ACTIVITY OF AND RESISTANCE TO CATIONIC ANTIMICROBIAL AGENTS IN
PSEUDOMONAS AERUGINOSA

by

KRISTEN NICOLE KINDRACHUK

B.Sc. (General), University of Manitoba, 2002
M.Sc. (Medical Microbiology), University of Manitoba, 2006

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Abstract

This thesis investigates mechanisms of adaptive resistance to the cationic antimicrobial agents tobramycin and polymyxin B in the opportunistic pathogen *Pseudomonas aeruginosa*. Using a comprehensive mutant library of *P. aeruginosa* to screen for genes capable of affecting tobramycin susceptibility, 135 genes were identified that caused increased resistance when insertionally inactivated. Transcriptional profiling studies demonstrated downregulation of 53 of these genes in response to tobramycin and significant up-regulation of a number of heat shock genes including an alternative lon protease, AsrA. Induced expression of *asrA* in trans demonstrated its ability to induce heat shock genes in the absence of tobramycin and also provided protection against tobramycin in the first hour after exposure to a lethal dose of 4 μg/ml. Upregulation of the known efflux pump MexXY was observed after prolonged exposure to sub-inhibitory concentrations of tobramycin but induction of this operon was not observed as part of the immediate response to lethal concentrations of tobramycin.

When investigating susceptibility testing methods for polymyxins, 24 *P. aeruginosa* clinical isolates were observed to have a distinct, reproducible phenotype in which skipped wells were observed during microbroth dilution testing for polymyxin B. Possible mechanisms underlying this phenotype were investigated in two of these isolates and one isolate demonstrating a constitutive resistance phenotype. The effects of varying concentrations of polymyxin B on growth, on expression of the resistance genes *phoQ, arnB* and PA4773 (*pmrAB* operon), and on outer membrane permeability were assessed. The isolates presenting the skipped well phenotype demonstrated adaptations in growth, gene expression and membrane permeabilization in response to specific concentrations of polymyxin B consistent with the involvement of Lipid A modifications in the adaptive resistance phenotype.

The results of this thesis highlight the complexity of the bacterial response to cationic antimicrobial agents, as we have demonstrated that adaptation conferring immediate protection (induction of heat shock) differs from that providing long term protection (induction of efflux and Lipid A modifications). Furthermore, the regulatory systems involved in conferring resistance through Lipid A modifications, PhoPQ and PmrAB, are complex and may vary between strains as they adapt further to the pressures imposed by antimicrobial treatment.

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List of Abbreviations

AAC	Aminoglycoside Acetyltransferases
ADP	Adenosine Diphosphate
ANT	Aminoglycoside Nucleotidyltransferases
APH	Aminoglycoside Phosphoryltransferases
ATP	Adenosine Triphosphate
AZLI	Inhaled Aztreonam and Lysine
CAMHB	Cation-Adjusted Mueller Hinton Broth
cAMP	Cyclic Adenosine Monophosphate
Carboxy-PTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
CCCP	Carbonyl Cyanide 3-Chlorophenylhydrazone
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Regulator
cfu	Colony Forming Units
CLSI	Clinical Laboratory Standards Institute
Dab	l-2,4-Diaminobutyric acid
DNA	Deoxyribonucleic Acid
ESBL	Extended Spectrum Beta-Lactamase
ETC	Electron Transport Chain
ExoS	Exotoxin S
ExoT	Exotoxin T
ExoU	Exotoxin U
ExoY	Exotoxin Y
GTP	Guanosine Triphosphate
HHQ	4-Hydroxy-2-Heptylquinoline
HQNO	2-Heptyl-4-Hydroxyquinoline N-Oxide
HSL	Homoserine Lactone
ICU	Intensive Care Unit
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Bertani
LES	Liverpool Epidemic Strain
LPS	Lipopolysaccharide
MBC	Minimum Bactericidal Concentrations
MFS	Major Facilitator Superfamily
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
mRNA	Messenger Ribonucleic Acid
MW	Molecular Weight
NADH	Nicotinamide Adenine Dinucleotide
NHSN	National Healthcare Safety Network
NNIS	National Nosocomial Infections Surveillance
NO	Nitric Oxide
NPN	1-N-Phenylnaphthylamine
OD	Optical Density

PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
PQS	*Pseudomonas* Quinolone Signal
PSE	Pseudomonas Specific Enzymes
qPCR	Quantitative PCR
RNA	Ribonucleic Acid
RND	Resistance Nodulation Cell Division
rRNA	Ribosomal Ribonucleic Acid
TCA	Tricarboxylic Acid
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TOB	Tobramycin
TOBI	Inhaled Tobramycin
tRNA	Transfer Ribonucleic Acid
VAP	Ventilator Associated Pneumonia
X-Gal	Bromo-Chloro-Indolyl-Galactopyranoside

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Co-Authorship Statement

The following thesis is composed of two published manuscripts (Chapters 2 and 4) and two manuscripts awaiting publication (Chapters 1 and 3). Copyright permission was granted for the published papers.

Chapter 1: The majority of this chapter was prepared and submitted in January 2009 for publication as a book chapter entitled "*Pseudomonas aeruginosa:* A persistent pathogen in cystic fibrosis and hospital-associated infections" in "Antibiotic Discovery and Development" to be published by Springer-Verlag. All content included here was written by Kristen Kindrachuk (Schurek) and contributing sections in the original manuscript by E. Breidenstein were either removed or re-written for publication in this thesis. R. Hancock was involved in editing the final review.

Chapter 2: This chapter was originally published as "Novel Genetic Determinants of Low-Level Aminoglycoside Resistance in *Pseudomonas aeruginosa*" in Antimicrobial Agents and Chemotherapy in 2008. The manuscript was written entirely by Kristen Kindrachuk (Schurek). L. Semenec and A. Marr were included as authors as they were involved in early investigations within our lab in aminoglycoside resistance. I. Wiegand contributed the *radA* mutant used in this study. P. Taylor assisted as a summer student in confirming MICs. All other experimental work was carried out by Kristen Kindrachuk (Schurek). R. Hancock was involved in discussing the research plan and experiments and constructively edited the manuscript.

Chapter 3: This chapter was prepared as a manuscript for submission is presently under review. M. Bains is a contributing author who carried out the microarray slide preparation on RNA samples provided to her by Kristen Kindrachuk. All other experimental work, analysis and writing of the first draft of the manuscript was done by Kristen Kindrachuck; R. Hancock was involved in discussing the research plan and experiments and constructively edited the manuscript.

Chapter 4: This chapter was originally published with minor additions added here as "Involvement of *pmrAB* and *phoPQ* in polymyxin B adaptation and inducible resistance in non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*" in 2009 in Antimicrobial Agents and Chemotherapy. All experimental work, analysis and writing of the manuscript was done by Kristen Kindrachuk (Schurek); R. Hancock was involved in discussing the research plan and experiments and constructively edited the manuscript. The other authors included were involved

in identifying the original phenotypes in the clinical isolates, instigated the collaboration and provided these strains prior to publication in any other forum.

1.0   Introduction

1.1   *Pseudomonas aeruginosa*[1]

Pseudomonas aeruginosa is a motile, non-fermenting, Gram-negative organism belonging to the family Pseudomonadaceae. Its history as a recognized human pathogen dates back to the 1850s when Sédillot observed that a blue-green discharge was frequently present and associated with infection in surgical wound dressings (198). The infectious organism, a rod-shaped, blue-green pigmented bacterium, was isolated in 1882 and initially named *Bacillus pyocyaneus* (185). The pathogenicity of the organism was demonstrated in animal models by Charrin who described it as a pyocyanic illness in his 1898 publication "La maladie pyocyanique" (185). By 1916, the ability of this organism to cause both severe acute and chronic infections was recognized (94). In 1925, Osler distinguished the organism as an opportunistic pathogen capable of infecting and invading only damaged tissue sites. As modern medicine advanced in the first half of the 20th century, extending the life expectancy of highly susceptible immunocompromised groups, *P. aeruginosa* established a more prominent role in hospital infections. In the 1960s *P. aeruginosa* emerged as a major human pathogen due to improved treatment of burn and wound victims, as well as surgical, neutropenic and cystic fibrosis (CF) patients (68). Despite anti-pseudomonas activity being one of the holy grails of pharmaceutical drug discovery for several decades, it still remains one of the most recalcitrant and difficult to treat organisms, and as therapeutic options run out, *P. aeruginosa* has achieved Superbug status.

Many of the difficulties with infection control of *P. aeruginosa* result from its superb adaptability. Its large genome encodes approximately 5,500 predicted genes and the proportion of predicted regulatory genes is greater than in all other sequenced bacterial genomes (299), thus lending to its adaptability to varying environments. *P. aeruginosa* is ubiquitous in nature, with many inert surfaces and moist environments serving as reservoirs including soil, water, plants and vegetables (117, 327). *P. aeruginosa* has a broad range of growth substrates, minimal nutrient requirements and is able to grow even in distilled water (87). The organism is tolerant of temperatures as high as 50°C and is capable of growing under both aerobic conditions, as well as anaerobic conditions using nitrate or arginine as a terminal electron acceptor (316). Despite possessing a large number of virulence factors, compromised host defenses are required for

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[1] A version of this section has been accepted for publication as a book chapter. Schurek, KN, Breidenstein EBM and REW Hancock. *P. aeruginosa*: a persistent pathogen in cystic fibrosis and hospital associated pathogens. In *Antibiotic Discovery and Development*.

1

initiation of infection with *P. aeruginosa.* In spite of this, *P. aeruginosa* is truly a challenging pathogen in the hospital setting as it is intrinsically resistant to many antibiotics and is capable of forming hardy biofilms, both within the body and on the surfaces of medical instruments (55, 128, 216).

1.1.1 Pathogenesis and Major Virulence Factors

P. aeruginosa stands out as a unique and threatening organism as it is both capable of causing severe invasive disease and of evading immune defenses causing persistent infections that are nearly impossible to eradicate. Furthermore, *P. aeruginosa* has been found to either colonize or cause infection at nearly every site of the body (247). A period of prior colonization at a local site is strongly associated with a later state of disease (19). Predisposing factors that present an opportunity for the colonizing bacterium to initiate infection include the breach of primary defense barriers either by use of invasive mechanical devices, wound trauma, broad-spectrum antibiotic use, chemotherapy or other host immunodeficiencies (57, 76, 261). Colonization and initial growth may lead to a larger local focus of infection, as is seen with CF lung infection, but in nosocomial infections can frequently precede dissemination either systemically or to another tissue site. The subsequent tissue damage, invasion, and dissemination of *P. aeruginosa* is likely attributed to the many virulence factors it produces. These virulence factors include pili, and flagella, which play an initial role in motility and adhesion to the epithelium, as well as the endotoxin lipopolysaccharide (LPS) and a number of secreted toxins including exotoxin A, exoenzyme S (ExoS), exoenzyme U (ExoU), exoenzyme T (ExoT), exoenzyme Y (ExoY), elastase, and alkaline protease. These factors are thought to be critical for maximum virulence of *P. aeruginosa*; however, based on observations in diverse plant and animal models, the relative contribution of any given factor may vary with the type of infection (250, 253, 304-306). Several of these virulence factors have also been studied for their roles as potential vaccine candidates although there is currently no generally accepted vaccine. The following section briefly outlines several prominent putative virulence factors produced by *P. aeruginosa* and their proposed roles in contributing to disease.

1.1.1.1 Lipopolysaccharide

The LPS is a predominant component in the outer membrane of *P. aeruginosa.* LPS plays a prominent role in activating the host's innate and acquired immune responses and eventually dysregulated inflammatory responses that contribute to morbidity and mortality (138). The

molecule produced by *P. aeruginosa* is a typical Gram-negative bacterial LPS, with a basic Lipid A structure inserted into the outer leaflet of the outer membrane, a relatively conserved core polysaccharide and a more variable O-antigen capping (Figure 1.1). The *P. aeruginosa* LPS tends to be less endotoxic to host cells compared to *Escherichia coli* LPS. This is thought to arise from the lower acylation of the *P. aeruginosa* Lipid A component, which comprises an N- and O-acylated diglucosamine bisphosphate backbone (6, 302). The inner core is composed of two D-*manno*-2-keto- octulosonic acid residues and two L-*glycero*-D-*manno*-heptose residues while the outer core contains an N-alanylated galactosamine residue, three D-glucose residues and one L-rhamnose, as well as variable substitution with amino acids, phosphate, pyrophosphate and ethanolamine (171, 244).

Significant variability in LPS composition can be observed in the Lipid A moiety and in the outer core. The Lipid A component can be either penta-, hexa- or hepta-acylated, and the phosphate residues variably capped with aminoarabinose. (81) The variations in the acylation patterns result in differing immune activation potencies due to varied binding to Toll-like receptor 4 (80) and together with the aminoarabinose capping influence susceptibility to host defense (antimicrobial) peptides, as well as aminoglycosides and polymyxins. The outer core can either be uncapped (85% of molecules, also lack the terminal core rhamnose molecule) or capped by a variable number of repeated saccharide (O-antigen) units of 3-5 sugars with defined linkage. The composition of this repeating unit determines the serotype of the *P. aeruginosa* isolate and there are 20 serotypes based on serological reactivity of the O-antigen (193). Despite the observation that only one in 6 molecules of LPS is capped by O-antigen in most isolates, these polysaccharide chains form a capsule-like layer on the surface of cells (182). In addition to this type of LPS (often called B-band LPS) there is a common antigen LPS (A-band LPS) that is capped by poly-D-rhamnose. Isolates lacking the O-antigen have a distinct rough colony morphology compared to the smooth isolates that predominantly produce an LPS containing the O-antigen (172). The O-antigen has been shown to be a major target for protective immunity in numerous studies. The relative amounts of O-antigen expressing and rough LPS that are produced can vary in a given strain dependant on the growth conditions, and can influence the pathogenicity of a given strain (82), with rough strains being less able to cause a systemic infection in experimental animals possibly due to their susceptibility to complement killing (130). Intriguingly, while blood stream isolates are normally smooth, O-antigen expressing, cystic fibrosis chronic respiratory isolates commonly express rough LPS, an observation that

Outer core (variable) | Inner core (conserved)

L-Rha-(α1→6)-α-D-Glc^{II}
1
↓
4
D-Glc^{III}-(α1→6)-D-Glc^{I}-(β1→3)-D-GalN-(α1→3)-Hep^{II}-(α1→3)-Hep^{I}-(α1→5)-Kdo^{I}-(α2→   Lipid A (variable)
2   2   4
| | ↑
L-Ala   Etn$P$---P 2
                                                          α-Kdo^{II}

P---P P---P
| |
6   4

b)

aminoarabinose

palmitate

Hexa-acylated
cystic fibrosis
lipid A

Penta-acylated
PAK lipid A
(lab isolate)
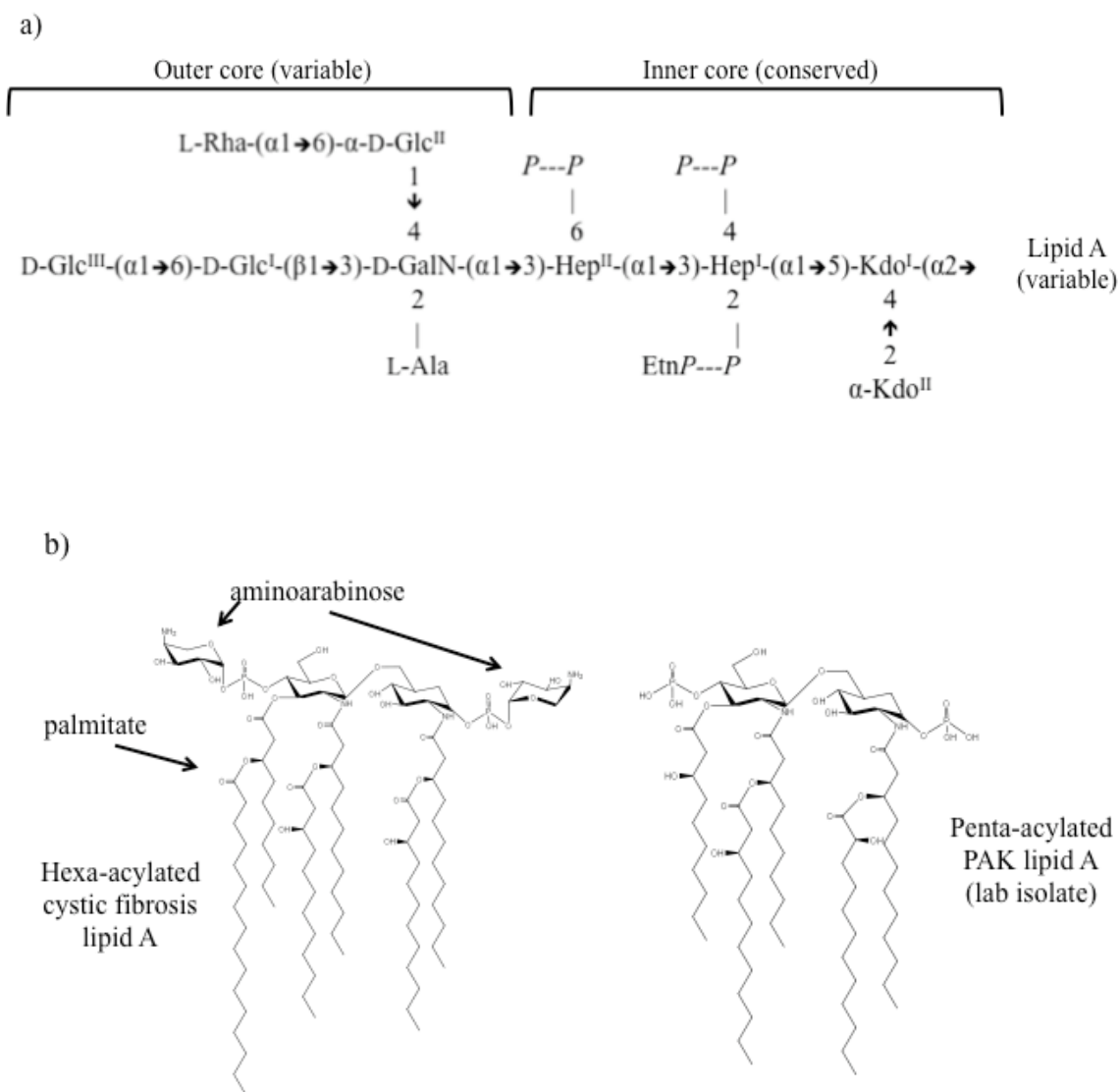
Figure 1.1 ***P. aeruginosa* lipopolysaccharide structure with modifications observed in cystic fibrosis isolates.** (a) Variable (rough) outer core with L-rhamnose attached to conserved inner oligosaccharide core. (b) Common variants of lipid A moiety of LPS.

may reflect the organism's attempt to evade the adaptive immune system and maintain chronic infections in the lung (130, 245).

A number of LPS vaccines have been investigated for use in CF patients in phase II and III clinical trials, however, these have not been successful (74, 132, 184, 239, 241). The LPS based vaccines provided little immunity and did not appear to protect the patients from infection with *P. aeruginosa* (243).

1.1.1.2 Flagellum

The single unsheathed polar flagellum of *P. aeruginosa* is responsible for the swimming motility of this organism and on semi-solid media flagella plays a role in swarming motility (174). Nonetheless, its role in virulence goes beyond simple motility. Flagellar proteins have been shown to play critical roles in attachment, invasion, biofilm formation and in the mediation of inflammatory responses. Flagellar protein synthesis, assembly and regulation involves more than 40 genes and is intricately controlled through transcriptional and post-translational events by the four main regulators RpoN, FleQ, FleR and FliA (59).

Although adhesion of *P. aeruginosa* is primarily mediated by type IV pili, flagella have been shown to mediate adhesion to corneal cells and have been implicated in the adhesion to cell bound mucins (254); in particular, FliD, the flagella cap protein, specifically binds to the oligosaccharides of respiratory mucins (5).

Non-flagellated mutants are often isolated from chronic infections in CF patients (204) due to the repressor activity of AlgT which acts on the FleQ regulator (307). The loss of flagella in these isolates is believed to be useful for the evasion of the host immune system, as flagellin mediates the inflammatory response via the innate immune system through its specific interaction with a leucine-rich repeat region of host Toll-like receptor 5 (TLR5) (320). Intriguingly, epithelial cells with the CFTR protein mutated are hyper-inflammatory, especially when stimulated through flagellin-TLR5 interactions (22).

Flagellar vaccines have been investigated in pre-clinical studies in mouse models and have reached phase III clinical trials for CF patients; however, limited protection was observed with a monovalent vaccine and development of a bivalent vaccine has been terminated (74).

1.1.1.3 Type IV Pili

The type IV pili of *P. aeruginosa* have a role in adhesion to many cell types and this is likely important in such phenomena as tissue tropism (attachment to particular tissues), initiation of biofilm formation and non-opsonic phagocytosis (12, 205, 252). These pili also mediate twitching motility, a factor found to be important in the formation of biofilms *in vitro* (170) as well as in the initiation of dissemination from an initial point of colonization (122, 169). Surface associated motility occurs by the coordinated extension and retraction of these polar pili (32). Although more than 50 genes have been identified to play either a direct or indirect role in the synthesis, functioning and control of the type IV pili of *P. aeruginosa,* the pili are composed of a

single type IVa pilin protein encoded by *pilA* (133). Five alleles of *pilA* have been identified, with group I pili being the most prevalent in CF and environmental isolates (181).

1.1.1.4 Type III Secretion

P. aeruginosa has a variety of secretion systems of which at least four likely play a role in virulence (Type I, II, III, and VI). One of the most intriguing is Type III secretion that involves a flagellum-basal-body related system for delivering proteins directly from the cytoplasm of *Pseudomonas* into the cytosol of host cells. A functional Type III secretion system contributes to the successful evasion of phagocytosis by *P. aeruginosa* as well as damage of host tissues, promotion of immune avoidance and bacterial dissemination. The Type III secretion system of *P. aeruginosa* delivers up to four cytotoxins ExoS, ExoT, ExoU and ExoY, directly to host cells. The gene clusters *psc, pcr, exs* and *pop* encode proteins of the *Pseudomonas* Type-III secretion apparatus and proteins involved in regulation of this apparatus (92, 110, 328).

ExoS and ExoT are bifunctional cytotoxins that possess both Rho GTPase-activating protein and ADP ribosyltransferase activities. These molecules can inhibit phagocytosis by disrupting actin cytoskeletal rearrangement, focal adhesins and signal transduction (10). ExoU is a phospholipase that contributes directly to acute cytotoxicity towards epithelial cells and macrophages. ExoY is an adenylate cyclase that affects intracellular cAMP levels and cytoskeleton reorganization (274, 329).

Recent evidence has implicated a role for the Type III secretion system in virulence in humans. The presence of large amounts of Type III secretion products, particularly ExoU, in *P. aeruginosa* cultures from intubated patients was linked to increased mortality regardless of whether these patients had symptoms or confirmation of ventilator-associated pneumonia (333).

1.1.1.5 Exotoxin

There are several key virulence factors that are secreted through Type II secretion mechanisms (which use a pilus-like apparatus to secrete proteins into the extracellular environment), including exotoxin A, lipases, phospholipases, alkaline phosphatase, and proteases; animal experiments have indicated the important role of these factors in model infections (237). Exotoxin A has been demonstrated to be involved in local tissue damage and invasion. This cytotoxin is encoded by the gene *toxA* and has been found to be present in most clinical isolates of *P. aeruginosa,* although its role in virulence is poorly understood. Exotoxin A is also an ADP-ribosylating factor although it is distinct from the ADP ribosylating activity of

ExoS and ExoT in that it inhibits protein biosynthesis in host cells by catalyzing the inactivation of elongation factor 2 (237).

1.1.1.6 Proteases

P. aeruginosa produces several secreted proteases including the zinc metalloprotease/elastase LasB, the metallo-endopeptidase LasA, and alkaline protease. These proteases work in a concerted fashion to destroy host tissue, thus playing an important role in both acute lung infections and in burn wound infections (21, 100, 267). LasB elastase is capable of degrading a number of connective tissue proteins including elastin, fibrin and collagen. Destruction of these proteins starts with LasB nicking the elastin, providing a substrate for degradation by other proteases including *Pseudomonas* LasA, elastin and alkaline protease, and host neutrophil elastase (100). A clear role for these destructive proteases in acute infections has been established. LasA and LasB elastases have also been found in the sputum of CF patients suffering from pulmonary exacerbations of infection (149, 160); although their role in chronic infection is not well understood.

1.1.1.7 Alginate

P. aeruginosa can produce a mucoid exopolysaccharide capsule, comprised of alginate, an acetylated random co-polymer of β1-4 linked D-mannuronic acid and L-guluronic acid (99). All *P. aeruginosa* strains investigated possess the *alg* genes for alginate production; however, most environmental and many clinical isolates, with the prominent exception of CF isolates, do not typically produce a mucoid phenotype. Overexpression of the alginate-producing genes is required for the mucoid phenotype. Conversion to mucoidy has been observed in the laboratory under nutrient limiting conditions, in the presence of the major lung surfactant, lecithin, and in response to sub-lethal concentrations of antibiotics (248, 310). Stable conversion to mucoidy in the clinic is typically due to mutations in the regulatory *muc* genes which act to negatively regulate the alternative sigma factor AlgU that is responsible for transcriptional activation of the *alg* genes (30, 31, 63). The overproduction of alginate is believed to play a role in cell adherence within the CF lung and is also thought to be involved in resistance to host defences by reducing susceptibility to phagocytosis (246).

1.1.1.8 Biofilm Formation

P. aeruginosa is capable of forming complex structures called biofilms. Biofilms are
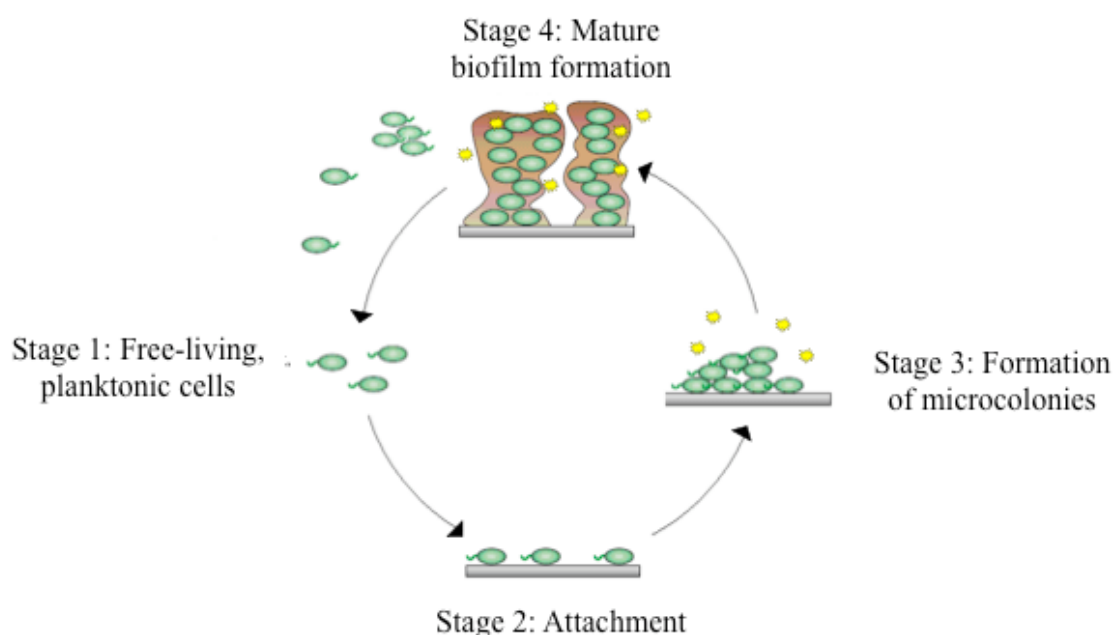
Figure 1.2 Development of a *P. aeruginosa* biofilm. Stage 1: Cells are free-swimming (planktonic), with predictable antimicrobial susceptibilities. Stage 2: Planktonic cells initiate attachment to a surface via their type IV pili and flagellum. Stage 3: Small aggregative communities begin to form and quorum sensing signals begin to accumulate. Stage 4: A critical threshold of quorum sensing signals is reached. Microcolonies become encased in an extracellular matrix. Cells enter a sessile phase of growth and become highly resistant to antimicrobials. Individual cells and small microcolonies slough from the mature biofilm initiating further biofilm development (98).

characterized by an extracellular matrix enclosing an aggregated population of slow growing cells, and adopt under most *in vitro* circumstances a heterogeneous, mushroom shape. Biofilm development is a complex process (Figure 1.2) and is in part controlled by quorum sensing signals. Type IV pili and flagella play a role in the initial attachment of cells to a surface (170). As the microcolony develops, the extracellular matrix forms and encloses the biofilm (Figure 1.2) . This matrix is composed of polysaccharides, proteins and nucleic acids. During biofilm formation, cell differentiation occurs, and oxygen and water filled channels are formed to provide nutrients to the deep-rooted cells of the mature biofilm (61, 95, 168, 268).

P. aeruginosa has been demonstrated to form biofilms on a variety of indwelling medical devices (48, 166). It is particularly problematic for patients requiring mechanical ventilation and catheterization, as the surfaces of medical devices can readily develop *P. aeruginosa* biofilms that are difficult to remove. Furthermore, *P. aeruginosa* has been demonstrated to grow as a biofilm within the body particularly at the site of burn wounds. It has been proposed that *P.*

aeruginosa exists as a biofilm in the CF lung (55, 283) and this has been observed in a mouse model of CF lung infection (146). Support for this belief has also come from reports detecting the presence of quorum sensing molecules in sputum from CF patients. The concept that *P. aeruginosa* exhibits a biofilm or biofilm-like mode of growth within the CF lung helps to further explain the persistence of these infections, as biofilms have demonstrated the ability to evade the host immune system and resist antibiotic treatment (152).

In addition to evasion of the host immune system, the highly antibiotic resistant nature of biofilms to killing by bactericidal antibiotics contributes to bacterial persistence in chronic infections (203). It is in part the slow growth and poor accessibility of biofilms that contributes to their high antibiotic resistance (36, 291), although adaptive mechanisms involved in biofilm differentiation may also be influential. It has been demonstrated that cells growing in a biofilm can be up to 1000 fold more resistant to antibiotics than free-swimming, planktonic cells (151). Biofilms present not only a diffusion barrier to antibiotics, but the cells in a biofilm have been demonstrated to have significantly different expression patterns compared to their planktonic counterparts; with the observed differentially expressed genes being dependent on the region of the biofilm where the cells are located (275).

1.1.1.9   Quorum Sensing

Quorum-sensing is a mechanism of cell to cell communication in which a critical number of bacteria (the quorum) are required to produce a sufficient amount of a secreted signal molecule (termed an autoinducer) to trigger expression of a large regulon. In *P. aeruginosa*, quorum-sensing systems regulate biofilm formation, swarming motility and a broad array of virulence factors via two separate secreted homoserine lactone (HSL) autoinducer molecules, N-(3-oxododecanoyl) homoserine lactone (Las system) and N-butyryl homoserine lactone (Rhl system), and three quinolines, 2-heptyl-4-hydroxyquinoline N-oxide (HQNO), 4-hydroxy-2-heptylquinoline (HHQ), and Pseudomonas quinolone signal (3,4-dihydroxy-2-heptylquinoline; PQS) (66, 140, 286). The HSL molecules are secreted from the cell, and as population density increases, as does the concentration of signaling molecules. Once a critical threshold concentration has been attained, the cells undergo broad changes in gene expression. The two HSL quorum-sensing regulatory systems, present in *P. aeruginosa*, LasIR and RhlIR (35), act through LasR and RhlR at the transcriptional level, which interact with a variety of sigma factors and transcriptional regulators. Regulation of these systems is also intertwined with the expression of the two-component regulatory system GacAS and the small regulatory RNAs

encoded by the *rsm* system, thus forming a complex network of regulation in *P. aeruginosa* (260). In addition to biofilm formation, these systems collectively regulate the production of hydrogen cyanide, rhamnolipid, pyocyanin, elastase, alkaline protease, superoxide dismutase, LasA and swarming motility (326).

Recent advances in the understanding of quorum-sensing in *P. aeruginosa* have generated interest in using quorum sensing as a target for therapeutics. The macrolide antibiotic, azithromycin, has been a promising candidate in this regard as it has been demonstrated to be capable of both penetrating biofilms and interfering with quorum sensing (145).

1.1.2 Antimicrobial Resistance

P. aeruginosa can be a particularly challenging organism to treat once infection has been established as it is intrinsically resistant to many of the available antibiotics. In addition, it readily acquires plasmids that can harbor multiple antibiotic resistance cassettes, and is capable of adapting and mutating leading to alterations in gene expression that can cause resistance. This section will provide an overview of the main mechanisms of resistance present in clinical isolates of *P. aeruginosa;* however resistance to aminoglycosides and polymxins will be addressed in more detail in Sections 1.2.2 and 1.3.2 respectively.

1.1.2.1   Intrinsic Resistance

P. aeruginosa exhibits intrinsic resistance to almost all of the available antibiotics, indicating that the wild type strain possesses a number of genetic mechanisms that contribute to reduced susceptibility of the organism. One of the major factors contributing to this intrinsic resistance is the low permeability of its outer membrane. The outer membrane is important for passively determining the rate of uptake of antibiotics and small molecules, and the outer membrane permeability of *P. aeruginosa* is 12-100 fold lower than that of *Escherichia coli* (128). However, by itself this is insufficient to mediate significant resistance, and antibiotics will equilibrate across the outer membrane. Thus, intrinsic resistance arises from the combination of slow uptake and secondary mechanisms that benefit from this slow uptake including degradative enzymes such as periplasmic β-lactamase, and particularly multidrug efflux (128).

The *P. aeruginosa* outer membrane is an asymmetric membrane composed of an inner leaflet of phospholipid, predominantly phosphatidylethanolamine, and an outer layer of polyanionic LPS. The latter presents a negatively charged surface which, together with the divalent cations bridging the individual LPS molecules, forms a matrix around the cell that is

relatively impermeable to polar compounds except polycations (127). Several classes of polycationic antimicrobials, including the aminoglycosides, polymyxins and cationic antimicrobial peptides employ the mechanism of self-promoted uptake wherein they competitively displace the divalent cations that bridge adjacent LPS molecules and are important for maintaining membrane integrity (131, 223). This causes a perturbation of membrane structure allowing the perturbing cationic antimicrobials to gain entry into the periplasm, located between the outer and cytoplasmic membranes. Conversely, many hydrophilic antibiotics, particularly the β-lactams, rely on the presence of the water filled channels of proteins termed porins, to gain entry to the periplasm. These porins are often an abundant (major) protein component of the outer membrane and are involved in controlling its selectivity as a barrier to extracellular molecules. Porins are divided into several classes including general porins, specific porins, and gated porins (226). OprF is an extensively studied general or non-specific porin found in *P. aeruginosa*. It has an exclusion limit of MW ~ 3000 and it is very inefficient as a channel, leading to the overall low outer membrane permeability of *Pseudomonas*. Reduced expression of this porin alone appears to have little effect on the susceptibility of the organism to β-lactams and fluoroquinolones (17). Another well characterized porin is the basic amino acid/peptide-specific porin, OprD, which is the major porin mediating the uptake of the carbapenem β-lactam imipenem (153, 154). Gated porins include the iron regulated outer membrane proteins that mediate the uptake of specific siderophore-iron complexes and are thus essential for virulence, as well as certain catechol containing antibiotics.

Resistance to many classes of antibiotics is also mediated by multidrug efflux pumps. The *P. aeruginosa* genome contains a large number of drug efflux systems (299), predominantly of the RND (Resistance-Nodulation-cell Division) and major facilitator superfamily (MFS) types. RND efflux pumps are comprised of an outer membrane channel-tunnel of distinct architecture, a cytoplasmic membrane pump and linker proteins. Four major RND efflux pump systems have been well characterized in *P. aeruginosa* as contributing to antimicrobial resistance: MexAB-OprM, MexXY-OprM/OpmG, MexCD-OprJ and MexEF-OprN, OprM together with the pump MexB and the linker protein MexA operate constitutively to mediate general efflux of antibiotics, including β-lactams, fluoroquinolones, tetracyclines, and macrolides (112, 175, 208, 209). Together with OprF, this system is primarily responsible for the high intrinsic resistance to these antibiotics in *P. aeruginosa*. The MexXY efflux proteins, together with channel-tunnel protein OprM and/or OpmG, mediates aminoglycoside intrinsic resistance (163, 195).

P. aeruginosa also expresses periplasmic β-lactamases to degrade many β-lactam antibiotics. B-lactamases are hydrolyzing enzymes that cleave the lactam ring of penicillins, carbapenems, cephalosporins and monobactams, thus leading to inactivation of the antibiotic (263, 273, 300). In *P. aeruginosa,* this activity is due to a chromosomally encoded AmpC β-lactamase. The AmpC β-lactamase of *P. aeruginosa* can degrade and contribute to intrinsic resistance to ticarcillin, piperacillin and the third generation cephalosporins. It is strongly induced by carbapenems, particularly imipenem, although these inducing carbapenems are stable against its hydrolytic activity.

1.1.2.2 Acquired Resistance

Hyperproduction of β-lactamases in *P. aeruginosa* can be the result of plasmid acquisition, but more usually results from mutations that derepress AmpC β-lactamase regulatory genes (183). In the latter situation, regulatory mutations lead to the overexpression of AmpC β-lactamase, increasing resistance to penicillins, cephalosporins and to a lesser extent meropenem. In *P. aeruginosa* clinical isolates, overexpression of AmpC β-lactamase has been predominantly associated with mutations in the *ampC* repressor gene *ampD* (183). Specific point mutations in the transcriptional regulator, *ampR*, have also been found to result in overexpression of *ampC,* but these mutations are relatively rare (196). Regarding plasmid encoded enzymes, a broad variety of β-lactamases, including *Pseudomonas* specific enzymes (PSE), OXA-type β-lactamases, extended spectrum β-lactamases (ESBLs) and metallo-carbapenemases have been detected in *P. aeruginosa* clinical isolates, and can each lead to high level resistance to specific β-lactams (270).

Although mutations leading to the overproduction of AmpC have little effect on the carbapenems, which are relatively unaffected by AmpC hydrolytic activity, mutations leading to the loss of the specific porin, OprD, can significantly affect this class of antibiotic (233). Mutations leading to the loss of OprD can be either in OprD itself or in one of the regulatory proteins controlling its expression *e.g.* MexT (NfxC). Interestingly, *mexT* regulatory mutations leading to the loss of this porin can be selected for by treatment with fluoroquinolones, but not by treatment with carbapenems themselves, since MexT controls a major efflux pump involved in fluoroquinolone resistance, MexEF-OprN, in addition to the OprD porin (229). Thus, mutations at the *mexT/nfxC* locus contribute not only to imipenem resistance via loss of a porin but also to multidrug resistance via overexpression of an efflux pump (97).

Similarly, over expression of the MexAB-OprM, and MexCD-OprJ operons, due largely to *nalB* and *nfxB* regulatory mutations respectively, lead to multidrug resistance (190, 207, 249), while overexpression of MexXY-OprM/OpmG is due to *amrR/mexZ* regulatory mutations and is thought to be a prominent cause of broad spectrum, low level (so-called impermeability-type) aminoglycoside resistance (195, 249, 324, 334).

In addition to selection of efflux regulatory mutations, fluoroquinolones select for mutations in the genes encoding DNA gyrase and topoisomerase IV. *P. aeruginosa* tends to exhibit reduced susceptibility to fluoroquinolones compared to the Enterobacteriaceae, and mutations in DNA gyrase and topoisomerase IV also occur at a higher rate in *P. aeruginosa.* In *P. aeruginosa* an individual mutation in DNA gyrase can lead to clinically relevant levels of resistance and is even greater when mutations in both DNA gyrase and topoisomerase IV are present (161).

1.1.2.3   Adaptive Resistance

Discrepancies in *in vitro* susceptibility of *P. aeruginosa* isolates and treatment outcomes in CF patients have been observed and can be attributed to the phenomenon of adaptive resistance. Adaptive resistance occurs when cell populations are exposed to non-lethal concentrations of antibiotic and undergo specific changes in gene expression that result in reduced susceptibility. It is a form of inducible resistance that does not require the presence of mutations and has been demonstrated both *in vitro* using CF isolates, and in mouse models, when isolates were pre-incubated with subinhibitory concentrations of antibiotics (11, 33, 105). The best-described mechanisms of adaptive resistance in *P. aeruginosa* overlap significantly with the above-described acquired mechanisms of resistance, and may in fact precede the development of mutational resistance. For example, β-lactamase production from the chromosomally encoded *ampC* β-lactamase is inducible by many β-lactams, particularly imipenem, and although this does not cause imipenem resistance, it can lead to resistance to other β-lactams including the third generation cephalosporins. Interestingly however, this strong induction may not result in large differences in susceptibility during MIC testing since, in the time required for induction, significant killing may still occur (194). Nonetheless the concern arises that this induction may allow small populations to survive and acquire stably resistant mutations. Similarly, adaptive resistance to polymyxins and antimicrobial peptides has been shown to occur through altered expression of the PhoPQ and PmrAB systems in response to these agents and leads to modulation of Lipid A fatty acid composition (220) which ultimately

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affects resistance to not only polymyxins and peptides but also the aminoglycosides which rely on the LPS binding for self-promoted uptake.

1.1.2.4   *P. aeruginosa* as a Superbug

The accumulation of multiple resistance mechanisms in clinical isolates of *P. aeruginosa* has resulted in strains that are resistant to all available antibiotics. This pan-drug resistance, together with high attributable mortality, has thrust *P. aeruginosa* into the spotlight as an emerging superbug. According to reports by the National Nosocomial Infections Surveillance (NNIS) System, which focused on nosocomial infections in intensive care units (ICU), not only were resistance rates increasing, but the incidence of occurrence of most infection types was also increasing (101). In 2003, the NNIS reported a 9% increase in resistance to the third generation cephalosporins, a 15% increase in ciprofloxacin resistance, and most alarming, a 47% increase in imipenem resistance over a five year period (228). According to the European Antimicrobial Resistance Surveillance System 18% of *P. aeruginosa* isolates were multidrug resistant with 6% of all isolates being resistant to piperacillin, ceftazidime, fluoroquinolones, aminoglycosides and carbapenems (289). The drug of last resort for infections with multidrug resistant *P. aeruginosa* is colistin (polymyxin E), and while resistance rates remain low (approximately 1% in most countries), mortality of 80% has been observed for infections caused by colistin resistant Gram negative bacilli (18).

1.1.3   Hospital-Associated *P. aeruginosa* Infections

Hospital-acquired (nosocomial) infections are those not present or incubating (i.e. non-obvious) at the time of hospital admission and usually developing more than 48 hours post admission. *P. aeruginosa* infections are typically of the late onset category, often presenting more than 5 days after admission. The 2006-7 report by the National Healthcare Safety Network (NHSN) at the Centers for Disease Control and Prevention ranked *P. aeruginosa* as the 6th most common healthcare associated pathogen causing infection, and it is typically found at even higher rank in studies focused on the ICU (228). The NHSN reports that in the US in 2006-2007, 8% of all hospital associated infections were due to *P. aeruginosa,* with *P. aeruginosa* causing 3% of central line associated bloodstream infections, 6% of surgical site infections, 10% of catheter associated urinary tract infections and 16% of ventilator associated pneumonia infections (141). It is worth noting however that difficulties in treatment of such infections, and

the associated morbidity and mortality, have made *Pseudomonas* one of the most feared hospital pathogens.

Of major concern with nosocomial infections are the high rates of antibiotic resistance. Recent reports from the NHSN annual update suggest that the highest resistance rates amongst pathogenic nosocomial isolates of *P. aeruginosa* were towards fluoroquinolones, with over 30% being considered resistant. Resistance rates for other therapeutics were as follows: carbapenems 25%, piperacillin-tazobactam 18%, and cefepime 11%, while the agent with the lowest rate was the aminoglycoside amikacin at 6% (141). However it is worth noting that in other countries rates vary dramatically and for some selected multidrug resistant isolates there are virtually no therapeutic options (289).

Mechanical ventilation, antibiotic therapy, surgery and chemotherapy are the major predisposing factors contributing to the acquisition of a *P. aeruginosa* infection in the hospital (311). The frequency with which patients come into contact with *P. aeruginosa* is high and this likely also plays a significant role in contributing to infection. Many inanimate surfaces which patients are exposed to, including countertops, sinks, toilets, flower vases and cleaning supplies, harbor *P. aeruginosa* (197). Furthermore patients themselves tend to have high colonization rates (311). In contrast to the minority of healthy individuals that carry *P. aeruginosa* in the gut, nasal mucosa, throat or on the skin, in the hospital *P. aeruginosa* frequently colonizes patients in the armpit, ear and perineum. Studies have shown that while only 7% of healthy individuals are colonized in the oropharynx and recovery rates of *P. aeruginosa* from stool approach only 24%, up to 50% of hospitalized patients may be colonized with *P. aeruginosa* (219). A period of prior colonization with *P. aeruginosa* is strongly associated with approximately 50% of all cases of invasive infection with *P. aeruginosa* (25).

1.1.3.1   Burn Wound Infections

A breach of the skin barrier is the hallmark of thermal injury allowing for the rapid colonization and infection of the dermal tissues by *P. aeruginosa*. *P. aeruginosa* is the leading cause of invasive infections in burn patients. The threat of invasive disease posed by *P. aeruginosa* in burn patients has been demonstrated in a mouse burn model where inoculation of the burned skin with as few as 10 organisms led to systemic dissemination and death (296). It has been observed that 75% of all deaths in patients with severe burns are related to sepsis from invasive burn wound infection (7, 9, 15). In addition to wounded skin injury, inhalation injury is common in burn patients. This results in edema and sloughing of the respiratory tract mucosa

and impairment of the normal mucociliary clearance mechanism, thus making these patients more susceptible to upper respiratory tract infections as well as *P. aeruginosa* pneumonia (51).

Although Gram-positive organisms such as *Staphylococcus aureus* and *Streptococcus pyogenes* are typically the first microorganisms to colonize the site of infection, after an average of 5 to 7 days other microbes including *P. aeruginosa* begin to colonize these wounds (2, 202). Success with early wound excision practices was shown to contribute to the prevention of invasive infections disseminating from the wound site (14). As well, topical antibacterials with anti-pseudomonal activity, such as silver nitrate, silver sulfadiazine, and mafenide acetate, have proven useful for controlling the colonization and growth on the surface of wound.

Prior to the inception of the practice of excision of necrotic wound tissue, burn wound sepsis was predominantly caused by invasive wound infections with *Pseudomonas* originating from the burn wound site. In addition, older treatment strategies such as immersion hydrotherapy contributed to increased exposure to *P. aeruginosa* that was frequently found growing in the water baths (43). During these pre-excision times, morbidity and mortality were extremely high as adequate penetration of the necrotic tissue at the wound site with either topical or systemic antimicrobials was difficult to achieve. Animal studies of partial-thickness cutaneous burns showed that mature biofilms could develop in 48 to 72 hours, indicating a major potential source of further difficulties in antimicrobial therapy at these sites (312).

In addition to the *P. aeruginosa* virulence factors described in Section 1.1.1, which undoubtedly contribute to the success of *P. aeruginosa* as a pathogen in the burn patients, the impairment of host immunity, beyond simple loss of the skin's physical barrier, plays a role in enhancing susceptibility to infection. Recent studies have demonstrated that thermal injury causes impaired production of the host defense peptides β-defensins in the tissues surrounding the wound. These immunomodulatory peptides have been proposed to play an important role in primary defense against *P. aeruginosa* and synthetic β-defensin was recently shown to be protective against *P. aeruginosa* infection in a burned mouse model (173).

While the rates of sepsis caused by dissemination from the wound site have now been significantly reduced due to early wound excision practices, *P. aeruginosa* sepsis still poses a major risk to burn patients, as many of these patients require mechanical ventilation or catheterization (51).

1.1.3.2   Bacteremia

P. aeruginosa is among the five leading causes of nosocomial bacteremia and frequently leads to sepsis. In the 1960s and early 1970s, aminoglycosides and polymyxins were the only options for treatment of *P. aeruginosa* bacteremia but were found to be fairly ineffective for these infections. Mortality of greater than 50% was reported when mortality was used as the end point (89, 325), and was as high as 70% in febrile neutropenic patients (26). Despite the introduction of effective anti-pseudomonal β-lactams and the associated reduction in mortality rates, *P. aeruginosa* bacteremia is still one of the most feared nosocomial infections. These infections are generally associated with higher mortality than with other infecting pathogens, and persistence, particularly associated with device-related bacteremia, continues to plague patients (262).

In the clinical presentation of bacteremia patients are typically febrile, although more severely ill patients may present signs of shock and hypothermia. The main distinguishing feature of *P. aeruginosa* sepsis is the presence of ecthyma gangrenosum and these infarcted skin lesions occur only in markedly neutropenic patients (247). As *P. aeruginosa* bacteremia rarely has notable distinguishing features, antimicrobial treatment tends to not be specific for the organism and standard empirical therapy for bacteremia and sepsis are usually administered until laboratory results are available; this may add to later complications as the antibiotics that can be successfully utilized to treat *P. aeruginosa* infections are often distinct and limited.

When *P. aeruginosa* disseminates from a site of local infection, it gains access to the bloodstream by breaking down the epithelial and endothelial tissue barriers (180). To evade the bactericidal activity of the serum complement, *P. aeruginosa* produces a smooth LPS (130, 245). Animal models have demonstrated that once in the blood stream, sepsis is related to the release of pro-inflammatory mediators like tumour necrosis factor-α (TNF-α). In a rabbit lung model of sepsis, provision of antibodies to TNF-α or the anti-inflammatory cytokine IL-10 lowered both the signs of septic shock and the levels of bacteremia (116, 277).

Standard treatment regimens for *P. aeruginosa* include the use of the anti-pseudomonal β-lactams ceftazidime, cefepime, meropenem, imipenem and piperacillin. Aztreonam may be used for patients with β-lactam allergy (23, 247). The addition of an aminoglycoside is frequently used but is generally at the discretion of the physician for non-neutropenic patients. When aminoglycosides and polymyxins were the only drugs available for treatment of *P. aeruginosa* infections, the prognosis for bacteremic patients was dismal. With the introduction of carbenicillin, the first antipseudomonal β-lactam, patient outcomes significantly improved (27,

276). However, it is worth noting that β-lactam and multi-drug resistance threaten to reverse these gains and, for example, carbenicillin is now rarely used therapeutically for such infections.

Analyses of treatment of *P. aeruginosa* bacteremia have included retrospective studies performed mostly for febrile neutropenic patients. These have included retrospective analyses of β-lactam monotherapy, β-lactam combination therapy, and the synergistic effects of combination therapy (26, 44, 188, 189, 238, 319). *In vitro* and animal experiments suggested a synergistic effect between the anti-pseudomonal β-lactams and aminoglycosides against *P. aeruginosa,* and so this, together with occasional reports of treatment failure with β-lactam monotherapy, was used to promote the practice of combination therapy (4, 150, 278). While a prospective trial of combination therapy with anti-pseudomonal β-lactams and aminoglycosides demonstrated superiority to monotherapy with aminoglycosides (142), there has not been strong evidence that combination therapy is superior to antipseudomonal β-lactam monotherapy.

In contrast to the concentration-dependent killing observed with aminoglycosides, β–lactams act in a time-dependent manner. Efficacy is correlated to the percentage of time for which tissue concentrations exceed the MIC, and for *P. aeruginosa* the ideal dosing would have a target attainment of 60-70% of the dosing interval (3).

Support for the use of combination therapy can also be found in the fight against the development of antimicrobial resistance, which is thought to be delayed when using two antibiotics with distinct mechanisms of action. Development of resistance is a major concern in treating *P. aeruginosa* infections, however, it is not known if combination therapy increases the rate of development of multi-drug resistance (e.g. through efflux pump upregulation), and progressive development of resistance to each of the two agents in a combination remains a risk.

Pharmacokinetic and pharmacodynamic studies of anti-pseudomonal therapeutics have offered insights as to effective dosing regimens, particularly for aminoglycosides (56). It is now understood that aminoglycosides kill in a concentration dependent manner and so it is predicted that a single daily high dose of aminoglycosides would result in more effective killing, without increased toxicity, than would multiple daily does or continuous infusion (77, 134). Although these pharmacokinetic and pharmacodynamic studies offer the hope of better outcomes than were observed in prior studies with aminoglycoside monotherapy, no prospective trials of single daily dosing with aminoglycosides have been conducted for *P. aeruginosa* bacteremia.

1.1.3.3   Hospital-Associated Pneumonia

The human respiratory tract presents a favourable environment to which *P. aeruginosa* has become particularly well adapted. *P. aeruginosa* has the formidable ability to cause both chronic infections in the lungs of CF patients and acute nosocomial pneumonia. Acute *P. aeruginosa* infections are rarely early onset and typical *P. aeruginosa* pneumonia is characterized by slow onset pneumonia, occurring after 5 days of hospitalization, and is associated with prior use of broad spectrum antimicrobial therapy, structural lung disease, or mechanical ventilation (23).

Animal model studies of *P. aeruginosa* pneumonia have demonstrated the involvement in virulence of proteases, flagella, pili, and LPS O side chains as well as the delivery of the extracellular toxins ExoS, ExoT and ExoU via a type III secretion system. For example, administration of anti-*pcrV* antibodies blocking the type III secretion system has been shown to offer protection against acute *P. aeruginosa* pneumonia when tested in animal studies (86, 282).

Adequate clinical trials focusing on appropriate antimicrobial therapy for acute nosocomial *P. aeruginosa* pneumonia have not been performed, and available studies are complicated by issues involving differentiation between colonization and infection as well as defining *Pseudomonas*-attributable mortality. Most recent studies have focused on the treatment of *P. aeruginosa* ventilator-associated pneumonia (VAP) and will be discussed in Section 1.1.3.4. The standard treatment for *P. aeruginosa* pneumonia is similar to treatment for *P. aeruginosa* bacteremia, although the inclusion of IV administration of an aminoglycoside in combination therapy for fully susceptible organisms is arguable given that aminoglycosides are not optimally active in the lungs at the concentrations normally used with IV administration (235, 240, 314). Aerosolized antibiotics, particularly aerosolized tobramycin, have been developed to circumvent the issues relating to the poor activity of aminoglycosides in the lungs and to deliver higher concentrations of drug directly to the respiratory tract (186). The inhaled aminoglycoside tobramycin is commonly used for treatment of respiratory tract infections in patients with CF, however, trials for non-CF patients with *P. aeruginosa* bronchiectasis were not as successful (13, 75, 232). Its efficacy in the treatment of acute pneumonia has not been demonstrated to date in controlled prospective trials with the exception of a few preliminary studies related to VAP.

1.1.3.4   Ventilator-Associated Pneumonia

     P. aeruginosa is commonly found to be the first or second major pathogen causing VAP (141). It is the most common multidrug resistant pathogen involved in this disease and recovery rate of *P. aeruginosa* is increased with increased duration of mechanical ventilation. *P. aeruginosa* VAP infections are characterized by their late onset, and early antimicrobial therapy with agents lacking anti-pseudomonal activity may provide an added factor predisposing a patient to *Pseudomonas* infections. In addition to being amongst the most common pathogens causing VAP, *P. aeruginosa* is also amongst the most lethal pathogens, since reports suggest up to 70-80% mortality when the organism remains confined to the lungs (46), with directly attributable mortality rates reaching 38% (83).

     Antimicrobial treatment of VAP has been the subject of recent studies investigating the optimal length of treatment. A comparison of 8 versus 15 day antibiotic therapy for VAP found that patients treated for only 8 days did not have significantly higher mortality and in general did not have significantly higher rates of recurring infection. However, in patients suffering from VAP caused by non-fermenting Gram-negative bacteria, including *P. aeruginosa,* the shorter treatment length was associated with a higher rate of recrudescent infections (45), highlighting one of the major issues specific to *P. aeruginosa*, namely persistence. Despite appropriate treatment of these infections, *P. aeruginosa* has been isolated from the alveolar space 8 days after the onset of VAP (79) and relapsing infections by susceptible isolates has been seen at a rate of 18% (135). This persistence is in fact one of the main challenges of treatment of VAP and has recently been shown to be associated with the expression of the Type III secretion systems (65, 79), although it is likely that growth in a biofilm mode plays a role in persistence in the face of seemingly appropriate treatment.

     Recent studies have begun to assess the use of aerosolized tobramycin for the treatment of VAP and have focused on the use of aerosolized antimicrobials as a preventative measure, and as an adjunct to systemic therapy due to its efficacy in treating multidrug resistant organisms (58). Earlier studies of preventative use of aerosolized polymyxin B administered every 4 hours revealed that although overall rates of pneumonia were reduced, there was an increase in pneumonia caused by multidrug resistant organisms and that these were associated with higher mortality rates (88). More recent investigations of the preventative use of aerosolized antimicrobials such as tobramycin, ceftazidime, and colistin for VAP have demonstrated that these agents could reduce the occurrence of VAP without promoting the emergence of drug resistant organisms. However, there was no reduction in mortality in patients receiving these

measures (85). Thus standard therapy for VAP involves treatment with broad-spectrum systemic antimicrobials upon establishment of VAP.

The use of adjunct aerosolized therapy for VAP is of arguable benefit; however, in studies focusing on *Pseudomonas* species and *Enterobacteriaceae* species, reports of 96% clinical cure rates have been cited when combination systemic and aerosolized antimicrobial therapy were used in conjunction with selective topical gastrointestinal decontamination (298). Unfortunately, these investigations were limited by the lack of a control group and other supportive data is limited to isolated case reports of successful treatment with combinations of systemic and aerosolized antibiotics (211, 288, 297). Although there is evidence that inhaled aminoglycosides can achieve drug levels in the tracheobronchial tree capable of reducing the density of *P. aeruginosa* and diminishing inflammatory markers, evidence to indicate that the inhaled antibiotics can penetrate further into the distal lung parenchyma for effective treatment of acute pneumonia has yet to be presented (64).

1.1.4 *Pseudomonas aeruginosa* Infections in Cystic Fibrosis

CF is an autosomal, recessive, multi-organ disorder affecting 1:2,500 in the Caucasian population (258). Mortality in this afflicted population is mainly attributed to chronic respiratory infections and the associated gradual deterioration of lung function. There are several pathogens known to play a role in CF lung infection, with *Staphlylococcus aureus* and *Haemophilius influenza* being the predominant pathogens colonizing younger patients, and *Stenotrophomonas maltophilia*, *Burkholderia cepacia* complex and *Pseudomonas aeruginosa* in adults. However, *P. aeruginosa* is often isolated from patients less than 2 years of age and is the most predominant concern in adults (102, 155). The nature of this disease is important in understanding why *P. aeruginosa* dominates as the primary pathogen in CF patients and so host pathology is addressed below.

The defective gene involved in CF encodes for the CF transmembrane conductance regulator (CFTR) resulting in pathological changes in organs that express CFTR, including secretory cells, the liver, the pancreas and the lungs. In a normal airway epithelial cell, the gene encoding for CFTR regulates the transport of chloride, sodium and water. Abnormalities of the CFTR gene product lead to a thick and dehydrated mucous secretion that impairs mucociliary clearance of bacterial pathogens (54). In the normal lung, the mucus layers function in binding and clearance of inhaled pathogens, and although the bacterial load can be quite high in the upper airways, the lower airways remain free of bacteria (8). Due to the characteristic thickened

mucus associated with CF lung disease, and resulting inability of ciliary beating to remove this mucus, the invading pathogens become trapped in the mucus layer and constant presence of bacteria with pathogen associated molecular patterns (PAMPs) lead to chronic inflammation consequently damaging the epithelial surface (72, 118, 176).

P. aeruginosa airway colonization frequently occurs after initial airway colonization with *S. aureus* and subsequent antimicrobial therapy (148). *P. aeruginosa* thereby replaces the other bacterium becoming the predominant bacterium in the CF lung. *P. aeruginosa* infections in the CF lung may differ from the classical definition of infection wherein symptoms arise primarily as a result of an invading pathogen. Instead, CF infection is reflects a state of chronic colonization characterized by high bacterial load that ultimately triggers a prolonged inflammatory response (221). Furthermore, the CF lung is characterized by excessive inflammation both in the absence of infection and in response to pathogens (137, 165). Acute exacerbations of infection add further respiratory distress and the number of acute exacerbations is associated with patient mortality (191).

1.1.4.1   Adaptations Occurring During Chronic Infection

CF patients frequently become colonized in the upper airways by unrelated environmental isolates of *P. aeruginosa*, although epidemic isolates (e.g. the Liverpool epidemic strain) are also known (272, 290). During the process of infection a number of adaptations occur leading to the characteristic persistence and antibiotic resistance of isolates found from chronic infection. Amongst the most common adaptations of *P. aeruginosa* found in CF isolates, is the conversion to mucoid phenotype due to overexpression of alginate (115). Environmental isolates usually present a non-mucoid phenotype, as *P. aeruginosa* penetrates the thickened mucus lining of the airways, travelling down the oxygen gradient, increased expression of alginate and a switch to a mucoid phenotype occur (120, 293). This phenotype often occurs coincidently with the establishment of chronic infection, and becomes stabilized by regulatory mutations as described above. The mucoid form of *P. aeruginosa* is associated with 90% of *P. aeruginosa* CF infections compared to only 2% of *P. aeruginosa* non-CF infections (67, 69). This phenotype is often coordinately regulated with a loss of flagella by the alternative sigma factor AlgT (307). The loss of flagella not only causes loss of motility but also decreased activation of host inflammatory mediators (52).

Other easily identified morphological adaptations of *P. aeruginosa* include the switch from smooth to rough colony morphology and the appearance of small colony variants. The

rough colony morphology is representative of strains that have lost the LPS O-antigen (130). As the O-antigen is the immunodominant portion of the LPS, this adaptation leads to a less virulent phenotype. It also makes rough isolates more susceptible to complement killing and perhaps explains in part why these organisms virtually never cause invasive infections. Modifications to the Lipid A moiety of the LPS are also observed. These include the addition of palmitate, aminoarabinose and the retention of 3-hydroxydecanoate (81). The small colony phenotype is less well understood, but is of considerable interest as these isolates exhibit increased antibiotic resistance. This phenotype is observed as pinpoint colonies after two days of incubation at 37ºC, and has been associated with prior aminoglycoside therapy. Isolates exhibiting this phenotype have been found to be hyperpiliated with increased abilities in twitching and biofilm formation, and with decreased ability for swimming (136).

Another phenotype of relevance to antimicrobial therapy and resistance is the hypermutator phenotype, which is frequently observed in CF isolates, but less commonly in nosocomial isolates of *P. aeruginosa* (231). This phenotype, characterized by an up to 1000-fold increased mutation frequency, has been attributed to mutations in genes encoding DNA replication and repair mechanisms, such as *mutS, mutL* and *mutY*. The higher mutation frequency gives an added advantage for survival in the CF lung since it facilitates the aforementioned adaptations, by stabilizing these changes through mutations. Most importantly, these hypermutator isolates have the ability to develop mutational resistance more readily during a course of antimicrobial therapy than do non-mutator isolates (e.g. at frequencies of 10^{-6} instead of 10^{-9}). The hypermutator phenotype can give rise to a variety of mixed morphologies, including those described above, within the lung (147). These mixed populations can colonize or infect different compartments within the lung and often have differing antimicrobial susceptibilities and virulence properties (91, 157, 201, 234).

Comparison of *P. aeruginosa* isolates from the CF lung to strains from non-CF patients showed clearly that CF isolates tend to demonstrate an overproduction of β-lactamase, loss of OprD and an overproduction of MexXY. This efflux pump overproduction leads to high-level aminoglycoside resistance and the overproduction of this and other efflux systems also lead to quinolone resistance, amongst which MexCD-OprJ was the most frequent (139).

1.1.4.2   Antimicrobial Therapy for Treatment of *P. aeruginosa*

Chronic colonization and infection with *P. aeruginosa* is an inevitable reality for the majority of adults with CF, as over 80% of adults over the age of 18 years return positive

cultures for *P. aeruginosa* (143). It is the state of chronic lung infection and the resulting inflammatory response that lead to their gradual deterioration of lung function. Although these chronic infections are generally deemed impossible to eradicate, antimicrobial therapy has been used to effectively delay the onset of chronic infection and to decrease bacterial load, thereby reducing the deleterious effects associated with increased inflammation in established infections (301, 315).

Treatment strategies have been developed for prevention of colonization, for eradication of early stage colonization and for clearance of established infections (102, 313). Common practices for treatment of infections during these stages are outlined in Table 1.1. Although prevention of colonization seems an appealing strategy, prophylactic treatment for prevention has gained only modest support as a therapeutic option. In fact, prophylactic antimicrobial use was initially developed for prevention of *S. aureus* infection, but was found to be associated with increased acquisition of *P. aeruginosa* (257).

The strategies used for patients with early stage infections differs from those adopted for individuals with established chronic infections, as the organism changes and adapts to the lung environment. Nonetheless, the drugs of choice for treatment of *P. aeruginosa* colonization and infection are all bactericidal agents including β-lactams, aminoglycosides, fluoroquinolones and colistin (93, 192, 295, 301, 315).

P. aeruginosa isolates from CF patients frequently develop multi-drug resistance. Combination therapy can be used to avoid resistance development and to exploit the synergistic effects of the bactericidal antibiotics.

The use of aerosols allows for drugs to be delivered directly to the lung in CF patients and a number of antibiotics including gentamicin, tobramycin, colistin, ceftazidime, carbenicillin, aztreonam and amikacin have been administered as aerosols to CF patients, although approved formulations and adequate controlled studies have not been performed for most of these (144, 294). Aminoglycosides have several advantages for use as aerosols. They are highly polar, chemically stable, and less toxic than when administered intravenously. The most promising aminoglycoside currently used for inhalation is the tobramycin formulation TOBI (Chiron, CA, USA) that is capable of delivering a high dose of tobramycin to the lower respiratory tract of CF patients (47, 255). Gilead Science has recently developed an aerosolized formulation of the monobactam aztreonam and lysine (AZLI) (212). Clinical trials have demonstrated the efficacy of AZLI as an adjunct therapy to TOBI. AZLI appeared to improve lung function and was well tolerated by the patients. AZLI was not approved after its phase III

Table 1. **Empiric therapy for the treatment of *P. aeruginosa* infections in patients with cystic fibrosis**

	Antibiotic	Pediatric dose (>6 years)	Adult dose	Daily dosing interval	Route of administration	Duration of treatment
First isolation of *P. aeruginosa* (without clinical symptoms)	Ciprofloxacin	15 mg/kg	15 mg/kg	12 hr	Oral	3-4 weeks
	+					
	Tobramycin	300 mg	300 mg	12 hr	Inhalation	
	or Colistin	150 mg	150 mg	12 hr	Inhalation	
Pulmonary exacerbations	Ceftazidime	50 mg/kg	2 g	8 hr	IV	2-3 weeks or longer if no signs of improvement
	or Piperacillin	100 mg/kg	3 g	6 hr	IV	
	or Imipenem	15-25 mg/kg	0.5-1 g	6 hr	IV	
	or Meropenem	40 mg/kg	2 g	8 hr	IV	
	or Aztreonam	50 mg/kg	2 g	8 hr	IV	
	+					
	Tobramycin	3 mg/kg	3 mg/kg	8 hr	IV	
	or Amikacin	5-7.5 mg/kg	2 g	8 hr	IV	
Mild exacerbations	Ciprofloxacin	15 mg/kg	2 g	8 hr	Oral	2-3 weeks
Maintenance therapy	Tobramycin	300 mg	300 mg	12 hr	Inhalation	28 day on/off cycle
	or Colistin	150 mg	150 mg	12 hr	Inhalation	
	+/-					
	Ciprofloxacin	10 – 15 mg/kg	0.5-0.75 g	12 hr	Oral	2-4 weeks cycled every 3-4 month

Based on references (42, 102).

clinical trial (Sept 2008) and additional studies over a longer time period are required before FDA approval.

1.1.4.2.1 Antimicrobial Therapy for Colonization and Initial Infection with *P. aeruginosa*

Eradication of *P. aeruginosa* from the CF lung is possible only in the early stages of colonization. At this stage the bacterial load tends to be low, and the organism is non-mucoid and has not begun to undergo significant morphological changes. Aggressive antimicrobial treatment upon first isolation of *P. aeruginosa* has been demonstrated in most cases to delay and occasionally prevent the onset of chronic infections resulting in a better quality of life and a greater life expectancy (227, 264). Successful eradication is judged by the observation of at least three consecutive negative cultures at intervals of at least one month. After one year of negative cultures following the onset of antimicrobial therapy, any isolation of *P. aeruginosa* is

considered to represent a new isolate (102). Aggressive antimicrobial use at the early stage has proven in certain cases to be successful, with a number of patients having remained culture negative for *P. aeruginosa* for several years after treatment (93, 259).

A number of differing antimicrobial regimens have been used for treatment of early colonization of *P. aeruginosa*. While earlier regimens included the use of intravenous ciprofloxacin, or nebulized tobramycin or colistin (192, 295), more current recommended therapies typically use a combination of oral and aerosolized antibiotics (93, 315). These latter two studies used a combination of oral ciprofloxacin and aerosolized colistin administered twice daily over a 3-week period. During the 27 months observed, 80% of the treated group did not go on to develop chronic infections with *P. aeruginosa*. Unfortunately, most investigations to date have been limited by small sample size and lack of proper control groups. Furthermore, microbiological samples are generally taken from the upper respiratory tract that gives a poor prediction of the microbiology of the lower airways. The only controlled study using lower respiratory tract samples investigated the effect of inhaled tobramycin (300 mg) over a 28-day period, but was terminated early due to adverse affects (103).

1.1.4.2.2 Antimicrobial Therapy for Chronic Infections

Once chronic infection has been established by *P. aeruginosa*, antimicrobial therapy becomes complicated by the high bacterial load present in the lung, as well as the phenotypic changes occurring in the pathogen. The high bacterial load and thickened mucus are barriers to the attainment of sufficient exposure of the entire bacterial population to bactericidal concentrations of antibiotics (215). Administration of insufficient concentrations of antibiotics adds increased selective pressure for resistant phenotypes, thereby enhancing the diversity of the population, lending further difficulties to effective treatment (201, 234).

Antimicrobial therapy is used during chronic infections in CF for two main purposes: maintenance therapy and treatment of acute exacerbations of infection (73). Maintenance therapy is recommended for CF patients with chronic *P. aeruginosa* infections in order to reduce bacterial load and maintain overall lung function. Unfortunately, a number of side effects are associated with long term antimicrobial use including loss of hearing, increased cough, alterations of the voice, and the appearance of antibiotic resistant strains. The use of on/off cycles of intermittent drug administration led to the reduced occurrence of these side effects (255).

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Multiple studies have investigated the effect of intermittent administration of TOBI on lung function in CF patients (47, 255). In one study, patients were treated with 300 mg of TOBI twice daily in a cycle of 28-days on/28-days off (47). This treatment regimen led to a significant improvement in lung function and a reduction of *P. aeruginosa* in the sputum. Similar observations were made by Ramsey et al (1999) who showed that patients receiving inhaled tobramycin for four weeks, followed by four weeks without tobramycin, over a period of 24 weeks, had better lung function compared to the placebo group. Patients receiving tobramycin in this study were also less likely to be hospitalized (26%) than the placebo group (255).

Oral ciprofloxacin has also been studied for its use in maintenance therapy and has shown promising results regarding improved lung function. The risk of developing fluoroquinolone resistance in both *S. aureus* and *P. aeruginosa* favours its intermittent use, and as with tobramycin no longer than 4 consecutive weeks on ciprofloxacin is recommended (42).

Intermittent delivery of aerosolized colistin has also been used, particularly in Europe, although randomized placebo controlled studies are lacking. Colistin is generally regarded as a last resort option for isolates that are resistant to the common antibiotics. Colistin exhibits high activity against multi-drug resistant strains and resistance of *P. aeruginosa* to colistin rarely occurs (269).

Recent interest in macrolide antibiotics for the treatment of CF infections has been sparked by the findings that these agents are capable of acting in a direct antimicrobial manner, penetrating biofilms and interfering with quorum sensing signals in *P. aeruginosa* (308, 309), as well as acting as anti-inflammatories. Phase III trials using thrice weekly azithromycin have demonstrated promising results with regards to decreased occurrence of pulmonary exacerbations, with only mild side effects including nausea, diarrhea and wheezing (271).

The high bacterial load that can accumulate within the CF lung in patients suffering from chronic infections with *P. aeruginosa* can cause frequent acute exacerbations characterized by strong inflammatory responses. Antimicrobial treatment can effectively reduce bacterial load and reduce the inflammatory response. Limited data is available for evaluation of effective treatments of pulmonary exacerbations and the results of antibiotic susceptibility profiling play an important role in guiding treatment. The use of aerosolized antimicrobials in the treatment of acute exacerbations requires further investigation (42, 102).

Mild exacerbations during chronic lung infections can typically be treated in an outpatient setting using oral ciprofloxacin (42). Moderate and severe exacerbations, however, require intravenous administration of two antimicrobial agents, generally, an antipseudomonal β-

lactam and either tobramycin or amikacin, which are recommended for use in combination for 2-3 weeks (284). While severe symptoms require the patient to be hospitalized to permit monitoring of renal function and for adjunct respiratory therapy, it is becoming more common to treat moderate exacerbations at home (42).

1.2 Aminoglycosides

1.2.1 Tobramycin Structure and Activity

Tobramycin belongs to a class of polycationic antimicrobial agents, the aminoglycosides. This class encompasses a number of structurally diverse, naturally produced and semi-synthetic derivative compounds composed of one or several aminated sugars joined to a dibasic cyclitol (ring II in Figure 1.3) via glycosidic linkages. The dibasic cyclitol of streptomycin, the first isolated aminoglycoside, is streptidine, however, in most clinically used aminoglycosides, including tobramycin, it is a 2-deoxystreptamine structure. The 2-deoxystreptamine aminoglycosides can be further divided into group based on 4,5 disubstitution of the deoxystreptamine as is seen for neomycin and paromomycin versus the 4,6 disubstituted structure of kanamycin, gentamicin and tobramycin (332). Compounds with the latter conformation have been found to have greater antibacterial activity. Tobramycin is the 4,6 disubstituted structure seen in Figure 1.3.
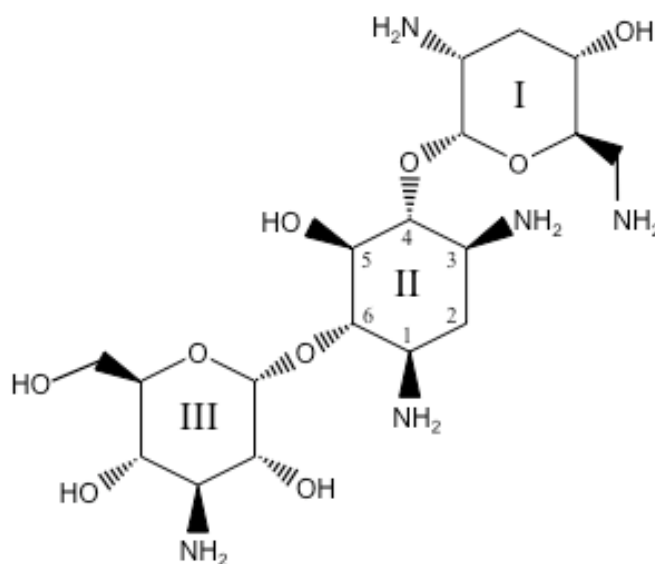
Figure 1.3   Chemical structure of tobramycin. Ring II illustrates the 4,6 disubstituted dibasic cylytol moiety.

As with other aminoglycosides, the antimicrobial activity of tobramycin is attributed to its ability to impair protein synthesis. It has been demonstrated that despite structural diversity, the aminoglycosides paromomycin and tobramycin bind a conserved sequence of rRNA in proximity to the site of codon–anticodon recognition in the aminoacyl tRNAsite (A site) of the 30S ribosomal subunit (90, 321). Upon binding of the aminoglycosides, tRNA–mRNA dissociation rate is reduced thereby interfering with the proofreading process and translational fidelity (164). Interestingly, this interaction does not explain the potent bactericidal activity of the aminoglycosides observed against Gram-negative organisms, as not all aminolgycosides are bactericidal nor are other inhibitors of protein synthesis, such as chloramphenicol and tetracycline, which exhibit only bacteriostatic activity.

Timing of events unfolding during bacterial killing by aminoglycosides varies in a concentration dependent manner (16). Uptake of these agents into the cell occurs in three distinct stages beginning with their passage across the outer membrane of the cell in a process known as self-promoted uptake (125, 129). In this first stage, the polycationic molecules competitively bind to Mg^{2+} binding sites on the bacterial LPS, thus disrupting bridges between adjacent LPS molecules, destabilizing the outer membrane and further promoting their uptake into the periplasmic space. From the periplasmic space aminoglycosides are actively transported across the cytoplasmic membrane in a process dependent on both electron transport and the proton motive force (38, 40). Once inside the cell, the bactericidal activity of aminoglycosides has been ascribed to mistranslated proteins inserting into the cytoplasmic membrane or possibly inhibiting the initiation of DNA synthesis at a protein synthesis dependent step causing dissociation of the replication origin from the membrane (60, 62, 111).

1.2.2 Mechanisms of Aminoglycoside Resistance

Aminoglycoside resistance occurs predominantly as a result of either decreased accumulation inside the bacterial cell or by aminoglycoside modification. Decreased accumulation can be attributed to changes in membrane permeability or upregulation of chromosomally encoded efflux pumps contribute to both intrinsic and adaptive resistance, and typically leads to moderate increases (2-8 fold increases in MIC) in aminoglycoside resistance (124, 225). High-level aminoglycoside resistance in *P. aeruginosa* is often caused by the presence of aminoglycoside modifying enzymes found on mobile genetic elements (243). Three major classes of modifying enzymes exist, including the aminoglycoside acetyltransferases (AAC), the nucleotidyltransferases (ANT) and the phosphoryltransferases (APH) (281). The

individual enzymes in each class are highly specific in their activity, affecting only specific subsets of aminoglycosides and no individual enzyme acts on all aminoglycosides. Nonetheless, the occurrence of strains possessing multiple enzymes is not uncommon and so the combined effect of the enzymes can inactivate a broad range of aminoglycosides. Recently, ribosomal methylating enzymes RmtA and RmtD have been demonstrated to cause high-level resistance to all aminoglycosides in *P. aeruginosa* (70, 71, 331).

Aminoglycoside resistance due to altered membrane permeability can be divided into mechanisms that affect the outer membrane, typically LPS modifications, and mechanisms that affect cytoplasmic membrane permeability. Regulation of LPS modifications occurs via the two two-component regulators PhoPQ and PmrAB in response to various stimuli including the presence of divalent cations and antimicrobials peptides (213, 214, 220). The genetic basis for the LPS modifications and regulatory systems will be discussed in further detail in section 1.4. Mechanisms affecting cytoplasmic membrane permeability are poorly understood; however it is generally accepted that decreases in membrane potential are the cause of decreased uptake across the cytoplasmic membrane. Support for this belief comes from early studies of aminoglycoside uptake and lethality, which demonstrated that inhibitors of electron transport were capable of antagonizing activity of streptomycin (40, 123). Additionally, work by Bryan *et al.* selected for aminoglycoside resistant mutants that were deficient in nitrite reductase and aerobic transport (37, 39).

RND efflux pumps play a major role in contributing to intrinsic, adaptive and mutational aminoglycoside resistance in *P. aeruginosa.* Though the *P. aeruginosa* genome encodes a number of these tripartite efflux systems, only the MexXY linker pump encoded by PA2019 and PA2018 respectively (also known as AmrAB and MexGH) is the only one to have been implicated in efflux mediated aminoglycoside resistance (162, 209, 218, 287). Unlike other well-studied RND efflux pumps such as MexAB-OprM, the cognate outer membrane pore component of this tripartite pump is not transcribed as an operon with the MexXY linker pump. It has been demonstrated that OprM is capable of functioning together with MexXY to form an operative tripartite pump effectively effluxing aminoglycosides (195, 209); however, it remains unknown as to whether this is the primary pairing of the linker pump *in vivo* and recent investigations have shown compatibility of OpmG with MexXY as well (50). Constitutive expression of MexXY contributes to a moderate level of intrinsic aminoglycoside resistance with knockouts of the individual pump components causing 2-4 fold increases in aminoglycoside susceptibility (209). The promoter region of *mexXY* is negatively regulated by MexZ (AmrR), a

TetR type transcriptional regulator that is divergently transcribed from *mexXY* (209, 210, 330). Induction of the *mexXY* operon, contributing to adaptive resistance, has been demonstrated in response to many of the antibiotic substrates of the MexXY pump, although induction is limited to those antibiotics that adversely affect ribosomal function (210). Direct interaction of the antibiotics with MexZ does not occur. Recently, a hypothetical gene, PA5471, has been demonstrated to play a role in relieving the negative regulation imposed by MexZ (217). Loss of function mutations in MexZ lead to constitutive up-regulation of *mexXY* and are amongst the most common aminoglycoside resistance mutations identified from *P. aeruginosa* isolates obtained from cystic fibrosis patients suffering from chronic infection (285).

1.3   The Polymyxins

1.3.1   Polymyxin Structure and Activity

The polymyxins comprise a group of closely related cationic polypeptide antibiotics with potent selective gram-negative activity. In 1947, the first polymyxins, polymyxin A and polymyxin D, were isolated from *Bacillus aerosporus* and *Bacillus polymyxa* respectively (1, 292, 322). Polymyxin B and polymyxin E (also known as colistin) were also isolated from different strains of *B. polymyxa* and were further developed for clinical use as antimicrobial agents used for treatment of gram-negative infections (322). The only difference between these latter two agents is at position 6, in polymyxin B this is occupied by d-phenylalanine and in colistin is d-leucine. Use of these agents has been limited predominantly to topical and intravenous use and more recently aerosolized use, as they are not absorbed through the gastrointestinal tract. Additionally, the high levels of neurotoxicity and nephrotoxicity exhibited by the polymyxins further restrict their use, making these agents a drug of last resort for multidrug resistant gram-negative infections (84).

The structures of most polymyxins, including the clinically relevant polymyxins, consist of a decapeptide containing a seven-membered ring with an α-connected side-chain ending with a fatty acid (322). The structures of polymyxin B is shown in Figure 1.4. Polymyxins are considered amphipathic in structure and the mode of interaction with the outermembrane follows the same principles as that of other amphipathic antimicrobial peptides (318). Briefly, the positively charged Dab residues of polymyxins interact with the negatively charged phosphate groups of the Lipid A displacing divalent cations, which normally stabilize adjacent LPS molecules (126, 251). This interaction brings the N-terminal hydrophobic fatty acyl chain of the
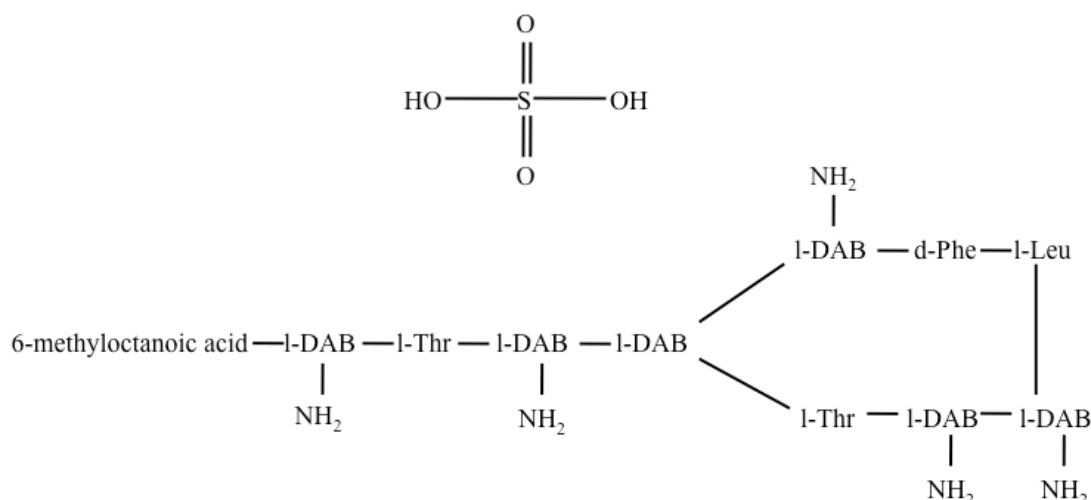
Figure 1.4   Chemical structure of Polymyxin B. Polymyxin B is formulated as a sulfate salt as depicted here. DAB, $\alpha,\gamma,$diaminobutyric acid; Thr, threonine, Phe, phenylalanine; Leu, leucine.

polymyxins in proximity of the outermembrane into which it inserts itself and physically disrupts the stability of the membrane. This destabilization allows for additional uptake of polymyxin which then inserts and disrupts the phopholipid bilayer of the inner membrane.

1.3.2   Polymyxin Resistance in *P. aeruginosa*

LPS modifications, specifically the addition of aminoarabinose to the Lipid A moiety in *P. aeruginosa*, have been identified in CF isolates and the presence of this modification is strongly associated with resistance to colistin (81). These modifications are capable of conferring resistance not only to the polymyxins but also to aminoglycosides and to other antimicrobial peptides including defensins by reducing the net negative charge of the Lipid A thereby reducing the binding affinity for polymyxins (199, 213, 214). Spontaneous polymyxin B resistant mutant strains of *P. aeruginosa* have been generated *in vitro* and mutations were mapped to the histidine box of PmrB (220). Resistance in these mutants was attributed to the addition of aminoarabinose to the Lipid A component of the LPS.

The *arnBCADTEF*-PA3559 operon encodes the genes for the addition of the aminoarabinose to the Lipid A. Regulation of the expression of this operon occurs through the activities of the two-component regulatory systems PmrAB and PhoPQ (109). Regulation of the *arn* operon has predominantly been studied in response to Mg^{2+} limiting conditions, as PhoPQ is known to be induced under these conditions (213); however, cationic antimicrobials have also

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been shown to induce both PhoPQ and PmrAB to varying degrees (214).

1.4   PhoPQ and PmrAB Two-Component Regulatory Systems

Two-component regulatory systems are sensory regulatory phospho-relay systems comprised of a cytoplasmic membrane bound sensor kinase (PhoQ and PmrB) and a response regulator (PhoP and PmrA) (109). Typically, the sensor kinase autophosporylates at a conserved histidine residue and then transfer of the phosphate to a conserved aspartate residue in the response regulator propagates the signal by allowing the DNA binding regulator to adopt a conformation favorable for binding the regulatory regions of interest. The PhoPQ system was first identified for its role in regulating the expression of a nonspecific acid phosphatase, PhoN (167), although it is in fact involved in regulation of a number of genes and a much broader role in both virulence and antimicrobial resistance has since been established (108, 109, 214). In *P. aeruginosa,* PhoPQ is encoded as an operon with OprH (*oprH-phoPQ)*. Under Mg^{2+} limiting conditions PhoP is phosphorylated and activates transcription of *phoPQ* from a promoter upstream of *oprH*. At the same time, PhoP also activates transcription of the *arnBCADTEF*-PA3559 operon thus mediating resistance to cationic antimicrobials. Similarly, PmrA regulates transcription of the PA4773-PA4775-*pmrAB*-PA4778 and *arnBCADTEF*-PA3559 operons in response to limiting Mg^{2+}.

In addition to regulation of LPS modifications by PhoPQ and PmrAB, the contribution of another kinase is suspected to be involved. As in the absence of PhoQ, PhoP has been demonstrated to be capable of activating a high level of *oprH–phoPQ* expression under both high- and low-Mg^{2+} conditions and resistance to cationic antimicrobials is observed (200). Furthermore, the complementation of PhoQ restored regulation and susceptibility. This would indicate that PhoP is active (ie. phosphorylated) in the absence of PhoQ thus suggesting a second kinase activates PhoP in *P. aeruginosa*, and is capable of phosphorylating PhoP regardless of the presence of Mg^{2+}. In *P. aeruginosa*, there are 64 predicted response regulators, 63 predicted histidine kinases, and 16 atypical kinases. The functions of the majority of these have yet to be established, and their roles in the phosphorylation of PhoP are currently being investigated in our lab.

1.5   Lon Proteases

1.5.1   Structure, Activity and Genetics of Lon Proteases

Lon proteases are widespread in many organisms from archaea and bacteria to humans. They belong to the diverse superfamily of AAA+ (ATPase Associated with cellular Activities) proteins (222). The AAA+ proteins were initially classified based on their ATPase domains containing conserved Walker A and B motifs (179, 323). Activities of this family of proteins typically involved facilitating energy dependent post-translational events such as protein folding and unfolding, complex assembly and disassembly, protein transport and protein degradation. More recently, the diversity of function of the AAA+ protein superfamily revealed many members were involved in DNA replication, recombination, repair and transcription (222). Furthermore not all AAA+ proteins necessarily contain the ATPase domain but typically form complex structures with other members that provide the ATPase domain (230). Lon is considered to be part of the AAA+ protease subfamily and can be further subdivided into LonA and LonB type classification based on structural differences (266). AAA+ proteases are oligomeric barrel-shaped structures with a chaperone ring containing the ATPase domain responsible for substrate binding, and a proteolytic core (28, 29). In both the LonA and LonB type proteases, the ATPase domain and the proteolytic domain are encoded on a single polypeptide. In addition to these two key domains, LonA type proteases contain a large N-terminal domain, while LonB type proteases possess a large transmembrane domain situated between the Walker A and B motifs but do not possess the N-terminal domain (266). The *E. coli* Lon protease is among the best-studied bacterial Lon proteases and is a LonA type protease. Further discussion of Lon proteases in this thesis will be in reference to LonA type proteases.

The oligomeric structure of bacterial Lon proteases have been reported to contain between four and eight subunits (106); however the most recent data suggests the *E. coli* Lon protease exists predominantly as a hexamer (187, 236). It has been speculated that the N-terminal domain is involved in substrate recognition, as deletion of this domain was shown to reduce or eliminate proteolytic activity and individual mutations were capable of enhancing substrate affinity for one substrate while decreasing affinity for another. The active site in the proteolytic domain of *E. coli* Lon protease has been identified as a Ser-Lys dyad (Ser679 and Lys722) (29). Peptidase activity in this domain does not require ATPase activity, however, proteolysis is significantly reduced in the absence of ATP binding and hydrolysis (256). This is consistent with the suggestion that ATP binding and hydrolysis is required for conformational

changes in either or both the protease and the substrate that would allow access of the substrate to the active site contained inside the pore of the barrel structure (265).

In addition to the proteolytic activity of Lon proteases, Lon is capable of sequence specific DNA binding (96). Although its role as a DNA binding protein has not been investigated in many studies, early investigations suggest involvement of *E. coli* Lon in transcriptional regulation of the galactose (*gal*) operon (41).

The *lon* gene of *E. coli* is transcribed as part of an operon with another housekeeping ATP-dependent protease, *clpXP*. This operon configuration is conserved throughout a number of species including *P. aeruginosa*. Though a few species such as *Streptococcus pneumonia, Staphylococcus aureus* and *Listeria monocytogenes* lack a *lon* orthologue (265), several organisms possess two Lon-type proteases as is the case for *Bacillus subtilis, Myxococcus xanthus, Borrelia spp.* and *Pseudomonas spp* (53, 156, 317). The second Lon-type protease in *P. aeruginosa* is encoded as a single gene PA0779 and shares 41% identity with the Lon protease (PA1803).

1.5.2   Role of Lon Protease in Stress, Pathogenesis and Motility

The *E. coli* Lon protease has long been recognized for its involvement in various stress responses including heat shock, UV and ionizing radiation, antibiotic and oxidative stress, and bacteriophage lysogeny (114, 119, 224, 242). Mutants in the *lon* gene have shown characteristic phenotypes such as increased mucopolysaccharide production and defective cell division resulting in filamentation (113, 114). Lon proteases are responsible for degrading damaged, misfolded and mistranslated proteins (121), but it is the stabilization and degradation of regulatory proteins by Lon that predominantly leads to the described phenotypes in the Lon deficient mutants. The *E. coli* Lon protease recognizes specific sequences on the stress-related regulatory proteins SulA (cell division regulator), SoxS (oxidative stress regulator), MarA (multiple antibiotic resistance regulator), RcsA (regulator of capsule synthesis) and UmuD (error-prone polymerase) (78, 107, 158, 280). Recognition of aberrant proteins by *E. coli* Lon occurs through recognition of the SsrA sequence tag attached to truncated proteins (49), and aromatic regions that are exposed on improperly folded proteins (121).

Roles for Lon proteases in the regulation of virulence factors in several Gram-negative organisms have been characterized including involvement in Type III secretion and in quorum sensing. In both *Salmonella enterica* serovar Typhimurium and *Pseudomonas syringae,* positive regulators of Type III secrection effector molecules are stabilized in Lon deficient mutants (24,

34). In contrast in *Yersinia pestis*, Type III secretion is repressed in the absence of Lon due to stabilization of the YmoA transcriptional repressor of Type III secretion that is normally degraded by Lon (159). Involvement of Lon in quorum sensing was demonstrated in both *P. aeruginosa* and *Pseudomonas putida* (20, 303). In *P. aeruginosa* both LasI and RhlI were stabilized in a Lon deficient mutant (303). Other complex adaptations in *P. aeruginosa* that are subject to regulation by Lon are biofilm formation and swarming motility and to a lesser extent swimming and twitching motility, all of which are reduced in Lon mutants (206). The *P. aeruginosa* Lon targets involved in the regulation of these adaptations are unknown.

In several organisms studied that possess a second Lon-type protease, including *B. subtilis, M. xanthus* and *B. burgdorferi,* the second Lon protease has been shown to be involved in life cycle differentiation (104, 279). *B. subtilis* and *M. xanthus* Lon mutants are defective in sporulation. In *B. burgdorferi* this alternate Lon (Lon-1) is induced by blood supporting a role in the mammalian host stage of this arthropod-borne pathogen (53).

In *P. aeruginosa*, the role of the second Lon-type protease, PA0779, in regulating nitrosative and oxidative stress has recently been investigated. Evidence suggests a role for PA0779 in modulating NO (nitric oxide) and ROS (reactive oxygen species) detoxification through binding (either directly or indirectly through protein-protein interactions) the intergenic region between the divergently transcribed flavohemoglobin regulatory gene (*fhpR*) and the flavohemoglobin gene (*fhp*) (177, 178). Post-translational control of NO levels by PA0779 is also suggested as reduced activity of nitrite reductase (NIR) and nitric oxide reductase (NOR) was observed in a PA0779 mutant despite mRNA levels remaining constant (177). The PA0779 protease mutant did not display any biofilm or motility defects observed for the PA1803 Lon protease of *P. aeruginosa* (177).

1.6   Rationale and Objectives

Despite the contrasting nature of *P. aeruginosa* pathogenesis observed in CF and nosocomial infections, there is common ground when faced with a diagnosis of *P. aeruginosa* infection. Aggressive antimicrobial therapy is essential. Cationic antimicrobials, particularly the aminoglycosides, form the foundation of most anti-pseudomonal regimens, and as multidrug resistance continues to rise, many countries have become reliant on the cationic polymyxins as drugs of last resort. These two classes of antimicrobials share common initial binding sites on the outermembrane and as such can be affected by common mechanisms of resistance.

Though acquired mechanisms of resistance leading to high-level aminoglycoside resistance are generally well understood, few mechanisms of adaptive resistance have been identified to date. Given that cationic antimicrobials such as the aminoglycosides and the polymyxins exert pleiotropic effects on the cell, it is likely that a number of factors can contribute to small increases in antibiotic resistance to these agents. In this thesis, chapters 2 and 3 focus specifically on indentifying novel contributors to aminoglycoside resistance, the 4th chapter focuses on identifying the roles of PhoPQ and PmrAB in adaptive resistance to cationic antimicrobials in nosocomial isolates exhibiting an adaptive resistance phenotype.

1.6.1 Hypothesis

In response to cationic antimicrobials, *P. aeruginosa* employs a set of complex adaptations to mediate antimicrobial resistance.

1.6.2 Overall Aim

The overall aim of this project was to investigate the mechanisms by which adaptive resistance to aminoglycosides and polymyxins occurs in *P. aeruginosa*.

1.6.3 Objectives

1. Identify potential genetic contributors to low-level aminoglycoside resistance.
2. Determine if low-level aminoglycoside resistance is associated with a particular phenotype.
3. Investigate the bacterial responses to lethal, inhibitory and sub-inhibitory concentrations of aminoglycosides.
4. Investigate the role of the alternate Lon protease encoded by PA0779 in the bacterial response to tobramycin.
5. Investigate the roles of PhoPQ and PmrAB in polymyxin adaptive resistance of nosocomial isolates of *P. aeruginosa*.

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2.0 Novel Genetic Determinants of Low-level Aminoglycoside Resistance in *Pseudomonas aeruginosa*[2]

2.1 Introduction

The majority of deaths in cystic fibrosis patients can be attributed to the progressive deterioration of lung function resulting from chronic infection by pathogens such as *Pseudomonas aeruginosa* (26). Antibiotic treatment of such chronic infections may temporarily suppress symptoms; however, they do not eradicate the pathogen. To overcome the inability of orally and parenterally administered antibiotics to adequately penetrate the lung tissue and secretions, the aerosolized aminoglycoside tobramycin was formulated to directly target the site of infection. Clinical trials of aerosolized tobramycin revealed that long term use of this agent against *P. aeruginosa* results in small, graduated increases in tobramycin MIC upon repeated isolation of the organism (21, 23), although the mechanisms contributing to this resistance have yet to be described.

Tobramycin is a bactericidal agent that targets the 30S ribosome and interferes with protein synthesis. Uptake occurs in three phases: an initial, reversible, ionic binding phase, a phase of slow energized uptake and finally a phase of very rapid energy-dependent uptake (3, 5, 9). Despite the traditionally held view that antibiotic action can be simplified to interaction with a single target, it is evident that aminoglycosides exert pleiotropic effects on the cell (8, 9) and these effects can be antagonized by a variety of compounds known to affect particular cellular metabolic processes. Furthermore, the bactericidal nature of aminoglycosides such as tobramycin cannot be accounted for simply by protein synthesis inhibition or misreading during translation as other protein synthesis inhibitors, such as chloramphenicol, and agents that promote misreading, including modified amino acids, are bacteriostatic. A limited number of cytochrome mutations leading to aminoglycoside resistance have previously been identified by selecting mutagenized *P. aeruginosa* on aminoglycoside containing media (2, 4). As aminoglycosides exert a number of effects on the bacterial cell, we believe the potential sites for development of resistance are numerous. The purpose of this study was to identify the extent of the tobramycin "resistome", i.e. potential genetic contributors to the graduated development of aminoglycoside

resistance in *P. aeruginosa* using a comprehensive mutant library for a tobramycin resistance screen.

2.2   Materials and Methods

2.2.1   Bacterial Strains

The PA14NR mutant library set described by Liberati *et al* (15) was used for screening genes related to tobramycin resistance. This mutant library comprises approximately 5,800 mutants representing around 4,600 genes. It was constructed using a mariner-based transposon, MAR2xT7, containing the resistance cassette *aacC1,* which confers resistance to the aminoglycosides gentamicin, astromicin and sisomicin, but not to tobramycin. In addition, two PAO1 mutant libraries, the mini-Tn5-*luxCDABE* mutant library described by Lewenza *et al* (14) and the University of Washington transposon mutant library (11), were used for verification and cross-referencing purposes.

2.2.2   Tobramycin Resistance Screening

PA14 mutants were inoculated into 100 μl of Mueller-Hinton broth (MHB) in 96-well plates and incubated at 37°C overnight. Overnight cultures were diluted 1:100 into MHB and were replica plated onto MH agar containing 0.5 μg/ml tobramycin (the MIC of the wild type strain under these conditions). Growth was assessed at 24 and 48 hours. Resistance was defined as a two-fold or greater increase in MIC compared to the parent strain PA14. MICs at 24 and 48 hours were determined in at least triplicate using microbroth dilution in cation-adjusted Mueller-Hinton broth (CAMHB) according to CLSI protocols (22).

2.2.3   Tobramycin Kill Curves

Kill curves were performed in triplicate, in 96-well plates to simulate the MIC conditions, using selected mutants representing the major gene class functions identified in the screen. Cultures were grown in CAMHB at 37°C to an OD_{600} of 0.4, and 1.25 ml were harvested by centrifugation. Pellets were resuspended in 1 ml CAMHB and then diluted 1:50. Fifty μl of each sample were inoculated into the wells of 96-well plates each containing 50 μl of a 4 μg/ml tobramycin solution yielding a starting inoculum containing approximately 3-4 x 10^6 cfu/ml in 2 μg/ml tobramycin. Separate 96-well plates were inoculated for each time point, and aliquots were plated at 0, 10, 20, 30, 40, 50 and 60 min. Plates were incubated at 37°C overnight and colony counts were performed to obtain cfu/ml at each time point.

2.2.4 Determination of Growth Rate

Growth rates were determined for all tobramycin resistant mutants related to energy metabolism. These mutants were inoculated and grown overnight in 3 ml CAMHB at 37°C. Two μl of each overnight culture were inoculated in 200 μl CAMHB and growth at 37°C was monitored using a TECAN Spectrafluor Plus by measuring the OD_{620} every 20 min.

2.2.5 Assessment of Aerobic and Anaerobic Growth Defects

Cell suspensions were prepared to a 0.5 McFarland standard in LB broth and diluted 1:20 into 200 μl of each LB (aerobic) and LB containing 15 mM KNO_3 (anaerobic) in a 96-well microtitre plate. Anaerobic plates were placed in an anaerobic jar containing a GasPak^{tm} Plus hydrogen and carbon dioxide generator envelope. All plates and jars were incubated at 37°C for 24 hours. The optical density (OD_{600}) of the mutants was measured at 600 nm in the 96-well plates and compared to the OD_{600} of PA14.

2.2.6 Tobramycin Outer Membrane Interaction Studies

The hydrophobic fluorescent probe 1-*N*-phenylnaphthylamine (NPN) was used as described by Loh *et al.* (16) to study the interaction of tobramycin with the outer membranes of the tobramycin resistant LPS mutants compared to PA14. Briefly, a 50 ml sample of mid-log phase cells was harvested by centrifugation at 3,000 *x g* for 10 min and resuspended in 5 mM sodium HEPES buffer (pH 7.2), with 5 μM CCCP, at an optical density at 600 nm of 0.5. NPN was added to a final concentration of 10 μM and baseline fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm using a Perkin Elmer LS 50B fluorescent spectrophotometer. Tobramycin was added to final concentrations of 1, 5, 10, 20, 40 and 60 μg/ml and increases in fluorescence were recorded.

2.2.7 Cloning of *cycB* Encoding Cytochrome c_5

To complement the PAO1 mutant strain *cycB*::*lux* and the PA14 *cycB* (PA5300) mutant, a PCR amplicon was generated using Phusion High-Fidelity DNA Polymerase (Finnzymes) from PAO1 genomic DNA. The amplicon was then ligated into the high copy number, broad-host-range plasmid vector pUCP18 and transformed into *E. coli* strain XL1BlueMRF'. Plasmids were isolated (QIAGEN) and transformed into both mutants as well as the wild types PAO1 and PA14 by electroporation (6).

2.2.8 Cloning of *radA*

To complement the PA14 *radA* mutant genomic DNA of *P. aeruginosa* PA14 was used as a template to amplify the whole *radA* gene using the Phusion High-Fidelity DNA Polymerase (Finnzymes). The PCR fragment was subcloned using the Topo Zero Blunt kit (Invitrogen). The vector was cut with XbaI and HindIII (Invitrogen) and the radA fragment was cloned into the low-copy number vector pBBR1MCS-1 (13) and transformed into *E. coli* Top10 (Invitrogen). Plasmids were isolated (Qiagen) and electroporated into the PA14 *radA* mutant as well as into the wildtype PA14 (6)

2.3   Results and Discussion

2.3.1   Tobramycin Resistance Screening and Confirmation

Screening of a pre-existing transposon mutant library allowed for the identification of a large number of potential genetic contributors to tobramycin resistance that have not been identified using the traditional method of selecting for resistance using randomly mutagenized *P. aeruginosa* on antibiotic media. This tobramycin resistance screen identified a total of 348 mutants with reduced susceptibility compared to the PA14 parent strain. MICs performed using broth microdilution confirmed that 186 of these mutants had an MIC at least two-fold greater than the MIC of the parent strain PA14. These 186 mutants represented a total of 135 genes as depicted in Figure 2.1. A major benefit of this type of screen is that even small changes in resistance may be detected. Here the highest MIC found in the screen was an eight-fold increase compared to PA14 for the *mutL* mutant (MIC of 4 μg/ml vs. 0.5 μg/ml for the wild type). Additionally, 21 of the mutants identified had four-fold increases in MIC while the remaining 165 mutants had a two-fold increase in MIC. Although two-fold changes in MIC are typically considered within the acceptable range of error, we were able to confirm the increased tobramycin MIC in nearly all of these mutants in at least duplicate either due to the redundancy of the PA14 mutant library or by the availability of corresponding mutants in either the mini-Tn5-*luxCDABE* library or the PAO1 University of Washington library. Of the 135 genes found in the PA14 screen, 44 genes had redundant mutants within the PA14 library, another 31 genes were available in corresponding mutants in the PAO1 mini-Tn5-*luxCDABE* library and four genes had corresponding mutants in the PAO1 University of Washington library. In total 70 genes were confirmed to have increased tobramycin resistance in at least two independent mutants. An additional 60 genes that were not represented by more than one mutant belonged to

Figure 2.1 Distribution of genes identified in the tobramycin resistance screen around the PAO1 genome.
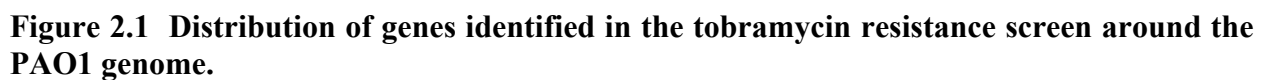
operons in which other mutants had confirmed resistance. Only six genes were not confirmed in duplicate nor belonged to an operon. In addition, wildtype tobramycin susceptibility in both the PAO1 *cycB* and PA14 *cycB* mutants was restored upon complementation. The *cycB* gene encodes for cytochrome c_5 and was selected for complementation as a representative of energy metabolism mutants.

2.3.2 Classification of Tobramycin Resistant Mutants Using PseudoCAP

Although two-fold changes in MIC are typically considered within the acceptable range of error for this assay, the study of El'Garch *et al* not only confirmed the reliability of two fold changes, but also demonstrated that independent mutants could act in a combinatorial fashion (7). Furthermore, slight increases in MIC may not only tip the MIC above the clinical breakpoint but may also be indicative of a genotype with enhanced adaptability to stress conditions such as exposure to antibiotics.

This enhanced adaptability has been observed in CF infections, where the isolation of antibiotic resistant strains with a hypermutator phenotype due to the presence mutations in DNA mismatch repair systems, particularly in *mutL*, *mutS* or *uvrD*, has previously been reported (18, 19). A number of isolates with unknown genotypes have also been found to exhibit this hypermutator phenotype. Similarly, mutations in regulatory genes are known to lead to a cascade of effects ultimately causing adaptive resistance (20). When the mutants exhibiting reduced susceptibility to tobramycin were categorized according to their PseudoCAP functional class according to www.pseudomonas.com (Figure 2.2), 7 regulatory genes were identified along with 8 distinct mutants known to be involved in DNA replication and repair processes, including mutants in *mutL*, *mutS*, *mutY*, *micA*, *mfd*, *nth*, *uvrA* and *radA*. Several of these mutants were also observed in similar antibiotic resistance screens performed in our lab (1, 27) as mutations in these genes promote the generation of secondary mutations capable of conferring resistance.

The most notable observation from the classification of the tobramycin resistant mutants was, however, the large proportion of energy metabolism mutants. To rule out decreased growth as the reason for reduced tobramycin susceptibility, all 186 mutants were assessed for major growth deficiencies using an endpoint OD_{600} at 24 hours as a measure for yield of growth. Of 186 mutants assayed, only mutations in 25 genes, comprising 10 individual genes and three operons (Table 2.1), were found to cause a significant reduction in growth yield under either aerobic or anaerobic conditions or both. Additionally, growth rates were determined for all energy metabolism mutants. With the exception of mutants belonging to the PA4429-PA4431
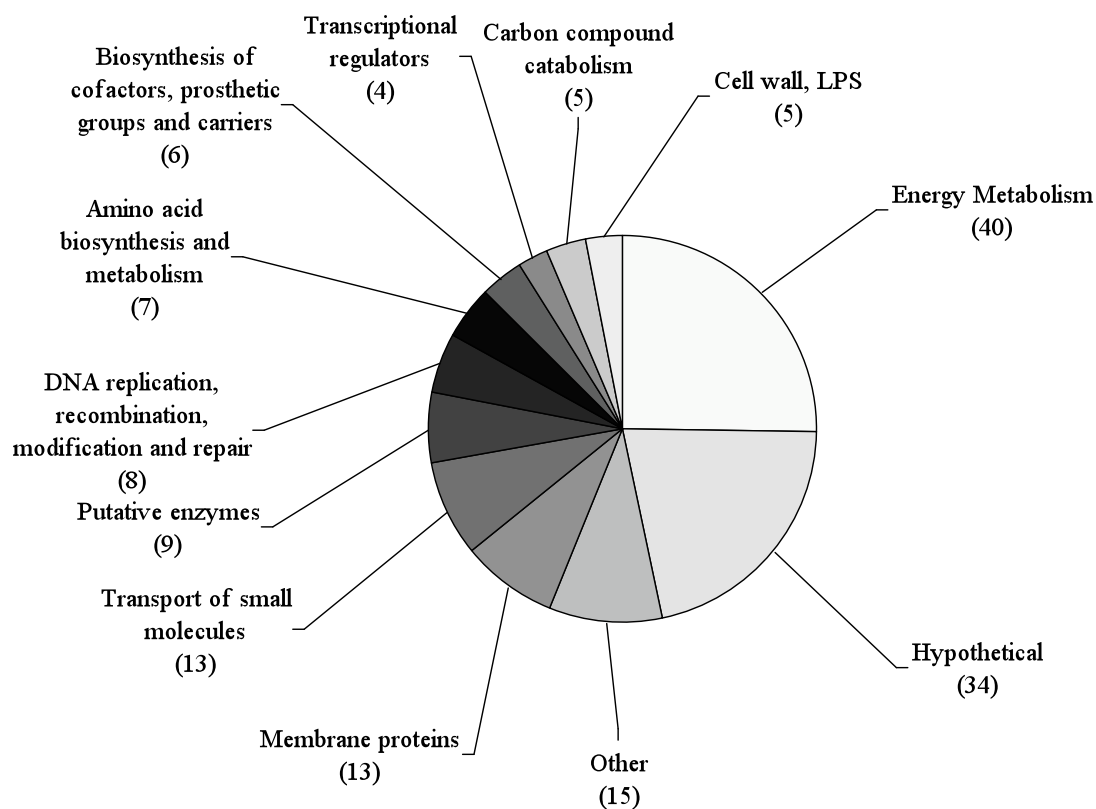
Figure 2.2 Distribution of the 135 genes found to be involved in tobramycin resistance by PseudoCAP functional class.

operon for which mutants had a doubling time of approximately 80 minutes, all of the energy metabolism mutants exhibited normal growth in CAMHB compared to PA14, with doubling times of approximately 50 minutes. Finally, growth on solid media was assessed for the 57 energy metabolism mutants by observing colony morphology. Only ten appeared as small colony variants (of which 3 also had an observed growth defect in liquid media); all ten of these had mutations in genes encoding cytochromes. Clinical isolates exhibiting this poorly-understood small colony phenotype have been shown to have increased antibiotic resistance compared to revertant colonies and recovery of these isolates is strongly associated with daily inhalation of tobramycin or colistin (10).

Several aminoglycoside resistant cytochrome mutants were previously isolated by selective passaging on antibiotic media (2, 4), however this study emphasizes the vast number of cytochrome genes that may impact aminoglycoside susceptibility. The definitive role that cytochromes play in aminoglycoside activity in *P. aeruginosa* is unknown. It is, however, known that the mechanism by which aminoglycosides cross the cytoplasmic membrane is an energy

Table 2.1 Tobramycin resistant mutants with defective growth in LB broth under aerobic and/or anaerobic conditions and small colony variants.

PAO1 Ortholog of Mutant Gene	Gene Name	Gene Description
General Growth Defect		
PA0432	*sahH*	S-adenosyl-L-homocysteine hydrolase
PA1547		Putative membrane protein
PA1548		Putative cytochrome oxidase maturation protein cbb3-type
PA1588	*sucC*	Succcinyl-CoA synthetase beta subunit
PA4429		Putative cytochrome c1 precursor
PA4430		Putative cytochrome c reductase, iron-sulfur subunit
PA5070	*tatC*	Sec-independent protein translocase TatC
Aerobic Growth Defect		
PA3818		Inositol-1-monophosphatase
PA3975		Possible phosphomethylpyrimidine kinase
Anaerobic Growth Defect		
PA0455	*dbpA*	RNA helicase DbpA
PA1480	*ccmF*	Cytochrome c-type biogenesis protein CcmF
PA2638-PA2648	*nuoBDEFGHIJKLM*	NADH dehydrogenase I
PA2667		Putative transcriptional regulator
PA2960	*pilZ*	Type IV fimbrial biogenesis protein PilZ
PA3173		Putative oxidoreductase, short-chain dehydrogenase/reductase family
PA3233		Putative signal-transduction protein
PA4673		Putative GTP-binding protein
PA4916		Putative ADP-ribose pyrophosphatase
Small Colony Variant (Energy Metabolism Mutants)		
PA1480	*ccmF*	Cytochrome c-type biogenesis protein CcmF
PA1483	*cycH*	Cytochrome c-type biogenesis protein
PA1552		Putative cytochrome c oxidase, cbb3-type, subunit III
PA1553		Putative cytochrome c oxidase, cbb3-type, subunit II
PA1554	*ccoN*	Cytochrome oxidase subunit (cbb3-type)
PA4429		Putative cytochrome c1 precursor
PA4430		Putative cytochrome b
PA4431		Putative cytochrome c reductase, iron-sulfur subunit
PA5300	*cycB*	Cytochrome c5

dependent process relying directly on the electron transport chain. Indeed it has been suggested that a threshold membrane potential is required to support aminoglycoside uptake (17). Thus, for these energy metabolism mutants we propose that resistance may result as a function of

decreased uptake due to altered membrane potential. Nonetheless cytochrome mutants such as the *cycB* mutant were not the only energy metabolism mutants identified in the tobramycin screen. Of particular note, was the identification of a number of genes related to NADH reduction, including the *nuo* and *nqr* operons as well as the putative NADH:ubiquinone oxidoreductase encoded by PA3493. These results along with the identification of several mutants involved in the TCA cycle and the cytochrome/ETC mutants were consistent with recent views suggesting that NADH depletion triggers production of free radicals which ultimately contribute to antibiotic killing (12) (see also Chapter 3).

2.3.3 Tobramycin Kill Curves

Tobramycin kill curves mimicking MIC conditions were performed for selected mutants of various functional classes including six energy metabolism mutants (PA1320, PA1480, PA1551, PA2638, PA2644, PA5300), two transcriptional regulators (PA0149, PA5438), one DNA replication and repair mutant (PA4609), two LPS mutants (PA5447, PA5450), two small molecule transport mutants (PA3408, PA5070), two membrane protein mutants (PA1767, PA3115) and two hypothetical protein mutants (PA0338, PA1588) (Figure 2.3). All selected
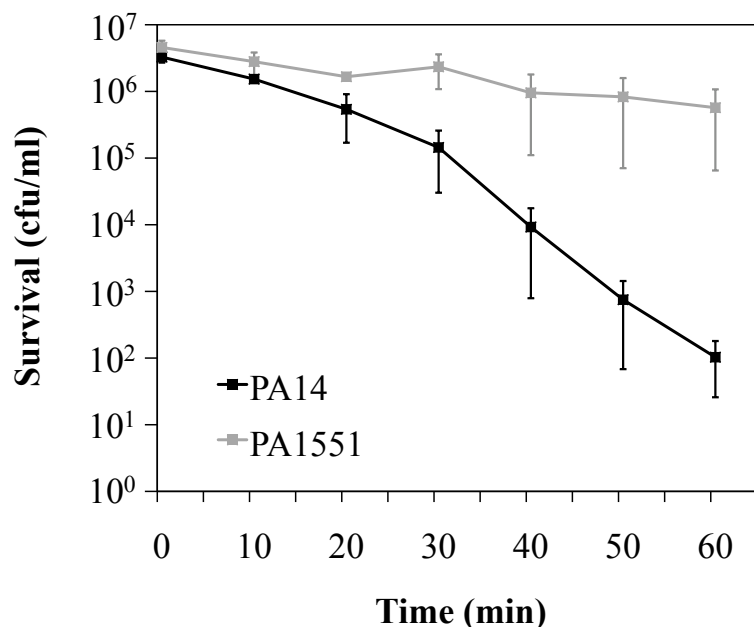
Figure 2.3   The impact of PA1551 gene mutation on killing of PA14 with 2 μg/ml tobramycin in CAMHB at 37°C.
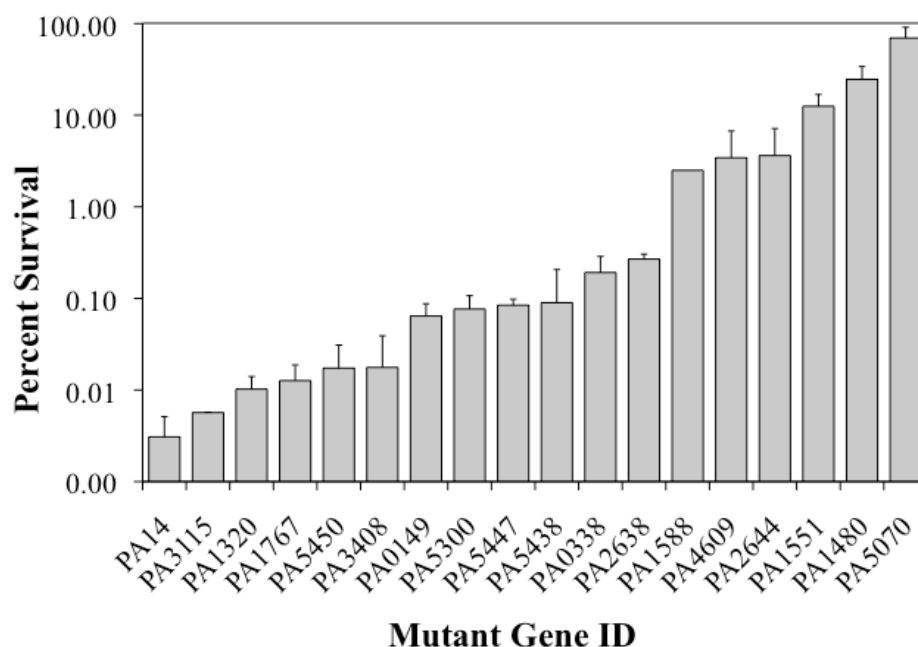
Figure 2.4 Survival of select PA14 mutants identified in the resistance screen at 60 min after treatment with 2 μg/ml tobramycin.

mutants showed a significant reduction (P<0.05 by one-tailed Student's t-test) in the extent of killing at 60 min by 2 μg/ml tobramycin compared to the wild-type with the exception of the PA3115 mutant (P=0.068) (Figure 2.4). As a number of mutants found in the tobramycin screen were impaired in DNA mismatch repair systems and many of these mutants are characterized by a hypermutator phenotype, it may be assumed that resistance in these mismatch repair mutants is due to a highly probable second site mutation. In other work that I collaborated in, we demonstrated that the *radA* mutant from the PA14 library showed an elevated mutation rate compared to PA14 and that the complemented mutant had a mutation rate similar to wildtype (27). The kill curves performed here for the same *radA* mutant show that the tobramycin resistance phenotype was stable under the conditions tested. As these kill curves were performed over a short period of time, only 60 minutes, this indicates that a second site mutation did not develop during antibiotic treatment. Tobramycin MICs were performed for the complemented *radA* mutant to determine whether resistance in this mutant was due primarily to the *radA* mutation or to a second site mutation. Tobramycin susceptibility was not restored in the complemented mutant indicating that resistance was due to a second mutation. In contrast, complementation of the *cycB* (PA5300) mutant, which did not exhibit a hypermutator phenotype,

restored susceptibility to the wild type MIC (MIC 0.5 µg/ml for complemented mutant and 1 µg/ml in *cycB* mutant).

2.3.4 Interaction of Tobramycin with the Outer Membranes of LPS Mutants

P. aeruginosa is capable of expressing two distinct forms of LPS known as the A-band and the B-band (O-antigen) that differ with respect to their O-polysaccharides. The O-polysaccharide of A-band LPS is a conserved poly-rhamnose molecule and genes involved in the assembly of the A-band LPS are encoded by the PA5447-PA5454 operon (24, 25). Several genes of this operon, namely *wbpZ, wbpY, wzt, wzm* and *wbpW* were identified in the tobramycin resistance screen. Tobramycin crosses the outer membrane of the bacterial cell via the process of self-promoted uptake (9). It competitively binds LPS, displacing divalent cations and disrupting the integrity of the outer membrane thus causing increased membrane permeability. NPN is a fluorescent probe that fluoresces weakly in aqueous solution but strongly in non-polar or hydrophobic environment. Under normal conditions, NPN is excluded from the membrane and does not fluoresce. Upon disruption of the outer membrane, e.g. by aminoglycosides (16) NPN partitions into the outer membrane interior and an increase in fluorescence can be observed. The
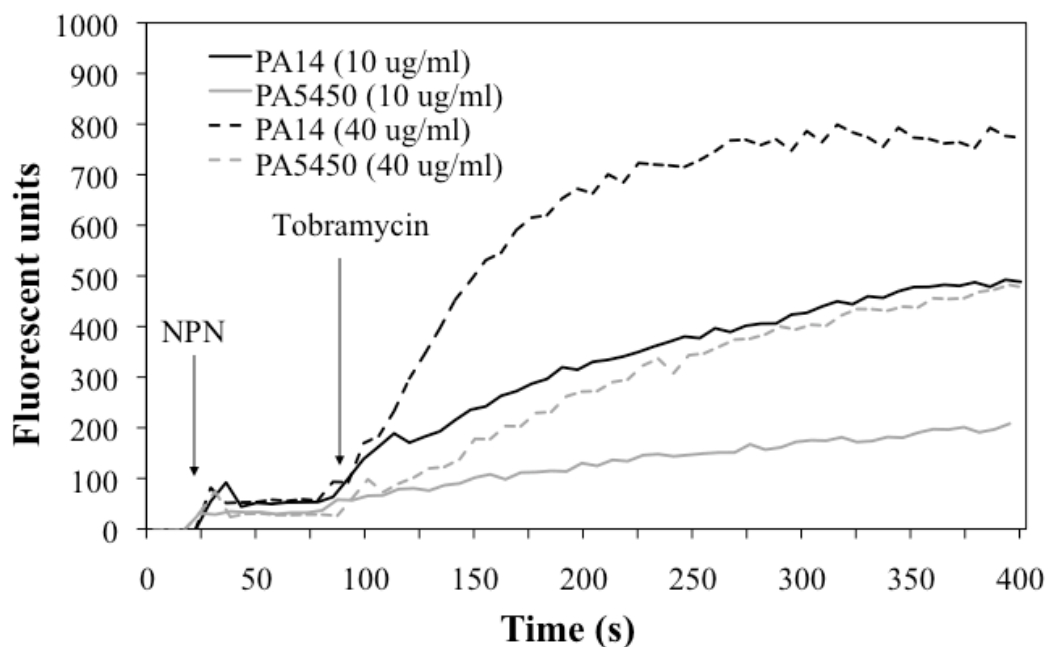

Figure 2.5 Permeabilization of the outer membrane of the tobramycin resistant mutant *wbpZ* compared to the wild type PA14 by 10 µg/ml and 40 µg/ml tobramycin as measured by membrane permeability to the lipophilic fluorescent probe NPN.

initial rate of increase in fluorescence varies with the concentration of aminoglycoside and defines the extent of outer membrane permeabilization, which in turn relates to the extent of self-promoted uptake. The NPN assay was used to determine if the mutations in A-band LPS genes were in fact contributing to resistance resulting in a reduced ability of tobramycin to interact with and permeabilize the outer membranes of these mutants. A maximum increase in fluorescence intensity was achieved using 40 μg/ml tobramycin for PA14 and 60 μg/ml for the six LPS mutants indicating higher concentrations of tobramycin were required to attain a similar level of disruption to the outer membrane. Differences in permeabilization were observed for all LPS mutants compared to the wildtype at all concentrations assayed above 1 μg/ml, at which concentration no permeabilization was observed for any of the mutants or the wildtype during the relatively short period that could be assessed in this assay. These findings reflect the substantially delayed killing (~1 hr) observed when using concentrations near the MIC. While a concentration of 5 μg /ml resulted in slight permeabilization of the wildtype outer membrane, this was not observed for any of the LPS mutants during the timeframe observed. At 10 μg/ml tobramycin, the initial rate of fluorescence increase was approximately two-fold higher in the PA14 parent strain than any of the LPS mutants tested (Figure 2.5) suggesting that the reduced ability of tobramycin to permeabilize the outer membrane of these mutants explains their reduced susceptibility.

2.4   Concluding Remarks

The results presented here indicate that a wide variety of genetic determinants may affect aminoglycoside resistance. This finding is in contrast to the previously held belief, supported by direct *in vitro* selection studies of randomly mutagenized *P. aeruginosa*, that mutations leading aminoglycoside resistance are relatively rare. In previous studies, aminoglycoside resistant mutants were selected on media containing >12 μg /ml gentamicin from *P. aeruginosa* subjected to ethyl methane sulfonate mutagenesis, while at a much lower selection concentration of 3.2 μg/ml gentamicin, non-mutagenized cultures did not produce any aminoglycoside resistant mutants (2, 4). The contrasting results demonstrated here are almost certainly because the ability to screen existing comprehensive libraries permitted us to identify many mutants with only two fold changes in resistance, which would be difficult to obtain and/or assess through direct selection. Despite the large number of genes identified here, it is of importance to note that this type of screen has the limitation of identifying only non-essential genes and so may in fact underestimate the size of the tobramycin resistome. Nonetheless, we believe that this screen is a

particularly sensitive method of detecting non-essential genes involved in resistance as we have not only successfully detected mutants in two genetic backgrounds, but additionally, we independently identified other known resistance genes such as the *nuo* (7) and cytochrome (2, 4) genes. Although the changes in MIC of the mutants are modest, in another study (7) such modest changes have been shown to be capable of acting cumulatively to result in higher levels of resistance when double, triple and quadruple mutants were generated for genes that when independently mutated lead to only two-fold increases in MIC. By including such modest changes here, we have provided strong evidence for a very extensive aminoglycoside "resistome." We have also related several phenotypes often associated with clinical aminoglycoside resistance with genes identified in this screen. Although the clinical relevance of the individual mutations found in this screen has yet to be elucidated, these results provide a large framework for future studies investigating the gradual development of aminoglycoside resistance due to potential cumulative effects of genetic mutations. As well, they may provide insights into the actual mechanisms by which aminoglycosides act upon the cell.

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3.0 Involvement of an ATP-Dependent Protease (PA0779/AsrA) in Inducing Heat Shock in Response to Tobramycin in *Pseudomonas aeruginosa*[3]

3.1 Introduction

Adaptive resistance to antimicrobials can be defined as conditional and reversible resistance that occurs due to prevailing growth conditions (including the presence of sub-effective antibiotic doses), and is increasingly being recognized as contributing to treatment outcome (21). Adaptive resistance to aminoglycosides is a major concern for CF patients undergoing longterm therapy with aerosolized tobramycin. Exposure of localized microcolonies of *P. aeruginosa* within the thick dehydrated mucus in the CF lung, to sub-inhibitory and bacteriostatic concentrations of tobramycin provides the organism with the opportunity to adapt to the presence of this antibiotic. Indeed, it has been observed that genotypically-related bacteria with a variety of phenotypes can be isolated simultaneously from the lungs of CF patients chronically infected with *P. aeruginosa* (14, 15, 23, 32). Though aminoglycosides such as tobramycin have potent bactericidal activity under aerobic conditions, this activity is significantly reduced under low oxygen conditions (3, 13, 16). This reduction of bactericidal activity in hypoxic and anoxic environments is of particular importance in the CF lung where *P. aeruginosa* resides within the lower levels of the steep oxygen gradients existing across the thickened mucus layer (44).

The most commonly observed aminoglycoside adaptive resistance phenotype of *P. aeruginosa* is an impermeability phenotype. This phenotype has largely been attributed to the induction of the MexXY-OprM tripartite efflux pump, although upregulation of MexXY-OprM does not account for all aminoglycoside adaptive resistance phenotypes observed (41). In an attempt to identify factors that may contribute to these aminoglycoside resistance phenotypes, we recently identified a large number of factors that are capable of contributing to aminoglycoside resistance *in vitro* (40), and work by others demonstrated a cumulative effect on MIC of several of these factors in double, triple and quadruple mutants (12), suggesting that these factors are capable of contributing to resistance through independent mechanisms; however, contributions to adaptive resistance were not investigated in either study.

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[3] A version of this chapter has been submitted for publication. Kindrachuk KN, Baines M and REW Hancock. (2010) Involvement of an ATP-dependent protease (PA0779/AsrA) in inducing heat shock in response to tobramycin in *Pseudomonas aeruginosa.*

Further highlighting the complexity of adaptive resistance, work by Overhage *et al.* has drawn a link between the complex adaptations occurring during swarming motility of *P. aeruginosa* and antibiotic resistance, including increased resistance of swarming cells to the aminoglycoside gentamicin (30). Moreover, the issue of adaptive resistance to aminoglycosides is complicated by the lack of understanding of the mechanism of bactericidal activity of aminoglycosides. Early research ascribed the action of aminoglycosides to their interaction with ribosomes either causing misreading and the incorporation of defective channel-forming proteins into the cytoplasmic membrane, or inhibiting the association of the origin of initiation of DNA synthesis with the membrane causing a defect in partitioning of daughter chromosomes during cell division (11, 25, 42). Conversely Kohanski *et al.* (17) have recently proposed a mechanism of lethal action of aminoglycsides and other antibiotics in *Escherichia coli,* involving hydroxyl radicals produced by hyperactivation of the TCA cycle and the electron transport chain. Components of the electron transport chain and the TCA cycle were found to be upregulated within 30 minutes after treatment with the aminoglycosides gentamicin and kanamycin (18). This response was shown to be mediated by the sensor kinase CpxA and the transcriptional regulator ArcA in response to envelope stress (18). In *P. aeruginosa*, the involvement of hydroxyl radicals has not been investigated, and no CpxA or ArcA homologues have been identified.

The purpose of this work was to characterize the effects of subinhibitory, inhibitory and lethal concentrations of tobramycin on gene expression in *P. aeruginosa* in an attempt to understand the nature of the adaptations that occur in response to this drug. We utilized aerobic and anaerobic conditions in this study as this allowed for a direct comparison of responses to the inhibitory and lethal concentrations of tobramycin using the same concentrations to elicit both responses, and also reflected a range of conditions more closely resembling those encountered within the CF lung.

3.2   Materials and Methods

3.2.1   Bacterial Strains and Plasmids

P. aeruginosa strains used included wildtype strain PAO1 and a transposon mutant in the PA0779 gene, PAO1_lux_15_F1 from the PAO1 mini-Tn5-*luxCDABE* transposon mutant library (22); this gene locus has been renamed here "*asrA*" for "<u>a</u>minoglycoside-induced <u>s</u>tress <u>r</u>esponse <u>A</u>TP-dependent protease" and the mutant is termed *asrA::lux*. One Shot® TOP10

Chemically competent *E. coli* (Invitrogen) and plasmid pHERD20T (36) were used for cloning experiments.

3.2.2 Tobramycin Susceptibility Assays

Minimal inhibitory concentrations (MICs) were performed in BBL™ Cation-Adjusted Mueller Hinton Broth (CAMHB) for aerobic susceptibility testing by microbroth dilution as described by CLSI protocols (10). For anaerobic conditions, MICs were performed in CAMHB according to the macrobroth dilution procedure described by CLSI (10) with modifications including placing cultures in anaerobic jars containing BBL GasPak™ Plus Disposable Hydrogen + Carbon Dioxide Generator Envelope (Becton Dickinson Biosciences) and supplementing the media with 15 mM KNO_3. Minimal bactericidal concentrations (MBC) were determined by spread plating 100 μl that was taken from each tobramycin dilution from the MIC assay plate after 20 hours incubation at 37^oC. The MBC was recorded as the lowest concentration at which no colonies formed.

Tobramycin kill curves were performed on cultures grown in CAMHB supplemented with 15 mM KNO_3 to an OD_{600} of 0.5-0.6. Fifty μl samples were serially diluted in ice cold CAMHB and plated at T=0 to obtain the starting cfu/ml. Tobramycin was added at concentrations of either 1, 2, 4 or 20 μg/ml and killing was assessed at specific time points by counting colonies and calculating percent survival relative to untreated cells at T=0.

3.2.3 Microarrays

Five separate microarray experiments were performed with 3-5 replicates assayed for each comparison. For each experiment described below, overnight cultures were diluted 1:50 into the appropriate media and incubated with shaking at 37 °C. Twelve ml samples of mid-log culture, OD_{600} of 0.5-0.6, were used for harvesting cells for the untreated controls. All cells were harvested by centrifugation at 5,000 x *g* at 4 °C. RNA was isolated using the RNeasy Midi Kit (QIAGEN) according to manufacturer's instructions. Microarray hybridization and analysis were performed using *P. aeruginosa* PAO1 DNA microarray epoxy coated slides from The Institute for Genomic Research (TIGR; recently renamed the J. Craig Venter Institute) Pathogenic Functional Genomics Resource Center, and ArrayPipe (version 1.7) as previously described (30, 46). Validation of the microarray data was performed using qPCR, as previously described (26) for select genes with altered expression from each microarray experiment, and a paired one-tailed Student's t-test was used to assess significance.

A subinhibitory aerobic tobramycin microarray was performed comparing gene expression of *P. aeruginosa* PAO1 grown to mid-log in CAMHB in the presence of 0.25 μg/ml tobramycin, to expression occurring in the absence of tobramycin. A lethal/bactericidal aerobic tobramycin microarray compared expression of *P. aeruginosa* PAO1 after a 30-minute treatment with 2 μg/ml tobramycin to its expression prior to addition of tobramycin. The CAMHB medium for this experiment was supplemented with 15 mM KNO_3 for consistency in comparing to subsequent anoxic microarrays. Anaerobic inhibitory and lethal microarray experiments each compared expression of *P. aeruginosa* PAO1 gene expression occurring under anoxic conditions after a 30-minute treatment with either 2 μg/ml or 20 μg/ml tobramycin respectively, to PAO1 gene expression under anaerobic conditions in the absence of tobramycin. Anaerobic growth of overnight cultures was achieved by placing culture tubes in an anaerobic jar containing a BBL GasPak™ Plus Disposable Hydrogen + Carbon Dioxide Generator Envelope (Becton Dickinson Biosciences). Dilutions were performed into 125 ml flasks filled to within 2 cm of the brim with CAMHB supplemented with 15 mM KNO_3 and plugged with rubber stoppers. To minimize the introduction of air, a syringe was used to remove control samples and to add tobramycin to the remaining cultures. A lethal tobramycin microarray of *asrA::lux* was performed comparing its expression in CAMHB supplemented with KNO_3 after a 30-minute treatment with 2 μg/ml tobramycin to its expression in the absence of tobramycin.

3.2.4   Plasmid Construction and Complementation

Genomic DNA from *P. aeruginosa* PAO1 was isolated using PureLink™ Genomic DNA mini kit (Invitrogen). The entire PA0779 gene was PCR amplified from the isolated genomic DNA using Phusion® High-Fidelity DNA Polymerase (New England BioLabs) and primers (AlphaDNA) containing flanking XbaI and HindIII restriction sites. The PCR amplicon and *Ecsherichia-Pseudomonas* shuttle vector pHERD20T were restricted with XbaI and HindIII, ligated and transformed into *E. coli*. Blue-white selection was carried out on Luria-Bertani broth (LB) containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), isopropyl β-D-1-thiogalactopyranoside (IPTG) and 100 μg/ml ampicillin. Plasmids were isolated using a QIAprep spin miniprep kit (QIAGEN) and were verified by restriction analyses with EcoRV, XbaI and HindIII (Invitrogen). Confirmed constructs were transformed by electroporation (9) into PAO1 and its *asrA::lux* mutant and selected for with 300 μg/ml carbenicillin. Empty pHERD20T plasmids were also transformed via electroporation into PAO1 and *asrA::lux* for controls.

3.2.5 Overexpression of *asrA*

Overexpression of the *asrA* gene from the *araC* regulated P_{BAD} promoter of pHERD20T was achieved by adding 1% arabinose to mid-log cultures grown in CAMHB supplemented with 15 mM KNO_3 and 300 μg/ml carbenicillin. qPCR was performed, as previously described (26), comparing relative expression of *asrA* and heat shock genes *htpG, ibpA, groES, clpB, dnaJ* and *hslV* before and after a 30 minute treatment with 1 % arabinose. The effect of overexpression of PA0779 on tobramycin susceptibility was assessed by comparing percent survival of PAO1 complemented with pHERD20T::*asrA* and pHERD20T and treated with 1% arabinose for 30 minutes prior to adding 4 μg/ml tobramycin. Significant increases in percent survival and expression of heat shock genes in response to overexpression of *asrA* was determined using a one-tailed, paired Student's t-test.

3.2.6 Swarming Motility

Swarming of PAO1 and *asrA::lux* was assessed in triplicate as previously described (31) by inoculating 1 μl of a mid-log culture grown in BM2 minimal media [62 mM potassium phosphate buffer, pH 7, 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.4% (wt/vol) glucose] onto BM2-swarm media (62 mM potassium phosphate buffer [pH 7], 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.4% [wt/vol] glucose, 0.5% [wt/vol] Casamino Acids, and 0.5% [wt/vol] Difco agar) and incubating at 37 °C. Diameters of the swarm zone were measured after 20 hours.

3.2.7 Scavenging of Nitric Oxide (NO) by Carboxy-PTIO

2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) is a known scavenger of NO (34). To assess the possible impact of NO production by *P. aeruginosa* on tobramycin susceptibility, a final concentration of 1 mM carboxy-PTIO was added to microbroth dilution MIC plates for PAO1 and *asrA::lux*.

3.3 Results

3.3.1 *P. aeruginosa* PAO1 Susceptibility to Tobramycin Under Aerobic and Anaerobic Conditions

Under aerobic conditions the MIC and MBC of tobramycin for *P. aeruginosa* strain PAO1 were both 1 μg/mL. In an anaerobic environment, tobramycin activity was bacteriostatic with a MIC for PAO1 of 1 μg/ml but a MBC of 16 μg/ml when nitrate was provided as a

terminal electron acceptor. In the absence of nitrate, killing was completely abolished, and a tobramycin MBC >64 μg/ml was observed.

Under aerobic conditions, kill curves using 2 μg/ml of tobramycin (2x MIC) demonstrated a 30-minute lag phase prior to a phase of rapid cell death (Figure 3.1). Under anaerobic conditions with 2 μg/ml of tobramycin, inhibition of growth without apparent cell death was observed over a period of 3 hours (Figure 3.1). Treatment of PAO1 with a tobramycin concentration of 20 μg/ml under anaerobic conditions resulted in a similar level of killing to that seen with aerobically grown cells treated with 2 μg/ml. However, the rate of killing was substantially accelerated during the first 30 min of anaerobic treatment. Based on these data, cells were harvested at 30 minute post-treatment for both the inhibitory and lethal microarrays.

3.3.2 Patterns of Altered Gene Expression in *P. aeruginosa* Strain PAO1 in Response to Lethal and Inhibitory Concentrations of Tobramycin

Gene expression of PAO1 was assessed by microarray analysis after 30 minutes treatment with tobramycin at concentrations of 2 μg/ml under aerobic conditions and 20 μg/ml

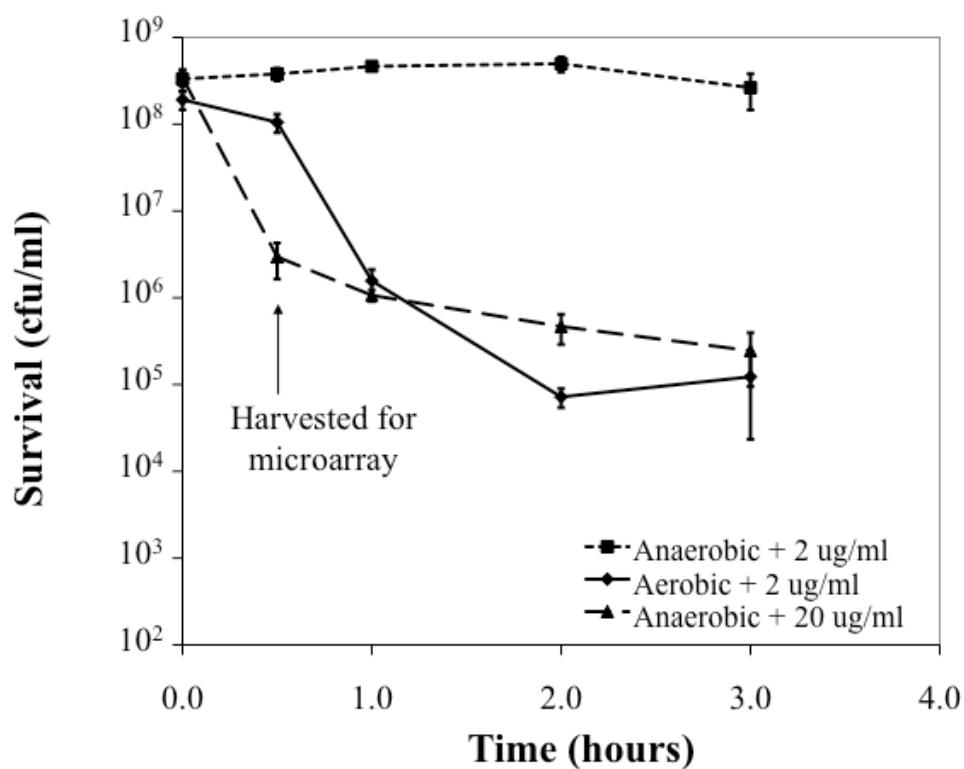**Figure 3.1   Effect of anaerobiosis on tobramycin activity against *P. aeruginosa* PAO1.** Log phase cultures of PAO1 grown in CAMHB + 15 mM KNO$_3$ at 37°C were treated with tobramycin. Error bars represent standard deviations of five replicates.

under anaerobic conditions. These concentrations were bactericidal with a typical lag before killing of PAO1 allowing the harvest of a sufficient number of bacteria for microarray analysis. Under aerobic conditions, expression of 422 genes was affected, with 205 genes upregulated and 217 genes downregulated. Under the anaerobic conditions tested, expression of 1,058 genes was affected with 477 genes upregulated and 581 genes downregulated. While the total number of genes affected under anaerobic conditions was more than double the number observed under aerobic conditions, the general pattern of altered gene expression, as assessed by the distribution of functional classes (Figure 3.2 a,b), was similar between the two conditions. The increased number of genes affected under lethal anaerobic conditions compared to lethal aerobic conditions might have been due to the more advanced stage of killing seen in the former at 30 minutes under the conditions assayed (Figure 3.1), in addition to the effect of anaerobicity. Under lethal conditions, the functional classes representing the majority of downregulated genes were related to energy metabolism, transport of small molecules, membrane proteins, while those most upregulated were transcriptional regulators. Genes classified as involved in the heat shock response were very highly upregulated in response to lethal concentrations of tobramycin but were either not upregulated or were only moderately upregulated in response to bacteriostatic concentrations. The *asrA* gene (PA0799), encoding a protein with 60% similarity (41% identity) to the Lon protease (PA1803), was also found to be upregulated in response to both bacteriostatic and lethal concentrations of tobramycin and was the most highly upregulated regulatory gene in the lethal microarrays.

Amongst the downregulated energy metabolism genes in the lethal microarrays were a number of genes that had been previously shown to increase tobramycin resistance when insertionally inactivated (40). These included the genes of the *nuo* operon, a number of cytochrome genes and a variety of dehydrogenases and oxidoreductases and denitrification related genes (Table 3.1). Genes involved in motility and attachment were also predominantly downregulated.

In contrast to these results, in the bacteriostatic anaerobic microarray expression of 81 genes were altered, with 50 genes upregulated and 31 downregulated. The distribution of functional class of affected genes in the inhibitory microarray was distinctly different from those of the lethal microarrays (Figure 3.2e), although membrane protein genes and genes related to transport of small molecules were largely downregulated as was seen with the lethal conditions.

Using PseudoCyc omics viewer, four major pathways/elements were over-represented. These included components of the TCA cycle and multidrug resistant pumps which were
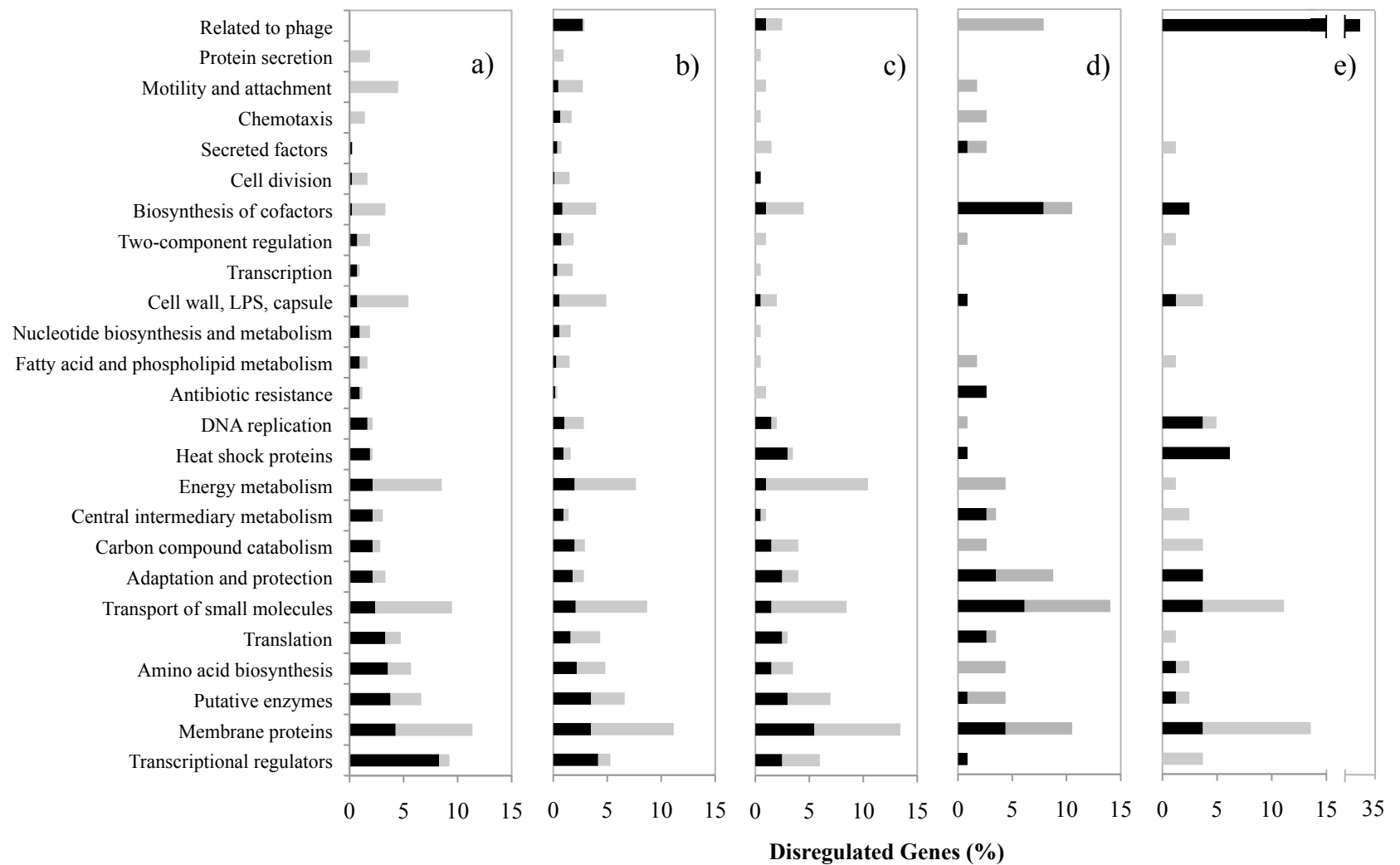
Figure 3.2 PseudoCAP functional class distribution of non-hypothetical genes with altered expression in response to tobramycin. a) PAO1 lethal aerobic b) PAO1 lethal anaerobic c) *asrA::lux* lethal aerobic d) PAO1 subinhibitory aerobic e) PAO1 inhibitory anaerobic . Black bars are upregulated, Grey bars are downregulated.

Table 3.1   Comparison of select genes and operons observed in tobramycin microarrays and resistant mutants.

Gene or Operon	Extent of Disregulation*					Resistant**
	Aerobic TOB 2 µg/ml	Anaerobic TOB 20 µg/ml	Aerobic TOB 0.25 µg/ml	Anaerobic TOB 2 µg/ml	PA0779 TOB 2 µg/ml	
Energy Metabolism						
PA1547-PA1551	-	-				Yes
PA1553-*ccoN*	-	-				Yes
ccoP	-					Yes
ccoO-PA1557	--	+				Yes
sdhCDAB		-				
sucABlpdG		-				
sucCD		-				Yes
PA1600-PA1602	--		---		-	
nuoABDEFGHIJKLMN	-	---				Yes
nqrABCDEF	-	-				Yes
Denitrification/Nitrogen Metabolism						
nirSMCFDLH-PA0512-*nirJEN*	--	--	-		-	
nirQ-PA0521-PA0522		+				
norCB		+	-		-	
norD		-				Yes
nosRZDFYL	-	--	--		-	Yes
narK1K2GHJI-PA3871	-	---	---	+	--	
narXL	-					
Flagella and Pili Biosynthesis						
pilGHI		+				
pilJKchpABCD	-	--				
flgBCDE	-					
flgFGHIJK	-	-/+				
flgL	-					
fliEFGHIJ		-				
PA1442-*fliMNOPQRflhB*	-	-				
PA3799-PA3807		-				
pilB	-					
pilCDcoaE-PA4530	-	-	-			
fimUpilVWXY1Y2E	-	--				
PA4958-*fimX*	-	-				
pilMNOPQ	-	--	-			
Heat Shock						
htpG-PA1597	+++	+++		+	+	
ibpA	++++	++++	+	+	++	
groESgroEL	++	++			+	
dnaJdapB	++	+++		+	+	
grpEdnaK	+++	+++		+	+	
hslVU-PA5055	++	+++			+	
Type III Secretion						
PA1697pscOPQRSTU	-	-				
popN-PA1699-PA1702-	-					
pcrDRGVH-popBD					-	
exsCEB	-					
exsDpscBCDEFGHIJKL	-					
Other						
asrA	++	++		+	+	
pqsABCDEphnA			+			
mexXY			+++			
hcnABC	-		+			

* Downregulated 2-4 fold (-), 5-10 fold (--), ≥10 fold (---). Upregulated 2-9 fold (+), 10-20 fold (++), 20-50 fold (+++), ≥ 50 fold (++++)

** Determined as resistant in a screen of inactivation mutants by Schurek *et al.* (40).

upregulated, and the iron transport and Lipid A biosynthesis pathways which were predominantly downregulated.

3.3.3 Changes in Gene Expression of *P. aeruginosa* Strain PAO1 in Response to Sub-Inhibitory Concentrations of Tobramycin

A total of 114 genes had at least a 2-fold change in expression in response to prolonged aerobic exposure to 0.25 μg/ml tobramycin, with 66 genes being downregulated and 48 genes upregulated. Similar to the lethal microarrays performed on cultures treated for only 30 minutes with tobramycin, the most downregulated genes were involved in energy metabolism, particularly those involved in nitrogen metabolism, and the aldehyde dehydrogenase and oxidoreductase genes encoded by the PA1600-PA1602 operon. In contrast, several upregulated operons were associated with pathogenesis and demonstrated multiple genes being upregulated. These included *pqsABCDE* (*Pseudomonas* quinolone signal) and *hcnABC* (hydrogen cyanide). The most highly upregulated genes (9-47 fold) encoded the known aminoglycoside efflux pump MexXY.

3.3.4 Gene regulation by *asrA*

The *asrA* gene product is homologous to Lon protease (PA1803) and was recently shown to be involved in regulation of protection from nitric oxide (19, 20). As this gene was among the most highly upregulated under lethal conditions and was also moderately upregulated in the inhibitory arrays, its role in the tobramycin induced regulon was investigated. A comparison of microarrays of *asrA::lux* treated aerobically with 2 μg/ml tobramycin to those of PAO1 treated with tobramycin, revealed that overall trends of gene expression by functional class were similar (Figure 3.2 a,c); however, notable differences were observed. The most significant difference in gene expression in *asrA*::*lux* compared to the wildtype was the reduced induction of the heat shock proteins in *asrA*::*lux* compared to the wildtype. For this reason we renamed the PA0779 gene *asrA* for aminoglycoside-induced stress response ATP-dependent protease. Also of note, the downregulation of flagella and pili genes that was observed in the wildtype PAO1 lethal microarrays was not observed in the *asrA*::*lux* mutant microarray. These differences were confirmed for selected genes by qPCR analysis (Figure 3.3).

The total number of genes affected was only 202 in *asrA::lux* compared to the 422 genes observed in the wildtype, this may be attributable to the lack of induction of the heat shock response which would likely cause significant changes in gene regulation.
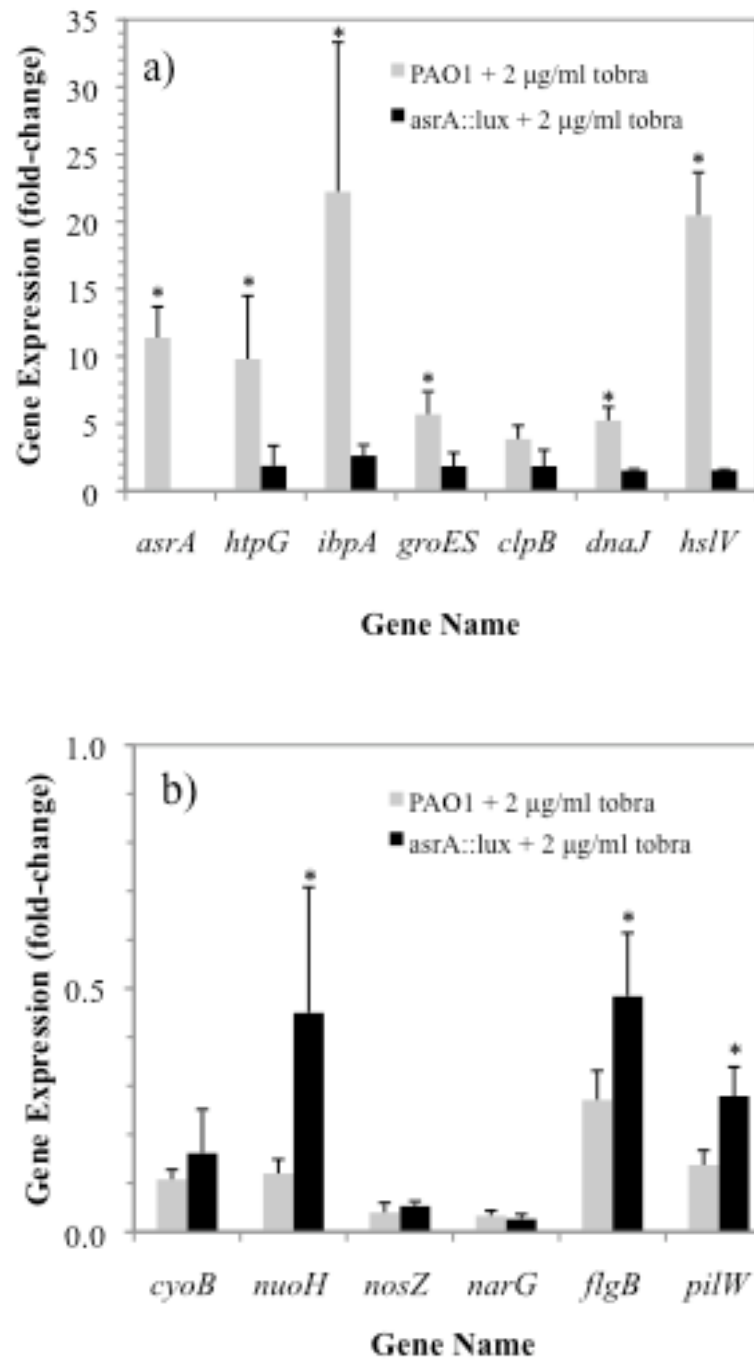
Figure 3.3   Comparison of responses in gene expression by qPCR to tobramycin of select genes in *P. aeruginosa* PAO1 and *asrA::lux.* a) upregulated heat shock genes b) energy metabolism and motility genes. * P-value ≤ 0.05.

3.3.5 Overexpression of *asrA* Led to Induction of the Heat Shock Response in the Absence of Tobramycin

The *asrA* gene was conditionally expressed in trans on the pHERD20T vector from the *araC*-P$_{BAD}$ promoter by induction for 30 minutes with 1% arabinose. Expression of *asrA* as assessed by qPCR was 35-fold greater than expression from its native promoter in PAO1 (p=0.006). The heat shock genes assayed (with the exception of *ibpA*) were found to be significantly (p < 0.05) upregulated in response to 1% arabinose, when *asrA* was overexpressed in trans but were not affected in the controls possessing the empty pHERD20T vector (Figure 3.4).

Figure 3.4 Effect of overexpression of *asrA* on expression of heat shock genes in *P. aeruginosa asrA::lux*. Expression from the pHERD20T P$_{BAD}$ was induced in mid-log cultures of *P. aeruginosa* for 30 min with 1% arabinose.

3.3.6 Effect of *asrA* Overexpression on Tobramycin Susceptibility

Killing by tobramycin was performed using 4 μg/ml tobramcyin to prevent an extended period of adaptation, since at this concentration the delay in killing was reduced to less than 10 minutes. A significant but quite modest differenc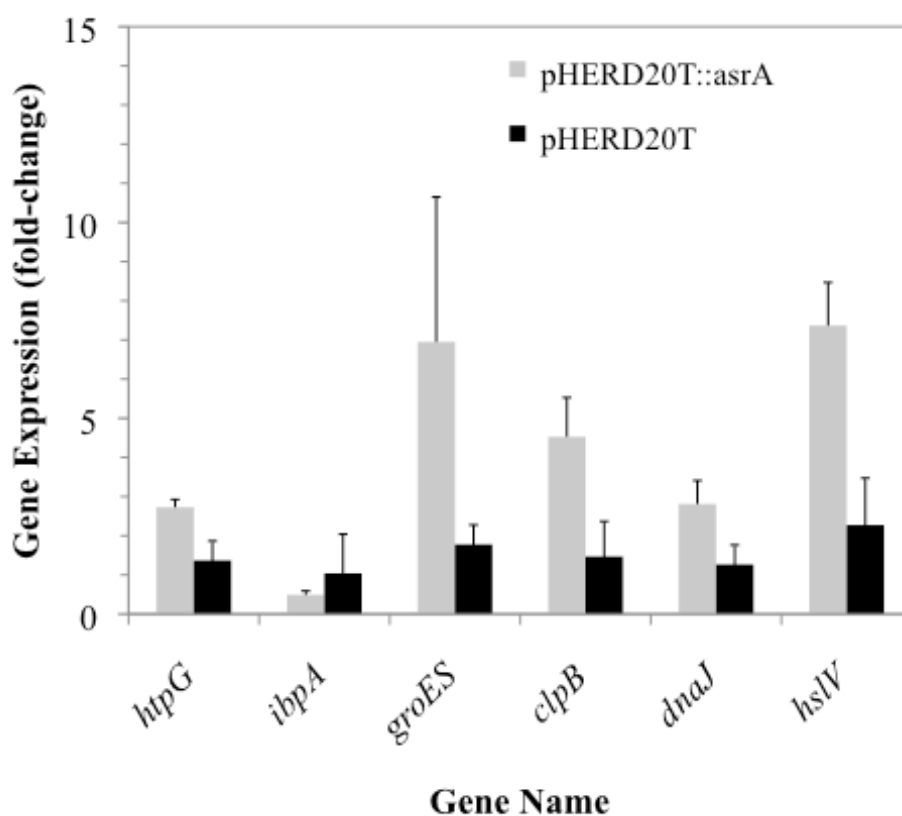e (p≤0.03) in percent survival was observed over the first 60 minutes in PAO1 overexpressing *asrA* due to arabinose induction (Figure 3.5); however, in an MIC assay read at 20 hours, no difference was observed.
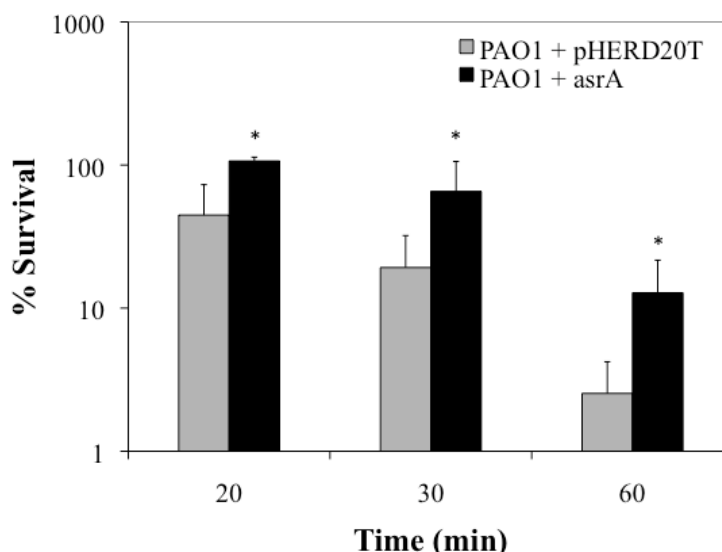
Figure 3.5 Effect of overexpression of *asrA* on expression on tobramycin susceptibility. Expression from the PBAD promoter of pHERD20T was induced with 1% arabinose for 30 min in mid-log cultures of *P. aeruginosa* PAO1 before treatment with 4 μg/ml tobramycin. * P-value ≤ 0.03.

3.3.7 Effect of *asrA* Mutation on Swarming

It was previously shown that in contrast to the significant changes in swarming motility resulting from mutations in the PA1803 Lon protease (24), mutations in the *asrA* Lon-like protease do not affect swarming motility (19). The affect of tobramycin on swarming motility was assessed as downregulation of flagella genes was observed in response to tobramycin in PAO1 but was not seen for the *asrA*::*lux* mutant. As previously reported (19), no difference in swarming was observed between PAO1 and *asrA::lux* in the absence of tobramycin (Table 3.2). Furthermore, consistent with previous findings, tobramycin did not alter PAO1 swarming (30). However, when tobramycin was included in the swarming media at concentrations of 0.5 μg/ml or 1 μg/ml, the swarm zone of *asrA::lux* increased. Similarly for *asrA::lux,* the swarm zone was

greater after 30 minute pre-treatment with tobramycin but was not significantly different in PAO1.

Table 3.2   Effect of tobramycin on swarming motility of *P. aeruginosa* strains PAO1 and *asrA::lux* at 20 hours.

[TOB]	Mean Diameter of Swarm Zone ± SD (mm)			
	PAO1		*asrA::lux*	
No drug	11	± 1	11	± 0
0.25 µg/ml	12	± 1	11	± 0
0.5 µg/ml	12	± 1	16	± 1
1 µg/ml	11	± 2	16	± 7
2 µg/ml	0	± 0	0	± 0
1 µg/ml (30 min)*	12	± 1	18	± 1

* Tobramycin was added to the *P. aeruginosa* culture for 30 min prior to inoculation on swarm media.

3.3.8   Effect of *asrA* Expression on Nitric Oxide Production

The *asrA* gene product was previously implicated in regulating flavohemoglobin and NO production (19, 20). Given the significant upregulation of *asrA* and downregulation of dinitrification genes in response to tobramycin (Table 3.1), the NO scavenger carboxy-PTIO was used to investigate whether altered NO levels played a role in tobramycin susceptibility, and if this was affected by the *asrA* gene product. No difference in tobramycin susceptibility was observed in the presence or absence of 1 mM carboxy-PTIO in either the PAO1 wildtype or the *asrA::lux* mutant. Carboxy-PTIO is a purple compound and a complete loss of colour was observed in growth control cultures of both PAO1 and its *asrA*::*lux* mutant indicating production of NO by *P. aeruginosa*. At concentrations 4-fold lower than the MIC, this loss of colour was also observed; however, at the MIC and concentrations 2-fold below the MIC no loss of colour was observed, indicating decreased production of NO in response to tobramycin. This decrease in NO production was not affected by the mutation of functional *asrA* in *asrA::lux*.

3.4   Discussion

In this study we have provided data regarding global gene expression responses to tobramycin under a variety of conditions ranging from sub-inhibitory to lethal. Recent studies based originally on analysis of gene expression responses led to the suggestion that aminoglycosides induce the production of lethal hydroxyl radicals in *E. coli* through hyperactivation of the electron transport chain and of the TCA cycle (17, 18). The results of the present study are in stark contrast to those found in *E. coli*. Here, the components of the TCA cycle and electron transport chain regulated by ArcA in *E.coli* (*nuo* and *suc* genes), specifically shown to be upregulated in the work by Kohanski *et al*, were found to be actually downregulated in response to tobramycin (Table 3.1). Although, use of the PseudoCyc omics viewer (38) showed upregulation of other components of the TCA cycle, the results observed here do not generally conform to Kohanski model for cell death in response to tobramycin. We hypothesized that a substantial proportion of the gene response to tobramycin represented the activation of defence mechanisms by *Pseudomonas* in an attempt to resist the lethal action of tobramycin. Indeed 53 of the genes affected by tobramycin treatment were previously found to be associated with tobramycin resistance when insertionally inactivated (40). Of these 53 genes, the majority were involved in energy metabolism and all but five genes (none of these five involved in energy metabolism) were downregulated in the presence of tobramycin. The major decrease in susceptibility to tobramycin observed between aerobically and anaerobically grown cells is indeed likely due to differences in uptake, as previously described (4, 5). Consistent with this suggestion we saw a strong overlap between global gene expression changes at lethal concentrations of tobramycin under both aerobic and anaerobic conditions.

Nonetheless, similarities were observed to the Kohanski study in *E. coli* with regards to the upregulation of heat shock response genes, particularly those involved in protein stabilization and protein folding. Here we demonstrated that this heat shock response mediated by *asrA* has a very modest effect on tobramycin susceptibility, with no change in MIC. A heat shock stress response would indeed be expected based on the traditionally described mechanism of action of aminoglycosides, which interfere with translation resulting in the production of faulty proteins and possibly affecting DNA initiation.

In addition to the heat shock genes, *asrA* encoding a putative Lon-type protease was found to be highly upregulated in response to lethal concentrations of tobramycin under both aerobic and anaerobic conditions, but was only moderately upregulated in response to inhibitory (bacteriostatic) concentrations. Lon proteases have previously been identified as participating in

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the heat shock response in *E. coli* and other organisms to degrade misfolded proteins (2, 29, 35). The results observed here suggest that the AsrA protein is not merely part of the heat shock response but is in fact a key mediator of this response, particularly in response to stress induced by tobramycin. Consistent with this conclusion, the induction of the heat shock genes did not occur in the *asrA::lux* mutant in response to tobramycin and expression of most heat shock genes assayed (with the exception of *ibpA*) was induced in the absence of tobramycin when *asrA* was overexpressed from an inducible promoter (Figure 3.4).

Lon proteases belong to the multifunctional family of AAA+ proteins (ATPases Associated with a variety of cellular Activities) that are involved in DNA replication, transcription, membrane fusion, and proteolysis (39). The *P. aeruginosa* PAO1 genome encodes a Lon protease PA1803 that is 84% similar to the *E. coli* Lon protease and one (AsrA/PA0779) that shows 53% similarity to *E. coli* Lon. It has been suggested that Lon proteases play a role in general stress responses (29); however, the presence of multiple Lon proteases in *P. aeruginosa* and the induction of only *asrA* and not other ATP-dependent proteases in response to tobramycin would suggest a more specialized role for these proteases. While the gene product encoded by *asrA* has only 60% similarity (41% identity) to the PA1803 Lon protease, close homologues of *asrA* include Lon-like proteases in the denitrifying bacteria *Azotobacter vinelandii* (93% similarity) and *Thiobacillus denitrificans* (81% similarity) which would appear to be consistent with recent studies demonstrating the involvement of *asrA* in regulating the protective response to NO (19, 20). Our studies show, however, that NO production by *P. aeruginosa* was decreased in response to tobramycin and this decrease was not affected by loss of a functional *asrA* product. Furthermore, downregulation of denitrification genes in *asrA::lux* occurred in response to tobramycin as it did in PAO1. This indicates that the involvement of *asrA* in regulating NO detoxification does not offer substantial protection against tobramycin. AsrA did however have an effect on swarming motility in response to tobramycin, although the increased swarming of the *asrA::lux* mutant in the presence of subinhibitory tobramycin did not result in growth on higher concentrations of antibiotic.

While *asrA* was a major player in the lethal and inhibitory responses to tobramycin, it did not appear to have a significant role in adaptive responses occurring during prolonged exposure to subinhibitory concentrations of tobramycin. Indeed, *mexX* and *mexY* were the most upregulated genes during prolonged exposure to subinhibitory tobramycin. It has been previously demonstrated that the MexXY-OprM tripartite multidrug efflux pump can mediate aminoglycoside efflux, and derepressed mutants in this system explain impermeability type

resistance to tobramycin. Upregulation of the MexXY pump in response to ribosome targeting agents, including the aminoglycosides, is mediated through the PA5471 gene product (27, 28, 45). Consistent with this, our subinhibitory microarray results revealed a 2.2-fold increase in PA5471 expression in response to tobramycin. No other genes were upregulated to as great an extent as *mexXY* consistent with this being the predominant mechanism of adaptive resistance to tobramycin.

Other genes and operons observed to have moderate upregulation under subinhibitory treatment conditions were involved in hydrogen cyanide production and quorum sensing. While these systems are typically associated with pathogenesis, their potential roles in adaptive resistance to aminoglycosides have not been investigated. Hydrogen cyanide is a respiratory inhibitor and its production by *P. aeruginosa* typically occurs under low oxygen conditions (7, 8) and has been associated with increased iron levels (1) as well as quorum sensing (33, 37). While HCN has been found in CF sputum (6), its physiological role in *P. aeruginosa* is not well understood. It has been suggested that the *P. aeruginosa* hydrogen cyanide synthase is merely a respiration-linked amino acid dehydrogenase (43) and consistent with this its synthesis is accompanied by production of a cyanide resistant electron transport chain. Given the findings of this study, which show a large number of anaerobic and aerobic respiratory elements are downregulated in response to lethal, inhibitory and subinhibitory concentrations of tobrmaycin, and are also related to tobramycin susceptibility, this may indicate a switch to an alternate pathway of energy generation that might impact on the efficiency of aminoglycoside respiration-dependent uptake and thus provide further protection against the effects of tobramycin.

3.4.1 Concluding remarks

Adaptive resistance is a complex phenomenon that remains poorly understood. While mechanisms imparting an impermeability phenotype, such as upregulation of antibiotic efflux pumps, likely provide the greatest adaptive protection against aminoglycosides over the long term, *P. aeruginosa* also alters its gene expression in a more immediate fashion in response to a lethal hit by aminoglycosides. We have shown here that some of these immediate adaptations include downregulation of energy metabolism genes (including those involved in nitrogen metabolism), as well as the upregulation of the heat shock response mediated via the *asrA* gene product. The combined effects of a number of adaptations, each providing some modest degree of resistance independently, may provide sufficient short-term protection until more effective long-term adaptations are established.

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4.0   Involvement of *pmrAB* and *phoPQ* in Polymyxin B Adaptation and Inducible Resistance in Non-Cystic Fibrosis Clinical Isolates of *Pseudomonas aeruginosa*[4]

4.1   Introduction

The emergence of multidrug resistant Gram-negative organisms and the simultaneous lack of new clinically-available antimicrobial agents have lead to a resurgence of older compounds such as the polymyxins (8). The polymyxins are cyclic cationic peptide with a fatty acyl tail (Figure 1.4) and as polycations share a common outer membrane self-promoted uptake mechanism with the aminoglycosides (as well as common resistance mechanisms (17)). These agents, including polymyxin B and colistin, have highly potent activity against Gram-negative organisms including *Pseudomonas aeruginosa*, but were previously abandoned due to a reported high incidence of nephrotoxicity and neurotoxicity (15).

Resistance to polymyxin B is predominantly associated with decreased uptake into the bacterial cell resulting from a reduced capacity for initial binding (26). Polymyxin B and other polycationic compounds such as the aminoglycosides enter the cell via a process known as self promoted uptake (13, 14). Polymyxin B binds to outer membrane lipopolysaccharide (LPS) displacing Mg^{2+} and disrupting the Mg^{2+} cross bridges between anionic LPS molecules in the outer membrane, thus leading to membrane destabilization. This leads to an increased permeability of the outer membrane allowing further uptake of the antibiotic. In *P. aeruginosa* the ability of polymyxin to permeabilize outer membranes reflects its ability to bind to LPS with higher affinity than the native cross-bridging cation Mg^{2+} (23, 25).
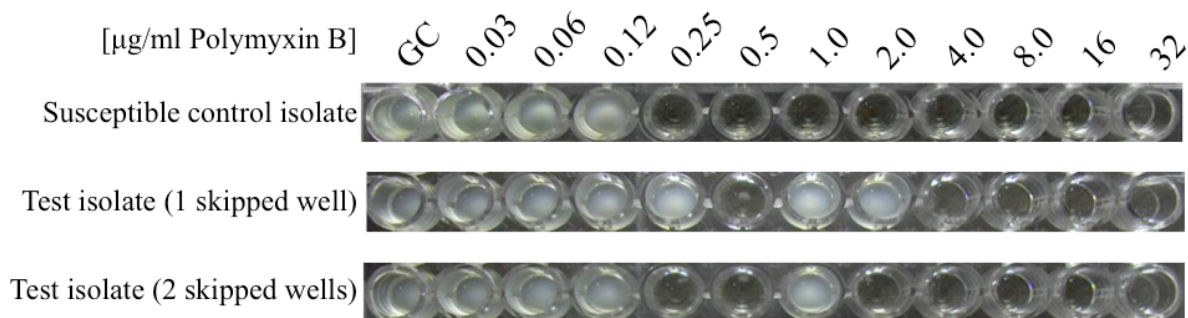
Figure 4.1 Representative images of polymyxin B MIC assays for Brazil isolates demonstrating skipped wells.

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[4] A version of this chapter has been published. Schurek KN, Sampaio JL, Kiffer CR, Sinto S, Mendes CM and REW Hancock. (2009) Involvement of *pmrAB* and *phoPQ* in polymyxin B adaptation and inducible resistance in non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 53:4345-51.

A polymyxin B adaptive resistance phenotype was first reported by Gilleland and colleagues in 1976 (11) when the wildtype PAO1 strain was passaged in minimal medium containing low Mg^{2+} and exposed to increasing concentrations of polymyxin B. Since this time, many studies have focused on the structural basis of adaptive resistance (9, 10). Under varying environmental conditions, *P. aeruginosa* has been found to synthesize different forms of the Lipid A component of LPS (7). In particular, under Mg^{2+} limiting conditions *P. aeruginosa* exhibits Lipid A modifications including the addition of aminoarabinose and palmitate. These modifications have been associated with polymyxin B resistance (20, 21).

It is well established that two distinct two-component regulators, PhoP-PhoQ and PmrA-PmrB, respond to limiting Mg^{2+} conditions, resulting in polymyxin B resistance in *P. aeruginosa* (17-19). Under Mg^{2+} limiting conditions PhoP-PhoQ autoregulates the *oprH-phoP-phoQ* operon (18) and similarly PmrA-PmrB autoregulates the PA4773-5-*pmrAB*-PA4778 operon (20). Furthermore, the *arnBCADTEF-PA3559* operon (PA3552-PA3559), which is responsible for the addition of aminoarabinose to Lipid A (21), is separately regulated by each of these two-component regulatory systems and is upregulated under Mg^{2+} limiting conditions (20). Both the PA4773-PA4778 and the PA3552-PA3559 operons have shown independent upregulation in response to various cationic antimicrobial peptides in laboratory mutants (21). Mutations in PmrB and the presence of aminoarabinose on the LPS have been directly associated with polymyxin B and aminoglycoside resistance (21).

During a comparative study of MIC methods for testing polymyxin B and colistin susceptibility, it was observed that 24 of 243 multidrug resistant clinical isolates of *P. aeruginosa* demonstrated skipped wells in the microbroth dilution method for either polymyxin B, colistin or both (a "skipped well" is an isolated well showing no growth of bacteria despite the fact that a well with a higher concentration demonstrates growth). According to Clinical and Laboratory Standards Institute (CLSI) guidelines, one skipped well is acceptable and MIC readings should be taken based on the well with the highest antibiotic concentration exhibiting growth (3). The assumption is that a technical error has occurred. The multi-drug resistant isolates described here skipped one to five wells (Figure 4.1), and bacteria recovered from wells at the higher polymyxin concentrations (i.e. after the skipped wells) also exhibited a polymyxin MIC profile containing skipped wells. The phenomenon of skipped wells has previously been described and was termed cocarde growth (1, 2, 26). In this study we set out to determine the potential causes for this type of resistance profile in the identified *P. aeruginosa* clinical isolates.

4.2 Materials and Methods

4.2.1 Bacterial Strain Selection and Identification

All isolates were collected as part of a Brazilian surveillance study between 2002 and 2004 which defined multidrug resistant as resistant to meropenem, ciprofloxacin and polymyxin B and at least one β-lactam from the set of cefepime, ceftazidime or piperacillin-tazobactam. Samples were collected from intensive care and oncology units from 21 hospitals throughout seven states. Each isolate represents a single sample from one patient. Isolates were identified as *P. aeruginosa* based on established protocols for each hospital and reconfirmed as *P. aeruginosa* according to identification procedures established by the Manual of Clinical Microbiology (22). Samples were maintained on glass beads and frozen at -80ºC. MICs were performed for meropenem, ciprofloxacin, cefepime, ceftazidime and piperacillin-tazobactam. A total of 243 isolates were identified as being multidrug resistant. Polymyxin B and colistin MICs were performed on these multidrug resistant isolates. Twenty four isolates skipped wells in the first round of MICs and three isolates (9BR, 19BR and 213BR) taken from different hospitals over the course of a three year period were selected for further analysis. *P. aeruginosa* strain PAO1 was used as a wildtype strain.

4.2.2 Antimicrobial Susceptibilities

MICs were determined as described by the CLSI guidelines (M7-A6 and M100-S15), using broth microdilution (3). Polymyxin B MICs were performed in triplicate for each isolate with concentrations ranging from 0.03 to 32 µg/ml in cation-adjusted Mueller Hinton broth (CAMHB) and were incubated at 37°C for up to 48 hrs. Gentamicin MICs were performed for concentrations ranges of 0.25 to 256 µg/ml in CAMHB and incubated for 24 hours.

4.2.3 Detection of *rmtA* and *rmtD*

Colony PCR was performed for the ribosomal methylating genes *rmtA* and *rmtD* using primers described by Doi and Arakawa (5).

4.2.4 Growth Curves

Growth curves were performed in triplicate for strains PAO1, 9BR, 19BR and 213BR in the absence of polymyxin B as well as in the presence of both 0.125 µg/ml and 2 µg/ml of polymyxin B. These concentrations of polymyxin B were chosen here and for all subsequent

experiments since growth appeared in the MIC wells at each of these concentrations, with 0.125 µg/ml being selected to represent a concentration below the liberal MIC and 2 µg/ml representing a concentration of polymyxin B occurring after the skipped wells, but below the conservative MIC. Overnight cultures were diluted 1:20 into CAMHB containing appropriate polymyxin B concentrations and OD_{600} was measured every 20 min for 24 hrs using the TECAN Spectrafluor Plus.

4.2.5 RNA Extraction and cDNA Synthesis

One hundred µl of each isolate was harvested directly from the MIC plate from (a) the well containing no polymyxin (b) the well containing 0.125 µg/ml polymyxin, and (c) the well containing 2 µg/ml polymyxin, and were inoculated into 10 ml CAMHB containing the respective concentrations of polymyxin (ie. no drug, 0.125 and 2 µg/mL). The isolates were incubated with shaking at 37°C and were grown to an OD_{600} of 0.5. RNA was isolated using a QIAGEN RNeasy mini kit according to manufacturer's instructions (QIAGEN Inc., Canada) and stored at -80°C with 0.2 U/µL SUPERase-In RNase inhibitor (Ambion Inc., Austin, TX). RNA quality was assessed by agarose gel electrophoresis and by spectrophotometry. Four µg of total RNA was added to a reaction mixture to make cDNA and used for quantitative Real Time PCR (qPCR). Quantitative PCR was performed on the genes PA1180 (*phoQ*), PA3552 (*arnB*) and PA4773 (first gene of the *pmrAB* operon), *rpsL* was used as a control. Each isolate was isolated from individual MIC plates in triplicate on separate days and qPCR was performed on each repeat in at least duplicate.

4.2.6 Polymyxin B Outer Membrane Interaction Studies

The hydrophobic fluorescent probe 1-*N*-phenylnaphthylamine (NPN) was used as described by Loh *et al.* to study the interaction of polymyxin B with the outer membranes of clinical isolates compared to PAO1 (16). Overnight cultures grown at 37°C in CAMHB containing either 0, 0.125 or 2 µg/ml polymyxin B were diluted 1:100 in fresh CAMHB containing the respective polymyxin B concentrations. Fifty ml samples of mid-log phase cells were harvested by centrifugation at 3,000 x g for 10 min, washed once with and resuspended in 5 mM sodium HEPES buffer (pH 7.2), with 5 µM carbonyl cyanide m-chlorophenylhydrazone (an uncoupler), at an optical density at 600 nm of 0.5. Fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm using a Perkin Elmer LS 50B fluorescent spectrophotometer. NPN was added to a final concentration of 10 µM to obtain

baseline fluorescence. Polymyxin B was added to final concentrations of 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 µg/ml.

4.2.7 Sequencing of *pmrAB* and *pmr* Promoter Region Including PA4773

Genomic DNA was isolated using a DNeasy Blood & Tissue kit (Qiagen Inc., Canada) according to the manufacturer's instructions from overnight cultures grown at 37ºC in CAMHB for isolates PAO1 and 9BR and CAMHB plus 2 µg/ml polymyxin B for isolates 19BR and 213BR. For the PA4773-PA4778 operon, a set of PCR primers was designed to encompass the entire non-coding upstream region of PA4773 as well as PA4773 itself (1,069 bp), and a second set of primers was designed to amplify the region flanking *pmrAB* (2,349 bp). The OprH-PhoPQ operon was amplified as three overlapping PCR fragments for coverage of all three genes as well as the upstream non-coding region. Sequencing was performed using Applied Biosystems BigDye v3.1 Terminator Chemistry and run on an Applied Biosystems PRISM 377 automated sequencer.

4.2.8 Genotyping

P. aeurinosa isolates PAO1, 9BR, 19BR and 213BR were typed by pulsed-field gel electrophoresis (PFGE) of *SpeI*-digested chromosomal DNA. *SpeI* restriction fragments were separated using the CHEF-DR® III System (Bio-Rad Laboratories, Hercules, CA) and PFGE profiles, then digitized on the Gel Doc 1000 (Bio-Rad Laboratories) with Quantity One® (Bio-Rad Laboratories) software, and the results were analyzed using Bionumerics™ version 3.5 (Applied Maths, Austin, TX) software. The dendrogram was constructed by the unweighted pair group method with arithmetic averages with the Dice coefficient (4). *SpeI* restriction analysis of *P. aeruginosa* has previously demonstrated that unrelated strains are accurately distinguished at a Dice coefficient < 85% (12).

4.3 Results and Discussion

4.3.1 Antimicrobial Susceptibilities and the Skipped Well Phenomenon

The occurrence of skipped wells during polymyxin B MIC testing was observed for 24 isolates. This did not coincide with the occurrence of skipped wells during colistin MIC testing since only eight of these isolates presented this phenotype. In comparison to the polymyxin B MICs, the MICs for the aminolgycoside gentamicin did not exhibit skipped wells. Furthermore,

all isolates were found to be PCR positive for the ribosomal modification enzyme genes *rmtA* and/or *rmtD* and deemed aminoglycoside resistant MIC >256 µg/ml.

Polymyxin B microbroth dilution assays producing skipped wells were found to be reproducible in only 10-15% of trials from the original stock cultures; however, when samples were harvested from the microtitre plate wells with growth at concentrations higher than the skipped wells, a reproducible skipped well phenotype was observed. This indicates that only a proportion of bacteria in the original stock cultures had the skipped well phenomenom, but that these bred true. Antimicrobial susceptibilities for the selected clinical isolates 9BR, 19BR and 213BR are presented in Table 4.1. Isolate 19BR did not grow in concentrations of 0.25 and 0.5 µg/ml polymyxin B and isolate 213BR did not grow in 0.5 and 1 µg/ml. To determine the effect of polymyxin B on the growth of these isolates, growth was assessed, in the presence of 0.125 and 2 µg/ml polymyxin B, for skipped well isolates 19BR and 213BR compared to a polymyxin B susceptible laboratory wild type strain PAO1 and isolate 9BR that was reproducibly polymyxin B resistant without skipped wells (Figure 4.2). The presence of 0.125 µg/ml polymyxin B, a concentration below the liberal MIC of each isolate, had no effect on the growth of any of the four isolates, while the presence of 2 µg/ml polymyxin B extended the lag phase of both skipped well isolates 19BR and 213BR by approximately 6 and 4 hours respectively. The extended lag phases observed for skipped well isolates 19BR and 213BR in the presence of 2 µg/ml polymyxin B were consistent with the need for a period of adaptation to permit growth at higher concentrations of polymyxin B.

4.3.2   Determining the Contributions of PhoP-PhoQ and PmrA-PmrB to the Skipped Well Phenomenon

As both adaptive and mutational resistance to polymyxin B have previously been linked to the 2 two-component response regulatory systems PhoP-PhoQ and PmrA-PmrB and their downstream regulatory effects on the *arnBCADTEF*-PA3559 operon (20, 21), we investigated the potential contributions of these operons to the skipped well resistance phenotype described here. The genes *phoQ, arnB* and PA4773 (first gene of the *pmrAB* operon) were selected for qPCR analysis as representative of transcripts of each of the three operons. Figure 4.3 depicts the expression of these three genes in the skipped well isolates 19BR and 213BR as well as the polymyxin B resistant isolate 9BR relative to their expression in susceptible PAO1 strain in the absence of polymyxin B. For the skipped well isolates 19BR and 213BR in the absence of polymyxin B, the expression levels of all three genes of interest were comparable to the

Table 4.1 Antibiotic susceptibilities of multidrug resistant clinical isolates of *P. aerugionsa* exhibiting a skipped well phenotype to polymyxin B.

Testing Order no.	Liberal MIC*		Conservative MIC**		E-test					
	Polymyxin B	Colistin	Polymyxin B	Colistin	Polymyxin B	Meropenem	Ceftazidime	Piperacillin/ Tazobactam	Ciprofloxacin	Cefepime
9BR	64	32	64	32	4	>32	>256	96	>32	>256
19BR	0.25	4	8	4	1.5	>32	>256	64	>32	>256
213BR	0.5	2	8	32	1.5	>32	>256	96	>32	>256

*Liberal MIC indicates the well containing the lowest concentration of antibiotic without visible growth.

**The conservative MIC is the MIC that takes into account the growth after the skipped wells.
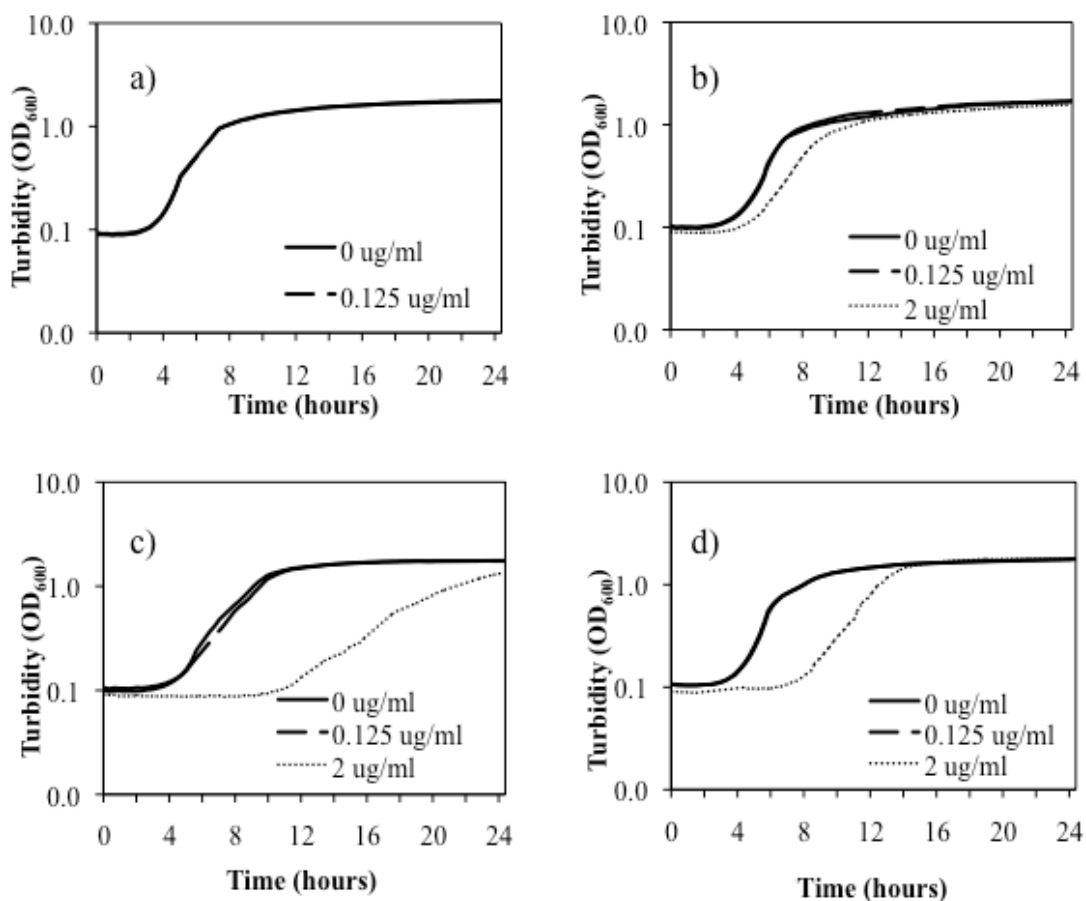
Figure 4.2 The effect of 0.125 μg/ml and 2 μg/ml polymyxin B on growth of *P. aeruginosa* isolates at 37°C in CAMHB (a) PAO1, (b) 9BR, (c) 19BR and (d) 213BR.

expression levels observed in PAO1. In contrast, the expression levels in the polymyxin B resistant isolate 9BR of *arnB* and PA4773, but not *phoQ* were significantly higher (p<0.05) than in PAO1, with 52- to 280-fold increases respectively. While expression was not affected by sub-inhibitory (0.125 μg/ml) polymyxin B treatment for any of the studied genes in the susceptible PAO1 strain (data not shown) or the two skipped well isolates, the polymyxin B resistant isolate exhibited a concentration dependent increase in expression of *arnB* and PA4773. At 2 μg/ml polymyxin B, although the wild type did not grow, at this concentration the skipped well isolates demonstrated a dramatic increase in the expression of *phoQ*, *arnB* and PA4773 (*pmrAB* operon) thus implicating a role for all three of these genes in the skipped well phenomenon. In a study investigating Lipid A modifications in CF, environmental and non-CF isolates of *P. aeruginosa,* Ernst *et al* found that aminoarabinose was only present in isolates obtained from CF patients (6).
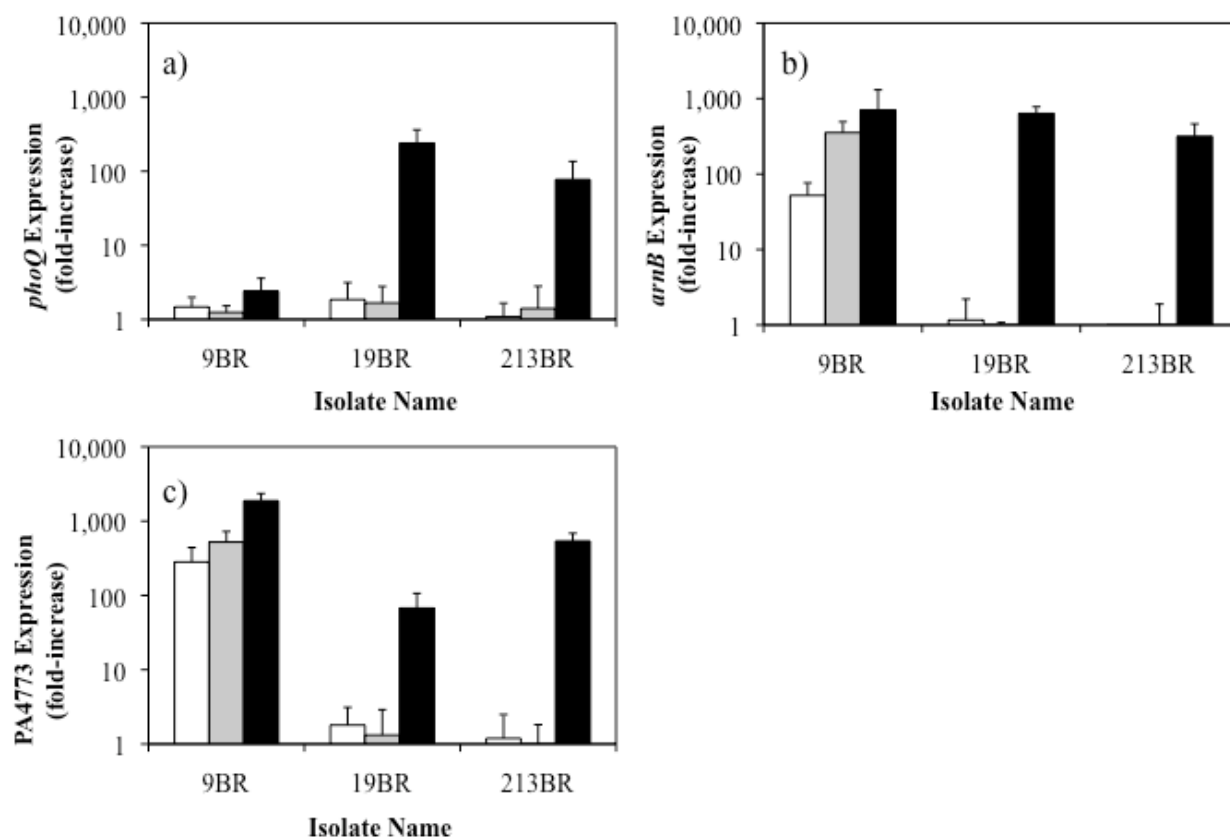
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Figure 4.3 The effect of polymyxin B on the expression of *phoQ* (a), *arnB* (b) and PA4773 (c) of isolates 9BR, 19BR and 213BR measured as a fold change compared to expression of the respective genes in PAO1 in the absence of polymyxin B. White bars represent 0 µg/ml, grey bars represent 0.125 µg/ml and black bars represent 2 µg/ml polymyxin B.

The authors further indicated that at the modification levels found in early CF isolates of *P. aeruginosa,* this modification alone did not appear to cause significant levels of resistance; however, the modification was always found to be present in colistin resistant CF isolates (6). The high levels of expression of the aminoarabinose modification operon seen in isolate 9BR suggests that this LPS modification is also likely to be present in polymyxin B resistant non-CF isolates.

The regulatory operon PA4773-PA4775-*pmrAB*-PA4778 and its downstream effector operon *arnBCADTEF*-PA3559 demonstrated increased expression in both the stably resistant isolate, 9BR, as well as in the two skipped well isolates, 19BR and 213BR, in response to polymyxin B, therefore, we believe that regulatory mutations in the upstream region of the PA4773-PA4775-*pmrAB*-PA4778 operon may be involved in the polymyxin B resistance observed for all of these isolates. When the non-coding region upstream of the PA4773-PA4775-*pmrAB*-PA4778 operon

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Table 4.2 Missense mutations in PA4773, PmrA, PmrB, OprH, PhoP and PhoQ and single nucleotide base pair changes in the promoter region of operons PA4773-PA4778 and *oprH-phoPQ* observed in isolates 9BR, 19BR and 213BR compared to wild type PAO1.

	Nucleotide Subtitutions		Amino Acid Substitutions					
Isolate #	Upstream Region of PA4773	Upstream Region of OprH	PA4773	PmrA	PmrB	OprH	PhoP	PhoQ
9BR	Δ 5361280 – 5361380 5361407 G>A	1276229 G>A 1276365 G>C[†] 1276493 C>T[†‡] 1276883 T>C[†‡]	None	L71R[†]	ΔD45 Y345H[†‡]	None	S179P[†]	*225E[†‡] T234A[†‡] S249P[†] P367S[†]
19BR	Δ 5361279 – 5361379 5361407 G>A	1276229 G>A 1276365 G>C[†] 1276493 C>T[†‡] 1276883 T>C[†‡]	None	L71R[†]	Y345H[†‡]	None	S179P[†]	*225E[†‡] T234A[†‡] S249P[†] P367S[†]
213BR	Δ 5361279 – 5361379 5361407 G>A	1276229 G>A 1276365 G>C[†] 1276493 C>T[†‡] 1276883 T>C[†‡]	None	L71R[†]	Y345H[†‡]	None	S179P[†]	*225E[†‡] T234A[†‡] S249P[†] P367S[†]

Δ = Deletion; * = Insertion

† These variants are also found in LESB58.

‡ These variants are also found in PA14.

was amplified and sequenced a large 101 bp deletion and a single base pair change were observed for all three clinical isolates when compared to the wildtype PAO1 strain (Table 4.2). To determine if these mutations were unique to these isolates a comparison was also made to other sequenced strains including another wildtype stain, PA14, and the earliest archived cystic fibrosis Liverpool epidemic strain, LESB58 (27). A number of single base pair changes were observed when comparing each of these strains (including the previously sequenced PA14 and LESB58 strains) to PAO1; however, neither the single base pair change, nor the large deletion were observed in any of PAO1, PA14 nor LESB58. Interestingly, LESB58 did have a 101 bp insertion compared to PAO1 and PA14 within the location where the deletion was observed in the Brazilian strains. A study of a second Liverpool epidemic strain, LES431, demonstrated moderate upregulation of *pmrA* and *pmrB* (6.7 and 9.2 fold respectively as determined by microarray) compared to PAO1 in LB media (24). The alterations in this promoter site, along with the increased expression of the PA4773-PA4775-*pmrAB*-PA4778 operon, in both the Brazilian and the LES strains implicate this region in regulation of this operon. The finding that all three clinical isolates from Brazil possessed the identical mutations in this region was surprising, as the isolates exhibited both distinct phenotypes and differing expression patterns. The PFGE genotyping seen in Figure 4.4 does indicate that these three strains are related with a Dice coefficient of >85%.

As previous work found two phenotypically-distinct polymyxin B resistant mutants selected from the wild type PAK strain on LB plates containing 20 to 50 µg/ml polymyxin B
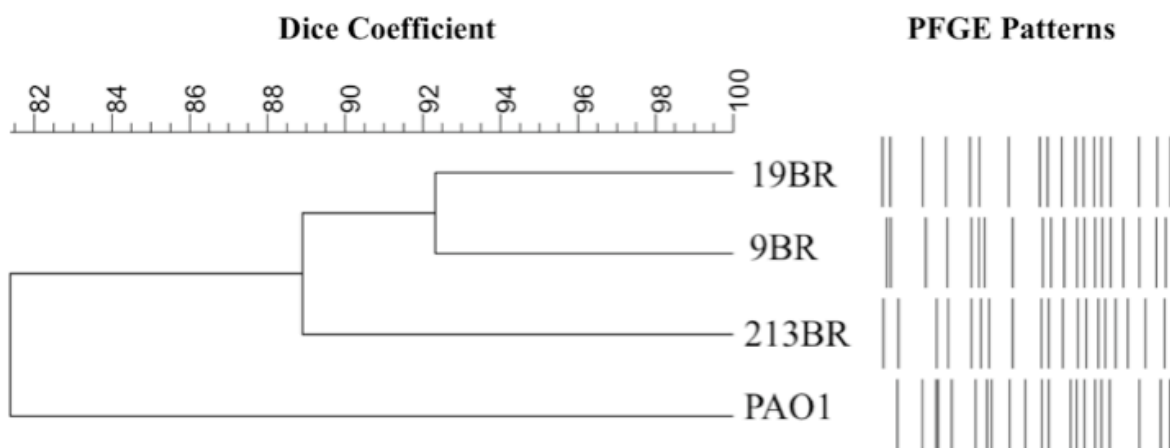

Figure 4.4 Genetic relatedness of isolates 9BR, 19BR, 213BR and PAO1 determined using *speI* restricted PFGE analysis.

each possessed a single mutation (L243Q and A248V) in the histidine box of PmrB near the active site H249 and contained aminoarabinose in their LPS under non-inducing conditions (21), we also sought to characterize the PmrAB locus of these isolates. Sequencing of PA4773, PmrA and PmrB, in 9BR, 19BR and 213BR showed that the 3 were identical, with the exception of a single amino acid deletion in 9BR (Table 4.2). When compared to PAO1 a missense mutation in PmrA (L71R) was observed, and this change was also found in LESB58. The missense mutation, Y345H, was observed in the wildtype strain PA14 as well as in LESB58 and so is not likely to be involved in polymyxin resistance. No mutations near the previously reported polymyxin B resistance mutations (21) in the histidine box of PmrB were observed.

As the major differences in the expression data between the skipped well polymyxin B resistant isolates and the constitutively polymyxin B resistant isolate was found to be in the induced expression of *phoQ,* we anticipated major differences in the sequence data at this locus; however, no differences between these three isolates were observed (Table 4.2).

4.3.3   Polymyxin B Interaction Studies

As modifications to LPS influence the ability of polymyxin B to interact with the outer membrane of *P. aeruginosa*, NPN assays were performed to compare the ability of polymyxin B to interact with the membranes of the skipped well isolates compared to the polymyxin B susceptible PAO1 strain and the polymyxin B resistant isolate 9BR. NPN is a fluorescent probe that fluoresces weakly in aqueous solution but strongly in non-polar or hydrophobic environments. Under normal conditions, NPN is excluded from the outer membrane due to the tight cross-bridging of LPS by divalent cations, and does not fluoresce. Upon disruption of the outer membrane, NPN partitions into the outer membrane interior and an increase in fluorescence can be observed. The initial rate of increase in fluorescence varies with the concentration of polymyxin B and defines the susceptibility of the outer membrane to permeabilization, which in turn relates to the efficiency of self promoted uptake. As expected, polymyxin B was able to permeabilize the membranes of all isolates tested in a concentration dependent manner (Figure 4.5), The outer membrane of PAO1 was permeabilized at a much lower concentration than that of 9BR (4 µg/ml versus 32 µg/ml polymyxin B) and to a greater extent at all concentrations tested compared to the outer membrane of 9BR. Permeabilization of the outer membrane of the skipped well isolate 19BR fell between the susceptible and the resistant isolate and was observed at polymyxin B concentrations ≥8 µg/ml.

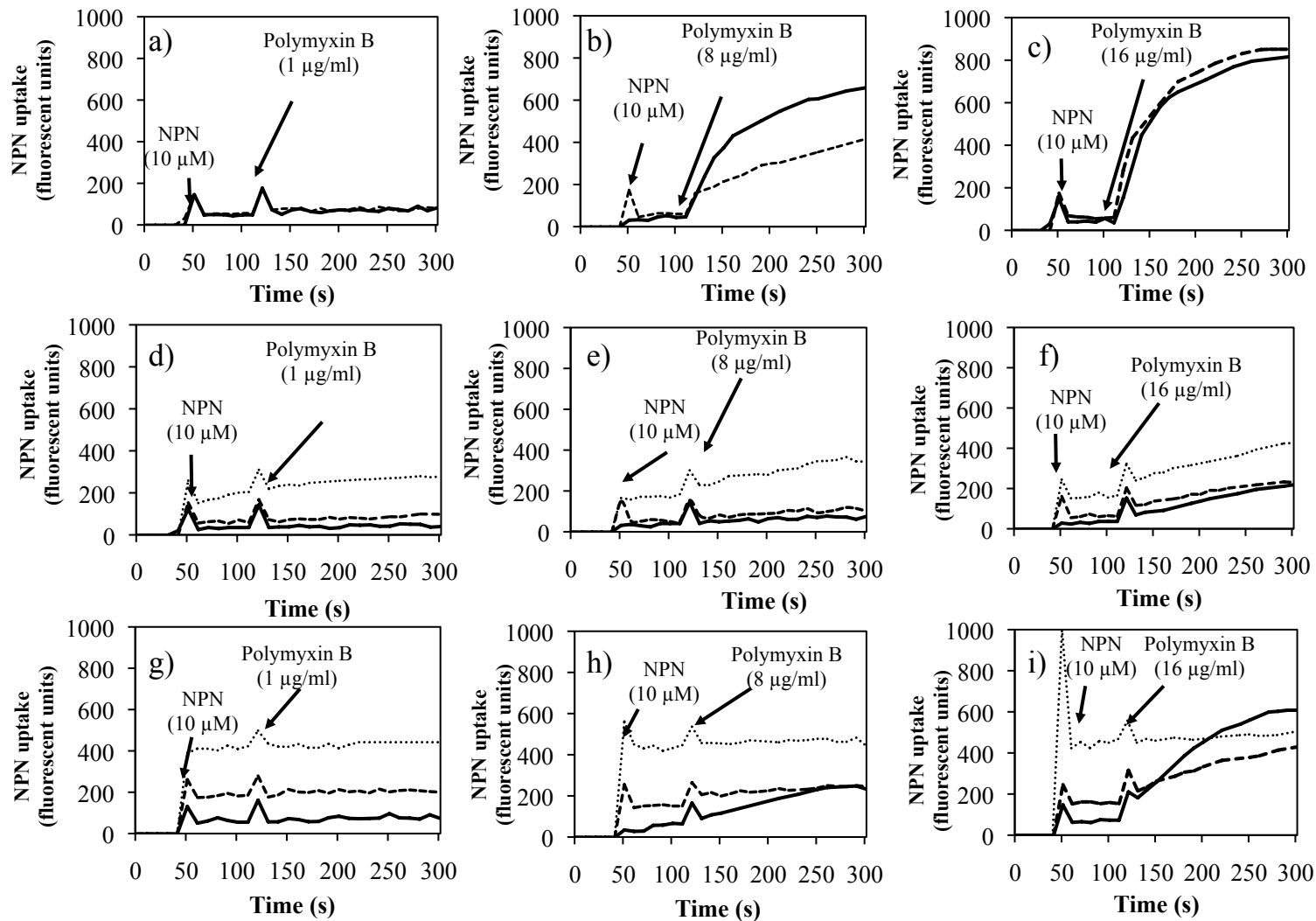**Figure 4.5 The effect of pre-incubation to mid-log phase with 0.125 µg/ml and 2 µg/ml polymyxin B on the membrane permeability of** *P. aerugionosa* **isolates PAO1 (a-c), 9BR (d-f) and 19BR (g-i).** Solid lines indicate unexposed cells, dashed lines indicate cells pre-incubated with 0.125 µg/ml polymyxin B and dotted lines indicate cells pre-incubated with 2 µg/ml.

Pre-incubation with polymyxin B had differing effects on the efficiency of self promoted uptake and the background levels of outer membrane permeability for each isolate. Pre-incubation with 0.125 μg/ml of polymyxin B had little to no effect on the background permeability of either the susceptible PAO1 strain (Figure 4.5 a-c) or on the resistant 9BR strain (Figure 4.5 d-f), while background permeability for 19BR increased significantly. Further increases in background outer membrane permeability were observed after pre-incubation with 2 μg/ml polymyxin B for both 9BR and 19BR. With regard to the effect on self promoted uptake, at intermediate concentrations of polymyxin B, as represented by 8 μg/ml polymyxin B in Figure 4.5, pre-incubation of PAO1 with 0.125 μg/ml did retard the initial rate of uptake. Similar results were observed for 19BR at 16 μg/ml as well as for 213BR (data not shown). These data indicate that exposure to sub-inhibitory concentrations of polymyxin B caused significant structural alterations in the outer membranes of the skipped well isolates 19BR and 213BR which affected their ability to interact with polymyxin B, consistent with the dramatic alterations in the expression levels of *arnB*, which is the first gene of the aminoarabinosylation operon that is known to influence polymyxin self promoted uptake and susceptibility by reducing the negative charge on LPS. While slight to moderate increases in baseline permeability of the outer membrane of 9BR to NPN were observed after pre-incubation with polymyxin B, no differences in the rate of NPN uptake were observed after further addition polymyxin B, thus indicating that, in contrast to the skipped well isolates, pre-exposure of this resistant isolate to polymyxin B had little effect on the ability of the outer membrane to bind additional polymyxin B.

4.5   Concluding Remarks

The data shown here support the notion that clinical isolates of *P. aeruginosa* have varying abilities to adapt to the presence of polymyxins. While the polymyxin B resistant isolate observed in this study constitutively overexpressed the PmrA-PmrB two-component regulatory system and was capable of only moderate adaptation as indicated by a modest concentration dependent upregulation of the PmrA-PmrB system and by minor changes in baseline NPN permeability upon exposure to polymyxin B, the skipped well isolates surveyed appeared to have a heightened ability to respond to higher concentrations of polymyxin B than to lower concentrations, although of note, the levels of expression of these genes did not reach the same high levels as 9BR. This enhanced adaptability was evidenced by the extended lag phase, by dramatic changes in expression of both the PhoP-PhoQ and the PmrA-PmrB systems, and by apparent structural changes in response to polymyxin B, affecting the permeabilization of outer

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membranes of these isolates. Of note, the structural changes in the outer membrane of the skipped well isolate, presumed on the basis the NPN results, occurred in a concentration dependent manner in response to pre-incubation with 0.125 µg/ml and 2 µg/ml polymyxin B, while the expression of *arnB, pho*Q and PA4773 only appeared to be increased upon exposure to 2 µg/ml polymyxin B. This discordance between the two assays may indicate the involvement of other systems affecting membrane permeability in response to low concentrations of polymyxin B in the skipped well isolates. A number of mutations were observed in the regulatory regions controlling *pmrAB* and *phoPQ* as well as in these genes themselves when the isolates were compared to wildtype isolates; however, only a single amino acid deletion in PmrB was observed in the constitutively resistant isolate compared to the skipped well isolates. The contributions of these mutations to the observed phenotypes have yet to be determined. The organisms displaying the skipped well phenomenon appeared to have the heightened ability to sense specific polymyxin B concentrations and induce the PhoP-PhoQ and PmrA-PmrB systems resulting in polymyxin resistance and the skipped well phenotype. Thus the ability of these organisms to grow at specific concentrations above those of the skipped wells relies on the differential effects of inhibition by particular polymyxin concentrations and the speed of induction of these operons. Most importantly, these data are the first to associate the PhoP-PhoQ, PmrA-PmrB systems and the LPS modification operon with polymyxin-inducible polymyxin B resistance in non-CF clinical isolates.

4.6   References

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5.0   Discussion

The body of work provided in this thesis sheds light on the overall complexity of antimicrobial action. Nonetheless, this work only begins to touch upon the potential mechanisms by which adaptive resistance may occur. In this section, I will discuss my work in the context of other recent publications identifying contrasting methodologies and discussing the benefits or limitations of various studies. Additionally, as the previous chapters were written and published (or submitted for publication) as independent works, this section will also serve to highlight and discuss observations on the work as a whole particularly focusing on the role of energy metabolism in aminoglycoside resistance, the importance of timing in antibiotic killing, and the connection between antibiotic use and common adaptations seen in cystic fibrosis.

5.1   Investigating Antimicrobial Resistance

The majority of antimicrobial resistance studies to date have focused on epidemiological analyses of acquired resistance genes and the emergence of high-level resistance (3, 15, 18, 31). Increasingly, the importance of low-level resistance and adaptive resistance are being recognized as important phenotypes (28, 29). In some cases a low-level resistance phenotype may serve as an indicator for an enhanced adaptability phenotype. My work further supports this notion, as I have demonstrated that isolates with mutations in DNA replication and repair genes may present a two-fold increase in MIC; however the hypermutability phenotype associated with this mutation is responsible for resistance (rather than the actual mutation) and can lead to further mutations leading to increases in resistance in the organism. Similarly in the case of fluoroquinolone resistance, it has been demonstrated that a single point mutation in one topoisomerase genes may cause only a two-fold increase in MIC but predisposes the organisms to elevated mutation rates in the second topoisomerase gene which indeed leads to high level resistance (16).

As few mechanisms contributing to low-level resistance have been identified to date, the study of low-level resistance in clinical isolates becomes a challenge. Such studies are further complicated by variability in the genotypes of clinical isolates making them difficult to compare. The use of large scale screening of mutant libraries has facilitated the identification of contributors to low-level antibiotic resistance and has the advantage of comparing mutations in a common genetic background. In recent years several antibiotic resistance screens have been published for *P. aeruginosa* (7, 12, 13). Overlap with the screen in Section 2 was minimal when

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comparing this to screens with other antibiotics, however the *nuo* operon, DNA replication and repair genes and denitrification genes were identified both in another aminolgycoside screen and in a fluoroquinolone resistance screen (7, 12). No other published screens to date have been as stringent or as extensive as the tobramycin screen reported here, as either partial libraries were used or higher antibiotic concentrations were employed (e.g. 4x MIC) which would lead to significantly fewer genes being identified. Nonetheless, the tobramycin screen suffers from the general limitations of transposon mutant screens. These screens cannot identify mutations in essential genes, nor do they identify point mutations which may have a different effect, such as enhanced target binding, compared to a complete gene deletion. To overcome the hurdle of investigating essential genes overexpression libraries are being employed for these types of investigations (25); however no such studies have been performed with *P. aeruginosa* to date.

Recent attempts have been made to use the results of published resistance screens to investigate the contributions of particular genes to low-level resistance in clinical isolates (19). Unfortunately, the study of resistance mechanisms in clinical isolates has mostly been limited to sequencing studies attempting to identify point mutations. An appreciation for the complexity of bacterial regulatory systems, as demonstrated in Section 4 with the PhoPQ/PmrAB/Arn circuit, provides the understanding that point mutations do not necessarily occur in the target resistance genes themselves but may be attributable to mutations in genes upstream in the regulatory or biochemical hierarchy. Investigations of both low-level resistance and adaptive resistance may necessitate the use of genome wide sequencing or RNASeq transcript detection methods for identification of resistance mechanisms in clinical isolates.

5.2   The Role of Energy Generation in Aminoglycoside Resistance

In chapters 2 and 3, a link was drawn between the genes that were dysregulated in response to tobramycin and those capable of conferring some degree of protection against tobramycin. The most obvious of these links appeared between the energy metabolism genes that conferred resistance when knocked out and were also predominantly downregulated in response to tobramycin. While the observation that knocking out energy metabolism genes conferred tobramycin resistance supports previous work that showed that aminoglycoside uptake is energy dependent (8-10, 17), it is interesting that such a wide range of energy metabolism processes could affect tobramycin susceptibility. *P. aeruginosa* is extremely versatile and possesses a number of complex respiratory chains (32). The effect, on the proton motive force, of a knockout in any single component of these pathways is unknown, and my attempts to measure

membrane potential were confounded by the presence of a very impermeable outer membrane. Nevertheless, I propose that these mutations influence the threshold membrane potential that is known to strongly influence aminoglycoside susceptibility (9). While it has been demonstrated that multiple mutations conferring low-level aminoglycoside resistance via independent mechanisms can act cumulatively in *P. aeruginosa* (12), the effect of multiple mutations in energy metabolism genes has not been determined. If these energy metabolism mutations do indeed cause resistance as a result of lowering membrane potential, mutations affecting distinct energy metabolism systems might further lower membrane potential thus further decreasing aminoglycoside uptake. This may be of importance given the finding that *P. aeruginosa* downregulated a wide variety of energy metabolism genes in response to tobramycin, notably three distinct NADH dehydrogenases (*nuo, nqr* and *pha*).

Consistent with the idea that aminoglycoside resistance is due to the production of hydroxyl radicals resulting from hyperactivation of the TCA cycle and electron transport chain (11, 20, 21), the PseudoCyc Omics viewer (26) showed that several genes of the TCA cycle were upregulated during lethal tobramycin treatment. However, the genes homologous to the *E. coli* ArcA-regulated genes (on which the Kohanski theory was based), that had been shown to be upregulated in Kohanski's work (21), were actually seen here to be downregulated in the lethal tobramycin *P. aeruginosa* arrays. The down regulation of these elements would suggest a mechanism different from the ArcA mediated mechanism of TCA cycle hyperactivation proposed by Kohanski *et al.* for *E. coli* (21), although this may also reflect the different mechanisms of energy metabolism in *P. aeruginosa* compared to *E. coli*. Nonetheless, we cannot rule out an increased production of hydroxyl radicals by other means, as no studies were performed which directly measured hydroxl radical levels in response to tobramycin. Furthermore, the *asrA* gene product, which is strongly induced in response to tobramycin, has 62% similarity to human mitochondrial Lon protease, which is known to be involved, amongst other things, in the protective response to oxidative damage (4-6). A similar role in the oxidative stress response of *P. aeruginosa* for AsrA has yet to be investigated. Nevertheless it is important to point out that the proposal of Kohanski *et al*, of a common oxidative mechanism of action for all bactericidal antibiotics, contradicts many years of research demonstrating independent and well defined mechanisms. It is also difficult to understand how such a notion is compatible with the ability of tobramycin to kill *P. aeruginosa* under completely anaerobic conditions.

5.3 Importance of Timing in Killing by Antibiotics and Development of Adaptive Resistance

The microarray studies performed in Chapter 3 were performed at a single timepoint after treatment with tobramycin. More powerful studies using a time course might shed light on the dynamics of gene regulation. For instance, despite the significant downregulation of 48 genes capable of conferring resistance when knocked out, as observed within 30 minutes in the lethal microarrays, there was still a rapid onslaught of killing occurring almost immediately after this 30 minute lag. Was then the extent of downregulation of these genes insufficient to provide adequate protection against tobramycin? Or, were the cells already committed to a path of cell death by the time this downregulation occurred, such that dysregulation was futile? Perhaps, a certain level of downregulation is required to provide adequate protection and the timing of the events dictates that only a small portion of the cell population (often termed "persistors") will be protected. Indeed, it is common to see a stabilization of the cell population and eventual re-growth after the phase of rapid cell death. This effect may due to the absence of residual aminoglycoside levels, or to the development of adaptive or mutational resistance. However, perhaps even the rapid occurrence of resistance mutations may be part of the adaptive resistance response since the DNA repair (mutator) genes *mutS, mutL* and *mutY* were all found to be downregulated in response to tobramycin, which would in turn increase the mutation rate.

5.4 Cystic Fibrosis Adaptations and Tobramycin Exposure

The overlap between the identified resistance genes and the genes downregulated in response to tobramycin provides a compelling argument for the relevance of many of the identified "resistance determinants" as contributing to adaptive resistance to aminoglycosides; however, the clinical relevance of these findings has yet to be assessed. In comparing the tobramycin microarray analyses performed here, to microarray analyses performed on the CF Liverpool Epidemic strains (27), a number of genes were found to be similarly dysregulated including upregulated genes such as the *pqsABCDE* quorum sensing genes, the hydrogen cyanide genes *hcnABC* and efflux pump genes *mexXY,* and downregulated genes including the nitrogen metabolism operons *nir, nar* and *nos.* The studies by Salunkhe *et al.* (27) also demonstrated that these nitrogen metabolism genes are further down regulated by oxidative stress induced by H_2O_2. In addition to the overlapping dysregulation of genes seen with the Liverpool Epidemic strains, other common adaptations such as loss of flagella (22) and the hypermutator phenotype (24) are reflected in our data that showed downregulation of flagella

and DNA repair genes in response to tobramycin. As common adaptations seen in CF isolates seem to be reflected in the microarray data, one might question whether these adaptations are occurring solely in response to antibiotic treatment. It has been suggested that these mutations occur as a result of the oxidative stress encountered within the CF lung (30). If the hypothesis is correct that aminoglycosides do in fact exert bactericidal activity by promoting production of hydroxyl radicals, then the oxidative stress present in the CF lung may predispose the organism to aminoglycoside resistance even in the absence of aminoglycoside exposure. Recent work by Fothergill *et al.* demonstrated variations in *P. aeruginosa* phenotypes during antimicrobial treatment for acute exacerbations of infection in CF patients (14). That study only focused on production of the exoproducts pyocyanin and elastase A; however, expression studies of a similar nature investigating the changes in gene expression seen in the tobramycin microarray would certainly be of use in assessing the relevance of these findings.

5.5   Involvement of LPS Modifications in Adaptive Resistance

Part of the difficulty in studying adaptive resistance phenotypes from clinical isolates results is due to the ability of isolates to revert or alter phenotypes when grown in conventional lab media. Furthermore monitoring global changes in gene expression relies on cultivating sufficient concentrations of organisms, again requiring cultivation within the lab. The use of clinical isolates exhibiting an unusual adaptive resistance phenotype in laboratory conditions in Chapter 4, provided a unique opportunity to investigate adaptive resistance. While the phenotype of skipped wells was not observed for aminoglycosides, adaptations affecting the LPS can similarly affect polymyxins and aminoglycosides, although it has previously been demonstrated for PAO1 that aminoglycosides do not have as great an ability to induce the systems involved in aminoarabinosaminylation of LPS as do the polymyxins (23).

The evidence provided in Chapter 4 suggests the involvement of PhoPQ and PmrAB in mediating adaptive resistance to polymyxins via the *arn* operon in the clinical isolates exhibiting the skipped well phenotype; however, it would seem likely that another regulatory system could be involved as only a single amino acid deletion in PmrB (ΔD45) was observed, when comparing the constitutively resistant isolate to the adaptively resistant isolates, even though the major differences in gene expression between these isolates lay in the different expression of *phoQ*. Nonetheless, the effect of this single amino acid deletion in PmrB might lead to the constitutive polymyxin B resistance observed in this isolate. Recent work by Barrow *et al.* found one polymyxin resistant clinical isolate to have two nucleotide substitutions in PmrB (A247T

and Y345H) (2). These investigators were able to restore polymyxin susceptibility by introducing wildtype PAO1 *pmrB* into the mutant, although the contributions of the individual mutations were not assessed. Similarly, they observed four polymyxin resistant clinical isolates, including three containing single amino acid substitutions (either V260G or H223R) and one frameshift mutation resulting in truncation. Susceptibility of these four isolates was also restored to wildtype levels with the introduction of wildtype PAO1 *phoQ*. A similar approach could be used to assess the contribution to polymyxin B resistance of the single amino acid deletion in PmrB in isolate 9BR.

The investigations performed here focused predominantly on gene expression and membrane permeabilization by polymyxin B, thus the addition of aminoarabinose or other LPS modifications was not directly assessed. Analysis of LPS composition would confirm the suggested mechanism of polymyxin B resistance in these isolates. Nonetheless, confirmation that aminoarabinosaminylation is occurring in these isolates might not fully explain the differential regulation seen in PhoPQ and PmrAB in response to polymyxin B. Full genome sequencing and microarray analysis of the transcriptomes of these isolates may provide more insights as to other factors involved in this complex regulatory system.

The adaptive resistance observed in these isolates appears to be in delicate balance with the lethal activity of polymyxin B against these strains. As complete killing is observed in the skipped wells, it would seem a threshold concentration of polymyxin B is required to activate the adaptive resistance. The clinical relevance of the polymyxin B adaptive resistance observed in these isolates is difficult to assess as the responses of the isolates to polymyxin B *in vivo* remains unknown.

5.6   Overall Conclusions

To address the initial hypothesis that complex adaptations leading to cationic antimicrobial resistance occur in response to cationic antimicrobial agents, I have identified a large number of genes that are capable of altering susceptibility to tobramycin and even more genes that are dysregulated in response to tobramycin, many of the latter coinciding with those that affect susceptibility. I was able to demonstrate the involvement of the heat shock response in mediating immediate protection against tobramycin, although induction of the MexXY aminoglycoside efflux pump at subinhibitory concentrations was observed and might play a more major role in long term protection. I have demonstrated distinct patterns of regulation of the *phoPQ, pmrAB* and *arn* operons in response to polymyxin B in nosocomial isolates of *P.*

aeruginosa exhibiting an intriguing "skipped well" adaptive resistance phenotype during *in vitro* susceptibility testing. These distinct patterns of regulation were not found to be attributable to mutations of PhoPQ or PmrAB, highlighting the potential contribution of an alternate system involved in regulating the regulatory network involved in LPS modification.

5.7   Future Directions

The work presented here has taken the initial steps of identifying a large number of factors capable of contributing to adaptive resistance; much of the interpretation of the data comes from interpretation of gene ontology, i.e. classification of genes as involved in energy metabolism or heat shock. A better understanding of the physiological roles of the various "resistance determinants" would provide more insight into their direct contributions to resistance. For example, little has been reported to date on the gene product of PA0779/*asrA*, and comparisons of the two Lon proteases encoded by *P. aeruginosa* would provide insight as to what type of stress conditions each of these proteases responds, and what are the resulting phenotypes observed due to the expression of each of these genes. An understanding of their normal physiological roles may help to identify why *asrA* was upregulated in response to tobramycin, under the tested conditions, while the other Lon proteases were not. Though it has previously been demonstrated that *asrA* plays a role in protection against nitric oxide, nitric oxide levels decreased in response to tobramycin, even in an *asrA* mutant. Instead, as the mitochondrial Lon protease exhibiting 62% amino acid similarity to *asrA*, is known to be important in oxidative stress, the role of oxidative stress in inducing transcription of *asrA* could be examined using oxidative agents such as hydrogen peroxide or cumene hydroperoxide. Transcriptional profiling of cells expressing *asrA* under conditions of oxidative stress, or other inducing conditions, would serve as a comparative to the transcriptional profiles presented in this thesis and direct effects of *asrA* could be distinguished from other effects of tobramycin. This could be performed not only for other stressors but also for other antibiotics, as to date most studies have focused on expression profiles in the presence of subinhibitory antibiotics. Again a simple comparative with other classes of bactericidal antibiotics may indicate which responses are universal stress responses and which are unique to aminoglycosides.

To further confirm, or rule out, a role for oxidative stress and the production of hydroxyl radicals in aminoglycoside activity, as suggested by Kohanski *et al.* for *E coli* (21)*,* direct measurement of hydroxyl radical production using hydroxyl radical scavengers could be

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performed. The use of the various mutants identified in Chapter 2 would identify which pathways, if any, were involved in altering hydroxyl radical production.

To assess the clinical significance of the findings of this work, RNASeq transcriptional profiling of *P. aeruginosa* from CF sputum samples treated during acute exacerbations would be a useful comparator. Most studies to date have focused on genetic profiling or sequencing of isolates over the course of chronic CF infections; however, one study demonstrated that tobramycin susceptibility is altered even in the short time frame of 4 hours post-treatment with inhaled tobramycin (1). The disadvantage of sequencing projects is that altered gene regulation due to environmental stimuli is not assessed. Transcriptional profiling of the events occurring during treatment in the acute exacerbation period may demonstrate similarities or differences to the profiling presented here.

Finally, with regards to the clinical isolates exhibiting the polymyxin adaptive resistance phenotype, it appears that our investigations of PhoPQ and PmrAB were too narrow to identify the key regulatory components contributing to the adaptive resistance observed. Again, large scale profiling either using whole genome sequencing or transcriptional profiling would be useful for identifying other regulatory components involved in controlling PhoPQ and/or PmrAB. Understanding the regulatory network at play here is of significance, as is understanding the effect of this skipped well phenotype on *in vivo* resistance. A *P. aeruginosa* animal model using these isolates would indicate whether treatment of isolates exhibiting this phenotype can be successful in eradicating the pathogen, thus indicating whether this adaptive resistance is simply a factor of the *in vitro* conditions presented or if it is of concern *in vivo* as well.

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