REGULATION OF POLYMYXIN B AND CATIONIC ANTIMICROBIAL PEPTIDE RESISTANCE IN *PSEUDOMONAS AERUGINOSA*

By

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ABSTRACT

Pseudomonas aeruginosa is a Gram-negative, opportunistic pathogen that is noted for its environmental ubiquity, its metabolic potential and its intrinsic resistance to a wide variety of antimicrobials, detergents, dyes, and biocides. These properties are consequences of a large (6.3 Mb) genome containing ~5500 genes of which 9.4% encode regulatory proteins. One of the largest classes of regulators in the *P. aeruginosa* genome is the two-component regulators. This work describes the contribution of two two-component regulatory systems, PhoP-PhoQ and PmrA-PmrB to Mg^{2+}-limitation induced polymyxin B and cationic antimicrobial peptide resistance. Both of these systems respond to limiting Mg^{2+} and cause increased transcription of an eight-gene operon, *pmrHFIJKLM-ugd*, that is responsible for the addition of aminoarabinose to 1 and 4' phosphates on Lipid A. In addition, the PmrA-PmrB system regulates a three gene operon, PA4773-PA4775 that also contributes to polymyxin B and cationic antimicrobial peptide resistance.

In addition to regulating polymyxin B and cationic antimicrobial peptide resistance, the PhoP-PhoQ system also directly regulates several small ORFs, one of which PA0921 contributes to swimming motility via an unknown mechanism. Similarly, PmrA-PmrB regulate other phenotypes, including the growth of *P. aeruginosa* in the presence of Fe^{3+}. This growth phenotype occurs through gene products encoded by the *feoAB* operon.

Interestingly, all genes identified in this study that are PmrA-PmrB regulated are also regulated by the presence of sub-inhibitory concentrations of cationic antimicrobial peptides. The regulation of PA4773-PA4775 and *pmrHFIJKLM-ugd* via cationic peptides is mostly independent of the PmrA-PmrB and PhoP-PhoQ systems. This observation explains why adaptive resistance to cationic antimicrobial peptides occurs and suggests that another, as yet unidentified, regulator is responsible for the detection of cationic antimicrobial peptides.

A third regulatory system, PxrRS, is also identified. Mutants in this system show increased susceptibility to cationic antimicrobial peptides and polymyxin B. This susceptibility was not due to loss of regulation of the PA4773-PA4775 or *pmrHFIJKLM-ugd*. Microarray analysis demonstrated downregulation of a number of heat-shock proteins, as well as two operons potentially involved in efflux. The combined downregulation of heat-shock proteins involved in response to cellular stress and efflux systems suggests that intrinsic cationic peptide resistance is altered in these mutants.

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LIST OF ABBREVIATIONS

°C – degrees Celsius

$\Delta\psi$ – transmembrane potential

ADP – adenosine diphosphate

ATP – adenosine triphosphate

CCCP – carbonyl cyanide m-chlorophenol hydrozone

CF – cystic fibrosis

CFTR – cystic fibrosis transmembrane regulator

DIG – digoxygenin

DNA – deoxyribonucleic acid

DTT – dithiothreitol

EDTA – ethylenediaminotetraacetate

GDP – guanodine diphosphate

GTP – guanidine triphosphate

HEPES – 2-(4-(2-hydroxyethyl)-1-piperazinyl) ethanesulfonic acid

LPS – lipopolysaccharide

MIC – minimal inhibitory concentration

NPN – 1-N-phenylnapthylamine

ORF – open reading frame

PCR – polymerase chain reaction

RND – resistance-nodulation-division

SDS – sodium dodecylsulphate

S. Typhimurium – *Salmonella enterica* sv. Typhimurium

TNFα – tumour necrosis factor alpha

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- S. Lewenza screened and mapped a library of mini-Tn5-*luxCDABE* mutants for those that showed strong responses to limiting Mg^{2+}-concentrations.
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- M. Bains purified His_6-PhoP and His_6-PmrA and performed gel-shift analysis on target gene promoters.
- G. Winsor and F. S. L. Brinkman performed bioinformatic analysis and computational prediction of putative PhoP and PmrA-regulated promoters.
- S. Lewenza contributed to experimental design and analysis.
- A. Kwasnicka was responsible for the construction of the His_6-PhoP expressing plasmid.
- R. E. W. Hancock edited the final draft of the manuscript.

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- M. Bains performed microarray experiments and analysis of *pxrR*::IS*lacZ* and wild-type PAO1

- R. E. W. Hancock edited the final draft of the manuscript.

Chapter 1 – Introduction

Pseudomonas aeruginosa

P. aeruginosa is a Gram-negative bacterium that is widely distributed throughout the environment and is commonly isolated from soil and water samples worldwide. It is an opportunistic pathogen of invertebrates, plants, and mammals, including humans. It is noted for its high metabolic diversity and its intrinsic resistance to a broad spectrum of antimicrobial compounds. In 2000, the genome of *P. aeruginosa* was sequenced, and this work greatly aided in the understanding of the basis for these observations (Stover, Pham et al. 2000).

The genome contains a large number (550) of genes encoding known and putative transporters. This number represents 10% of the entire coding capacity of the genome, and indicates that *P. aeruginosa* has the ability to transport a large variety of small molecules across its cell envelope (Stover, Pham et al. 2000). This is believed to be, in part, an adaptation to the intrinsically low permeability of the *P. aeruginosa* outer membrane (Hancock and Nikaido 1978). Unlike *E. coli*, where transport across the outer membrane occurs primarily through the general porins OmpC and OmpF, the major *P. aeruginosa* porin, OprF, is a poorly functional pore (Brinkman, Bains et al. 2000). This is likely due to the presence of a salt-bridge within the porin monomer that restricts the size of the pore opening, and therefore limits diffusion (Brinkman, Bains et al. 2000). To compensate for the low permeability of the outer membrane, *P. aeruginosa* has several families of specific porins, including one large family with homology to the basic amino acid porin, OprD (Tamber and Hancock 2005). Unlike conventional porins, where the rate of diffusion across the outer membrane increases with increasing concentrations of solute, the specific porins contain a substrate-specific binding site that acts as a selectivity filter, and transport of specific substrates via these proteins is saturable (Tamber and Hancock 2003). The genome also encodes a large family of TonB-dependent iron-siderophore receptors

(Stover, Pham et al. 2000). This family is believed to be important in allowing *P. aeruginosa* to scavenge iron from a wide variety of iron-siderophore complexes that may be present in dilute aqueous environments. Both of these families of proteins serve to increase transport across the outer membrane when solute concentrations are limiting.

In addition to this unique outer membrane strategy, *P. aeruginosa* also contains a large family of resistance-nodulation-division (RND) efflux pumps (Poole and Srikumar 2001). These are tripartite complexes, which span the bacterial inner membrane, cytoplasm, and outer membrane. They consist of an inner membrane spanning proton-substrate antiporter component, an outer membrane spanning outer membrane channel and a membrane linker protein that links the inner membrane pump to the outer membrane channel (Eswaran, Koronakis et al. 2004). Each RND system has a fairly broad substrate range, and any given substrate may be effluxed by a number of different RND systems (Poole and Srikumar 2001). *P. aeruginosa* contains a family of 19 outer member efflux proteins, 14 membrane linker protein, and 13 inner membrane pumps that appear to encode subunits of RND-transporters (Stover, Pham et al. 2000).

This combination of low outer membrane permeability and active efflux enables *P. aeruginosa* to maintain a low intracellular concentration of many antimicrobial compounds. Both of these general mechanisms, combined with the ability of *P. aeruginosa* to acquire resistance elements from the environment (Morrison, Miller et al. 1978; Saye, Ogunseitan et al. 1987), results in an organism that is relatively resistant to the action of many antimicrobial compounds.

P. aeruginosa is also noted for a large proportion (~9.3%) of ORFs that encode regulatory proteins (Stover, Pham et al. 2000). This is believed to contribute to the ability of *P. aeruginosa* to occupy a large number of niches by contributing to the bacterium's metabolic flexibility. Of these regulatory proteins, a large number are two-component regulators. As

suggested by the name, two-component regulatory systems generally consist of two different proteins, one a sensor histidine kinase responsible for signal detection and transduction and the other a response regulator that transmits the signal detected by the sensor kinase and transmits it to some biological output (usually gene regulation) (Wolanin, Thomason et al. 2002).

The *P. aeruginosa* genome contains 63 sensor kinases and 64 response regulators. Of the 63 sensor kinases, 42 are typical, in that they possess a signaling domain attached to a histidine-containing transmitter domain. Following signal detection, the histidine residue is autophosphorylated, permitting subsequent transfer of the phosphate from the histidine residue of the sensor kinase to an aspartate residue contained within a receiver domain in the cognate response regulator (Fig 1.1A) (Wolanin, Thomason et al. 2002). Despite the conserved signaling pathway leading to response regulator phosphorylation, the mechanism by which transcription is modulated is quite diverse and may include increased contact between the response regulator and a particular σ-factor or RNA polymerase subunit (Garrett and Silhavy 1987; Makino, Amemura et al. 1993; Stock, Robinson et al. 2000).

In addition to these simple systems, the genome also contains other less orthodox sensor/regulator hybrids (Fig. 1.1B and 1.1C) (Rodrigue, Quentin et al. 2000). These types of systems differ primarily in the structure of the sensor kinase. For the so-called hybrid systems, signal detection still leads to autophosphorylation at a conserved histidine residue, however this phosphate is then transferred to a receiver domain within the sensor kinase (Tsuzuki, Ishige et al. 1995). The phosphate can then be transferred to an Hpt (histidine phosphotransfer) domain at the C-terminus of the sensor kinase. A final phosphotransfer reaction leads to phosphorylation of a receiver domain in a response regulator protein. This final phosphotransfer leads to gene modulation by the phosphorylated response regulator. *P. aeruginosa* contains five of these types of response regulators (Rodrigue, Quentin et al. 2000),

and one, GacS has been shown to be crucial in regulating virulence (Kitten, Kinscherf et al. 1998; Goodman, Kulasekara et al. 2004). A third type of so-called unorthodox system, is very similar to the hybrid two-component regulators, although the Hpt module is a free protein, rather than a module at the C-terminus of the sensor histidine kinase (Fig. 1.1C) (Rodrigue, Quentin et al. 2000). These additional phosphotransfer reactions in the hybrid and unorthodox two-component systems allow the integration of multiple signals into a single biological output (Matsushika and Mizuno 1998; Goodman, Kulasekara et al. 2004). These types of systems have been well-characterized in *Bacillus subtilis* sporulation where phosphate flux through the Spo0F

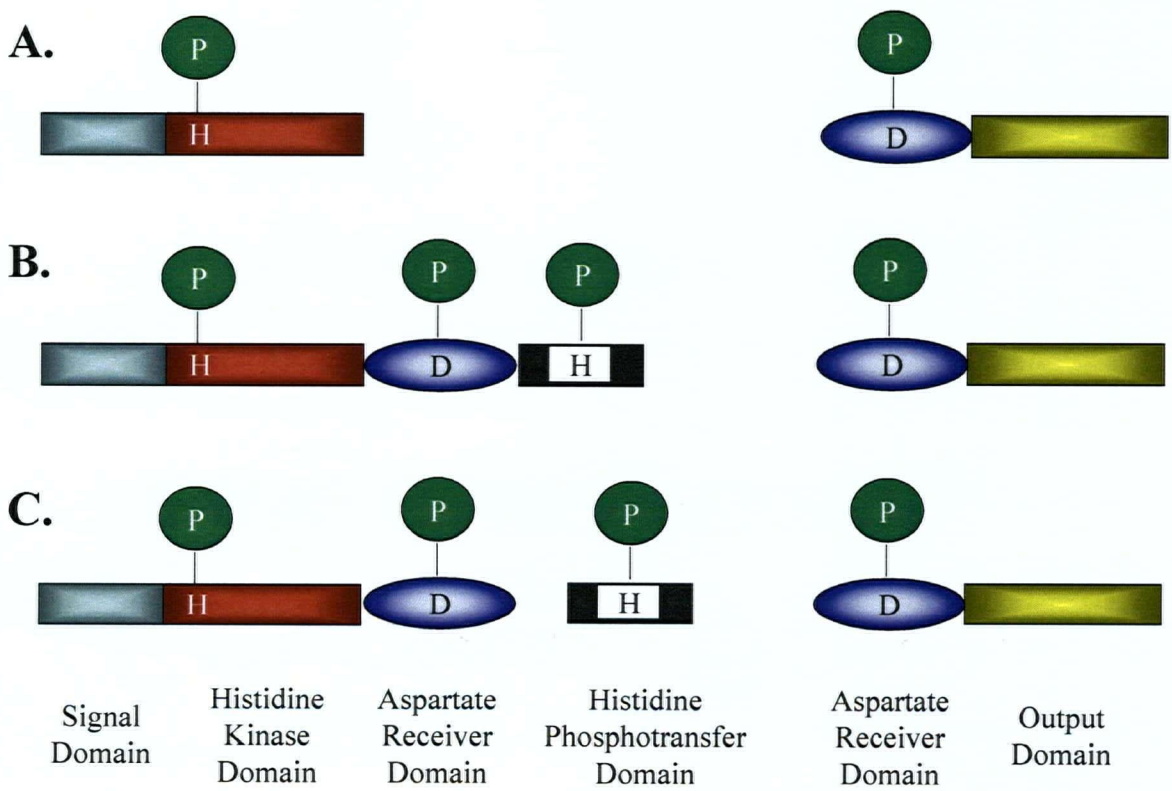

Figure 1.1. Two-component regulatory systems. A) simple two-component regulatory system B) unorthodox two-component regulatory system C) hybrid two-component regulatory system. Adapted from (Rodrigue, Quentin et al. 2000).

-Spo0B-Spo0A system is modulated by a series of dedicated phophatases (Perego and Hoch 1996), thereby preventing sporulation unless environmental conditions are ideal (Stephenson and Hoch 2002).

P. aeruginosa in cystic fibrosis

P. aeruginosa causes high rates of infections in individuals with cystic fibrosis (CF). Cystic fibrosis is a common genetic disease caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) (Riordan, Rommens et al. 1989). This protein is a chloride ion channel that when mutated results in a number of clinical symptoms including decreased nutrient absorption in the intestinal epithelium, thickening and scarring of the vas deferens, leading to sterility in males, as well as dehydration of the periciliary mucous of the bronchial epithelium (Ratjen and Doring 2003; Staab 2004). In spite of the other systemic effects of the CFTR mutation, it is this dehydration and thickening of the bronchial airway fluid that reduces the ability of the periciliary beating to remove mucous from the lung. These thickened mucous secretions and the consequent reduction in the efficiency of mucociliary clearance lead to ready infection by a range of pathogens. There appears to be some degree of bacterial succession in early infection, with bacteria like *Staphylococcus aureus* and *Haemophilus influenzae* causing the first infections (Rajan and Saiman 2002). These are eventually displaced by non-mucoid strains of *P. aeruginosa*, which over a period of months to years, will convert to a mucoid phenotype. The emergence of mucoidy has been proposed to be a consequence of oxidative bursts of hyperactive neutrophils that are a hallmark of the CF lung (Mathee, Ciofu et al. 1999). This may be due to the ability of alginate to scavenge oxygen free radicals, like those produced by macrophages (Simpson, Smith et al. 1989). The appearance of mucoid strains also correlates with a loss of lung function, exacerbated by the increased resistance of mucoid *P. aeruginosa* to neutrophil-mediated killing (Martin, Schurr et al. 1994).

In addition to the pronounced conversion to mucoidy, *P. aeruginosa* from chronic CF infections also have several alterations in their LPS. These include a conversion to serum-sensitive rough LPS (Kelly, MacDonald et al. 1990). This conversion to serum sensitivity is genetically separable from conversion to mucoidy and has been linked to the *rfb* locus (Evans, Pier et al. 1994). It is thus probable that this conversion represents a separate adaptation to the environment of the CF lung.

Some patients progress from *P. aeruginosa* infection to become colonized by the highly antibiotic resistant organisms *Burkholderia cepacia* and/or *Stenotrophomonas maltophilia*, developments that are also associated with poor clinical outcome (Gladman, Connor et al. 1992; Whiteford, Wilkinson et al. 1995). In the case of *Burkholderia*, the poor prognosis may be due to the ability of this strain to efficiently invade bronchial epithelium and to disseminate from the site of initial infection, leading to sepsis (Hutchison and Govan 1999).

Although chronic inflammation is a hallmark of the CF lung, it is not entirely clear whether or not inflammation precedes the initial infection, or whether the initial inflammation predisposes the individual to infection. A number of studies have suggested that infants with CF who have not yet been infected still display several hallmarks of inflammation, including increased pro-inflammatory cytokine production (Balough, McCubbin et al. 1995; Khan, Wagener et al. 1995; Hilliard, Konstan et al. 2002). Other groups have published contradictory data, suggesting that there is little difference in inflammation in uninfected infants with cystic fibrosis as compared with control groups (Armstrong, Grimwood et al. 1997; Dakin, Numa et al. 2002). Additionally, at least in the early stages of infection, antimicrobial treatment can significantly reduce both bacterial load and inflammation, supporting the hypothesis that the CF lung is not hyperinflamed before the onset of chronic infection. Also, the authors of one of these studies noted a 78% correlation between the number of neutrophils in the lung and bacterial load and a 34% correlation between bacterial load and IL-8 levels in the bronchial

airway lavage fluid (Dakin, Numa et al. 2002), supporting the hypothesis that infection precedes inflammation. The reasons for the discrepancies among these data are not currently known, however the authors noted that there were dramatic differences in sampling methodology between studies that reached differing conclusions.

Regardless of whether inflammation precedes infection, once infected, individuals with CF are unable to clear the infection and lung damage is caused by the chronic hyperinflammation that results at the site of infection (Kharazmi, Schiotz et al. 1986). Bronchial epithelial cells from CF patients secrete higher than normal levels of the chemokine, IL-8 (Corvol, Fitting et al. 2003). Additionally, the presence of pathogens within the lung also serves to increase production of other pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) that serve to further increase neutrophil recruitment to the lungs. These neutrophils themselves are also a significant source of IL-8 production within the lung (Corvol, Fitting et al. 2003). This positive-feedback loop results in a massive neutrophil recruitment to the site of infection (Suter, Schaad et al. 1984; Kharazmi, Schiotz et al. 1986; Fick, Sonoda et al. 1992). These neutrophils appear to be systemically primed before entering the lung, making them more likely than usual to degranulate (Conese, Copreni et al. 2003). This increased neutrophil recruitment and degranulation together with hyperinflammation leads to scarring of the bronchial epithelium and loss of lung function.

In addition to its prominent role in CF pathology, *P. aeruginosa* is currently the third leading cause of nosocomial (hospital-acquired) infections in North America, and the leading causative agent of nosocomial ventilator-acquired pneumonia (VAP) (Shorr, Sherner et al. 2005). These acute infections differ significantly from the chronic infections that *P. aeruginosa* mediate in CF, in that the bacteria are generally highly cytotoxic, due to the expression and

secretion of various virulence factors including type II and III effector proteins (Sadikot, Blackwell et al. 2005).

Virulence factors of *P. aeruginosa*

A number of *P. aeruginosa* virulence factors have been identified that appear to be important within the CF lung. There appears to be a succession of changes that occur after an individual first becomes infected with *P. aeruginosa*. In the early stages, environmental *P. aeruginosa* strains are believed to be inhaled to the lower airway and are not cleared due to the defective mucociliary system described above (Robinson and Bye 2002). At this stage it is presumed that adherence to the bronchial epithelium plays an important role in this initial colonization, as animal studies have pointed to a number of potential bacterial adhesins that contribute to virulence, including flagella, pili, surface outer membrane proteins, and recently described fimbria (Carnoy, Scharfman et al. 1994; Alm, Hallinan et al. 1996; Vallet, Olson et al. 2001; D'Argenio, Calfee et al. 2002).

Pseudomonas aeruginosa secretes a number of toxins that are involved in host tissue damage. Additionally, some *P. aeruginosa* signaling proteins including siderophores (Coffman, Cox et al. 1990; Britigan, Roeder et al. 1992; Britigan, Railsback et al. 1999) and quorum sensing molecules (Smith, Harris et al. 2002; Smith, Kelly et al. 2002) have been shown to have some toxicity toward eukaryotic cells, independent of their roles in iron-scavenging and quorum sensing respectively. In addition to these toxins, during acute infections, a type III secretion system plays an important role in determining the outcome of infection. This system is responsible for the secretion of up to four different effector proteins. These proteins, ExoS, ExoT, ExoU, and ExoY are involved in extensive modification of the host response to infection. ExoS and ExoT are closely related bifunctional cytotoxins with an ADP-ribosyltransferase domain as well as a RhoGAP domain, with ExoS being the most important bacterial determinant of dissemination of infection (Lee, Smith et al. 2005). ExoT is involved in the inhibition of

phagocytosis by polarized epithelial cells and macrophages (Garrity-Ryan, Shafikhani et al. 2004). The RhoGAP domains of both proteins appear to have similar activity, affecting GTPase activity of Rho, Rac, and Cdc42 (Barbieri and Sun 2004). However, the ADP ribosyl transferase activities are different, with ExoS interacting with a wider variety of host proteins than ExoT, the activity of which appears to be quite substrate specific and limited to several proteins involved in the formation of focal adhesion plaques (Barbieri and Sun 2004). ExoY is an adenylate cyclase that has been implicated in cytoskeletal rearrangements of the host cells, although a clear role in virulence for this protein has not yet been established (Cowell, Evans et al. 2005). ExoU is a potent phospholipase that is associated with acute cytotoxicity in strains that possess this protein (Sato, Frank et al. 2003; Sato and Frank 2004).

Interestingly, during the conversion from acute to chronic pathogen in the CF lung, *P. aeruginosa* undergoes several dramatic morphological changes. Although, type III secretion appears to be extremely important during acute infection (Roy-Burman, Savel et al. 2001), it is also becoming increasingly clear that isolates from CF patients exhibit dramatically reduced secretion of type III effectors (Jain, Ramirez et al. 2004; Lee, Smith et al. 2005). A recent publication has demonstrated that *P. aeruginosa* possesses a complex regulatory hierarchy that controls whether or not a bacterium is in the "acute" or the "chronic" infection mode (Goodman, Kulasekara et al. 2004). When in the acute phase, the RetS response regulator is activated, leading to increased type II and III toxin secretion and production of type IV pili responsible for twitching motility, while the synthesis of exopolysaccharides responsible for biofilm formation are repressed. When in the chronic phase, the RetS regulator is repressed, and the state of the cell is dominated by the GacAS*rsmZ* system. This leads to a reversal of the phenotypes listed above (i.e. decreased toxin production, decreased twitching motility, and upregulation of biofilm-associated processes). This reversal is presumed to increase resistance to antimicrobial compounds via promotion of biofilm formation while the decrease in exotoxin production

reduces the toxicity observed in acute infections. The exact signal(s) to which the bacterium is responding in these situations is not yet clear.

In addition to the dramatic phenotypic switching mentioned above, a major phenotypic change during chronic infection is the increased production of alginate. Alginate is a highly hydrated exopolysaccharide composed of long chains of β-D mannuronate and its C-5 epimer α-L-guluronate . The genetics and regulation of alginate production in *P. aeruginosa* are complex, but have been well described (Govan and Deretic 1996; Ramsey and Wozniak 2005). *In vivo*, several mutations lead to increased alginate production, but the most common involves a point mutation within the gene encoding the anti-σ factor, MucA. This *mucA22* mutation leads to activation of AlgU (also called AlgT) and activation of AlgU-dependant promoters (Fyfe and Govan 1983). Among these is the *algD-alg8-alg44-algKEGXLIJFA* operon, which encodes the enzymes necessary for the biosynthesis and secretion of alginate. Although metabolically expensive for the bacterium, this mutation almost always appears during chronic infection of the CF lung and the consequent appearance of anti-alginate antibodies correlate with poor clinical outcome (Govan and Deretic 1996).

A number of hypotheses have been advanced as to why *P. aeruginosa* in the CF lung converts to mucoidy. Mucoid *P. aeruginosa* are more resistant to opsonization, to reactive oxygen intermediates, and to the activity of complement (Govan and Deretic 1996). There has been some suggestion in the past that the presence of alginate, a polyanionic compound, can make bacteria more resistant to the activity of cationic antimicrobial peptides (Friedrich, Scott et al. 1999; Chan, Burrows et al. 2004; Chan, Burrows et al. 2005), however, it is not entirely clear if this is true for all cationic peptides, or how relevant this observation is in the context of the CF lung since the majority of endogenous cationic host defence peptides are only weakly antimicrobial or are very salt-sensitive (Yan and Hancock 2001; Starner, Agerberth et al. 2005).

Although the reasons for the conversion to mucoidy are not entirely clear, it has been shown, *in vitro*, that exposure of *P. aeruginosa* biofilms to low levels of hydrogen peroxide or to activated human peripheral polymorphonuclear neutrophils (PMNs) results in the emergence of strains containing *mucA22* mutations identical to those found in the strains most commonly isolated from CF patients (Mathee, Ciofu et al. 1999). This data strongly suggests that within the lung, the influx of neutrophils associated with persistant *P. aeruginosa* infection is directly responsible for the observed conversion to mucoidy.

In addition to these well-characterized phenotypic changes, *P. aeruginosa* cells isolated from the CF lung also possess a number of LPS alterations that are consistent with increased resistance to cationic antimicrobial peptides (Ernst, Yi et al. 1999). These include the presence of altered acylation patterns compared with those cells grown in laboratory media. These cells are also noted for the presence of one or two aminoarabinose groups at the 1 or 4' positions of Lipid A. The addition of these aminoarabinose groups is consistent with adaptations that occur when *P. aeruginosa* is grown under conditions of divalent cation limitation, and are associated with increased resistance to cationic antimicrobial peptides and polymyxin B (McPhee, Lewenza et al. 2003; Moskowitz, Ernst et al. 2004).

Treatment options for *Pseudomonas* infections in CF patients

When cystic fibrosis was first clearly described in the late 1930s it was an untreatable disease (Anderson 1938). Indeed, survival rates were very low before the advent of antimicrobial therapy. The last 40 years have been noteworthy for the dramatic improvement in both treatment options for pulmonary infections of CF patients, and in the survival rates for the disease. Currently, an individual diagnosed with CF has a median age of survival of 38 years, as compared with less than four years in 1961 (CCFF, 2005). This improvement is directly related to the improved antimicrobial therapies that have been introduced. The progress and

treatment of CF lung disease is often divided into a series of stages, corresponding to both the type and number of bacterial pathogens isolated from lung cultures.

The lungs of CF patients are often infected early in life with a variety of pathogenic bacteria, including *Haemophilus influenzae* and *Staphylococcus aureus*. In spite of this association, studies documenting a link between infection with *H. influenzae* and *S. aureus* and lung pathogenesis are sparse. Indeed, there is some evidence that eradication of *S. aureus* is actually a risk factor for early colonization with *P. aeruginosa* (Ratjen, Comes et al. 2001). In spite of this controversy, early treatment of CF patients often includes anti-Staphylococcal therapy, a treatment which often leads to bacterial clearance (Rajan and Saiman 2002; Solis, Brown et al. 2003).

P. aeruginosa infections also go through a series of stages beginning with the appearance of the first *P. aeruginosa* positive culture. At this stage, aggressive treatment can apparently eradicate the infection (Rosenfeld, Ramsey et al. 2003). This treatment usually involves treatment with oral ciprofloxacin in combination with aerosolized tobramycin or colistin (Wiesemann, Steinkamp et al. 1998; Rosenfeld, Ramsey et al. 2003). Successful eradication has also been observed following 14-21 day courses of intravenous ceftazidime or cefipime combined with intravenous tobramycin (Marchetti, Giglio et al. 2004). If this treatment is unsuccessful (i.e. *P. aeruginosa* is still isolated from sputa), the patient is assumed to be chronically colonized with *P. aeruginosa* (Rosenfeld, Ramsey et al. 2003). At this stage, the goal of antimicrobial treatment is to reduce the bacterial load in the lungs, thereby reducing the levels of inflammation. Although maintenance therapy has not yet been standardized, continuous treatment with inhaled colistin may decrease the density of *P. aeruginosa* in the sputum (Jensen, Pedersen et al. 1987). Cyclic, 28-day treatment with TOBI, a tobramycin formulation designed for inhalation has also been shown to be effective at reducing bacterial load and increasing pulmonary function (Pai and Nahata 2001).

Following chronic colonization, CF patients are often subject to sporadic exacerbations in which the bacterial load suddenly increases with a concomitant decrease in pulmonary function. This stage of infection is also correlated with the rise of hypermutable strains of *P. aeruginosa*, including mucoid strains (Oliver, Canton et al. 2000; Oliver, Baquero et al. 2002; Macia, Blanquer et al. 2005). As mentioned above, the clearest correlation with decreasing clinical outcome is the isolation of mucoid *P. aeruginosa* from the lower respiratory tract, and aggressive antimicrobial therapy is usually undertaken when mucoid strains are detected . This treatment is usually a combination therapy involving anti-*Pseudomonal* penicillin or cephalosporin (ticarcillin, piperacillin, ceftazidime, or cefepime) in combination with a monobactam (aztreonam) or a carbapenem (imipenem or meropenem) and tobramycin (Canton, Cobos et al. 2005). These aggressive antimicrobial treatments have been successful in decreasing the bacterial load, resulting in a prolonged period where there is intermittent positive and negative *P. aeruginosa* cultures, and a corresponding delay in the onset of lung deterioration (Smith, Doershuk et al. 1999; Doring, Conway et al. 2000).

In recent years, there has been a dramatic increase in the infection rate of CF patients by other, even more aggressive pathogens (Gladman, Connor et al. 1992). The most important of these emerging pathogens is *Burkholderia cepacia*. This species is actually a complex of nine different *Burkholderia* genomovars, only some of which are associated with human infection (Speert 2002; Cunha, Leitao et al. 2003; Petrucca, Cipriani et al. 2003). Infections by this species are found in ~20% of patients and these infections are even more difficult to treat than are *P. aeruginosa* infections (Speert 2002). These bacteria are invasive toward lung epithelial cells and in ~20% of *B. cepacia* infected patients, can lead to a condition known as cepacia syndrome (Mahenthiralingam, Urban et al. 2005). This condition is characterized by necrosis of the lung sometimes leading to systemic dissemination of the infection, causing sepsis and death. Infection by *B. cepacia* is strongly correlated with poor clinical prognosis. Indeed, it has been

estimated that infection with *B. cepacia* causes an average 50% decrease in residual life expectancy (Hutchison and Govan 1999).

Cationic antimicrobial peptides (host defense peptides)

Cationic antimicrobial peptides are generally defined as being less than 50 amino acids in length, with an overall charge ranging from +2 to >+10 due to the presence of excess lysine and/or arginine residues and are further delineated here as demonstrating antimicrobial activity under physiological conditions. Cationic antimicrobial peptides are also usually capable of folding into amphipathic structures, with a clear separation of hydrophobic and hydrophilic amino acid residues, thereby interacting with biological membranes and exerting antimicrobial activity. A related class of peptides is the host defense peptides, which have similar physical properties, but generally these peptides are only weakly antimicrobial under physiological concentrations of monovalent and divalent cations (Bals, Wang et al. 1998; Garcia, Krause et al. 2001). This definition serves to discriminate between peptides for which antimicrobial activity is the most important function, and those for which immunomodulatory activity is more important (McPhee, Scott et al. 2005). It is important to note that these activities are not mutually exclusive, and a single peptide can express these different activities at separate tissue sites; however recent studies have demonstrated that these activities are separable (Wu, Hoover et al. 2003; Braff, Hawkins et al. 2005). Both antimicrobial and immunomodulatory cationic peptides are widespread throughout Life, being found in bacteria, plants, insects, arthropods, and mammals.

Peptides as antimicrobial agents

Cationic antimicrobial peptides are important antibiotic candidates because they are broad-spectrum, with activity against both Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses. They also have rapid killing kinetics . There is a tendency to label peptides as "potent" when activities have only been tested in dilute media such as 10 mM

phosphate buffer or highly diluted growth medium. Indeed when tested in either 100 mM NaCl (the concentration of NaCl in the blood) or even more importantly 2 mM Mg^{2+} or Ca^{2+} these antimicrobial activities are often revealed to be rather weak (Friedrich, Scott et al. 1999). There are however some peptides, such as polymyxin B, polyphemusin I, and tachyplesins that maintain their antimicrobial activity in a physiologically relevant environment (Tam, Lu et al. 2002; McPhee, Lewenza et al. 2003). Cationic peptides selected for commercial development can have potent activity against bacterial cells, but generally have reduced toxicity towards eukaryotic cells. This selectivity is achieved by exploiting the intrinsic differences between eukaryotic and prokaryotic membrane structure. Eukaryotic membranes typically have 45%-55% phosphatidylcholine and 15-25% phosphatidylethanolamine lipids on their surfaces, lipids which have no net charge at pH 7 (Yeaman and Yount 2003). Mammalian membranes also contain 10-20% cholesterol (Yeaman and Yount 2003). In contrast, Gram-negative bacteria contain a highly negatively charged polyanionic lipopolysaccharide (LPS) on their outer membrane surface, while the cytoplasmic membrane of all bacteria contains greater than 30% negatively charged lipids like phosphatidylglycerol, and cardiolipin (Yeaman and Yount 2003). These features would tend to attract the binding of peptides to bacterial membranes. Another difference is the large transmembrane electrochemical potential gradient ($\Delta\psi$) across bacterial cytoplasmic membranes (~ -130 to -150 mV) whereas most eukaryotic cells possess modest transmembrane potential gradients of around –15 mV. This greater bacterial $\Delta\psi$ is oriented interior negative, such that it would effectively "electrophorese" these peptides into cells and thus may be a major factor in determining cationic peptide susceptibility (Yeaman and Yount 2003).

Cationic antimicrobial peptides – mechanisms of action

The mechanism of action of many cationic peptides is still not well-characterized. To date, most studies have focused on their interactions with bacterial membranes as well as with various model membrane systems (Jelinek and Kolusheva 2005). It is clear that all cationic antimicrobial peptides interact with biological membranes but it is less clear whether this is directly responsible for their antimicrobial activity.

Two membranes surround Gram-negative bacteria. The inner cytoplasmic membrane has a typical bilayer structure composed of phospholipids, with a number of integral and peripheral membrane proteins. The outer membrane is asymmetric, with the inner leaflet composed of phospholipids and the outer leaflet comprising polyanionic glycolipid LPS (Erridge, Bennett-Guerrero et al. 2002; Raetz and Whitfield 2002). The negative charges on LPS, due to a high content of phosphates and acidic sugars, are bridged by divalent cations that serve to partially neutralize the negative charge and stabilize the outer membrane (Nicas and Hancock 1980). These divalent cations bind with moderate affinity to the LPS, and such sites are the location at which self-promoted uptake of polycations, like the cationic antimicrobial peptides, occurs.

The polycationic lipopeptide polymyxin B was demonstrated to increase the permeability of the outer membrane and to sensitize Gram-negative cells to antibiotics that are normally unable to cross the outer membrane (Vaara 1992; Vaara 1993; Morris, George et al. 1995) and thus has been utilized as a model for the activity of other cationic antimicrobial peptides. The ability of polymyxin B to bind to and neutralize lipopolysaccharide was first described in the 1960s (Rifkind and Palmer 1966; Rifkind 1967; Rifkind 1967). In addition, mutant strains resistant to polymyxin B binds less polymyxin B and bind it with lower affinity than wild-type cells (Hancock, Irvin et al. 1981; Vaara and Vaara 1981; Vaara 1992). Based on these observations, and the isolation of a mutant that mimicked cells grown on low Mg^{2+} (constitutive expression of Mg^{2+}-regulated protein OprH) and was cross resistant to polymyxin

B, gentamicin and EDTA (Nicas and Hancock 1983), the self-promoted uptake hypothesis was proposed (Nicas and Hancock 1980). This hypothesis proposes that polycationic molecules bind to the divalent cation binding sites on LPS at the surface of the outer membrane by displacing native divalent cations such as Mg^{2+} or Ca^{2+}. This leads to the disruption of the stabilization of LPS by divalent cation cross-bridging, leading to localized disruption of the bilayer. The disrupting polycation is then taken up through the membrane it has destabilized, hence the name for the process, self-promoted uptake (Nicas and Hancock 1980). Self-promoted uptake explains the preferential activity of many cationic peptides against Gram-negative bacteria. It also explains the observation that divalent cations, such as Mg^{2+} and Ca^{2+}, are far more antagonistic to peptide activity than are monovalent cations like Na^{+} or K^{+}, because of the peptide binding to a site that is normally occupied by a divalent cation.

All cationic peptides must interact with the cytoplasmic membrane (in both Gram negative and Gram positive bacteria) to lead to lethality. Indeed, if high enough concentrations of cationic amphipathic peptides are used (i.e. well above the MIC), these usually cause membrane disruption. Despite this observation, it is also quite clear that at the minimal lethal concentration not all peptides kill through membrane disruption.

Structural classes of cationic antimicrobial peptides

The most abundant class of cationic peptides (~50% of sequences in the antimicrobial peptides database (Tossi 2003)) is the amphipathic α-helical class, which upon interaction with target membranes, folds into amphipathic α-helices with one face of the helix containing the majority of hydrophobic amino acids, and the opposite face containing the majority of polar or charged amino acids. This class includes the human cathelicidin LL-37 (Oren, Lerman et al. 1999) (hCAP-18) which although a relatively weak antimicrobial agent, plays an extremely important role in immune system signaling/modulation (Yang, Chertov et al. 2001; Scott, Davidson et al. 2002; Davidson, Currie et al. 2004). Although structurally conserved, the mode

of action of this class of peptides appears to be quite diverse. A model of pleurocidin, an α-helical cationic antimicrobial peptide isolated from the winter flounder is shown in Fig 1.3A (Syvitski, Burton et al. 2005).

Due to the relatively simple secondary structure of the α-helical peptides, large numbers of synthetic peptide variants have been designed on this template. These include simple non-natural peptides such as the KLAL peptides. Most of these peptides have been used to quantitate the relative contribution of particular biophysical features to the activity of a given peptide, including charge, hydrophobicity, hydrophobic moment and amphipathicity (Dathe, Schumann et al. 1996; Wieprecht, Dathe et al. 1996; Wieprecht, Dathe et al. 1997). In practice, it is very difficult to alter one of these features without also causing changes in other properties, but strong correlations can be drawn between alterations of each property and the effect these have on antimicrobial and hemolytic activity, and such variants are thus useful in testing hypotheses regarding mechanism of action (Dathe, Schumann et al. 1996; Wieprecht, Dathe et al. 1996; Wieprecht, Dathe et al. 1997).

The second large class of peptides includes the β-stranded peptides, also isolated from diverse sources. These peptides are stabilized by two or more disulfide bonds or by cyclization. They include the relatively short and highly antimicrobial β-hairpin tachyplesins (Park, Lee et al. 1992; Yang, Chertov et al. 2001) and polyphemusins (Miyata, Tokunaga et al. 1989) from the Asian and American horseshoe crabs (Fig 1.3B), and protegrins from pig neutrophils (Chen, Falla et al. 2000) which each contain two disulfide bonds stabilizing a two-stranded β-hairpin. Gramicidin S, an already commercialized cyclic β-stranded decapeptide antibiotic produced by *Bacillus brevis*, has been extensively characterized and indeed has spawned many derivatives (Jones, Sikakana et al. 1978; Gibbs, Kondejewski et al. 1998). Overall charge, hydrophobicity,

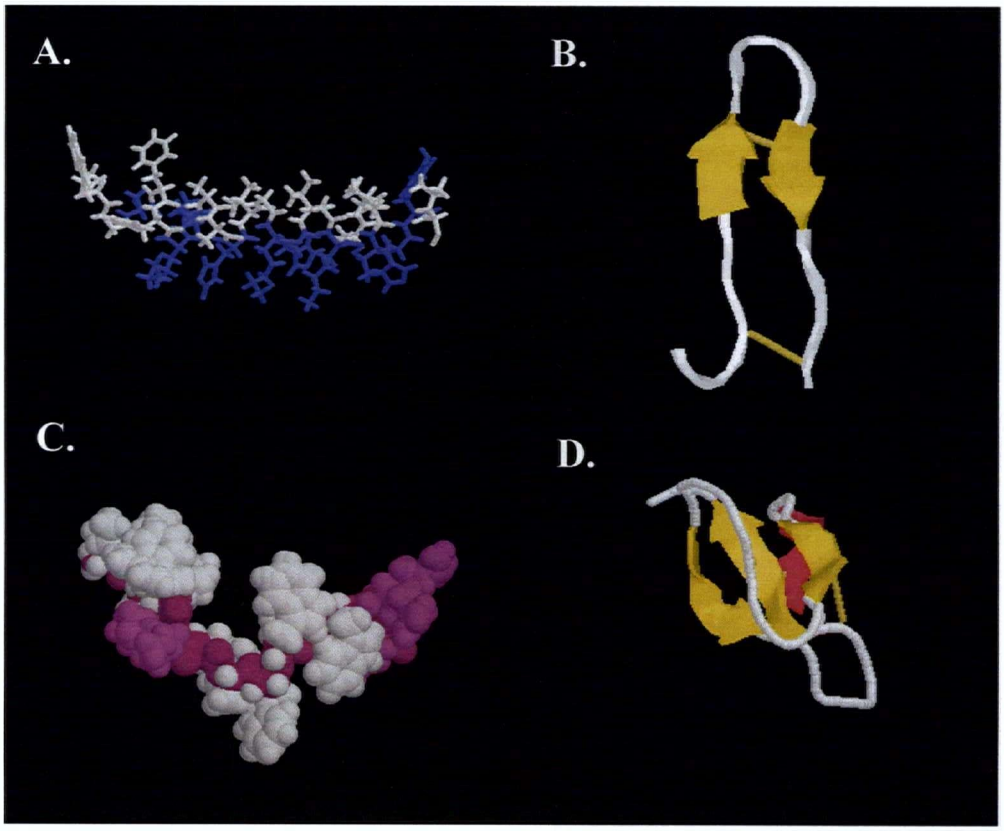

Figure 1.2. Examples of structural classes of cationic antimicrobial peptides. Illustrations were prepared from PDB structural files using Protein Explorer 2.0 beta. A) α-helical pleurocidin, PDB ID 1Z64 (Syvitski, Burton et al. 2005). B) β-sheet peptide polyphemusin I, PDB ID 1RKK (Powers, Rozek et al. 2004). C) atypically structured indolicidin, PDB ID 1G89 (Rozek, Friedrich et al. 2000). D) mixed α-β structure peptide human β-defensin 3 (hBD-3), PDB ID 1KJ6 (Schibli, Hunter et al. 2002).

and amphipathicity are also important in determining the activity of this class of peptides, although due to the added structural constraints, it is somewhat more difficult to make accurate predictions of what effect a particular substitution will have on activity (Kondejewski, Jelokhani-Niaraki et al. 1999; Jelokhani-Niaraki, Kondejewski et al. 2000; McInnes, Kondejewski et al. 2000). There are also a vast number of 3 to 4 disulphide bond-stabilized peptides, the most prominent of which are the defensins, a major component of innate immunity in plants, insects and mammals (Fig 1.3D) (Lehrer, Barton et al. 1989; Goldman, Anderson et al. 1997; Bals, Wang et al. 1998; Imler and Bulet 2005; Lay and Anderson 2005).

A number of other cationic antimicrobial peptides are characterized by their unusual composition, containing high proportions of particular amino acids such as tryptophan, histidine, or proline. Some of these (indolicidin and tritrpticin) appear to adopt extended structures upon interaction with membranes such that the structure is stabilized by hydrogen bonding and Van der Waals forces with lipids, rather than intra-peptide interactions (Schibli, Hwang et al. 1999; Rozek, Friedrich et al. 2000), although others (histatin) do form well-defined structures (Raj, Marcus et al. 1998). The peptide indolicidin is relatively small (13 residues) and contains a large proportion of tryptophan residues. In SDS micelles, it forms a boat-like structure (Fig. 1.3C) that is unique among peptides examined to date (Rozek, Friedrich et al. 2000). The histatin family of peptides (Oppenheim, Xu et al. 1988; Xu, Telser et al. 1990) of humans and other primates contains ~27% histidine residues and is involved in protection of the buccal mucosa from pathogenic yeast (Tsai and Bobek 1998). The mode of action of many of these types of peptides is not well characterized, although indolicidin appears not to cause bacterial membrane disruption at its effective concentration (Gennaro, Zanetti et al. 2002).

Due to the fact that colistin and polymyxin B may be effective anti-Pseudomonal therapeutics, and that this class of drugs has a practical advantage of low rates of spontaneous resistance to the drug (Bratu, Quale et al. 2005; Li, Nation et al. 2005), there is currently a great deal of interest in developing variant cationic antimicrobial peptides that will offer even greater therapeutic success than polymyxin or colistin. Although, to date, there has been only limited success in the clinical trials that have been undertaken, this class of compounds still represents a potential therapeutic option (Zhang and Falla 2004; Zhang, Parente et al. 2005).

Bacterial resistance to cationic antimicrobial peptides

Growth of many bacterial species under conditions of divalent cation limitation leads to increased cationic antimicrobial peptide resistance via the activation of the PhoP-PhoQ two-component regulatory systems (Macfarlane, Kwasnicka et al. 1999; Groisman 2001; Llama-

Palacios, Lopez-Solanilla et al. 2003; Winfield, Latifi et al. 2005). This system has been well-characterized in *Salmonella enterica* sv. Typhimurium (*S.* Typhimurium), and is diagrammed in Figure 1.3. In this species, activation of the PhoP-PhoQ system leads to increased transcription of a number of genes, many of which could cooperate to increase bacterial resistance to cationic antimicrobial peptides. Although this system is also involved in regulating genes that play no known role in resistance to cationic antimicrobial peptides, these genes are omitted from Figure 1.3.

Divalent cations serve a number of important roles within bacterial cells, including outer membrane stabilization (Nicas and Hancock 1980; Nicas and Hancock 1983; Moore, Chan et al. 1984), cofactors for ATP within the cells, stabilization of polyanions like DNA and RNA, and as a cofactor for many metabolic enzymes. In order to maintain a sufficient intracellular concentration of divalent cations, a number of transport proteins have evolved to transport Mg^{2+} across the cytoplasmic membrane. In S. Typhimurium, these include the MgtA, MgtB, and CorA proteins (Moncrief and Maguire 1999). MgtA and MgtB are homologs of one another, belonging to the P-type ATPase class of transporters (Kehres and Maguire 2002). CorA is expressed constitutively, while both *mgtA* and *mgtB* are induced upon exposure to limiting Mg^{2+} via the *phoPQ* system (Tao, Snavely et al. 1995). *P. aeruginosa* contains homologs of *corA* and one MgtA/B homologue (Stover, Pham et al. 2000). In addition, *P. aeruginosa* contains a gene encoding another putative Mg^{2+} transporter, MgtE. While this protein was identified based on its ability to complement a Mg^{2+} transport deficiency, its role in Mg^{2+} transport is not well understood (Townsend, Esenwine et al. 1995; Stover, Pham et al. 2000).

The most obvious phenotypic changes upon PhoP-PhoQ activation are the alterations that occur in the Lipid A moiety of the *S.* Typhimurium LPS. The PhoP-PhoQ system directly activates transcription of the *pagP* gene as well as the *ugtL* gene. The *pagP* gene encodes an outer membrane localized acyltransferase that transfers palmitate from a phospholipid molecule

to the N-linked myristoyl residue of the proximal sugar (Bishop, Gibbons et al. 2000). The presence of this extra acyl chain increases the hydrophobicity of the lipidic face of the LPS and this increased hydrophobicity may serve to increase the stability of the outer membrane to cationic peptide-induced membrane distortion (Guo, Lim et al. 1998). The *ugtL* gene encodes an inner membrane localized protein that promotes the formation of monophosphorylated Lipid A (i.e. only phosphorylated at the 1 or the 4' position, rather than at both positions (Shi, Cromie et al. 2004). This deficit of a phosphate reduces the charge on the Lipid A moiety and would therefore reduce the interaction of the cationic peptide with the LPS.

Another PhoP-PhoQ activated gene is that encoding the small protein, PmrD. This protein induces the activation of a second two-component regulatory system, PmrA-PmrB. This activation occurs because the PmrD protein binds to the phosphorylated (activated) form of PmrA and protects it from dephosphorylation by its cognate response regulator, PmrB (Kato and Groisman 2004). Once activated, the PmrA-PmrB system causes increased transcription of the *ugd* gene, and a seven gene operon, *pmrHFIJKLM* (Gunn, Lim et al. 1998; Gunn, Ryan et al. 2000; Mouslim and Groisman 2003). These genes are responsible for the addition of N_4-aminoarabinose to the 1 and 4' positions of the *S.* Typhimurium Lipid A (Nummila, Kilpelainen et al. 1995; Gunn, Ryan et al. 2000). In addition to this, the PmrA-regulated gene, PmrC, is involved in the addition of ethanolamine to the same 1 and 4' phosphates as are modified by *pmrHFIJKLM* and *ugd* (Lee, Hsu et al. 2004). A model showing the known Lipid A changes observed in S. Typhimurium and regulated by the PhoP-PhoQ or PmrA-PmrB system is shown in Fig. 1.4 (Zhou, Ribeiro et al. 2001; Lee, Hsu et al. 2004; Shi, Cromie et al. 2004). The net result is a heterogeneous mixture of Lipid A molecules, which possess reduced charge, due to the removal or blocking of the

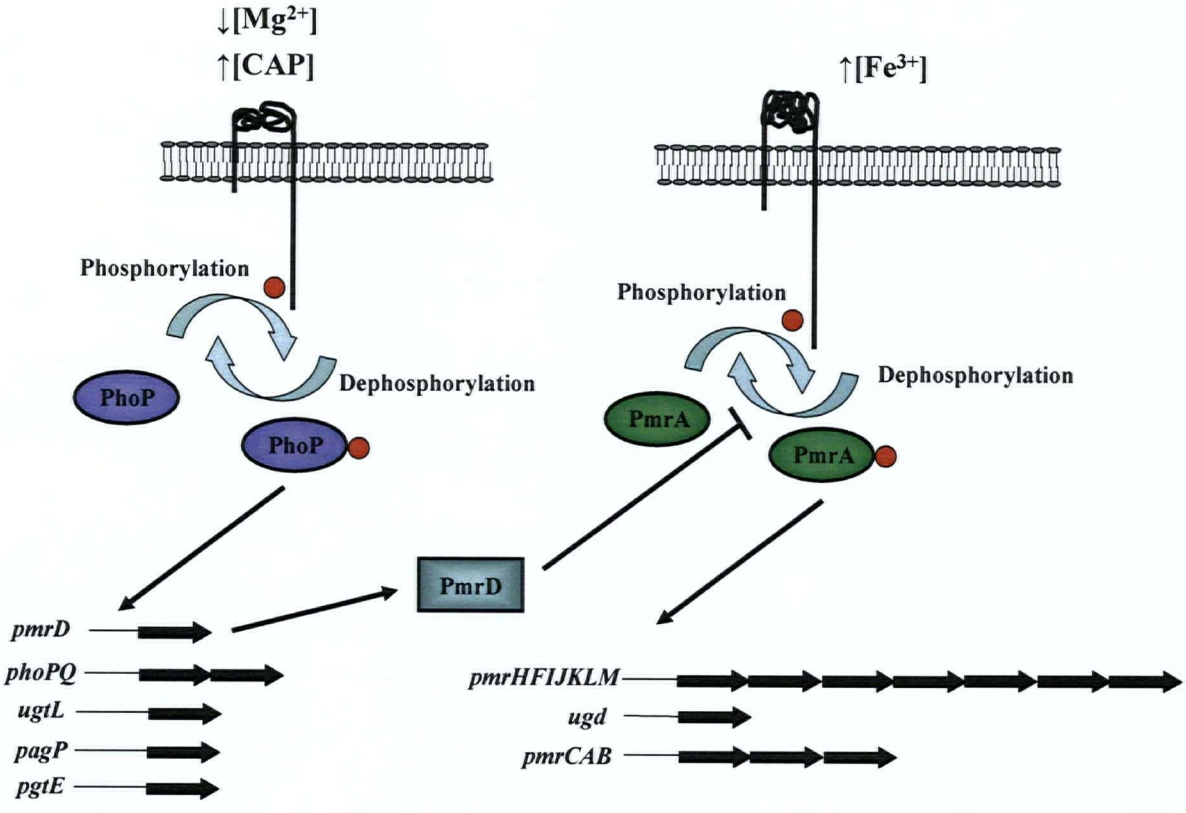

Figure 1.3. PhoP-PhoQ and PmrA-PmrB signaling in *Salmonella enterica* serovar Typhimurium. Limiting concentrations of Mg^{2+} or elevated concentrations of cationic antimicrobial peptides induce autophosphorylation of the PhoQ protein. This phosphate is then transferred to a conserved aspartate residue on PhoP. Phospho-PhoP then promotes transcription from a number of promoters, including those shown. The *pmrD* gene encodes a small basic protein that binds to PmrB, inhibiting PmrB-catalyzed dephosphorylation of PmrA. This leads to elevated levels of phospho-PmrA. Phospho-PmrA promotes transcription of the *pmrHFIJKLM* and *ugd* genes, leading to the addition a N_4-aminoarabinose to the Lipid A. Transcription of PmrCAB leads to increased levels of PmrA and PmrB as well as PmrC, a protein responsible for addition of ethanolamine to Lipid A. PmrA-PmrB also respond to the presence of elevated concentrations of Fe^{3+} independently of PmrD, leading to similar Lipid A alteration. Figure adapted from Kato & Groisman, 2004.

phosphates normally found at the 1 and 4' positions and/or altered acylation patterns. The modification of phosphates results in the reduction of the anionic charge on LPS, which in turn, reduces the requirement for divalent cation-mediated outer membrane stabilization and makes

the bacteria containing these modifications more resistant to the activity of cationic antimicrobial peptides.

Although it is clear that growth of *Salmonella* under conditions of limiting Mg^{2+} is capable of stimulating the PhoP-PhoQ and PmrA-PmrB mediated LPS changes, it is unclear as to whether this is a relevant mechanism with respect to pathogenesis. Within most tissues of the human body, including the bronchioles of the lung (Matsui, Grubb et al. 1998), and the intestinal lumen, divalent cation concentrations are in the low millimolar range, more than enough to repress the PhoP-PhoQ mediated signaling (Vescovi, Ayala et al. 1997; McPhee, Lewenza et al. 2003). It has long been argued that the divalent cation concentration within the *Salmonella*-containing vacuole is limiting, and therefore, *in vivo*, *Salmonella* is responding to this intracellular condition (Garcia-del Portillo, Foster et al. 1992; Groisman 2001). Recently however, this assertion has also been questioned and an alternative model has been presented for intramacrophage activation of the PhoP-PhoQ system (Bader, Sanowar et al. 2005; Hancock and McPhee 2005).

The PhoQ protein of a number of bacteria, including *E. coli*, *Salmonella* sp., *Shigella* sp., *Yersinia* sp., contains a patch of acidic aspartate and glutamate residues that is proposed to be important for both divalent cation and cationic peptide sensing (Bader, Sanowar et al. 2005). In this work, the authors provide an elegant explanation for their previous observation that cationic antimicrobial peptides directly activate the PhoQ protein (Bader, Navarre et al. 2003). The acidic patch, which is located directly adjacent to the plane of the membrane *in vivo*, is normally bound by divalent cations, thereby stabilizing the kinase in a non-active conformation. Upon divalent cation limitation, or upon divalent cation displacement by cationic peptides, the sensor kinase is activated, leading to phosphorylation of PhoP and activation of target promoters (Bader, Sanowar et al. 2005; Hancock and McPhee 2005). This new hypothesis has also been supported by recent work that directly measured divalent cation concentrations within the SCV,

showing that the Mg^{2+} concentration is ~1 mM, a normally non-inducing concentration (Orozco, Touret et al. 2005).

Interestingly, several non-enteric bacteria such as *Pseudomonas*, *Photorhabdus*, *Providencii*, and *Agrobacterium*, have PhoQ homologues that respond to limiting divalent cation concentration, but that do not contain this acidic patch, suggesting that the actual mechanism by which this protein detects limiting divalent cation concentration may differ depending upon the species examined. Indeed, there is some support for this in the literature as when divalent cations are removed from the *P. aeruginosa* PhoQ periplasmic domain, the monomeric state of the sensor is promoted, while the *E. coli* sensor protein remains in a dimeric conformation regardless of the divalent cation concentration (Lesley and Waldburger 2001). Additionally, the cation-bound periplasmic domain of the PhoQ protein has a dramatically different 2D-structure when compared with the *E. coli* protein (Lesley and Waldburger 2001). When Mg^{2+} is added to the *P. aeruginosa* periplasmic domain, a conformational shift occurs as assessed by circular dichroism and fluorescence spectroscopy. In constrast, no such shift occurs for the purified *E. coli* PhoQ periplasmic domain (Lesley and Waldburger 2001). In spite of these differences in the signaling domain, hybrid PhoQ proteins containing the periplasmic domain from *P. aeruginosa* and the signaling domain from *E. coli* show similar ability to respond to divalent cation limitation as native *E. coli* PhoQ (Lesley and Waldburger 2001).

In other organisms, other LPS alterations have been identified that lead to increased resistance to cationic antimicrobial peptides. *Yersinia pestis* produces a modified LPS termed lipooligosaccharide, differing primarily by the lack of repeating O-antigen subunits. In *Yersinia pestis*, PhoP regulates the addition of a galactose moiety to the LOS, in addition to regulating the addition of aminoarabinose to Lipid A (Hitchen, Prior et al. 2002). Mutants of *Y. pestis* lacking

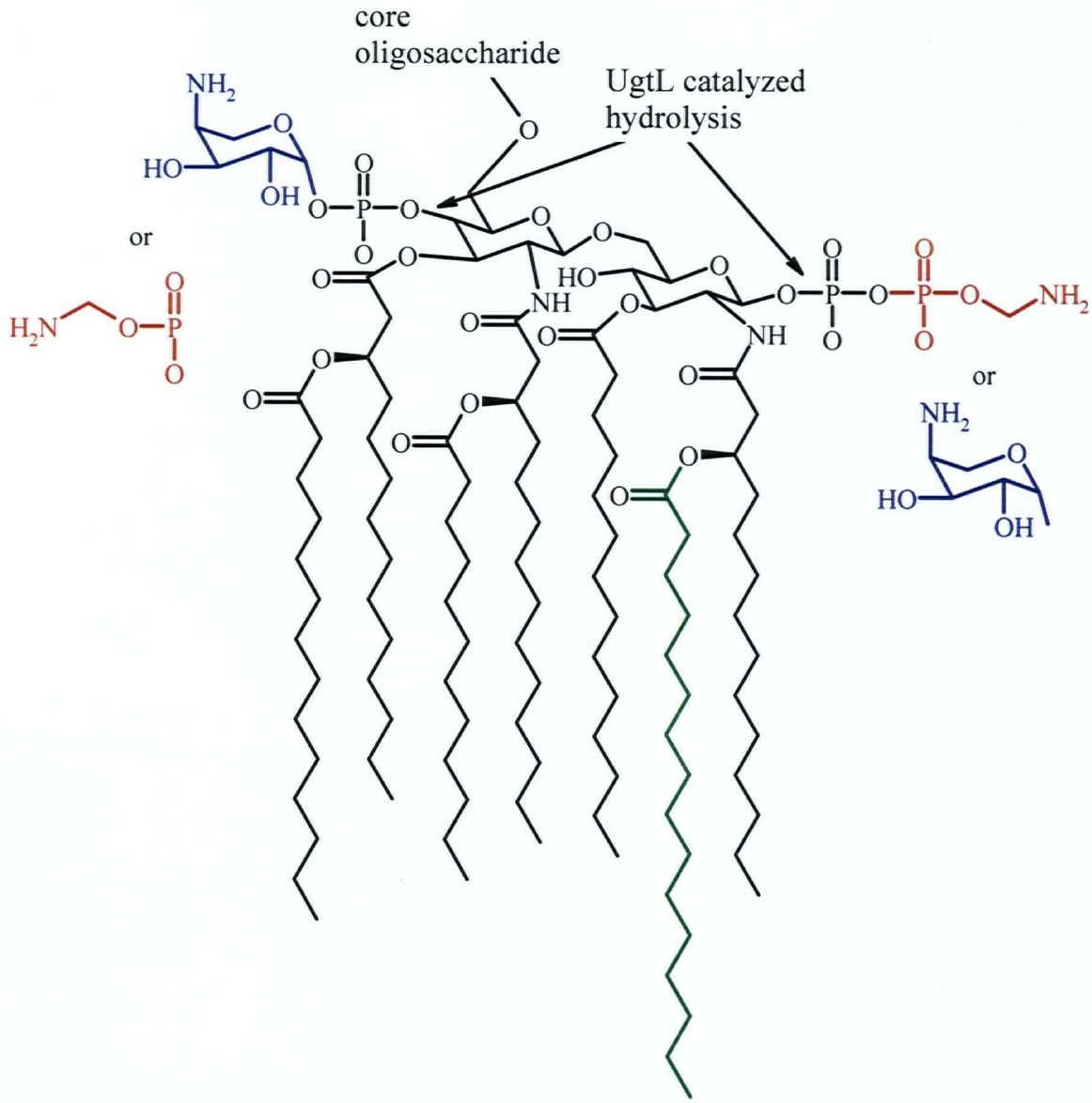

Figure 1.4. Structure of Lipid A from *Salmonella enterica* serovar Typhimurium. Modifications to Lipid A that are associated with PhoP-PhoQ and PmrA-PmrB mediated signaling are shown. Modifications shown in red represent the PmrC catalyzed addition of ethanolamine to the 1 or 4' phosphates of Lipid A. The structure of N_4-aminoarabinose is indicated in blue. The PagP-catalyzed addition of palmitic acid to the N_2-myristoyl group is indicated in green. Additionally, the sites of phosphate hydrolysis catalyzed by the UgtL protein are also indicated.

phoP exhibit >100-fold increased sensitivity to polymyxin B and 8-fold higher sensitivity to cecropin P1, an insect-derived cationic antimicrobial peptide (Hitchen, Prior et al. 2002). The authors of this work did not observe any of the Lipid A changes seen in *S.* Typhimurium, and attributed the increased sensitivity of the *phoP* mutant to the loss of LOS modification. These

results have been contradicted by several groups that have observed Lipid A modification by aminoarabinose in *Y. pestis*, as well as in other yersiniae (Marceau, Sebbane et al. 2004; Rebeil, Ernst et al. 2004; Winfield, Latifi et al. 2005). This issue is currently unresolved, although the suggestion that LOS, as well as Lipid A modifications can lead to increased resistance to cationic peptides is supportive of the strong influence that the characteristics of the outer membrane have on cationic antimicrobial sensitivity.

In addition to the LPS/LOS modifications described above, there are a number of other types of systems that contribute to increased cationic peptide resistance in a number of organisms. In *S.* Typhimurium, for example, another PhoP-regulated gene, *pgtE*, encodes an OmpT-family outer membrane protease (Guina, Yi et al. 2000). This protein catalyzes the degradation of α-helical cationic peptides, giving rise to increased resistance. In *E. coli*, deletion of *ompT* results in increased susceptibility of the bacterium to killing by protamine, likely via a mechanism similar to that observed with *pgtE* (Stumpe, Schmid et al. 1998). A *Yersinia pestis* homologue of PgtE called Pla, is known to be expressed at 37°C, but not at 25°C and is a virulence factor involved in the movement of the bacteria from a subcutaneous wound to distal sites, although what role, if any it plays in cationic antimicrobial peptide resistance is unknown (Lahteenmaki, Kukkonen et al. 2001). Furthermore, studies that looked at the susceptibility of both Gram-positive and Gram-negative bacteria in the presence of protease inhibitors suggested that a number of intracellular proteases including DegP, also contributed to the intrinsic resistance of a number of bacteria to cationic peptides (Ulvatne, Haukland et al. 2002).

In addition to the impermeability-based mechanisms of cationic peptides resistance described above, there is a precedent for the ability of certain proteins to efflux cation antimicrobial peptides. As described on page 2, efflux is a very common mechanism used by

various bacteria to reduce the concentration of a given antibiotic within the cytoplasm. In *Yersinia* species, the *rosA-rosB* genes encode a temperature-regulated potassium-efflux pump antiporter that shows specificity for several cationic antimicrobial peptides (Bengoechea and Skurnik 2000). This system also appears to respond to the presence of cationic antimicrobial peptides, demonstrating that the presence of these compounds may induce systems responsible for increasing peptide resistance (Bengoechea and Skurnik 2000).

Goals of this study

P. aeruginosa infections represent the third leading cause of nosocomial infections in North America. Furthermore, they are the leading cause of increased morbidity in cystic fibrosis patients. Treatment of these *P. aeruginosa* infections often involves the administration of colistin, a cationic peptide antimicrobial. Furthermore, a number of novel cationic antimicrobial peptide therapies are being developed for the treatment of infections that are recalcitrant to conventional antimicrobial therapy. Given the clinical importance of *P. aeruginosa* infections in cystic fibrosis patients, this study was undertaken to identify and characterize resistance determinants to cationic antimicrobial peptides. At the time of the commencement of these studies, the PhoP-PhoQ system of *P. aeruginosa* had been identified, but the pathway by which this system led to resistance was unknown. Furthermore, some unusual regulatory phenomena identified in the initial studies characterizing PhoP-PhoQ from *P. aeruginosa*, strongly suggested that other regulatory systems were involved in regulating resistance to polymyxin B and cationic antimicrobial peptides (Macfarlane, Kwasnicka et al. 1999; Macfarlane, Kwasnicka et al. 2000). Therefore the major hypotheses and goals for this work included:

1. A novel regulatory system is involved in the regulation of cationic antimicrobial peptide resistance in response to limiting concentrations of Mg^{2+}. The first goal was to identify this system.

2. This new system and the previously identified PhoP-PhoQ system form a regulatory network that contributes to resistance via the regulation of unknown effector genes. The goal here was to identify these genes and characterize the role they play in contributing to cationic antimicrobial peptide resistance.

3. Resistance to cationic antimicrobial peptides in many bacterial species occurs via mechanisms that are unrelated to PhoP-PhoQ or PmrA-PmrB signaling. Examples of this include active efflux of the cationic peptide and degradation by endogenous proteases. I hypothesized that these types of systems would be present in *P. aeruginosa* and sought to identify them through a screening approach.

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CHAPTER 2 - PmrA-PmrB of *Pseudomonas aeruginosa*[*]

INTRODUCTION

Infections with *Pseudomonas aeruginosa* are particularly difficult to cure through antimicrobial therapy due to the bacterium's intrinsically impermeable outer membrane and active efflux of toxic agents from the cytoplasm (Nikaido 1996). Virtually no novel antibiotics are available for this organism although cationic antimicrobial peptides hold the promise of improving the success of anti-*Pseudomonas* therapy (Hancock 1997; Zhang, Parente et al. 2005).

Cationic antimicrobial peptides are a structurally diverse group of molecules that are found in virtually all eukaryotes examined to date (Hancock, Falla et al. 1995; Hancock and Chapple 1999). In addition to their proven role in killing a wide variety of potential pathogens including Gram-positive and Gram-negative bacteria, fungi, and viruses, they are also multifunctional modulators of innate immunity (Scott and Hancock 2000). They are known to interact with the outer membrane via the self-promoted uptake pathway permitting activity against Gram-negative bacteria (Hancock, Falla et al. 1995; Hancock and Chapple 1999).

As a widespread environmental isolate, *P. aeruginosa* has evolved mechanisms for responding to many different stimuli. This diversity of responsiveness is reflected in the genome sequence, in which 9.4% of ORFs encode regulatory proteins (Stover, Pham et al. 2000). This compares to ~6% for *E.coli* or *B. subtilis* (Stover, Pham et al. 2000) and ~12% for *S. coelicolor* (Bentley, Chater et al. 2002). One major class of regulatory systems found in *P. aeruginosa* is

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the two-component regulatory system family. These systems generally involve a cytoplasmic membrane-spanning histidine kinase sensor protein and a cytoplasmic response regulator. Generally, upon engagement of a periplasmic binding site on the sensor kinase by an effector molecule, this protein autophosphorylates at a histidine residue on the cytoplasmic domain and transfers this phosphate to an aspartate residue of the response regulator leading to increased transcription of multiple genes, and/or repression of others (Rodrigue, Quentin et al. 2000; Stock, Robinson et al. 2000; West and Stock 2001). The *Pseudomonas* genome contains 64 response regulators and 63 histidine kinases as well as sixteen atypical kinases (Rodrigue, Quentin et al. 2000). The function of most of these regulatory proteins is undetermined.

In *Salmonella*, the PhoP-PhoQ two-component regulatory system is a global regulatory system that responds to limiting concentrations of Mg^{2+} and other divalent cations to activate virulence, as well as polymyxin B and cationic antimicrobial peptide resistance, by affecting the transcription of more than 40 genes (Soncini, Garcia Vescovi et al. 1996; Gunn, Belden et al. 1998; Heithoff, Conner et al. 1999; Monsieurs, De Keersmaecker et al. 2005). In *P. aeruginosa*, the PhoP-PhoQ system has been shown to control resistance to aminoglycosides, polymyxin B, and cationic antimicrobial peptides (Macfarlane, Kwasnicka et al. 1999; Macfarlane, Kwasnicka et al. 2000). Insertional inactivation of PhoQ, but not PhoP, also decreases the virulence of *P. aeruginosa* in a burned mouse model by 100-fold relative to a wild-type parent strain (Brinkman, Macfarlane et al. 2001). LPS isolated from the sputum isolates of patients with cystic fibrosis chronically infected with *P. aeruginosa* showed modifications that reflect those observed in *Salmonella* when grown in Mg^{2+} limiting conditions (Ernst, Yi et al. 1999).

In *Salmonella*, PhoP-PhoQ-mediated resistance to polymyxin B and cationic antimicrobial peptides largely occurs through changes in the structure of Lipid A, including the addition of N_4-aminoarabinose, ethanolamine, and palmitic acid (Zhou, Ribeiro et al. 2001). The addition of aminoarabinose to the Lipid A-phosphates of LPS is catalyzed by the seven-

gene operon, *pmrHFIJKLM* (Trent, Ribeiro et al. 2001; Trent, Ribeiro et al. 2001) which is controlled indirectly by PhoP-PhoQ via another two-component regulatory system, PmrA-PmrB (Soncini, Garcia Vescovi et al. 1996; Kox, Wosten et al. 2000; Wosten, Kox et al. 2000). In addition to this regulatory hierarchy between the two response regulators, *Salmonella* PmrB can be independently activated by high (100 μM) concentrations of Fe^{3+} or by reduced pH (possibly by influencing Fe^{3+} solubility) (Soncini, Garcia Vescovi et al. 1996; Wosten, Kox et al. 2000). Homologues of the *pmrHFIJKLM* LPS modification system (PA3552-PA3558) are present in the genome of *P. aeruginosa.* In *Pseudomonas*, the operon contains an eighth gene (PA3559) that is homologous to the *ugd* gene (UDP-glucose dehydrogenase) that in *Salmonella* is required for LPS modification and is regulated by the PhoP-PhoQ, PmrA-PmrB, and the YojN-RcsA-RcsB systems (Mouslim and Groisman 2003).

P. aeruginosa possesses several closely related homologues of the response regulator PmrA, and the signal sensor kinase, PmrB. However, the most closely related *Pseudomonas* homologues of the PmrB sensor kinase share similarity only in the C-terminal kinase domain and, until this study was undertaken, the identity of PmrA and PmrB in *P. aeruginosa* had not been determined.

In this chapter, the identification of the PmrA-PmrB encoding operon of *P. aeruginosa* is described. Additionally, this work describes a target operon of the PmrA-PmrB system that is homologous to the *pmrHFIJKLM* and *ugd* operons of *S.* Typhimurium. Mutation of *pmrH* results in hypersusceptibility to cationic antimicrobial peptides and polymyxin B. The work also demonstrates that certain cationic antimicrobial peptides and the polymyxins are capable of inducing the *pmrA-pmrB* genes, and the *pmrHFIJKLM-ugd* operon, increasing the resistance of *Pseudomonas* to these agents. This argues that the development of cationic peptide derivatives that do not induce these genes will represent an important improvement in therapeutic treatment of *Pseudomonas* infections of cystic fibrosis patients. We also show that Mg^{2+} regulates the LPS

modification operon via a mechanism that is dependent upon both PmrA-PmrB and PhoP-PhoQ systems. These and other observations, demonstrate that the PmrA-PmrB and PhoP-PhoQ systems, while sharing several features, operate differently in *Pseudomonas* and *Salmonella.*

EXPERIMENTAL PROCEDURES

Bacterial strains, primers, and growth conditions.

The bacterial strains and plasmids used in this study are described in Table 2.1. Cultures were routinely grown in Luria-Bertani broth or BM2-glucose minimal medium containing low (20 μM) or high (2 mM) $MgSO_4$ concentrations. Antibiotics for selection were used at the following concentrations: tetracycline, 100 μg/ml for *P. aeruginosa* and 10 μg/ml for *E. coli*; ampicillin 100 μg/ml for *E. coli*; carbenicillin, 300-500 μg/ml for *P. aeruginosa*; gentamicin 50 μg/ml for *P. aeruginosa* and 15 μg/ml for *E. coli.* Routine genetic manipulations were carried out according to Maniatis et al. (Maniatis, Fritsch et al. 1989).

DNA manipulations.

A bank of *luxCDABE* fusion strains were made by mobilizing pUT mini-Tn5 *luxCDABE* (Winson, Swift et al. 1998) into H103 and selecting for resistance to 100 μg/ml tetracycline. Resulting mutants were screened for differential expression of the fusion in response to high (2mM) or low (20 μM) $MgSO_4$ concentration. Briefly, a 48-pin replicator stamp was used to subculture overnight LB cultures into the left and right half of 96-well plates containing low Mg^{2+} and high Mg^{2+} conditions, respectively. After growth for 4–6 h, the luminescence was observed using a ChemiGenius^2 Bio-Imaging System (Syngene). In total, 10,000 mutants were screened for differential expression. Interrupted genes were identified by arbitrarily-primed PCR using primers Tn5luxout (5' GTCATTCAATATTGGCAGGTAAACACTATTATCACC) and ARB1 (5' GGCCA- CGCGTCGACTAGTACNNNNNGATAT) and the following cycling conditions: 95°C for 5 min followed by 5 cycles of 95°C for 30 s, 45°C for 30 s, and 72°C for

1.5 min Thirty cycles were then performed with cycling at 95°C for 30 s, 60°C for 30 s, and 72°C for 2 min followed by an extension step at 72°C for 5 min. The products from this PCR reaction were then used as the template in another PCR reaction using primers ARB2 (GGCCACGCGTCGACTAGTAC) and Tn5luxout. The cycling conditions for this second reaction were 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min followed by an extension step at 72°C for 5 min. All PCR reactions used 150 μM each dNTP, 5% DMSO, 1X polymerase buffer and 0.5 μM each primer with 1.25U of Vent polymerase (NEB, Mississauga, Canada). These amplicons were then sequenced using BigDye sequencing chemistry (PE Biosystems, Foster City, CA) on a BaseStation51 DNA fragment analyzer (MJ Research, Reno, NV). Strain H974 was identified as having an insertion in the intergenic region between PA4773 and PA4774 and is described hereafter as a PA4773::*lux* mutant.

For construction of a *pmrB* interposon mutant, a fragment containing the *pmrA* and *pmrB* genes was PCR amplified using primers designed from the genome sequence. These amplicons were then cloned into pCR2.1-TOPO using the TOPO-TA cloning kit (Invitrogen, Carlsbad CA) according to the manufacturer's instructions. An *Eco*RI fragment containing the *pmrA-pmrB* genes was then ligated into *Eco*RI digested pEX18Tc (Hoang, Karkhoff-Schweizer et al. 1998) creating pEX*pmrAB*. A *Bam*HI fragment from pX1918GT containing a *xylE*-Gm^R cassette was cloned into the unique *Bgl*II site in the *pmrB* gene of pEX*pmrAB* creating pEX*pmrB-xylE*. The inserted cassette was shown to be in the forward orientation by restriction analysis. For construction of strain H973, a *pmrB* merodiploid, pEX*pmrB-xylE* was mobilized into the chromosome without selection on 5% sucrose to maintain the plasmid vector sequence. To produce the *pmrA* interposon mutant, *pmrA* was PCR amplified and cloned into pEX100T (Schweizer and Hoang 1995) producing pEX*pmrA*. A *xylE*-Gm^R cassette from plasmid pX1918GT was removed with *Pst*I and ligated into *Pst*I digested pEX*pmrA*.

Table 2.1. *P. aeruginosa* strains, plasmids, and peptides used in this study.

Strain	Genotype, Characteristics, or Sequence	Reference
H103	wild-type *P. aeruginosa* PAO1	
H851	*phoP::xylE*-Gm^R derivative of H103	(Macfarlane, Kwasnicka et al. 1999)
H854	*phoQ::xylE*-Gm^R derivative of H103	(Macfarlane, Kwasnicka et al. 1999)
H970	*pmrB*:: *xylE*-Gm^R derivative of H103	This study
H973	*pmrB*$^+$; *pmrB*::*xylE*-Gm^R merodiploid derivative of H103	This study
H974	PA4773::*luxCDABE* derivative of H103; Tc^R	This study
H975	PA4773::*luxCDABE*; *phoQ*::*xylE*-Gm^R derivative of H103	This study
H976	PA4773::*luxCDABE*; *pmrB*::*xylE*-Gm^R derivative of H103	This study
H980	PA4773::*luxCDABE; phoP::xylE*-Gm^R derivative of H103	This study
H981	PA4773::*luxCDABE*; *pmrA::xylE*-Gm^R derivative of H103	This study
H988	*pmrA::xylE*-Gm^R derivative of H103	This study
H993	*pmrH*::*xylE*-Gm^R derivative of H103	This study
Plasmids		
pCR2.1-TOPO	PCR cloning vector, Ap^R	Invitrogen
pCS26	source of a 6 kb NotI *luxCDABE* cassette	(Bjarnason, Southward et al. 2003)
pEX100T	suicide vector containing *sacB* gene,Ap^R	(Schweizer and Hoang 1995)
pEX18Tc	suicide vector containing *sacB* gene, Tc^R	(Hoang, Karkhoff-Schweizer et al. 1998)
pX1918GT	source of *xylE*-Gm^R cassette; Gm^R, Ap^R	(Schweizer and Hoang 1995)
pUC*phoP*	formerly pEMR3, *phoP* cloned into pUCP19	(Macfarlane, Kwasnicka et al. 1999)
pEX*pmrA*	pEX100T containing *pmrA* gene	This study
pEX*pmrAB*	pEX100T containing *pmrA-pmrB* genes	This study
pUC*pmrA*	*pmrA* cloned into pUCP23	This study
pEX*pmrA::xylE*	suicide vector containing *pmrA::xylE*-Gm^R fusion	This study
pEX*pmrB::xylE*	suicide vector containing *pmrB::xylE*-Gm^R fusion	This study

pEXQ-*xylE*F1	suicide vector containing *phoQ::xylE*-Gm^R fusion	(Macfarlane, Kwasnicka et al. 1999)
pUT mini-Tn*5* *luxCDABE*	mini-Tn*5*-*luxCDABE* containing plasmid	(Winson, Swift et al. 1998)
pUC*pmrH::* *luxCDABE*	pUCP23 containing the entire intergenic region between PA3551 (*algA*) and PA3552 (*pmrH*) fused to *luxCDABE*	This study
Peptides		
CP10A	ILAWKWAWWAWRR-NH_2*	(Friedrich, Rozek et al. 2001)
CEMA	KWKLFKKIGIGAVLKVLTTGLPALKLTK*	(Macfarlane, Kwasnicka et al. 2000)
CP208	KKKSFIKLLTSAKVSVLTTAKPLISS*	(Friedrich, Scott et al. 1999)
indolicidin	ILPWKWPWWPWRR-NH_2*	(Selsted, Novotny et al. 1992)
CP11CN	ILKKWPWWPWRRK-NH_2*	(Friedrich, Moyles et al. 2000)
cycCP11	$IC_1LKKWPWWPWRRC_1K$	(Rozek, Powers et al. 2003)
LL-37	LLGDFFRKSKEKIFKEFKRIVQRIKDFLRNL VPRTES*	(Gudmundsson, Agerberth et al. 1996)
polyphemusin	$RRWC_1FRVC_2YRGFC_2YRKC_1R$*	(Zhang, Scott et al. 2000)
linear polyphemusin	RRWAFRVAYRGFAYRKAR*	(Zhang, Scott et al. 2000)
polymyxin B	fa-$B^LT^LB(B^LB^LFL^LB^LB^LT^L)$†	(Windholz, Budavari et al. 1976)
PMBN	$B^LT^LB(B^LB^LFL^LB^LB^LT^L)$†	(Windholz, Budavari et al. 1976)
colistin	fa-$B^LT^LB(B^LB^LLL^LB^LB^LT^L)$†	(Windholz, Budavari et al. 1976)

* sequence in the one letter amino acid code; -NH_2 indicates amidation of the carboxyl terminus; numbered cysteines represent residues joined by disulphide bonds.

† sequence in the one letter amino acid code; B indicates α, γ diamino butyrate; a superscripted L indicates that amino acid is the L-enantiomer; fa indicates a 6-methyloctanoyl or 6-methylheptanoyl fatty acid chain

A similar approach was used to construct a *pmrH*::*xylE*-Gm^R allele. PA3552 encodes a homolog (61% identity, 78% similarity) of the S. Typhimurium *pmrH* gene. Gene-replacement constructs were mobilized into *P. aeruginosa* H103 using biparental mating and resolved by successive selection on 50 μg/ml gentamicin and 5% sucrose. The allelic exchanges were confirmed by PCR. The PCR amplified *pmrA* gene was cloned into pUCP23, producing pUC*pmrA*.

Plasmid pUCP*lux* was created by first replacing the MCS of pUCP23 (West, Schweizer et al. 1994) with a new linker (*Eco*RI-*Bam*HI-*Sma*I-*Xho*I-*Not*I-*Hin*dIII). The *luxCDABE* genes from plasmid pCS26 (Bjarnason, Southward et al. 2003) were cloned as a *Not*I fragment into the new MCS. The orientation of the *luxCDABE* cassette was confirmed by restriction digestion. To determine how the eight-gene operon encoding the LPS aminoarabinose modification system (*pmrHFIJKLM-ugd*) of *Pseudomonas* is regulated, a plasmid-encoded promoter fusion to the entire intergenic region between PA3551 (*algA*) and PA3552 (*pmrH*) was created by PCR amplifying the promoter for *pmrH* and cloning it in front of the *luxCDABE* cassette, to produce pUC*pmrH*::*luxCDABE*.

Gene reporter assays.

XylE assays were performed as described previously (Macfarlane, Kwasnicka et al. 1999). Briefly, a 25 ml *P. aeruginosa* culture was grown in 125 ml Erlenmeyer flasks to an OD_{600} of 0.3-0.6. The cells were pelleted by centrifugation, resuspended in 750 μl of 50 mM potassium phosphate buffer pH 7.5 containing 10% v/v acetone, and broken by sonication. Unbroken cells and debris were removed by centrifugation. The protein content of the crude extracts was determined by the modified Lowry assay. Aliquots of the cell extracts were then added to 1 ml of 50 mM potassium phosphate buffer, pH 7.5 containing 0.3 mM catechol. The absorbance change of the solution was monitored at 375 nm and the rate of change over 5 minutes was used

to determine the enzyme activity in the sample using an E_{375} for 2-hydroxymuconic semialdehyde of 44,000.

Induction of the *luxCDABE* fusion in liquid media was measured using a SPECTRAFluorPlus luminometer (Tecan, San Jose, CA). Luminescence was corrected for growth by simultaneously monitoring the absorbance at 620 nm. Results presented show the average and standard deviation of 6-8 replicate measurements. Image analysis of colonies grown on agar plates was performed with a Luminograph LB980 photon-imaging video system (EG&G Berthold, Bundoora, Australia).

Killing curves.

P. aeruginosa cultures were grown to OD_{600} of 0.3-0.6 in BM2-glucose minimal media containing 20 μM Mg^{2+}. These cultures were then diluted 1:100 into pre-warmed sodium phosphate buffer, pH 7.5 containing 2 μg/ml polymyxin B sulphate (Sigma). Samples were shaken at 37°C and aliquots were withdrawn at specified times, and then assayed for survivors by plating onto LB agar.

Minimal inhibitory concentrations (MICs).

MICs were assessed using standard broth microdilution procedures in BM2 glucose minimal medium containing 20 μM or 2 mM Mg^{2+} (Macfarlane, Kwasnicka et al. 1999). Growth was scored following 24 hr incubation at 37°C. For measuring MICs against cationic antimicrobial peptides, a modified assay was used to prevent artificially high MICs due to aggregation of peptides and binding to polystyrene (Zhang, Scott et al. 2000).

Outer membrane permeability assays.

P. aeruginosa was grown to mid-logarithmic phase in BM2-glucose minimal media supplemented with 20 μM $MgSO_4$. The cells were then harvested, washed, resuspended to $OD_{600} \sim 0.5$ in buffer containing 5 mM HEPES (pH 7.0), 5 mM glucose, and 5 mM KCN and

incubated at 20°C for 10 minutes. Bacterial suspensions were placed in a quartz cuvette with a magnetic stir bar. 1-N-phenyl napthylamine (NPN) was added to the cuvette at a concentration of 5 μM and the baseline fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The assay was initiated by the addition of various concentrations of polymyxin B. Fluorescence was measured until a stable signal was observed, indicating that additional NPN entry into the membrane had stopped.

RESULTS

Identification of *pmrA-pmrB*.

To permit screening for Mg^{2+} responsive promoters, a library of mini-Tn*5luxCDABE* mutants was constructed. Strain H974 contained a transposon insertion between ORFs PA4773 and PA4774 and luminescence was strongly activated under Mg^{2+}-limitation (Fig. 2.1A, Table 2.2).

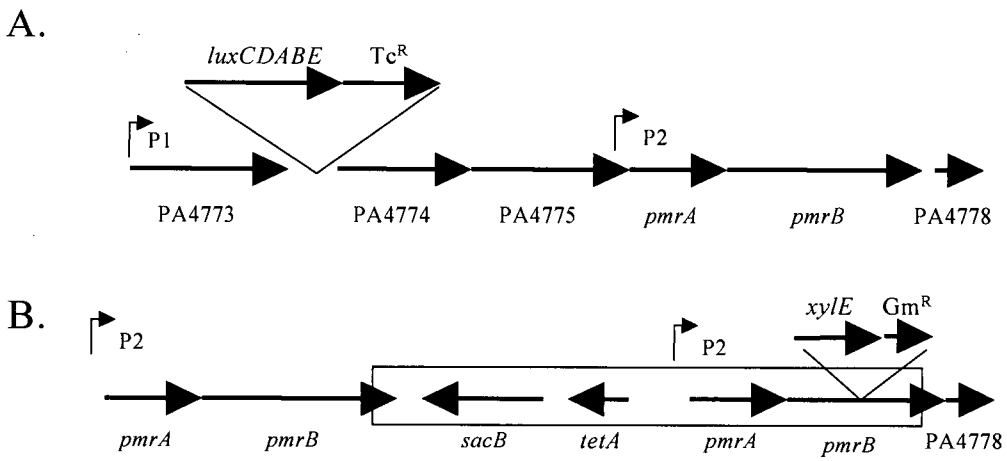

Figure 2.1. A) Structure of the *pmrA-pmrB* containing operon. The site of transposon insertion in strain H974 is shown. P1 and P2 represent hypothetical, unmapped, PmrA-regulated and weakly constitutive promoters, respectively, positioned approximately based on the data presented in the text. B) Structure of the merodiploid fusion in strain H973. Plasmid encoded sequences are boxed or the site of insertion of the *xylE*-Gm^R cassette is indicated.

These open reading frames are situated in an operon upstream of a two-component regulatory system PA4776 (response regulator) and PA4777 (sensor kinase), and a MerR-type regulator, PA4778. The sensor kinase and response regulator pair exhibited significant similarity at the amino acid level to the PmrA-PmrB system of *Salmonella* (PmrA: 45% identical, 60% similarity; PmrB: 27% identical, 48% similarity), although the periplasmic sensing domain of the sensor protein shared very little homology with that of PmrB from *Salmonella.*

Regulation of *pmrA-pmrB* by Mg^{2+}.

As shown in Table 2.2 and Fig. 2.2, the PA4773::*lux* fusion in strain H974 was strongly induced by low (20 μM) Mg^{2+} and repressed by high (2 mM) Mg^{2+}. To determine if the Mg^{2+}-regulation observed for this fusion was controlled by the PhoP-PhoQ system or other Mg^{2+} responsive elements, a series of double mutants were created. Since PhoQ-null mutants are known to express PhoP-activated genes under normally repressing conditions (Macfarlane, Kwasnicka et al. 1999), *phoQ* mutants were constructed in strain H974, producing strain H975 (*phoQ::xylE*-Gm^R; PA4773::*lux*). This strain exhibited increased activation of the *luxCDABE* cassette under high Mg^{2+} conditions, suggesting that the *luxCDABE* fusion in this strain was controlled in part by *phoQ* (Table 2.2, Fig. 2.2), although some level of Mg^{2+} regulation was still observed. A *phoP* mutant was also created in the H974 background. This strain (H980) showed wild-type response to Mg^{2+} indicating that the effect of *phoQ* deletion was not mediated via PhoP (Fig. 2.2).

An interposon mutant, H970, was created in the putative *pmrB* sensor kinase gene by cloning the *pmrA-pmrB* genes and interrupting the *pmrB* gene with a cassette containing the reporter gene *xylE* prior to recombining the *pmrB::xylE*-Gm^R gene back into *P. aeruginosa*. The presence of the *xylE* cassette allowed examination of the response of *pmrB* transcription to changing environmental conditions. Initial examination of this response showed very little

activity regardless of Mg^{2+} concentration, with only 6.4 ± 0.4 pmols·μg^{-1}min^{-1}

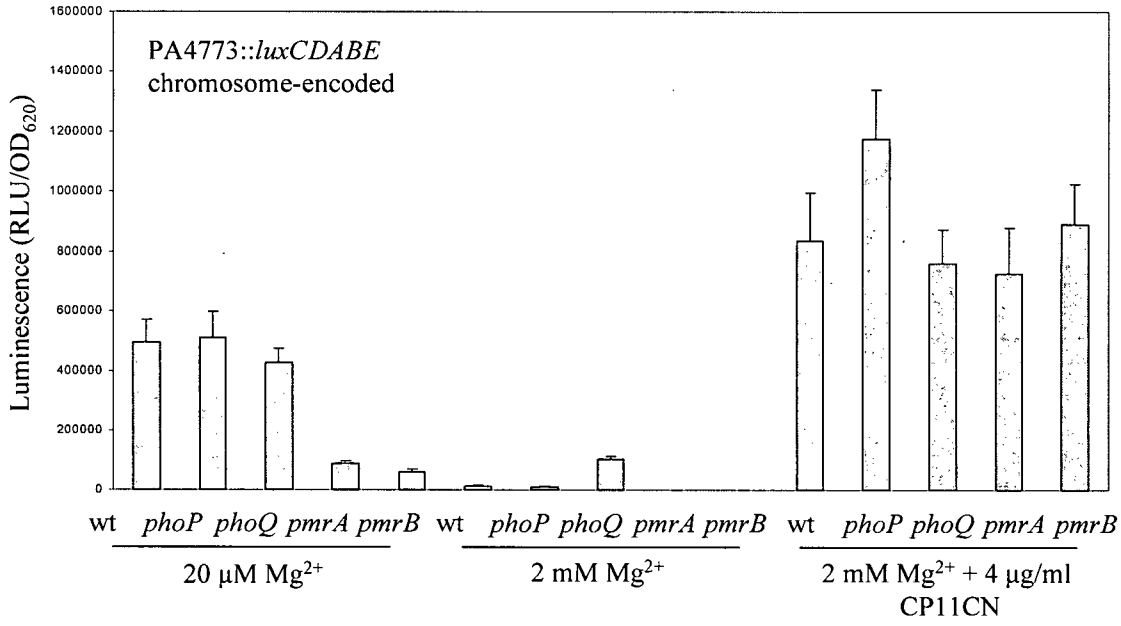

Figure 2.2. Regulation of PA4773 in *phoP*, *phoQ*, *pmrA*, and *pmrB* mutant strains. Induction of PA4773::*lux* fusion in BM2-glucose minimal media supplemented with 20 μM Mg^{2+}, 2 mM Mg^{2+}, and 2 mM Mg^{2+} with 4 μg/ml CP11CN in H974 (PA4773::*lux*), H980 (PA4773::*lux*; *phoP*), H975 (PA4773::*lux*; *phoQ*), H981 (PA4773::*lux*; *pmrA*) and H976 (PA4773::*lux*; *pmrB*).

Table 2.2. Effect of overexpression of *phoP* or *pmrA* on PA4773 expression. Luminescence of the PA4773::*luxCDABE* fusion in strains H974 (PA4773::*lux*), H975 (PA4773::*lux*, *phoQ*), and H976 (PA4773::*lux*, *pmrB*) fusion under high and low Mg^{2+} conditions were measured. Measurements are expressed in thousands of RLU/OD_{620}.

	Plasmid	H974	H975	Fold-change[a]	H976	Fold-change[b]
2 mM Mg^{2+} (high)	none	16.6±3.1	78.8±7.3	4.7	2.0±0.8	0.12
	pUC*phoP*	8.8±3.6	52.8±4.2	6	1.3±0.5	0.14
	pUC*pmrA*	20.2±1.7	ND	ND	1.8±0.8	0.09
2 μM Mg^{2+} (low)	none	876±23	829±14	0.94	91.8±1.3[c]	0.10
	pUC*phoP*	951±38	650±18	0.68	40±1.1[c]	0.04
	pUC*pmrA*	2630±30	ND	ND	16.7±0.6[c]	0.006

[a] indicates the fold-change of H975 compared to H974

[b] indicates the fold-change of H976 compared to H974

[c] The modest 2.5 to 5.5 fold decrease in RLU in the *pmrA*-overexpressing strain H976 compared to the same strain with no plasmid or the phoP-overexpressing strain H976 may be due to the overexpression of PmrA in the absence of phosphorylation by PmrB, causing this PmrA to act as a repressor.

of 2- hydroxymuconic semialdehyde produced under high Mg^{2+} conditions and 21.7 ± 5.0 pmols·$\mu g^{-1}min^{-1}$ produced under low Mg^{2+} conditions. We reasoned that this could be due to a strict requirement for the PmrA and PmrB proteins to activate the operon. To determine whether or not this was the case strain H973 was created (Fig. 2.1B), a co-integrate merodiploid mutant that contained both a wild-type *pmrB* gene in addition to a *pmrB::xylE* fusion. Strain H973 (merodiploid strain) produced 29.6 ± 1.3 pmols·$\mu g^{-1}min^{-1}$ 2-hydroxymuconic semialdehyde when grown in high Mg^{2+}, increasing 39-fold to 1150 ± 30 pmols·$\mu g^{-1}min^{-1}$ when grown in low Mg^{2+}. This demonstrates that a functional copy of *pmrB* was required for maximal expression of *pmrB*.

To further confirm the strict requirement for *pmrB,* strain H976 was constructed, in which *pmrB* was interrupted by the *xylE*::Gm^R cassette in a H974 (PA4773::*lux*) background. Strain H976 exhibited 10-16 fold lower expression of the *luxCDABE* reporter (Table 2.2 and Fig. 2.2) compared to H974. However both H974 and H976 were inducible in low Mg^{2+} concentrations, which may be due to PhoQ mediated regulation of PmrA phosphorylation as discussed below. Strain H981, a *pmrA* mutant also showed abrogation of the induction of the PA4773:*lux* fusion under low Mg^{2+} conditions (Fig 2.2).

When *pmrA* was supplied on a plasmid (pUC*pmrA*) to strain H974 (PA4773::*lux*), luminescence was increased three-fold in low Mg^{2+} media (Table 2.2). When pUC*pmrA* was added to strain H976, which lacked a functional copy of *pmrB*, very little expression was observed from the PA4773::*lux* fusion, even in low Mg^{2+} media. These results were consistent with the hypothesis that PmrB was required to activate PmrA, leading to increased expression of the PA4773::*lux* fusion. Addition of pUC*phoP* did not have any effect on expression of the PA4773::*lux* fusion (Table 2.2).

Table 2.3. Minimal inhibitory concentrations (μg/ml) of peptides and aminoglycosides toward *P. aeruginosa* grown in low Mg^{2+} medium. MICs were determined by two-fold serial dilution in BM2-glucose minimal media with 20 μM Mg^{2+} added. Results shown are the mode of 4-8 independent experiments.

	MIC (μg/ml)				
Antibiotic	H103 (wt)	H974 (PA4773::*lux*)	H970 (*pmrB::xylE*)	H988 (*pmrA::xylE*)	H993 (*pmrH::xylE*)
CP10A	32	2	2	2	8
indolicidin	32	4	16	16	16
CP11CN	>32	4	32	32	2
cycCP11	16	2	16	16	4
LL-37	16	8	8	16	8
polyphemusin	1	0.5	0.5	0.5	0.25
linear polyphemusin	>32	>32	>32	>32	ND
CEMA	2	1	0.5	1	2
CP208	>32	>32	>32	>32	ND
polymyxin B	8	0.25	1	0.25	<0.03
PMBN*	>32	>32	>32	>32	ND
colistin	32	16	4	8	<0.03
tobramycin	2	1	2	2	2
amikacin	4	1	4	2	1

*polymyxin B nonapeptide

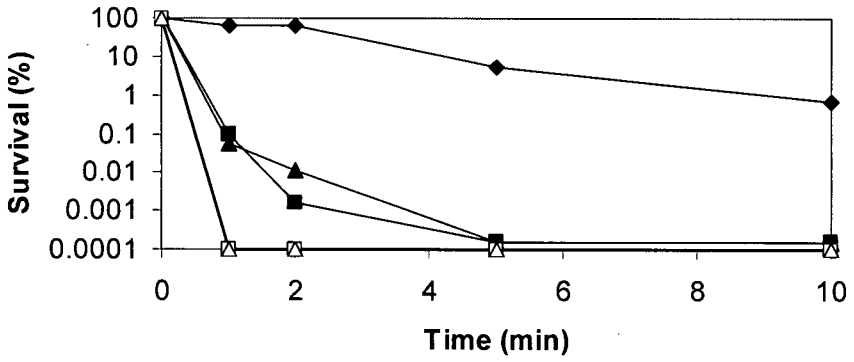

Figure 2.3. Killing of *P. aeruginosa* by 2 μg/ml polymyxin B. Strains are ◇ H103 (WT) grown in 2 mM Mg^{2+}; Δ H970 (*pmrB::xylE*), 2 mM Mg^{2+}; □ H974 (PA4773::*luxCDABE*), 2 mM Mg^{2+}; ◆ H103, 10 μM Mg^{2+}; ▲ H970, 10 μM Mg^{2+}; ■ H974, 10 μM Mg^{2+}.

The *pmrA-pmrB* genes and the *pmrH* (PA3552) gene regulate resistance to polymyxin B and cationic antimicrobial peptides.

Strains H103 (wt), H970 (*pmrB::xylE*-Gm^R), and H974 (PA4773::*lux*) were examined for sensitivity to killing by 2 μg/ml polymyxin B after growth in medium containing high (2mM) or low (20 μM) Mg^{2+} (Fig. 2.3). Under high Mg^{2+} conditions, all strains exhibited complete susceptibility to polymyxin B, with 6 log orders of killing taking place within two min. In low Mg^{2+} medium, the parent strain was more resistant to polymyxin B, exhibiting only two-log orders of killing in 10 min, while the mutants, H970 and H974 were completely susceptible to killing (i.e. >6 log orders reduction in colonies within 5 min) upon exposure to this concentration of antibiotic.

MIC data also confirmed the increased susceptibility of strains H970 (*pmrB::xylE*), H974 (PA4773::*lux*), H988 (*pmrA*::*xylE*), and H993 (*pmrH*::*xylE*) to polymyxin B, and certain cationic antimicrobial peptides (Table 2.3). The mutant strains showed 8-32-fold lower MICs to polymyxin B. Strains H974 (PA4773*::lux*), H970 (*pmrB::xylE*), and H988 showed marginal MIC changes to peptides LL-37, polyphemusin, linear polyphemusin and polymyxin B nonapeptide. Linear polyphemusin and CP208 showed no antimicrobial activity towards any of the strains at the concentrations examined. Strain H974 showed 8-fold decreases in MIC to indolicidin, CP11CN, and cycCP11CN, while strains H970 and H988 showed no significant change in MIC to these compounds. CP10A exhibited a 16-fold reduced MIC to all mutant strains. Interestingly, interruption of *pmrH* resulted in a different susceptibility pattern to that of either the PA4773, *pmrA* or *pmrB* mutants. This strain showed a modest 4-fold decrease in MIC to CP10A. However, the strain was supersusceptible to both polymyxin B and colistin, with more than a 1000-fold decrease in the MIC to these drugs. There was little effect of these mutations on the susceptibility of the strains to amikacin and tobramycin. Overall, strain H974

was generally more sensitive than H970 (*pmrB::xylE*-Gm^R) or H988 (*pmrA::xylE*-Gm^R) to most of the antimicrobial agents examined. This may be due to a polar effect of the transposon on the downstream genes PA4774 and PA4775. The functions of the PA4773, PA4774, and PA4775 genes are unknown, but they share some similarity with polyamine biosynthetic genes.

PA4773 and *pmrH* affect the permeability of the outer membrane.

The functions of PA4773, PA4774, and PA4775 are unknown. Therefore, the sensitivity of the outer membrane to permeabilization with polymyxin B was examined measuring NPN uptake (Fig. 2.4). NPN is a hydrophobic dye that is normally excluded from the interior of the lipid bilayer by the tight association of the hydrophilic LPS headgroups. Antibiotics that interfere with this packing arrangement allow NPN to access the interior of the bilayer. The fluorescence of the molecule increases due to the increased hydrophobic character of this region of the bilayer. The results demonstrated that permeability of the outer membrane correlated with the susceptibility of each strain to polymyxin B. Following exposure to 4 μg/ml polymyxin B, the *pmrH::xylE* strain exhibited an immediate and dramatic increase in NPN fluorescence. The isogenic parent strain did not show any increased NPN fluorescence, while the PA4773::*luxCDABE* strain showed an increase that was intermediate between those of the *pmrH::xylE* strain and the wild-type strain.

Induction of the *pmrA-pmrB* containing operon by cationic antimicrobial peptides.

Two types of antibiotic resistance that can influence clinical outcome are mutational resistance and adaptative resistance. Adaptive antibiotic resistance can involve multiple mechanisms one of which could involve pre-exposure to sub-inhibitory concentrations of the antibiotic. In the case of polymyxin B resistance in *Salmonella* sp., detection of low Mg^{2+} by PhoQ or high Fe^{3+}/low pH by PmrB leads to induction of genes responsible for the LPS modifications that reduce self-promoted uptake across the outer membrane. Reporter fusions in strains H973 and H974 were utilized to determine whether or not these signals and/or

polymyxin B and cationic antimicrobial peptides could induce expression of *pmrB::xylE* or PA4773::*luxCDABE* in *P. aeruginosa.*

No induction was observed in response to increased Fe^{3+} concentrations or lowered pH, the signals responsible for activation of PmrB in *Salmonella* (Fig. 2.5B). However, a dose-dependent induction of *pmrB::xylE* in the merodiploid strain H973 was observed over a range of

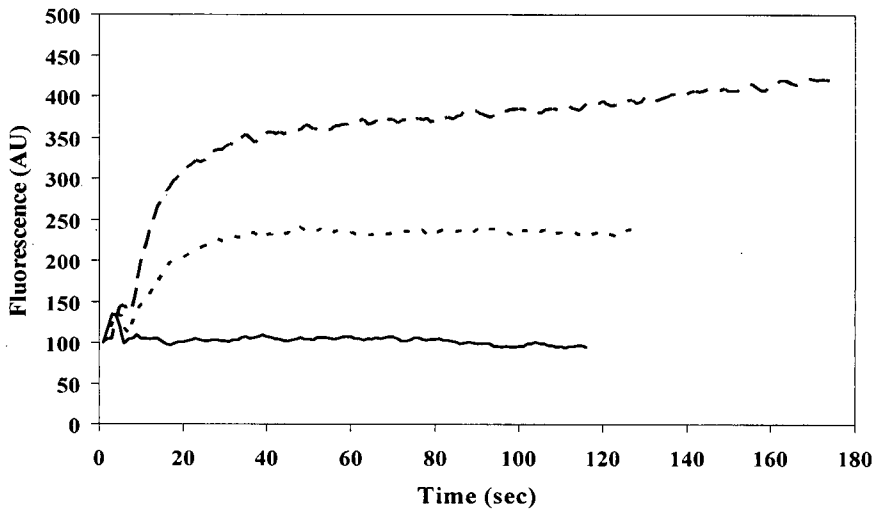

Figure 2.4. Outer-membrane permeability of *P. aeruginosa*. Mid-logarithmic phase cultures of H103 (WT, solid line), H974 (ΔPA4773, dotted line) and H993 (Δ*pmrH*, dashed line) were exposed to 1 μg/ml polymyxin B and the increase in NPN fluorescence was measured.

concentrations from 0 to 750 ng/ml of polymyxin B (Fig. 2.5A). Similarly, growth of the *pmrB::xylE* fusion in high (2 mM) Mg^{2+} with the addition of sub-inhibitory concentrations of cationic antimicrobial peptides led to a very strong induction of the *pmrB::xylE* fusion in response to certain cationic peptides (Fig. 2.5B). Cattle indolicidin caused a 50-fold increase in expression while CP11CN, an improved indolicidin variant, increased expression of the fusion by 45-fold. Polyphemusin was considerably less effective with only a four-fold increase.

Utilization of the *lux* fusion as a reporter in H974 permitted rapid and sensitive analysis of the effects on the *pmrA-pmrB* operon of a variety of peptides at different concentrations (Table 2.4). The patterns observed using the *lux* reporter matched very closely to those observed using the *xylE* reporter although the fold-changes for induction appeared to be higher when

measured by *lux* expression, probably because baseline expression could be more reliably determined by luminescence. Thus, both of these reporters appeared to be regulated in the same manner. The PA4773::*lux* fusion in H974 was strongly induced in high Mg^{2+} by indolicidin and the indolicidin variants CP10A, cycCP11 and especially CP11CN (Table 2.4). These sequence related peptides appeared to affect the expression to similar degrees and importantly, very low concentrations (31-250 ng/ml; <1% of MIC) still caused significant induction of this fusion.

The α-helical peptide CEMA was also capable of causing modest induction of luminescence in the PA4773::*lux* fusion, although the lowest concentration causing induction was significantly higher (0.5 μg/ml) than that observed for CP11CN (31 ng/ml), despite the fact that the MIC of CEMA toward *P. aeruginosa* is 16-fold lower than that for CP11CN (Table 3). Conversely, the inactive α-helical variant, CP208 still caused induction of the fusion at extremely high concentrations, but induction fell off rapidly as the concentration of CP208 fell below 8 μg/ml. The weakly antimicrobial human cathelicidin peptide LL-37 demonstrated similar effects to CP208. Nevertheless, it is clear that induction of this operon was not related to the ability of the peptides to kill bacteria, implying that induction of resistance and cellular damage may be separate events.

Horseshoe crab polyphemusin and related peptides were also examined for their ability to induce luminescence in the PA4773::*lux* fusion, since polyphemusin displays strong antibacterial activity toward *Pseudomonas*. Interestingly, the β-hairpin peptide polyphemusin induced only a 17-fold increase in luminescence in the PA4773::*lux* fusion in H974 (Table 2.4), despite the fact that *P. aeruginosa* is >32 fold more sensitive to killing by this peptide compared to indolicidin and its derivatives (Table 2.3). Interestingly, the non-bactericidal linear

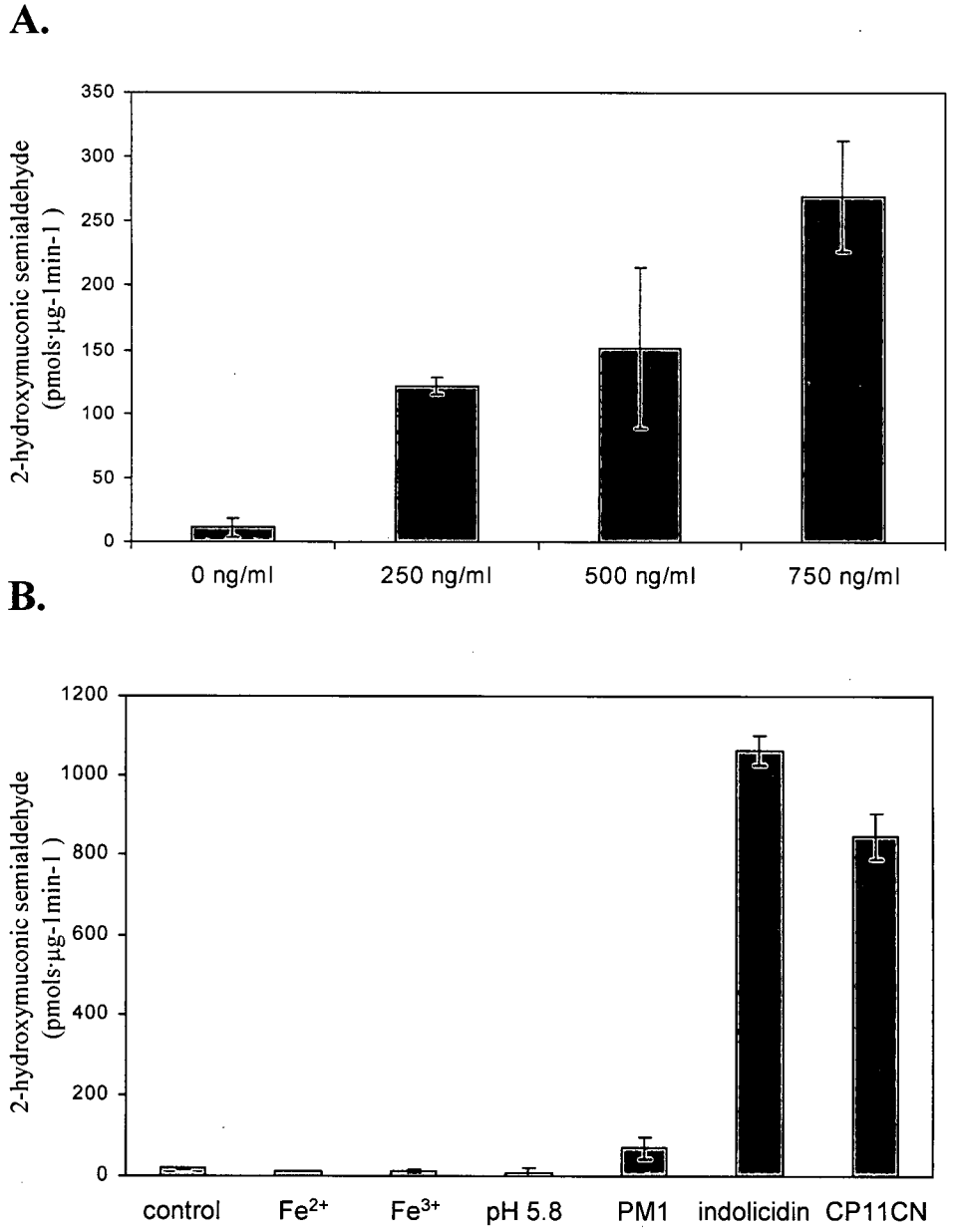

Figure 2.5. Induction of the *pmrA-pmrB* containing operon by cationic antimicrobial peptides. A) Dose-dependent response to increasing concentration of polymyxin B of the *pmrB::xylE* fusion in strain H970. B) Induction of *pmrB::xylE* fusion in strain H970 by various stimuli. Control - BM2 medium supplemented with 2 mM Mg^{2+} pH 7.0; Fe^{2+} - addition of 100 µM $FeSO_4$ + 300 µM desferroximine mesylate; Fe^{3+} - addition of 100 µM $FeCl_3$ + 300 µM 2, 2 dipyridyl; pH 5.8 - altering the pH of BM2 medium to pH 5.8; PM1 - addition of 62 ng/ml polyphemusin; indolicidin - addition of 500 ng/ml indolicidin; CP11CN – addition of 500 ng/ml CP11CN.

polyphemusin variant P1L caused very high induction of the fusion at concentrations from 0.5-4 μg/ml, equivalent to that observed with the indolicidin-like peptides.

The response to a number of aminoglycoside antibiotics and polymyxins was also examined. None of the aminoglycosides studied were able to induce luminescence in the PA4773::*lux* fusion strain. Five to eleven-fold induction was observed in response to polymyxin B concentrations between 0.125-1 μg/ml, to colistin (polymyxin E) between 0.25-2 μg/ml, and polymyxin B nonapeptide between 0.5-2μg/ml. Thus, induction by polymyxins was substantially lower that that observed with certain peptides.

A series of mutants were created in strain H974 to determine whether or not the induction, by peptides, of the PA4773:*lux* fusion depended upon the products of the *phoP*, *phoQ*, *pmrA*, or *pmrB* genes. We used the indolicidin variant peptide, CP11CN, as a representative peptide, due to the fact that it demonstrated the strongest effect on transcription of PA4773::lux in our comparison of different peptides (Table 2.4). Interestingly, the fusion showed strong induction by CP11CN in all of these strains (Fig 2.2), implying that the regulator controlling peptide-mediated activation of this operon is not known.

Induction of LPS modification operon (*pmrHFIJKLM-ugd*) by cationic antimicrobial peptides.

The genes responsible for LPS modifications in *Salmonella* are directly regulated by PmrA and PmrB. In order to determine how the eight-gene operon encoding the putative LPS modification system (*pmrHFIJKLM-ugd*) of *Pseudomonas* is regulated, we created a plasmid-encoded promoter fusion to the entire intergenic region between *algA* (PA3551) and *pmrH* (PA3552). This *pmrH*::*lux* fusion was then mobilized into strains H103 (WT), H851 (*phoP*), H854 (*phoQ*), H988 (*pmrA*), and H970 (*pmrB*) and the luminescence of these strains under varying Mg^{2+} and peptide conditions was examined. As shown in Figure 2.6, the expression of this fusion was induced 86-fold in low Mg^{2+} in a wild-type background. Expression was

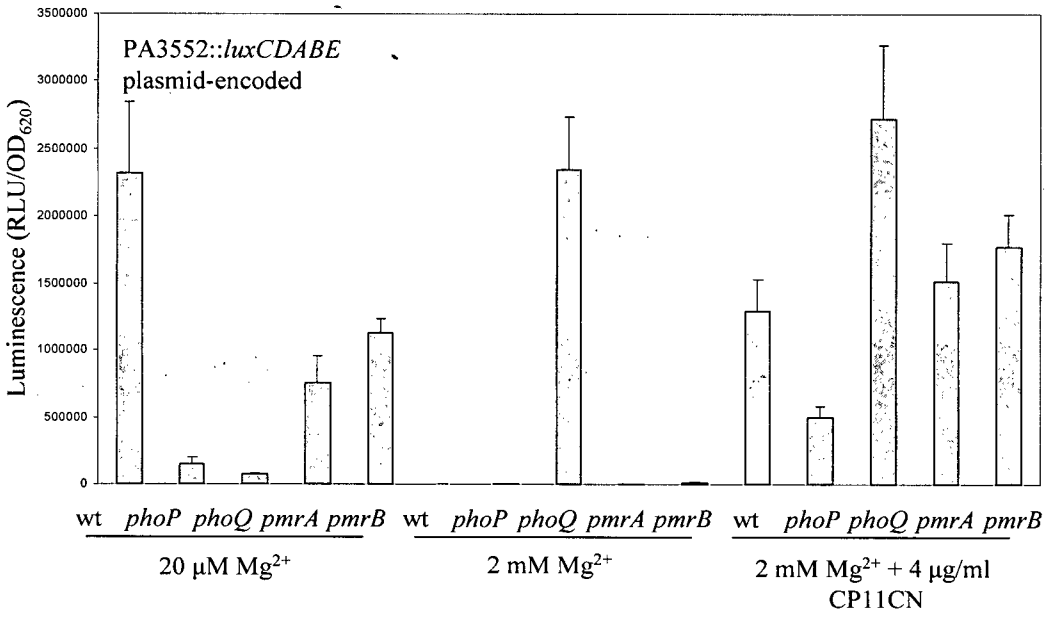

Figure 2.6. Regulation of *pmrH* (PA3552) in *phoP*, *phoQ*, *pmrA*, and *pmrB* mutant strains. Induction of p_{PA3552}::*lux* fusion in BM2-glucose minimal media supplemented with 20 μM Mg^{2+}, 2 mM Mg^{2+}, or 2 mM Mg^{2+} with 4 μg/ml CP11CN in H103 (wt), H851 (*phoP*), H854 (*phoQ*), H988 (*pmrA*) and H970 (*pmrB*). The p_{PA3552}::*lux* fusion contained a *lux* cassette fused to the entire intergenic region between PA3551 and PA3552 and reports on the transcription of the eight-gene operon encoding the LPS aminoarabinose modification system (PA3552-PA3559) of *Pseudomonas*. In *Salmonella* this operon is PmrA-PmrB regulated.

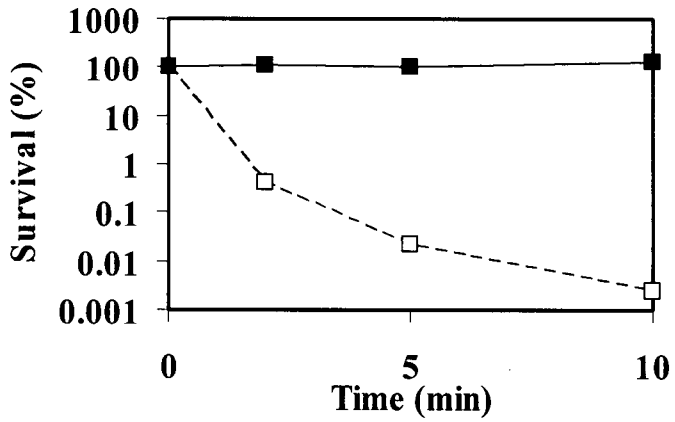

Figure 2.7 Adaptive resistance to polymyxin B induced by CP11CN in *P. aeruginosa*. Cultures of *P. aeruginosa* H103 were grown to mid-logarithmic phase in the presence (■) or absence (□) of 2 μg/ml CP11CN. Killing was initiated by the addition of 1 μg/ml polymyxin B at 0 min. Aliquots were withdrawn and serial dilutions were plated to determine the percentage of cells that survive the polymyxin B exposure.

Table 2.4. Induction of PA4773:*luxCDABE* fusion in strain H974 in response to cationic peptides

Antibiotic	Fold-induction	Maximal luminescence (RLU/OD_{620})	Peptide Concentration (μg/ml) leading to 10% luminescence	50% luminescence	100% luminescence
CP10A	82	81500	0.25	1	4
indolicidin	86	85600	0.25	2	8
CP11CN	130	130000	0.031	1	4
cycCP11	119	119000	0.031	1	4
LL-37	25	24500	2	4	16
polyphemusin	17	16800	0.015	0.062	0.5
linear polyphemusin	132	132000	0.5	2	4
CEMA	7	6800	0.5	0.5	2
CP208	16	16100	8	16	32
polymyxin B	5	5200	0.125	0.25	0.5
PMBN*	6	5600	0.5	2	4
colistin	11	11000	0.25	0.5	2
gentamicin	1	1260	-	-	-
tobramycin	1	1220	-	-	-

*polymyxin B nonapeptide

reduced 13-fold in strain H851 (*phoP*) while in strain H988 (*pmrA*) expression was reduced by 2-fold, indicating that the PhoP activator is an important determinant of low Mg^{2+} induced activation of the fusion. In high Mg^{2+}, the *pmrH::lux* fusion in the *phoQ* mutant was strongly derepressed, similar to the phenotype observed for the PA4773::*lux* fusion in strain H976 (Fig. 2.2) and for OprH expression (Macfarlane *et al.*, 1999). In addition to this regulation by Mg^{2+}, the *pmrH::lux* fusion was also induced 50-80 fold by 4 μg/ml CP11CN in all strains examined (Fig. 2.6).

Adaptive resistance is induced by preexposure to sub-inhibitory concentrations of cationic antimicrobial peptides.

To confirm that the observed response to sub-inhibitory concentrations of cationic antimicrobial peptides actually leads to increased cationic antimicrobial peptide resistance, killing assays were

performed in cells that had been pre-exposed to the indolicidin variant peptide, CP11CN. These conditions were chosen based on the observed high level of induction of a PA4773::luxCDABE fusion in the presence of this peptide (Table 2.4). As demonstrated in Figure 2.7, this preexposure to sub-MIC CP11CN resulted in a 4 log-order decrease in killing by 1 μg/ml polymyxin B as compared to a culture that was not preexposed to CP11CN.

DISCUSSION

Previous work has demonstrated that the PhoP-PhoQ system of *P. aeruginosa* plays a role in regulating resistance to polymyxin B, α-helical peptides, and aminoglycosides (Ernst, Yi et al. 1999; Macfarlane, Kwasnicka et al. 1999; Macfarlane, Kwasnicka et al. 2000) and hinted at the possibility that other regulatory systems are likely involved in regulation of these phenotypes. We have shown here an additional role for PmrA-PmrB. This is conceptually similar to the situation for *Salmonella* but this study illustrates several key differences between the PhoP-PhoQ and PmrA-PmrB systems of *Pseudomonas* compared to those of *Salmonella* (Table 2.5).

In *Salmonella*, the PhoP-PhoQ system responds to conditions that are found within the *Salmonella*-containing vacuole (SCV) and is essential for intramacrophage survival (Garcia-del Portillo, Foster et al. 1992; Groisman 2001). The PmrA-PmrB system of *Salmonella* is interconnected with PhoP-PhoQ, but also independently activated by increased concentrations of Fe^{3+} (Soncini and Groisman 1996; Wosten, Kox et al. 2000). The concentration of iron in the mouse stomach and small intestine may approach the levels required for PmrB-signaling (Wosten, Kox et al. 2000). Thus, the *Salmonella* PhoP-PhoQ and PmrA-PmrB systems appear to be involved in adapting to the many environments encountered during the lifestyle of *Salmonella* species.

P. aeruginosa however, is primarily a pathogen of mucosal surfaces. As such, it does not usually encounter decreases in pH, nor, generally speaking, are the Mg^{2+} concentrations within

the airway surface fluid, which can be as high as 2.2 mM, limiting (Cowley, Govindaraju et al. 1997; Baconnais, Tirouvanziam et al. 1999). The airway surface fluid of CF patients can possess a very high concentration of cationic peptides and proteins (300 μg/mL) (Soong, Ganz et al. 1997), yet the lungs of CF patients are often chronically infected with *P. aeruginosa*, which is capable of resisting these high concentrations of peptides. A previous report indicated that *P. aeruginosa* from the lungs of patients with cystic fibrosis had LPS modifications similar to those associated with the *Salmonella* PhoP-PhoQ-regulated changes and this was taken as evidence of activation of PhoP-PhoQ (Ernst, Yi et al. 1999). The data presented here provide an alternative explanation for these modifications since endogenous lung peptides and/or colistin treatment of *P. aeruginosa* infection could have resulted in direct activation of the putative LPS modification operon (*pmrHFIJKLM-ugd*) and the *pmrA-pmrB* operon. *In vitro* data demonstrates that LL-37, a peptide constitutively expressed in the human lung, is capable of inducing the *pmrA-pmrB* fusion at concentrations in the range of 2-16 μg/ml. This may be clinically relevant, since it is known that newborns with cystic fibrosis complicated by pulmonary infections have levels of approximately 6-8 μg/ml of LL-37 in their bronchoalveolar lavage fluid (Schaller-Bals, Schulze et al. 2002), a level higher than that observed in non-CF individuals.

In this work the identification of a two-component regulatory system, PmrA-PmrB, in *P. aeruginosa* has been described. The PmrA-PmrB system is regulated by Mg^{2+} and this Mg^{2+} regulation is largely independent of the PhoP-PhoQ system, which also responds to Mg^{2+}. PmrA and PmrB are required for high-level expression of this operon under Mg^{2+}-deficient conditions (Fig. 2.2). The promoter for the *pmrA-pmrB* operon contained an imperfect direct repeat that may form a binding site for PmrA (Fig. 2.8). Moreover, the fact that the PA4773::*lux* fusion is expressed despite a polar insertion indicates that there must be a second promoter in front of the downstream *pmrA-pmrB* genes.

Overexpression of *phoP* in strain H974 (PA4773::*lux*), H975 (PA4773::*lux*; *phoQ::xylE*) and H976 (PA4773::*lux*; *phoP::xylE*) had only a marginal effect on induction of the operon by low Mg^{2+} (Table 2). In contrast PhoQ was capable of affecting operon expression since a *phoQ* interposon mutant (H975) lead to 5-fold increased expression of a PA4773::*luxCDABE* fusion (Table 2.2, Fig. 2.2). This latter result was consistent with the suggestion that in addition to the ability of PhoQ to act as both a kinase and phosphatase toward PhoP (Macfarlane, Kwasnicka et al. 1999), it may also be a phosphatase toward PmrA.

As mentioned above, certain classes of cationic antimicrobial peptides and polymyxin B were shown to activate the PA4773-4775-*pmrAB* and *pmrHFIJKLM-ugd* operons in a Mg^{2+}-independent manner. The activation of the PA4773::*lux* fusion by peptides appeared to take place in the absence of both the PhoP-PhoQ system and the PmrA-PmrB system. This contrasts with the situation in *Salmonella*, where LPS modification induced by cationic antimicrobial peptides requires the PhoP-PhoQ system (Bader, Navarre et al. 2003). This suggests that another regulatory system exists in *P. aeruginosa* that responds to peptides by inducing this operon. The relationship between inducibility of the system and resistance to a given peptide was incomplete because induction did not require peptide activity *per se*, and since the peptides used have different structures and mechanisms of action and are apparently differentially affected by the PmrAB-regulated resistance mechanism. Examination of the data in Tables 3 and 4 shows that when the antibacterial activity of an antibiotic was high, (e.g. polyphemusin I, CEMA, polymyxin B) its ability to induce PmrA-PmrB was relatively weak. The converse did not hold true, indicating that there can be other reasons for weak antibacterial activity, but the generally poor activity of the indolicidins against *P. aeruginosa* might be explained in part by their strong ability to induce PmrA-PmrB. It is important to note that the ability to induce the *pmrA-pmrB* operon is not related to the mechanism of action of specific peptides, as completely non bactericidal peptides like CP208 and polymyxin B nonapeptide were still able to induce this

operon. Similarly there was no strong relationship between peptide structure and the ability to induce the *pmrA-pmrB*-containing operon as all cationic peptides, including lipopeptides, α-helical, cyclic, and β-hairpin molecules had some ability to induce.

Adaptive antibiotic resistance can involve multiple mechanisms one of which would involve pre-exposure to antibiotic. *P. aeruginosa* exhibits a phenotype known as adaptive polymyxin B resistance (Gilleland and Farley 1982). This phenotype occurs when *P. aeruginosa* is passaged in medium containing serially increasing concentrations of polymyxin B, after which growth can be observed at concentrations 1000 times greater than the MIC. However, the resistance is not stable, so that when these cells are grown in medium containing no antibiotic, the cells revert to normal susceptibility. The mechanism by which this takes place is unknown, although the regulation of this type of resistance suggests that the cells may be responding directly to the presence of polymyxin B, thereby inducing resistance. The activation of the *pmrA-pmrB* and the *pmrHFIJKLM-ugd* operons by cationic peptides is consistent with the adaptive polymyxin B resistance phenotype. Furthermore, this hypothesis is supported by the demonstration that pre-exposure to CP11CN resulted in ~5 log orders increased survival in the presence of a subsequent polymyxin B exposure.

In *Salmonella*, the PmrA-PmrB system directly controls the *pmrHFIJKLM* and *ugd* operons, which are responsible for LPS modifications involved in resistance to cationic antimicrobial peptides. Since *Pseudomonas* possesses orthologs of this system, the regulation of these genes (PA3552-PA3559) was examined. The *pmrH::lux* fusion was activated by growth on low (20 μM) Mg^{2+}. Deletion of either *phoP* or *phoQ* resulted in ~95% less expression under low Mg^{2+} conditions, while deletion of *pmrA* or *pmrB* resulted in ~50% less expression than wild-type cells, but still substantially more than in the *phoP* or *phoQ* mutants. Under high Mg^{2+}, this fusion was 700-fold derepressed in the *phoQ* mutant (Fig. 2.6).

Table 2.5. Differences between the PhoP-PhoQ and PmrA-PmrB systems of *Salmonella* and *Pseudomonas*.

Phenotype /property	*Salmonella enterica* sv. Typhimurium	*Pseudomonas aeruginosa*
Lifestyle	Intracellular pathogen; SCV is an acidified, low Mg^{2+} environment	Extracellular pathogen associated with mucosal surfaces; Commonly found in soil and water samples
Cationic peptide resistance	LPS modifications are important for resistance; PhoP mutants are susceptible	Unique three-gene operon (PA4773-PA4775) also contributes to resistance; PhoP mutants do not differ from wild-type
Cationic peptide signaling	Occurs via the PhoP-PhoQ systems	Is independent of both the PhoP-PhoQ and PmrA-PmrB systems
Virulence	PhoP and PhoQ mutants exhibit decreased virulence; PmrA mutants are also less virulent; constitutive PhoP expression attenuates virulence	PhoQ mutants show 100-fold less virulence than wild-type; PhoP mutants have similar virulence to wild-type strain
PhoP	Indirectly regulates PmrA-PmrB via PmrD	Directly regulates OprH and LPS modification operon; 70% similar to *Salmonella* PhoP throughout entire sequence
PmrA	Directly regulates LPS modification operon	Directly regulates LPS modification operon and the *pmrA-pmrB* operon; *pmrA-pmrB* genes are induced by cationic peptides; 60% similar to *Salmonella* PmrA throughout entire sequence
PhoQ	Activated by limiting Mg^{2+}	Activated by limiting Mg^{2+}; 52% similar to *Salmonella* PhoQ throughout entire sequence, 23 residue insertion in signaling domain
PmrB	Activated by lowered pH and increased Fe^{3+}	Activated by limiting Mg^{2+}; 48% similar to *Salmonella* PmrB in the C-terminal sequence, no similarity in the N-terminal 170 residues

The promoter structure of *pmrH*, contains three binding motifs, two that resemble a PhoP-like binding sequence (Macfarlane, Kwasnicka et al. 1999), and one that resembles a putative PmrA-like binding sequence (Fig. 2.8). This putative PmrA-motif is also found in the promoter of PA4773 and both types of motifs are direct repeats with conserved spacing between them. This is a common motif for other known two-component regulators and thus may represent the binding sites for PhoP and PmrA.

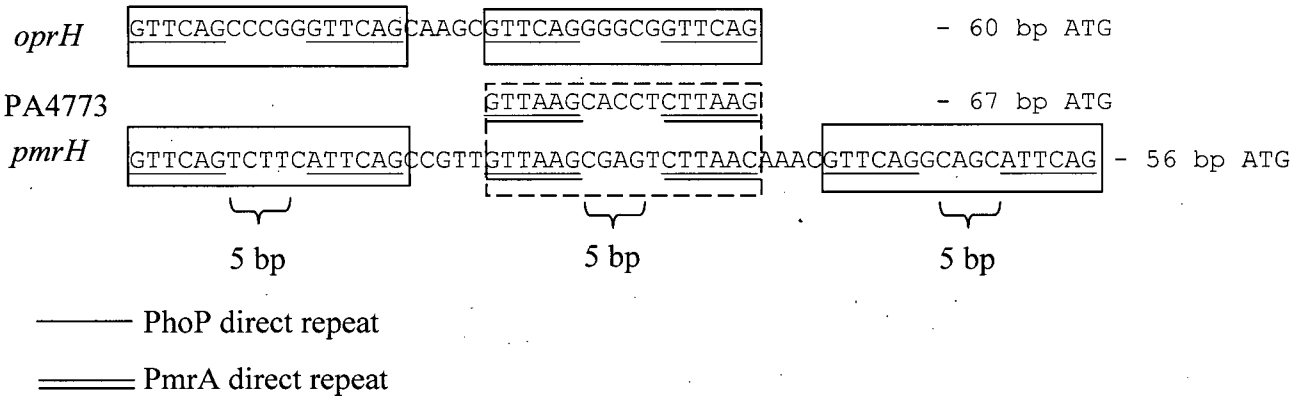

Figure 2.8. PhoP and PmrA binding sites. Alignment of the promoter regions of *P. aeruginosa oprH*, PA4773 and *pmrH* depicting putative PhoP and PmrA binding sites (boxed regions). The PhoP binding site (solid line) contains two 6 bp direct repeats separated by a 5 bp spacer region which resembles the known *E. coli* PhoP binding site (Yamamoto, Ogasawara et al. 2002) and that identified in Macfarlane *et al.* (Macfarlane, Kwasnicka et al. 1999). The PmrA binding site (dashed line) contains similar but distinct, direct repeats with a 5 bp spacer region that resembles the *Salmonella* PmrA binding site (Aguirre, Lejona et al. 2000). The distance between the predicted binding sites and the putative start codon (ATG) of the gene is indicated.

Previous studies in *Pseudomonas* have shown that while deletion of *phoQ* results in resistance to cationic antimicrobial peptides under Mg^{2+}-replete conditions where the bacteria would normally be sensitive, *phoP* mutants had no susceptibility phenotype (Macfarlane, Kwasnicka et al. 1999; Macfarlane, Kwasnicka et al. 2000). Data presented in this paper suggests that in addition to PhoQ acting as a kinase under low Mg^{2+} growth conditions, it also acts as a phosphatase under Mg^{2+}-replete conditions. This difference is consistent with the

observation that the PhoQ sensing domain of *P. aeruginosa* undergoes a large conformational shift when Mg^{2+} is non-limiting, while the sensing domain of *E. coli* PhoQ does not (Lesley and Waldburger 2001). Intriguingly, the data also suggest that PhoQ exerts an effect on genes regulated by PmrA exclusively, since *phoQ* mutants exhibit derepression of a PA4773::*lux* fusion (Fig. 2.2).

PmrD is a small basic protein that regulates the activity of PmrA via a post translational mechanism. It connects the PhoPQ system with the PmrAB system in *S.* Typhimurium and is strictly required for Mg^{2+} regulation of polymyxin B resistance (Kox, Wosten et al. 2000). The possibility that PhoQ acts via a PmrD-like intermediate has not been formally excluded, however, unlike the situation observed in *Salmonella* (Kox, Wosten et al. 2000), the derepression/activation is not dependent upon PhoP (Fig. 2.2) and there is no gene homologous to *pmrD* in *P. aeruginosa.* Thus, although there are many similarities between the PhoP-PhoQ and PmrA-PmrB systems of *Pseudomonas* and *Salmonella*, there are many differences as well (Table 2.5).

Collectively, these data indicate that the regulation of cationic peptide resistance is very complex. The data indicate *P. aeruginosa* possesses at least two regulatory systems that respond independently to Mg^{2+}, and PmrB appears to also respond to signals other than limiting Mg^{2+} and different from those signals that *Salmonella* PmrB respond to. More work is needed to elucidate the mechanism for cationic peptide-mediated gene induction. Although the data presented here suggest a potential problem with induced resistance to cationic peptides, the results also point to one group of peptides, the polyphemusins, that are almost unaffected by PmrA-PmrB-mediated resistance and have a relatively poor ability to induce the *pmrA-pmrB* operon. Thus, this provides clear evidence that it is possible to design cationic antimicrobial peptides which are unaffected by the resistance mechanisms discussed here.

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Chapter 3 – Identification of PhoP and PmrA regulated genes: Role of PhoP and PmrA in Mg^{2+}-regulated phenotypes[1]

INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic pathogen, capable of infecting a large number of hosts including nematodes, insects, plants, animals, and especially humans. It is the third-leading cause of nosocomial infection and is also the leading cause of morbidity and mortality in cystic fibrosis (CF) patients (Rajan and Saiman 2002). This organism is also noted for its metabolic diversity, which allows it to colonize a large number of environmental habitats. Its versatility is believed to be related to the large number of regulatory proteins found in its genome (469 of 5570 ORFs) (Stover, Pham et al. 2000).

The two-component response regulators constitute one of the largest families of regulatory proteins in *P. aeruginosa* (Stover, Pham et al. 2000). These systems typically contain a sensor protein that responds to some chemical or physical stimulus, leading to phosphorylation of the sensor protein at a conserved histidine residue, thus altering the conformation of the sensor and promoting interaction with a cognate response regulator protein (Stock, Robinson et al. 2000). Following binding, there is a phosphotransfer to a conserved aspartate residue in the response regulator and the phosphorylated response regulator then recognizes and binds to a specific DNA sequence leading to modulation of transcription from that promoter (Stock, Robinson et al. 2000). Alternatively, some regulation occurs through phosphatase action on the response regulator (Stock, Robinson et al. 2000). In *P. aeruginosa*, there are 64 response regulators and 63 histidine kinases as well as sixteen atypical kinases (Rodrigue, Quentin et al. 2000). The function of the majority of these regulatory proteins has not yet been established.

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[1] A version of this chapter will be submitted for publication as McPhee, JB, Bains, M, Winsor, G, Kwasnicka, A, Lewenza, S, Brinkman, FSL, and REW Hancock. Contribution of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems to Mg^{2+}-induced gene expression in *Pseudomonas aeruginosa*.

In *Salmonella* sp., the PhoP-PhoQ and PmrA-PmrB systems are involved in cationic peptide and polymyxin B resistance, as well as in coordinating virulence (Miller, Kukral et al. 1989; Miller and Mekalanos 1990; Guo, Lim et al. 1998; Gunn, Ryan et al. 2000). Although the systems appear to regulate some similar phenotypes in *P. aeruginosa* (Brinkman, Macfarlane et al. 2001; McPhee, Lewenza et al. 2003; Lewenza, Falsafi et al. 2005), there are some major differences between the two species. In both, the PhoP-PhoQ system responds strongly to limiting concentrations of divalent cations. Studies have suggested however that the mechanism by which the PhoQ proteins in these species sense the divalent cation limitation may differ (Lesley and Waldburger 2001). Indeed, in addition to responding to limiting concentrations of divalent cations, the PhoQ protein of *S. typhimurium*, unlike that of *P. aeruginosa* (McPhee, Lewenza et al. 2003), also responds directly to the presence of cationic antimicrobial peptides (Bader, Sanowar et al. 2005).

In *P. aeruginosa*, the PmrA-PmrB system is required for regulating polymyxin B and cationic antimicrobial peptide resistance in response to limiting Mg^{2+} conditions (McPhee, Lewenza et al. 2003). These alterations in cationic peptide resistance take place primarily via the regulation of the *pmrH-ugd* LPS modification operon. Mutants in *pmrH-ugd* or in the *pmrA-pmrB* regulator exhibit increased sensitivity to cationic antimicrobial peptides under Mg^{2+} limiting conditions (McPhee, Lewenza et al. 2003). Furthermore, mutants containing constitutively active PmrB show constitutive addition of aminoarabinose to Lipid A which results in increased resistance to cationic peptides (Moskowitz, Ernst et al. 2004). In addition to this Mg^{2+}-regulated response, both operons are also directly activated by cationic peptides via a mechanism that is independent of PmrA-PmrB and PhoP-PhoQ (McPhee, Lewenza et al. 2003).

Mg^{2+} dependent regulation of cationic peptide and polymyxin B resistance in *P. aeruginosa* is also regulated by the PhoP-PhoQ system (Macfarlane, Kwasnicka et al. 1999; Macfarlane, Kwasnicka et al. 2000). This is likely because the *pmrH-ugd* LPS modification

operon, that encodes enzymes responsible for the addition of aminoarabinose to the Lipid A moiety of LPS, is directly regulated by both the PmrA-PmrB systems and the PhoP-PhoQ systems (McPhee, Lewenza et al. 2003; Moskowitz, Ernst et al. 2004). Previous work established that there are two conserved binding motifs within the promoters that control the *phoP-phoQ* and *pmrA-pmrB* containing operons and which have been implicated to be involved in autoregulation of the expression of the respective two-component regulators (Macfarlane, Kwasnicka et al. 1999; McPhee, Lewenza et al. 2003). The promoters of the PA4773-*pmrAB* and *pmrH-ugd* operons contain the sequence $CTTAAGN_5CTTAAG$ that was proposed to be the PmrA-binding sequence (McPhee, Lewenza et al. 2003). The promoter upstream of the PhoPQ, and PmrAB regulated *pmrH-ugd* operon also contains two PhoP-like binding sites that are very similar to those previously identified in the *oprH-phoP-phoQ* promoter (Macfarlane, Kwasnicka et al. 1999).

Given the importance of the PhoP-PhoQ and PmrA-PmrB genes in regulating virulence and host antimicrobial and cationic antimicrobial peptide resistance in *P. aeruginosa*, we searched the *P. aeruginosa* genome for promoters that contained the putative PhoP or PmrA binding sites. Using a combination of genetic and biochemical analyses, a series of new PhoP and PmrA regulated genes were identified.

MATERIALS AND METHODS

Bacterial strains, primers, and growth conditions.

The bacterial strains and plasmids used in this study are described in Table 3.1. All primers were synthesized by AlphaDNA (Montreal, QC) and the sequences of these primers are shown in Table 3.2. Mutants in PA0921 and PA1343 were obtained from the University of Washington (Jacobs, Alwood et al. 2003). Cultures were routinely grown in Luria-Bertani broth or BM2-glucose minimal medium containing low (20 μM) or high (2 mM) $MgSO_4$ concentrations. Antibiotics for selection were used at the following concentrations: tetracycline,

Table 3.1. *P. aeruginosa* strains and plasmids used in this study.

Strain or plasmid	Genotype or characteristics	Reference
H103	wild-type *P. aeruginosa* PAO1	Macfarlane et al., 1999
H851	*phoP::xylE:aacC1*; Gm^R	Macfarlane et al., 1999
H974	PA4773::*luxCDABE* derivative of H103; Tc^R	McPhee et al., 2003
H988	*pmrA::xylE:aacC1*; Gm^R	McPhee et al., 2003
H1025	*feoA*::*luxCDABE* derivative of H103	Lewenza et al., 2005
UW-WT	Wild-type *P. aeruginosa* PAO1,	Jacobs et al., 2003
UW-44235	PA1343:IS*phoA*	Jacobs et al., 2003
UW-47583	PA0920-PA0921:IS*phoA*	Jacobs et al., 2003
p*phoP*-His_6	His_6-*phoP* cloned into pET28a	This study
p*pmrA*-His_6	His_6-*pmrA* cloned into pET28a	This study

Table 3.2. Sequences of primers used in this study.

Primer	Sequence (5' – 3')
RTrpsL-F	TGCGTAAGGTATGCCGTGTA
RTrpsL-R	CAGCACTACGCTGTGCTCTT
RTPA4773-F	GCACCTGGCGATCCATAC
RTPA4773-R	CTGGGCGCCATCGAGTA
RTPA3552-F	CACTGGACTTTCTGCCATTCT
RTPA3552-R	TGTTCGAGCTCCTGGTTCTT
RTPA1249-F	TACGCCGTGGAAGTATGTCA
RTPA1249-R	GCGTCGACGAAGTGGATATT
RTPA1343-F	CCCTGAACACAACAATCACC
RTPA1343-R	CCAGTAGGCGTAGTTGGAG
RTPA0921-F	ATTGGGAACAGTCGTTGCAG
RTPA0921-R	CGAATAGACCAGCAGGGAAC
RTPA4782-F	GTGTGGTCCTGGTGTTCCTT
RTPA4782-R	GAAACGCAGTGGCGAATC
RTPA4359-F	GCACCCTGAACACCCTCTAC
RTPA4359-R	AGGCTGAAGGTATCGACCAG
RTPA1559-F	GCGATTTCCTCGACACCTC
RTPA1559-R	GATGGTCGGGTTCTTCGAG
RToprH-F	CGAAGGCGGCTATCGTTAC
RToprH-R	AGAATTGCGAGCTGCTGTG

100 μg/ml and gentamicin, 50 μg/ml. Routine genetic manipulations were carried out according to standard molecular biology procedures (Maniatis, Fritsch et al. 1989).

Identification of PhoP- and PmrA-binding sites.

Sequence logos for the proposed PhoP binding sites upstream of the *oprH-phoPQ* and *pmrH-ugd* operons, as well as the proposed PmrA-binding sites upstream of the PA4773-*pmrB* and *pmrH-ugd* operons were first constructed using Weblogo. Frequency matrices were then constructed using the Emboss program, "Prophecy". These matrices were used to parse the *P. aeruginosa* genome for additional matching binding sites, using a conservative cut-off score of 0.82-0.85. Any sites that were present within an ORF, or in a promoter between convergent genes were excluded from further analysis based on the likelihood that these did not represent real promoters. Regulation of the putative target genes was then examined by semi-quantitative PCR (qPCR) in strains H103 (WT), H851 (*phoP::xylE*) and H988 (*pmrA::xylE*) in response to limiting Mg^{2+}. Genes identified as being regulated by PhoP and/or PmrA were then used to refine the binding motif and carry out a second iteration of frequency matrix construction and genomic searches for matching motifs.

His_6-PhoP and His_6-PmrA purification.

The constructs used to overexpress and purify the His_6-PmrA and His_6-PhoP were created by PCR by amplifying the *phoP* or *pmrA* genes and cloning them separately into pET28a (Invitrogen, Carlsbad, CA) as NdeI-BamHI fragments. The plasmids containing the His_6-*phoP* or His_6-*pmrA* genes were transformed into *E. coli* BL21. Cells were grown to OD_{600} ~0.5 before being induced with 1 mM IPTG for 3 hr. Cells were harvested and resuspended in sonication buffer containing 500 mM NaCl, 5 mM $MgCl_2$, 50 mM $NaPO_4$ pH 7.8 and 10 mM imidazole. Cells were lysed by sonication on ice (3 X 1 min). Cell debris and unbroken cells were removed by centrifugation at 7,500 X g. The supernatant was filtered through a 0.8 μm

filter (Nalgene, Rochester, NY). The filtered supernatant was mixed with Ni^{2+}-NTA resin (Qiagen, Mississauga, ON) and gently shaken at 23°C for 1 hr. The resin was washed sequentially with 5-ml sonication buffer containing 30, 50, 100 or 200 mM imidizole. His_6-*phoP* was eluted with sonication buffer containing 300 mM imidazole and 15 % glycerol. Protein was collected on ice, aliquoted and frozen in a dry ice and ethanol bath. Frozen aliquots were stored at -80C. Purified proteins are shown in Fig. 3.4.

Semi-quantitative PCR assays (qPCR).

Total RNA was isolated using RNeasy mini columns (Qiagen, Mississauga, ON) from mid-log phase *P. aeruginosa* grown in BM2-glucose minimal media with 20 μM Mg^{2+} or 2 mM Mg^{2+}. RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA) to remove contaminating genomic DNA). Four μg of total RNA was combined with 0.5 μM dNTPs, 500 U/ml SuperaseIN (Ambion, Austin, TX), 10 μM DTT, in 1X reaction buffer and reverse transcribed for 1 hour at 37°C and 2 hr at 42°C with 10,000 U/ml Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The RNA was subsequently destroyed by the addition of 170 mM NaOH and incubation at 65°C for 10 minutes. The reaction was then neutralized by addition of HCl and the cDNA was used as a template for PCR. The number of cycles used to amplify each gene of interest was chosen to ensure that the PCR reaction was not saturated. All reactions were normalized to the *rpsL* gene encoding the 30S ribosomal protein S12.

DNA-binding assays.

These were done according to previously published methods (Haydel et al., 2002). PCR-amplified promoter fragments of the entire intergenic region of the genes of interest were purified by excision from agarose gels using the Qiaquick column purification system (Qiagen, Mississauga, ON). These were DIG-labeled using a DIG gel shift kit (2^{nd} generation) from Roche Applied Science. The purified protein (His_6PhoP or His_6PmrA) was mixed with 40 pg of

DIG-labeled probe in buffer consisting of 20 mM 2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid (HEPES), 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.2% Tween 20, 30 mM KCl and 0.5 mM $MgCl_2$. For samples containing unlabelled probe, 100 ng of the probe was allowed to bind for 15 min at 20°C before adding DIG-labeled probe and incubating for another 20 min. All other samples were allowed to incubate for 20 min at 20°C before electrophoresis. Following electrophoresis, the sample were blotted to nylon membranes and exposed to film overnight at 23°C.

Growth assays.

Overnight cultures of *P. aeruginosa* PAO1 strain H103 (WT) and H1032 (*feoA*::*luxCDABE*) were grown in HEPES buffered minimal medium containing containing 20 mM glucose, 2 mM $MgSO_4$ and 10 μM $FeSO_4$. These were washed in sterile 0.85% NaCl and diluted to OD_{600} = 0.2 in 0.85% NaCl. Five μl of these cultures were added to 200 μl of minimal medium containing high (2 mM) or low (20 μM) $MgSO_4$ and 10 μM $FeSO_4$ or $FeCl_3$. Media containing $FeSO_4$ was also supplemented with 1 mM sodium ascorbate to maintain the iron in the ferrous form and with 300 μM desferrioxamine mesylate to chelate any contaminating ferric ions. Similarly, media containing ferric iron was supplemented with 300 μM 2, 2, dipyridyl to chelate ferrous ions. The growth of the cultures at 37°C was monitored in a TECAN Spectrofluor Plus by measuring the A_{620} every 20 min for 36 h. Growth experiments were carried out twice, with eight replicates per experiment. A representative example is shown in Figure 3.6.

Motility assays.

Swimming assays were performed by inoculating 1 μl of an overnight bacterial culture onto a BM2-glucose plate containing 0.3% agar. Mg^{2+} was added to 2 mM for the high Mg^{2+} media, while 200 μM EDTA was added for the Mg^{2+}-limiting media. Twitching assays were

carried out LB media containing 1% agar. Mg^{2+}-replete conditions were created by supplementing the media with 2 mM $MgSO_4$. Mg^{2+}-deficient media was created by adding 200 μM EDTA to the media. As with the swimming assays, 1 μl of an overnight culture was inoculated into the agar/plastic interface. After 24 hours of growth the agar was removed and the twitch zones were measured. For swarming assays, brain-heart infusion broth (BHI) containing 0.5% agar and either 2 mM $MgSO_4$ or 200 μM EDTA were inoculated with 1 μl of an overnight culture. After 24 hr the swarm zones were measured.

RESULTS AND DISCUSSION

Identification of putative PhoP-PhoQ and PmrA-PmrB regulated genes.

As the PhoP-PhoQ and PmrA-PmrB systems both regulate transcriptional changes upon growth in limiting Mg^{2+}, and microarray experiments suggest that growth in limiting Mg^{2+} affects transcription of ~3% of the genome (Bains and Hancock, unpublished), a bioinformatic search for novel target genes of the PhoP-PhoQ and PmrA-PmrB systems was undertaken. Putative binding sites for PhoP and PmrA were identified in previous studies (Macfarlane et al., 1999; McPhee et al., 2003). These sequences were used to generate frequency matrices with the Emboss program "Prophecy". These matrices were then used to search the *P. aeruginosa* genome sequence for sequences with high similarity to the PmrA or PhoP matrix. As new sequences were found and confirmed by q-PCR, they were incorporated into the matrix to provide a larger list of putatively regulated genes. A number of PmrA-like and PhoP-like sequences were identified suggesting that these genes may be regulated by either of these systems. The promoters identified via this analysis are shown in Table 3.3 (PhoP) and Table 3.4 (PmrA).

Transcript levels of the candidate genes were examined in wild-type, *phoP::xylE* and *pmrA::xylE* strains grown in minimal media under high (2 mM) and low (20 μM) Mg^{2+} conditions. Analysis of q-PCR results (Fig. 3.1) confirmed PmrA-dependent Mg^{2+} regulation for

the *feoAB* (PA4359-8), PA4782 and PA1559-60 operons as well as the previously identified *pmrH-ugd* (PA3552-3559) and PA4773-*pmrAB* operons. PhoP-dependent Mg^{2+} regulation was observed for PA0921 and PA1343, as well as for the previously identified *oprH-phoPQ* and *pmrH-ugd* operons. Interestingly, the *aprA* gene encoding alkaline protease, was regulated by Mg^{2+}, although this regulation was not strongly dependent on PhoP or PmrA. This indicates that there may be other, as yet unidentified regulators that are involved in the *P. aeruginosa* response to divalent cation limitation. No Mg^{2+}- or PhoP-dependent regulation was observed for PA1851, PA3925, PA0918, PA2775, or PA0297 (*spuA*) (data not shown). Similarly, no Mg^{2+}- or PmrA-dependent regulation of PA0327, PA0545, PA0053, PA4500, PA2505, PA2506, PA3868, PA4498, PA4499, or PA5106 was observed (data not shown). These results suggest that these genes are

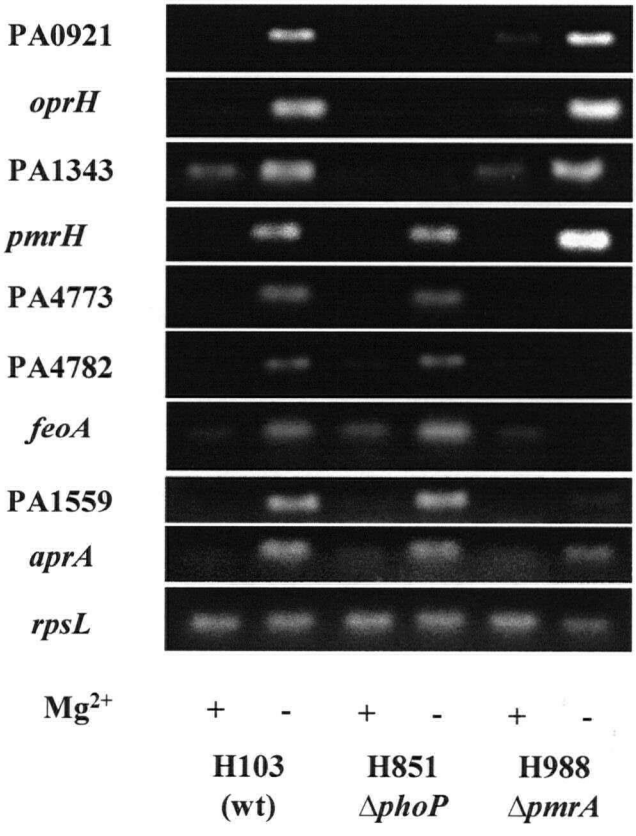

Figure 3.1. PmrA- and PhoP-regulated genes as assessed by RT-PCR. Primers were designed to amplify a 100-150 bp fragment in the 5' region of each gene indicated. RNA was isolated from H103 (wild-type), H851 (*phoP::xylE*) and H988 (*pmrA::xylE*) under Mg^{2+}-limiting (-) and Mg^{2+}-replete (+) conditions.

Table 3.3. PhoP-like promoters identified in this study. Patterns of regulation detected by q-PCR and results of gel-shift assays are also indicated. Positions that differ from the consensus are indicated with bold type. Abbreviations: N – A, C, G, or T; R – A or G

Operon identified	**Consensus sequence CGTTCAGNNNNNRTTCAG**	Mg^{2+} regulated?[a]	PhoP-regulated?[a]	Gel-shift
oprH-phoP-phoQ	CGTTCAGCCCGGGTTCAG and CGTTCAGGGGCGGTTCAG	Yes	Yes	Yes
pmrH-ugd	CGTTCAGTCTTCATTCAG and CGTTCAGGCAGCATTCAG	Yes	Yes	Yes
PA1343	CGTTCAGAAATTGTTCAG and CGTTCAGGCCCGATTCAG	Yes	Yes	Yes
PA0921	CGTTCAGCGATGGTTCAG	Yes	Yes	Yes
PA4457-4461	CGTTCAGCTTGGATTCAG	No	-[b]	-
PA1851	CGTCCAGGCCTG**T**TTCAG	No	-	-
PA3925	CGTTCAGACCCTAT**C**CAG	No	-	-
PA0918	C**A**TTCAGGCTGG**C**TTCAG	No	-	-
PA2775-2774	C**C**TTCA**C**GGATGATTCAG	No	-	-
spuABCD (PA0297-0301)	CGTT**G**AGGCCGT**T**TTCAG	No	-	-

[a] Determined by q-RT-PCR
[b] not tested
[c] "-" signifies not tested, as no Mg^{2+} regulation could be demonstrated

either not regulated by Mg^{2+} and PhoP/PmrA at all, or their expression is co-dependent upon the presence of a second regulatory signal (e.g. release from repression) that was not present under the conditions assayed.

Examination of the sequence logos generated from these data (Fig 3.2A and 3.2B) indicated that there was a higher level of plasticity within the PmrA-binding site than within the PhoP-binding site. It was also clear that both the PmrA and PhoP consensus sequences are quite strongly related to one another. Prokaryotic transcription factor binding sites are often direct or inverted repeats and consistent with this, the PhoP consensus sequence approximately consisted of two GTTCAG half sites separated by 5 nucleotides while the PmrA consensus was approximately two CTTAAG half-sites separated by five nucleotides. The PhoP consensus from *Pseudomonas* is quite different from that of *Salmonella* [(G/T)GTTTA(A/T)

Table 3.4. PmrA-like promoters identified in this study. Positions that differ from the consensus are indicated with bold type. Abbreviations: N – A, C, G, or T; S – C or G

Operon identified	Consensus sequence NTTAASNNNNNCTTAAS	Mg^{2+} regulated?[a]	PmrA-regulated?[a]	Gel-shift
pmrH-ugd	GTTAAGCGAGTCTTAAC	Yes	Yes	Yes
PA4773-*pmrAB*	GTTAAGCACCTCTTAAG	Yes	Yes	Yes
feoAB-PA4357	CTTAAGCGAGCCTTAAG	Yes	Yes	Yes
PA1559-1560	ATTAACGGTTCCTTAAG	Yes	Yes	Yes
PA4782-4781	CTTAAGCGATCC**C**TAAG	Yes	Yes	ND[b]
PA0327	TTTAAGCTTGGCTT**C**AG	No	-[c]	-
algD-8-44-KEGXLIGFA	CTTAAGGTTTGCTTAAG	No	-	-
PA0545	GTTAAGGGACAATTAAG	No	-	-
PA0053	TTTAAGCATGTC**GC**AAG	No	-	-
aprA (PA1249)	TTTAAGTGCAGCTTAA**T**	Yes	No	ND[b]
PA2050-2051	TTTAAGTGCAGCTTAA**T**	No	-	-
PA4500	TTT**C**AGCATGACTTAA**T**	No	-	-
PA2505	ATTAAGTGCGA**G**TTAAG	No	-	-
PA2506	CTTAACTCGCACTTAA**T**	No	-	-
PA3868	ATTAAGGCCTGCT**C**AAC	No	-	-
PA4498	TTTAAGCTCGACTTAA**A**	No	-	-
PA4499	TTTAAGTCGAGCTTAA**A**	No	-	-
PA5106	TTTA**C**GCACCGCTTAA**T**	No	-	-

[a] Determined by q-RT-PCR
[b] not tested
[c] "-" signifies not tested, as no Mg^{2+} regulation could be demonstrated

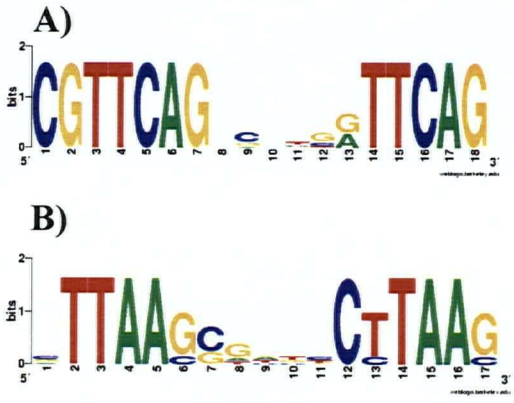

Figure 3.2. Weblogos generated from the conserved sequences identified in the promoters of the PhoP- and PmrA-regulated genes. (A) PhoP weblogo; (B) PmrA weblogo

(G/T)GTTTA(A/T)] (Yamamoto, Ogasawara et al. 2002) while that for PmrA is almost identical (C/T)YTTAA(G/T)-N_5-(C/T)YTTAA(G/T) (Aguirre, Lejona et al. 2000; Tamayo, Ryan et al. 2002)

Studies described in Chapter 2 indicated that two known PmrA-regulated genes, *pmrH* and PA4773 were also regulated by sub-inhibitory concentrations of cationic antimicrobial peptides. Here, these observations were extended to all of the genes that were confirmed to be PmrA-regulated in this study. In addition to PA4773 and *pmrH*, cationic antimicrobial peptides

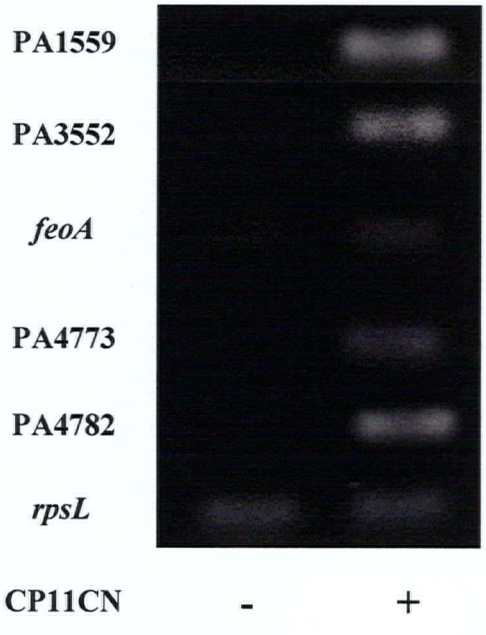

Figure 3.3. Exposure to 2 µg/ml CP11CN causes induction of PmrA-dependent promoters. Primers were designed to amplify a 100-150 bp fragment in the 5' region of each gene indicated. RNA was isolated from H103 (wild-type) in BM2-glucose medium supplemented with 2 mM $MgSO_4$ in the absence (-) or presence (+) of 2 µg/ml CP11CN.

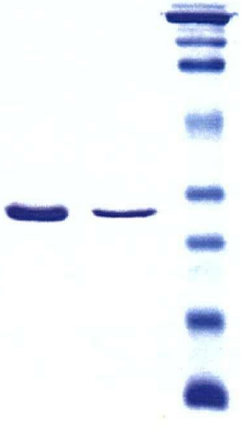

Figure 3.4. Purified His_6-tagged PmrA and PhoP from *P. aeruginosa*. Lane 1- His6-PmrA; Lane 2 – His6-PhoP; Lane 3 MW markers.

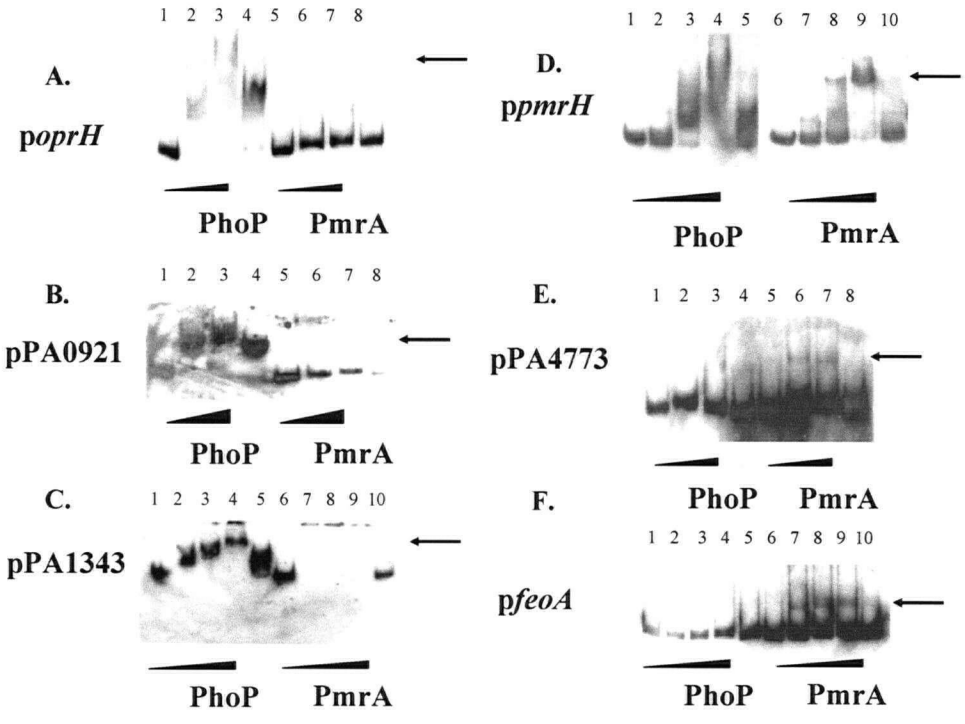

Figure 3.5. PhoP and PmrA bind to promoters that contain PmrA and/or PhoP motifs. Examples of results of gel shift assays are displayed. All lanes contain 40 pg of labeled probe prepared as described in Materials and Methods. **A.** The entire intergenic region between *napE* (PA1177) and *oprH* (PA1178) was amplified by PCR and end labeled with DIG. Lane 1 – no protein; lane 2 – 2.5 μg His_6-PhoP; lane 3 – 5 μg His_6-PhoP; lane 4 - 5 μg His_6-PhoP preincubated with 100 ng unlabeled probe; Lane 5 – no protein; lane 6 – 2.5 μg His_6-PmrA; lane 7 – 5 μg His_6-PmrA; lane 8 - 5 μg His_6-PmrA preincubated with 100 ng unlabeled probe; **B.** The entire intergenic region between PA0921 and PA0921 was used as a probe. Lane 1 – no protein; lane 2 – 2.5 μg His_6-PhoP; lane 3 – 5 μg His_6-PhoP; lane 4 - 5 μg His_6-PhoP preincubated with 100 ng unlabeled probe; Lane 5 – no protein; lane 6 – 2.5 μg His_6-PmrA; lane 7 – 5 μg His_6-PmrA; lane 8 - 5 μg His_6-PmrA preincubated with 100 ng unlabeled probe. **C.** The entire intergenic region between PA1343 and PA1343 was used as a probe. Lane 1- no protein; lane 2 – 1.25 μg His_6-PhoP; lane 3 – 2.5 μg His_6-PhoP; lane 4 – 5 μg His_6-PhoP; lane 5 - 5 μg His_6-PhoP preincubated with 100 ng unlabeled probe; Lane 6- no protein; lane 7 – 1.25 μg His_6-PmrA; lane 8 – 2.5 μg His_6- PmrA; lane 9 – 5 μg His_6- PmrA; lane 10 - 5 μg His_6-PmrA preincubated with 100 ng unlabeled probe. **D.** The entire intergenic region between *algA* (PA3551) and *pmrH* (PA3552) was used as a probe. Lane 1- no protein; lane 2 – 1.25 μg His_6-PhoP; lane 3 – 2.5 μg His_6-PhoP; lane 4 – 5 μg His_6-PhoP; lane 5 - 5 μg His_6-PhoP preincubated with 100 ng unlabeled probe; Lane 6- no protein; lane 7 – 1.25 μg His_6-PmrA; lane 8 – 2.5 μg His_6- PmrA; lane 9 – 5 μg His_6- PmrA; lane 10 - 5 μg His_6- PmrA preincubated with 100 ng unlabeled probe. **E.** The entire intergenic region between PA4772 and PA4773 was used as a probe. Lane 1 – no protein; lane 2 – 2.5 μg His_6-PhoP; lane 3 – 5 μg His_6-PhoP; lane 4 - 5 μg His_6-PhoP preincubated with 100 ng unlabeled probe; Lane 5 – no protein; lane 6 – 2.5 μg His_6-PmrA; lane 7 – 5 μg His_6-PmrA; lane 8 - 5 μg His_6-PmrA preincubated with 100 ng unlabeled probe. **F.** The entire intergenic region between PA4360 and PA4359 was used as a probe. Lane 1- no protein; lane 2 – 1.25 μg His_6-PhoP; lane 3 – 2.5 μg His_6-PhoP; lane 4 – 5 μg His_6-PhoP; lane 5 - 5 μg His_6-PhoP preincubated with 100 ng unlabeled probe; Lane 6- no protein; lane 7 – 1.25 μg His_6-PmrA; lane 8 – 2.5 μg His_6- PmrA; lane 9 – 5 μg His_6- PmrA; lane 10 - 5 μg His_6- PmrA preincubated with 100 ng unlabeled probe.

also increased transcription of the PA4782, *feoA*, and PA1559, genes containing PmrA-motifs (Fig. 3.3). This regulation strongly supports the hypothesis that the recognition site for the, as yet unknown, cationic antimicrobial peptide regulator overlaps that of the PmrA response regulator.

Promoters identified in bioinformatic screens interact specifically with purified PhoP or PmrA.

To further investigate the role of PhoP in the apparent PhoP-regulation of the PA0921, PA1343, *oprH-phoPQ*, and *pmrH-ugd* operons, gel-mobility shift assays were performed using a PCR-amplified upstream intergenic region of these operons. When recombinant PhoP was incubated with the promoter upstream of the *oprH*, *pmrH*, PA0921, or PA1343 genes (Fig. 3.5), a DNA fragment with reduced mobility was observed. Similarly, mobility shift analysis of the putative PmrA-regulated promoters revealed that those upstream of the *pmrH* (Fig. 3.5), PA4773, PA1559, and *feoA* (Table 3.4) promoters were all bound by the PmrA protein, and this binding was specifically inhibited by an excess of unlabelled probe. Promoters regulated by PmrA did not interact with His_6PhoP, nor did PhoP-regulated genes interact with His_6PmrA, with the exception of that upstream of the *pmrH-ugd* operon, which is known to be regulated by both regulatory systems (McPhee, Lewenza et al. 2003). When the PA1343 promoter was mixed with His_6-PhoP, an insoluble precipitate formed that did not enter the gel at all (Fig. 3.5C). These results indicate that the PhoP and PmrA systems of *P. aeruginosa* regulate separate gene sets (with a single overlapping target operon) and that these gene sets independently respond to the Mg^{2+} limitation signal. These results also indicate that, in contrast to the situation in *Salmonella*, the PhoP-PhoQ and PmrA-PmrB systems of *P. aeruginosa* regulate a relatively small subset of the total number of Mg^{2+}-limitation responsive genes. This observation, and the observation that the alkaline protease gene is regulated by neither PhoP nor

PmrA, indicates that there may be other, as yet unidentified, regulators contributing to the total Mg^{2+} stimulon.

Mutants in *P. aeruginosa feoB* are defective for growth with Fe^{2+} as an iron source.

It has been demonstrated that *phoQ* knockout mutants of *P. aeruginosa* are considerably less virulent in a mouse infection model and that PhoP-PhoQ regulates motility (Brinkman, Macfarlane et al. 2001). Therefore we examined selected Mg^{2+}-limitation responsive genes for virulence related phenotypes. Since this study demonstrated that PmrA and limiting Mg^{2+} regulated the *feoAB* operon, the function of *feoAB* in iron-dependent growth was examined. FeoAB is a well conserved system that is involved in the transport of ferrous iron (Marlovits, Haase et al. 2002). While the function of the FeoA protein is currently unknown FeoB, a GTP-requiring G protein, has been well studied (Kammler, Schon et al. 1993; Boyer, Bergevin et al. 2002). Deletion of the GTP binding motif eliminates Fe^{2+} transport activity (Marlovits, Haase et al. 2002). However due to the lack of similarity of *E. coli* FeoB to other known metal transporters, and the low rates of GDP/GTP binding and hydrolysis, it has been suggested, that FeoB may not act alone as a membrane-spanning Fe^{2+} transporter (Marlovits, Haase et al. 2002). Previous studies have shown that *feoB* mutants of *E. coli* are defective for colonization and have decreased virulence in mouse models of infection (Kammler, Schon et al. 1993; Boyer, Bergevin et al. 2002), while *Helicobacter pylori feoB* mutants are also less virulent in mouse models (Velayudhan, Hughes et al. 2000).

To investigate the role of *P. aeruginosa* FeoAB, growth studies were performed in media containing only Fe^{2+} or Fe^{3+} as the sole source of iron comparing a polar knockout mutant in the *feoA* gene with an isogenic wild type strain (Fig. 3.6). These studies clearly demonstrated that the *feoA* mutant had a severe growth defect when Fe^{2+} was provided as the iron source, but showed no change in growth when Fe^{3+} was the sole iron source, consistent with analogous observation made for *H. pylori* (Velayudhan, Hughes et al. 2000). This defect was apparent

under both Mg^{2+}-limiting and Mg^{2+}-replete conditions, but the phenotype was more pronounced when Mg^{2+} was limiting, as under these conditions growth was completely abrogated in the *feoA* mutant (Fig. 3.6).

It is not yet clear whether *P. aeruginosa* utilizes Fe^{2+} or Fe^{3+} as its primary iron source during infection . There is clear evidence for the presence of Fe^{3+}-chelating siderophores like pyoverdine and pyochelin in the sputum of CF patients indicating that iron is limiting in the CF lung (Haas, Kraut et al. 1991). Also mutants in Fe^{3+}-siderophore uptake, especially ferri-pyoverdine, have somewhat reduced virulence in a number of model systems (Meyer, Neely et al. 1996; Takase, Nitanai et al. 2000). However pyoverdine deficient mutants have been found in the CF lung indicating that other iron-uptake systems may be functional in these bacteria (De Vos, De Chial et al. 2001).

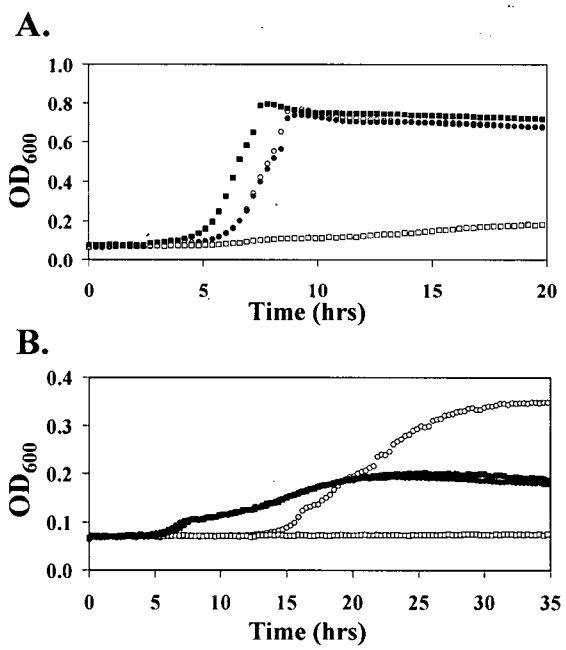

Figure 3.6. Demonstrated involvement of *feoAB* in growth on ferrous iron. The growth of H103 (wt) and H1025 (*feoA::luxCDABE*) were measured under Mg^{2+}-limiting and Mg^{2+}-replete conditions with only Fe^{2+} or Fe^{3+} available as an iron-source. A) Mg^{2+}-replete conditions. B) Mg^{2+}-limiting conditions. ●, H103 + Fe^{3+}; ○, H103 + Fe^{2+}; ■, H1025 + Fe^{3+}, □, H1025 + Fe^{2+};

Recent results demonstrate that the thickened and dehydrated mucous in the CF lung has reduced oxygen tension and direct measurements of O_2 concentrations within this layer indicate

that it approaches complete hypoxia (~5 mmHg), even though the pO_2 within the lung is normal (~200 mmHg) elsewhere in the CF lung (Worlitzsch, Tarran et al. 2002). Indeed *P. aeruginosa* is known to grow anaerobically in the CF lung (Yoon, Hennigan et al. 2002). This decreased oxygen tension would tend to stabilize iron in the Fe^{2+} state. Furthermore, the sputa from the CF lung is noted for its higher than normal concentrations of ferritins, being up to 70-fold higher than observed in healthy individuals (Stites, Plautz et al. 1999; Reid, Withers et al. 2002), and CF individuals have 20 fold higher proportions of H-type ferritins, which are important for detoxifying Fe^{2+} ions, oxidizing them to Fe^{3+} before they are sequestered within the ferritin shell (Harrison and Arosio 1996). In spite of this circumstantial evidence for the presence of Fe^{2+} in the lung, little is known about the ratio of ferrous iron to ferric iron within the lung. This is due to the fact that assays for the presence of iron in the lung usually involve the chemical reduction of transferrin-bound iron making it impossible to determine the Fe^{2+}/Fe^{3+} ratio (Stites, Plautz et al. 1999). In spite of this uncertainty, the results presented suggest that *Pseudomonas aeruginosa* in the mucous layer may utilize Fe^{2+} to satisfy their iron requirement for growth. Consistent with this the *P. aeruginosa* multicopper oxidase, a ferroxidase that forms a crucial part of the ferrous iron uptake pathway, was detected in all of the 35 respiratory clinical isolates surveyed although considerable variation was observed in siderophore expression (Huston, Jennings et al. 2002; Huston, Potter et al. 2004).

PA0921 plays a role in swimming motility.

The PA0921 and PA1343 genes, shown here to be regulated by PhoP and Mg^{2+}-limitation, encode small basic proteins that are unique to *P. aeruginosa.* Resistance to polymyxin B was unchanged in strains containing mutantions in these genes (data not shown). The PhoP-PhoQ system of *P. aeruginosa* has been shown to regulate swarming motility in *Pseudomonas aeruginosa* (Brinkman, Macfarlane et al. 2001). Due to this link between Mg^{2+}-status and motility, we examined the swimming, swarming, and twitching motility of a wild-type strain

and of a ΔPA1343 (UW-44235 and a ΔPA0921 (UW-47583) mutant to determine if either of these genes was involved in regulating motility. A significant swimming defect was observed in the ΔPA0921 mutant on swimming media when medium was supplemented with 2 mM $MgSO_4$, but only a marginal defect was observed under Mg^{2+}-deficient conditions (Table 3.5). There were no significant differences observed with respect to swarming or twitching in either of these mutants, nor was there any motility defect in the ΔPA1343 strain (Table 3.5).

The reasons for the swimming defect in a ΔPA0921 strain are not known, however, Western immunoblots on a wild-type strain and on the ΔPA0921 strain showed no difference in flagellin production under either Mg^{2+}-replete or Mg^{2+}-limited conditions (data not shown), indicating that the regulation may be due to a chemosensory defect, rather than down-regulation of flagellar components, as has been described for *P. aeruginosa* growth in CF mucin (Palmer, Mashburn et al. 2005).

Table 3.5. Swimming, swarming, and twitching motility of *P. aeruginosa* strains grown on BM2-glucose medium supplemented with 2 mM $MgSO_4$ (high Mg^{2+}) or with 200 μM EDTA (low Mg^{2+}). Results shown are the average of 4-6 different measurements.

Phenotype	Strain	Phenotype	Motility Zone (mm)	
			High Mg^{2+}	Low Mg^{2+}
Swimming (mm)	UW-WT	Wild-type	23.4 ± 0.8	8.5 ± 0.4
	UW-47583	ΔPA0921	16.9 ± 0.7*	7.4 ± 0.3*
	UW-44235	ΔPA1343	25.4 ± 1.2	8.1 ± 0.4
Swarming (mm)	UW-WT	Wild-type	13.7 ± 3.2	13.8 ± 1.1
	UW-47583	ΔPA0921	15.4 ± 4.1	12.3 ± 3.5
	UW-44235	ΔPA1343	15.4 ± 4.6	14.1 ± 2.7
Twitching (mm)	UW-WT	Wild-type	20.0 ± 2.1	15.3 ± 4.3
	UW-47583	ΔPA0921	18.7 ± 1.5	16.1 ± 0.6
	UW-44235	ΔPA1343	18.9 ± 0.8	17.7 ± 1.6

* - significantly different from WT-strain (Student's t-test, $p < 0.05$).

Little is known about the PA0921 protein. It is unique to *P. aeruginosa*, with no other homologues found in other bacterial genomes sequenced to date. It is predicted to be a small (12.8 kDa) protein with a slightly basic pI of 9.05. These properties bear some similarity to the PmrD protein of *S. typhimurium* (Kox, Wosten et al. 2000). This protein interacts with the PmrA protein, preventing its dephosphorylation by PmrB under non-inducing conditions (Winfield, Latifi et al. 2005). PSORT-b analysis suggested that the protein is localized to the cytoplasmic membrane (Gardy, Laird et al. 2005) with 4 transmembrane helices identified.

In summary, this work has expanded the number of known targets of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems. In addition to the previously identified *pmrH-ugd* operon responsible for the addition of aminoarabinose to the Lipid A moiety of LPS, and the PA4773-*pmrAB* operon, we have shown that the PmrA-PmrB system also regulates three other operons. It regulates the PA1559-PA1560 operon, encoding two conserved hypothetical proteins, the *feoAB* operon, encoding a system involved in the uptake of Fe^{2+}, and the PA4782 gene. Interestingly, the PA4782 gene encodes a small protein of unidentified function that is just upstream of PA4781, an uncharacterized response regulator. This apparent regulation of a second two-component response regulator by PmrA-PmrB suggests that there may be hierarchical control over some, currently unknown, members of the Mg^{2+} stimulon. While the function of the PA4781 protein is unknown, it does contain a conserved HD-GYP domain. This domain is proposed to be involved in cyclic-di-GMP turnover, a known regulator of diverse functions in *P. aeruginosa*, including biofilm formation, antibiotic resistance, and production of surface adhesins (D'Argenio, Calfee et al. 2002; Hoffman, D'Argenio et al. 2005; Meissner, Wild et al. 2005).

This work has also demonstrated that all PmrA-regulated genes identified in this work were also regulated by exposure to sub-inhibitory concentrations of CP11CN. The regulator responsible for this phenotype is currently unknown. This observation indicates that the PmrA

regulon may have evolved in such a way as to permit its stimulation in environments where Mg^{2+} limitation does not occur. The observation of regulation by cationic antimicrobial peptides is consistent with conditions that are found within the surface airway liquid of the lung, where Mg^{2+} concentration is non-limiting (~2 mM) but there is a high concentration of cationic host defence peptides, due to the inflammation and chronic neutrophil degranulation that characterize the CF lung.

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CHAPTER 4 – PxrRS of *Pseudomonas aeruginosa*[2]

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterial pathogen, responsible for high infection rates in the immunocompromised and cystic fibrosis patients (Gibson, Burns et al. 2003; Park 2005). This bacterium is also noted for its high intrinsic resistance to a wide variety of antimicrobials due to a combination of a generally impermeable outer membrane and secondary resistance mechanisms including multi-drug efflux systems and inducible chromosomal β-lactamases (Hancock and Nikaido 1978; Poole and Srikumar 2001; Ciofu 2003). This combination of features has contributed to making *P. aeruginosa* one of the leading causes of nosocomial infections in Western society (Fagon, Chastre et al. 1993; Richards, Edwards et al. 1999).

One of the most effective treatments in use is inhaled colistin, either alone or in combination with ciprofloxacin (Marchetti, Giglio et al. 2004). Colistin (polymyxin E) is a cationic lipopeptide produced by *Bacillus polymyxa* that has been used as an anti-Pseudomonal drug for over 40 years (Gordon and M. 1960). Polymyxins interact with the outer membrane of Gram-negative bacteria by binding to the Lipid A component of lipopolysaccharide, thereby displacing divalent cations that normally stabilize the outer membrane (Nicas and Hancock 1983; Hancock and Wong 1984; Moore, Chan et al. 1984; Moore, Bates et al. 1986). This self-promoted uptake across the outer membrane gives the drug access to internal targets. Polymyxins kill bacteria rapidly and clinically significant levels of resistance have not yet emerged (Fish, Piscitelli et al. 1995; Littlewood, Koch et al. 2000; Li, Nation et al. 2005). Polymyxins share certain properties with a large class of natural antibiotics termed cationic

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antimicrobial peptides. This class of compounds holds great potential as a novel class of antibiotics, with wide-spectrum activity against Gram-positive and Gram-negative bacteria, enveloped viruses, and fungi (Brogden 2005; Jenssen 2005). Several highly active variants have been described and are in various stages of clinical and preclinical trials (Zhang and Falla 2004; Zhang, Parente et al. 2005).

In *P. aeruginosa*, the PhoP-PhoQ and PmrA-PmrB systems regulate polymyxin and cationic antimicrobial peptides resistance in response to limiting concentrations of Mg^{2+} (Macfarlane, Kwasnicka et al. 2000; McPhee, Lewenza et al. 2003). This resistance occurs primarily via the coregulation of an LPS modification operon, *pmrHFIJKLM-ugd* (PA3552-PA3559), that encodes proteins that catalyze the addition of aminoarabinose to the 1 and 4' phosphates of Lipid A (McPhee, Lewenza et al. 2003; Moskowitz, Ernst et al. 2004). This modification reduces the negative charge normally found on Lipid A and is associated with cationic antimicrobial peptide and polymyxin B resistance (Zhou, Ribeiro et al. 2001; Moskowitz, Ernst et al. 2004).

P. aeruginosa also undergoes an adaptive response to the presence of sub-inhibitory concentrations of cationic antimicrobial peptides and polymyxin B (Gilleland and Farley 1982; McPhee, Lewenza et al. 2003). This response results in increased transcription of the *pmrHFIJKLM-ugd* and PA4773-4775-*pmrAB* operons when the cells are exposed to sub-inhibitory concentrations of cationic antimicrobial peptides (McPhee, Lewenza et al. 2003). The adaptive response is independent of both PmrA-PmrB and PhoP-PhoQ, and presumably depends on an unknown regulator. In an effort to identify novel regulatory systems that contribute to polymyxin B and cationic antimicrobial peptide resistance, a panel of mutants in two-component response regulators was screened for an altered response to the presence of the sub-inhibitory cationic antimicrobial peptide, CP11CN. Through this approach we were able to identify a previously uncharacterized two-component regulatory system, named here PxrRS

(PA1799-1798), which is responsible for intrinsic resistance to polymyxin B and cationic antimicrobial peptides in *P. aeruginosa*. In addition to its role in polymyxin B resistance, mutants in *pxrR* also have a loss of functional type IV pili, due to down-regulation of the *fimUpilVWXY1Y2E* operon.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions.

Bacterial strains and plasmids used in this study are described in Table 4.1. Sequences of primers used in this study are shown in Table 4.2. *P. aeruginosa* was routinely grown in BM2-glucose minimal medium supplemented with 20 μM (low) or 2 mM (high) Mg^{2+}, containing

Table 4.1. Strains and plasmids used in this study.

Strain	Phenotype	Reference
UW-WT	Wild-type PAO1 strain	(Jacobs, Alwood et al. 2003)
42767	PA0756::IS*phoA*	(Jacobs, Alwood et al. 2003)
10580	PA1798::IS*lacZ*	(Jacobs, Alwood et al. 2003)
13144	PA1799:: IS*lacZ*	(Jacobs, Alwood et al. 2003)
39547	PA2479::IS*phoA*	(Jacobs, Alwood et al. 2003)
5552	PA2523::IS*lacZ*	(Jacobs, Alwood et al. 2003)
44882	PA2657::IS*phoA*	(Jacobs, Alwood et al. 2003)
17142	PA2809::IS*lacZ*	(Jacobs, Alwood et al. 2003)
4715	PA3204::IS*lacZ*	(Jacobs, Alwood et al. 2003)
1772	PA3205::IS*lacZ*	(Jacobs, Alwood et al. 2003)
43428	PA4885::IS*phoA*	(Jacobs, Alwood et al. 2003)
H103	Wild-type PAO1 strain	(Lewenza, Falsafi et al. 2005)
PAO1_lux_1_E7	*htpG::luxCDABE*	(Lewenza, Falsafi et al. 2005)
PAO1_lux_32_C12	PA3523::*luxCDABE*	(Lewenza, Falsafi et al. 2005)
PAO1_lux_97_E3	PA3522::*luxCDABE*	(Lewenza, Falsafi et al. 2005)
PAO1_lux_11_G4	PA4065::*luxCDABE*	(Lewenza, Falsafi et al. 2005)
Plasmid	Relevant characteristics	
pUCP22	*E. coli-Pseudomonas* shuttle vector	(West, Schweizer et al. 1994)
pUC*pmrH*:: *luxCDABE*	Entire intergenic region between *algA* and *pmrH* cloned upstream of a *luxCDABE* cassette	(McPhee, Lewenza et al. 2003)
pUC*pxrR*	*pxrR* gene and promoter cloned into pUCP22	This study

Table 4.2. Sequences of primers used in this study.

Primer	Sequence (5'-3')
RTrpsL-F	TGCGTAAGGTATGCCGTGTA
RTrpsL-R	CAGCACTACGCTGTGCTCTT
RTPA4773-F	GCACCTGGCGATCCATAC
RTPA4773-R	CTGGGCGCCATCGAGTA
RTPA3552-F	CACTGGACTTTCTGCCATTCT
RTPA3552-R	TGTTCGAGCTCCTGGTTCTT
RTpilV-F	CTTCAGCATGATCGAAGTGC
RTpilV-R	GCCTTGAAGAAGTCGGATTG
PA1799-F	GAATCAAGGGCATGCATTCTA
PA1799-R	CGTACCACCAGCAGGTTCTT

antibiotics at the following concentrations: carbenicillin, 200 μg/ml; gentamicin, 50 μg/ml; tetracycline, 50 μg/ml. Routine genetic manipulations were carried out according to Maniatis et al. (Maniatis, Fritsch et al. 1989).

Luciferase assays.

Briefly, 18 hr cultures of *P. aeruginosa* containing pUC*pmrH*::*luxCDABE* were diluted to ~5 X 10^6 cfu/ml in BM2-glucose high Mg^{2+} either in the presence or absence of 2 μg/ml CP11CN (Friedrich, Moyles et al. 2000; McPhee, Lewenza et al. 2003). These cultures were incubated in a Tecan Spectrafluor Plus at 37°C with shaking. Luminescence measurements were taken using at 18 minute intervals throughout the growth of the bacteria in the same instrument.

Semi-quantitative PCR assays (qPCR).

Total RNA was isolated using RNeasy mini columns (Qiagen, Mississauga, ON) from mid-logarithmic (OD_{600} ~0.4-0.6) phase *P. aeruginosa* grown in BM2-glucose minimal media with 20 μM Mg^{2+} or 2mM Mg^{2+}. RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA) to remove contaminating genomic DNA. Four μg of total RNA was combined with 0.5 μM dNTPs, 500 U/ml SuperaseIN (Ambion, Austin, TX), 10 μM DTT, in 1X reaction buffer and reverse transcribed for 1 hour at 37°C and 2 hr at 42°C with 10,000 U/ml Superscript II reverse

transcriptase (Invitrogen, Carlsbad, CA). The RNA was subsequently destroyed by the addition of NaOH to 170 mM and incubation at 65°C for 10 minutes. The reaction was then neutralized by addition of HCl and the cDNA was used as a template for PCR. The number of cycles used to amplify each gene of interest was chosen to ensure that the PCR reaction was not saturated via trial and error. All reactions were normalized to the *rpsL* gene encoding the 30S ribosomal protein S12.

Outer membrane permeability assays.

P. aeruginosa cells were grown to mid-logarithmic phase (OD600~0.4-0.6) in BM2-glucose minimal media. Cells were washed then resuspended to OD600 in 5 mM HEPES buffer pH 7.0 containing 5 mM glucose and 5 mM KCN. The cells were incubated for 10 minutes. Two ml of cell suspension was added to a quartz cuvette and placed in a Perkin-Elmer LB 50B luminescence spectrometer. NPN (5 μM) was then added to the cuvette containing the KCN-treated *P. aeruginosa* cells. Fluorescence was monitored at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. After the baseline stabilized, polymyxin B was added to the cell suspension at various concentrations and the fluorescence was monitored until it had stabilized.

Motility assays.

Swimming assays were performed by inoculating 1 μl of an overnight bacterial culture onto a BM2-glucose plate containing 2 mM Mg^{2+} and 0.3% agar. Twitching assays were carried out on LB media containing 1% agar. Inoculation was done with 1 μl of an overnight culture that was stabbed into the agar/plastic interface. After 24 hours of growth, the agar was removed and the twitch zones were measured. For swarming assays, brain-heart infusion broth (BHI) containing 0.5% agar were inoculated with 1 μl of an overnight culture. After 24 hr the swarm zones were measured. Results shown are the average and standard deviation of 4-6 independent measurments.

Biofilm assays.

Stationary phase cultures of UW-WT and *pxrR::ISlacZ* were diluted to OD_{600} ~0.1 and 100 µl of this culture was added to a polystyrene microtitre dish. This was incubated at 37°C for 24 hours. The media was removed and the adherent cells were extensively washed with deionized water. The biofilm was then stained with 1% crystal violet for 10 minutes. The crystal violet was removed and the plate was extensively washed with deionized water. The biofilm was then solubilized by washing each well with 100 µl of ethanol. This ethanol was removed and quantitated by measuring the absorbance at 595 nm.

RNA extraction, cDNA synthesis and hybridization to DNA microarrays

Five biological samples each of *Pseudomonas aeruginosa* PAO1 and *pxrR::*IS*lacZ* (Jacobs, Alwood et al. 2003) were grown overnight in BM2-glucose medium supplemented with 2 mM $MgSO_4$ in acid washed glassware. Cultures were diluted 1/100 into the fresh media and cells were harvested at mid-log phase, (OD_{600} 0.5-0.6). RNA was prepared according to Qiagen RNeasy Midi RNA isolation kit according to the manufacturers instructions (Qiagen Inc., Canada). The isolated RNA was treated with DNA-free kit (Ambion Inc., Austin,TX, USA) to remove any contaminating genomic DNA. RNA was stored at -80°C with 0.2 units of SUPERase-In RNase Inhibitor (Ambion Inc., Austin, TX). RNA quality was assessed by running on 1% Agarose-Le gel (Ambion Inc., Austin, TX), as well as spectrophotometrically.

Ten µg of total RNA was treated using the Microbe Express kit with *Pseudomonas* module (Ambion) to remove ribosomal RNA via short magnetic-bead linked oligonucleotides that are specific for *P. aeruginosa* 16s and 23s rRNA . Messenger RNA was reverse transcribed according to TIGR protocols (http://pfgrc.tigr.org/protocols/M007.pdf). Briefly, reverse transcription reactions (20 µL) were combined with 42.5 µg/ml mRNA, 300 µg/ml random hexamers (Invitrogen, Mississauga, ON), 300 U/ml Superase In (Ambion, Austin, TX), 15 mM

DTT, and 20000 U/ml Superscript III (Invitrogen, Mississauga, ON). Removal of unincorporated aminoallyl-dUTP and free amines, labeling using Cy-dyes, removal of free dyes and analysis of labeling reaction was performed as in the TIGR Microbial RNA aminoallyl labeling for microarrays protocol, http://pfgrc.tigr.org/protocols/M007.pdf, cDNA from *pxrR*::IS*lacZ* was labeled with cyanine-5 (GE Healthcare Canada) and all PAO1 cDNA was labeled with cyanine-3 (GE Healthcare Canada). Yield and fluorophore incorporation was measured using a Lamda 35 UV/VIS fluorimeter (PerkinElemer Life and Analytical Sciences, Inc., USA). *P. aeruginosa* PAO1 microarray slides were provided by The Institute of Genomic Research-Pathogenic Functional Genomics Resource Center (http://pfgrc.tigr.org/). Hybridization of labeled cDNA was done as per the protocol for TIGR hybridization of labeled DNA probes, http://pfgrc.tigr.org/protocols/M008.pdf. Two hundred pmoles of each cyanine labeled sample from PAO1 and *pxrR*::IS*lacZ* cells were combined and hybridized to the array slides overnight at 42°C. Slides were scanned using the ScanArray™ Express scanner/software (Packard BioScience BioChip Technologies) and quantified using ImaGene 6.0 Standard Edition software (BioDiscovery, Inc., El Segundo, CA, USA).

Analysis of DNA Microarrays

Assessment of slide quality, data normalization, detection of differential gene expression and statistical analysis was carried out using ArrayPipe version 1.7, a web-based, semi-automated software specifically designed for processing of microarray data (Hokamp, Roche et al. 2004) with gene annotation from TIGR and linked with the *Pseudomonas aeruginosa* genome database (http://www.pseudomonas.com). The following processing steps were applied: flagging of markers and control spots; subgrid-wise background correction using the median of the lower 10% foreground intensity as an estimate for the background noise; data-shifting to rescue most of the negative spots; printTip LOESS normalization; merging of duplicate spots;

two-sided one-sample Student's t-test on the $\log_2$-ratios within each experiment; and finally, averaging of biological replicates to yield overall fold-changes for each treatment group.

RESULTS

Identification of PxrRS.

Mutants in two-component response regulators were obtained from the University of Washington Genome Sequencing Center (Jacobs, Alwood et al. 2003). The plasmid pUC-*pmrH::luxCDABE* was mobilized into these strains by electroporation. Luminescence of the plasmid-containing strains was then measured in the presence and absence of 2 μg/ml CP11CN. One strain, containing a transposon insertion in PA1799 exhibited markedly lower luminescence in the presence of CP11CN than either the isogenic parent strain, or any of the other response regulator mutants tested. A sample of the screening results is shown in Fig. 4.1. Minimal inhibitory concentrations to polymyxin B and CP11CN were determined for UW-WT, PA1799::IS*lacZ* and PA1798::IS*lacZ* using the broth microdilution method (Table 4.3). These results showed that mutants in PA1798 and PA1799 were consistently 2-4 fold more sensitive to polymyxin B than the wild type strain, regardless of Mg^{2+} concentration in the growth medium. When PA1799 was provided on a plasmid to PA1799::IS*lacZ*, the resistance to polymyxin B and CP11CN was restored to wild-type levels or greater. Based on these observations, PA1799 and PA1798 were named *pxrR* and *pxrS*, for polymyxin resistance *r*egulator and *s*ensor, respectively.

PxrR mutants show increased sensitivity to cationic antimicrobial peptides.

Killing assays were carried out with CP11CN on both *pxrR::*IS*lacZ* and an isogenic wild-type strain, as these assays are more sensitive that inhibition assays. Although MIC assays (Table 4.3) showed a sensitivity phenotype only for polymyxin B, killing assays showed that the *pxrR::*IS*lacZ* mutant was more sensitive to CP11CN compared to an isogenic wild-type strain

(Fig. 4.2). In addition, complementation of the strain with pUC*pxrR* restored killing to wild-type levels, while providing pUCP22 (vector control) did not.

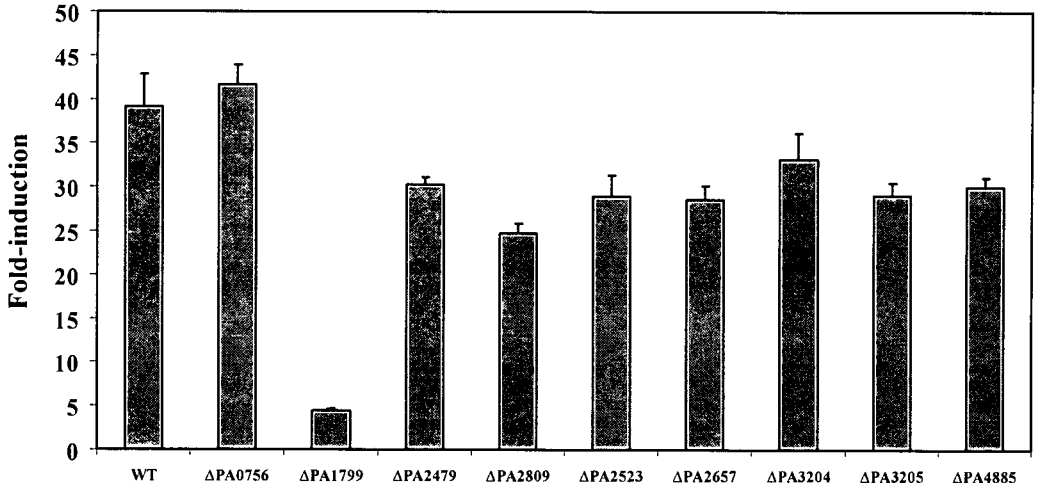

Figure 4.1. Induction of a *pmrH::luxCDABE* fusion in response to 2 μg/ml CP11CN in regulatory mutant strains. Strains containing transposon insertion mutations in the genes indicated were transformed with a plasmid containing a fusion between the pmrH promoter and a promoterless luxCDABE cassette. Luminescence was measured following 5 hours growth in the presence of 2 μg/ml CP11CN. The fold-change was calculated by normalizing to the luminescence of cells grown in the absence of CP11CN.

PxrR mutants show normal transcriptional response to CP11CN.

To confirm the results of the initial screen, semi-quantitative RT-PCR (q-PCR) was conducted on wild-type and *pxrR::*IS*lacZ* mutants in the presence and absence of 2 μg/ml CP11CN. The *pxrR::*IS*lacZ* strain strain showed a transcriptional response to cationic antimicrobial peptides that was indistinguishable from that of the wild-type strain (Fig 4.3). Taken together with the killing assays it seems apparent that *pxrRS* mutants failed to demonstrate sustained *pmrH::luxCDABE* expression in the presence of CP11CN because the subinhibitory level of CP11CN inhibited bacterial growth and ATP production required for luminescence, rather than causing specific loss of cationic peptide induced PA4773 and *pmrH* expression.

PxrR mutants have normal outer membrane permeability.

To examine the basis of increased susceptibility, the outer membrane permeability of a *pxrR::*IS*lacZ* mutant was assessed using the NPN uptake assay. No differences in outer membrane permeability induced by polymyxin B were observed between the strains (Fig. 4.4).

PxrR mutants undergo adaptive resistance to polymyxin B following pre-exposure to cationic antimicrobial peptides.

Exposure to cationic antimicrobial peptides induces the *pmrHFIJKLM-ugd* and the PA4773-PA4775 operons independently of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems (McPhee, Lewenza et al. 2003). This adaptive resistance leads to increased resistance to a subsequent exposure to polymyxin B (Fig. 2.7). In a similar fashion to the wild-type strain, when the *pxrR::*IS*lacZ* strain is grown in BM2-glucose medium in the presence of 2 μg/ml CP11CN, specific induction of the PA4773 and *pmrH* genes was observed (Fig. 4.3). Additionally, both the UW-WT strain and the *pxrR::*IS*lacZ* strain become adaptively resistant to a subsequent exposure to polymyxin B (Fig. 4.5). However, consistent with the observed increased susceptibility of the *pxrR::*IS*lacZ* mutant to polymyxin B, even the adaptively resistant *pxrR::*IS*lacZ* cells demonstrated approximately 10-fold reduced survival to 1 μg/ml polymyxin B compared to a similarly treated wild-type strain.

Microarray analysis shows downregulation of several potential intrinsic cationic peptide resistance genes in *a pxrR::ISlacZ* mutant.

The somewhat surprising observation that a *pxrR::*IS*lacZ* strain had normal responses to subinhibitory concentrations of cationic antimicrobial peptides led to the hypothesis that there were other, novel genes involved in intrinsic resistance to this class of compounds. Microarray analysis indicated that PxrR regulated a number of heat shock proteins in *P. aeruginosa* (Table 4.4). These include the *htpG*, *dnaK*, PA3601-3600 ribosomal genes, and the *ibpA* gene, all of which have been described as encoding heat shock proteins in several other bacterial species. The DnaK protein has been directly implicated as a target of cationic antimicrobial peptides

(Kragol, Lovas et al. 2001), while both *htpG* and *ibpA* have been shown to affect protein aggregation and refolding in *E. coli* (Thomas and Baneyx 2000; Lethanh, Neubauer et al. 2005). Additionally, two efflux systems were observed to be downregulated in the *pxrR::*IS*lacZ* strain. One is an ABC transporter operon PA4063-PA4066 and the other is an RND efflux-type transporter system, *mexPQ-opmE* (PA2523-PA2521).

Downregulation of pili biosynthesis genes in *pxrR* mutant.

Microarray analysis also showed that the *pxrR::*IS*lacZ* strain exhibited significant downregulation of an operon containing the *fimUpilVWZY1Y2E* genes. This operon has been shown to be involved in type IV pili biosynthesis using a combination of transposon mutagenesis and some functional analysis (Alm, Hallinan et al. 1996; Alm and Mattick 1996; Jacobs, Alwood et al. 2003). In order to confirm this, the transcription of the first gene in the operon, *pilV* was examined via semi-quantitative RT-PCR (4.6A). This analysis confirmed the downregulation of the *pilV* gene observed in the microarray analysis.

PxrR mutants are defective in pilin-dependent phenotypes.

In order to further characterize the consequences of the strong down-regulation of the *fimUpilVWZY1Y2E* operon, a number of pili-dependent phenotype were examined. Twitching motility is characterized as the movement of bacteria over surfaces due to the progressive extension and retraction of type IV pili (Mattick 2002). This motility is strictly dependent upon the presence of functional type IV pili (Mattick 2002). In turn, twitching motility is required for the early stages of biofilm production, and pili deficient mutants are incapable of forming the robust biofilms that are characteristic of *P. aeruginosa* (O'Toole and Kolter 1998; Heydorn, Ersboll et al. 2002; Mattick 2002; Klausen, Heydorn et al. 2003). As shown in Fig. 4.6B and 4.6C, mutants in *pxrR* are completely deficient in both twitching motility and biofilm formation. In addition, the *pxrR::*IS*lacZ* mutant was completely resistant to the pilin-specific phage PO4 (data not shown).

Table 4.3. Minimal inhibitory concentration (MIC) of cationic peptides and polymyxin B in BM2-glucose medium under 2 mM (high) and 20 μM (low) Mg^{2+} conditions.

	Minimal inhibitory concentration (μg/ml) in BM2-glucose containing high (2 mM) or low (20 μM) Mg^{2+}									
	Polymyxin B		CP10A		CP11CN		Indolicidin		CEMA	
$[Mg^{2+}]$	high	low	high	low	high	low	high	low	high	low
UW-WT	2	8	64	8	>64	64	>64	>64	8	8
Δ*pxrS*	0.5	2	64	8	>64	64	>64	>64	8	4
Δ*pxrR*	0.5	4	64	16	>64	>64	>64	>64	8	4
Δ*pxrR* + pUC*pxrR*	4	16	64	64	>64	>64	>64	>64	8	16
H103	1	ND*	32	ND	ND	ND	ND	ND	4	ND
PAO1_11_G4 (PA4065::*lux*)	1	ND	32	ND	ND	ND	ND	ND	4	ND
PAO1_97_E3 (*mexP::lux*)	1	ND	32	ND	ND	ND	ND	ND	4	ND
PAO1_32_C12 (*mexQ::lux*)	1	ND	32	ND	ND	ND	ND	ND	4	ND
PA01_1_E7 (*htpG::lux*)	0.5	ND	32	ND	ND	ND	ND	ND	4	ND

*ND – not determined

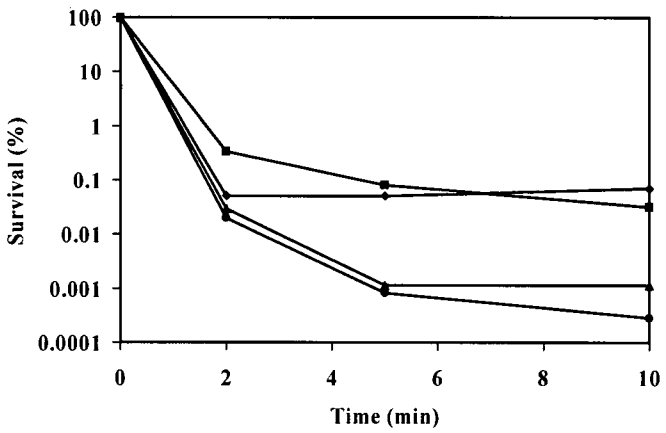

Figure 4.2. Δ*pxrR* mutants of *P. aeruginosa* have increased susceptibility to CP11CN. Killing curves were carried out using 8 μg/ml CP11CN on cells grown to mid-logarithmic phase in BM2-glucose medium supplemented with 2 mM $MgSO_4$. UW-WT, ■; *pxrR*::IS*lacZ*, ●; *pxrR*::IS*lacZ* + pUC*pxrR*, ♦; Δ*pxrR* + pUCP22, ▲.

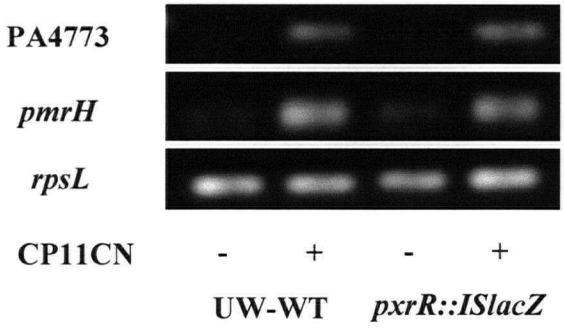

Figure 4.3. A *pxrR::*IS*lacZ* mutant show normal responses to the presence of subinhibitory concentrations of cationic antimicrobial peptides. UW-WT or *pxrR::*IS*lacZ* strains were grown to mid-logarithmic phase in the presence or absence of 2 μg/ml of CP11CN. Cells were harvested, RNA was extracted, and cDNA synthesized from the RNA. The relative levels of the PA4773 and *pmrH* mRNAs was then assessed by qPCR.

Figure 4.4. Mutants of *pxrR* have normal outer membrane permeability. Outer membrane permeability was measure using the NPN uptake assay. No significant differences were observed.

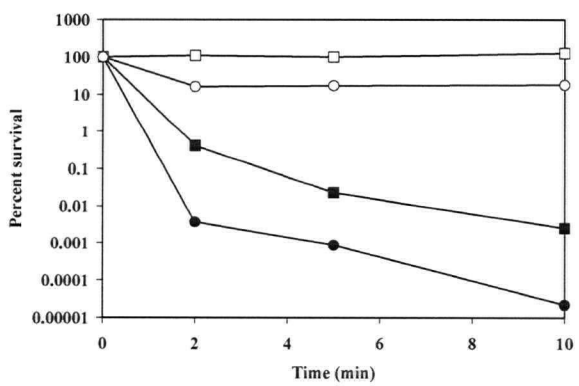

Figure 4.5. *pxrR* mutants undergo adaptive resistance following exposure to sub-MIC levels of cationic antimicrobial peptides. UW-WT and *pxrR::*IS*lacZ* strains were grown to mid-logarithmic phase in the presence or absence of 2 μg/ml CP11CN. Cells were then harvested, washed, and resuspended in 1X BM2 salts buffer. Killing was initiated by the addition of 1 μg/ml polymyxin B. Cells were removed and serial dilutions plated and counted to determine the percentage of cells that survived the exposure. □ – UW-WT cells preexposed to CP11CN before the onset of killing; ■ – UW-WT cells without prexposure; ○ - *pxrR::*IS*lacZ* cells preexposed to CP11CN before the onset of killing ● – *pxrR::*IS*lacZ* cells without prexposure.

Table 4.4. Genes identified as being significantly regulated in a *pxrR::*IS*lacZ* mutant via microarray analysis

PAID	Gene name	Fold change	Gene description
PA0527	*dnr*	1.7	transcriptional regulator Dnr
PA0718		-1.6	hypothetical protein of bacteriophage Pf1
PA0781		-1.8	hypothetical protein
PA1596	*htpG*	-1.7	heat shock protein HtpG
PA1922		-2.2	probable TonB-dependent receptor
PA2343		-1.7	xylulose kinase
PA2862	*lipA*	1.6	lactonizing lipase precursor
PA3006	*psrA*	-1.7	probable transcriptional regulator
PA3126	*ibpA*	-1.7	heat-shock protein IbpA
PA3600		-2.1	ribosomal protein L36
PA3601		-2.2	ribosomal protein L31
PA3920		-4.7	probable metal transporting P-type ATPase
PA4063		-2.0	hypothetical protein
PA4066		-1.9	hypothetical protein
PA4528	*pilD*	-2.0	type 4 prepilin peptidase PilD
PA4551	*pilV*	-1.8	type 4 fimbrial biogenesis protein PilV
PA4552	*pilW*	-1.6	type 4 fimbrial biogenesis protein PilW
PA4553	*pilX*	-1.5	type 4 fimbrial biogenesis protein PilX
PA4554	*pilY1*	-1.7	type 4 fimbrial biogenesis protein PilY1
PA4555	*pil2*	-1.6	type 4 fimbrial biogenesis protein PilY2
PA4556	*pilE*	-1.6	type 4 fimbrial biogenesis protein PilE
PA4761	*dnaK*	-2.2	DnaK protein
PA4836		-2.1	hypothetical protein
PA4837		-1.6	probable outer membrane protein
PA4919		-1.6	nicotinate phosphoribosyltransferase
PA5172	*arcB*	1.7	ornithine carbamoyltransferase, catabolic
PA5173	*arcC*	1.6	carbamate kinase
PA5534		-2.1	hypothetical protein
PA5535		-5.4	conserved hypothetical protein
PA5538	*amiA*	-1.7	N-acetylmuramoyl-L-alanine amidase

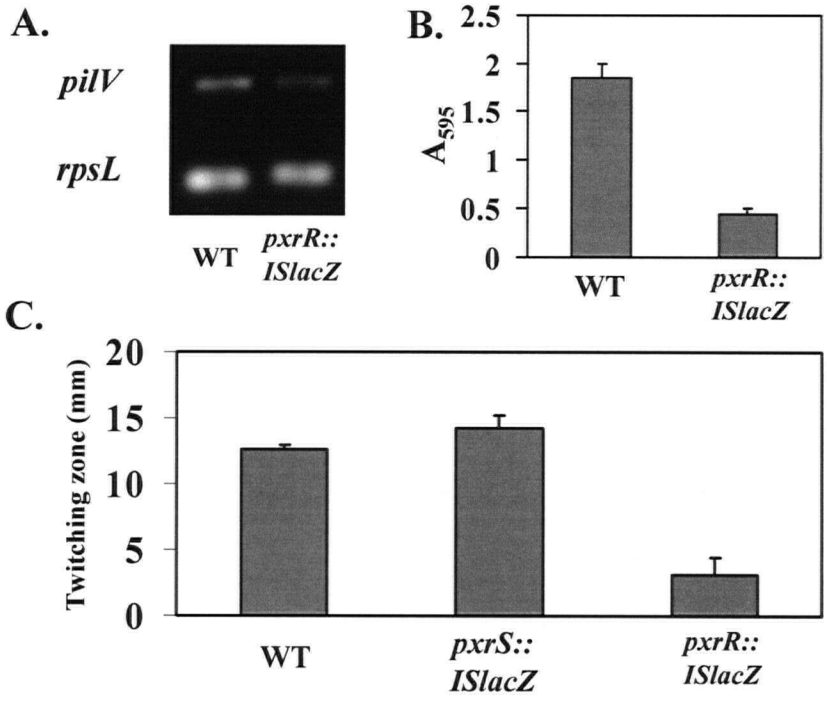

Figure 4.6. Mutants of *pxrR* exhibit pili-negative phenotypes. A. *pxrR::*IS*lacZ* mutants have decreased expression of *pilV*, a gene involved in assembly of pili B. *pxrR::*IS*lacZ* mutants are defective for biofilm formation after 24 hours. C. Mutants in *pxrR*, but not *pxrS*, are defective for twitching.

DISCUSSION

Previous work in *P. aeruginosa* has demonstrated the existence of two separate two-component regulatory systems that contribute independently to polymyxin B and cationic antimicrobial peptide resistance in response to limiting concentrations of Mg^{2+} in the growth media. These systems, PhoP-PhoQ and PmrA-PmrB contribute to cationic peptide and polymyxin B resistance through the regulation of two separate operons, PA4773-PA4775 and *pmrHFIJKLM-ugd* (PA3552-PA3559) (Macfarlane, Kwasnicka et al. 1999; Macfarlane, Kwasnicka et al. 2000; McPhee, Lewenza et al. 2003). The *pmrHFIJKLM-ugd* operon is directly regulated by both the PmrA-PmrB and PhoP-PhoQ systems. It contributes to resistance via the addition of aminoarabinose to the 1 and 4' positions of lipid A, which results in the

neutralization of the negative charge on the lipid A, thereby decreasing the strength of the interaction between Lipid A and cationic antimicrobial peptides (Moskowitz, Ernst et al. 2004). The PA4773-PA4775 genes of the *pmrAB* operon also contribute to resistance via an as yet uncharacterized mechanism.

In addition to this Mg^{2+} regulation of cationic peptide resistance, work described in Chapters 2 and 3 has also demonstrated that the presence of sub-inhibitory concentrations of cationic antimicrobial peptides causes increased transcription of the PmrA-dependent genes, including the PA4773 operon and the *pmrHFIJKLM-ugd* operon, even in the presence of 2 mM Mg^{2+}. In contrast to the situation in *Salmonella enterica* sv Typhimurium, this cationic antimicrobial peptide mediated regulation was largely independent of PhoP-PhoQ and was also independent of the PmrA-PmrB system, strongly supporting the existence of another regulatory system that specifically responds to the presence of cationic antimicrobial peptides (Bader, Navarre et al. 2003; McPhee, Lewenza et al. 2003; Bader, Sanowar et al. 2005).

The initial screen carried out in this study was designed to identify this regulator by looking for a mutant that exhibited diminished response to the presence of sub-inhibitory concentrations of CP11CN. A *pxrR::*IS*lacZ* mutant showed only a 5-fold increase in luminescence of a *pmrH::luxCDABE* plasmid fusion following six hours of incubation, compared with 30-40 fold induction in an isogenic parent strain. Interestingly, mutants in both *pxrR* and *pxrS* showed 4-fold lower MICs to polymyxin B than the isogenic parent strain, even in the absence of CP11CN (Table 4.3). The strain also showed evidence of increased resistance under Mg^{2+}-limiting conditions (i.e. PhoPQ and PmrAB regulated resistance), indicating that the mutation in *pxrR* did not interfere with Mg^{2+}-signaling. The resistance of the *pxrR* mutant was restored to that of the wild-type strain when *pxrR* was overexpressed from a multi-copy plasmid. Altered MICs for *pxrR* and *pxrS* mutants were observed only for polymyxin B and no differences were observed in the MIC to indolicidin, the indolicidin variants CP10A or

CP11CN, or an α-helical cecropin-magainin hybrid, CEMA. However, overexpression of *pxrR* did lead to significant increases in resistance to CP10A and moderate increases to CP11CN under Mg^{2+} limiting conditions. Consistent with this, killing experiments confirmed that, although *pxrR* mutants showed the same MIC as an isogenic parent strain, higher levels of killing were observed for the *pxrR* strain following treatment with 8 μg/ml CP11CN (Fig. 4.2).

In spite of these changes, examination of the transcript levels of *pmrH* and PA4773 in CP11CN-treated cells did not show any significant differences between a *pxrR::*IS*lacZ* strain and an isogenic wild-type strain. This result suggested that the defect in *pxrR::*IS*lacZ* mutant bacteria was not due to a direct regulatory effect on the *pmrH* promoter, but rather, may have been an indirect effect due to increased sensitivity to the treatment.

In an effort to understand why *pxrR::*IS*lacZ* mutants were more sensitive to cationic antimicrobial peptides and polymyxin B, microarray experiments were conducted. Genes with significantly altered gene expression are indicated in Table 4.4. These experiments showed that a large number of heat-shock proteins, including *dnaK*, *htpG*, *ibpA*, and PA3601-3600, two small ribosomal proteins that have been shown to be heat-shock regulated in *E. coli*, were downregulated in a *pxrR* mutant. This might indicate that either one of these proteins is the direct target for cationic peptides or that the *pxrRS* mutants failed to initiate heat-shock repair/adaptation mechanisms in response to peptide-mediated damage.

Several studies have suggested that some insect-derived cationic antimicrobial peptides may function by binding to both DnaK and GroES, two heat-shock systems that are responsible for the refolding of heat-damaged proteins (Otvos, O et al. 2000; Kragol, Lovas et al. 2001). The binding to DnaK occurs via a specific interaction between pyrrhocoricin, an antimicrobial peptide found in the haemolymph of the sap sucking aphid, *Pyrrhocoris apterus* (and presumably apidaecin and drosocin, peptides from the honeybee and fruit fly, respectively)

(Casteels, Ampe et al. 1989; Bulet, Dimarcq et al. 1993) and the lid region of DnaK (Otvos, O et al. 2000; Kragol, Lovas et al. 2001). This inhibition causes an increase in the level of misfolded intracellular proteins, leading to cell death. This interaction is stereospecific, and bacteria with mutations in the lid region of DnaK, are resistant to the action of pyrrhocoricin (Kragol, Hoffmann et al. 2002). These studies were among the first to suggest specific interactions between a cationic antimicrobial peptide and an intracellular target.

Due to the observation of increased transcription of a number of heat-shock proteins, we examined the sensitivity of several heat shock protein mutants to polymyxin B. Our studies however, failed to demonstrate any link between the absence of *htpG* and polymyxin B sensitivity (Table 4.3). Neither was this strain, nor the *pxrR*::IS*lacZ* strain more sensitive to a 46°C heat shock than wild-type PAO1 (data not shown). This result indicates that differences in sensitivity may only be seen after the loss of multiple heat-shock proteins, as has been previously described in *E. coli* (Thomas and Baneyx 1998; Thomas and Baneyx 2000).

Efflux has been described as a resistance mechanism against cationic antimicrobial peptides in several bacterial species. In *Neisseria meningitidis*, mutants in the *mtrCDE* multidrug efflux system exhibit a 6-20 fold increase in susceptibility to protegrin, a cationic antimicrobial peptide of porcine neutrophils (Shafer, Qu et al. 1998). Recently, this system has also been shown to be involved in susceptibility to polymyxin B and the human host defense peptide, LL-37 (Tzeng, Ambrose et al. 2005). Similarly, in *Staphylococcus aureus*, mutants in the *qacA* gene are more sensitive to a small antimicrobial peptide from platelet microbicidal protein (Kupferwasser, Skurray et al. 1999). The RosAB system in *Yersinia* sp. is a potassium/drug antiporter system that increases resistance to polymyxin B (Bengoechea and Skurnik 2000). In spite of these examples, to date, no efflux systems involved in resistance to cationic antimicrobial peptides have been observed in *P. aeruginosa*. The RND family of

multidrug efflux systems has been well described in a wide number of bacterial species and *P. aeruginosa* has several RND efflux systems that are involved in resistance to aminoglycosides, β-lactams, quinolones, chloramphenicol, tetracycline, rifampicin, and a wide variety of other unrelated compounds (Poole 2004). In spite of the downregulation of the *mexPQ-opmE* efflux system observed in the *pxrR::*IS*lacZ* strain, MIC assays did not demonstrate any difference between mutants in *mexPQ-opmE* and an isogenic wild-type strain (Table 4.3).

In addition to the *mexPQ-opmE* RND efflux system, the PA4063-PA4066 operon was also observed to be downregulated in a *pxrR::*IS*lacZ* strain. This operon contains homologs of proteins involved in the efflux of an endogenous lytic cationic peptide in *Streptococcus pneumoniae* (Novak, Charpentier et al. 2000). Again however, despite the observed downregulation of PA4063-PA4066 in the *pxrR::*IS*lacZ* mutant, no differences in MIC were observed between a PA4065::*luxCDABE* mutant and an isogenic parent strain.

In addition to the effects on polymyxin B resistance, mutants in *pxrR* were completely defective for functions requiring type IV pili. Type IV pili are polarly localized appendages that are involved in adherence to host epithelial cells (Alm, Hallinan et al. 1996), biofilm formation (O'Toole and Kolter 1998; Klausen, Heydorn et al. 2003), twitching motility (Mattick 2002) and sensitivity to phage PO4 (Hobbs, Collie et al. 1993). Pili contribute to motility via extension and retraction over smooth surfaces. The pilus is composed primarily of the PilA protein, forming a helical fibre extending from a basal structure that is not yet well characterized. Pili extension and retraction occurs via regulation of pilin assembly and disassembly, likely catalyzed by the PilB, PilT, and PilU proteins (Turner, Lara et al. 1993; Merz, So et al. 2000; Graupner, Weger et al. 2001). A number of other proteins are also involved in pilus biogenesis, including PilQ, which forms the outer membrane pore (secretin) through which the assembled pilus in inserted (Bitter, Koster et al. 1998; Collins, Frye et al. 2005). A number of other

accessory proteins are involved in pilin assembly, including PilE, PilV, PilW, PilX, FimU and FimT (Russell and Darzins 1994; Alm, Hallinan et al. 1996; Alm and Mattick 1996).

Microarray analysis demonstrated that the *pxrR*::IS*lacZ* strain showed a reduction of expression of the *fimUpilVWXY1Y2E* operon, and this was confirmed by measuring RNA for the *pilV* gene by q-PCR. Although little is known about this operon, the PilW and PilX proteins show similarities to proteins of the general secretion pathway from a number of Gram-negative bacteria (Alm, Hallinan et al. 1996). Briefly, they possess leader sequences similar to the prepilin-like class of proteins and are membrane localized. The PilY1 and PilY2 proteins share similarity to the gonococcal PilC protein, which forms the tip of the pilus (Alm, Hallinan et al. 1996). These similarities suggest that the *fimUpilVWXY1Y2E* operon is involved in the secretion and assembly of pilin monomers. Additionally, transposon mutant screens have demonstrated that mutants in each of the genes of this operon lead to a loss of twitching motility (Jacobs, Alwood et al. 2003).

This work expands the number of known two-component regulators that contribute to polymyxin B and cationic antimicrobial peptide resistance in *P. aeruginosa* to three. Unlike the PhoP-PhoQ and PmrA-PmrB systems however, the PxrRS system appears to regulate resistance via a different mechanism than the alteration of expression of the *pmrHFIJKLM-ugd* and PA4773-PA4775 operons. These latter operons are normally regulated in a *pxrR*::IS*lacZ* mutant. Transcriptional profiling suggested possible reasons for this increased sensitivity including downregulation of several intrinsic resistance mechanisms such as active efflux systems and heat-shock proteins.

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Chapter 5 – Concluding remarks

Introduction

The rise of antibiotic resistance will be one of the greatest challenges facing the medical profession for the foreseeable future. The introduction of antibiotics into general clinical practice has resulted in the greatest increase to average life expectancy since the introduction of improved public sanitation in the late 19th century . However, since the very first introduction of antimicrobial chemotherapy in the 1940s, bacteria that are resistant to those antimicrobials have appeared, usually within only a few years of the introduction of said drug into the clinic. This trend can result in rates of resistance that approach 50-90% of isolates, rendering the drug essentially useless for therapy. Indeed, the increased rates of appearance of methicillin resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* are particularly troubling public-health problems, as these infections are difficult to eradicate (Menichetti 2005; Zetola, Francis et al. 2005).

The problem of increasing antibiotic resistance is compounded by the lack of research and development of new antimicrobial therapies (Shah 2005). Only two novel classes of antibiotics have been introduced since the 1960s; these being the oxazolinidones, of which linezolid represents the fist approved example and the lipopeptide daptomycin (Strahilevitz and Rubinstein 2002; Carpenter and Chambers 2004). These antibiotics have broad spectrum activity against Gram-positive bacteria, and their use is generally limited to treatment of multi-drug resistant infections, serving as a drug of last resort for these infections (Kauffman 2003; Cunha 2005). In spite of linezolid's novel mechanism of action, resistant strains have already emerged, indicating the continuing need for novel antibiotics (Mutnick, Enne et al. 2003).

Cationic antimicrobial peptides represent a potentially new class of antibiotics. These peptides seem to have several natural advantages as antibiotics, they kill rapidly and do not easily engender resistance and they have broad-spectrum activity against Gram-negative and

Gram-positive bacteria, fungi, and enveloped viruses. Although there has been a great deal of research on cationic antimicrobial peptides in the last 15 years, to date the promise of antimicrobial peptides as therapeutic agents has been hampered by difficulties in bringing them through clinical trials (Zhang and Falla 2004; McPhee and Hancock 2005).

One of the leading causes of nosocomial and other opportunistic infections is the Gram-negative bacterium *Pseudomonas aeruginosa* (Fagon, Chastre et al. 1993; Richards, Edwards et al. 1999). This bacterium is noted for its environmental ubiquity, its intrinsic resistance to many antibiotics, and its high metabolic diversity. This diversity is a consequence of a large genome size (6.3 Mb) with many metabolic and transport gene, and the large proportion of this genome that is devoted to gene regulation (Stover, Pham et al. 2000). Of the 5570 ORFs in the annotated *P. aeruginosa* genome, 592 are involved in transcriptional regulation, including 123 two-component response regulatory proteins (Stover, Pham et al. 2000). Of these two-component systems, the majority remain uncharacterized.

It has long been known that growth in medium depleted of divalent cations leads to increased resistance to polymyxin B (Brown and Melling 1969; Brown and Melling 1969). This has been observed in a large number of bacterial species, including *Salmonella*, *P. aeruginosa*, *E. coli*, *Yersinia*, *Photorhabdus*, *Meningococcus*, and *Erwinia* (Guo, Lim et al. 1997; Llama-Palacios, Lopez-Solanilla et al. 2003; Derzelle, Turlin et al. 2004; Newcombe, Jeynes et al. 2005). Although the best characterized two component regulatory systems that influence cationic peptide/polymyxin resistance, namely the PhoP-PhoQ and PmrA-PmrB systems are those studied in *Salmonella*, it is a mistake to assume that they are identical in every species examined. In fact, while there are similarities, the way in which signals from these systems are integrated differs in every species examined (McPhee, Lewenza et al. 2003; Winfield, Latifi et al. 2005). In fact, even between *S.* Typhimurium and *E. coli* species examined, there is

significant heterogeneity in the way in which these systems work (Winfield and Groisman 2004).

PhoP-PhoQ of *P. aeruginosa*

In *P. aeruginosa*, mutants generated by chemical mutagenesis were isolated that showed constitutive resistance to EDTA, polymyxin B and aminoglycosides (Nicas and Hancock 1980). These mutants also showed constitutive expression of an outer membrane protein, OprH and lower concentrations of divalent cations in the cell envelope than wild-type strains. These results suggested that OprH was involved in polymyxin B resistance. However, in 1999, the PhoP-PhoQ two-component regulatory system of *P. aeruginosa* was identified, located just downstream of the *oprH* gene, forming an operon with it (Macfarlane, Kwasnicka et al. 1999; Macfarlane, Kwasnicka et al. 2000). Interestingly, interposon mutants in *phoP* showed a complete loss of OprH expression under both Mg^{2+}-replete and Mg^{2+}-limiting conditions, but resistance to cationic antimicrobial peptides was unaffected in limiting Mg^{2+} (Macfarlane, Kwasnicka et al. 1999; Macfarlane, Kwasnicka et al. 2000). Mutants of PhoQ on the other hand demonstrated constitutive expression of OprH and constitutive resistance to polymyxin B and cationic antimicrobial peptides (Macfarlane, Kwasnicka et al. 1999; Macfarlane, Kwasnicka et al. 2000). These results strongly suggested that the PhoP-PhoQ system was solely responsible for regulating OprH expression, but that another unidentified regulatory system contributed to the Mg^{2+}-induced polymyxin B and cationic peptide resistance in *P. aeruginosa.* It was hypothesized that this unidentified regulatory system would be regulated by Mg^{2+}, would interact somehow with the PhoP-PhoQ regulatory system, and would be involved in resistance to polymyxin B.

PmrA-PmrB of *P. aeruginosa.*

Chapter 2 of this work describes the identification of the PmrA-PmrB system of *P. aeruginosa.* This regulatory system has all of the characteristics that were predicted in that it is

Mg^{2+} regulated, is upregulated in PhoQ mutants, and is involved in regulating polymyxin B resistance. The PmrA-PmrB system has been well-described in *S.* Typhimurium. In *Salmonella*, the PmrA-PmrB system lies in a regulatory hierarchy beneath the PhoP-PhoQ system (Fig 1.3). In *S.* Typhimurium, in the absence of the PhoP-PhoQ system, there is no divalent cation or cationic antimicrobial peptide induced response (Bader, Navarre et al. 2003; Bader, Sanowar et al. 2005). This occurs because a small PhoP-regulated protein, PmrD, is strictly required for signal transduction from the PhoP-PhoQ system of *Salmonella*, to the PmrA-PmrB system. In turn, the PmrA-PmrB system is directly responsible for the regulation of the *pmrHFIJKLM* operon, which catalyzes the addition of aminoarabinose to Lipid A, leading to polymyxin B resistance (Fig. 5.2).

It is clear from results in *P. aeruginosa*, that while there are many similarities to the S. Typhimurium system, there are also many distinct differences. One of the most prominent differences is that the PhoP protein is dispensible for Mg^{2+} induced polymyxin B resistance. This occurs, in part, because the PmrA-PmrB regulatory system also responds to limiting divalent cation concentrations, independently of the PhoP-PhoQ system. Signals from both systems lead to increased transcription of the *P. aeruginosa pmrHFIJKLM-ugd* operon. While both systems are required for maximal transcription of the *pmrHFIJKLM-ugd* operon, either one can be deleted and some Mg^{2+} induced transcription still occurs via the remaining system, leading to increased resistance.

This work also implicated the three genes preceeding the *pmrA-pmrB* genes in resistance to cationic antimicrobial peptides. Knockouts of PA4773, PA4774, and PA4775 all showed altered sensitivity to cationic antimicrobial peptides and polymyxin B. Furthermore, the patterns of resistance are dramatically different from those of *pmrHFIJKLM-ugd* knockout mutants. Mutants in *pmrHFIJKLM-ugd* were exquisitely supersusceptible to polymyxin B and colistin exposure under Mg^{2+}-limitation, while the effect on CP10A and other cationic

antimicrobial peptides was less striking. Conversely, mutants in PA4773-4775 showed a stronger supersusceptibility towards certain cationic antimicrobial peptides (CP10A, CP11CN, and indolicidin) than they did toward polymyxin B or colistin. This altered sensitivity pattern suggested that the phenotypes observed in the PA4773-PA4775 mutants were not due to polar effects on transcription of *pmrA-pmrB*, but rather were a consequence of the biological activity of these novel proteins. These genes are unique to *P. aeruginosa*, although PA4773 and PA4774 have high similarity to enzymes involved in polyamine biosynthesis in many bacterial and eukaryotic species. PA4773 is similar to S-adenosylmethionine decarboxylases (SpeD) while PA4774 is similar to polyamine aminopropyltransferases (SpeD). *P. aeruginosa* also contains more related homologs of the SpeD and SpeE proteins, perhaps indicating that the PA4773 and PA4774 proteins might be involved in novel polyamine-associated phenotypes.

Polyamines are organic polycations that have been found in virtually every species examined. The most common polyamines in biological systems are putrescine, cadaverine, spermidine, and spermine (Fig 5.1). They are often found associated inside cells with anionic cellular components like DNA, RNA, and ribosomes (Tabor and Tabor 1976). In addition to these intracellular roles for polyamines, there is some evidence that polyamines are involved in membrane stability in erythrocytes (Ballas, Mohandas et al. 1983), with significant increases in resistance to shear-stress when the erythrocytes are also exposed to spermine. Studies with *E. coli* and *Salmonella* have also demonstrated that polyamines are a constituent of the bacterial outer membrane that can be preferentially displaced by washing cells with 1 M NaCl (Koski and Vaara 1991).

Of specific interest to polyamine involvement in polymyxin B or cationic antimicrobial peptide resistance are studies that examined the interactions of purified LPS with a variety of cationic compounds (Peterson, Hancock et al. 1985). This study measured the displacement of a cationic spin-labeled probe, 4-dodecyl dimethyl ammonium-1-oxyl- 2,2,6,6-tetramethyl

piperidine bromide (CAT_{12}), from purified LPS. These studies permitted the simultaneous examination of the head group motility of LPS and the actual ability of the probe to be displaced from the LPS. Head group motility is a measure of the fluidity of the membrane, while measurement of the dissociation constant permit a description of the ability of cations to compete for a specific binding site. Addition of polymyxin B, polymyxin B nonapeptide, or EM49 (a cationic antimicrobial peptide) caused the greatest displacement of CAT_{12} from the LPS samples as compared to aminoglycosides or polyamines (Peterson, Hancock et al. 1985). When the splitting parameter (a function of spin-label mobility) was measured following the addition of cationic compounds however, the change in motility did not vary as a function of charge, but rather varied depending upon the class of compound examined. In this way, addition of aminoglycosides or cationic antimicrobial peptides caused a pronounced rigidification of the LPS samples while addition of Mg^{2+}, spermine, spermidine, cadaverine, or putrescine increased the fluidity of the LPS samples. These results demonstrate that although cationic antimicrobial peptides and polyamines both bind to LPS with high affinity, polyamines are able to bind without rigidifying the LPS and presumably permit the cell to maintain normal membrane-associated processes.

These results are intriguing and they suggest that it would be possible for *P. aeruginosa* to regulate susceptibility to cationic antimicrobial peptides, via the regulation of endogenous polyamine production. The fact that the PA4773-PA4775 operon is regulated by the PmrA-PmrB system, which is also involved in cationic antimicrobial peptide resistance via the *pmrHFIJKLM-ugd* operon supports this hypothesis. It must be stated however, that a great deal more research is required to test this hypothesis extensively.

PhoP-PhoQ and PmrA-PmrB in virulence.

In *P. aeruginosa*, mutants in *phoP* did not have any real phenotype, showing similar virulence as an isogenic wild-type strain (Brinkman, Macfarlane et al. 2001). In contrast to this,

mutants of PhoQ, which show constitutive resistance to cationic antimicrobial peptides, are ~100-fold less virulent than wild-type *P. aeruginosa* (Brinkman, Macfarlane et al. 2001). The reasons for this are not yet clear, although it is interesting to note that PhoQ mutants appear to have altered regulation of both PhoP- and PmrA-dependent phenotypes (McPhee, Lewenza et al. 2003). Thus, mutants in *phoQ* exhibited derepression of both the *pmrHFIJKLM-ugd* and modestly PA4773-PA4775-*pmrAB*. These results suggested that the PhoQ protein was capable of dephosphorylating PhoP and PmrA, modulating the activity of these proteins. Thus, in the case of a *phoQ* mutant, the loss of this phosphatase activity would lead to constitutive activation of PmrA- and PhoP-dependent promoters.

In addition to the role these systems play in polymyxin B and cationic antimicrobial peptide resistance, the work described in Chapter 3 expanded on the role of these systems in regulating phenotypes that may be associated with virulence. Using a combination of bioinformatic and genetic analysis, the regulons of the PhoP-PhoQ and PmrA-PmrB systems were expanded. This analysis demonstrated that the PhoP system regulates two small ORFs, unique to *P. aeruginosa*, PA0921 and PA1343, as well as the *pmrHFIJKLM-ugd* operon and the *oprH-phoPQ* operon. One of these proteins, PA0921 modulated swimming motility under Mg^{2+}-replete conditions. Since swimming motility has been associated with early stages of virulence in *P. aeruginosa* (Montie, Doyle-Huntzinger et al. 1982), this may play a role in the *P. aeruginosa* infectious process.

In a similar approach, the PmrA regulon was also expanded to include the PA4782, the *feoAB* operon, and the PA1559-PA1560 operon, in addition to the *pmrHFIJKLM-ugd* and PA4773-4775-*pmrAB* operons. Although little is known about the PA4782 or PA1559-PA1560 genes, the *feoAB* genes are involved in the ability of *P. aeruginosa* to grow using Fe^{2+} as an iron source (Chapter 3). This would tend to be an advantage in environments where Fe^{2+} is stabilized, such as environments that are anoxic or acidic. Recently, evidence has emerged

suggesting that within the CF lung, *P. aeruginosa* grows as an anaerobic biofilm within the thickened dehydrated airway surface liquid (Yoon, Hennigan et al. 2002). Direct measurement of the oxygen concentrations within this thickened layer shows that even though the pO_2 within the lung is normal, the interior of the mucous layer can be hypoxic (Worlitzsch, Tarran et al. 2002).

Cationic antimicrobial peptide induction of PmrA-regulated promoters.

Transcriptional responses to cationic antimicrobial peptides have been described in a number of species, including *E. coli* (Hong, Shchepetov et al. 2003), *Yersinia* (Bengoechea and Skurnik 2000), *Salmonella* (Bader, Navarre et al. 2003), *Bacillus subtilis* (Pietiainen, Gardemeister et al. 2005), and *P. aeruginosa* (McPhee, Lewenza et al. 2003). Although each species examined to date shows significant differences from one another, a general observation is that they all include adaptations that serve to ameliorate cell wall induced damage caused by the cationic antimicrobial peptide exposure.

One of the most interesting findings to have come out of this research was the observation that the presence of sub-inhibitory concentrations of cationic antimicrobial peptides causes transcriptional regulation of the PA4773 and *pmrH* operons that are responsible for increased resistance to cationic antimicrobial peptides when grown under Mg^{2+} limitation. This response occurred independently of the PmrA-PmrB and PhoP-PhoQ systems, as strains that have these genes deleted show similar transcriptional responses to the presence of cationic antimicrobial peptides as the wild-type strain.

This observation suggested a mechanism by which adaptive resistance to polymyxin B and other cationic antimicrobial peptides might take place. The initial sub-inhibitory exposure to polymyxin B or cationic antimicrobial peptides might cause increased transcription of the PA4773-PA4775 and *pmrHFIJKLM-ugd* operons, thereby increasing the resistance to a later, even higher concentration of the compound (Fig. 2.7). In the same issue of Molecular

Microbiology that this work was published, a study in *Salmonella* documented a similar phenotype (Bader, Navarre et al. 2003). In contrast to the situation in *Pseudomonas*, in *Salmonella* the response required the PhoP-PhoQ system, implying that this system acted directly in the detection of cationic antimicrobial peptides. This observation was later confirmed, and a model for cationic antimicrobial peptide detection in *Salmonella* was described, in which the binding of cationic antimicrobial peptides to the cytoplasmic membrane led to displacement of divalent cations from the PhoQ sensor kinase (Bader, Sanowar et al. 2005). A model for the cationic peptide response of *S.* Typhimurium is shown in Figure 5.1.

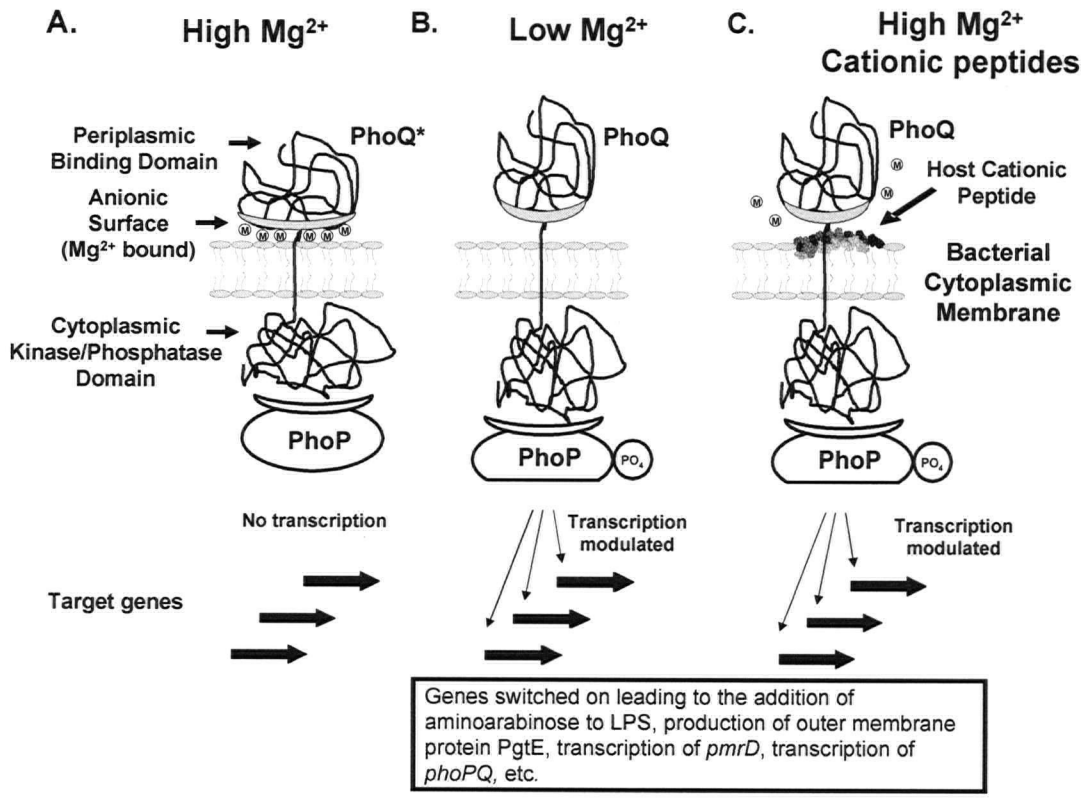

Figure 5.1: Model of PhoQ activation by low Mg^{2+} and peptides in *S.* Typhimurium A. In the presence of high Mg^{2+} concentrations in the medium in the absence of peptides, Mg^{2+} ions bridge the negatively charged cytoplasmic membrane and a prominent anionic surface of PhoQ, stimulating phosphatase activity and leading to dephosphorylation (inactivation) of the transcriptional regulator PhoP. At low Mg^{2+} concentrations in the medium (**B.**) or in the presence of cationic host defence peptides (**C.**), Mg^{2+} ions can no longer bridge between the negatively charged cytoplasmic membrane and a prominent anionic surface of PhoQ, causing a conformational shift that stimulates kinase activity and leads to phosphorylation of the transcriptional regulator PhoP, stimulating transcription of the PhoPQ regulon

This conformational shift is similar to that observed when PhoQ is subjected to divalent cation limitation (Bader, Sanowar et al. 2005). This leads to phosphorylation of PhoP and activation of PhoP regulon.

In *P. aeruginosa*, the mechanism of sensing cationic antimicrobial peptides is likely different. It does not involve the PmrB or PhoQ sensor kinases, but rather yet another, as- yet unidentified regulatory protein. However, data presented in Chapter 3, shows that all members of the PmrA regulon that have been identified, are also upregulated in response to CP11CN, including those genes that do not play a role in cationic antimicrobial peptide or polymyxin B resistance. Since there are no other obvious similarities in the promoters of these genes, it is likely that the unknown regulator involved in the response to CP11CN recognizes the same binding site as the PmrA protein. A model for PhoPQ, PmrAB, and cationic antimicrobial peptide sensing in *P. aeruginosa* is presented in Figure 5.2.

Intrinsic resistance of *P. aeruginosa* to cationic antimicrobial peptides

In addition to the inducible types of cationic antimicrobial peptide resistance, a number of intrinsic resistance mechanisms have been described in several species. These mechanisms tend to be constitutively expressed, but otherwise do not differ from the adaptive resistance mechanisms that have been described in other species.

Proteases within bacterial cells are crucial to a number of processes, including intrinsic resistance to cationic antimicrobial peptides. This mechanism of intrinsic resistance is active in both Gram-negative and Gram-positive bacteria. *S. aureus* mutants in the *degP* protease gene, show increased susceptibility to lactoferricin B (Haukland, Ulvatne et al. 2001). Similarly, the constitutively active outer membrane protease OmpT has been implicated in resistance to protamine in *E. coli*, under both high/low Mg^{2+} conditions (Stumpe, Schmid et al. 1998). The importance of this has been further highlighted through the development of a protease stabilized variant of the cationic antimicrobial peptide CP11CN, itself a variant of indolicidin. Cyclo-

CP11 was engineered by introducing a disulphide bridge between the N- and C-terminus, thereby restricting the mobility of the protease-sensitive site (Rozek, Powers et al. 2003). This variant showed similar activity to CP11CN but this activity was retained following incubation with trypsin, whereas the activity of the parent peptide was completely lost.

Efflux systems, although described as inducible systems in *Yersinia* (Bengoechea and Skurnik 2000), form part of the intrinsic cationic peptide resistance system of several bacterial species. In *Neisseria meningitidis*, the MtrCDE efflux system is responsible for ~5 fold increased resistance to protegrin, a cationic peptide from porcine neutrophils (Shafer, Qu et al. 1998). Similarly, the *sapABCDF* system of *S.* Typhimurium has also been shown to be involved in resistance to cationic peptides (Parra-Lopez, Baer et al. 1993).

The work described in Chapter 4 of this thesis points to a regulatory system, PxrRS, that was involved in intrinsic resistance to cationic antimicrobial peptides. Mutants of *pxrR* and *pxrS* had a 2-4-fold increased susceptibility to polymyxin B as assessed by MIC. Similarly, mutant strains were also more sensitive to exposure to the cationic antimicrobial peptide CP11CN as assessed by killing curves. These strains showed normal PmrA and PhoP dependent Mg^{2+} regulation as well as a normal response to subinhibitory concentrations of cationic antimicrobial peptides. This strongly supports the hypothesis that the PxrRS system is involved in a process controlling susceptibility to cationic antimicrobial peptides and polymyxin B. Microarray analysis comparing the *pxrR*::IS*lacZ* strain to an isogenic parent further suggested that the resistance phenotype may have occured in part through the activation of certain efflux systems. *P. aeruginosa* is noted for the large number of active efflux systems, but none have, to date, been implicated in cationic antimicrobial peptide resistance.

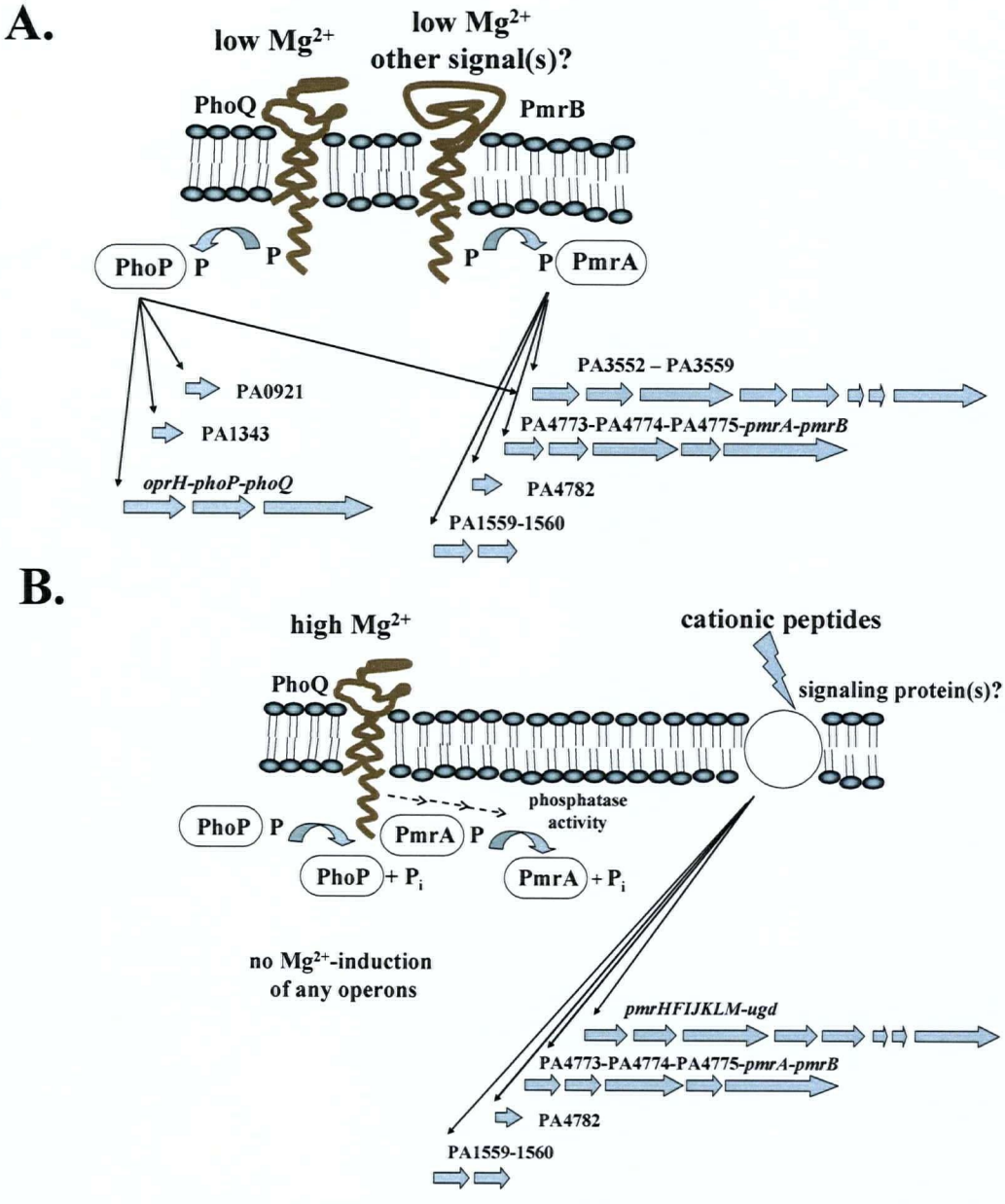

Figure 5.2. The PhoP-PhoQ and PmrA-PmrB systems of *P. aeruginosa*. **A.** Under Mg^{2+} limitation, phospho-PhoP causes increased transcription of the PA0921, PA1343, *oprH-phoPQ*, and the *pmrHFIJKLM-ugd* operons. Similarly, Phospho-PmrA also causes increased transcription of the *pmrHFIJKLM-ugd* operon independently of PhoP, and it also increases transcription of PA4773-PA4774-PA4775-*pmrAB*, PA4782, and PA1559-PA1560. **B.** Under Mg^{2+}-replete conditions the PhoP and PmrA protein are dephosphorylated (by PhoQ and/or other unidentified phosphatases) reducing transcription from PmrA- and PhoP-dependant promoters. In the presence of cationic antimicrobial peptides however, an unidentified signaling protein causes increased transcription of PA4773-PA4774-PA4775-*pmrAB*, PA4782, PA1559-PA1560, and *pmrHFIJKLM-ugd*, largely independently of the PmrAB or PhoPQ system.

Future research directions

The work described in this thesis has opened up a novel field of *Pseudomonas aeruginosa* research. It describes both the first identification of the Mg^{2+}-regulated PmrA-PmrB two-component regulatory system of *P. aeruginosa*, as well as the PxrR-PxrS two-component regulatory system, contributing to intrinsic cationic peptide resistance. It also describes the first demonstration of a biological basis for the development of adaptive resistance in *P. aeruginosa*. With these discoveries in mind, several novel research avenues have been opened.

The first and most pressing question that needs to be addressed is to identify the regulatory protein that is responsible for cationic peptide sensing. While this has been accomplished in *S.* Typhimurium (Bader, Sanowar et al. 2005), it is clear that *P. aeruginosa* is different in this respect. The protein may be induced upon exposure to sub-inhibitory concentrations of cationic antimicrobial peptide, and therefore, this question may lend itself to microarray analysis. Indeed, microarray analysis in the presence of sub-inhibitory cationic antimicrobial peptides has revealed certain candidate regulators. In the event that the gene is not transcriptionally induced by cationic peptides, but rather regulation occurs post-transcriptionally, simultaneous screening of transposon mutant libraries for mutants that fail to induce known peptide-regulated *luxCDABE* fusions, is also being carried out. This work is currently in progress in the Hancock lab, where it will form the core study of another Ph.D student.

A further point of interest that has come out of these studies is the intriguing link between a single histidine sensor kinase (PhoQ) and two separate response regulators (PhoP and PmrA). This suggests a novel signalling pathway consisting of cross-talk between PhoQ and PmrA/PhoP. Indeed, preliminary microarray studies have suggested that the PhoQ protein regulates a much larger repertoire of bacterial genes than would be predicted based solely upon the PmrA or PhoP regulons alone. This hints at the possibility that PhoQ is a highly

promiscuous regulatory protein that affects the relative phosphorylation states of a number of response regulators. A more precise definition of this interaction is required to fully appreciate why this system differs so much from other simple two-component regulatory systems. This research would ideally be conducted using purified PhoQ, PhoP, PmrB, and PmrA proteins to study the rates of phosphate flux through each system under different conditions.

Further research will be needed to out to understand the contribution of the PxrRS system to intrinsic cationic antimicrobial resistance. Microarray studies point to both active efflux and modulation of heat-shock protein levels as potential contributors to this phenotype, but functional studies of the target genes did not show a strong phenotype for any of the efflux systems or individual heat-shock proteins examined. This may indicate that each target gene contributes only a small amount to the observed phenotype. In order to study this further, a series of multiple mutants may need to be constructed in order to explore this phenomenon more completely.

It is clear from the results discussed in this thesis that the response of *P. aeruginosa* to both limiting Mg^{2+} and to cationic antimicrobial peptides is a complex developmental process. While this work has delineated the contributions of three two-component regulatory systems to cationic antimicrobial peptide resistance, it is likely that the actual process is even more complex and that we have only scratched the surface.

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