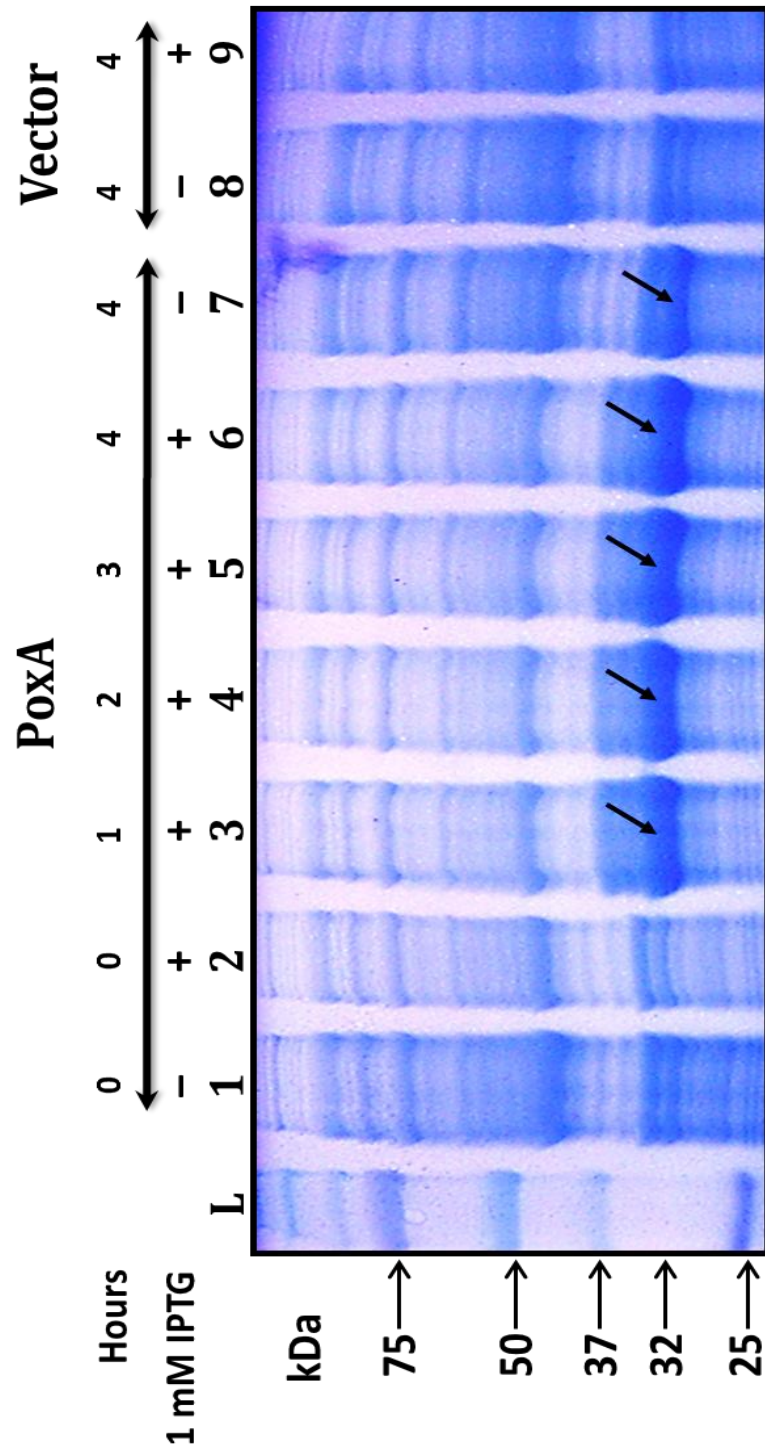


Figure 2.2. *poxA* is translated into a 32.4-kDalton protein. Whole cell extracts were obtained from *E. coli* BL21(DE3) cells carrying pET15bVP-PoxA in the absence (Lane 1) and presence of IPTG (Lane 2) at 0 time point of induction and at the 1-, 2-, 3-, and 4-hour mark of IPTG induction (Lanes 3-6). Uninduced cells grown for 4 hours (Lane 7) as well as cells

similarity (777, 778). Proteins carrying the canonical α/β fold include lipases, esterases, proteases, peroxidases as well as transporters and hormone precursors. The role of PoxA and its relation to PoxB are yet to be determined.

2.4.3 *poxA* and *poxB* deletions do not alter β -lactamase activity or β -lactam susceptibility. To investigate the role of *poxA* and *poxB* in *P. aeruginosa*, single in-frame deletions of each of the genes were generated. The β -lactamase and β -lactam susceptibility profiles were compared with that of the parent strain PA01. An in-frame deletion of *poxB* was also introduced into a previously generated PA0 Δ *ampC* strain (760). Single deletions of *poxA* or *poxB* did not alter the β -lactamase or susceptibility profiles of the strains as compared to the wild-type PA01 (Table 2.2 and Figure 2.3). Since the AmpC β -lactamase was present in these backgrounds and potentially obscuring the role of PoxB, the susceptibility and β -lactamase activity were also examined in strains lacking *ampC*.

Little to no β -lactamase was detected in both PA0 Δ *ampC* and PA0 Δ *ampC* Δ *poxB* suggesting AmpC is responsible for the activity observed in wild-type and in deletion *poxA* and *poxB* strains (Figure 2.3). Interestingly, a deletion of *ampC* alone significantly increased susceptibility against the aminopenicillins (ampicillin and amoxicillin), but produced only minor changes against the carbapenems (imipenem and doripenem), with no effect observed against the cephalosporins and the rest of the penicillins (Table 2.2). When overexpressed, however, AmpC was capable of restoring the phenotype and/or increasing resistance against all penicillins and cephalosporins tested (Table 2.3), in most

cases past clinical breakpoints, while having little to no effect on the carbapenems. These results are in agreement with the known hydrolysis spectrum of AmpC that includes penicillins and cephalosporins but rarely carbapenems (625, 626, 779, 780). Resistance against penicillins and cephalosporins has been observed in the clinical setting and correlates with different levels of AmpC derepression (379-381, 722, 781-783). Lastly, deleting *poxB* from an already *ampC*-deficient background did not further alter susceptibility suggesting either that PoxB plays no role in β -lactam resistance or that it is not expressed under the tested experimental conditions (Table 2.2).

Table 2.2: Susceptibility profiles of *poxA*, *poxB* and *ampC* deletion mutants

β -lactam	Minimum Inhibitory Concentration (μ g/ml)				
	PA01	Δ <i>poxA</i>	Δ <i>poxB</i>	Δ <i>ampC</i>	Δ <i>ampC</i> Δ <i>poxB</i>
Ampicillin	>256	>256	>256	16-24	24
Ampicillin/sulbactam	>256	>256	>256	16-32	16-32
Amoxicillin	>256	>256	>256	8-12	16-24
Amoxicillin/clavulanate	>256	>256	>256	8	8
Ticarcillin/clavulanate	8-12	12	8-12	8	12
Piperacillin/tazobactam	3-4	3	3	3	3-4
Piperacillin	4-6	4	4		
	4-6				
Aztreonam	1.5-3	2	2	1.5-3	2-3
Cefepime	1-1.5	1-1.5	1	0.75-2	1
Cefotaxime	4-6	6	6	4-6	4
Ceftazidime	1-1.5	1.5	1.5	1.5	1
Imipenem	1-1.5	1.5	1.5	0.25-0.5	0.38-0.5
Meropenem	0.25-0.5	0.28-0.50	0.28	0.25-0.75	0.25-0.28

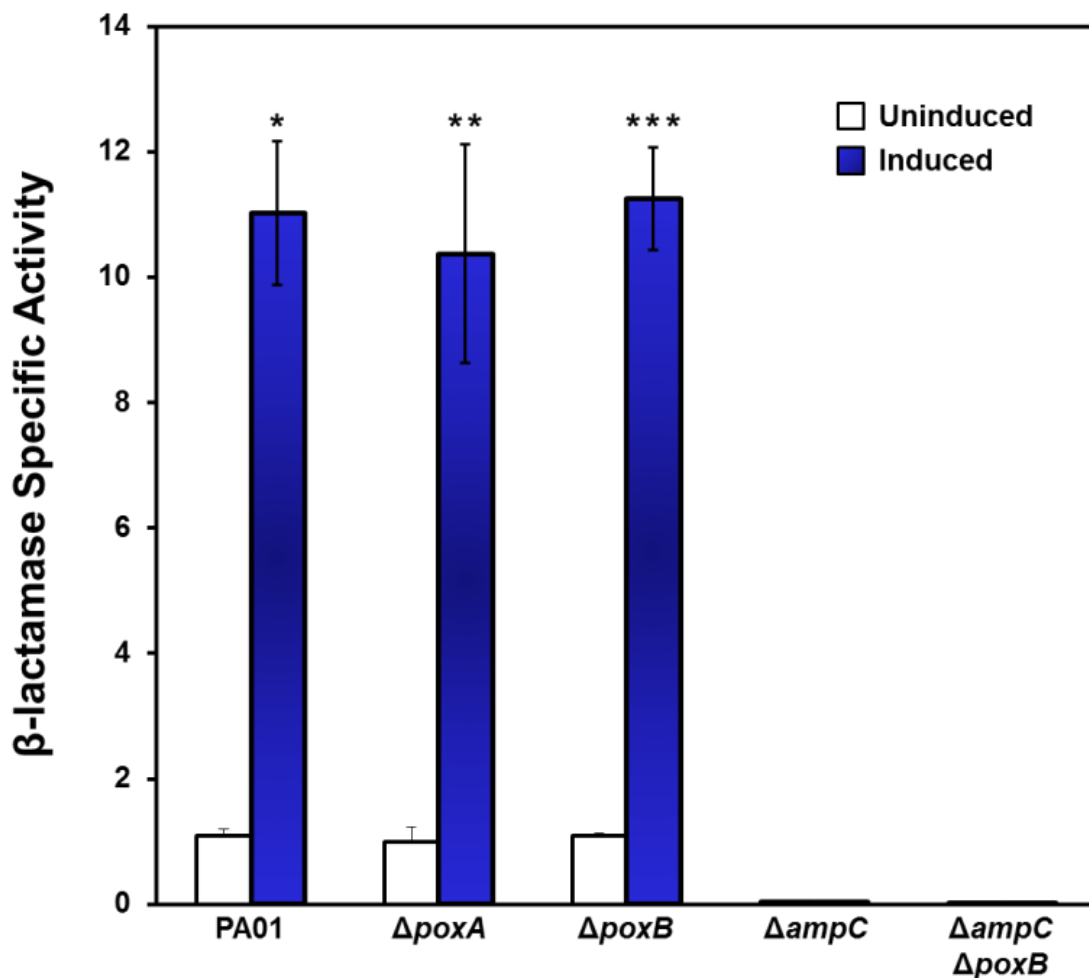


Figure 2.3. β -lactamase activity profile of *pox* and *ampC* deletion mutants. The β -lactamase activities of PA0 Δ *poxA*, PA0 Δ *poxB*, PA0 Δ *ampC* and PA0 Δ *ampC* Δ *poxB* were quantified in the presence (blue bars) and absence (white bars) of the inducer (benzylpenicillin at 50 μ g/ml for 1h). Assays were carried out on cell lysate with 100 μ M of nitrocefin as chromogenic substrate. One milliunit of β -lactamase was defined as 1 nanomole of nitrocefin hydrolyzed per minute per microgram of protein. * p -value < 0.005 for β -lactamase activity in PA01 induced versus activity in PA01 uninduced; ** p -value < 0.05 for β -lactamase activity in PA0 Δ *poxA* induced versus activity in PA0 Δ *poxA* uninduced; *** p -value < 0.005 for β -lactamase activity in PA0 Δ *poxB* induced versus activity in PA0 Δ *poxB* uninduced, as determined by paired t -test.

2.4.4 PoxB is a carbapenemase. To determine the role of PoxB in *P. aeruginosa*, we overexpressed *poxB* in a low-copy plasmid under the control of an IPTG-inducible promoter in both PA0 Δ *ampC* and PA0 Δ *ampC* Δ *poxB*. Overexpression of PoxB provided little to no resistance against the penicillins and the cephalosporins (Table 2.3). There was, however, a significant decrease in susceptibility (increase in MIC) against the carbapenems, meropenem and doripenem, as determined by E-test (Table 2.3). A four-fold increase in MIC was also observed against meropenem (not tested against doripenem) using the broth microdilution method (Figure 2.4). PoxB, however, did not appear to alter susceptibility against imipenem in an *ampC*- or *ampCpoxB*-deficient background suggesting PoxB may not be able to hydrolyze this carbapenem.

In carbapenems, the signature β -lactam ring, composed of one nitrogen and three carbons, is fused to an unsaturated five-atom ring that binds sulfur at position C2 (Figure 2.5). The basic structural differences between the carbapenems (imipenem, doripenem, and meropenem) are imparted by the varying side chains extending from the sulfur at C2. Meropenem and doripenem have bulkier side groups than imipenem. Additionally, there is a methyl group at C1, absent from imipenem that could be the basis for the lack of activity of PoxB against this compound. However, beyond structural differences, resistance/susceptibility to imipenem in *P. aeruginosa* must also take into account the role of the outer membrane porin OprD.

Table 2.3: Overexpression of <i>AMP^RC_{ampC}</i> and <i>PoxB_{ampC}</i> in <i>PA0ΔampCΔpoxB</i> and <i>PA0ΔampCΔpoxB</i>									
β-lactam	(vector)	(vector)	(vector)	<i>PA0ΔampC</i> (pPoxB)	<i>PA0ΔampCΔpoxB</i> (vector)	<i>PA0ΔampCΔpoxB</i> (pPoxB)	<i>PA0ΔampCΔpoxB</i> (pAmpC)		
Ampicillin	>256	24	24	24	32	64	>256		
Ampicillin/sulbactam	>256	12	16	16	12-16	16	>256		
Amoxicillin	>256	8-16	16	16	8-16	24	>256		
Amoxicillin/clavulanic acid	>256	8-12	12	12	8-12	16	>256		
Ticarcillin/clavulanic acid	12	6-12	8	8	8	12	64		
Piperacillin/tazobactam	3	2-3	1.5-2	1.5-2	3	3	64		
Piperacillin	3	3-4	4	4	4	3	>256		
Cefepime	1-1.5	1.5	1-1.5	1-1.5	1-1.5	1.5	4		
Cefotaxime	6	6	6	6	8	8	>256		
Ceftazidime	1.5	1	1	1	1	1-1.5	12-16		
Imipenem	1.5	0.5	0.5-0.75	0.5-0.75	0.5-0.75	0.5-0.75	0.5		
Meropenem	0.38	0.25-0.38	1.0-1.5	1.0-1.5	0.25-0.38	1.5-2	0.5		
Doripenem	0.38	0.094	1.5	1.5	0.094-0.125	1.5-2	0.75		

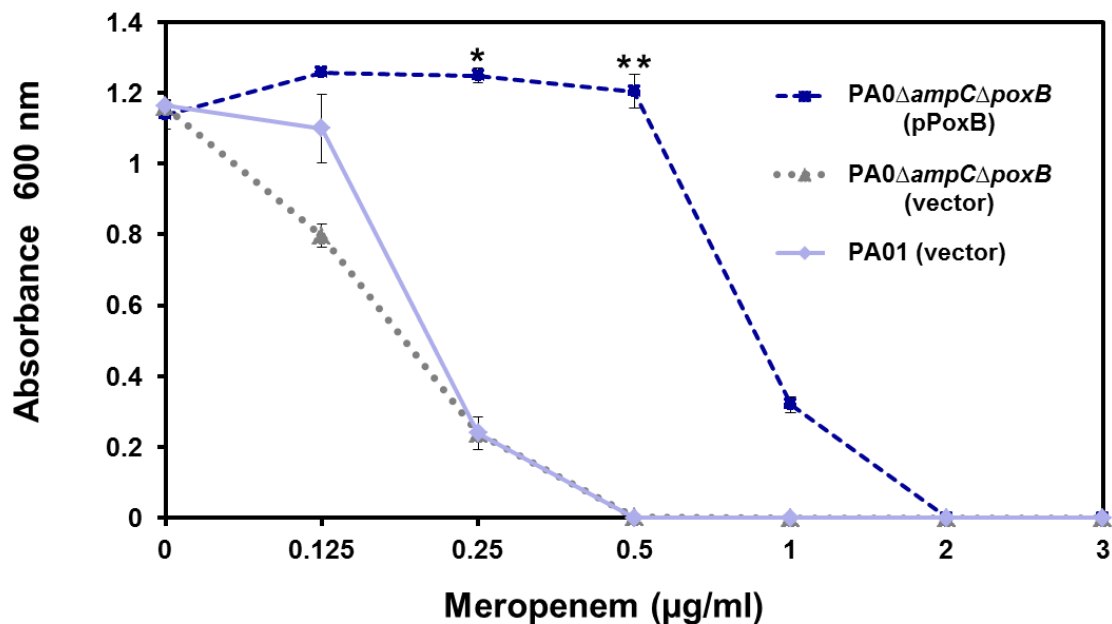


Figure 2.4. Determination of meropenem MIC in a PoxB-overexpressing background. The MIC of meropenem was determined by the microbroth dilution method in PA0ΔampCΔpoxB (pPoxB) (blue square), PA0ΔampCΔpoxB (vector) (grey triangles) and in PA01 (vector) (light blue diamond). Cells were grown O/N in the presence of 1 mM IPTG with increasing concentrations of meropenem (0-3 µg/ml). The final cell density measured at OD₆₀₀ after 18 hours of growth is reported. * p -value < 0.0001 for OD₆₀₀ of PA0ΔampCΔpoxB (pPoxB) versus optical density for PA0ΔampCΔpoxB (vector) with 0.25 µg/ml meropenem; ** p -value = 0.0005 for OD₆₀₀ of PA0ΔampCΔpoxB (pPoxB) versus optical density for PA0ΔampCΔpoxB (vector) with 1 µg/ml meropenem, as determined by unpaired t -test.

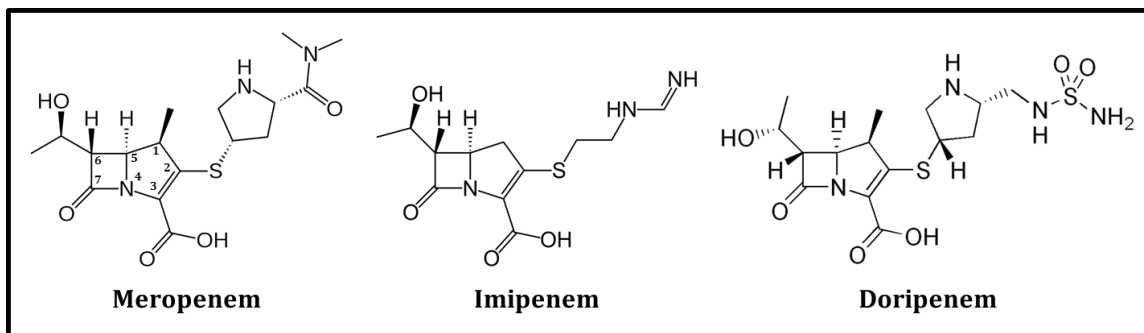


Figure 2.5. Chemical structures of carbapenems: meropenem, imipenem and doripenem. The positions of atoms in the core β -lactam ring and the carbapenem nucleus structure are numbered and depicted in meropenem.

OprD is a substrate-specific porin that mediates the diffusion of basic amino acids and imipenem into the cell (757, 784, 785). Imipenem remains a viable and powerful treatment option against *P. aeruginosa*, however, mutations in or loss of *oprD* and subsequent resistance to this β -lactam, often arise after imipenem treatment (313, 315, 786). In the absence of *oprD*, the MIC for imipenem increases to ~ 8 -16 $\mu\text{g/ml}$ as the antibiotic cannot penetrate the cell as fast as it could in the wild-type (305, 314, 315). Imipenem will still enter the cell, most likely through other pores and porins, albeit at a much lower rate (L. Silver, Personal communication). This slower rate of diffusion makes imipenem a better substrate for the AmpC β -lactamase, which is active in the periplasm of the cell. Thus, in an *oprD*-deficient background, the activity of imipenem is determined by the expression of *ampC* and potentially *poxB*, but not by the *mex* efflux system, as imipenem, unlike meropenem and doripenem, is not subject to efflux (312, 787, 788). If *ampC* is also removed from the equation, the activity of imipenem should be determined by PoxB. Previous studies have shown PoxB has some affinity against

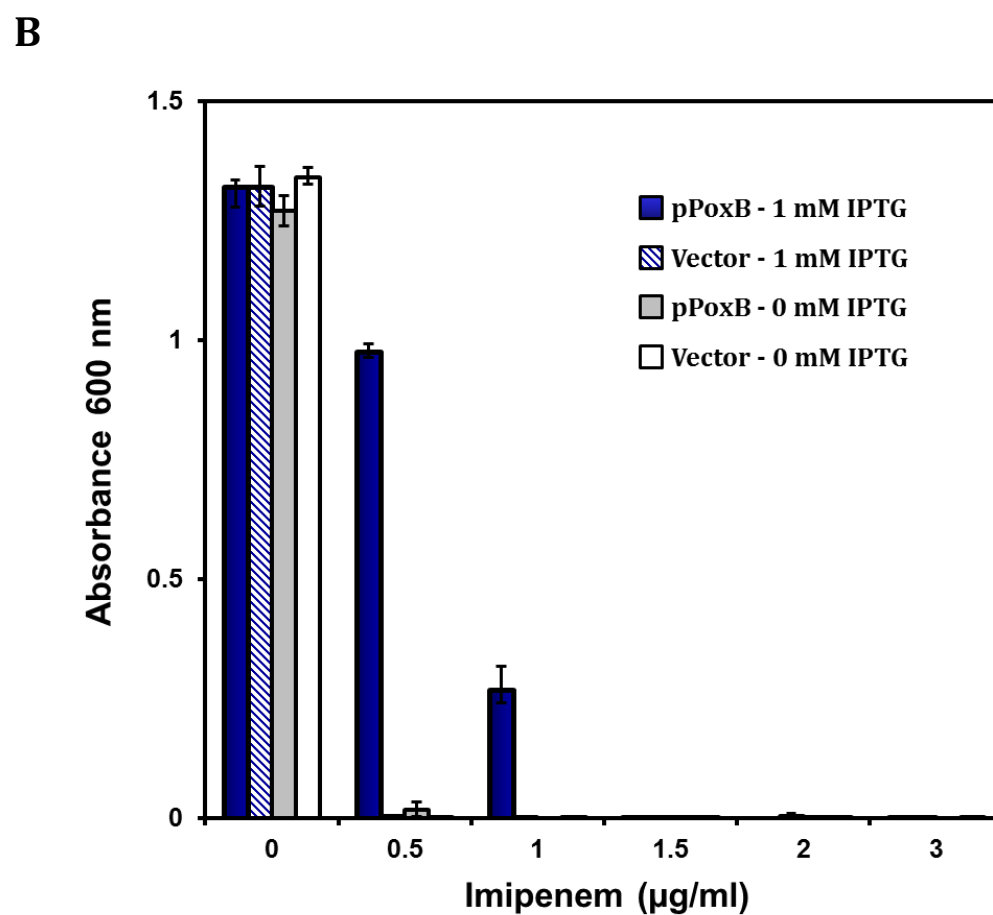
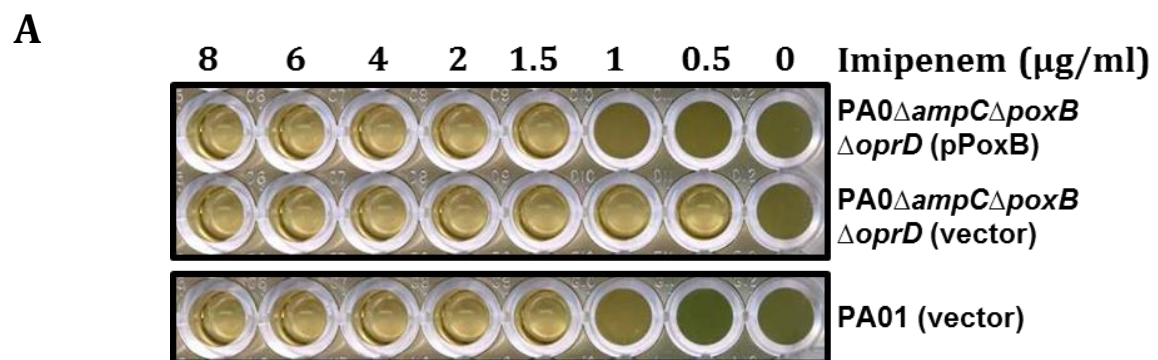
imipenem (741), however, PoxB activity against imipenem has not been examined in an *oprD*-deficient background. We thus set out to test the ability of PoxB to provide resistance against imipenem in the absence of *oprD*.

As expected, deletion of *oprD* alone significantly decreased susceptibility of the strains against the carbapenems, with the greatest increase in MIC observed for meropenem followed by imipenem (Table 2.4). In such a background, the MIC is presumably dictated by the action of AmpC and PoxB, along with efflux for meropenem and doripenem. AmpC plays a major role in providing resistance here, since in its absence, the strain becomes very susceptible to imipenem, but less so to meropenem and doripenem (Table 2.4). This work corroborates previous studies where imipenem resistance was found to be dependent upon both the loss of *oprD* and the expression of AmpC β -lactamase (305). Similarly, in our study AmpC afforded greater protection against imipenem in a less permeable background, e.g., loss of *oprD* (Table 2.4). Specifically the loss of *oprD* raised the MIC from 1 to 8 $\mu\text{g/ml}$, with a further deletion of *ampC* restoring susceptibility to that of wild-type levels (MIC = 1 $\mu\text{g/ml}$). Resistance against meropenem and doripenem in our study, was found to be affected mainly by the loss of *oprD*, with only minor changes observed upon deletion of *ampC*, suggesting these two carbapenems are not good substrates for AmpC and/or they are efficiently effluxed. In agreement with these results, our single *ampC* deletion mutant also showed a slight increase in susceptibility against imipenem but no change against meropenem (Table 2.2).

		Minimum Inhibitory Concentration (µg/ml)			
Carbapenems	Concentration (mM)	PA0ΔampCΔpoxB profile			
		PA0ΔampCΔpoxB (vector)	PA0ΔampCΔoprD (vector)	PA0ΔampCΔpoxB ΔoprD (vector)	PA0ΔampCΔpoxB ΔoprD(pPoxB)
Imipenem	0	1.5	8-12	0.75-1	0.75-1.5
	1	1.5	8-16	1	4-8
Meropenem	0	0.38-0.75	6	2-4	4
	1	0.50-0.75	6	2-4	6
Doripenem	0	0.38	0.75-1	0.75	1-1.5
	1	0.38	1.5	0.5-1	1
					8-12

A further deletion of *poxB* from an *ampCoprD*-deletion mutant did not alter susceptibility (Table 2.4). However, overexpression of PoxB in an *oprDampCpoxB*-deficient background significantly increased the MICs of all the carbapenems, including imipenem (Table 2.4), showing that in fact PoxB can hydrolyze all of these β -lactams. In particular, the increase in MIC observed for meropenem reached the CLSI clinical resistant breakpoint for this carbapenem ($\geq 8 \mu\text{g/ml}$) (767). Determination of MIC by the microbroth dilution method corroborated the E-test results (Figure 2.6 A-D).

Previous biochemical studies showed that the supposed preferred substrates of oxacillinases, namely oxacillin and cloxacillin, were hydrolyzed very poorly, if at all by PoxB (741). Additionally, PoxB exhibited very low affinity towards most of the substrates tested, which included benzylpenicillin, ampicillin, piperacillin and cephalothin. PoxB, however, did exhibit the highest affinity towards imipenem, although its ability to hydrolyze it was low (741). Against meropenem, PoxB exhibited low affinity and weak hydrolysis, while overexpression *in trans* did afford the cells a decreased in susceptibility (increase in MIC) against meropenem but not imipenem (741). Similarly, our work shows that PoxB does not significantly hydrolyze penicillin and cephalosporin β -lactams. In contrast to previous work, however, we show PoxB is capable of hydrolyzing not only meropenem but also imipenem and doripenem producing a decrease in susceptibility (higher MIC) towards these β -lactams when overexpressed. Although PoxB biochemically may not have the greatest affinity towards some of these carbapenems, its expression does afford the cell greater protection against these β -lactams. PoxB thus is a carbapenemase with a narrow spectrum of hydrolysis.



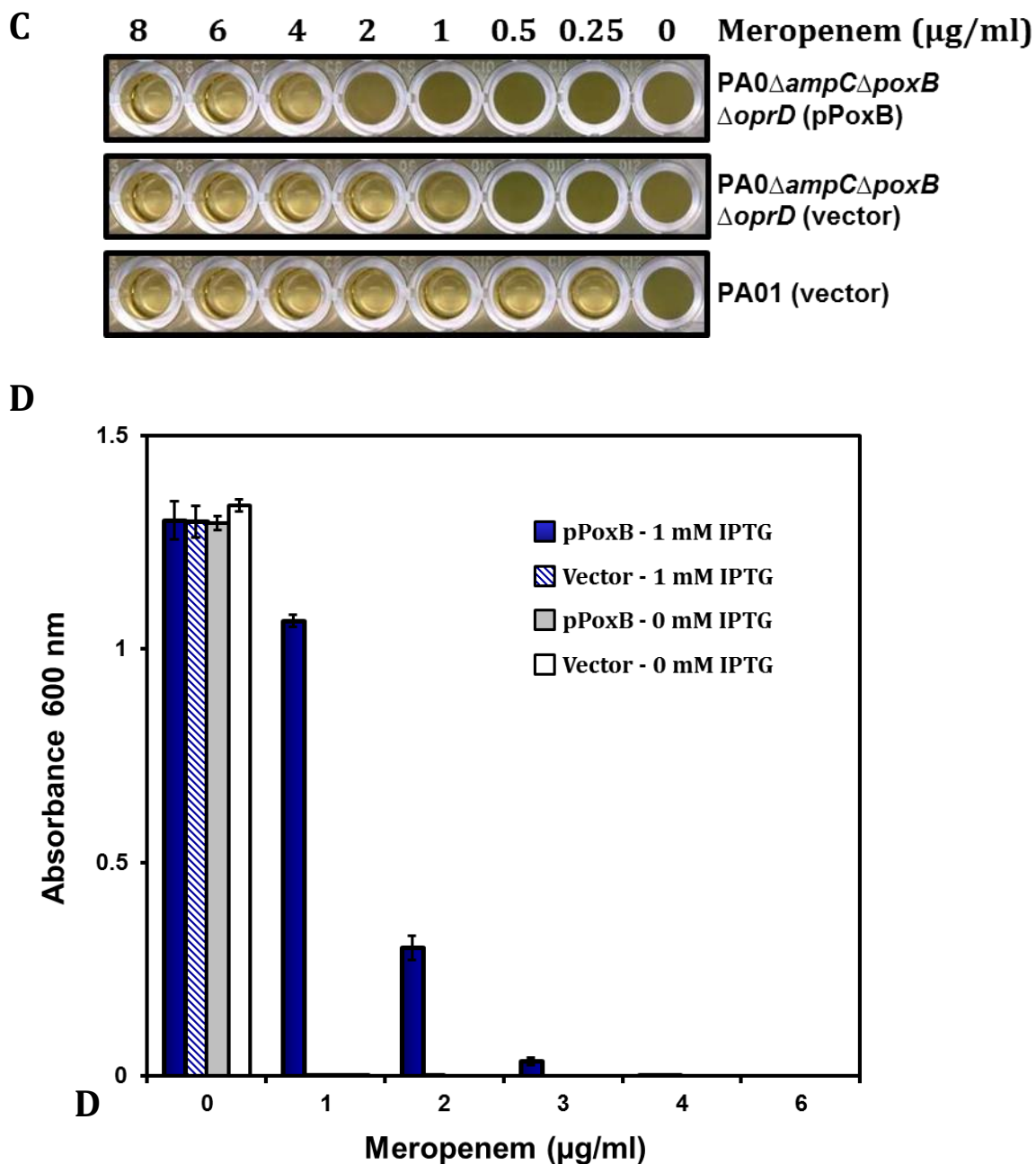


Figure 2.6. Determination of carbapenem MICs in *P. aeruginosa* *oprD*-deficient strains by the microbroth dilution method. (A) MIC of imipenem in the presence of 1 mM IPTG. (B) Optical density of cells grown O/N with increasing concentrations of imipenem (0-3 $\mu\text{g/ml}$) in the presence and absence of IPTG (1 mM). (C) MIC of meropenem in the presence of 1 mM IPTG. (D) Optical density of cells grown O/N with increasing concentrations of meropenem (0-3 $\mu\text{g/ml}$) in the presence and absence of IPTG (1 mM).

2.4.5 Expression of PoxB does not alter efflux. It is expected that the significant decrease in susceptibility against carbapenems observed above is caused by the expression of PoxB and not by an increase in the activity of efflux pumps, especially as imipenem is not the subject of efflux. Nonetheless, the activity of efflux pumps was checked by examining the susceptibility of PoxB-overexpressing strains against known efflux substrates. As expected, the MIC values did not significantly differ between wild-type and PoxB-overexpressing strains (Table 2.5), suggesting efflux systems were neither enhanced nor compromised by the increased expression of PoxB. Thus, these results clearly argue that the increase in carbapenem MIC (Tables 2.3 and 2.4) is provided by the expression of PoxB.

Table 2.5: MIC in the presence of efflux substrates

Antibiotic	Range (µg/ml)		
	PA01 vector	PA0Δ <i>ampC</i> Δ <i>poxB</i> (vector)	PA0Δ <i>ampC</i> Δ <i>poxB</i> (pPoxB)
Chloramphenicol	32	32	32
Trimethoprim	150	150	100
Novobiocin	512	512	512
Tetracycline	8	8	8
Ofloxacin	1	1.5	1
Norfloxacin	0.75	0.75	0.75
Ciprofloxacin	0.19	0.19	0.19
Levofloxacin	0.38	0.38	0.38

2.4.6 Inhibitors of PoxB. β-lactam antibiotics are often co-administered with β-lactamase inhibitors to improve the efficacy of treatment against β-lactamase-expressing pathogens (423, 428, 557). β-lactamase inhibitors are β-lactams themselves with only

minor antibacterial activity (789-792) that can either reversibly or irreversibly inhibit the β -lactamase thus preventing it from hydrolyzing the co-administered antibiotic (423, 793-796). Currently, three irreversible β -lactamase inhibitors, clavulanic acid (797, 798), sulbactam (426, 799, 800) and tazobactam (801, 802) are approved for clinical use. Their activity is limited to most class A enzymes, being generally ineffective against classes B, C and D β -lactamases (428, 467). PoxB has been shown to be only poorly inhibited by these (741). A new β -lactamase inhibitor, termed avibactam, with a broader spectrum of activity has been developed and is currently undergoing phase III clinical trials in combination with ceftazidime (803-805). Unlike the aforementioned compounds, avibactam is not a β -lactam, although its structure closely resembles one (804). The ability of avibactam to inhibit PoxB activity was examined and compared to that of weak inhibitors of oxacillinases, namely tazobactam, sulbactam and clavulanic acid.

Subinhibitory concentrations of imipenem (0.5 $\mu\text{g/ml}$) and meropenem (1 $\mu\text{g/ml}$) that only allowed *PA0 Δ ampC Δ poxB Δ oprD* to grow when PoxB was overexpressed (Figure 2.6A and 2.6C) were used to examine the inhibitory action of tazobactam, sulbactam and clavulanic acid against PoxB. A slight inhibition of growth was apparent with tazobactam in the presence of imipenem, and to a smaller extent with sulbactam (Figure 2.7). In the presence of clavulanic acid, growth either remained unaltered or was slightly enhanced. Overall, as expected, the ability of PoxB-overexpressing strains to grow on imipenem or meropenem was not significantly affected by the presence of these inhibitors at the tested clinical concentrations, indicating these compounds did not inhibit PoxB activity (Figure 2.7).

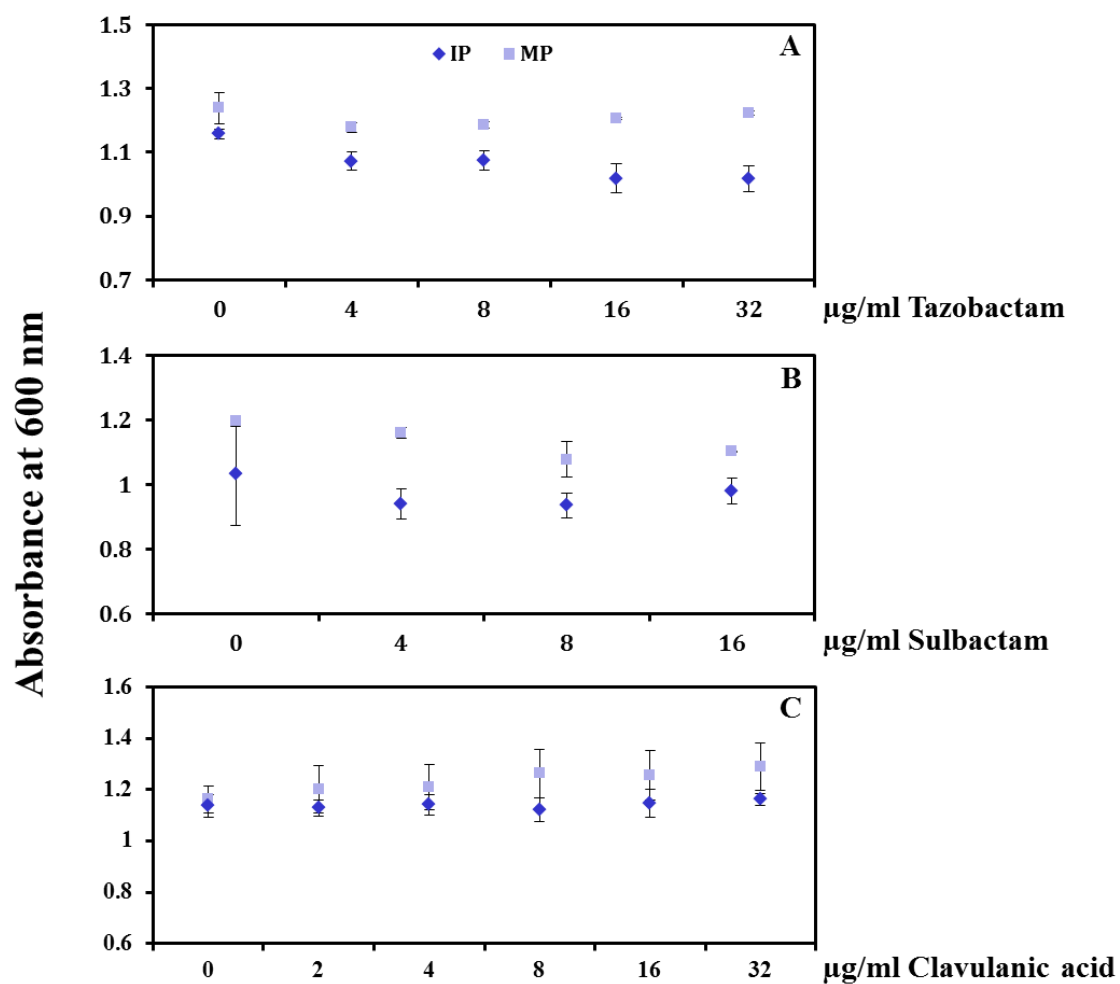


Figure 2.7. Effect of tazobactam, sulbactam and clavulanic acid on PoxB activity. The ability of *PA0ΔampCΔpoxBΔoprD* (pPoxB) to grow in the presence of subinhibitory concentrations of imipenem (0.5 µg/ml) or meropenem (1 µg/ml) was examined in the presence of the class A enzyme inhibitors tazobactam (**A**), sulbactam (**B**) and clavulanic acid (**C**). OD₆₀₀ readings after O/N growth with aeration at 37°C are reported.

Similarly, the ability of avibactam to inhibit PoxB activity was tested by growing PoxB-expressing strains in CAMH agar with IPTG in the presence and absence of avibactam at a clinically used concentration (4 µg/ml). The presence of PoxB increases the MIC against imipenem, meropenem and doripenem (as detailed in Section 2.4.4, Table 2.4) since PoxB can hydrolyze these carbapenems. Inhibition of PoxB by avibactam, should then produce a decrease in MIC against the carbapenems. Our results show, however, that the MIC of PoxB-expressing cells was not altered in the presence of this inhibitor (Table 2.6), suggesting avibactam does not significantly inhibit PoxB activity.

Table 2.6: Effect of avibactam on carbapenem MIC

Carbapenems	Minimum Inhibitory Concentration (µg/ml)		
	CAMH	PA0Δ <i>ampC</i> Δ <i>poxB</i> Δ <i>oprD</i> (vector)	PA0Δ <i>ampC</i> Δ <i>poxB</i> Δ <i>oprD</i> (pPoxB)
Imipenem	Alone	0.25	0.75
	IPTG	1.5	6
	IPTG + AV	1.5	4, 6
Meropenem	Alone	6	4
	IPTG	4, 6	32
	IPTG + AV	6, 4	32
Doripenem	Alone	0.75-1	0.75-1
	IPTG	0.75-1	8
	IPTG + AV	1-1.5	8

AV- Avibactam at 4 µg/ml

The inhibitory action of these compounds was also tested directly on cell lysate from PoxB-overexpressing cells by quantification of the β -lactamase activity. Concentrations as high as 700 μ M of tazobactam failed to reduce the β -lactamase activity in cell lysates from PA0 Δ *ampC* Δ *poxB* (pPoxB) cells, while only a minor reduction was observed with 4000 μ M of sulbactam (Figure 2.8A and 2.8B). A general downward trend of inhibition was observed with clavulanic acid starting at 50 μ M, but a 50% inhibition was not reached even at high levels of the inhibitor (4000 μ M). Avibactam decreased PoxB activity by more than half (Figure 2.8C) at a concentration of 2000 μ M (530.488 μ g/ml), which is 75-fold higher than that of the highest concentration of avibactam used in clinical studies (0.5-7 μ g/ml) (806-808). Notwithstanding that avibactam produced greater inhibition than the other tested inhibitors, the large quantities of the compound needed to inhibit PoxB activity, makes avibactam a weak inhibitor.

Avibactam at a concentration of 4 μ g/ml has proven sufficient and efficient at increasing susceptibility and/or eliminating resistance against a number of β -lactams in a large number of clinical isolates (809-811). For instance, in a study of 1466 Gram-negative isolates collected from patients hospitalized with pneumonia, bloodstream, intra-abdominal and urinary tract infections, avibactam in combination with ceftazidime effectively reduced MICs from 16 or 32 to 0.25 μ g/ml in 90% of Enterobacterial isolates (809). About 99.8% of such isolates exhibited an MIC of ≤ 4 μ g/ml, the CLSI clinical susceptible breakpoint for ceftazidime (809). Similarly, avibactam reduced the MIC anywhere from 16 or 32 to 4-8 in 90% of *P. aeruginosa* isolates, with an average of

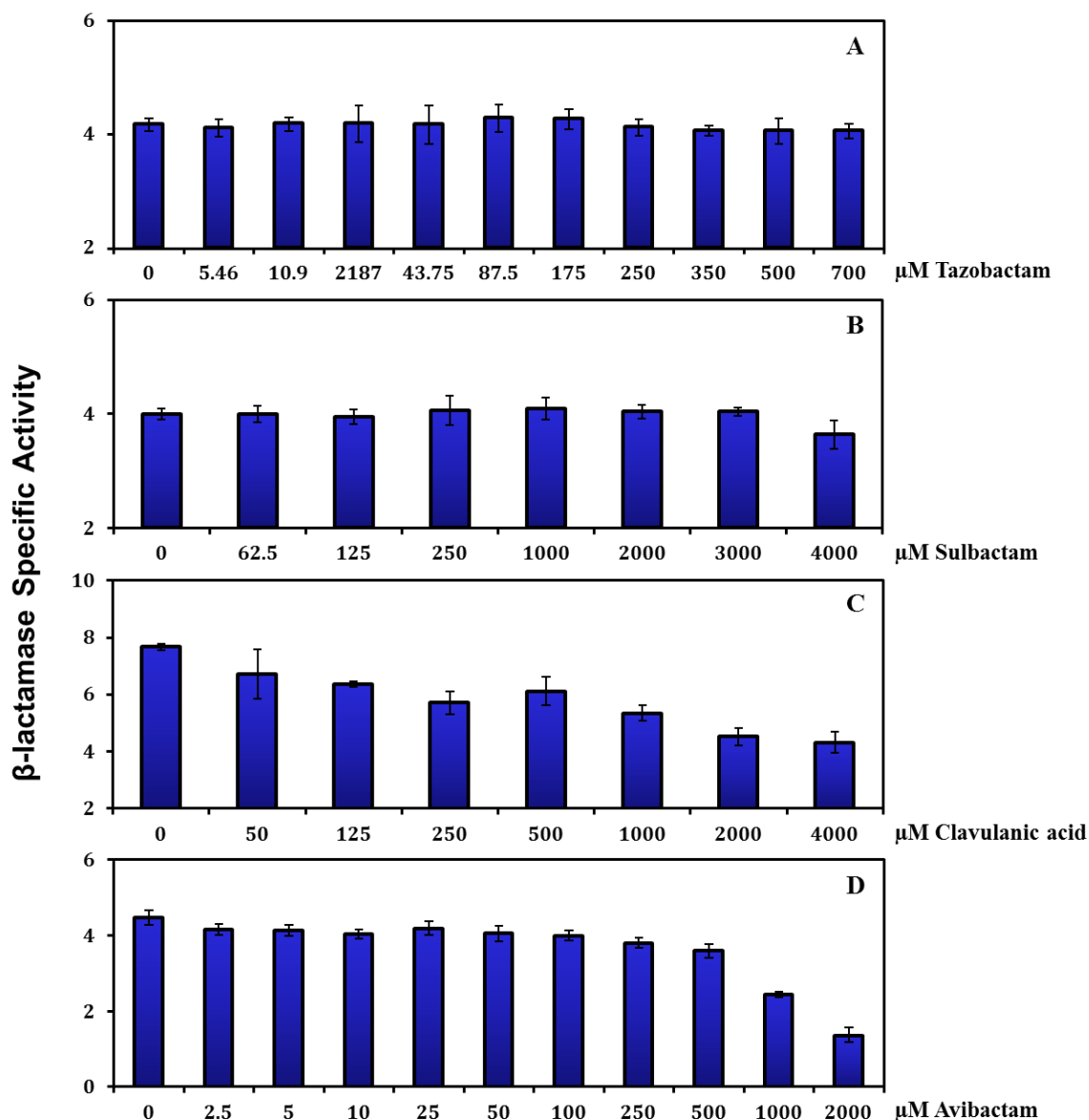


Figure 2.8. PoxB β -lactamase activity in the presence of tazobactam, sulbactam, clavulanic acid and avibactam. The β -lactamase assay was performed on lysate from IPTG-induced (1 mM) PA0 $\Delta amp^C \Delta poxB$ (pPoxB) cells. Lysate was incubated for 20 min with increasing concentrations of tazobactam (A), sulbactam (B), clavulanic acid (C), and avibactam (D). Chromacef was used as a chromogenic substrate. One milliunit of β -lactamase was defined as 1 nanomole of chromacef hydrolyzed per minute per microgram of protein.

96.8% of all isolates showing a ceftazidime/avibactam MIC of ≤ 8 $\mu\text{g/ml}$, the CLSI susceptible breakpoint for ceftazidime in this organism (809).

Although avibactam has shown variable activity against class D enzymes, it remains a more potent and effective inhibitor of class A (805-808, 812) and C (805, 807, 812, 813) enzymes, including the problematic class A *Klebsiella pneumoniae* carbapenemases or KPCs (814, 815). A study found that avibactam potentiated the activity of both ceftazidime and aztreonam against meropenem-nonsusceptible Gram-negative bacilli expressing multiple β -lactamases of the class A and C types (811). Avibactam, however, was less successful at reducing the MIC of *Acinetobacter baumannii* isolates carrying OXA-23-like genes (MIC₉₀ fold reduction of 2-4 vs fold reduction of 32-256 against class A, B and C enzymes in that particular study) (811). Similarly, avibactam did not significantly improve the activity of cefepime, ceftazidime or imipenem in *A. baumannii* isolates carrying PER-1, OXA-51 and OXA-58, with only a minor increase in susceptibility (~ 2 -fold decrease in resistance) observed (816). The efficacy of ceftazidime against an OXA-5-expressing *P. aeruginosa* clinical isolate was also not improved by the presence of avibactam (817).

OXA-48 appears to be just one of a few class D enzymes against which avibactam is active. A study showed that *K. pneumoniae* and *E. coli* isolates harboring the carbapenemase OXA-48 exhibited significant reduction in the MIC of imipenem, cefepime and ceftazidime when combined with avibactam, leading to susceptibility in 90% of isolates (816). In another study, the number of *K. pneumoniae* OXA-48-producers showing resistance was eliminated and/or susceptibility was enhanced by the

addition of avibactam (818). However, a kinetics study also found the rates of reaction of avibactam with OXA-48 and OXA-10 to be much slower than that of the other tested class A and C enzymes (804). The *P. aeruginosa* OXA-10 was actually found to be poorly inhibited by avibactam (804). Although avibactam has not been tested against very many OXA enzymes and while the structure and hydrolytic activities of oxacillinases is wide and varied, the emerging trend suggests that avibactam is not a very good inhibitor of class D enzymes. It is thus not surprising avibactam is only a weak inhibitor of PoxB (OXA-50).

2.4.7 Inhibition by NaCl. Oxacillinases are known to be inhibited by NaCl *in vitro* (669, 674, 682). This characteristic is commonly attributed to the presence of a Tyr residue at position 144, with replacement by Phe leading to resistance to inhibition (703, 819). Inhibition is often showed directly by measuring enzyme activity (β -lactamase assay), or indirectly by determining MIC changes (820). Previous work showed inhibition of purified PoxB by NaCl (IC_{50} , 50 mM), which not surprisingly carries Tyr at position 144 (741). We here further test if the previously reported NaCl inhibition of PoxB can translate into quantifiable changes that can affect the ability of PoxB to provide resistance. To that end, PA0 $\Delta ampC\Delta poxB\Delta oprD$ (pPoxB) was grown in the presence of subinhibitory concentrations of imipenem (0.5 μ g/ml) or meropenem (1 μ g/ml) that only allowed growth if PoxB was expressed. Growth was monitored O/N and inhibition of growth was used as an indirect indicator of PoxB activity.

NaCl produced a small, but steady increase in growth up to a concentration of 50 and 100 mM in the presence of meropenem or imipenem, respectively (Figure 2.9). A slight growth decrease was detected in the presence of meropenem at NaCl concentrations of 100 mM or higher. Concentrations of ≥ 200 mM decreased growth below the level of untreated cells, suggesting NaCl was compromising PoxB-dependent growth (Figure 2.9). In the presence of imipenem, growth never fell below that of untreated cells, but the enhancement of growth seen with up to 100 mM was lost at NaCl concentrations of 200 mM or higher, also suggesting that this higher range of solute was affecting the ability of PoxB to hydrolyze the β -lactam.

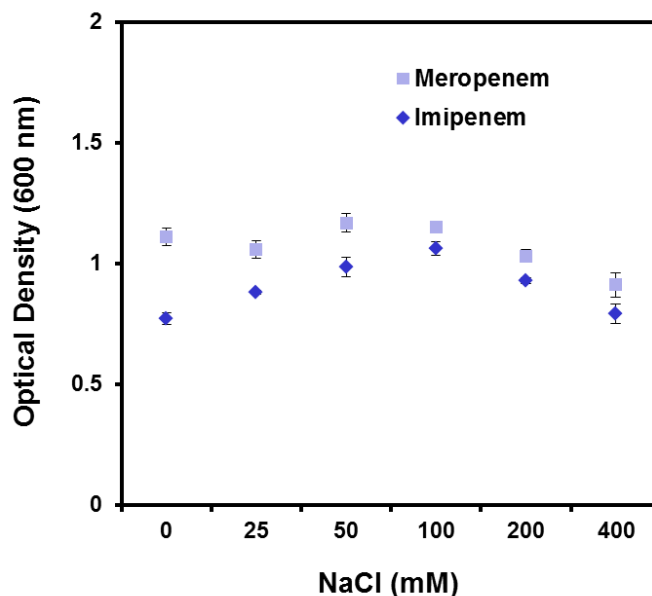


Figure 2.9. PoxB activity in the presence of NaCl. Inhibition of PoxB by NaCl was examined by growing *PA0 Δ ampC Δ poxB Δ oprD* (pPoxB) O/N in the presence of subinhibitory concentrations of meropenem or imipenem with IPTG (1 mM) and with increasing concentrations of NaCl. The subinhibitory concentrations of meropenem and imipenem selected were those previously shown to allow growth of only PoxB-overexpressing cells.

Although small inhibition of growth is detected with NaCl, particularly at higher concentrations, NaCl did not completely abolish growth of PA0 Δ *ampC* Δ *poxB* Δ *oprD* (pPoxB) in LB or CAMH broth containing meropenem or imipenem (Figure 2.9). In short, PoxB activity did not appear to be inhibited by NaCl to levels significant enough to prevent hydrolysis of the carbapenems and subsequently growth in media containing these β -lactams. The slight decrease in growth observed, however, was determined to be mostly unrelated to osmolarity, since cells grown in the presence of NaCl only lysed at a concentration of 800 mM of solute (Figure 2.10). A concentration of 400 mM does appear to slightly affect osmolarity leading to a <2-fold reduction in absorbance as compared to growth in 200 mM of NaCl.

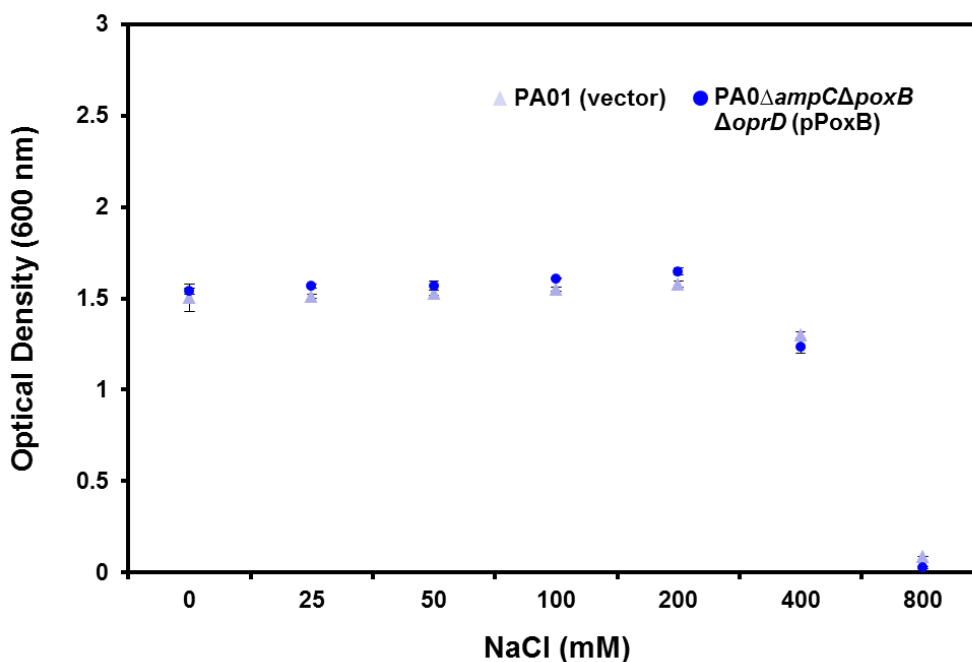


Figure 2.10. Determination of MIC for NaCl. The concentration of NaCl needed to lyse cells was determined by growing PA01 (vector) and PA0 Δ *ampC* Δ *poxB* Δ *oprD* (pPoxB) O/N with increasing concentrations of NaCl in CAMHB media.

Inhibition by NaCl, however, was apparent on cell lysate from PA0 Δ *ampC* Δ *poxB* (pPoxB) cells (Figure 2.11). Specifically, β -lactamase activity decreased with increasing concentrations of NaCl. The phenotype was readily observed in the absence of IPTG. However, in the presence of IPTG, where the levels of PoxB are significantly higher, NaCl inhibition could not be appreciated (Data not shown).

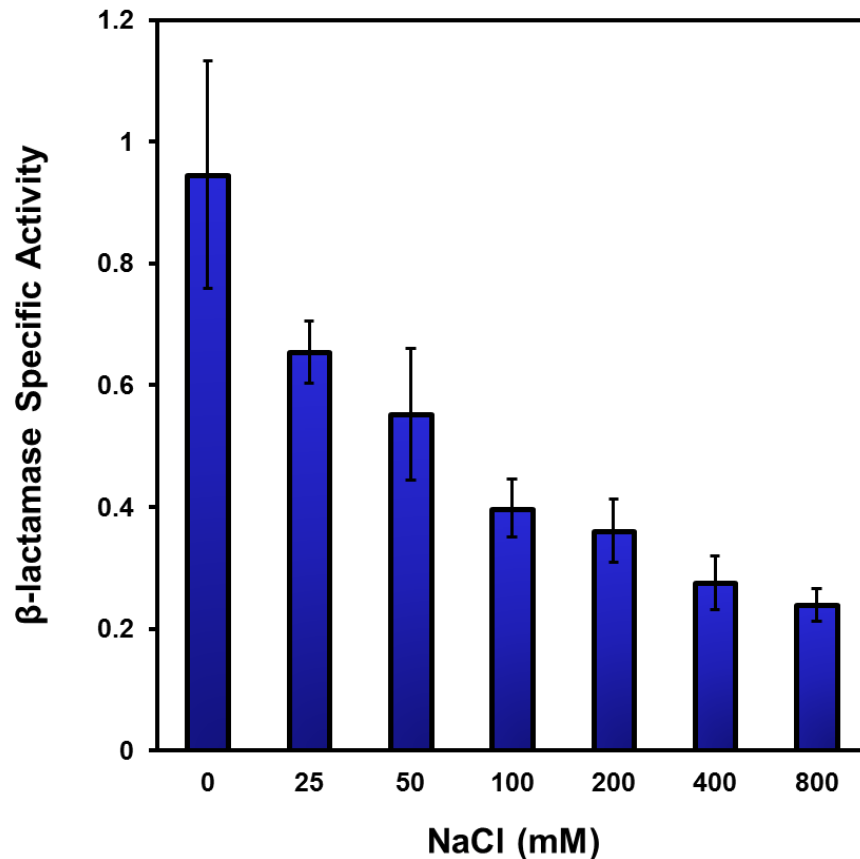


Figure 2.11. Inhibition of PoxB by NaCl. PoxB inhibition was measured *in vitro* as a function of β -lactamase activity. The β -lactamase assay was performed on lysate from IPTG-induced and uninduced (1 mM) PA0 Δ *ampC* Δ *poxB* (pPoxB) cells. Lysate was incubated for 20 min with increasing concentrations of NaCl (0-800 mM). Only data for uninduced lysate is shown. Nitrocefin was used as a chromogenic substrate. One milliunit of β -lactamase was defined as 1 nanomole of nitrocefin hydrolyzed per minute per microgram of protein.

The CF lung is notoriously susceptible to bacterial infections and in particular to colonization by *P. aeruginosa* (122, 123). The most widely accepted hypothesis of CF pathobiology, the low volume hypothesis, states that mutations in the *CFTR* gene cause an excessive intake of NaCl in the lung epithelium leading to surface airway dehydration and impaction of mucus thus setting up the stage for the chronic and persistent bacterial infections that are the hallmarks of CF lung disease (155, 177, 189). The CF lung, with its imbalance of Na⁺ and Cl⁻ ions transport, may confer an advantage to OXA-producing organisms susceptible to inhibition by NaCl than otherwise provided by healthy lung. By the same token, OXA-enzymes susceptible to NaCl inhibition may exhibit decreased activity in the normal lung. However, it has been difficult to measure the salt content on surface airway epithelium. Mice and epithelial culture studies, although substantiating the low volume hypothesis, have not shown a difference in ion content between healthy and CF models (174, 176, 821-823). Thus, it is difficult to ascertain the ramification of the imbalance of Na⁺ and Cl⁻ transport caused by *CFTR* mutations on the activity of OXA-type β -lactamases, if at all.

2.4.8 PoxA and Congo red binding phenotype. A Biolog microarray phenotypic assay pointed to a growth defect for the PA0 Δ *poxA* mutant in the presence of 10 mM NaNO₃. Since *P. aeruginosa* is a facultative anaerobe capable of using NO₃ as a terminal electron acceptor (824), we investigated the colony morphology of *poxA* mutants on CR and CB plates with NaNO₃ in aerobic and anaerobic conditions. Interestingly, a phenotype was observed in the presence of oxygen and in the absence of NaNO₃ (Figure 2.12).

Specifically, overexpression of PoxA produced colonies with an outer dark ring and a darker inner ring, while the center of the colony remained white (Figure 2.12). The observed red phenotype here is indicative of hyperbinding to CR and suggests enhanced production of extracellular polysaccharides such as Pel or Psl (768, 770).

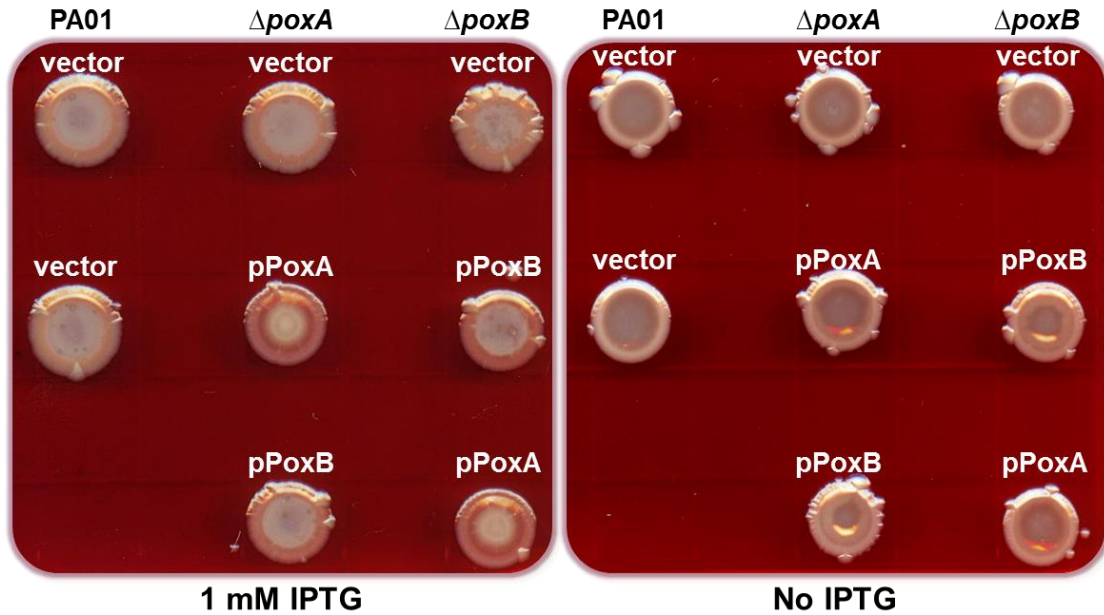


Figure 2.12. Colony morphology on CR. Fresh cultures were diluted to an OD₆₀₀ of 0.05 and 10 μ l of each were spotted on agar plates containing 1% tryptone and 1% agar supplemented with 40 μ g/ml CR and 20 μ g/ml CB in the presence of gentamycin (75 μ g/ml) and IPTG (1 mM). Plates were incubated at 25°C in the dark for five days. The colonies were imaged every 24 hours using an Epson scanner. Fifth-day colonies are shown.

Non-mucoid *P. aeruginosa* strains can secrete two extracellular polymeric substances, Pel (768, 825, 826) and Psl (769, 827) polysaccharides, that are known to contribute to biofilm formation. Although the *P. aeruginosa* strain PA01 has all the genes necessary for production of both Pel and Psl, the latter has been shown to play a bigger

role in biofilm formation and maturation in this background (768, 769, 826, 827). The PA14 strain, on the other hand, can only produce Pel due to a three-gene deletion in the *psl* locus (768, 825, 826). Nonetheless, a recent study showed that both Pel and Psl polysaccharides were involved in micro- and macrocolony formation and in subpopulation interactions in the PA01 biofilms (828), while a PA01 *psl* mutant was able to form biofilm after acquiring mutations that allowed it to overexpress Pel (829).

CR is known to bind polysaccharides containing β -1,3- or β -1,4-glycosidic linkages (830), and thus has been used to detect the carbohydrates-rich components in the extracellular matrices of a number of bacteria (831-833). CR is in fact routinely used to detect the presence of the Pel and Psl polysaccharides in *P. aeruginosa* (768-770, 825). In order to determine if hyperbinding to CR observed upon expression of PoxA (Figure 2.12), is the result of increased production of either Pel or Psl polysaccharides, pellicle formation, autoaggregative properties and binding to CR were examined in liquid media. All strains were able to form pellicle at the liquid-air interphase and there was no difference by visual inspection in the amount of pellicle being formed (Figure 2.13). Similarly, not much difference was observed in the ability of the pellicles to bind CR (Figure 2.14) or in the ability of the strains to form aggregates on the walls of the tubes (Data not shown).

Expression of PoxA thus, does not appear to contribute to an increased production of Pel or Psl in liquid media. Thus, at first glance, the CR-binding phenotype observed in Figure 2.12, does not appear to be the result of an increased production of Pel or Psl polysaccharides. Clearly the surface attached biofilm growing on plates are able to bind

CR there, but the basis for binding remains unknown. Given that CR is known to bind polysaccharides, it is likely that the CR phenotype observed here is due to increased production of a carbohydrate or polysaccharide. Alternatively, the surface attached biofilms that grow on plates may be conducive to the production of Pel or Psl in plates but not in liquid media. CR, however, has also been shown to bind non-cellulose material of the extracellular matrix, such as the proteinaceous thin aggregative fimbriae and curli polymers produced by *Salmonella* spp. and *E. coli* isolates, respectively (834-836).

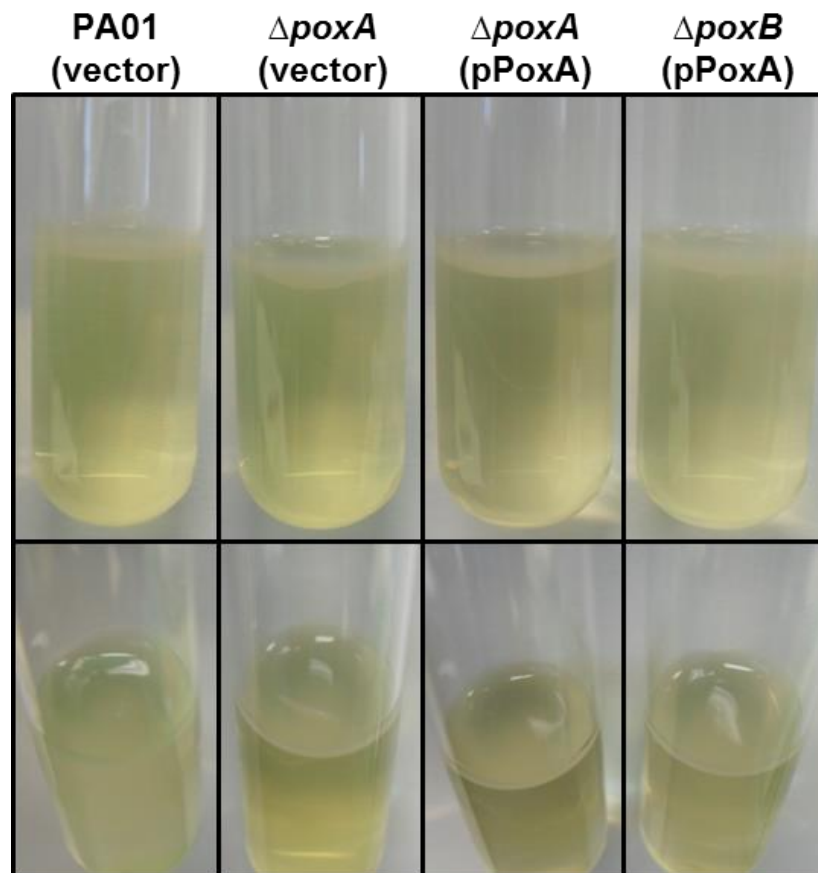


Figure 2.13. Pellicle formation in PoxA-expressing cells. Stationary phase cultures were diluted to an OD₆₀₀ of 0.025 in T-broth and pellicle formation was monitored at room temperature without shaking for seven days. Seventh-day cultures are shown.

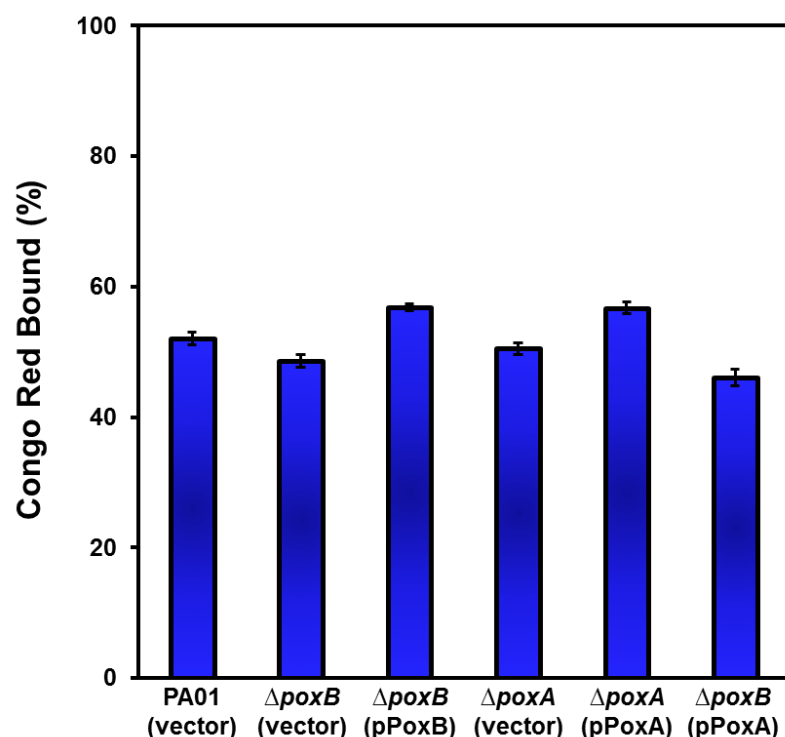


Figure 2.14. CR-binding assay. Two-day old cells were pelleted, resuspended in T-broth containing CR (40 μ g/ml) and grown with aeration at 37°C for 90 minutes. The cells along with bound CR were pelleted by centrifugation and the OD₄₉₅ of the supernatant was used to calculate the percentage of bound CR.

2.5 Concluding Remarks

P. aeruginosa carries two chromosomal β -lactamases, AmpC, a penicillinase and cephalosporinase; and PoxB, a carbapenemase. The role of AmpC and its contribution to β -lactam resistance are well-established and evident here. In our study, AmpC expression readily afforded protection against the cephalosporins and penicillins but not against the carbapenems. PoxB, on the other hand, had a narrow spectrum of hydrolysis that mostly included the carbapenems. Similar to other class D enzymes, PoxB was only poorly

inhibited by the currently available inhibitors: clavulanic acid, tazobactam and sulbactam. A novel inhibitor, avibactam, was slightly more effective at reducing PoxB activity but clinical concentrations failed to decrease MIC of carbapenems by E-test. Additionally, *poxB* was shown to form an operon with an upstream ORF of yet unknown function, termed *poxA*. Expression of PoxA *in trans* enhanced CR-binding abilities suggesting the production of a CR-binding substance likely rich in carbohydrates.

PoxB appears to be constitutively expressed, but its relevance in the clinical setting is still unknown. Mutational derepression of AmpC is a common mechanism of resistance. Thus, it is plausible that mutations in *poxB* regulatory genes may lead to increased expression and carbapenem resistance in the clinical setting. Expression and regulation of *poxB* are examined in Chapter 3.

2.6 Acknowledgments

We would like to thank Lars Dietrich and Hassan Sakhtah (Columbia University for insightful discussions on Congo Red and Pel polysaccharides and Dr. Lynn Silver for suggestions on inhibitor studies. This study was supported in part by the National Institutes of Health – Minority Biomedical Research Support SCORE (SC1AI081376; KM), Research Initiative for Scientific Enhancement graduate student fellowship (NIH/NIGMS R25 GM61347; DZ), NIH/NIAID R37 AI021451 (SL), National Science Foundation IIP-1237818 [PFI-AIR: CREST-I/UCRC-Industry Ecosystem to Pipeline Research] (KM), Florida International University Teaching Assistantship (DZ) and Florida International University Dissertation Year Fellowship (DB). We are additionally

thankful for the funds provided by the Biomedical Research Initiative student summer research award in biomedical sciences (DZ) and to the Cystic Fibrosis Foundation student traineeship grant (DZ).

Chapter 3

Regulation of the *poxAB* operon encoding a class D β -lactamase in *Pseudomonas aeruginosa*

3.1 Abstract

Regulation of AmpC β -lactamase in *Pseudomonas aeruginosa* is a tightly controlled process that involves the global transcriptional regulator AmpR and the recycling of muramyl peptides of the peptidoglycan layer. AmpC is normally produced at low basal levels, but β -lactams such as benzylpenicillin, cefoxitin and imipenem, can induce its high level expression. A second chromosomally encoded carbapenemase, PoxB, has been characterized in *P. aeruginosa*, but its regulation has not yet been examined. Although expression of *poxB* appears to be constitutive, studies in a heterologous host suggest AmpR could also be involved in its regulation. In the present work regulation of *poxB* by AmpR was detected at the transcriptional level by RT-PCR. Quantification of β -lactamase activity, however, did not show regulation at the protein level. Further, AmpR failed to bind the *pox* promoter suggesting indirect regulation. A global genetic screen for transposon insertions into *pox* regulators identified a possible link between polyamine transport and *pox* promoter activity.

Key Words: PoxB, AmpR, β -lactamase regulation

Abbreviations: Electrophoretic mobility shift assay, EMSA; Open reading frame, ORF; *pox* promoter, P_{*pox*}; RT-PCR, Real-time polymerase chain reaction; Transposon, Tn

3.2 Introduction

Pseudomonas aeruginosa PoxB is a class D β -lactamase with a narrow spectrum of hydrolysis that mostly includes the carbapenems (741, 837). It is poorly inhibited by the clinically used class A enzyme inhibitors and by avibactam, a new non- β -lactam inhibitor that is currently undergoing clinical trials (Chapter 2) (741). Additionally, PoxB forms a two-gene operon with the upstream open reading frame (ORF), *poxA*, whose function is still unknown. The *pox* operon appears to be constitutively expressed but its regulation has not been investigated.

It is not uncommon for regulatory proteins to be encoded in the general vicinity of the genes they regulate (730, 739). For instance, the transcriptional regulator AmpR is encoded immediately upstream of, and divergently transcribed from, the *ampC* structural gene that it regulates (711, 776). It was our previous hypothesis that a two-component regulatory system encoded by *mifSR*, located immediately upstream of the *pox* operon and divergently transcribed, could be involved in its regulation. The response regulator MifR has recently been implicated in the formation of biofilm (838). Deletion of either or both *mifS* and *mifR*, however, did not affect β -lactam and carbapenem susceptibility or *poxB* expression (G. Tatke, Personal communication).

Previous work from our lab, however, seems to implicate AmpR as possible regulator of *pox* expression (710). Specifically, expression of *ampR* in an *Escherichia coli* heterologous host resulted in decreased PoxB β -lactamase activity (710). AmpR is an interesting candidate for regulator of *pox* expression. As a LysR-type transcriptional regulator, AmpR regulates *ampC* expression by binding to the promoter region in the

presence and absence of β -lactam inducers in both the Enterobacteriaceae and *P. aeruginosa* (839, 840). In this manner, AmpR can accomplish either repression or induction given the presence/absence of activating inducer. Recently, AmpR has been redefined as a global regulator, differentially controlling expression of various other genes involved in virulence, quorum sensing, biofilm formation as well as various transcriptional regulators (841-844). The AmpR regulon is quite extensive and possibly includes both direct and indirect interactions. It would thus not be surprising if AmpR also regulates expression of the *pox* operon.

In the present work we analyze the role of AmpR as potential regulator of *poxB* expression. Our findings suggest AmpR regulates *poxB* but does not directly interact with the *pox* promoter (P_{pox}) which indicates indirect regulation. A *P. aeruginosa* transposon library was constructed and screened for insertions into regulators of *pox* expression. Two transposon mutants exhibiting increased P_{pox} activity carried insertions in genes involved in the transport and uptake of polyamines, essential molecules implicated in a wide range of cellular functions (845-847). Polyamines reduced the susceptibility of *P. aeruginosa* towards various β -lactams but provided resistance only against the carbapenems. The mechanism by which polyamines are able to induce carbapenem resistance is not well understood but could be related to a decrease in cell permeability or to the increased expression of the carbapenem-hydrolyzing β -lactamase PoxB. Polyamines have, in fact, been shown to modulate gene expression through binding of both DNA and RNA (848-851). Thus, polyamine-dependent regulation, although unprecedented for β -lactamases, is plausible.

3.3 Materials and Methods

3.3.1 Bacterial strains, plasmids and growth media. Bacterial strains and plasmids used in this study are listed in Table 3.1. *E. coli* and *P. aeruginosa* were cultured regularly in Luria-Bertani medium (10 g tryptone, 5 g yeast extract, and 5 g NaCl, per liter) at 37°C. Antibiotics were used at the following concentrations: ampicillin (Ap) at 100, gentamicin (Gm) at 15 and tetracycline (Tc) at 20 µg/ml for *E. coli*; Gm at 75 and Tc at 60 µg/ml for *P. aeruginosa*. For construction of PA0Δ*ampR*Δ*poxB*, a previously generated in-frame deletion of *poxB* was introduced into PA0Δ*ampR* (843) using overlap extension PCR and homologous recombination as previously described (758). The presence of the deletion was checked by gene-specific PCR.

Table 3.1: Bacterial strains and plasmids used in this study

Strains/Plasmids	Relevant phenotype and genotype	Reference/ Source
<i>Escherichia coli</i>		
DH5α	F ⁻ Φ80 <i>lacZ</i> Δ <i>M15</i> Δ(<i>lacZYA-argF</i>) <i>U169 deoR</i>	New England
	<i>recA1 endA1 hsdR17</i> (r _k ⁻ m _k ⁺) <i>phoA supE44</i> λ ⁻	Biolabs
	<i>thi-1 gyrA96 relA1</i>	
TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>)Φ80 <i>lacZ</i> Δ <i>M15</i>	Life
	Δ <i>lacX74 recA1 araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU</i>	Technologies
	<i>galK rpsL</i> (Str ^R) <i>endA1 nupG</i>	
SM10λ <i>pir</i>	Km ^R , <i>thi-1, thr, leu, tonA, lacY, supE,</i>	Stephen Lory
	<i>recA::RP4-2-Tc::Mu, pir</i>	

<i>Pseudomonas aeruginosa</i>		
PA01	Wild-type	(762)
PKM201	PA0Δ <i>ampC</i> , PA01 containing in-frame deletion of <i>ampC</i>	(760)
PKM315	PA0Δ <i>ampR</i> , PA01 containing in-frame deletion of <i>ampR</i>	(843)
PKM332	PA0Δ <i>ampR</i> Δ <i>poxB</i> , PA01 containing in-frame deletions of <i>ampR</i> and <i>poxB</i>	This study
PKM106	PA0 <i>attB</i> ::mini-CTX- <i>lacZ</i> ; Tc ^R	(852)
PKM117	PA0 <i>attB</i> ::P _{<i>ampC</i>} - <i>lacZ</i> ; Tc ^R	This study
PKM118	PA0 <i>attB</i> ::P _{<i>pox</i>} - <i>lacZ</i> ; Tc ^R	This study
PKM319	PA0Δ <i>ampR</i> <i>attB</i> ::mini-CTX- <i>lacZ</i> ; Tc ^R	This study
PKM335	PA0Δ <i>ampR</i> <i>attB</i> ::P _{<i>pox</i>} - <i>lacZ</i> ; Tc ^R	This study
Plasmids		
mini-CTX- <i>lacZ</i>	Tc ^R ; Integration-proficient vector for single-copy chromosomal <i>lacZ</i> fusion at the <i>attB</i> site	(853)
pMMB67EH-Gm	Gm ^R ; IncQ, RSF1010, <i>lacI</i> ^q P _{<i>tac</i>} expression vector with <i>ampR</i> :: <i>aacCI</i> insertion at <i>DraI</i>	(763)
pBTK24	Ap ^R , Gm ^R ; transposon delivery vector, <i>ori</i> R6K	Stephen Lory
pPoxA	Gm ^R ; pMMB67EH-Gm carrying a 946-bp fragment containing <i>poxA</i> ORF	Chapter 2
pOC19	Gm ^R ; pMMB67EH-Gm containing His-tagged AmpR at C-terminus	(852)
pDZ29	Ap ^R ; pCR2.1-TOPO derivative containing a 217-bp <i>EcoRI</i> - <i>BamHI</i> <i>pox</i> promoter fragment	This study
pDZ197	Tc ^R ; P _{<i>pox</i>} fused to the <i>lacZ</i> of mini-CTX- <i>lacZ</i>	This study

3.3.2 Construction of promoter fusions. A Φ CTX-based system was used to construct single-copy promoter fusions, where the expression of the promoterless reporter gene *lacZ* is under the control of P_{pox} . Briefly, P_{pox} was amplified using primers KM_PpoxAFov (5'-GAATTCTCACAGGCCGCGCAGCAGCGGCAG-3') and KM_PpoxARev (5'-GGATCCCTCACCAGCCGCGCAGGGTGAAACC-3'). The amplicon was cloned into the TA cloning vector pCR2.1-TOPO, sequenced and subcloned into the *EcoRI*-*Bam*HI sites of the integrative vector mini-CTX-*lacZ* and resequenced for fidelity. The transcriptional fusion was subsequently introduced into *P. aeruginosa* PA01 and the isogenic mutant PA0 Δ *ampR* as a single copy at the *attB* site. A previously generated PA0:: P_{ampC} -*lacZ* was used as positive control for promoter-driven expression of *lacZ* (710).

3.3.3 Quantification of *poxB* mRNA expression. Total RNA was extracted from *P. aeruginosa* PA01 and a previously generated PA0 Δ *ampR* (843) in the presence and absence imipenem (0.15 μ g/ml) using the RNeasy mini kit (Qiagen). cDNA synthesis was performed with Superscript III (Invitrogen) and the (NS)₅ random primer as previously described (764). qPCR was performed with the ABI 7500 cyclor (Applied Biosystems) using the *Power* SYBR Green PCR Master Mix with ROX (Applied Biosystems). *clpX* (*PA1802*) was used as endogenous control and amplified with DBS_qRT_clpXF (5'-TGCGATTACGATGTGGAGA-3') and DBS_qRT_clpXR (5'-CCCTCGATGAGCTTCAGCA-3') (843). DZRTpoxBFord1 (5'-AATCGGCCAGGTTGTGGATAA-3') and DZRTpoxBRev1 (5'-GGAGCAGAAAGCGGGTCTGT-3') were

used for the real time amplification of *poxB*. Gene expression was normalized to that of the PA01 uninduced value.

3.3.4 β -lactamase assay. The β -lactamase assay was performed as previously described with some modifications (765). Briefly, stationary-phase cultures of PA01, PA0 Δ *ampR* and PA0 Δ *ampR* Δ *poxB* were diluted in 25 ml of LB broth to an OD₆₀₀ of 0.01 and incubated with shaking at 37°C. At OD₆₀₀ of 0.6-0.8, cells were induced with 0.15 μ g/ml of imipenem and reincubated for 1 hour at 37°C. Ten milliliters of cells were harvested by centrifugation and resuspended in 800 μ l of 1x BugBuster solution (Novagen). Benzonase (1 μ l/ml of BugBuster used) (Novagen) and rLysozyme (1 KU (Novagen) were then added and the mixture was rotated for 20 min at room temperature. The lysate was centrifuged at 4°C for 15 minutes to collect the β -lactamase-containing supernatant. A 2- μ l aliquot of cell lysate was added to nitrocefin (final concentration, 100 μ M) in 250 μ l of 50 mM sodium phosphate buffer. The reaction mixture was incubated at 37°C for 20 minutes and the hydrolysis of nitrocefin was measured spectrophotometrically at 482 nm. The Bradford assay was used to determine total protein concentration of each sample. β -lactamase activity was expressed as nmol of nitrocefin degraded per minute per microgram of total protein.

3.3.5 β -galactosidase assay. The β -galactosidase activity was quantified as a measure of P_{pox} activity as previously described (854). Briefly, fresh cultures of PA01 and PA0 Δ *ampR* harboring P_{pox} -*lacZ* were grown at 37°C and induced with benzylpenicillin

(50 µg/ml) at an OD₆₀₀ of 0.5 for 1 hour. The assay was performed on 1-ml cell lysate using ortho-nitrophenyl-β-galactoside (ONPG) as a chromogenic substrate and the activity was expressed in Miller units. Assays performed as part of transposon screening were routinely done in the absence of induction.

3.3.6 Electrophoretic mobility shift assay (EMSA). The *pox* promoter region was amplified using *P. aeruginosa* PA01 genome as template with primers EM-PpoxAFor (5'-CGATCAGGGTCTGCAGCGAGC-3') and EM-PpoxARev (5'-GGCTTGCCGCTC-GGTTCGC-3') and the 335-bp amplicon was used to perform EMSA. Briefly, the promoter fragment was radiolabelled at the 5' end by incubation with T4 polynucleotide kinase (NEB) and [γ -³²P]ATP (3,000 Ci mmol⁻¹; Perkin Elmer). The labeled fragment was diluted to a final concentration 100 nM and unincorporated nucleotides were removed by sephadex G-25 (Biorad, Hercules, CA) spin chromatography. DNA binding reactions containing 50 fmol of ³²P-labeled DNA probe and varying amounts of total protein membrane fractions were incubated for 20 minutes and loaded thereafter in a non-denaturing 5% PAGE. Protein membrane fractions were used as AmpR was shown to be membrane-associated (852). Radioactive signals were detected by scanning a phosphorstorage cassette with the GE Healthcare Typhoon 9400 scanner.

3.3.7 Pilot experiment to construct transposon library. Before attempting to generate a transposon library it was necessary to determine the conditions needed to obtain good coverage over the PA01 genome. About 20,000 to 40,000 mutagenized colonies, which is

roughly 5-7 times the number of genes (~5500) in PA01, are necessary. A pilot study was thus conducted by setting matings between the donor *E. coli* SM10 λ pir (pBTK24) and the recipient PA0 Δ ampR::P_{pox}-lacZ. PA0 Δ ampR::P_{pox}-lacZ was grown overnight (O/N) at both 37°C and 42°C, while *E. coli* SM10 λ pir (pBTK24) was grown O/N only at 37°C. The donor and recipient were then diluted (1/5) and grown at 37°C in LB for about 4 hours. The recipient strain that was grown O/N at 42°C was also diluted and incubated in a dry bath at 42°C for 4 hours. When the cultures reached an OD₆₀₀ of 2.5 or higher, after approximately 4½ hours, 6 mls of both the donor and recipient were centrifuged at 13,000 rpm for a minute. After discarding the supernatant, the cells were washed in 1 ml of LB broth, repelleted and resuspended again in 1 ml of LB broth. One set of matings was set up using the recipient that was grown at 42°C (Matings A-C), while the others were done with the recipient that had grown at 37°C (Matings D-E) (Table 3.2).

Table 3.2: Mating volumes used in pilot study

Strains	Pilot Matings (μl)					Controls (μl)		
	A	B	C	D	E	Recipient (37°C)	Recipient (42°C)	Donor
PA0 Δ ampR::P _{pox} -lacZ at 37°C	-	-	-	200	400	400	-	-
PA0 Δ ampR::P _{pox} -lacZ at 42°C	200	600	400	-	-	-	600	-
<i>E. coli</i> SM10 λ pir (pBTK24)	1000	600	800	1000	800	-	-	1000

All mating mixtures were centrifuged, the supernatant discarded and the pellet resuspended in 30 µl of LB. The suspensions were then spotted on LB plates and incubated at 37°C for 4 hours. All spots were resuspended in 200 µl of LB. Fifty and 150 µl of each were plated on selective media (LB-PIA-Gm⁷⁵). For the controls, only 100 µl of each strain were plated (Table 3.3). The most efficient matings had the recipient grown at 42°C at a 1:1 ratio of donor to recipient (Table 3.2 and 3.3).

3.3.8 Transposon mutagenesis and screening for regulators of the *pox* operon. The reporter strains, PA0::P_{*pox*}-*lacZ* and PA0Δ*ampR*::P_{*pox*}-*lacZ*, displaying distinctly different levels of *pox* promoter activity, were each mutagenized on four different occasions. Twenty matings were set-up each time with the objective of obtaining enough transposon-mutagenized colonies to cover the entire PA01 genome. Matings were set-up as described in the pilot study. Briefly, O/N cultures of recipients and donor grown at 42 and 37°C, respectively, were freshly diluted 1/5 in LB broth and grown for four hours at 42 and 37°C, respectively. At the four-hour time point, enough culture of both donor and recipient strains were centrifuged at 13,000 rpm for one minute (1 ml culture/eppendorf tube). The supernatants were discarded and the pellets resuspended in 1 ml of LB. Matings were set up in donor to recipient ratios of 5:1, 2:1, and 1:1 (Table 3.4). All mating mixtures were centrifuged, the supernatant discarded and the pellet resuspended in 30 µl of LB, which were then spotted on LB plates and incubated at 37°C for an additional four hours. All spots were individually resuspended in 200 µl of LB and 50 and 100 µl were plated on selective media (LB-PIA-Gm⁷⁵ or LB-PIA-Gm⁷⁵-X-gal¹⁰⁰).

Table 3.3: Approximate colony count obtained for pilot study

Pilot Matings										Controls		
	A		B		C		D		E	Recipient (37°C)	Recipient (42°C)	Donor
	50	150	50	150	50	150	50	150	50	150	100	100
Volume plated (μl)	290	625	592	895	437	480	1	6	0	3	0	0
Colony count												

Table 3.4: Mating ratios used for generation of *P. aeruginosa* library

Strains	Matings			Controls	
	1-7	8-14	15-20	Recipient	Donor
PA01::P _{pox} -lacZ or PA0ΔampR::P _{pox} -lacZ (μl)	200	600	400	600	-
<i>E. coli</i> SM10 λpir (pBTK24) (μl)	1000	600	800	-	1000

For the PA0::P_{pox}-lacZ library, colonies obtained with the 1st and 2nd mating events, were all pooled and frozen into five 96-well plates since selection was done only in LB-PIA-Gm⁷⁵. Screening of these pools for dark blue colonies remains to be done on LB-PIA-Gm⁷⁵-X-gal¹⁰⁰ (Figure 3.1). Colonies for the 3rd and 4th mating events were directly selected on LB-PIA-Gm⁷⁵-X-gal¹⁰⁰. The blue colonies resulting from this screening were individually frozen in 4 x 96-well plates and further analyzed as described in later sections.

For the PA0ΔampR::P_{pox}-lacZ library, all colonies obtained were pooled and frozen with 10% skim milk in 57 x 2-ml-vials (mating events 1-3) and in two 96-well plates (4th mating event) (Figure 3.2). Screening for white colonies has so far been done on 34 wells (A1-C10, Plate #1, mating event 4). The screening was done by inoculating a scraping from a well into 10 ml of LB broth and growing at 37°C for 45 minutes. Each culture was then serially diluted and the 10⁻⁵ and 10⁻⁶ dilutions were plated onto LB-PIA-Gm⁷⁵-X-gal¹⁰⁰. White to light blue colonies were selected for further analysis and individually frozen on 10% skim milk.

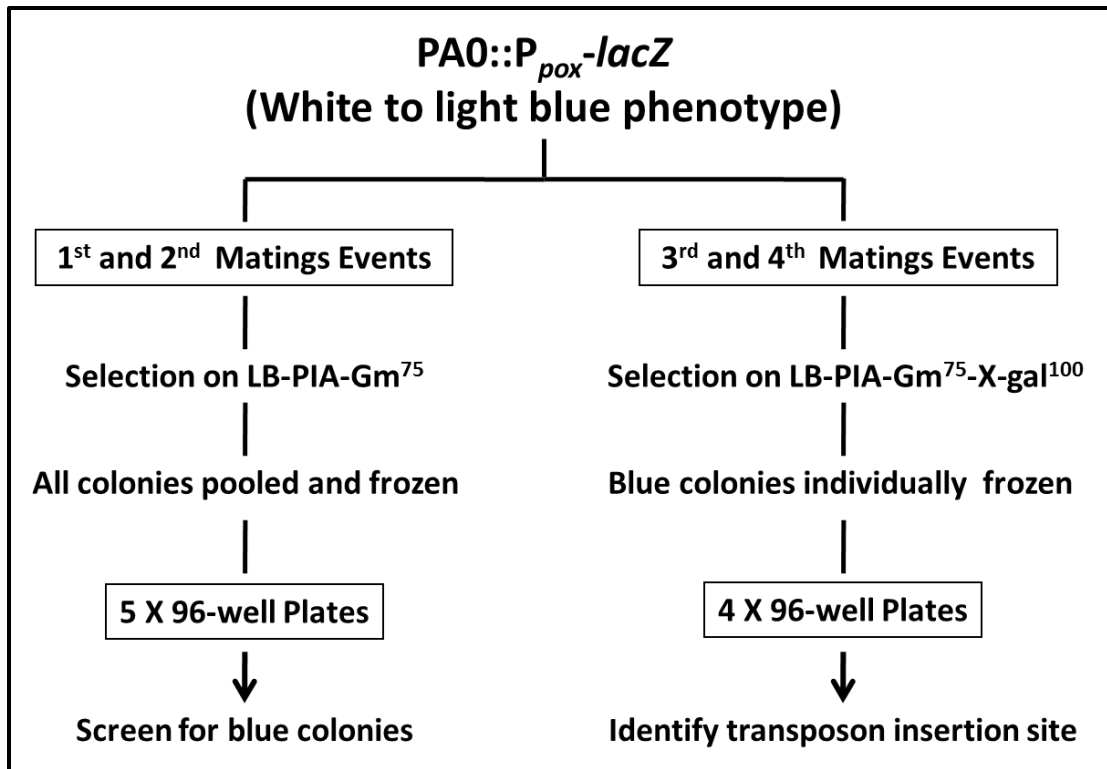


Figure 3.1. Selection and screening of the PA01 library

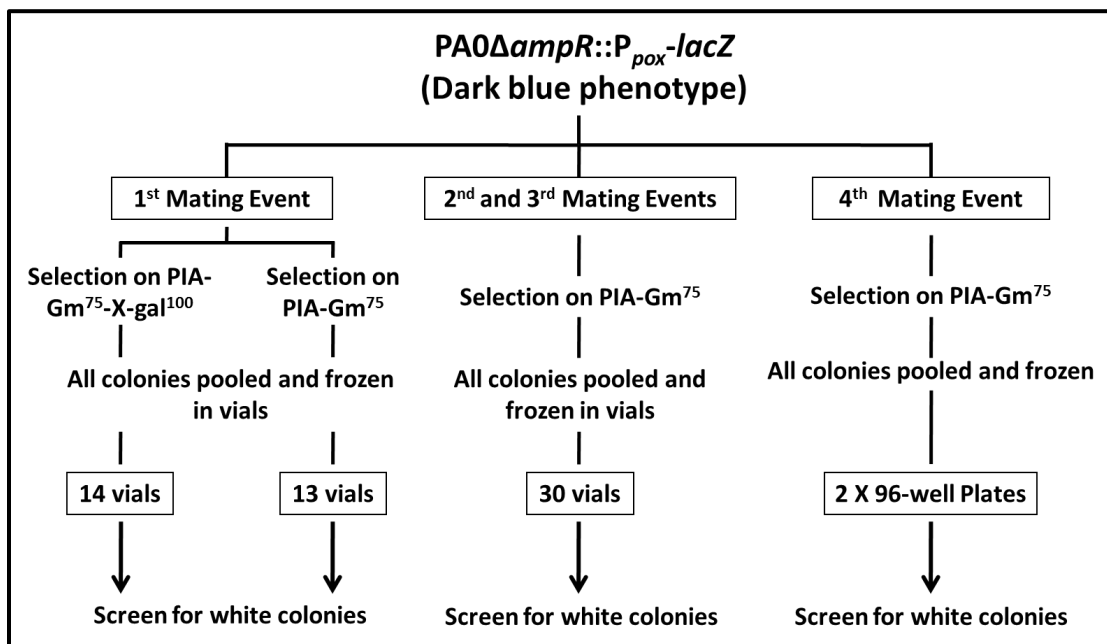


Figure 3.2. Selection and screening of the PA0ΔampR library

3.3.9 Mapping of the transposon (Tn) insertion site. Insertion sites were mapped by a two-round semi-random PCR (825) using either pure colony or purified DNA as template. The first round of PCR was performed using the Tn-specific primer Round1-BTK24 (5'-GTCGTAAATCACTGCATAATTTCG-3') and the random primer Round1-Pa(1) (5'-GGCCACGCGTCGACTAGTACNNNNNNNNNGATAT-3') (855), that can anneal anywhere on the genome and has a nonrandom tail. One microliter of purified product from the first round of PCR was used as template for a nested PCR with primers Round2-BTK24 (5'-GCACTCCCGTTCTGGATAAT-3') and Round2-Pa (5'-GGCCACGCGTCGACTAGTAC-3') (855). The products were gel-purified and sequenced with another nested Tn-specific, outward-facing primer BTK24-seq (5'-GCGGATAACAAT-ATAATGTGTGGA-3'), that will read through the junction and into the genome. Mapped insertions were subsequently confirmed by gene-specific PCR.

3.4 Results and Discussion

3.4.1 AmpR downregulates *poxB* expression. Previous work in an *E. coli* heterologous background showed increased PoxB activity in the absence of AmpR suggesting the transcriptional regulator normally acts to downregulate *poxB* expression (710). Regulation of the *pox* operon, however, has not yet been examined in *P. aeruginosa*. The expression of *poxB* was thus quantified in the presence and absence of β -lactams in an *ampR* deletion strain (PA0 Δ *ampR*) by RT-PCR. In the absence of the inducer, equivalent amounts of *poxB* mRNA were observed in both wild-type and PA0 Δ *ampR* (Figure 3.3). In the presence of the inducer, however, significantly higher amount of *poxB* mRNA was

detected in an *ampR*-deficient background. This finding confirms previous work done in a heterologous host (710) and suggests AmpR negatively regulates *pox* expression in the presence of β -lactams.

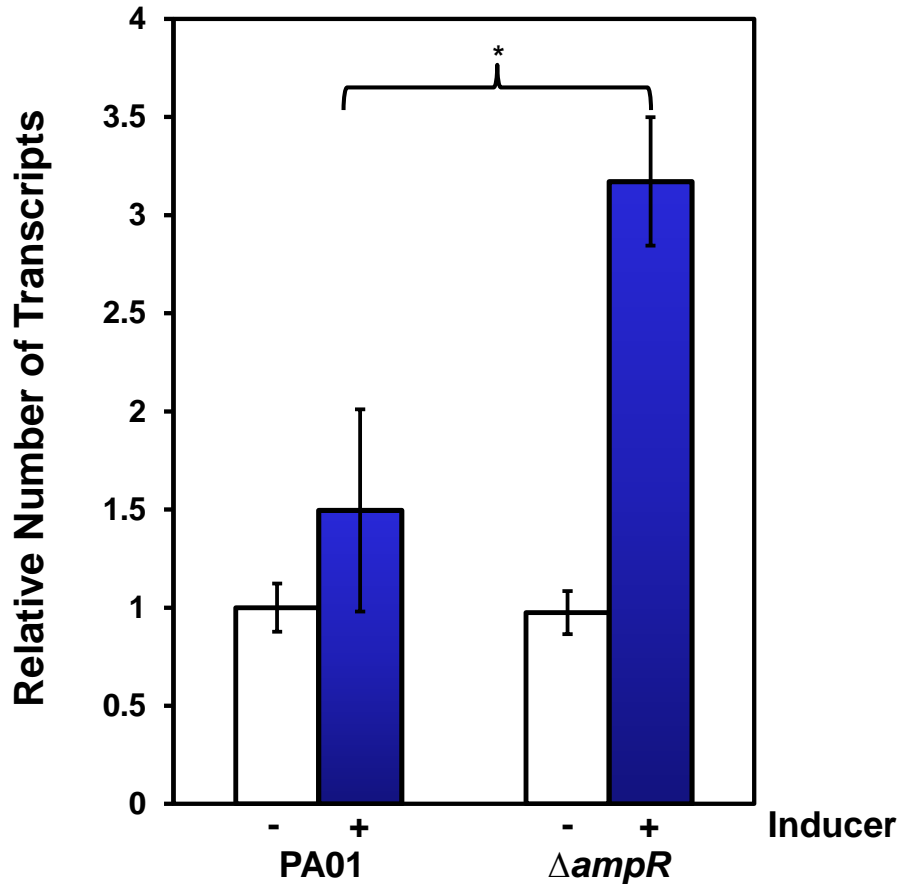


Figure 3.3. Expression of *poxB* in the absence of AmpR. RNA was isolated from PA01 and PA0 $\Delta ampR$ in the presence and absence of imipenem (0.15 μ g/ml), reversed transcribed to cDNA and tested by qPCR with *poxB* specific primers, as described in the *Materials and Methods*. Values were normalized to the expression of the wild-type uninduced sample and represent the mean (\pm SD) of two experiments conducted in triplicates. * p -value < 0.01 for *poxB* expression in PA0 $\Delta ampR$ induced as compared to PA01 induced, as determined by unpaired t -test.

3.4.2 PoxB β -lactamase activity. To determine if the differential RNA expression observed in the absence of *ampR* actually translates into quantifiable changes at the protein level, the β -lactamase activity of PA0 $\Delta*ampR* and PA0 $\Delta*ampR* $\Delta*poxB* strains was measured in the presence and absence of the inducer. As expected, in the wild-type β -lactamase levels are detectable and induced upon β -lactam challenge (Figure 3.4). This activity could be due to either AmpC alone or AmpC and PoxB combined.$$$

The β -lactamase activity did not significantly increase in the absence of induction in PA0 $\Delta*ampR*, the levels being equivalent to that of wild-type uninduced. In the presence of the inducer, however, deletion of *ampR* resulted in the loss of induction, suggesting the activity observed is likely due to the constitutive expression of *ampC*, which requires AmpR for induction. A further deletion of *poxB* from an already *ampR*-deficient strain yielded β -lactamase levels similar to that of PA0 $\Delta*ampR*, with no inducibility being detected upon exposure to β -lactams. A PA0 $\Delta*ampC* $\Delta*ampR* strain behaved similarly to an *ampC* deletion strain characterized in Chapter 2, in that it produced negligible amounts of β -lactamase. The activity of PoxB thus, appears to be very low and masked by the expression of *ampC*. The inducibility of *poxB* observed at the mRNA level (Figure 3.3) does not translate into a detectable difference in terms of β -lactamase activity in the conditions tested.$$$$

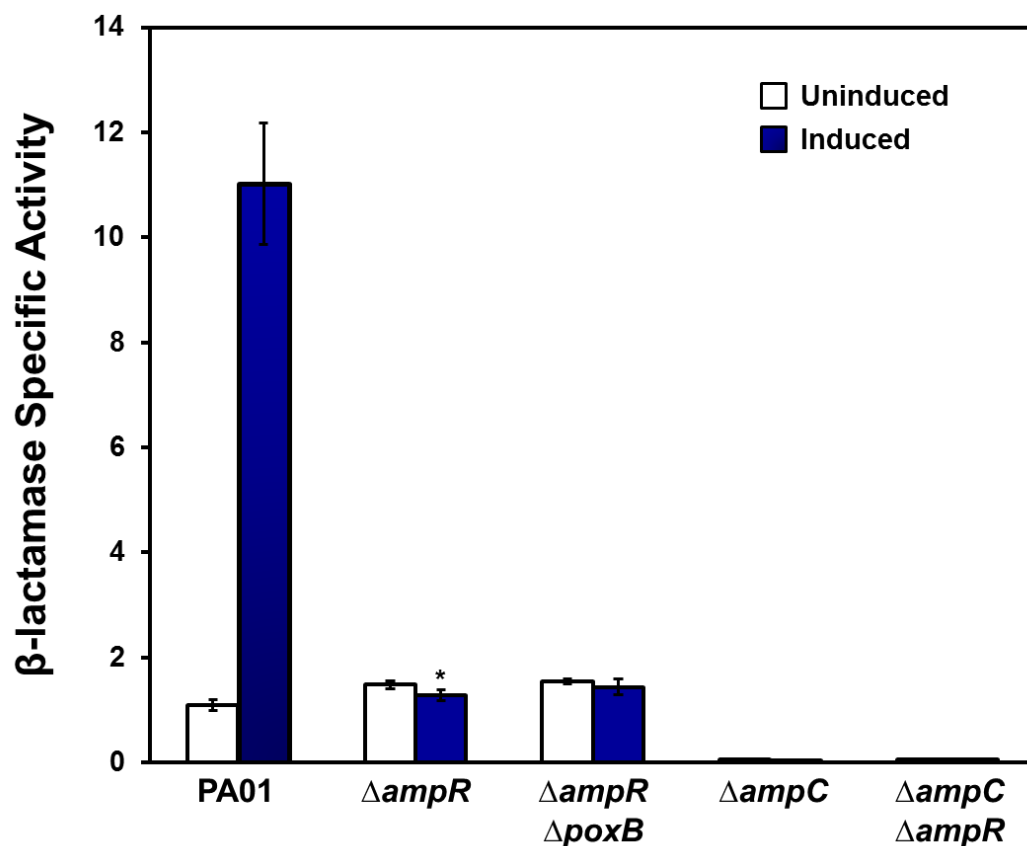


Figure 3.4. β -lactamase expression of PA01 and its isogenic mutants. The β -lactamase activity was quantified for the wild-type strain PA01 and the isogenic mutants PA0 $\Delta ampR$, PA0 $\Delta ampR \Delta poxB$, PA0 $\Delta ampC$ and PA0 $\Delta ampC \Delta ampR$, in the presence and absence of the inducer. Logarithmic-phase cultures were induced with 0.15 μ g/ml of imipenem for 1 hour before harvesting. Assays were performed in triplicates on BugBuster and rLysozyme-treated lysate using nitrocefin as chromogenic substrate. One milliunit of enzyme is defined as 1 nmol of nitrocefin hydrolyzed per minute per microgram of total protein. * p -value= 0.0001 for β -lactamase activity in PA0 $\Delta ampR$ induced versus activity in PA01 induced, as determined by unpaired t -test.

3.4.3 AmpR does not bind P_{pox} . As a LysR-type transcriptional regulator, AmpR regulates gene expression by binding to DNA with its canonical N-terminal HTH motif (856). Binding to the *ampC* promoter has been found to occur in both the presence and absence of β -lactams (839, 840, 852). An EMSA was thus performed to determine if AmpR directly binds the *pox* promoter. A [γ - 32 P] ATP radiolabeled P_{pox} fragment was incubated with increasing concentrations of total membrane fractions extracted from cells overexpressing a C-terminal His-tagged AmpR that was previously shown to be functional (852). Cell extracts were recovered in the presence and absence of benzylpenicillin. A 193-bp fragment corresponding to the *ampC* promoter region was used as positive control as it was been previously shown to bind AmpR (852).

As expected and previously shown, AmpR was able to shift P_{ampC} in the presence and absence of the inducer (Figure 3.5, Lanes 16 and 18), indicating EMSA conditions were working properly. AmpR, however, did not shift a 335-bp P_{pox} fragment in the presence (Lanes 8-12) or absence (Lanes 2-6) of benzylpenicillin, indicating AmpR does not bind the *pox* promoter. If AmpR is in fact regulating expression of *poxB*, as suggested by previous work and by qPCR here, then this regulation is indirect. This hypothesis is further substantiated by the absence of an AmpR-binding site in the *pox* promoter region.

Previous work from our lab has shown AmpR is global regulator controlling expression of not just *ampC*, but also of various other genes including a number of putative and characterized transcriptional regulators and sigma factors such as *creB*, *pprB*, *algB*, *algT/U* and *rpoS* (710, 842, 843). Thus it is possible that AmpR regulates *poxB* through these or other, as of yet, unidentified regulators.

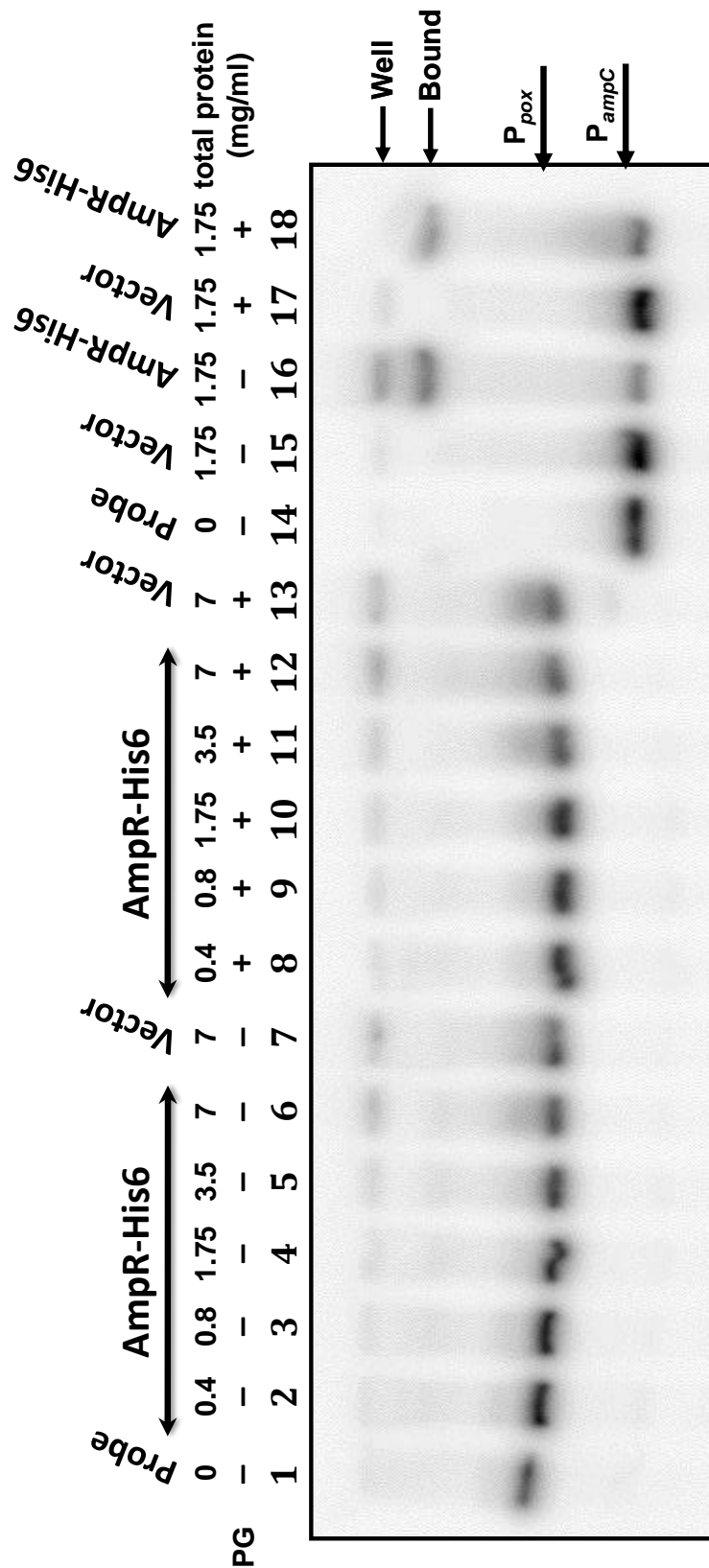


Figure 3.5. *P. aeruginosa* AmpR does not bind to P_{pox} . Fifty fmol of a 300-bp radio-labeled P_{pox} fragment (Lane 1) were mixed with membrane fractions recovered from PA0 $\Delta ampR$ cells carrying pAmpR-His₆ (Lanes 2-6 and 8-12) or pMMB67EH-Gm (Lanes 7 & 13) in the presence (Lanes 8-13) and absence (Lanes 2 to 7) of 200 μ g/ml of penicillin G. A 193-bp 32 P-labeled P_{ampC} fragment (Lane 14) was used as positive control and also incubated with extracts carrying pAmpR-His₆ (Lanes 16 and 18) or pMMB67EH-Gm (Lanes 15 & 17) in the presence (Lanes 17-18) and absence (Lanes 15-16) of the inducer.

3.4.4 PoxA autoregulates its own promoter. Autogenous regulation is a common regulatory mechanism in which a gene product regulates expression of the very gene that encodes it (857). Autoregulation, especially negative autoregulation, is a common theme among transcriptional factors and regulators, with over 40% of *E. coli* transcriptional factors regulating their own synthesis (857-859). Autoregulation however, also occurs in non-regulatory structural genes including those coding for enzymes involved in a myriad of metabolic processes, often with the first gene regulating the expression of the rest of the genes in that operon (857, 860).

Since the role of PoxA was unclear, we postulated that PoxA may autoregulate the *pox* operon. In order to determine if this was the case, *poxA* was expressed in a low-copy plasmid under an IPTG inducible promoter and introduced into PA01 carrying a chromosomal P_{pox} -*lacZ* fusion. In the absence of induction, the *pox* promoter exhibited high basal, constitutive levels in both the presence and absence of *poxA in trans* (Figure 3.6). After one hour induction with IPTG, a small but significant decrease in P_{pox} activity was observed in the presence of *poxA*, suggesting PoxA at high levels can negatively regulate its own transcription. The trend, although no longer statistically significant, was also observed 2 hours after IPTG induction. In wild-type, PoxA may contribute to maintaining low levels of PoxB. Indeed *poxB* mRNA levels are relatively low and appear for the most part to be uninducible in PA01 (Figure 3.3). PoxA is a putative hydrolase of yet uncharacterized function.

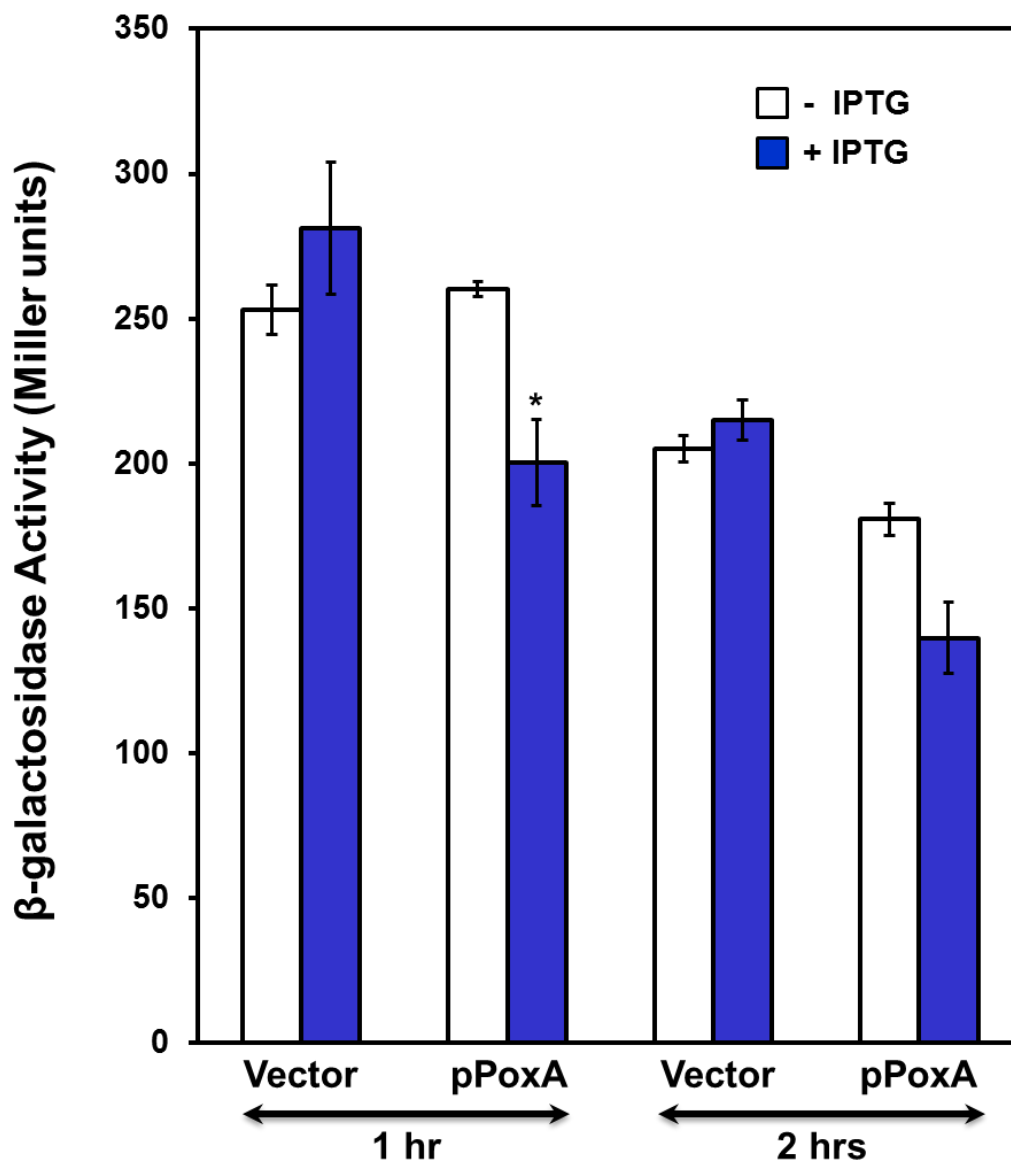


Figure 3.6. Activity of the *pox* promoter in the presence of pPoxA. The activity of the *pox* promoter activity was quantified in PA01 carrying a chromosomal *lacZ* fusion in the presence and absence of PoxA *in trans*. Exponentially growing cultures were induced with 1 mM IPTG for 1 or 2 hours before harvesting for quantification of β -galactosidase activity. * *p*-value <0.05 for promoter activity of PA0::P_{*pox*}-*lacZ* (pPoxA) in the presence of IPTG vs P_{*pox*} activity in the same strain in the absence of IPTG, as determined by paired *t*-test.

3.4.5 Approach to identifying regulators of the *pox* operon. To identify other possible regulators of the *pox* operon we have taken a global approach that involves construction of a *P. aeruginosa* mutant library with potential transposon insertions into regulators of *pox* expression. In order to screen for desired insertions, two reporter strains, PA0::P_{*pox*}-*lacZ* and PA0Δ*ampR*::P_{*pox*}-*lacZ*, were constructed and the conditions needed for a successful genetic screening were investigated by examining promoter activity in the presence of X-gal or ONPG.

As expected from previous work, the *pox* promoter was found to be more active in the absence of *ampR* as evidenced by the distinctly dark blue phenotype displayed by PA0Δ*ampR*::P_{*pox*}-*lacZ* on plates containing X-gal (Figure 3.7 B and D). PA0::P_{*pox*}-*lacZ*, on the other hand, having an intact AmpR that can repress P_{*pox*}, displayed a greenish to light blue colony phenotype (Figure 3.7 A and C). For subsequent selection and screening of the mutant library, 100 µg/ml of X-gal was used, however, lower concentrations of 40 and 60 µg/ml of X-gal were also effective at showing color differences.

The promoter activity was also investigated by the β-galactosidase assay. In the positive control P_{*ampC*} activity was detected in the absence of induction and induced 3-fold upon β-lactam challenge (Figure 3.8). On the other hand, P_{*pox*} activity was detected in both PA0::P_{*pox*}-*lacZ* and PA0Δ*ampR*::P_{*pox*}-*lacZ*, with higher activity observed in the absence of *ampR* upon induction with β-lactams (Figure 3.8). These findings are in agreement with the mRNA study (Figure 3.3) and the visual analysis of P_{*pox*} activity (Figure 3.7).

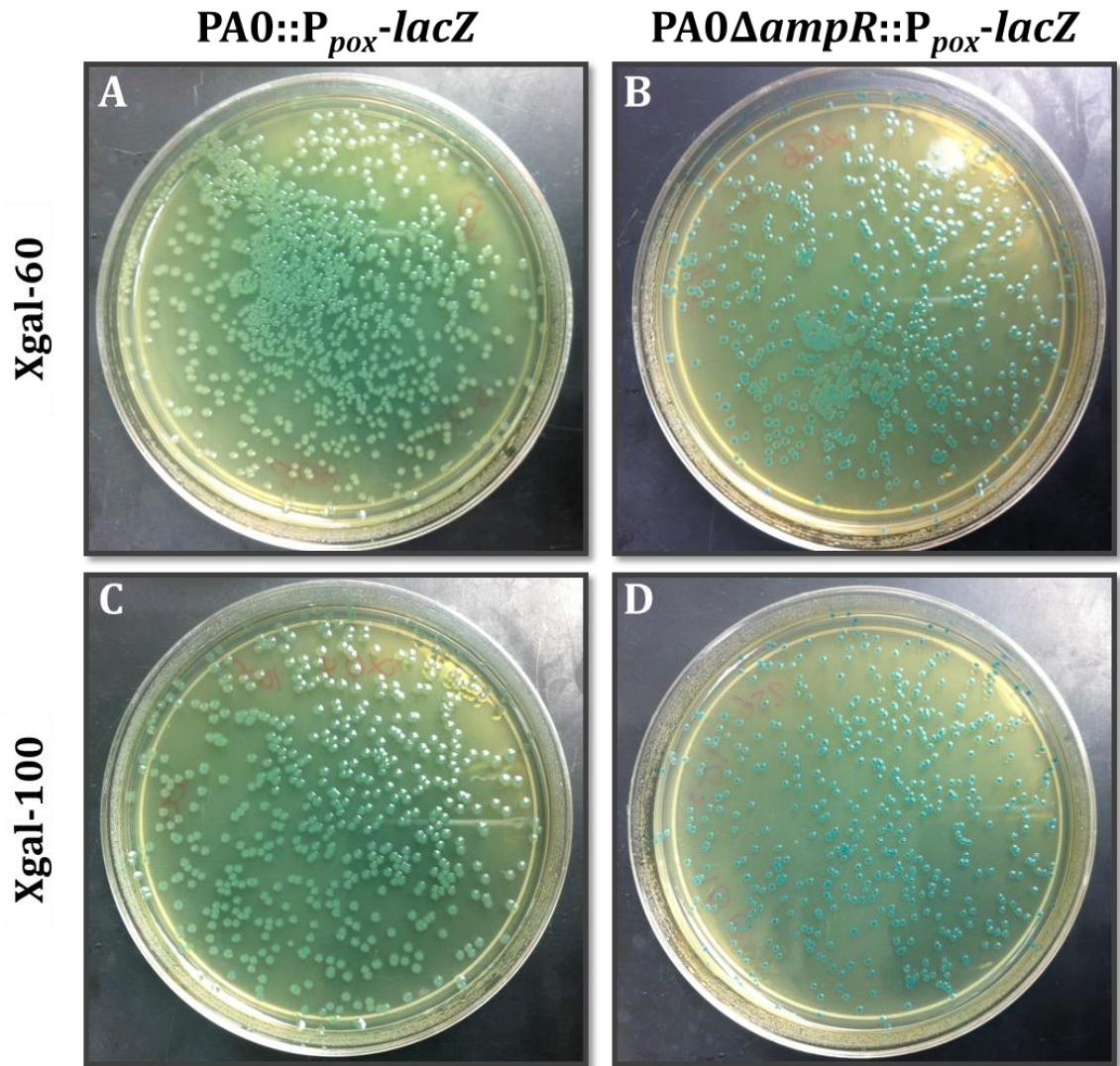


Figure 3.7. Activity of the *P. aeruginosa* *pox* promoter. The 10⁻⁵ and 10⁻⁶ dilutions of freshly grown PA01 (Panels A and C) and PA0ΔampR (Panels B and D) cells carrying P_{pox}-lacZ were plated on LB agar plates containing 40 (data not shown), 60 or 100 μg/ml of X-gal. The promoter activity was ascertained by visually inspecting colony color phenotype after an O/N incubation at 37°C.

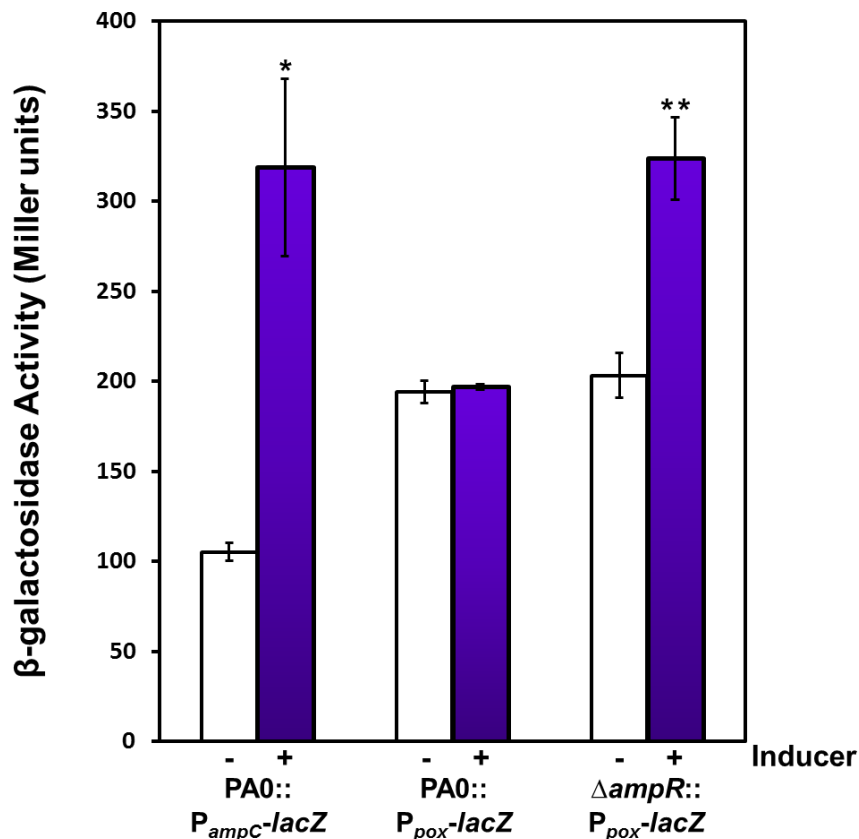


Figure 3.8. P_{pox} activity in the presence and absence of *ampR*. PA01 and PA0Δ*ampR* cells carrying P_{pox} -*lacZ* were grown at 37°C and induced with benzylpenicillin at an OD₆₀₀ of 0.5 for 1 hour. The assay was performed on cell lysate using ONPG as a chromogenic substrate. β-galactosidase activity was quantified in Miller units in the presence and absence of β-lactams. * p -value<0.05 versus uninduced PA0:: P_{ampC} -*lacZ*; ** p -value<0.02 versus uninduced PA0Δ*ampR*:: P_{pox} -*lacZ* as determined by paired t -test.

3.4.5.1 Identifying negative regulators. Having an intact *ampR*, PA0:: P_{pox} -*lacZ* displays a light blue phenotype on X-gal (Figure 3.8). Thus we postulate that the dark blue colonies resulting from the transposon mutagenesis of this strain are likely to have insertions into negative regulators of *pox* expression (Figure 3.9). Alternatively, a false-negative dark blue colony could result from insertions immediately upstream of *lacZ* such

that transcription from the Tn drives expression of the β -galactosidase gene. Only mapping can determine the exact Tn insertion site.

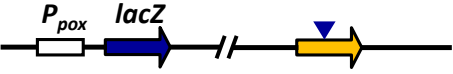
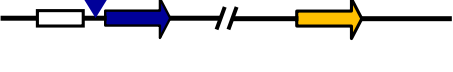
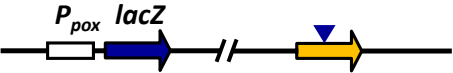
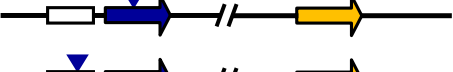
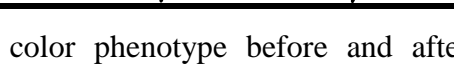
Strain	Colony color on X-gal	Transposon insertion	Resulting phenotype	Regulator
PA0:: P_{pox} - <i>lacZ</i>	White - light blue		Dark Blue	Negative
				False Negative
$\Delta ampR$:: P_{pox} - <i>lacZ</i>	Dark Blue		White-light blue	Positive
				False Negative
				False Negative

Figure 3.9. Predicted color phenotype before and after Tn insertion into putative regulators of the *pox* operon. Blue triangles indicate transposon insertion sites; yellow arrows denote any positive or negative regulator-encoding genes.

3.4.5.2 Identifying positive regulators. PA0 $\Delta ampR$:: P_{pox} -*lacZ* displays a distinctly dark blue phenotype on X-gal (Figure 3.8). Upon Tn mutagenesis of this strain, colonies that display a white to light blue color, indicative of a decrease in P_{pox} activity, potentially have insertions in genes encoding positive regulators of *pox* expression (Figure 3.9). There are, however, two other possible Tn insertions that can lead to repression of *lacZ* resulting in a white phenotype: (a) Tn insertion into P_{pox} that controls the transcription of *lacZ*, or (b) Tn insertion into *lacZ* itself. To determine where in the genome the insertion occurred, Tn-specific primers will be used to sequence through the transposon junctions and into the genome.

3.4.6 Construction of mariner Tn library. A *P. aeruginosa* PA01 mutant library was constructed by mutagenesis of PA0::P_{pox}-lacZ and PA0ΔampR::P_{pox}-lacZ with the Tn delivery vector pBTK24 (Figure 3.10). This vector is derived from the pBT20 minitransposon (861) and like its predecessor, carries the *Himar1* mariner transposase element isolated from the horn fly *Haematobia irritans* (855, 862, 863).

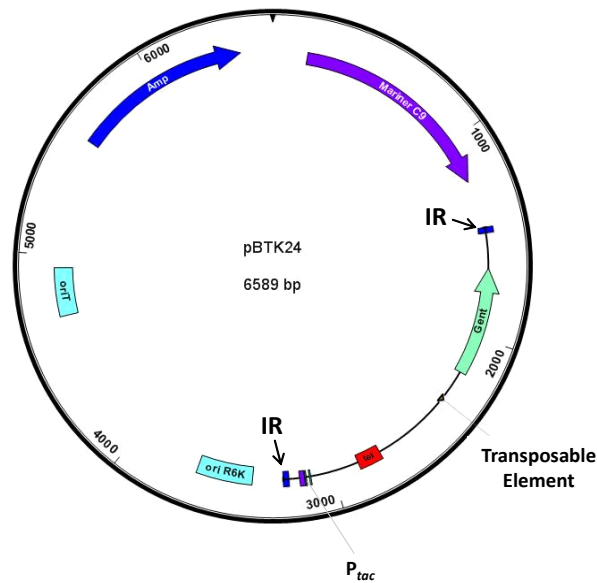


Figure 3.10. Plasmid map and features of the mariner transposon vector, pBTK24. pBTK24 has an *ori* R6K origin for replication in *E. coli* and an ampicillin marker for plasmid propagation. The transposase-encoding gene, *mariner* C9, is responsible for excision and recombination of the transposable element. Inverted repeats depicted in blue flank the transposon, which carries a gentamicin cassette for selection and an outward-facing P_{tac} promoter for transcription of downstream genes.

The pBTK24 vector also carries an *oriT* origin for transfer into *Pseudomonas*, but having only an *E. coli* origin of replication (*ori* R6K), is a suicide delivery vector that cannot be propagated in a *P. aeruginosa* background. The *mariner* C9 transposase is encoded on the plasmid backbone to promote transposition and to prevent transposase integration into the genome. The transposable element itself is flanked by 29-bp inverted

terminal repeats (ITRs), which are recognized by the transposase for transposition by a cut-and-paste mechanism (861). Additionally, the transposable element contains a gentamicin cassette for selection and an outward-facing P_{tac} promoter for transcription of downstream genes to prevent polarity.

3.4.6.1 Generation of the transposon library. Transposon libraries of PA0::P_{pox}-*lacZ* and PA0Δ*ampR*::P_{pox}-*lacZ* were obtained by mutagenesis with *E. coli* SM10 λpir (pBTK24). Each recipient strain was mutagenized on four different occasions (mating events), with a total of 20 matings per event, in order to generate a library with coverage over the entire PA01 genome. A total of 163,266 colonies were obtained for the PA0Δ*ampR*::P_{pox}-*lacZ* library and more than 503,988 colonies were obtained for PA0::P_{pox}-*lacZ* (Table 3.5).

Table 3.5. Approximate colony count after Tn mutagenesis

Strains	Mating Events	Colony Count
PA0::P _{pox} - <i>lacZ</i>	1	387,625
	2	86,427
	3	299,36
	4	ND
	<i>Total</i>	> 503, 988
PA0Δ <i>ampR</i> ::P _{pox} - <i>lacZ</i>	1	973
	2	3,329
	3	19,766
	4	139,198
	<i>Total</i>	163,266

ND-Not determined

3.4.7 Mapping insertion sites and identification of regulators

3.4.7.1 PA0::P_{pox}-lacZ library. Mapping was first attempted on 44 blue colonies obtained from the PA0::P_{pox}-lacZ library (3rd and 4th mating events, Plate # 8, Figure 3.1). Out of these, 15 insertions could be confirmed by gene-specific PCR and were selected for further analysis. The majority of the Tn insertions were mapped to genes and operons involved in the biosynthesis of lipopolysaccharide (LPS) (Table 3.6). Often insertions occurred twice in the same gene but at different positions within the ORF (e.g., *wbpJ*, *wzt*, *wbpY*, and *rmlA*). Two clones had a Tn insertion that mapped to the same location within *wzt*, suggesting these two clones were siblings. Three different insertions were found within the *PA3488* ORF, coding for a hypothetical, unclassified protein.

Although these 15 colonies displayed a dark-blue phenotype on X-gal indicative of increased *lacZ* expression, the promoter activity as determined by β -galactosidase assay was equivalent to that of wild-type (Figure 3.11 A and B). Thus, determination of promoter activity by blue/white screening alone was deemed to be insufficient for identification of regulators.

It is likely that disturbances in LPS created by Tn insertions in such genes, led to porosity of the membrane and leakage of the enzyme where it could readily cleave X-gal. Subsequently, all blue PA01 colonies frozen in the 4 x 96-well plates (Plates 8-11) from the 3rd and 4th mating events (Figure 3.1) were further screened by assaying the level of β -galactosidase activity before selecting for mapping. Colonies producing significantly different levels of enzyme as compared to the control strain were selected for mapping.

Table 3.6. Mapped insertions from PA01 library, Plate # 8

Well ID	Insertion Site	Insertion Coordinate	Gene	Function
A4	PA3147	3530530	<i>wbpJ</i>	Glycosyltransferase
B4	PA3147	3530649	<i>wbpJ</i>	Glycosyltransferase
E1	PA5450	6140010	<i>wzt</i>	ABC subunit of A-band LPS efflux transporter
F1	PA5450	6140164	<i>wzt</i>	ABC subunit of A-band LPS efflux transporter
E9	PA5450	6140164	<i>wzt</i>	ABC subunit of A-band LPS efflux transporter
E11	PA5448	6137971	<i>wbpY</i>	Glycosyltransferase
H1	PA5448	6137221	<i>wbpY</i>	Glycosyltransferase
C1	PA5451	6141320	<i>wzm</i>	Membrane subunit of A-band LPS efflux transporter
A1	PA5163	5812349	<i>rmlA</i>	dTDP-glucose pyrophosphorylase
E6	PA5163	5812475	<i>rmlA</i>	dTDP-glucose pyrophosphorylase
D3	PA4999	5616945	<i>waal</i>	O-antigen ligase
F7	PA0795	874014	<i>prpC</i>	Citrate synthase
B5	PA3488	3906941	-	Hypothetical protein
E4	PA3488	3906772	-	Hypothetical protein
F5	PA3488	3907035	-	Hypothetical protein

Screening did not reveal any noteworthy colonies in Plates 9 and 11, however, in Plate 8, two colonies were identified, B6 and G7, as having 8-fold higher promoter activity than the control (Figure 3. 12). These, however, turned out to be false negative as mapping revealed the Tn inserted immediately upstream of the *lacZ* ORF and mapping

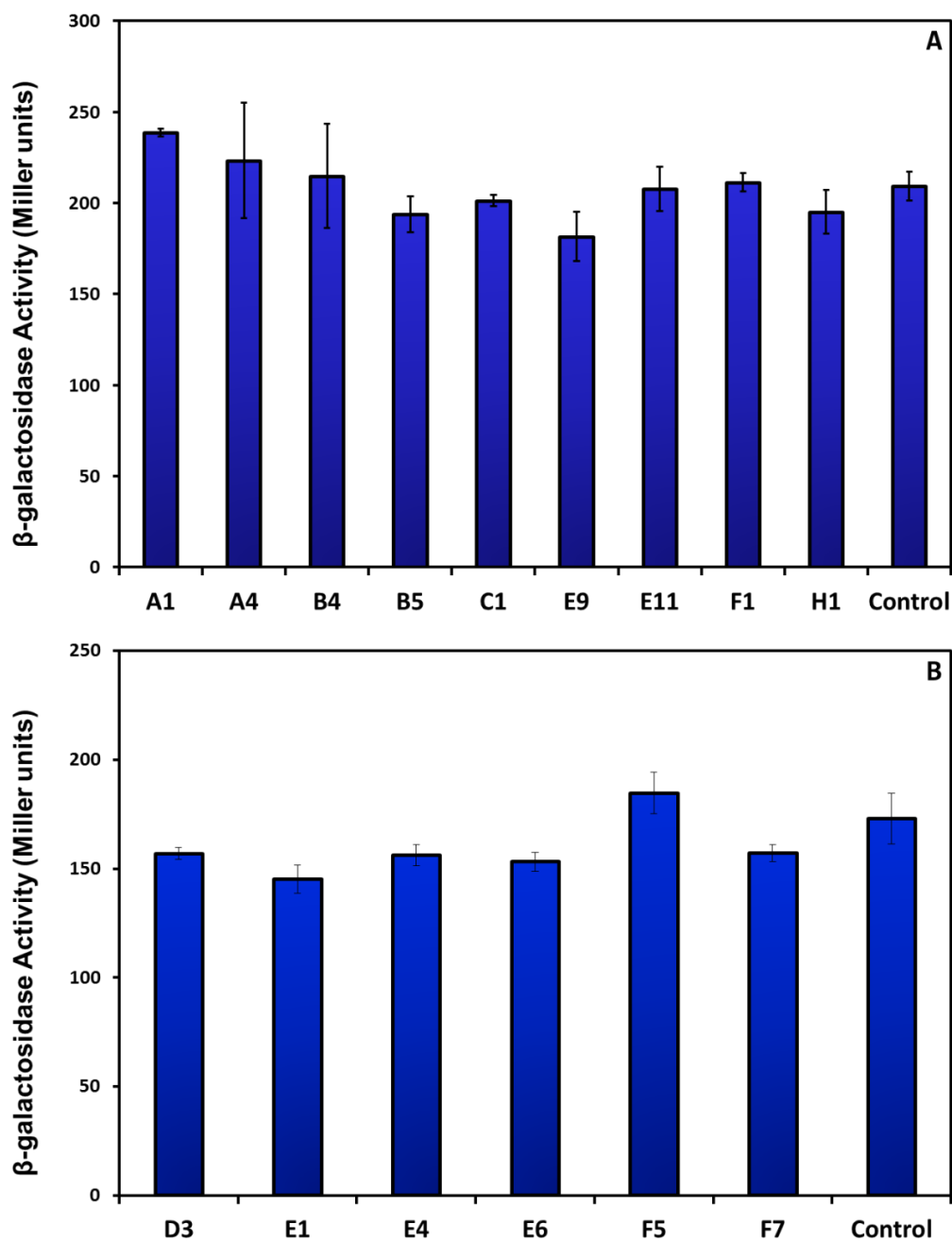


Figure 3.11. β -galactosidase activity of Tn mutants. The β -galactosidase activities of 15 Tn mutants showing a dark-blue phenotype on X-gal were quantified in the absence of induction as described in *Materials and Methods*. The *pox* promoter activity of the mutants (panel A and B) was found to be similar to the unmutagenized parent strain PA0::P_{pox}-lacZ (Control).

revealed the Tn inserted immediately upstream of the *lacZ* ORF and downstream of the cloned *pox* promoter, with the -35 and -10 sequences of the Tn inserted in the direction of *lacZ*. Thus, the outward-facing promoter of the transposable element appears to be driving the high level expression of *lacZ* observed in these clones.

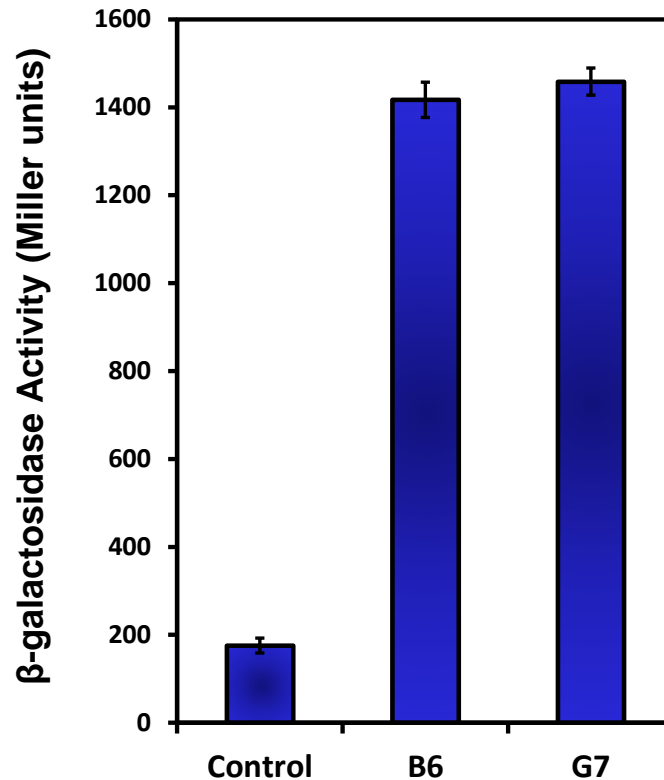


Figure 3.12. β -galactosidase activity of Tn mutants. The β -galactosidase activities of 96 blue colonies from the PA01 library (Plate 8) were quantified in the absence of induction as described in *Materials and Methods*. Only two colonies from plate 8, B6 and G7, were identified as having significantly higher *lacZ* expression than the unmutagenized control, PA01::P_{pox}-*lacZ*.

Screening of Plate 10 revealed that colonies A3, A4, A5, A6, B8 and B11 had both a darker blue and higher β -galactosidase activity than the control (Figure 3.13 K and L). Similar to clones B6 and G7 above, colonies A3, A4 had the Tn inserted immediately upstream of the *lacZ* ORF and downstream of the cloned *pox* promoter. Colonies A6 and A12 had insertions in the LPS genes *wbpY* and *wbpL*. As these two colonies only had slightly higher β -galactosidase activity than control, they were not pursued further. Clones A5 (DZ478) and B8 (DZ481) had insertions in *PA0322* and in *PA0301* (*spuE*), respectively (Figure 3.14). The role of these two genes will be explored in a later section.

Although the data from Plate 8 failed to identify any regulators, these data serve to validate the study and screening methods. In general, colonies displaying high *lacZ* activity were also dark in color. On the other hand, having a dark blue phenotype does not always correlate with increased *lacZ* expression. Specifically, disturbances in the LPS layer can allow for the color phenotype to be enhanced as a leakier, more porous membrane presumably allows escape of LacZ or uptake of X-gal (J. Goldberg, personal communication). Lastly, the outward-facing promoter of the transposable element was found to be very efficient at transcribing downstream genes, with expression of *lacZ* increasing up to 8-fold when such promoter was located upstream (Figure 3.12). Thus evaluation of potential clones must take into account not only the contribution of the Tn-disrupted gene but also the transposon-driven expression of nearby genes.

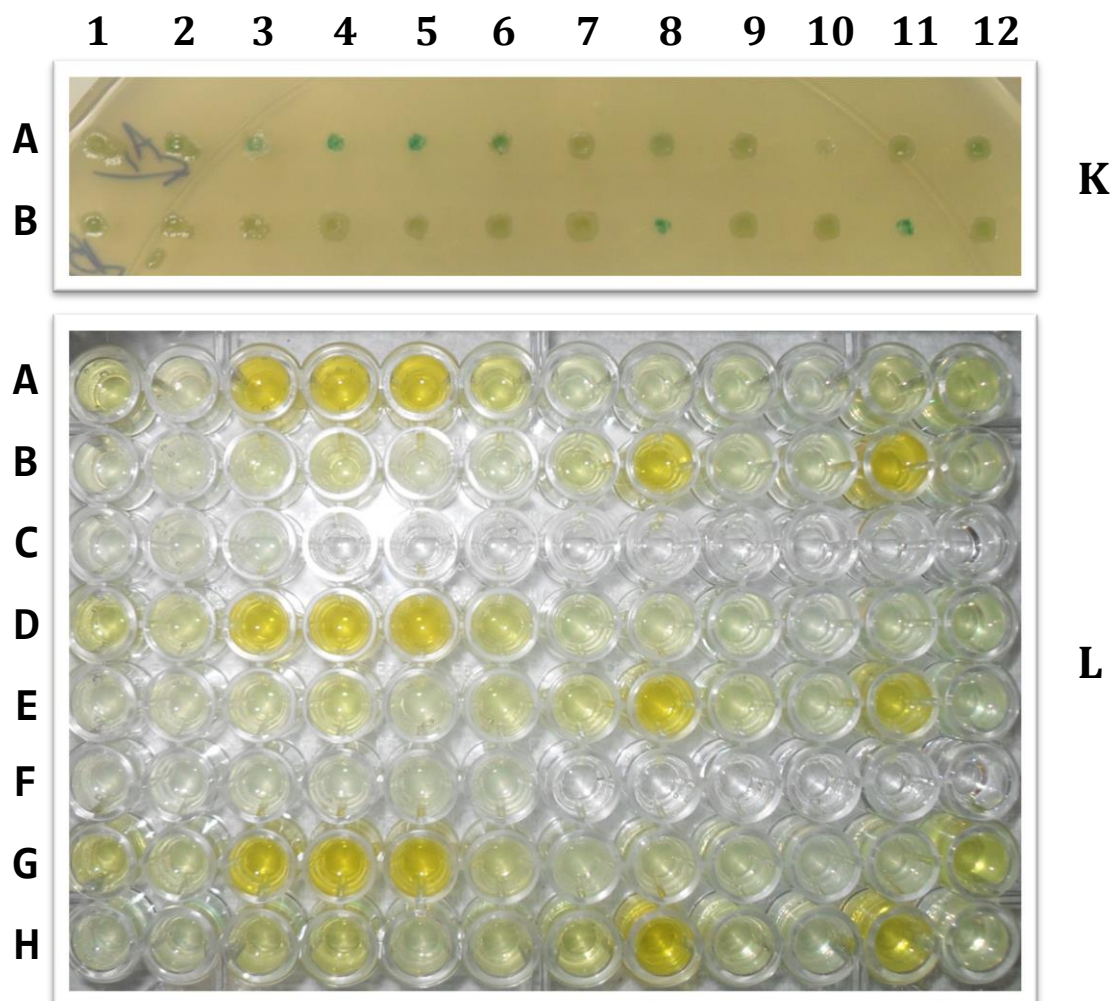


Figure 3.13. Qualitative and quantitative screening of PA0::P_{pox}-lacZ transposon mutants. Twenty-four dark blue colonies from Plate 10, wells A1 through B12, were patched onto an LB plate containing imipenem (0.15 µg/ml), Gm (75 µg/ml) and X-gal (100 µg/ml) and incubated O/N at 37°C for qualitative determination of promoter activity (Panel K). The β-galactosidase activities were also quantified in triplicates in the absence of induction as described in *Materials and Methods* (Panel L). Rows D-E and G-H are technical replicates of rows A-B. Wells C1-C3 and F1-F6 represent activity of the control unmutagenized strain PA0::P_{pox}-lacZ. The promoter activity, as measured by β-galactosidase assay (Panel L), is consistent with the activity observed in X-gal plates (Panel K). Colonies A3, A4, A5, A6, B8 and B11 have both a dark blue phenotype and increased hydrolysis of ONPG as evidenced by the darker yellow color. Although colonies A1 and A12 appear to have slightly more β-gal activity than the control (Panel L), only data from A3, A4, A5, A6, B8 and B11 could be subsequently reproduced.

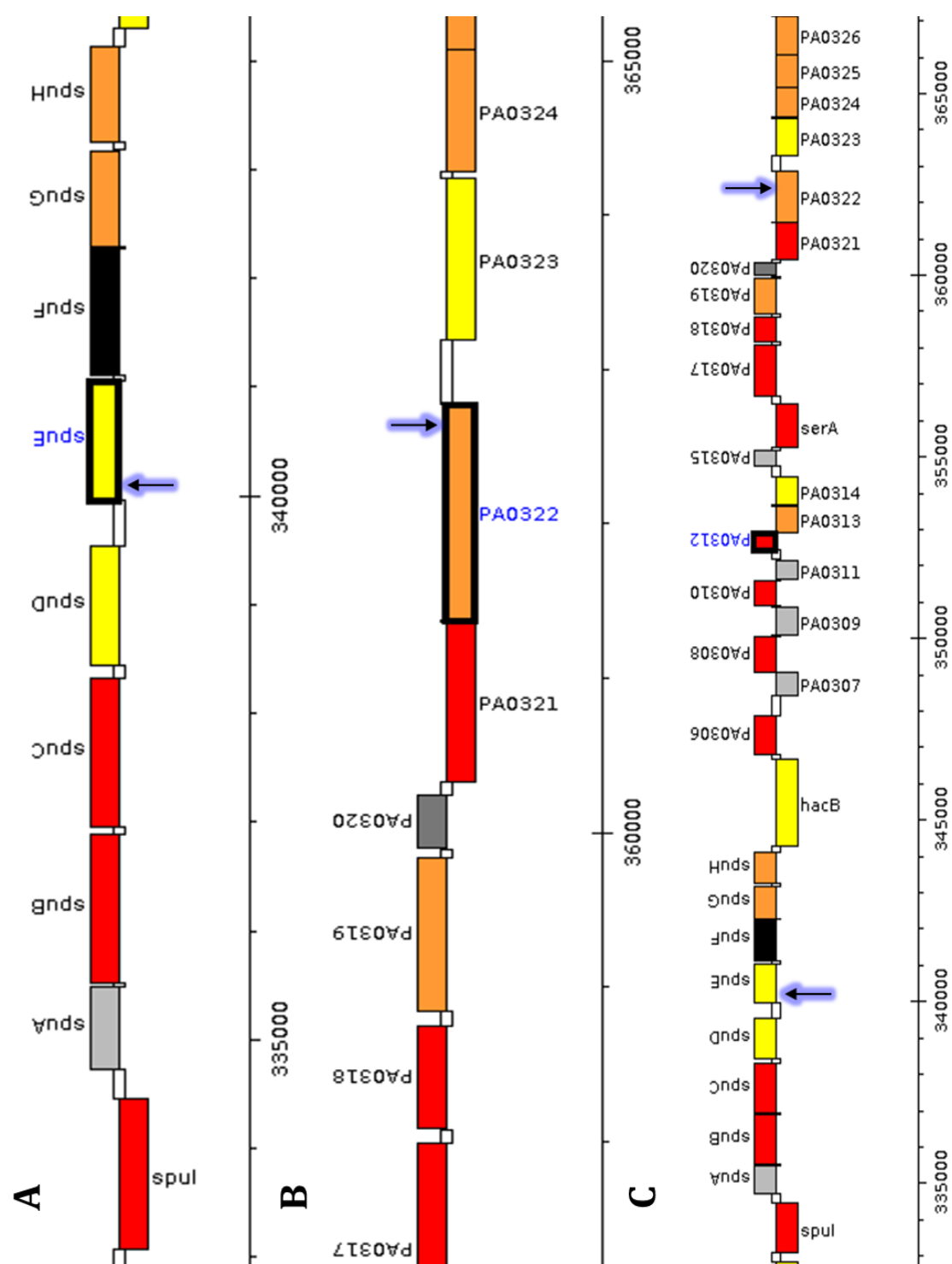


Figure 3.14. Genomic localization of *PA0322* and *spuE*. *PA0322* and *SpuE* were identified as possible regulators of the *pox* operon using genome-wide genetic screen. *spuE* encodes a polyamine transport protein and is part of a transcriptional unit containing two operons involved in the biosynthesis and transport of polyamines (Pannel A). *PA0322*, is a probable amino acid transporter with homology to the *E. coli* amine transport protein YcjJ, also implicated in a polyamine-utilizing pathway (Pannel B). These two genes are about 25, 000 bp and 21 ORFs away from each other (Pannel C) and localized in a clusters of genes mostly involved with polyamine transport, utilization and metabolism.

3.4.7.2 *PA0ΔampR::P_{pox}-lacZ* library. Screening of *PA0ΔampR::P_{pox}-lacZ* library has thus far identified 77 white to light blue colonies (Figure 3.15A), 14 of which showed varying degrees of mucoidy. Additionally, 21 colonies displaying a dark blue phenotype were selected for further analysis as they appeared to be darker than the unmutagenized parent strain, *PA0ΔampR::P_{pox}-lacZ* (Figure 3.15B).

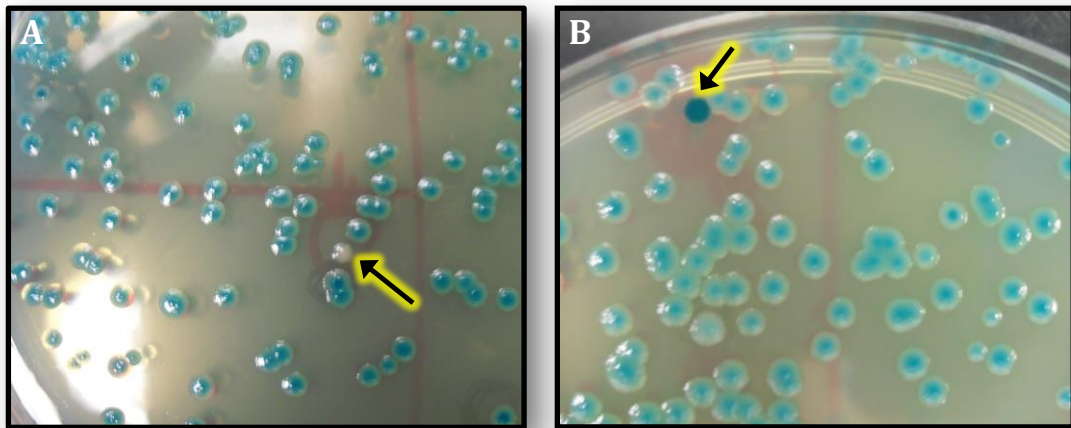


Figure 3.15. Screening of *PA0ΔampR::P_{pox}-lacZ* library. Colonies obtained after Tn mutagenesis of *PA0ΔampR* were all pooled and frozen in 10% skim milk in vials and 96-well plates. Screening was performed on a number of wells (A1-C10) from Plate #1 by growing scrapings in LB at 37°C for 45 minutes. The 10^{-5} and 10^{-6} dilutions were plated on LB-PIA-Gm⁷⁵-X-gal¹⁰⁰. Seventy-seven white (Panel A) and 21 darker blue (Panel B) colonies were selected for further analysis.

The 77 white to light blue colonies were further screened by patching them onto LB-Gm⁷⁵-X-gal¹⁰⁰ plates. Ten of these colonies displayed a clear white phenotype, and not surprisingly carried insertions within various positions of *lacZ*. The rest of the 77 colonies, ranging in color from light blue to the typical light green of *P. aeruginosa*, were further analyzed by assaying β -galactosidase activity. Only two colonies, CR88 and 90, were found to produce low β -galactosidase activity but similarly carried insertions in *lacZ*.

The 21 dark blue colonies showing a darker phenotype than that of the unmutagenized parental strain, were suspected of having the insertion immediately upstream of *lacZ*, with the promoter from Tn likely responsible for intense blue color observed. Alternatively, the intense blue color could be due to increased leakage from the cell, as previously hypothesized. A region immediately upstream of *lacZ* was thus PCR amplified to check for transposon insertion. All but six of these colonies had insertions in the MCS of mini-ctx-*lacZ*, thus explaining the intense dark color observed. The remaining six colonies were assayed for promoter activity, however all colonies produced β -gal similar to that of the isogenic strain. Thus far, the PA0 Δ *ampR* library has not yielded any potential leads, however, screening has not yet been completed for this library.

3.4.8 Role of PA0301 and PA0322. Since colonies B8 and A5 (plate 8, PA01 library) had increased *lacZ* activity, a darker blue phenotype and carried Tn insertions in *spuE* and PA0322, respectively, we decided to further explore the role of these two genes and

their possible link to β -lactam resistance. SpuE (*PA0301*) forms part of the *spuABCDEFGH* gene cluster (Figure 3.14A) involved in the utilization and transport of polyamines (putrescine, cadaverine, spermidine, spermine) (864). Polyamines are small polycationic organic compounds having more than one primary amine ($-\text{NH}_2$ group) and ubiquitously found in prokaryotes and eukaryotes (Figure 3.16) (845, 865). They are essential for growth and due to their cationic nature they are able to interact with polyanionic molecules such as nucleic acids (848, 866-868). They have also been shown to stabilize and condense DNA (869-871). Their ability to bind DNA and RNA translates into a range of pleiotropic effects such as modulation of protein-DNA interaction, transcription and translation among many others (846, 849-851, 872-874). They have also been reported to affect the activity of a number of ion channels such as the inwardly rectifying potassium channel and to decrease outer membrane permeability in *E. coli* (875-877).

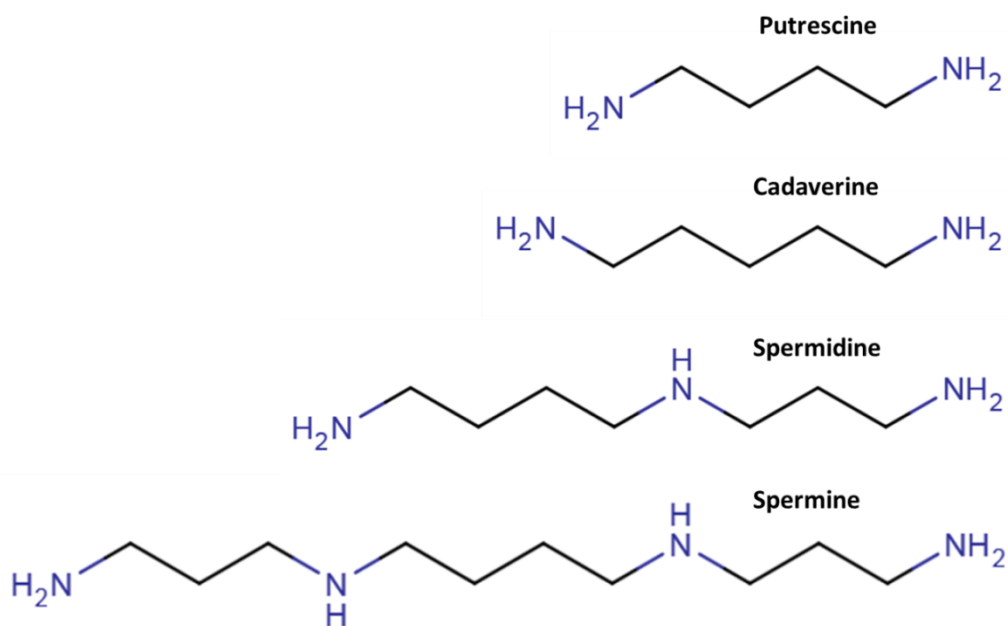


Figure 3.16. Structures of some commonly encountered polyamines

Besides their synthesis and catabolism, bacteria are able take up polyamines from the environment (847, 878). *P. aeruginosa* can use polyamines as sole source of carbon and nitrogen (864, 879). Two main ABC-transporter-type polyamine uptake systems have been reported in *E. coli*: the PotA/B/C/D or the spermidine-preferential pathway, and the PotF/G/H/I or the putrescine-specific system (880-882). A homologous system has been found in *P. aeruginosa* encoded by the *spuABCDEFGH* gene cluster, where *spuD* and *spuE* encode the periplasmic polyamine binding proteins for putrescine-preferential and spermidine-specific binding, respectively (864, 883). SpuG and SpuH are the pore-forming permeases in the inner membrane and SpuF is the ATPase for energy-driven transport. SpuA, B and C encode putative enzymes for the utilization of polyamines.

Previous work showed that polyamines decreased expression of T3SS secretions and virulence genes in PA01 (884). Polyamines were also shown to increase resistance in *P. aeruginosa* against aminoglycosides, with resistance against quinolones and cationic peptides being mediated by the expression of the *oprH-phoPQ* operon (885). In contrast, polyamines significantly increased susceptibility of *P. aeruginosa* against a number of β -lactams including the penicillins ampicillin, carbenicillin, piperacillin and ticarcillin, and the third generations cephalosporins ceftazidime, cefoperazone and ceftriaxone (886). This reduction in resistance was not linked to a decrease in expression of *ampC* or to disruption of the outer membrane as expected of polycationic ions which are known to be outer membrane disorganizers (886). Similarly, *A. baumannii* clinical and lab strains were shown to be more susceptible to the β -lactams aztreonam, carbenicillin, piperacillin

and ticarcillin when treated with spermine and spermidine, but only a slight to moderate effect was observed against ceftazidime or meropenem (887).

PA0322 also encodes a probable amino acid transporter sharing 61% homology with the *E. coli* YcjJ (PuuP) enzyme, which coincidentally is a putrescine importer (888-890). The Puu system is one of several putrescine utilization pathways reported in *E. coli* besides the Pot system. Not much is yet known about the role of *PA0322* as it still remains uncharacterized. *PA0322* does appear to form a putative operon with the upstream ORF *PA0321*, which displays 67% similarity to an acetylpolyamine aminohydrolase from *Mycoplana ramosa* involved in putrescine catabolism (891).

While identifying two targets that involve polyamine transport/utilization is promising, the range of pleiotropic effects these molecules have can obscure the real connection to the *pox* promoter. The observed phenotype may simple be an indirect effect with no actual connection to P_{pox} . Additionally, membrane perturbations cannot be ruled out as the main cause of the observed phenotype.

Increased *poxB* expression has been shown to decrease susceptibility against the carbapenems (Chapter 2). In order to see if carbapenem susceptibility was altered in these clones and if polyamines can affect susceptibility towards these β -lactams, the MIC against carbapenems was determined in the presence and absence of the polyamine spermidine. Addition of 1 mM of spermidine produced little to no change in susceptibility across strains and β -lactams, while a concentration of 20 mM spermidine was bactericidal to all strains tested (Table 3.7).

Table 3.7. β -lactam MIC in the presence of spermidine

β -lactam	Polyamine (mM)	PA0::P _{pox} - <i>lacZ</i>	PA0::P _{pox} - <i>lacZ</i> PA0322::Tn	PA0::P _{pox} - <i>lacZ</i> <i>spuE</i> ::Tn
Meropenem	0	1.5	1.5	1
	1	1.5	2	1.5
	5	>32	>32	>32
	10	>32	>32	>32
Doripenem	0	0.19	0.19	0.19
	1	0.25	0.25	0.50
	5	0.75	1	1
	10	2	2	2
Imipenem	0	0.75	1	1
	1	1.5	1.5	1.5
	5	6	8	12
	10	>32	>32	>32
Ceftazidime	0	1.5	1.5	1.5-2
	1	1.5	1.5	1.5
	5	2	1.5	2
	10	8	8	8
Piperacillin	0	4	3	4
	1	4	3	3
	5	4	4	4
	10	12	12	12
Ticarcillin/ Clavulanate	0	12	12	12
	1	12	12	8
	5	6	8	8
	10	8	8	8
Aztreonam	0	2	2	1.5
	1	2	1.5	1.5
	5	1	1	1
	10	1.5	1.5	1.5

The presence of 5 or 10 mM of spermidine rendered all strains very resistant towards meropenem (MIC >32 µg/ml). This change however, occurred in both the unmutagenized and mutagenized strains and thus is likely unrelated to altered P_{pox} activity in Tn mutants. Similarly, spermidine at 5 mM produced a significant increase in MIC against imipenem, with 10 mM leading to resistance against this β -lactam in a strain-independent manner (MIC >32 µg/ml). A smaller but significant decrease in susceptibility was also produced against doripenem that, although not leading to resistance, raised MIC values 4-5-fold and 10-fold in the presence of 5 and 10 mM of spermidine, respectively. Spermidine also produced a 2-3-fold increase in MIC against ceftazidime and piperacillin, while having no effect on aztreonam. Overall, although spermidine was able to alter the MIC values against various β -lactams tested, the most dramatic increase in MICs were produced against the carbapenems, leading to resistance against meropenem and imipenem. This experiment does not reveal if *poxB* expression is altered or enhanced by the presence of polyamines, merely that polyamines significantly raise the MIC values against the carbapenems and other β -lactams.

The mechanism by which carbapenem MIC is raised is not clear but it could be related to a decrease in cell permeability. Previous studies in *E. coli* have shown that polyamines decrease outer membrane permeability to the β -lactam cephaloridine by inhibiting permeation through the porin channels OmpF and OmpC (875, 877). Similarly, the polyamine cadaverine induced closing of liposome-reconstituted porins in *E. coli* and was proposed to be a modulator of porin activity *in vivo* (876). Under conditions of oxidative stress, polyamines also increased resistance in *E. coli* against

fluoroquinolones, antibiotics that are known to traverse the cell via porins (892). Thus a decrease in outer membrane permeability could account for the decrease in susceptibility to carbapenems that is observed in the presence of polyamines in the current study. However, other studies show that polyamines can sensitize *E. coli*, *S. aureus*, *Salmonella* and *P. aeruginosa* to most β -lactams effectively increasing susceptibility against these and other drugs (886, 893). The reduction in MIC suggested that any possible decrease in permeability, caused by polyamine inhibition of porin transport, did not play a major role or was not enough of a factor to generate resistance against most β -lactams in those studies (886, 893).

Porins blockage by polyamines in *P. aeruginosa* does appear to be a factor contributing to resistance at least against imipenem (886). Kwon and Lu showed that while polyamines increased susceptibility against most β -lactams (decreased MIC), the MIC against imipenem is increased in the presence of polyamines (886, 893). This increase appeared to occur by blockage of the OprD porin. Inhibition of OprD activity may explain the results of the present work showing that the MIC against all carbapenems tested is increased in the presence of polyamines. However, MICs of various other non-carbapenem β -lactams such as ceftazidime and piperacillin, were also slightly altered in this study. These β -lactams, however, are not known to use the OprD porin for passage into the cell. Thus, if polyamines affect susceptibility by blocking of porins, they may be able to block more than one type of porin besides OprD. In the present study, spermidine however, appears to be more effective at blocking OprD than other porins, since it is the carbapenem MIC that is mostly altered. Alternatively,

although permeability may be an issue contributing to resistance, other polyamine intracellular effects may be responsible for the increase in MIC observed. Future studies may elucidate on the expression of *poxB* in the presence of polyamines and the mechanism by which polyamines are able to induce resistance against the carbapenems.

3.5 Concluding Remarks

Regulation of class D chromosomal β -lactamases has only been previously described in *Ralstonia pickettii* and *Aeromonas* spp. (567, 730). In *R. pickettii*, the induction of two chromosomally encoded β -lactamases, OXA-22 and OXA-60, requires the regulatory element Rp3 encoded upstream of *bla*_{OXA-60} and divergently transcribed (730). *Aeromonas* spp. can produce two–three inducible and unlinked β -lactamases, one of which is an oxacillinase (566, 734-736). Expression of all is coordinately regulated by a two-component system, BlrAB, encoded immediately upstream of the *bla*_{OXA} gene (567, 739). Unpublished studies from our lab showed a two-component system, MifSR, encoded upstream of *poxAB* and divergently transcribed, played no role in *poxB* expression or carbapenem resistance.

Previous work suggested the transcriptional regulator AmpR may be involved in the regulation of the *pox* operon (710). Our transcription studies reveal *poxB* mRNA levels increase in the absence of AmpR. The increase in *pox* mRNA levels, however, do not translate into quantifiable changes at the protein levels as measured by PoxB β -lactamase activity. Additionally, binding of AmpR to the *pox* promoter was not detected indicating a possible indirect regulation by AmpR. As genes can often regulate their own

synthesis and that of the operon they are encoded in, the activity of P_{pox} was investigated in the presence of PoxA. PoxA was found to decrease P_{pox} activity when expressed *in trans* from an IPTG inducible promoter. This finding suggests PoxA is capable of regulating the expression of the *pox* operon. Autoregulation by PoxA may account for the low PoxB activity that is normally observed.

Interestingly, this work has unexpectedly connected the role of the small, essential molecules polyamines with carbapenem resistance. Specifically, a genome search for *pox* regulators identified Tn insertions in two polyamine transport genes leading to increased P_{pox} activity. Although the link between polyamine transport and *pox* expression is not yet clear, polyamines were found to drastically raise MICs against carbapenems leading to clinical resistant phenotypes. Whether or not the polyamine-induced carbapenem resistance is connected to enhanced *pox* expression, polyamine research may be a new and worthwhile area of study in the lab.

3.6 Acknowledgments

We would like to thank Dr. Stephen Lory for kindly providing mini-transposon strains for mutagenesis and Camille de Rimonteil for the invaluable help with the transposon mutagenesis project. This study was supported in part by the National Institutes of Health –Minority Biomedical Research Support SCORE (SC1AI081376; KM), Research Initiative for Scientific Enhancement graduate student fellowship (NIH/NIGMS R25 GM61347; DZ), and Florida International University Teaching Assistantship (DZ).

Chapter 4

Structural and functional characterization of *Pseudomonas aeruginosa* global regulator AmpR

As published in the Journal of Bacteriology

November 2014

Volume 196, Pages 3890-3902

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4.1 Abstract

Pseudomonas aeruginosa is a dreaded pathogen in many clinical settings. Its inherent and acquired antibiotic resistance thwarts therapy. In particular, derepression of the AmpC β -lactamase is a common mechanism of β -lactam resistance among clinical isolates. The inducible expression of *ampC* is controlled by the global LysR-type transcriptional regulator (LTTR) AmpR. In the present study we investigated the genetic and structural elements that are important for *ampC* induction. Specifically, the *ampC* (P_{ampC}) and *ampR* (P_{ampR}) promoters and the AmpR protein were characterized. The transcription start sites (TSSs) of the divergent transcripts were mapped using 5' rapid amplification of cDNA ends-PCR (RACE-PCR), and strong σ^{54} and σ^{70} consensus sequences were identified at P_{ampR} and P_{ampC} , respectively. Sigma factor RpoN was found to negatively regulate *ampR* expression possibly through promoter blocking. Deletion mapping revealed the minimal P_{ampC} extends 98-bp upstream of the TSS. Gel shifts using membrane fractions showed AmpR binds to P_{ampC} *in vitro* whereas *in vivo* binding was demonstrated using ChIP-qPCR. Additionally, site-directed mutagenesis of the AmpR helix-turn-helix (HTH) motif identified residues critical for binding and function (Ser38, and Lys42), and critical for function but not binding (His39). Amino acids Gly102 and Asp135, previously implicated in the repression state of AmpR in the enterobacteria, were also shown to play a structural role in *P. aeruginosa* AmpR. Alkaline phosphatase fusion and shaving experiments suggest that AmpR is likely to be membrane associated. Lastly, *in vivo* cross-linking study shows that AmpR dimerizes. In conclusion, a potential membrane-associated AmpR dimer regulates *ampC* expression by direct binding.

Key Words: β -Lactam resistance, LysR-type transcriptional regulator, AmpC β -lactamase

Abbreviations: EMSA, electrophoretic mobility shift assay; HTH, helix-turn-helix; LTTR, LysR-type transcriptional regulator; PG, peptidoglycan; RACE-PCR, rapid amplification of cDNA ends-PCR; TSS, transcriptional start site

4.2 Introduction

Pseudomonas aeruginosa, a Gram-negative opportunistic pathogen, causes severe and life-threatening infections in susceptible individuals. This pathogen is primarily associated with morbidity and mortality in patients with cystic fibrosis, a deadly genetic disease (742). The bacterium's innate ability to counteract the action of antibiotics often complicates treatment strategies. Intrinsic resistance is conferred by its low membrane permeability, the expression of efflux pumps and hydrolyzing enzymes, the alteration of antimicrobial targets and the ability to form biofilm (246, 436, 781). In particular, resistance to β -lactam antibiotics is mediated by the expression and overproduction of a chromosomally-encoded class C β -lactamase AmpC (379-381).

Ambler class C β -lactamases were first described in members of the Enterobacteriaceae family where expression is either constitutively low or inducible (375, 376, 630, 894). In species where expression is inducible, such as *Citrobacter freundii* and *Enterobacter cloacae*, the induction requires β -lactam challenge and the presence of a transcriptional regulator AmpR (375, 712, 895, 896). AmpR is a member of the LysR family of transcriptional regulators and as such is a DNA-binding protein with a predicted helix-turn-helix (HTH) motif at the N-terminus and an inducer-binding domain at the C-terminus (856, 897). Comprehensive studies in the Enterobacteriaceae have established the critical role of AmpR as the regulator of *ampC* expression and the paradigm of β -lactamase induction.

In the Enterobacteriaceae, *ampC* inducibility is intimately linked to the recycling of the peptidoglycan (PG) of the murein sacculus (713, 716, 718, 721). During normal

physiological growth, N-actylglucosaminyl-1,6-anhydromuropeptides (GlcNAc-1,6-anhydro-MurNAc tri, tetra, and pentapeptides) are continuously being released from the murein sacculus due to remodeling (716, 718). The permease, AmpG, transports the metabolites into the cytoplasm where the glycosidase NagZ removes the GlcNAc moiety and the amidase AmpD removes the stem peptides from either the incoming GlcNAc-1,6-anhydro-MurNAc-peptides or from the NagZ-processed product (716, 717, 719, 898-901). The resulting muramyl peptides are recycled back into the PG biosynthetic pathway to form the PG precursor UDP-MurNAc-pentapeptide (902). It has been proposed that during normal cell growth, the cytosolic concentrations of UDP-MurNAc-pentapeptide maintain AmpR in an inactive conformation that represses the expression of *ampC* (716, 718). In the presence of β -lactams, however, there is an excessive breakdown of murein leading to accumulation of 1,6-anhydromuropeptides in the cytoplasm, which in turn overwhelm the hydrolytic activity of AmpD (714, 716, 718, 903). The increased number of AmpD-unprocessed muramyl peptides presumably displaces the repressor UDP-MurNAc-pentapeptide from AmpR and induce a conformational change in the protein to promote expression of *ampC* (714, 718, 903).

All *amp* gene homologs (*ampC*, *ampR*, *ampD* and *ampG*) have been identified and studied in *P. aeruginosa* (643, 710, 711, 776, 782, 904-906). Whether a similar induction mechanism is employed by *P. aeruginosa* is not yet known, however, recent work illustrates significant departures from the classical enterobacterial induction system. In particular, there are three *ampD* homologs in *P. aeruginosa* that are responsible for a stepwise up-regulation mechanism leading to constitutive β -lactamase hyperexpression

(246, 643, 904). Additionally, *P. aeruginosa* harbors two AmpG homologs, PA4218 (AmpP) and PA4393 (AmpG) that appear to be required for induction of *ampC* (837, 906). Further, our lab has shown that *P. aeruginosa* AmpR is a global transcriptional regulator involved in the control of *amp* and various other genes (710, 842-844).

AmpR exhibits high sequence identity with its counterparts in *C. freundii* and *E. cloacae*, and as in the Enterobacteriaceae, *ampR* is located immediately upstream of *ampC* and divergently transcribed (711, 776). Such similarities suggest a common regulatory mechanism among the species, however, the *P. aeruginosa ampR-ampC* intercistronic region bears little resemblance to that of the enterobacteria. *In vitro* studies using crude extracts have shown *P. aeruginosa* AmpR binds to this region, but the exact binding site and the identity of the amino acids involved in the interaction have not yet been determined (776). In essence, not much is known about the structural elements that are critical to the functioning of *P. aeruginosa* AmpR as regulator of β -lactamase expression.

In the present work, we define some of the genetic elements in the *ampR-ampC* intergenic region including the *ampR* and *ampC* transcriptional start sites, as well as, the minimal length of the *ampC* promoter needed for induction of the *ampC* system. We further show AmpR specifically binds to the 193-bp P_{ampC} fragment identified by promoter mapping as being required for induction. We also identify amino acids in the AmpR HTH motif that are critical for the interaction with the promoter. Additionally, we examine the role of two amino acids, Gly102 and Asp135, previously implicated in the repression state of AmpR from the Enterobacteriaceae. Lastly, we show that *P.*

aeruginosa AmpR likely functions as dimers as previously seen for *C. freundii* and is potentially a membrane protein.

4.3 Materials and Methods

4.3.1 Bacterial strains, plasmids and media. Bacterial strains, plasmids and primers employed in this study are shown in Table S1 of the Supplemental Material. *E. coli* and *P. aeruginosa* were routinely cultured in Luria-Bertani medium (10 g tryptone, 5 g yeast extract, and 5 g NaCl, per liter). *Pseudomonas* Isolation Agar (Difco) was used with LB at a 1:1 ratio in triparental mating experiments. Antibiotics were used at the following concentrations (per milliliter): ampicillin (Ap) at 100 µg, tetracycline (Tc) at 15 µg, and gentamycin (Gm) at 15 µg for *E. coli*; Gm and Tc each at 75 µg for *P. aeruginosa*. PA0Δ*ampC* and PA0Δ*ampR* strains used in this work were previously constructed using overlap extension PCR and homologous recombination (760, 843).

4.3.2 P_{ampC} promoter deletions. To characterize the minimal promoter necessary for full activity, 5'-end deletions of P_{ampC} were constructed and transcriptionally fused to a promoterless *lacZ*. Briefly, 352-, 193-, 171-, 151-, 130-, 111-, 90-, 70-, and 51-bp fragments were generated by PCR with the following primer pairs respectively: SBJ03*ampCR*For-OC*P_{ampC}*RevBc, OC*P_{ampC}*For193-OC*P_{ampC}*RevBc, OC*P_{ampC}*For173-OC*P_{ampC}*RevBc, OC*P_{ampC}*For151-OC*P_{ampC}*RevBc, OC*P_{ampC}*For131-OC*P_{ampC}*RevBc, OC*P_{ampC}*For111-OC*P_{ampC}*RevBc, OC*P_{ampC}*For91-OC*P_{ampC}*RevBc, OC*P_{ampC}*For71-OC*P_{ampC}*RevBc, and OC*P_{ampC}*For51-OC*P_{ampC}*RevBc (Table S1, Supplemental material).

The fragments were sequenced, then cloned into the *EcoRI-BamHI* sites of the integrative vector mini-CTX-*lacZ* and integrated into PA01.

4.3.3 Construction of His-tagged AmpR. Primers OCAmpR-His-For and OCAmpR-His-Rev (Table S1) were used to amplify PA01 genomic *ampR*. The 933-bp amplicon, carrying a His₆ sequence at the 3'-end was cloned into pBluescriptSK(+) and sequenced (Table S1). The fragment was subsequently subcloned into the *EcoRI-BamHI* sites of pMMB67EH-Gm, a broad-host range expression vector (763). His-tagged AmpR was shown to be functional by β -lactamase assay and E-test (Text S1 and Table S2, Supplemental material).

4.3.4 Expression and purification of AmpR-His₆. AmpR-His₆ was purified according to standard protocols. Briefly, stationary-phase cultures of PA0 Δ *ampR* (pAmpR-His₆) were diluted to an OD₆₀₀ of 0.02 in 2 liters of LB broth and incubated with shaking at 37°C until the culture density reached an OD₆₀₀ of 0.2. Cells were then induced with 1 mM of isopropyl β -D-1-thiogalactopyranoside (IPTG) and incubated for an additional six hours before harvesting. The cells were recovered by centrifugation at 6,000 X g for 10 minutes at 4°C and resuspended in 25 ml of lysis buffer (20 mM HEPES pH8, 0.5 M NaCl, 10% glycerol, 1 mM PMSF, 2 pellets of EDTA-free protease inhibitor cocktail tablets complete, 100 μ l of 0.1 mg ml⁻¹ of lysozyme and 5 μ l of DNase1). Following disruption of the cells on ice with sonication (20-s pulse ON and 20-s pulse OFF for 20 minutes, amplitude 50%), the cell lysate was centrifuged at 10,000 X g for 10 minutes at

4°C. The supernatant was further ultracentrifuged at 36, 000 rpm for 1 h at 4°C. Membranes pellets were resuspended in 20 ml of solubilization buffer (20 mM HEPES pH8, 0.5 M NaCl, 10% glycerol, 5 mM imidazole, 1 mM PMSF, 2 pellets of EDTA-free Protease Inhibitor cocktail tablets complete, and 2 % of CHAPS) and loaded onto a HisTrap FF 1 ml column. AmpR-His₆ was eluted with buffer B (20 mM HEPES pH8, 0.5 M NaCl, 10% glycerol, 500 mM imidazole and 0.6% of CHAPS) by using an FPLC chromatograph (Akta, Amersham Biosciences). About 20 ml were recovered and dialyzed to remove imidazole. This AmpR preparation was used to make AmpR-specific antibodies.

4.3.5 Construction of *P. aeruginosa* AmpR HTH and point mutants. Site-directed mutagenesis was used to replace various amino acid residues in AmpR. Briefly, Ser38, His39, Lys42, Ser43, and Glu46 were replaced with Ala; Gly102 and Asp135 were replaced with Glu and Asn, respectively. Substitutions were constructed by PCR using the following primer pairs: AmpRSer38AlaFor-AmpRSer38AlaRev, AmpRHis39AlaFor-AmpRHis39AlaRev, AmpRLys42AlaFor-AmpRLys42AlaRev, AmpRSer43AlaFor-AmpRSer43AlaRev, AmpRGlu46AlaFor-AmpRGlu46AlaRev, AmpRGly102GluFor-AmpRGly102GluRev, and AmpRAsp135AsnFor-AmpRAsp135AsnRev (Table S1, Supplemental material).

4.3.6 Membrane fraction purification. Preliminary studies showed pAmpR-His₆ expression and β -lactamase induction were achieved with a 2-hour incubation at a 1 mM-IPTG concentration. Thus, PA0 Δ *ampR* (pAmpR-His₆) cells at OD₆₀₀ of 0.2 were induced with 1 mM of IPTG and incubated for two hours before harvesting for membrane fractionation. For β -lactamase induction, cells were further treated with 200 μ g ml⁻¹ of penicillin G an hour after IPTG addition. Cells were recovered by centrifugation at 6,000 X g for 10 minutes at 4°C and resuspended in 50 ml of lysis buffer (20 mM HEPES pH8, 0.1 M NaCl, 1 mM EDTA, 1 mM PMSF, and 50 μ l of DNase1). Following disruption of the cells on ice with sonication (15 cycles of 10 second pulse ON and 30 S pulse OFF, amplitude 40%), the cell lysate was centrifuged at 5,000 rpm for 10 minutes at 4°C. The supernatant was ultracentrifuged at 36,000 rpm (Rotor Ti70) for one hour at 4°C and the pellets were resuspended with 2 ml of membrane buffer (25% sucrose, 20 mM Tris pH8, and 0.5 mM PMSF). Two-hundred milliliters of membrane fractions were aliquoted and stored at -80°C.

4.3.7 Electrophoretic mobility shift assay (EMSA). The 193-bp PCR fragment containing the *ampR-ampC* intergenic region plus a small part of *ampR* ORF was used to perform EMSA. Ten pmol of this fragment were radiolabelled at the 5' end by incubation with T4 polynucleotide kinase (NEB) and [γ -³²P]ATP (3,000 Ci mmol⁻¹; Perkin Elmer). The labeled fragment was diluted to a final concentration 100 nM and unincorporated nucleotides were removed by sephadex G-25 (Biorad) spin chromatography. DNA binding reactions containing 50 fmol of ³²P-labeled DNA probe

and varying amounts of total protein membrane fractions were incubated for 20 minutes and loaded thereafter in a non-denaturing 5% PAGE. Radioactive signals were detected by scanning a phosphostorage cassette with the GE Healthcare Typhoon 9400 scanner.

For competition assays, 50 fmol of the 193-bp ^{32}P -labeled probe were mixed with the unlabeled 193-bp fragment in 10-, 100- and 500-fold molar excess in the EMSA assay. For nonspecific assays, 50 fmol of the 193-bp ^{32}P -labeled probe were mixed with a PCR-amplified 233-bp fragment (*alg44*) in 10-, 100- and 500-fold molar excess in the EMSA assay.

4.3.8 5' RACE PCR. The *ampC* and *ampR* TSSs were mapped using a classical 5' RACE-PCR on total mRNAs extracted from PA01, PA0 Δ *ampR* and PA0 Δ *ampC* (907). Stationary-phase cultures of PA01, PA0 Δ *ampR* and PA0 Δ *ampC* were diluted to an OD₆₀₀ of 0.02 and incubated with shaking at 37°C until the cultures reached an OD₆₀₀ of 0.6. The cultures were then induced with 200 $\mu\text{g ml}^{-1}$ of penicillin G for one hour and subsequently blocked on ice for 15 minutes with 1/5th of the final culture volume in 5% acidic phenol-95% ethanol, pH 4. One milliliter of the cells were recovered by centrifugation and resuspended with 3 mg ml^{-1} of lysozyme (Tris EDTA, pH 8). RNA was then extracted according to the RNeasy mini kit protocol (Qiagen), treated with 10 U of RQ1 DNase (Promega) for one hour, extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform, precipitated and dried. Superscript III (Invitrogen) was then used to reverse transcribe 10 μg of RNA as previously described (907) using primers

5RA-P_{ampC}233 and 5RA-P_{ampR}229 for determination of the *ampC* and *ampR* TSS, respectively. In the first round of PCR amplification (Pfu, Stratagen), primers Q_t, Q₀, and 5RA-P_{ampC}154 were used for *ampC* TSS determination, while Q_t, Q₀, and 5RA-P_{ampR}169 were used for determination of the *ampR* TSS. In the second round of PCR amplification (Pfu, Stratagen), primer pairs Q_i-5RA-P_{ampC}113, and Q_i-5RA-P_{ampR}99 were used for *ampC* and *ampR* TSS determination, respectively (Table S1). PCR products were cloned into TOPO (Invitrogen), blue colonies were selected for screening and clones were sequenced.

4.3.9 qPCR analysis of *ampR* and *ampC* mRNAs. Total RNA was extracted from PA01, PA0Δ*rpoN* and PA0Δ*rpoN* (pRpoN) in the presence and absence of the inducer (0.2 μg ml⁻¹ imipenem) using the RNeasy mini kit (Qiagen). RNA was reverse transcribed into cDNA with Superscript III (Invitrogen) and an (NS)₅ random primer using standard methods (764). For qPCR, the ABI 7500 (Applied Biosystems) cyclor was used with *Power* SYBR Green PCR Master Mix with ROX (Applied Biosystems). The reading was normalized to *clpX* (PA1802), whose expression remains constant in all the samples and conditions tested. Melting curves were generated to ensure primer specificity. Gene expression of each sample was normalized to PA01 uninduced value, to see the effect of induction and mutation at the same time. Primer pairs DBS_QRTAmpRFwd-DBS_QRTAmpRRev and qRT_*ampCF*-qRT_*ampCR* were used for the real time amplification of *ampR* and *ampC*, respectively.

4.3.10 Construction of VSV-G-tagged AmpR. A 540-bp fragment corresponding to the 3' end of *ampR*, minus the stop codon, was amplified using primers DB_ampR3'_F and DB_ampR3'_R. The amplicon was cloned into pP30ΔFRT-MvaT-V (908), a replication-incompetent vector in *P. aeruginosa*, such that the 3' end of *ampR* was fused in frame with three alanines and the VSV-G tag (YTDIEMNRLGK). The construct was then introduced into PA01 as single copy by mating and clones were selected for gentamycin resistance.

4.3.11 Chromatin immunoprecipitation quantitative PCR (ChIP-qPCR). Cells harboring the VSV-G-tagged AmpR were harvested after sub-MIC β -lactam exposure (843) and treated with formaldehyde to cross-link proteins with DNA as previously described (908). DNA was sheared by sonication to an average length of 0.5-1.0 kb and AmpR was immunoprecipitated with anti-VSV-G agarose beads (BETHYL Laboratories, Inc.). ChIP-qPCR was performed using *Power* SYBR Green PCR Master Mix (Applied Biosystems) with primers DBS_ChIP_ampCF and DBS_ChIP_ampCR. Fold enrichment was normalized to *clpX* (PA1802).

4.3.12 Protein cross-linking. PA0Δ*ampR* (pAmpR-His₆) was grown to an OD₆₀₀ of 0.3 and induced with 1 mM IPTG for 2 hours. Cells were grown for two additional hours in the presence and absence of β -lactam antibiotics (0.1 $\mu\text{g ml}^{-1}$ of imipenem). Cultures were then treated with 0.1% formaldehyde for 20 and 40 minutes at room temperature. Crude extracts containing 10 μg of total protein were separated on an SDS-

polyacrylamide gel and AmpR was visualized using anti-His antibody. The blot was subsequently stripped and reprobed using anti- σ^{70} antibody (NeoClone).

4.3.13 Polyclonal anti-AmpR-His₆ antibody production. Purified AmpR-His₆ was used as antigen to raise anti-AmpR-His₆ rabbit polyclonal antibodies (Covance, Princeton, NJ). AmpR-His₆ antibody was affinity-purified as previously described (909).

4.3.14 Western Blotting and EMSA of HTH mutants. Concentration of purified AmpR-His₆ was determined by the bicinchoninic acid (BCA) method (910). A calibration Western blot was generated using FujiFilm LAS-3000 imager to correlate intensities with the concentration of purified AmpR-His₆. Membrane fractions were purified from PA0 Δ *ampR* pAmpR-HTH mutants and their concentrations were determined using the BCA method, whereas the exact quantity of AmpR was deduced from Western blotting. Preliminary gel shifts with increasing concentrations of membrane fractions of AmpR-HTH mutants showed that a 0.4 mg ml⁻¹ is sufficient to shift the 193-bp P_{*ampC*} PCR fragment (Data not shown). For a second gel shift, a 0.4 mg ml⁻¹ of total membrane fraction (8.44 ng of AmpR), recovered from PA0 Δ *ampR* overexpressing AmpR HTH mutants in the presence and absence of 200 μ g ml⁻¹ penicillin G, was hybridized with the 193-bp PCR fragment spanning the *ampC-ampR* intergenic region (Fig. 7). As this concentration was not enough to visualize AmpR, a higher quantity (33 ng) was used for Western blotting of the HTH mutants to show that the amount of AmpR-His₆ is equivalent under all conditions and thus in the EMSA

experiment (Data not shown). Further, the stability of AmpR-His₆ mutants was verified by Western using equal amounts of total protein with AmpR-specific antibodies (Figure 4.8). Sigma⁷⁰ (NeoClone) was used as control to show the same amount of total protein was loaded per sample. All Western blots were developed according to standard protocols. Briefly, proteins were transferred to a PVDF membrane (Bio-Rad) and blocked with TBST (TBS 0.1 % Tween) and 5 % non-fat dry milk at 4°C overnight or for four hours, followed by rinsing with the same solution and probing with rabbit anti-AmpR antibody (1:3000). Membranes were subsequently washed with TBST, incubated with goat anti-rabbit IgG (H+L)-horseradish peroxidase-conjugated antibody (1:5000) (Bio-Rad), rinsed and developed using Enhanced Chemiluminescence Western Blotting Substrate (Pierce).

4.3.15 β -galactosidase assay. β -galactosidase assays were performed as previously described (854).

4.3.16 AmpR-LacZ and -PhoA fusion construction and analysis. The topology of AmpR was investigated using *phoA* and *lacZ* fusions. The plasmid pSJ01 (710) carrying a 1220-bp fragment containing *ampR* was digested at *HindIII*, *HincII* and *PstI*, corresponding to the amino acid positions, Gln15, Val134 and Gln186, respectively (Fig. S2A). The resultant fragments were ligated in-frame upstream of *phoA* and *lacZ*-containing plasmids, pTrecphoA and pTrelacZ (Table S1, Supplemental material), (911).

The *phoA* and *lacZ* activities were qualitatively determined in *E. coli* according to standard protocols (854).

4.3.17 Protease protection (shaving) assay. A stationary-phase culture of PA0 Δ *ampR* (pAmpR-His₆) was diluted to an OD₆₀₀ of 0.02 and incubated with shaking at 37°C until the culture reached an OD₆₀₀ of 0.4. The cells were then induced with 1 mM IPTG for four hours and chloramphenicol (500 μ g ml⁻¹) was added 30 minutes prior to harvesting to stop protein synthesis. The cells were harvested by centrifugation and resuspended in 40 mM Tris-Cl pH 8.0, 0.5 M sucrose. Spheroplasts were obtained by adding 1 mg ml⁻¹ of lysozyme and 4 mM EDTA for 10 minutes in a 30°C water bath followed by the addition of 20 mM MgCl₂. The formation of spheroplasts was monitored by light microscopy. Spheroplasts were harvested by centrifugation at 4,000 X g for 10 minutes and resuspended in 40 mM Tris-Cl pH 8.0, 0.5 M sucrose. Proteinase K (10 μ g ml⁻¹) was added to 1 ml aliquot of the spheroplasts and incubated in a 30°C water bath. Samples were taken at different time points and added to 2 mM PMSF and 4X SDS PAGE Sample Buffer. Samples were then boiled for 5 minutes and ran in a 4-20% SDS-PAGE (Criterion Biorad). Proteins were transferred to a nitrocellulose membrane (Biorad) and identified using AmpR (Covance), σ^{70} (NeoClone) and His-tag (Qiagen) antibodies. The immunoblot was developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

4.4 Results and Discussion

4.4.1 Analysis of the *P. aeruginosa* *ampC-ampR* regulatory region. *P. aeruginosa* AmpR shares high identity with its homologs in *C. freundii* and *E. cloacae* (58.3 and 62.5%, respectively), as well as, the same gene organization, with *ampR* located upstream of *ampC* and divergently transcribed (711, 776). The *P. aeruginosa* *ampR-ampC* intercistronic region, however, shares no significant similarities with that of *C. freundii* and *E. cloacae*, with the exception of an inverted 38-bp sequence that in *C. freundii* is protected by AmpR (711, 840). The lack of conservation in the promoter region could point to different sigma factor requirements and/or different regulatory mechanisms. To elucidate the transcriptional regulatory elements of *P. aeruginosa* *ampC* and *ampR*, their 148-bp intergenic region was characterized.

The transcriptional start sites (TSS) were determined using 5' RACE-PCR on total mRNAs isolated from PA01, PA0 Δ *ampR* and PA0 Δ *ampC* (Figure 4.1). A strong similarity to RpoN (σ^{54}) sigma factor sequences was detected at -12 and -24 positions of the *ampR* TSS, whereas strong σ^{70} promoter sequences were observed at the -10 and -35 positions of *ampC*. Alignment of the *C. freundii*, *E. cloacae* and *P. aeruginosa* intergenic region reveals a fair conservation for the *ampC* -10 and -35. However, there is a downstream shift in the *P. aeruginosa* *ampR* TSS that could contribute to the change in sigma factor control observed here (Figure S1, Supplemental material).

The presence of putative -12 and -24 recognition sequences in the *ampR* promoter (P_{ampR}) suggested that its expression might be RpoN or σ^{54} -dependent. Quantitative real-time PCR was used to validate this hypothesis. Total RNA was extracted from β -lactam

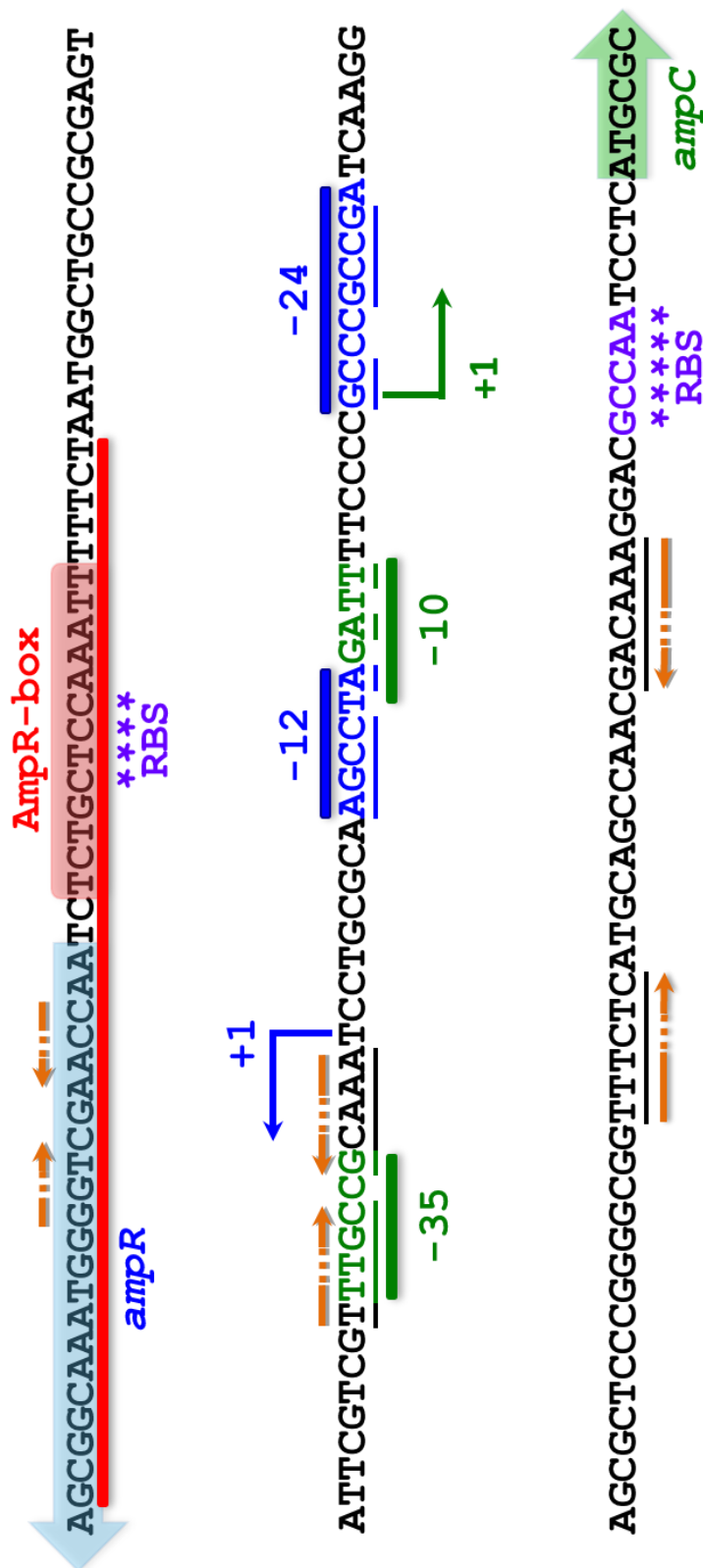


Figure 4.1. Intergenic region of *P. aeruginosa* *ampC* and *ampR*. A 148-bp region separates *ampR* from *ampC*. The *ampR* and *ampC* transcriptional start sites are indicated with a blue and green +1, respectively, whereas the colored arrows designate the beginning of the ORFs. Strong σ^{54} (blue) and σ^{70} (green) sequences are observed in the *ampR* and *ampC* promoter regions, respectively. The underlined bases correspond to conserved sequences. The Shine-Delgarno sequences or ribosome binding sites (RBS) are marked by asterisks. A putative AmpR-binding site was identified based on sequence homology and is indicated by the AmpR-box. Orange arrows denote palindromic sequences in close proximity with the *ampC* and *ampR* RBSs indicative of hairpin formation for regulating translation through blocking of ribosome binding.

induced and uninduced PA01, PA0 $\Delta rpoN$ and PA0 $\Delta rpoN$ (pRpoN) strains and reverse transcribed into cDNA using standard methods (764). In the wild-type, exposure to β -lactams increased *ampR* and *ampC* expression approximately 100-fold over the uninduced samples (normalized to 1) (Figure 4.2). A further, significant increase in expression of both genes was observed in PA0 $\Delta rpoN$ upon β -lactam challenge that could be rescued by pRpoN. In the absence of β -lactams, a small, but significant reduction in expression was observed in the PA0 $\Delta rpoN$ that could not be complemented.

In spite of the presence of a probable binding site on P_{ampR} , σ^{54} is not required for *ampR* expression. The significant increase in the absence of *rpoN* and in the presence of the β -lactams suggests σ^{54} negatively impacts *ampR* expression. RpoN may exert this negative effect indirectly by enabling transcription of a negative regulator or directly by competing for the RNA polymerase holoenzyme with another sigma factor. Alternatively, *P. aeruginosa* RpoN can directly repress transcription by blocking promoter access to a different sigma factor in a phenomenon referred to as σ -factor antagonism as previously reported (912). Specifically, σ^{54} has been shown to repress σ^{22} -dependent transcription from P_{algD} by directly binding to the overlapping promoter sequences in the absence of an external stimulus (912). Similarly, σ -factor antagonism has also been reported in *E. coli*, where mutagenesis of a σ^{54} -dependent promoter created a new TSS with σ^{70} requirement that exhibited decreased transcriptional activity in the presence of σ^{54} (913).

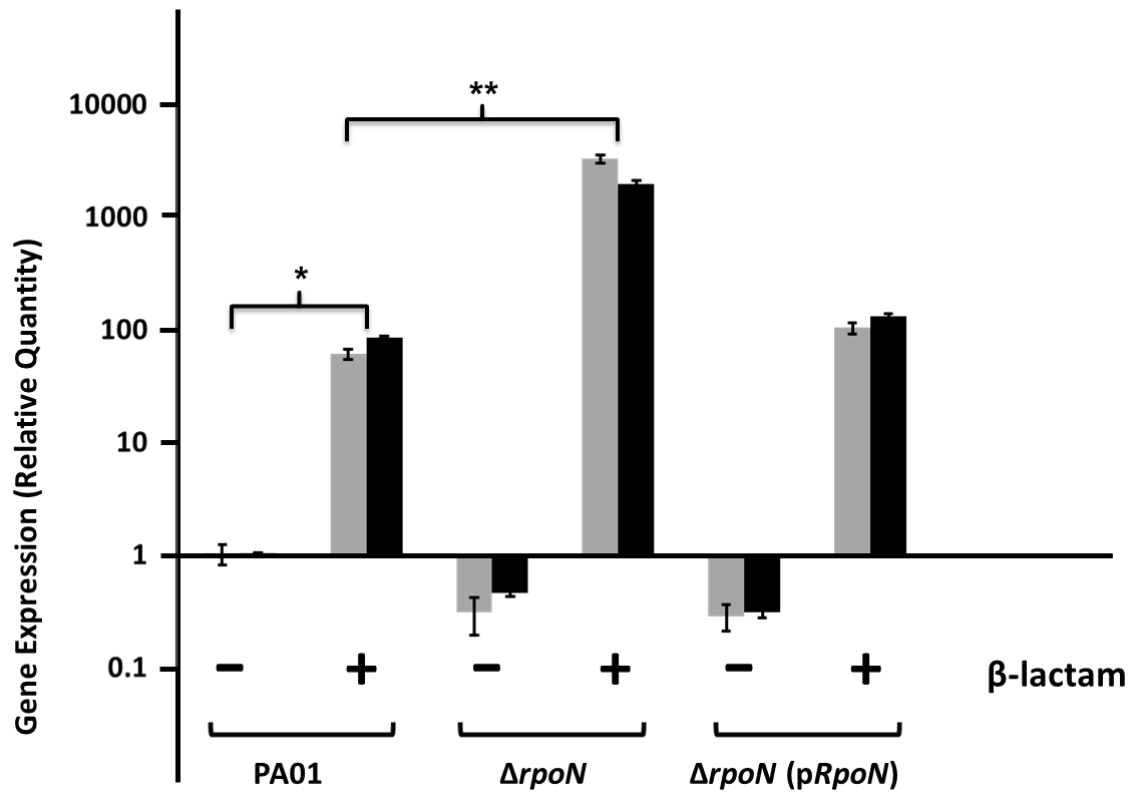


Figure 4.2. RpoN downregulates *P. aeruginosa ampR* expression in the presence of β -lactams. RNA was isolated from PA01, PA0 Δ *rpoN* and PA0 Δ *rpoN*(pRpoN) in the presence and absence of β -lactams, reversed transcribed to cDNA and tested by qPCR with *ampC* (grey bars) and *ampR* (black bars) specific primers, as described in the *Experimental Procedures*. Values were normalized to the expression of the wild-type uninduced sample and represent the mean (\pm SD) of two experiments conducted in triplicates. * p -value < 0.001 for both *ampR* and *ampC* expression in induced PA01 versus expression in uninduced PA01, ** p -value < 0.005 for *ampR* and *ampC* expression in induced PA0 Δ *rpoN* versus expression in PA01 induced as determined by unpaired t -test.

A model of repression by σ^{54} through promoter blocking is conceivable, where in the absence of some external stimuli, in this case β -lactams, σ^{54} binds P_{ampR} and prevents access and thus transcription by the sigma factor. In the presence of the inducer, however, σ^{54} may be partially or completely displaced by the sigma-RNA polymerase complex to promote *ampR* transcription. Thus, the loss of *rpoN* leads to complete de-repression of *ampR* expression in the induced condition.

Lastly and very interestingly, the expression of *ampC* followed a pattern similar to that of *ampR* in all backgrounds. Previously, *ampR* expression in *P. aeruginosa* was shown to be low and not significantly induced upon exposure to the β -lactam benzylpenicillin (710). Similarly, expression from the *C. freundii ampR* promoter in *E. coli* was found to be constitutive in the presence and absence of the inducer (6-aminopenicillanic acid) (840). In *E. cloacae*, induction with cefoxitin significantly increased transcription of *ampC* but had no effect on *ampR* expression (895). Recent work from our lab however, showed that in the presence of very powerful known inducers, namely imipenem and meropenem, expression of both *ampC* and *ampR* is equally and very significantly induced in wild-type *P. aeruginosa* (760). In light of such results, it is not surprising that our current data shows that wild-type *P. aeruginosa* has similar *ampC* and *ampR* mRNA levels in the presence of imipenem. Similar induction profiles in the absence of *rpoN* suggest that the *ampC* and *ampR* promoters can reach their full induction potential upon removal of the restricting negative effect imposed by RpoN. It is not clear how exactly RpoN, or its absence, can accomplish this, but it is worth noting that in the *ampC* and *ampR* TSSs, the -12 sequence of *ampR* overlaps the -

10 sequence of *ampC*. Thus, if σ^{54} is in fact blocking promoter access to RNA polymerase, it could be blocking access to both the *ampR* and *ampC* promoters until such time as inducers lead to its partial or complete displacement from the intergenic region. Further studies are needed to elucidate the mechanism of *ampC* and *ampR* downregulation by RpoN.

4.4.2 Mapping of *P. aeruginosa* P_{ampC} . To map the minimal promoter needed for AmpR-dependent activity, a series of 5'-end deletions of P_{ampC} were transcriptionally fused to the promoterless *lacZ* gene of the integrative vector mini-CTX-*lacZ* (853). The activity of each promoter deletion was then analyzed in PA01 by assaying β -galactosidase activity in the presence and absence of 200 $\mu\text{g ml}^{-1}$ of penicillin G. The minimum length of the promoter needed for full P_{ampC} activity is 193-bp (Figure 4.3). This fragment consists of the full *ampR-ampC* intergenic region plus small parts of the *ampR* (22 bp) and *ampC* (23 bp) ORFs. The high activity seen with the 193-bp fragment in the absence of β -lactams as compared to the wild-type may be the result of the partial or complete removal of the repressor-binding site. Subsequent loss of a 22-bp *ampR* fragment from the 5' end of the 193-bp segment resulted in a 2-fold decrease in induction likely indicating partial removal of the activator-binding site. This 22-bp fragment corresponding to the beginning of the *ampR* ORF seems to be necessary for full induction of P_{ampC} . *In silico* analysis reveals that this segment is very well conserved among other species but has no real identifiable features. Induction was abolished with a further 20-bp deletion (151-bp fragment). Thus, the 42-bp region (denoted red in Figures 4.1 and 4.3),

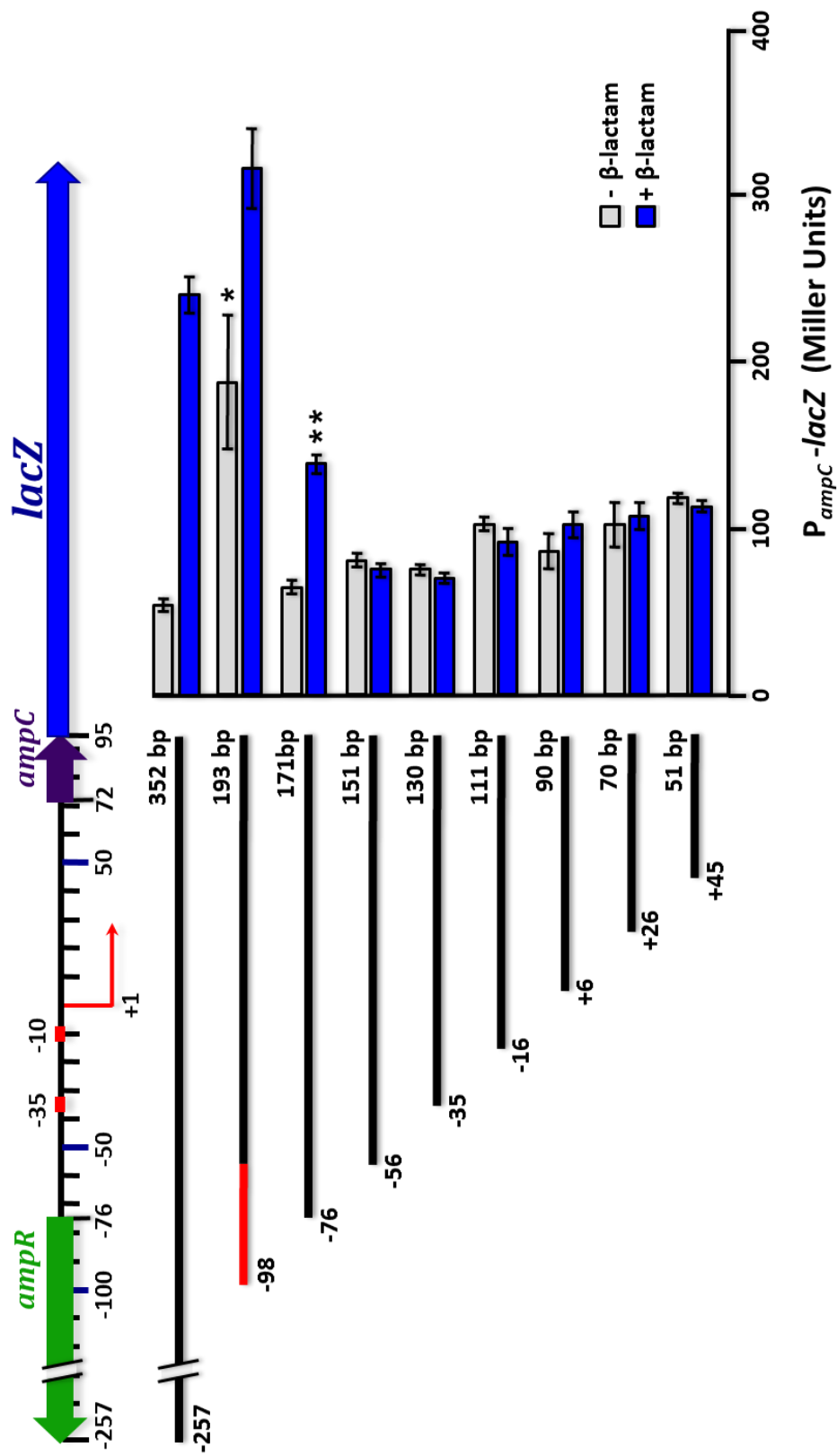


Figure 4.3. Mapping of the minimal *P. aeruginosa ampC* promoter. 5'-end deletions of P_{ampC} were constructed and transcriptionally fused to a promoterless *lacZ* gene in the integrative vector mini-CTX-*lacZ* as described in the *Materials and Methods*. Constructs were introduced into PA01 for integration at the *attB* site to generate single-copy promoter fusions. Promoter activities are expressed in Miller units. The +1 denotes the *ampC* transcriptional start site. The 42-bp segment, missing from the 151-bp construct, that appears to be necessary for activator binding, is depicted in red at the 5' end of the 193-bp fragment. * p -value < 0.05 as compared to uninduced PA0attB:: $P_{ampC352-lacZ}$; ** p -value < 0.05 versus uninduced PA0attB:: $P_{ampC171-lacZ}$ as determined by unpaired t -test using ANOVA.

present at the 5' end of the 193-bp fragment but deleted from the 151-bp construct, demarcates the outer bounds of the functional promoter needed for activation of P_{ampC} . Since this 42-bp fragment includes the AmpR box, an *in silico* derived putative AmpR-binding site (Figure 4.1), this region could be critical for activator binding.

4.4.3 *P. aeruginosa* AmpR binds to P_{ampC} . Previously, *P. aeruginosa* AmpR has been shown to bind P_{ampC} using AmpR-overexpressing *E. coli* whole cell extracts (776). Similarly, crude preparations of *C. freundii* AmpR were also shown to retard a radio-labeled *ampR-ampC* intergenic region (840). Since preliminary work from our lab suggested that *P. aeruginosa* AmpR is likely to be a membrane-associated protein (See Localization studies of *P. aeruginosa* AmpR section below and Figure. S2), we tested the ability of PA01 membrane fractions to bind P_{ampC} . EMSA was performed using AmpR-His₆ enriched membrane fractions and a [γ -³²P] ATP radiolabeled P_{ampC} fragment. Shift was observed with increasing concentrations of total membrane protein up to 0.4 mg ml⁻¹ (Figure 4.4). The binding was competed-out by mixing labeled DNA with unlabeled promoter DNA in 100-fold molar excess confirming AmpR binding to P_{ampC} (Figure 4.4).

Additionally, competition with a nonspecific, unlabeled fragment (233-bp *alg44* PCR fragment) mixed in 10-, 100-, and 500-fold molar excess with the labeled P_{ampC} fragment, failed to displace AmpR-His₆ from P_{ampC} illustrating the binding specificity.

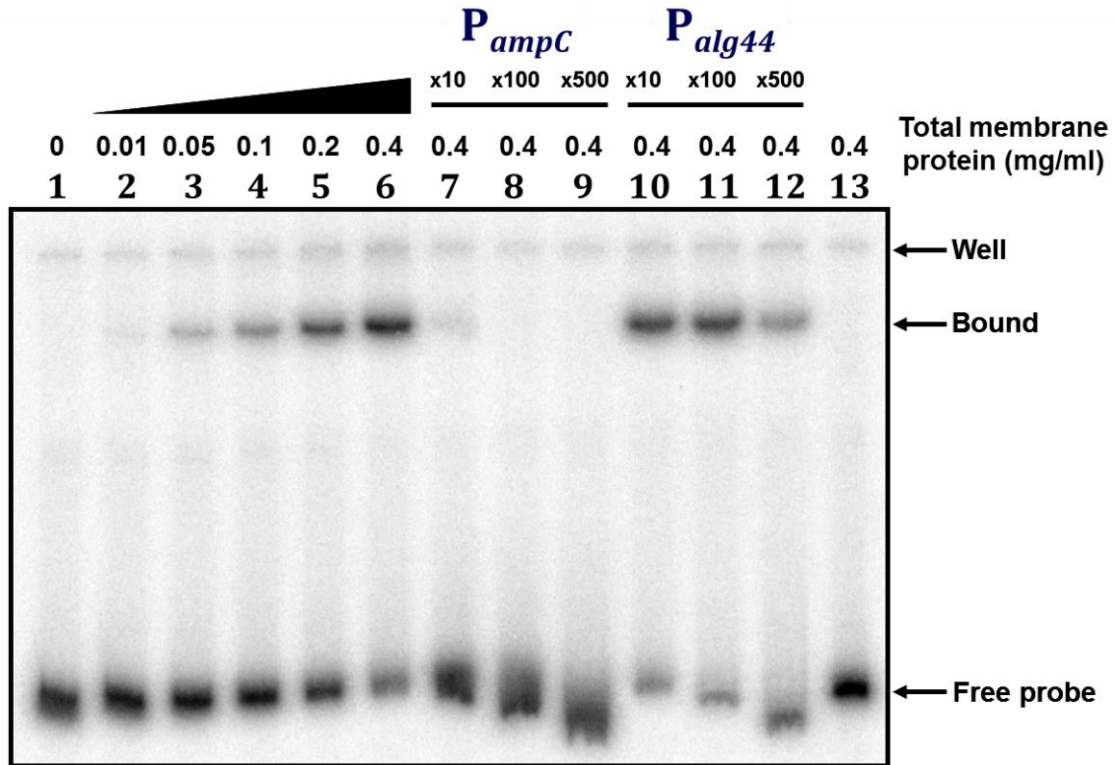


Figure 4.4. *P. aeruginosa* AmpR binds P_{ampC} . Fifty fmol of a 193-bp radio-labeled P_{ampC} fragment (Lane 1) spanning the *ampC-ampR* promoter region were mixed with increasing concentrations of AmpR-His₆ enriched membrane fractions extracted from PA0 $\Delta ampR$ (pAmpR-His₆) in the presence of 200 $\mu\text{g ml}^{-1}$ of penicillin G (Lanes 2 to 6). Competition assays were carried out with the cold P_{ampC} fragment in 10- (Lane 7), 100- (Lane 8) and 500-fold (Lane 9) molar excess. To characterize the binding specificity, a nonspecific fragment (233-bp *alg44* PCR fragment) was mixed in 10- (Lane 10), 100- (Lane 11) and 500-fold (Lane 12) molar excess with the radio-labeled reaction mix. Lane 13 is the control showing the 193-bp radio-labeled P_{ampC} fragment in the presence of membrane fractions extracted from PA0 $\Delta ampR$ containing the plasmid backbone alone.

To determine if AmpR interacts with P_{ampC} *in vivo*, ChIP-qPCR was employed (908). A functional VSV-G-tagged AmpR (Table S3, Supplemental material) was introduced into PA01 as single copy and then immunoprecipitated with anti-VSV-G antibody. Sequence-specific primers were used to detect the presence of P_{ampC} DNA with qPCR. Promoter occupancy was detected in the presence and absence of β -lactams as expected of LTTRs (856) (Fold enrichment over *clpX* control- Uninduced: 10.6 ± 1.73 , Induced: 13.3 ± 4.63 ; Fold enrichment for negative control target *aprX*- Uninduced: 1.30 ± 0.02 , Induced: 1.34 ± 0.22). AmpR thus binds P_{ampC} *in vivo* in the presence and absence of the inducer.

4.4.4 HTH is important for AmpR function. The majority of prokaryotic DNA-binding proteins, including LTTRs, use the HTH motif to interact with DNA (897, 914). In LTTRs, this domain is often found at the N-terminus (856, 897). The canonical HTH motif is comprised of three helical bundles where the second and third helices interact with the DNA, and the third makes the essential contacts with the major groove to provide recognition (897, 914, 915). A multiple alignment of the AmpR family HTH motif shows the highest degree of conservation is found in the first two helices, with the most variation in the third helix that provides specificity (Figure S3, Supplemental material). Although *P. aeruginosa* AmpR has been shown to bind the *ampR-ampC* intercistronic region (776), the amino acids involved in the interaction have not been identified.

An amphipathic wheel of the third helix (residues Gln34 to Leu48), generated using DNASTAR Protean, identified polar and charged amino acids potentially facing the major groove of the DNA (Figure 4.5). Point mutations corresponding to these residues were generated by site-directed mutagenesis of AmpR-His₆. Residues Ser38, His39, Lys42, Ser43, and Glu46 were thus replaced with alanine and the mutants were overexpressed in PA01 and PA0Δ*ampR* strains carrying the chromosomal *P_{ampC}-lacZ* fusion (Figure 4.6). The *P_{ampC}* activity observed in PA01 is the result of both *ampR* alleles, from the chromosome (*ampR_{chr}*) and from the plasmid (*ampR_{pls}*), whereas in PA0Δ*ampR* only *ampR_{pls}* contributes.

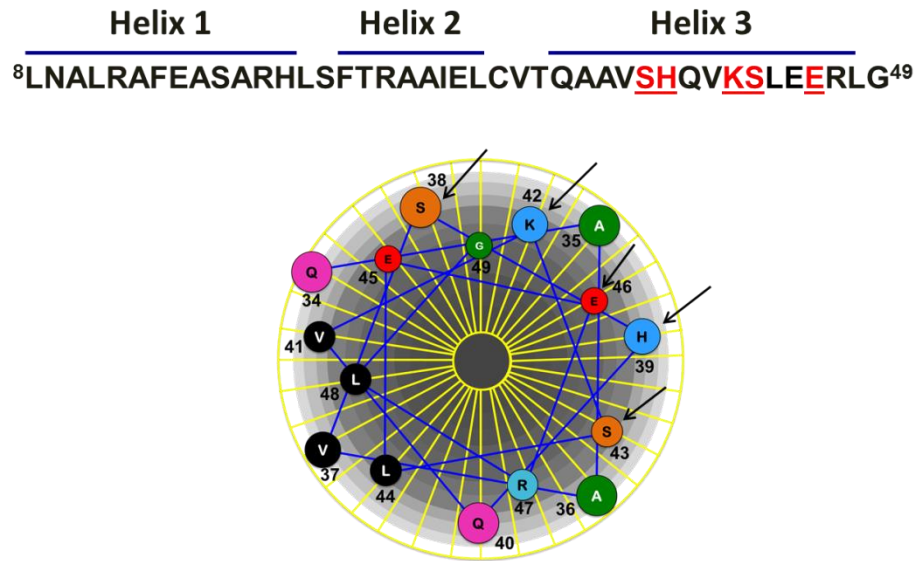


Figure 4.5. Analysis of the third helix of the *P. aeruginosa* AmpR HTH motif. An amphipathic wheel of the third helix (residues Q34 to L48) was generated using DNASTAR Protean in order to identify polar and charged amino acids likely facing the major groove of the DNA. The AmpR residues Ser38, His39, Lys42, Ser43, and Glu46, were identified as amino acids likely to interact with the DNA and were thus targeted for mutagenesis. They are indicated by the black arrows and denoted as S5, H6, K9, S10 and E13 in the helical wheel, respectively. The N-terminal sequence of *P. aeruginosa* AmpR illustrates the HTH motif and the location of the above amino acids (in red) in the third helix of AmpR.

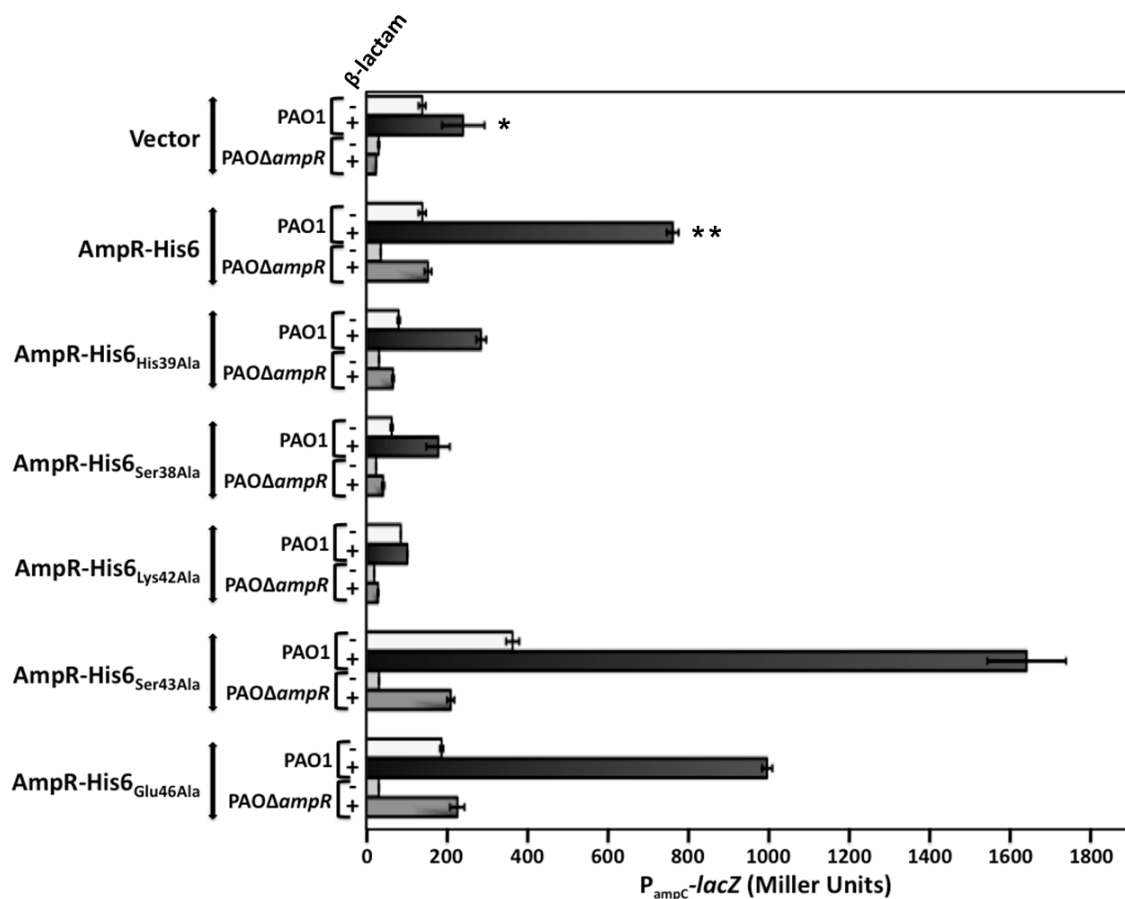


Figure 4.6. Functional analysis of the *P. aeruginosa* AmpR HTH motif. Site-directed mutagenesis was used to replace Ser38, His39, Lys42, Ser43, and Glu46 with Ala in AmpR-His₆. The mutant proteins were overexpressed in PAO1 and PAOΔampR strains carrying a single copy of the P_{ampC}-lacZ fusion integrated at the attB site. β-galactosidase activity was quantified in the presence and absence of β-lactams. * *p*-value < 0.05 versus uninduced PAO1 vector control; ** *p*-value < 0.005 versus induced PAO1 vector control as determined by unpaired *t*-test using ANOVA.

Alanine substitutions at Ser43 and Glu46 did not affect the ability of AmpR to activate P_{ampC} in PAOΔampR (Figure 4.6). In addition, both AmpR_{Ser43Ala} and AmpR_{Glu46Ala} were able to bind P_{ampC} in the presence and absence of β-lactams (Figure 4.7). These two findings suggest Ser43 and Glu46 are not critical for AmpR function. However, expression of AmpR_{Ser43Ala} and AmpR_{Glu46Ala} in PAO1 that carries AmpR_{chr},

significantly increased P_{ampC} activity by more than 2-fold in the presence of inducers suggesting a possible interaction between chromosomal encoded $AmpR_{chr}$ and the variants (Figure 4.6). In particular, the Ser43Ala substitution increased basal levels in the absence of inducers, while leading to hyperinduction in the presence of β -lactams. Similarly, significant activation of P_{ampC} in PA01 in the presence of $AmpR$ -His₆ further strengthens the idea that $AmpR$ functions as a multimer.

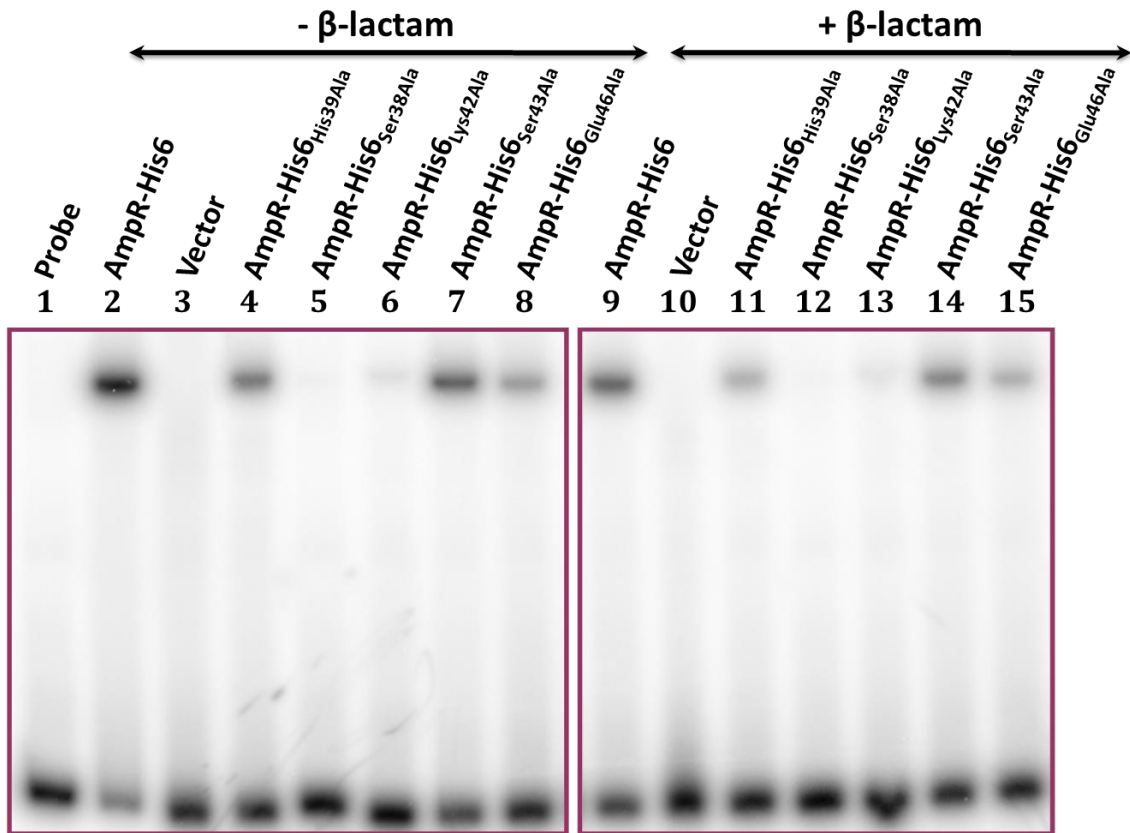


Figure 4.7. Electromobility shift assay of *P. aeruginosa* $AmpR$ HTH mutants. A 50 fmol of the 193-bp radio-labeled P_{ampC} fragment (Lane 1) were mixed with membrane fractions recovered from PA0 $\Delta ampR$ in the absence (Lanes 2 to 8) and presence of β -lactams (Lanes 9 to 15) and carrying pMMB67EH-Gm (Lanes 3 & 10), pAmpR-His₆ (Lanes 2 & 9), pAmpR-His₆(His39Ala) (Lanes 4 & 11), pAmpR-His₆(Ser38Ala) (Lanes 5 & 12), pAmpR-His₆(Lys42Ala) (Lanes 6 & 13), pAmpR-His₆(Ser43Ala) (Lanes 7 & 14), and pAmpR-His₆(Glu46Ala) (Lanes 8 & 15).

AmpR mutant proteins failed to activate P_{ampC} when Ser38, His39, or Lys42 were substituted with alanine. These three residues are thus essential for AmpR activity and are presumably involved in the binding to P_{ampC} . A multiple alignment reveals Ser38 and Lys42 are well-conserved in members of the AmpR family as expected of amino acids that play a critical role in the functionality of a protein (Figure S3, Supplemental material).

The loss of P_{ampC} activity in AmpR_{Ser38Ala}, AmpR_{His39Ala} and AmpR_{Lys42Ala} could be attributed to the destabilization of the proteins. Their expression was thus analyzed using Western blotting with anti-AmpR antibody (Figure 4.8). Interestingly, not only are these three mutant AmpR proteins made, it appears that they, and in particular AmpR_{Ser38Ala} and AmpR_{Lys42Ala}, are made in large quantities. These amino acid substitutions, therefore, appear to stabilize rather than destabilize the proteins. Thus, we argued that the loss of P_{ampC} activity may be due to their inability to bind DNA. Gel-shifts revealed that AmpR_{Ser38Ala} failed to bind to P_{ampC} while AmpR_{Lys42Ala} bound very poorly correlating well with the loss of P_{ampC} transcriptional activity (Figure 4.7). Surprisingly, the His39Ala substitution did not prevent AmpR from binding to P_{ampC} in the presence or absence of β -lactams, although it clearly prevented it from activating transcription from P_{ampC} (Figures 4.6 and 4.7). AmpR_{His39Ala} is thus a positive control mutant that can bind DNA but cannot activate transcription from the promoter to which it binds.

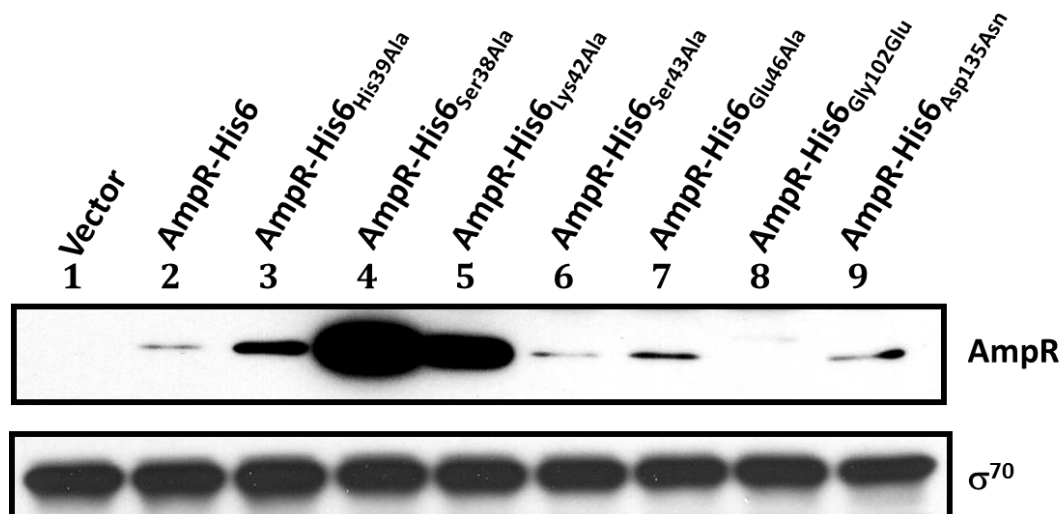


Figure 4.8. Stability of *P. aeruginosa* AmpR mutant proteins. Total protein extracts were recovered from AmpR HTH and point mutants after a 1½-hour incubation with 1 mM of IPTG. The stability of each mutant was verified by Western blotting using AmpR-specific antibodies. Equal amounts of total membrane protein were loaded per well; σ^{70} was used as a loading control and detected with anti- σ^{70} antibody.

Positive control (*pc*) mutants are proteins that are defective in transcriptional activation but retain the ability to bind DNA. The *pc* phenotype is caused by the disruption of favorable protein-protein interactions between the activator protein and the RNA polymerase (916-918). Several *pc* mutants of other proteins have been characterized with mutations in or near the DNA-binding domain (916-921). Mutations away from this region have also been reported (922). In particular, mutations in the DNA-binding region have been mapped to the second helix of the HTH motif and to the junction between the second and third helix of the same domain in the activator proteins λ cI, 434 cI, and P22 c2 (916-919). AmpR_{His39Ala} is different from previously reported *pc* mutants in that its mutation is found in the helix of the DNA-binding domain that is

thought to directly interact with the major groove of the DNA (helix 3), and not in the helix which usually lies across the major groove (helix 2), and makes contacts with the DNA backbone. Although it is not clear whether this third helix can contact the RNA polymerase, it may interact with other sites in the nearby helix to indirectly affect transcription. Our work here does not reveal how the disruption of His39 affects activation of transcription, but merely that it is required for it.

In the present work we show that the highly conserved residues Ser38 and Lys42 (not conserved in *Klebsiella pneumoniae*), in the HTH motif are critical for binding and function of AmpR. The less conserved His39 is also necessary for promoter activation but not for binding to the DNA.

4.4.5. Gly102 and Asp135 are critical for AmpR function. Previous work identified *C. freundii ampR* mutants that constitutively express β -lactamase (839, 923). Specifically, a change in Gly102 to Glu (Gly102Glu) resulted in high constitutive β -lactamase expression in an inducer-independent manner, while a Gly102Asp substitution yielded a similar but less pronounced phenotype (839, 923). In addition, Asp135 was also found to play a role in the function of *C. freundii* and *E. cloacae* AmpR (923, 924). The expression of *C. freundii* AmpR_{Asp135Tyr} led to constitutive β -lactamase hyperexpression in an *ampG* mutant background. In *E. cloacae* AmpR Asp135 substitutions to Val or Asn resulted in higher β -lactamase activity in the presence and absence of inducers and contributed to increased β -lactam resistance in two different *E. coli* backgrounds (923,

924). Gly102 and Asp135, thus appear to play important roles in the activation/repression state of AmpR in, at least, the Enterobacteriaceae.

To investigate the role of these amino acids, *P. aeruginosa* AmpR Gly102 and Asp135 were replaced with Glu and Asn, respectively. The two mutant AmpR proteins were overexpressed in PA01 and PA0 Δ *ampR* strains carrying *P_{ampC}-lacZ* (Figure 4.9). Unlike in *C. freundii*, in *P. aeruginosa* the Gly102Glu substitution resulted in the loss of *P_{ampC}* activity in the presence and absence of β -lactams. The loss of activity is due to destabilization of the protein (Figure 4.8), suggesting a structural role for Gly102 in *P. aeruginosa* AmpR.

On the other hand, the Asp135Asn substitution led to an inducer-independent increase in *P_{ampC}* transcriptional activity in both PA01 and PA0 Δ *ampR* with no concomitant increase in the amount of protein being made (Figures 4.8 and 4.9). Thus, we postulate that the Asp135Asn substitution in the effector binding domain appears to stabilize the active conformation effectively turning AmpR into a constitutive activator of *ampC* transcription. The Asp135Asn substitution has also been reported in AmpR from a *P. aeruginosa* clinical variant that exhibited hyper-constitutive β -lactamase expression and high resistance to β -lactams (722). The importance of Asp135 corroborates the previous work in *E. cloacae* and *C. freundii* (923, 924). Gly102, on the other hand, clearly plays different roles in the *P. aeruginosa* and *C. freundii* AmpR proteins.

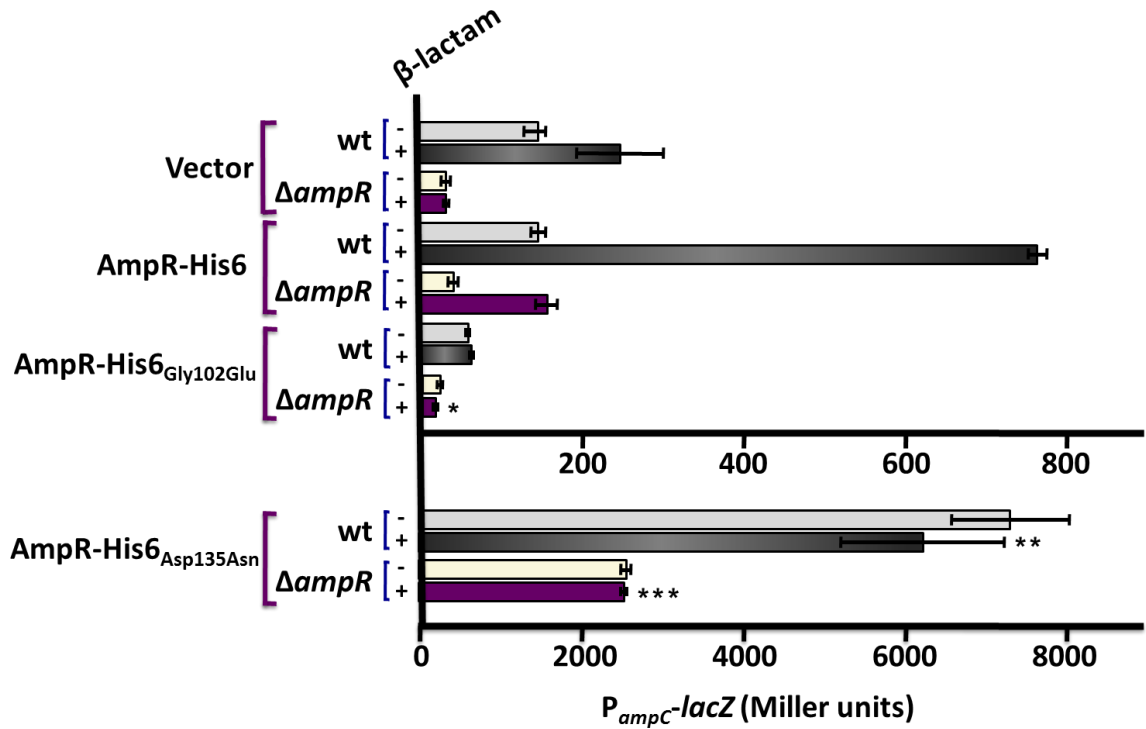


Figure 4.9. Activity of the *P. aeruginosa* *ampC* promoter in the presence of AmpR-His6_(Gly102Glu) and AmpR-His6_(Asp135Asn) mutants. Site-directed mutagenesis was used to replace Gly102 and Asp135 of *P. aeruginosa* AmpR with Glu and Asn, respectively. Mutant AmpRs were expressed in wild-type PA01 and PA0Δ*ampR* strains carrying *P_{ampC}-lacZ*. β-galactosidase activity was quantified in the presence and absence of β-lactams and is represented in Miller units. * *p*-value<0.01 versus induced PA0Δ*ampR* pAmpR-His6; ** *p*-value<0.01 versus induced PA0*attB*::mini-CTX-*lacZ*; *** *p*-value<0.001 versus induced PA0Δ*ampR* pAmpR-His6 as determined by unpaired *t*-test using ANOVA.

4.4.6 Cross-linking studies suggest *P. aeruginosa* AmpR dimerizes. The *P_{ampC}* activity in the presence of both *ampR_{chr}* and *ampR_{pls}* is always considerably higher than that in PA01 and PA0Δ*ampR* (*ampR_{pls}*) (Figure 4.6). These findings suggest a possible interaction between the wild-type and His-tagged AmpR. This is not surprising as LTTRs exist and/or function as dimers or tetramers (856, 897, 925-929). To determine if in fact

P. aeruginosa AmpR can dimerize, proteins from PA0 Δ *ampR* (pAmpR-His₆) were cross-linked and AmpR was visualized with anti-His antibody. The detection of a 64-kD and a 32 kD species in cross-linked and non-cross-linked samples, respectively, suggests that AmpR dimerizes *in vivo* (Figure 4.10). Only the monomeric form of σ^{70} was detected after stripping and reprobing the blot. Our findings corroborate previous work in *C. freundii* where both AmpR and its effector binding domain were shown to dimerize in solution and in the crystallized form, respectively (930, 931).

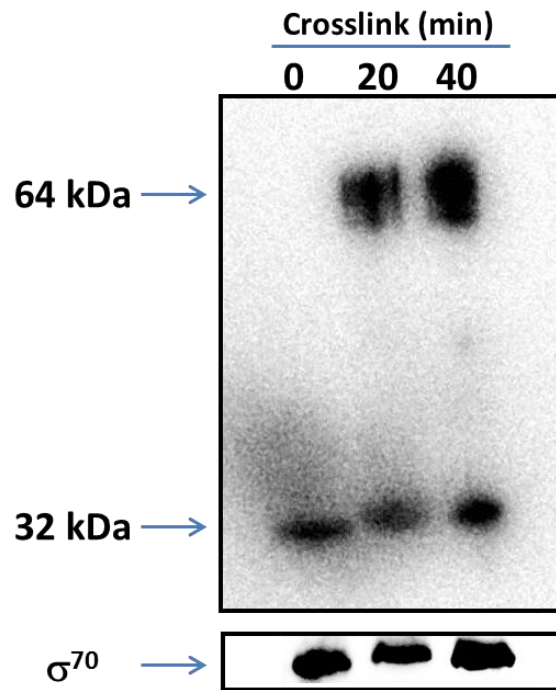


Figure 4.10. *P. aeruginosa* AmpR appears to dimerize *in vivo*. A fresh culture of PA0 Δ *ampR* strain harboring pAmpR-His₆ was treated with 0.1% formaldehyde for 20 and 40 minutes at room temperature to achieve protein cross-linking. Crude extracts containing 10 μ g of total protein were separated on an SDS-polyacrylamide gel and AmpR was visualized using anti-His antibody. The blot was later stripped and reprobed using anti- σ^{70} antibody. Monomeric AmpR is detected in non-cross-linked samples at zero time point, while AmpR dimeric entities (64KDa) are detected 20 and 40 minutes after protein cross-linking.

4.4.7. Localization studies of *P. aeruginosa* AmpR. Although it is generally accepted that AmpR is a cytoplasmic protein, our bioinformatics analyses suggested that AmpR may be membrane associated. More specifically, a Kyte-and-Doolittle hydrophobicity plot (932) and the topology prediction softwares TopPred2 (933), DAS (934), MEMSAT (935), TMpred (936), and SCAMPI (937) suggested the presence of a transmembrane domain somewhere between amino acids 92 and 114 of *P. aeruginosa* AmpR. The crystal structure of *C. freundii* AmpR, however, reveals this segment is near the protein-protein interface of the dimer and thus unlikely to traverse the membrane (930).

In order to localize AmpR, *phoA* and *lacZ* were fused in-frame at amino acid positions Glu15, Val134 and Gln185 (Figure S2A, Supplemental material). Fusions at Glu15 were LacZ-positive and PhoA-negative, whereas fusions at Val134 and Gln185 were PhoA-positive and LacZ-negative suggesting AmpR may traverse the inner membrane with the N and C termini in the cytoplasm and periplasm, respectively (Figure 4.11). Since this data is only qualitative, localization of *P. aeruginosa* AmpR was further investigated with a protease protection assay using a C-terminal His-tagged AmpR that was shown to be functional (Table S2, Supplemental material). Full length AmpR (32 kDa) was detected in whole cell extracts, as well as, in spheroplasts preparations treated with Proteinase K (0-min incubation) that were immediately processed for immunoblotting (Figure S2B, Supplemental material). Incubations with Proteinase K of 5 minutes or longer resulted in the visible reduction of full length AmpR and the appearance of the degradation product (10-kDa fragment). However, slight degradation of σ^{70} was observed. The evidence provided here is suggestive of AmpR being an inner

membrane-associated protein. If confirmed, this would be an important finding and could have major implications regarding the regulation of two *ampD* amidase homologs that have now been localized to the periplasm (938, 939). The identity of the muramyl-peptides that are important for regulating AmpR will further confirm its localization and is the subject of ongoing work in the lab.

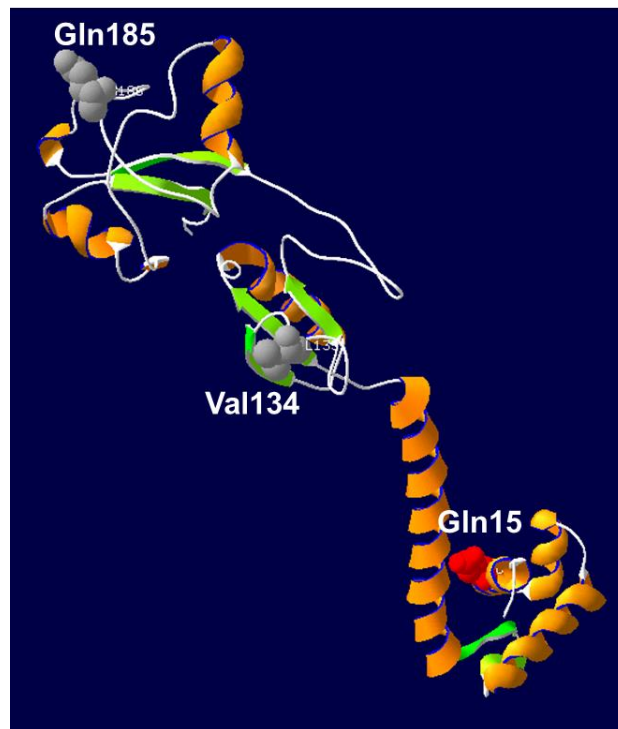


Figure 4.11. AmpR model. A three-dimensional structure of AmpR was generated using RasMol (<http://www.umass.edu/microbio/rasmol/>). The topology of AmpR was investigated by introducing *phoA* and *lacZ* fusions at amino acid positions Glu15, Val134, and Gln185. Fusions at Glu15 were LacZ positive but PhoA negative, whereas fusions at Val134 and Gln185 were PhoA positive and LacZ negative.

Although the majority of purified LTTRs appear to be soluble cytoplasmic proteins, the nodulation factor, NodD, from *Rhizobium* species appears to be a peripheral membrane protein associated with the inner leaflet of the cytoplasmic membrane (940). A

few membrane-bound non-LTTRs have also been reported, such as the *Salmonella enterica* serovar Typhimurium acid-sensing CadC, the streptococcal CpsA involved in regulation of capsule production, and the *Vibrio cholera* toxin activator, ToxR (941-943). Since these proteins act as both signal sensor and response regulator, they form a simple but sophisticated form of transmembrane signaling system.

4.5 Concluding Remarks

The role of AmpR as regulator of *ampC* expression has been clearly established in both the Enterobacteriaceae and *P. aeruginosa*. Our recent work has further redefined AmpR as a major global regulator, playing an important role in acute infections through its regulation of virulence, biofilm formation, quorum sensing and non- β -lactam resistance (710, 841-844). Regulation of *ampC*, however, remains one of its most critical roles, as AmpC derepression is a prevalent mechanism of β -lactam resistance in *P. aeruginosa*.

In the present work we characterize some of the genetic and structural elements necessary for induction of *ampC* and important for the functioning of AmpR as regulator of AmpC β -lactamase expression. The presence of strong σ^{54} consensus sequences in the *ampR* promoter led us investigate its possible involvement in the regulation of *ampR*. However, contrary to what was expected, RpoN was not required for *ampR* expression in the conditions tested. Instead, RpoN was found to downregulate expression of both *ampR* and *ampC*, although the exact mechanism is yet unknown.

Like other LTTRs, AmpR has two important regions critical for its functioning as activator/repressor of *ampC* expression: the HTH motif for binding to DNA and the effector binding domain for ligand interaction. Analysis of polar and charged amino acids on the AmpR HTH revealed two residues, Ser38 and Lys42, important for binding of AmpR to the promoter region and consequently for *ampC* promoter activation. A third residue, His39, was shown to be important for function but not for binding to P_{ampC} . In the effector binding domain, we examined the role of two amino acids, Gly102 and Asp135, previously shown to be important for maintaining AmpR in an inactive conformation in the enterobacteria. In *P. aeruginosa*, Gly102 appears to be responsible for maintaining a stable structural conformation, while Asp135 is responsible for keeping AmpR in an inactive state that represses *ampC* expression. Additionally, our work suggests that AmpR dimerizes and that it is likely to be membrane associated. This is the first comprehensive look at the *P. aeruginosa* AmpR and the promoter elements that it regulates.

4.6 Acknowledgments

This study was supported in part by the National Institutes of Health – Minority Biomedical Research Support SCORE (SC1AI081376; KM), Research Initiative for Scientific Enhancement graduate student fellowship (NIH/NIGMS R25 GM61347; DZ), NIH/NIAID R37 AI021451 (SL), National Science Foundation IIP-1237818 [PFI-AIR: CREST-I/UCRC-Industry Ecosystem to Pipeline Research] (KM), and Florida International University Dissertation Year Fellowship (DB). The content is solely the

responsibility of the authors and does not necessarily represent the official views of the funding agencies.

We thank Dr. D. Haas from UNIL, Switzerland for kindly providing PA0 Δ *rpoN* and PA0 Δ *rpoN* (pRpoN), Dr. G. Plano from University of Miami, Miami, FL for sharing reagents for Western blotting and Nikolay Atanasov Nachev from Florida International University, Miami, FL for bioinformatics analysis of *P. aeruginosa* AmpR.

Chapter 5

General Discussion and Summary

5.1 Overview

The Infectious Diseases Society of America classified *Pseudomonas aeruginosa* as one of the troublesome ESKAPE¹ pathogens that, along with *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Enterobacter* spp., causes the majority of nosocomial infections and effectively evades the action of antibiotics (944, 945). The intractability of *P. aeruginosa* is owned to its intrinsic low membrane permeability, the presence of multidrug efflux pumps that can export more than one type of antimicrobial, and the expression of β -lactamases, enzymes which can hydrolyze and inactivate the commonly used β -lactam antibiotics (246, 270, 317, 549, 946). In addition, *P. aeruginosa* can evolve resistance after treatment with antibiotics through mutations in resistance genetic determinants or their regulator-encoding genes (247, 313, 336, 749, 947-949).

P. aeruginosa also has an innate ability to acquire resistance genes from the environment and is often a natural reservoir of multiple plasmid-encoded β -lactamases (549, 950). Chromosomally, *P. aeruginosa* carries AmpC, a class C β -lactamase that plays a major role in intrinsic resistance against penicillin- and cephalosporin-type β -lactams (711). Mutational derepression of this enzyme, whereby AmpC is produced at high levels irrespective of the presence or absence of β -lactams, is currently one of the most common mechanisms of β -lactam resistance in the clinical setting (379, 380, 722, 749).

¹ ESKAPE is an acronym for the six pathogens, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp., which are currently a major problem in the hospital setting.

This dissertation delved into the role of a second chromosomally encoded β -lactamase in *P. aeruginosa* termed PoxB. This work established PoxB is an oxacillinase-type β -lactamase with a narrow spectrum of hydrolysis that includes the carbapenems imipenem, meropenem and doripenem. The currently available β -lactamase inhibitors did not significantly affect PoxB activity as commonly seen for class D enzymes. In addition, PoxB was shown to form a two-gene operon with the upstream open reading frame *poxA*, whose expression exerted negative control on the *pox* promoter. The transcriptional regulator AmpR negatively impacted *pox* expression but this regulation did not occur by binding to the promoter. A mutant transposon library, constructed to screen for regulators of *pox* expression, identified two clones with insertions in genes involved in polyamine transport. Polyamines coincidentally were shown to provide resistance against imipenem and meropenem. In summary, two chromosomal-encoded β -lactamases with complementing hydrolytic spectrums have the potential to provide *P. aeruginosa* with resistance against most classes of β -lactams including the penicillins, cephalosporins and carbapenems.

5.2 PoxB is a chromosomal-encoded class D carbapenemase. Class D β -lactamases are predominantly found in *P. aeruginosa* and in *A. baumannii*, but have also been reported in *Escherichia coli*, *K. pneumoniae*, and *Proteus mirabilis*, among others (606, 685). Three distinct oxacillinase categories are now recognized: the narrow-spectrum, the extended-spectrum and the carbapenem-hydrolyzing oxacillinases (680). Carbapenem-hydrolyzing class D β -lactamases (CHDLs), are widespread in the nosocomial pathogen

A. baumannii but have also been occasionally reported in *K. pneumoniae* and *Acinetobacter* spp., as well as and in a few of the Enterobacteriaceae (680, 685). CHDLs are less common in *P. aeruginosa* and to date only three CHDLs have been reported there: OXA-40, OXA-198 and, of course, OXA-50 or PoxB (549, 740, 741, 951, 952).

Specifically, a plasmidic carbapenemase was detected in two unrelated imipenem-resistant *P. aeruginosa* isolates from northern Spain where OXA-40-producing *A. baumannii* were found to be common (951, 953, 954). The nucleotide sequence in fact showed 100% identity to the previously described *bla*_{OXA-40} from an *A. baumannii* isolate endemic to this area (951). Thus, one of the first OXA-type carbapenemases detected in *P. aeruginosa* was termed OXA-40 (954). Similarly, OXA-198 was identified in the imipenem-resistant clinical isolate PA41437 (952). OXA-198 was encoded in a plasmid-borne integron but was not closely related to other OXA enzymes. Biochemically, OXA-198 behaved similarly to other CHDLs, exhibiting a strong affinity for carbapenems but low rate of hydrolysis (952). Its expression in a heterologous host provided resistance against ticarcillin and decreased susceptibility against piperacillin, cefepime, and the carbapenems and but not against aztreonam and extended-spectrum cephalosporins (952). Although the clinical strain PA41437 was resistant to imipenem and displayed a high minimum inhibitory concentration (MIC) against meropenem (12 µg/ml), OXA-198 expression *in trans* only decreased susceptibility against these drugs, suggesting other mechanisms of resistance were operating in the PA41437. Sequencing of *oprD* revealed a number of mutations and an 88-bp deletion in this strain (952).

To our knowledge PoxB (OXA-50), is the first carbapenem-hydrolyzing class D β -lactamase reported in *P. aeruginosa* (740, 741). Similar to OXA-198, OXA-50 is only weakly related to other class D enzymes, displaying 43-47% homology to CHDLs from the OXA-23 and OXA-229 group found in *A. baumannii* and *Acinetobacter* spp. Unlike the other two OXA enzymes reported in *P. aeruginosa*, OXA-50 is carried on the chromosome and ubiquitously found in environmental and clinical *P. aeruginosa* isolates (740). The goal of this dissertation was to characterize the naturally occurring class D β -lactamase PoxB and determine its contribution to intrinsic resistance in *P. aeruginosa*.

Since the class C, chromosomal encoded AmpC β -lactamase is known to play a major a role in both the intrinsic and acquired resistance against β -lactams in *P. aeruginosa*, the role of PoxB was investigated in strains carrying clean deletions of the β -lactamase-encoding genes (PA0 Δ *ampC*, PA0 Δ *poxB* and PA0 Δ *ampC* Δ *poxB*). This work clearly showed AmpC confers protection against penicillins and cephalosporins. Specifically, an *ampC* deletion rendered strains susceptible to the aminopenicillins (ampicillin and amoxicillin) but produced only a small decrease in MIC for imipenem and doripenem (Chapter 2, Table 2.2). Overexpression of AmpC in PA0 Δ *ampC* restored resistance against the aminopenicillins and additionally provided resistance or significantly increased MICs (decreased susceptibility) against the cephalosporins and the rest of the penicillins (Chapter 2, Table 2.3). A slight decrease in susceptibility was also observed against doripenem but not against imipenem or meropenem suggesting the carbapenems are not good substrates of the AmpC β -lactamase.

The role of PoxB, on the other hand, was not readily observed in the natural background. For instance, a *poxB* deletion failed to alter susceptibility in the presence or absence of *ampC* (Chapter 2, Table 2.2). Similarly, the amount β -lactamase activity produced by a *poxB* deletion strain (PA0 Δ *poxB*) was comparable to that of wild-type PA01 (Chapter 2, Figure 2.3). PA0 Δ *ampC*, on the other hand, failed to produce any detectable β -lactamase activity in the presence or absence of chromosomal *poxB*. This suggests that the activity detected in wild-type and *poxB* deletion strains was due to the presence of AmpC. PoxB thus appears to be expressed at very low, marginal levels in the lab strain PA01 under the tested experimental conditions.

Expression of *poxB* from an IPTG-inducible promoter in an *ampC*-deficient strain (PA0 Δ *ampC*) or in an *ampCpoxB* deletion strain (PA0 Δ *ampC* Δ *poxB*), however, increased the MIC, thereby decreasing susceptibility, against the carbapenems doripenem and meropenem, but produced little to no change against the penicillins and cephalosporins (Chapter 2, Table 2.3). It is well-documented that clinical strains can develop resistance to imipenem after treatment with said carbapenem (304, 313, 314, 955). Mutations, deletions and insertions usually occur in *oprD*, encoding an outer membrane porin specific for the uptake of imipenem, and result in impaired/lost function of the porin (313, 315, 947, 955). Similarly, in our studies loss of the outer membrane porin, OprD, specific for the uptake of imipenem, extended the hydrolysis spectrum of PoxB to include imipenem and enhanced the carbapenem-hydrolyzing phenotype leading to clinical intermediate and resistant phenotypes (Chapter 2, Table 2.4). Thus, PoxB is a carbapenem-hydrolyzing class D β -lactamase.

In agreement with our studies, previous work by Girlich *et. al* examining the biochemical properties of PoxB, failed to detect hydrolysis of oxacillin, cloxacillin, cefepime, cefotaxime, ceftazidime and aztreonam, among others (741). Hydrolysis of various other penicillins and cephalosporins, however, was detected, as well as of nitrocefin, meropenem and imipenem. PoxB, however, displayed low affinity, high K_m , for most β -lactams tested, with the exception of imipenem and nitrocefin for which K_m values of 20 and 200 μ M were obtained, respectively (741). Hydrolysis of both imipenem and meropenem, nonetheless, was very low, with meropenem exhibiting even weaker hydrolysis than that of imipenem. PoxB expression did afford the cell decreased susceptibility against meropenem in the previous and present study (741). In the current work, PoxB expression additionally increased MIC against doripenem, and in the absence of OprD against imipenem. Protection against imipenem was not previously detected in the work of Girlich *et. al* despite PoxB exhibiting high affinity for this β -lactam, as experiments were conducted in *oprD*⁺ strains.

The current study further tested the effect of class A β -lactamase inhibitors, tazobactam, sulbactam and clavulanic acid, on the activity of PoxB (Chapter 2, Figures 2.7 and 2.8). These compounds did not significantly affect PoxB activity and thus were considered poor inhibitors and likely also poor substrates of the enzyme. That OXA-50 is resistant to inhibition is not surprising as in general, class D enzymes are not significantly inhibited by clavulanic acid, sulbactam, and tazobactam (428, 467). Exceptions include OXA-53 (956), OXA-18 (957) and OXA-45 (958) all inhibited by clavulanic acid, OXA-

12 (735) inhibited by both clavulanic acid and tazobactam, and OXA-2 and its point mutant OXA-32 that are susceptible to inhibition by tazobactam (959).

Avibactam, a new non- β -lactam-type inhibitor, was also a poor inhibitor of PoxB (Chapter 2, Table 2.6). Avibactam has been shown to successfully inhibit class A and C enzymes, but has generally lacked activity against *A. baumannii* isolates expressing OXA-type β -lactamases (804, 805). Significant activity has been observed against the CHDL OXA-48, but a kinetics study showed that the rate of inhibition was lower than that for class A and C enzymes (805, 816). Thus inhibition of class D enzymes still remains a problem in the clinical setting where the commercially available inhibitors are not generally active against OXA enzymes. In this respect, OXA-50 is a typical class D β -lactamase. *In vitro* inhibition by NaCl, often used as a defining characteristic of most oxacillinases, was also observed for PoxB. This inhibition, although readily detected in crude extracts from PoxB-expressing cells, was only slightly noticeable by the broth microdilution method (Chapter 2, Figures 2.9 and 2.11).

In summary, although exhibiting relatively low homology to other class D enzymes, in most respects, PoxB is a typical class D carbapenemase. In general, CHDLs do not significantly hydrolyze extended-spectrum cephalosporins but do exhibit some level of hydrolysis against the penicillins, mainly benzylpenicillin, ampicillin, piperacillin and ticarcillin (680, 693, 960). Additionally, CHDLs display low K_m values for imipenem, lower than for other β -lactams, suggesting very high affinity for this substrate (680, 685, 960). Hydrolysis rates for both imipenem and meropenem, however, remain very low.

In its natural host and under the conditions tested, *poxB* seems to be expressed at very low levels which do not contribute to the intrinsic resistance of PA01. This raises the question of the natural role of this operon in *P. aeruginosa*. Does the *poxAB* operon play a yet unidentified role besides that of resistance? Further, we know that increasing the expression of PoxB can lead to decreased susceptibility or resistance against the carbapenems, but the relevance of OXA-50 in the clinical setting is not yet known.

Although *poxB* expression appears to be constitutive and unregulated, it is possible that mutations in the promoter region or insertion elements could lead to its expression in a way that contributes to resistance as seen in other bacteria. For example, *A. baumannii* often carries the chromosomal *ampC* but lacking *ampR*, expression is normally non-inducible (961, 962). Ceftazidime resistance in this pathogen however, has been linked to increased expression of *ampC* due to the presence of the upstream insertion element *ISAbal* with strong promoter sequences (963, 964). *ISAbal* was absent from ceftazidime-susceptible strains (724, 965). This insertion sequence seems to be prevalent in carbapenem-resistant *A. baumannii* isolates where it has been reported in association with *bla*_{OXA-51-like} and *bla*_{OXA-23-like} genes (700, 725, 727). Although *ISAbal* has not been reported in *P. aeruginosa*, promoter sequences in *ISPa12* have been found to drive expression of the extended-spectrum β -lactamase-encoding gene *bla*_{PER-1} in *P. aeruginosa*, *Providencia stuartii*, *A. baumannii* and *Salmonella enterica* serovar Typhimurium (728). Similarly, expression of the extended-spectrum β -lactamase-encoding gene, *bla*_{VEB-1}, in ceftazidime-resistant *P. aeruginosa* isolates was found to be driven by promoter sequences within the upstream-located *IS1999* (966). Thus,

upregulation of gene expression by insertion sequences is a plausible mechanism of PoxB overexpression.

As a sole mechanism of resistance, *poxB* expression may be easy to overlook as the changes in MIC against carbapenems may not be substantial. Moreover, carbapenem resistance is often linked to mutations or insertions in *oprD*, and screening of the *poxB* ORF and its promoter are not routinely done. This work showed that, when combined, the concerted expression of *poxB* and the loss of *oprD* can lead to resistance and/or significant MIC changes. Thus, although constitutively expressed in PA01, the role of PoxB in *P. aeruginosa* in the clinical setting may currently be overlooked.

5.3 Regulation of *poxB*. After establishing the role of PoxB as a carbapenem-hydrolyzing class D β -lactamase, its regulation was investigated (Chapter 3). As previous work pointed to the involvement of the transcriptional regulator AmpR (710), expression of *poxB* was examined by RT-PCR in the presence and absence of *ampR* (Figure 3.3). A small but significant increase in expression was observed in the absence of *ampR* and in the presence of β -lactams suggesting AmpR normally acts to downregulate *poxB* expression. Similarly, promoter studies using reporter strains carrying P_{pox} -*lacZ* fusions were in agreement with the mRNA data (Figure 3.8). Specifically, induction of *poxB* could be achieved only in the absence of *ampR* and in the presence of β -lactams (Chapter 3). Interestingly these changes could not be observed at the protein level in terms of β -lactamase activity.

AmpR is an interesting candidate for regulator of *pox* expression. As a LysR-type transcriptional regulator, AmpR carries an N-terminal HTH motif to interact with DNA and an effector binding domain in the C-terminus for ligand interaction (852, 856, 897). As detailed in Chapter 4 of this dissertation, AmpR activates transcription of *ampC* in the presence of β -lactams but maintains expression at low basal levels in the absence of such compounds, thus acting as both a negative and positive regulator of *ampC* expression (710, 711, 776). AmpR has additionally been shown to have an extensive regulon that includes a number of characterized and putative transcriptional and sigma factors, as well as genes involved in quorum sensing and virulence (710, 841, 843). That the regulon of AmpR includes transcriptional regulatory proteins suggests AmpR-dependent expression may occur by indirect regulation. Our EMSA studies in fact, failed to show binding of AmpR to the *pox* promoter (Figure 3.5), suggesting regulation by AmpR is indirect.

In an effort to identify possible *pox* regulators, a genome-wide approach was undertaken using the reporter strains PA0::P_{*pox*}-*lacZ* and PA0 Δ *ampR*::P_{*pox*}-*lacZ* (Chapter 3, Section 3.4.5). The strains were mutagenized with a random mariner transposon (Tn) in order to identify clones with increased or decreased *lacZ* expression indicative of altered *pox* promoter expression. This study generated a wealth of clones that are still being screened and evaluated. Two noteworthy clones however, identified based on their increased *lacZ* expression, had insertions in *spuE* (PA0301) and PA0322, genes involved in a characterized and a putative polyamine uptake system, respectively.

Polyamines (putrescine, cadaverine, spermidine, spermine) are essential molecules found in all domains of life (845, 847). Bacteria can synthesize, catabolize and

uptake these molecules for a number of diverse processes as described in Chapter 3 (845, 847, 878). Interestingly, polyamines have been found to sensitize a number of bacterial species to multiple β -lactams including penicillins, cephalosporins and monobactams (886, 893). This sensitization did not appear to occur by an increase in outer membrane permeability as expected of polyamines given their cationic nature. On the other hand, polyamines provided resistance against the β -lactam imipenem in *P. aeruginosa* seemingly through blockage of OprD, the major route of entry for imipenem into the cell (893).

In order to determine if the increased *lacZ* expression in mutants containing Tn insertions in *spuE* and *PA0322*, was due to an increase in *pox* expression, the susceptibility of the mutants to carbapenems was investigated in the presence and absence of polyamines (Chapter 3, Table 3.7). Our studies show that polyamines significantly increased MICs, or decreased susceptibility, against ceftazidime, piperacillin and the carbapenems, but not against aztreonam, in both unmutagenized and Tn-mutagenized strains. This increased in MIC is likely the result of blockage of OprD as well as other porins, as ceftazidime and piperacillin are not known to use the OprD porin for passage into the cell. If resistance is occurring by blocking of porins, then the polyamines do not necessarily need to come into the cell to exert their action. This would explain why polyamines could raise MICs in what appear to be a strain-independent manner. The resistance provided was independent of polyamine uptake pathways and as such the *spuE* and *PA0322* Tn insertions did not affect susceptibility. That the polyamine spermidine (10 mM) could induce resistance against both imipenem and meropenem

(>32 µg/ml) likely suggests that polyamines more efficiently block OprD than other porins and that meropenem and imipenem preferentially enter the cell via this route.

Alternatively, although the function of these genes may be impaired, since there are other polyamine transport systems, both putative and characterized, the disruptions imposed by the Tn insertions could potentially be overcome by other such systems. Thus, polyamines could still enter the cell to exert their action, e.g., modulation of gene expression. For instance, SpuE is a periplasmic binding protein that specifically binds spermidine, but the *spuABCDEFGH* gene cluster that encodes it also encodes SpuD, another periplasmic binding protein that although preferentially binds to putrescine, can also bind and transport spermidine (864, 883). Thus, this system is also likely involved in the uptake and transport of both spermidine and putrescine. The presence of *spuABCDEFGH* probably also compensates for the loss of *PA0322* a putative, not yet proven putrescine transporter. Other putative polyamine transport and utilization systems are also encoded in the general vicinity of the *spu* gene cluster. The redundancy of such system may illustrate how essential these molecules are for survival.

It is worth noting that the Tn insertion into *spuE* likely also led to disruption of transcription for the rest of the operon, *spuFGH*, encoding the ATPase for transport and the two permeases, respectively. Since the Tn contained an outwardly-directed promoter and the insertion occurred with the promoter in the direction of transcription of these genes, it is likely that transcription of these downstream ORFs did occur. As experiments revealed in Chapter 3, the outward-facing promoter of this mariner transposon was very strong.

An alternative explanation for the polyamine-dependent MIC increase could be the induction of genes that themselves confer resistance, such as those involved in efflux. Although polyamines have not been shown to induce expression of efflux pumps, spermidine did provide resistance against cationic peptides and quinolones by inducing expression of *oprH-phoPQ* and the LPS modification operon (885). It is likely we don't yet know all the current targets of polyamines.

The β -lactamase profiles of *spuE* (PA0301) and PA0322 Tn mutants were not altered as compared to wild-type as would be expected if such insertions affected *pox* expression. Although the increased *lacZ* expression phenotype displayed by these mutants appears to be unconnected to P_{pox} expression, that we have stumbled upon yet another condition that contributes to carbapenem resistance is very interesting. To conclusively prove *pox* involvement, the transcript levels would have to be quantified in *spuE* and PA0322 mutants.

5.4 Future directions. Previous studies showed *poxB* is ubiquitous in *P. aeruginosa* clinical and environmental isolates (741), yet its expression and contribution to β -lactam resistance has not been analyzed in such backgrounds. Traditionally, carbapenem resistance in *P. aeruginosa* has been provided by the loss of OprD and the expression of class B β -lactamases, especially the VIM and IMP-types (549, 947, 955, 967-969). OXA-50, however, is not routinely screened for and thus its true contribution in the clinical setting is unknown.

Future studies should elucidate on the role of PoxB in clinical isolates, especially for those that are shown to be carbapenem non-susceptible (intermediate and resistant phenotypes). Using PCR-based methods, the gold standard for identifying β -lactamases, the presence of metallo- β -lactamases and OXA-type enzymes, including OXA-50, could be detected. Since the mere presence of PoxB does not imply its expression, its mRNA levels should be quantified. Alternatively, the actual protein could be quantified by doing Western blot. Sequencing of the *oprD* ORF should also be done in carbapenem non-susceptible isolates to determine contribution of this porin to resistance.

Another area of this research to expand could involve identification of other potential roles for PoxB and the *pox* operon besides that of resistance. Phenotypic microarrays (Biolog, Hayward, CA) could help identify other such functions. Such arrays typically measure the rate of respiration over a period of time under a number of environmental conditions including ranges of salinity, pH, nitrogen, carbon, phosphorus and sulfur sources, as well as many antimicrobial drugs and chemical compounds. In this respect two strains will be critical: *poxB* and *poxAB* deletion mutants.

A phenotypic microarray has been carried out in PA0 Δ *poxA* and pointed to a growth defect in the presence of NaNO₃, leading us to investigate morphology in anoxic and oxic environments. While the NaNO₃ phenotype could not be reproduced, we did observed PoxA-expressing strains were able to bind the dye Congo red (CR) better presumably because of increased production of an extracellular polymeric substance. The CR-binding phenotype was not reproducible or readily observed in liquid media as is often seen with strains that overproduce Pel and Psl polysaccharides. Thus, it is likely

that the CR-binding phenotype is due to production of a polysaccharide or a CR-binding substance other than Pel or Psl. In order to rule out the involvement of Pel or Psl polysaccharides, expression of some of the genes of *pel* and *psl* operons could be quantified by RT-PCR. Additionally, the carbohydrate composition of the CR-binding substance being produced in PoxA-expressing cells could be investigated by gas chromatography/mass spectrometry as previously described (768, 825).

Although screening and confirmation of the Tn mutant library was well underway, more than 50% of the library still remains to be screened and/or evaluated. Thus, a continuing project could involve further screening with the clones that still remain to be investigated. Although the relationship between *pox* expression and polyamine transport identified by the transposon screening is not yet clear, polyamine-induced resistance and susceptibility in *P. aeruginosa* is by itself an interesting and worthy area of study. A microarray could elucidate on the targets of these essential molecules and the link to carbapenem resistance.

Carbapenem resistance has become an increasing problem in the nosocomial pathogen *A. baumannii* with the predominant mechanism of resistance being the expression of OXA enzymes (622, 692, 708, 709, 726, 820). Carbapenems are often the drug of choice for treating organisms expressing extended-spectrum β -lactamases (ESBLs) which can hydrolyze penicillins and cephalosporins. There is a real concern that treatment with such compounds can lead to ESBLs that additionally can hydrolyze carbapenems. Thus, there is a need to develop effective class D β -lactamase inhibitors. Although not yet prevalent in *P. aeruginosa*, CHDLs have the potential to become

widespread, as they have in the closely related Pseudomonadales species *A. baumannii*, given its genomic plasticity and ability to acquire resistance genes from the environment. *P. aeruginosa* already carries PoxB, a chromosomal carbapenem-hydrolyzing β -lactamase, ubiquitous in clinical and environmental isolates, and with the potential to become yet another tool in the arsenal of this intractable pathogen against the β -lactam antibiotics.

SUPPLEMENTAL MATERIAL

Table S1: Bacterial strains, plasmids, and primers used in this study

Strains/ Plasmids	Relevant phenotype and genotype	Source
<i>Escherichia coli</i>		
<i>E. coli</i> DH5 α	F ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (<i>r_k⁻ m_k⁺</i>) <i>phoA supE44 λ⁻ thi-1 gyrA96 relA1</i>	New England Biolabs
<i>Pseudomonas aeruginosa</i>		
PA01	Wild-type	(762)
PA06358	PA0 Δ <i>rpoN</i> , PA01 containing in-frame deletion of a 0.9-kb fragment from the <i>rpoN</i> gene (PA4462)	(970)
PKM106	PA0 <i>attB</i> ::mini-CTX- <i>lacZ</i> ; Tc ^R	This study
PKM107	PA0 <i>attB</i> ::P _{ampC352} - <i>lacZ</i> ; Tc ^R	This study
PKM108	PA0 <i>attB</i> ::P _{ampC193} - <i>lacZ</i> ; Tc ^R	This study
PKM109	PA0 <i>attB</i> ::P _{ampC171} - <i>lacZ</i> ; Tc ^R	This study
PKM110	PA0 <i>attB</i> ::P _{ampC151} - <i>lacZ</i> ; Tc ^R	This study
PKM111	PA0 <i>attB</i> ::P _{ampC-130} - <i>lacZ</i> ; Tc ^R	This study
PKM112	PA0 <i>attB</i> ::P _{ampC-111} - <i>lacZ</i> ; Tc ^R	This study
PKM113	PA0 <i>attB</i> ::P _{ampC90} - <i>lacZ</i> ; Tc ^R	This study
PKM114	PA0 <i>attB</i> ::P _{ampC70} - <i>lacZ</i> ; Tc ^R	This study
PKM115	PA0 <i>attB</i> ::P _{ampC51} - <i>lacZ</i> ; Tc ^R	This study
PKM116	PA0 <i>attB</i> ::P _{ampR193} - <i>lacZ</i> ; Tc ^R	This study
PKM201	PA0 Δ <i>ampC</i> , PA01 containing in-frame deletion of <i>ampC</i>	(760)
PKM315	PA0 Δ <i>ampR</i> , PA01 containing in-frame deletion of <i>ampR</i>	(843)
PKM318	PA0 <i>ampR</i> _{VSV-G} ; VSV-G tag added in-frame to AmpR	This study
Plasmids		
pBSK(+)	Ap ^R ; ColE1 <i>ori lacZ</i> α	Stratagene
pEXG2	Gm ^R ; pMB1 <i>ori sacB</i>	(759)
pP30 Δ FRT-MvaT-V	Gm ^R ; ColE1 <i>ori</i> RP4 (<i>mob</i>) <i>aacC1</i> , integration-proficient vector containing VSV-G epitope tag	(908)
pSJ01	Ap ^R , pGEMEX-1 with a 1220-bp <i>EcoRI-BamHI</i> -flanked fragment containing <i>ampR</i>	(710)
pTrc <i>phoA</i>	Ap ^R , low-copy <i>trc</i> promoter expression vector carrying <i>lacI</i> ^q and <i>phoA</i>	(911)
pTrc <i>lacZ</i>	Ap ^R , low-copy <i>trc</i> promoter expression vector carrying <i>lacI</i> ^q and <i>lacZ</i>	(911)
Mini-CTX- <i>lacZ</i>	Tc ^R ; Integration-proficient vector for single-copy chromosomal <i>lacZ</i> fusion at the <i>attB</i> site	(853)
pMMB67EH-Gm	Gm ^R ; IncQ, RSF1010, <i>lacI</i> ^q P _{tac} expression vector with <i>ampR</i> :: <i>aacC1</i> insertion at <i>DraI</i>	(763)

pP30ΔFRT-AmpR-V	pP30ΔFRT-MvaT-V digested with <i>Hind</i> III and <i>Not</i> I to release <i>mvaT</i> and carrying 540-bp 3' fragment of <i>ampR</i> fused in-frame with VSV-G tag	This study
pOC1	Tc ^R ; P _{ampC352} fused to the <i>lacZ</i> of mini-CTX- <i>lacZ</i>	This study
pOC2	Tc ^R ; P _{ampC193} fused to the <i>lacZ</i> of mini-CTX- <i>lacZ</i>	This study
pOC3	Tc ^R ; P _{ampC171} fused to the <i>lacZ</i> of mini-CTX- <i>lacZ</i>	This study
pOC4	Tc ^R ; P _{ampC151} fused to the <i>lacZ</i> of mini-CTX- <i>lacZ</i>	This study
pOC5	Tc ^R ; P _{ampC130} fused to the <i>lacZ</i> of mini-CTX- <i>lacZ</i>	This study
pOC6	Tc ^R ; P _{ampC111} fused to the <i>lacZ</i> of mini-CTX- <i>lacZ</i>	This study
pOC7	Tc ^R ; P _{ampC90} fused to the <i>lacZ</i> of mini-CTX- <i>lacZ</i>	This study
pOC8	Tc ^R ; P _{ampC70} fused to the <i>lacZ</i> of mini-CTX- <i>lacZ</i>	This study
pOC9	Tc ^R ; P _{ampC51} fused to the <i>lacZ</i> of mini-CTX- <i>lacZ</i>	This study
pOC10	Tc ^R ; P _{ampR193} fused to the <i>lacZ</i> of mini-CTX- <i>lacZ</i>	This study
pOC11	Ap ^R ; pBSK-AmpR-His ₆ (933-bp <i>Eco</i> RI- <i>Bam</i> HI fragment containing AmpR His-tagged at C-terminus)	This study
pOC12	Ap ^R ; pOC11-AmpR _{Ser38Ala}	This study
pOC13	Ap ^R ; pOC11-AmpR _{His39Ala}	This study
pOC14	Ap ^R ; pOC11-AmpR _{Lys42Ala}	This study
pOC15	Ap ^R ; pOC11-AmpR _{Ser43Ala}	This study
pOC16	Ap ^R ; pOC11-AmpR _{Glu46Ala}	This study
pOC17	Ap ^R ; pOC11-AmpR _{Gly102Glu}	This study
pOC18	Ap ^R ; pOC11-AmpR _{Asp135Asn}	This study
pOC19	Gm ^R ; pMMB67EHGM containing 933-bp <i>Eco</i> RI- <i>Bam</i> HI <i>ampR</i> -His ₆ fragment from pOC11	pAmpR-His ₆ , This study
pOC20	Gm ^R ; pMMB67EHGM with <i>Eco</i> RI- <i>Bam</i> HI fragment containing AmpR-His ₆ -Ser38Ala from pOC12	This study
pOC21	Gm ^R ; pMMB67EHGM with <i>Eco</i> RI- <i>Bam</i> HI fragment containing AmpR-His ₆ -His39Ala from pOC13	This study
pOC22	Gm ^R ; pMMB67EHGM with <i>Eco</i> RI- <i>Bam</i> HI fragment containing AmpR-His ₆ -Lys42Ala from pOC14	This study
pOC23	Gm ^R ; pMMB67EHGM with <i>Eco</i> RI- <i>Bam</i> HI fragment containing AmpR-His ₆ -Ser43Ala from pOC15	This study
pOC24	Gm ^R ; pMMB67EHGM with <i>Eco</i> RI- <i>Bam</i> HI fragment containing AmpR-His ₆ -Glu46Ala from pOC16	This study
pOC25	Gm ^R ; pMMB67EHGM with <i>Eco</i> RI- <i>Bam</i> HI fragment containing AmpR-His ₆ -Gly102Glu from pOC17	This study
pOC26	Gm ^R ; pMMB67EHGM with <i>Eco</i> RI- <i>Bam</i> HI fragment containing AmpR-His ₆ -Asp135Asn from pOC18	This study
pKK862	Ap ^R , pTrclacZ derivative with a 1220-bp fragment containing <i>ampR</i> and the <i>lacZ</i> gene at <i>Hind</i> III site	This study
pKK863	Ap ^R , pTrcphoA derivative with a 1220-bp fragment containing <i>ampR</i> and the <i>phoA</i> gene at <i>Hind</i> III site	This study
pKK864	Ap ^R , pTrclacZ derivative with a 1220-bp fragment containing <i>ampR</i> and the <i>lacZ</i> gene at <i>Hinc</i> II site	This study

pKK865	Ap ^R , pTrc <i>phoA</i> derivative with a 1220-bp fragment containing <i>ampR</i> and the <i>phoA</i> gene at <i>HincII</i> site	This study
pKK866	Ap ^R , pTrc <i>lacZ</i> derivative with a 1220-bp fragment containing <i>ampR</i> and the <i>lacZ</i> gene at <i>PstI</i> site	This study
pKK867	Ap ^R , pTrc <i>phoA</i> derivative with a 1220-bp fragment containing <i>ampR</i> and the <i>phoA</i> gene at <i>PstI</i> site	This study
Primers		
DZampCUF1	5'-GGAATTCAAGACGATGCTCCGGGTCAGTG-3'	(760)
DZampCUR1	5'-GATACCAGATTCCCCTGCCTGTCTAGCTAGCTAGAATGCTC-3'	(760)
DZampCDF2	5'-CTAGCTAGCTAGAATGCTCAAGCGCGCTCGCGAGGCGGA CGGA-3'	(760)
DZampCDR2	5'-CGGGATCCGACCCTGCATACCATCAAGG-3'	(760)
KMampRUF1	5'-GGAATTCAGCCTGGAAGTTCGCAATCTCGACGAGA-3'	(843)
KMampRUR1	5'-GAGCATTCTAGCTAGCTAGGTTGCCACCGAAGTGAGTACC-3'	(843)
KMampRDF2	5'-CTAGCTAGCTAGAATGCTCAATGGGGTCGAACCAATCTCT GCTCC-3'	(843)
KMampRDR2	5' CGGGATCCGATGCTCGGGTTGGAATAGA-3'	(843)
OCampR-His-For	5'-ACAGAATTCGAAGGAGATATACCTTGGTTCGACCCCATTT GCC-3'	This study
OCampR-His-Rev	5'-GAGGATCCTTAGTGATGATGATGATGTCTCCC CCGCGCCTCAACGGCAGCC-3'	This study
SBJ03 <i>ampCR</i> For	5'-GGAATTCTGAGGCCGCGCGGCAGACGCTTGAACA-3'	This study
OCP _{ampC} For193	5'-CGGGAATTCGCGGCAAATGGGGTCGAACC-3'	This study
OCP _{ampC} For173	5'-CGGGAATTTCTCTCTGCTCCAAATTTTTTCT-3'	This study
OCP _{ampC} For151	5'-CGGGAATTCATGGCTGCCGCGAGTATTCG-3'	This study
OCP _{ampC} For131	5'-CGGGAATTTCTGTTTGCCGCAAATCCTGC-3'	This study
OCP _{ampC} For111	5'-CGGGAATTCGCAAGCCTAGATTTTCCCCG-3'	This study
OCP _{ampC} For91	5'-CGGGAATTTCCCGCCGATCAAGGAGCGCT-3'	This study
OCP _{ampC} For71	5'-CGGGAATTTCCCGGGGCGGTTTCTCATGC-3'	This study
OCP _{ampC} For51	5'-CGGGAATTCAGCCAACGACAAAGGACGC-3'	This study
OCP _{ampC} RevBc	5'-CGGGGATCCTCAGGGGAATCTGGTATCGCG-3'C	This study
OCP _{ampR} Rev193	5'-CGGGGATCCGCGGCAAATGGGGTCGAACC-3'	This study
OCP _{ampR} ForEc	5'-CGGGAATTCAGGGGAATCTGGTATCGCGC-3'	This study
<i>alg44</i> For	5'-CGAAGCCCAGCGCCAGTTCG-3'	This study
<i>alg44</i> Rev	5'-GGTCCACCGAGCGCACCTGG-3'	This study
AmpRSer38AlaFor	5'-ACCCAGGCGGCGGTGCCCCACCAGGTGAAGAGC-3'	This study
AmpRSer38AlaRev	5'-GCTCTTCACCTGGTGGGCGACCGCCGCTGGGT-3'	This study
AmpRHis39AlaFor	5'-CAGGCGGCGGTGAGCGCCAGGTGAAGAGCCTC-3'	This study
AmpRHis39AlaRev	5'-GAGGCTCTTCACCTGGGCGCTGACCGCCGCTG-3'	This study
AmpRLys42AlaFor	5'-GTCAGCCACCAGGTGGCGAGCCTCGAGGAGCGT-3'	This study

AmpRLys42AlaRev	5'-ACGCTCCTCGAGGCTCGCCACCTGGTGGCTGAC-3'	This study
AmpRSer43AlaFor	5'-AGCCACCAGGTGAAGGCCCTCGAGGAGCGTCTC-3'	This study
AmpRSer43AlaRev	5'-GAGACGCTCCTCGAGGGCCTTCACCTGGTGGCT-3'	This study
AmpRGlu46AlaFor	5'-GTGAAGAGCCTCGAGGCGCGTCTCGGCGTGGCC-3'	This study
AmpRGlu46AlaRev	5'-GGCCACGCCGAGACGCGCCTCGAGGCTCTTCAC-3'	This study
AmpRGly102GluFor	5'- CACCGTCGGCGCGGTGCGAAACCTTCACGGTCGGTT-3'	This study
AmpRGly102GluRev	5'- AACCGACCGTGAAGGTTTCGACCGCGCCGACGGTG-3'	This study
AmpRAsp135Asn For	5'-CCCACAACAACCGCGTCAACATCGCCGCCGAGGG- 3'	This study
AmpRAsp135Asn Rev	5'-CCCTCGGCGGCGATGTTGACGCGGTTGTTGTGGG- 3'	This study
5RA-P _{ampC} 233	5'-GGGTCTCCGGCGTCACCCGG-3'	This study
5RA-P _{ampC} 154	5'-GGCTACGGCCAGGCCCGG-3'	This study
5RA-P _{ampC} 113	5'-CCGGTTGTACGGCGGCGTCG-3'	This study
5RA-P _{ampR} 229	5'-GCAGGCCGCGGATGCGGTCG-3'	This study
5RA-P _{ampR} 169	5'-GGTCAGCATGAGGCCGCGCG-3'	This study
5RA-P _{ampR} 99	5'-CCTGGTGGCTGACCGCCGCC-3'	This study
Q _t	5'- GCCAGGTGAGCAGGAGTGACGGGAATTCAGCTGGAT CCTGCACCCCTTTTTTTTTTTTTTTTTT-3'	(764)
Q ₀	5'-GCCAGGTGAGCAGGAGTGACG-3'	(764)
Q _i	5'-GGAATTCAGCTGGATCCTGCACCC-3'	(764)
DBS_ChIP_ampCF	5'-GGTCGAACCAATCTCTGCTCCAAA-3'	(841)
DBS_ChIP_ampCR	5'-TTGTGCTTGGCTGCATGAGAAACC-3'	(841)
DBS_qRT_clpXF	5'-TGCGATTACGATGTGGAGA-3'	(843)
DBS_qRT_clpXR	5'-CCCTCGATGAGCTTCAGCA-3'	(843)
DBS_QRTAmpRFwd	5'-CATTGGCCTTCATCACCGGTTGTA-3'	(760)
DBS_QRTAmpRRev	5'-GGTTTCTCATGCAGCCAACGACAA-3'	(760)
qRT_ampCF	5'-CGCCGTACAACCGGTGAT-3'	(971)
qRT_ampCR	5'-CGGCCGTCTCTTTTCGA-3'	(971)

His-tagged AmpR is functional. PA0 Δ *ampR* was complemented with the vector, pMMB67EH-Gm, or with the His-tagged AmpR. β -lactamase assays show that in the absence of AmpR there is low constitutive *ampC* expression (Table S2). However, overexpression of AmpR-His₆ in PA0 Δ *ampR* in the presence of β -lactams restores the β -lactamase activity to that of the wild-type induced strain. Additionally, the inherently resistant PA01 strain shows a significant increase in sensitivity to both amoxicillin and ampicillin when *ampR* is deleted, as determined by E-test (Table S2). The resistance is restored upon complementation with AmpR-His₆. The AmpR-His₆ construct thus complements the *ampR* deletion and retains its native function.

Table S2: Complementing assays showing His-tagged AmpR is functional.

Strains	Minimum Inhibitory Concentration ($\mu\text{g ml}^{-1}$) *		β -Lactamase Specific Activity ($\text{nmol min}^{-1} \text{mg protein}^{-1}$) [†]	
	Amoxicillin	Ampicillin-Sulbactam	- β -lactam	+ β -lactam
PA01	>256	>256	0.040 ± 0.012	0.872 ± 0.032
PA0 Δ <i>ampR</i> (pVector) [^]	7	14	0.035 ± 0.007	0.031 ± 0.011
PA0 Δ <i>ampR</i> (pAmpR-His ₆)	>256	>256	0.042 ± 0.002	1.295 ± 0.008

*Etests were performed in the presence of 1 mM of IPTG.

[^]The vector used to construct pAmpR-His₆ was pMMB67EH-Gm.

[†]Cultures were induced with 1 mM of IPTG and β -lactamase activity was quantified in presence and absence of β -lactams (penicillin G at $200 \mu\text{g ml}^{-1}$ for 1h). Assays were performed in triplicates using sonicated lysate and nitrocefin as a chromogenic substrate.

VSV-G-tagged AmpR is functional. The VSV-G-tagged AmpR was introduced into PA0 Δ *ampR*. The inherently resistant PA01 strain shows a significant increase in sensitivity to both amoxicillin and ampicillin-sulbactam when *ampR* is deleted, as determined by E-test (Table S3). The resistance is restored upon integration of *ampR*-VSV-G. The AmpR-VSV-G complements the *ampR* deletion and retains its native function.

Table S3: Complementing assays showing VSV-G-tagged AmpR is functional.

Strains	Minimum Inhibitory Concentration ($\mu\text{g ml}^{-1}$) *	
	Amoxicillin	Ampicillin-Sulbactam
PA01	>256	>256
PA0 Δ <i>ampR</i>	8	48
PA0 Δ <i>ampR</i> :: <i>ampR</i> -VSV-G	>256	>256

*MIC was determined by Etest.

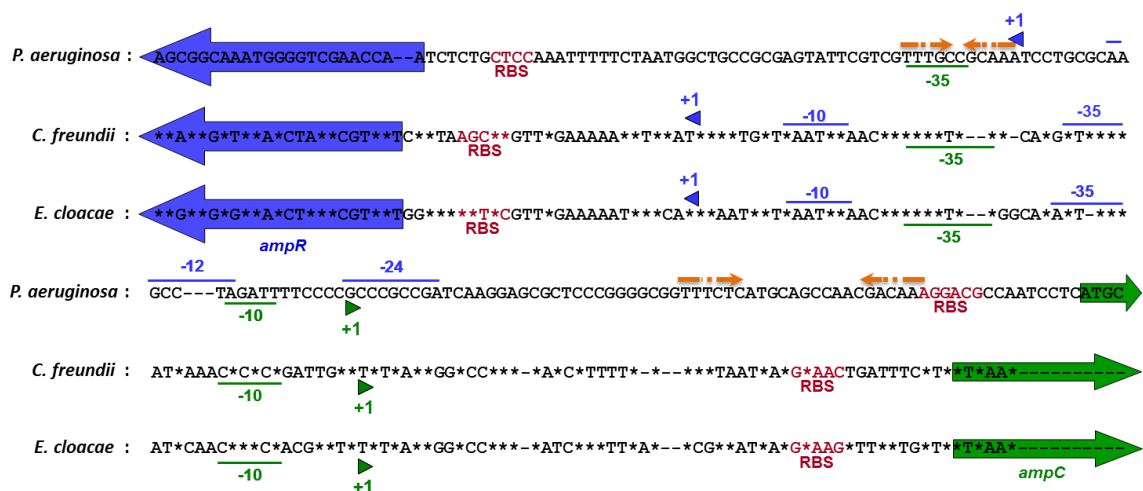
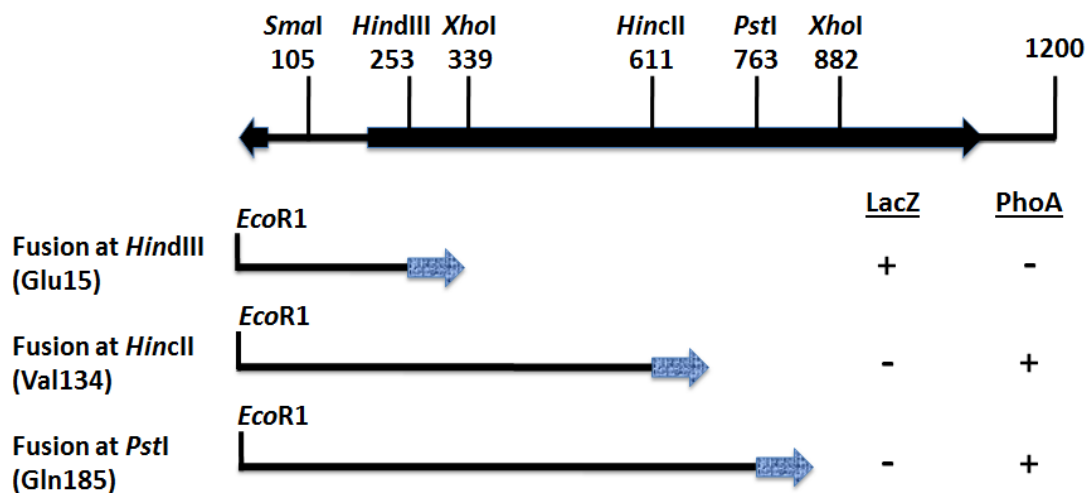


Figure S1. Multiple alignment of the *ampR-ampC* intergenic region. The *C. freundii*, *E. cloacae* and *P. aeruginosa* *ampR-ampC* intergenic regions were aligned using ClustalW2. The *ampR* and *ampC* ORFs (arrows), TSSs (triangles), and sigma sequences (underlined) are depicted in blue and green, respectively. A downstream shift in the *P. aeruginosa* *ampR* TSS is likely responsible for the change in sigma factor control observed here. Orange arrows denote palindromic sequences.

A



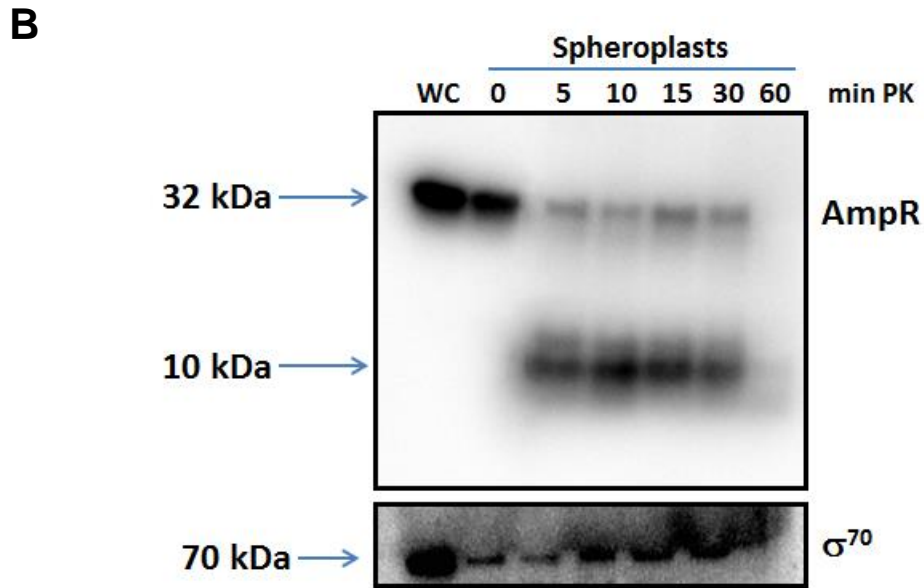


Figure S2. Localization studies of *P. aeruginosa* AmpR. (A) Restriction map and topology of AmpR. The topology of AmpR was investigated by introducing *phoA* and *lacZ* fusions at the restriction sites *HindIII*, *HincII* and *PstI* restriction sites corresponding to amino acid positions Glu15, Val134 and Gln185, respectively. Fusions at Glu15 were LacZ-positive but PhoA-negative, whereas fusions at Val134 and Gln185 were PhoA-positive and LacZ-negative. (B) Spheroplasts of PA0 Δ *ampR* (pAmpR-His₆) were obtained and treated with 10 μ g/ml of Proteinase K for varying lengths of time. Anti-AmpR antibodies were used to monitor the presence of the full-length AmpR (32 kDa) and the shaved product (10 kDa) by Western blotting. The membranes were stripped and re-probed with anti- σ^{70} antibodies.

		Helix 1		Helix 2		Helix 3	
<i>P. aeruginosa</i>	LVRPHLP	LNALRAFEASARH	LS	FTRAAIEL	CVT	QAAVSHQVKSLEERL	GVALFKRLPR 58
<i>C. freundii</i>	MT*SYI*	**S*****A***	**	*****	N**	HS*I*QH*****QQ*	NCQ**V*GS* 58
<i>E. cloacae</i>	MT*SY**	**S*****A***	**	**H*****	N**	HS*I*QH**T**QH*	NCQ**V*VS* 58
<i>K. pneumoniae</i>	M**RY**	**P*****A***	**	*****	N**	H****Q**RA***Q*	*CV**T*VS* 58
<i>A. tumefaciens</i>	M**QF**	**G*****	**	*****	***	*****Q***G**K**	*****Q**** 58
<i>O. anthropi</i>	M*****	**S*****	**	**K*****	***	*****T**	N*T**E**** 58
<i>L. hongkongensis</i>	M***F**	*****	**	*****	N**	*****A**QQ*	N*M***** 58

Figure S3. Multiple alignment of the AmpR HTH motif. N-terminal sequences from *P. aeruginosa*, *C. freundii*, *E. cloacae*, *K. pneumoniae*, *A. tumefaciens*, *O. anthropic*, and *L. hongkongensis* AmpR were aligned using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Asterisks denote conserved residues that do not deviate from those in the reference sequence of *P. aeruginosa*. Residues denoted in red were replaced with alanine in *P. aeruginosa* AmpR-His₆ by site-directed mutagenesis.

SUPPLEMENTAL MATERIALS AND METHODS

β-lactamase assay. The β-lactamase assay was modified from a previously published protocol (765). Briefly, stationary-phase cultures were diluted in 30 ml of LB broth to an OD₆₀₀ of 0.02 and incubated with shaking at 37°C. At an OD₆₀₀ of 0.2, cells containing the expression plasmid were induced with 1 mM IPTG for an hour. At an OD₆₀₀ of 0.6, the remaining cultures were induced with 200 μg ml⁻¹ of penicillin G for an hour before harvesting. Cells were harvested by centrifugation, resuspended in 1 ml of 50 mM of sodium phosphate buffer and sonicated on ice for 1 min 20 s (20-sec pulse ON, 20-sec pulse OFF, amplitude 30%). The sonicated cells were centrifuged at 10,000 X g at 4°C for 30 minutes to collect the β-lactamase-containing supernatant. A 2-μl aliquot of cell lysate is added to nitrocefin (final concentration, 100 μM) in 1 ml of assay buffer. The reaction was incubated at 37°C for 20 minutes and the hydrolysis of nitrocefin was

measured spectrophotometrically at 482 nm. Total protein concentration was determined with Bradford on the same supernatant. The activity was expressed as nmol of nitrocefin degraded per minute per milligram of total protein.

Minimum Inhibitory concentration (MIC). The MIC was determined by the E-test system according to the manufacturer's instructions (bioMérieux, Marcy l'Etoile).

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PUBLICATIONS AND PRESENTATIONS

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107th Annual Meeting of the American Society for Microbiology in Boston, MA. June 1st - 5th, 2008. D. Zincke, D. Balasubramanian, K-F.Kong, L. Schneper, K. Mathee. Characterization of the *poxAB* operon in *Pseudomonas aeruginosa*. Abstract #A-083

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