REGULATION OF VIRULENCE AND ANTIMICROBIAL PEPTIDE RESISTANCE IN *PSEUDOMONAS AERUGINOSA*

by

WILLIAM JAMES GOODERHAM

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ABSTRACT

Pseudomonas aeruginosa is a ubiquitous environmental Gram-negative bacterium that is also a major opportunistic human pathogen in nosocomial infections and cystic fibrosis chronic lung infections. These *P. aeruginosa* infections can be extremely difficult to treat due to the high intrinsic antibiotic resistance and broad repertoire of virulence factors, both of which are highly regulated.

It was demonstrated here that the *psrA* gene, encoding a transcriptional regulator, was up-regulated in response to sub-inhibitory concentrations of antimicrobial peptides. Compared to wild-type and the complemented mutant, a *P. aeruginosa* PAO1 *psrA*::Tn*5* mutant displayed intrinsic super-susceptibility to polymyxin B, a last resort antimicrobial used against multi-drug resistant infections, and indolicidin, a bovine neutrophil antimicrobial peptide; this super-susceptibility phenotype correlated with increased outer membrane permeability. The *psrA* mutant was also defective in simple biofilm formation, rapid attachment, and normal swarming motility, phenotypes that could be complemented by the cloned *psrA* gene. The role of PsrA in global gene regulation was studied by comparing the *psrA* mutant to wild-type by microarray analysis, demonstrating that 178 genes were up- or down-regulated by greater than 2-fold ($P \leq 0.05$). Dysregulated genes included those encoding known PsrA targets, the type III secretion apparatus and effectors, adhesion and motility genes and a variety of metabolic, energy metabolism and outer membrane permeability genes. This indicates that PsrA is a central regulator of antimicrobial peptide resistance and virulence.

P. aeruginosa containing a mutation in the PhoQ sensor kinase-encoding gene was highly attenuated for persistence in a rat chronic lung infection model. In addition, the polymyxin B hyper-resistant *phoQ* mutant displayed reduced type IV pili-dependent twitching motility and was less cytotoxic towards human bronchial epithelial cells, indicating that the virulence defect observed could be due at least in part to these phenotypes. Using microarrays it was further demonstrated that PhoQ regulates a large number of genes that are PhoP-independent and that the *phoQ* mutation leads to up-regulation of PhoP- and PmrA-regulated genes as well as other genes consistent with its virulence phenotypes.

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LIST OF ABBREVIATIONS

°C – degrees Celsius
ATP – adenosine triphosphate
CCCP – carbonyl cyanide m-chlorophenol hydrozone
CFU – colony forming unit
CI – competitive index
DNA – deoxyribonucleic acid
EDTA – ethylene diamine tetra acetic acid
FBS – fetal bovine serum
HEPES – 2-(4-(2-hydroxyethyl)-1-piperazinyl) ethanesulfonic acid
LB – Luria-Bertani
LDH – lactate dehydrogenase
LPS – lipopolysaccharide
MEM – minimal essential medium
MIC – minimal inhibitory concentration
NPN – 1-*N*-phenylnapthylamine
ORF – open reading frame
PA – *Pseudomonas aeruginosa* locus of identity
PAGE – polyacrylamide gel electrophoresis
PBS – phosphate buffered saline
PCR – polymerase chain reaction
RNA – ribonucleic acid
RPM – revolutions per minute
SDS – sodium dodecylsulphate
TSB – tryptic soy broth
UW – University of Washington
WT – wild-type

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CHAPTER 1 – Introduction

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium capable of inhabiting diverse soil and water habitats. Not solely a harmless environmental bacterium, *P. aeruginosa* is also an opportunist capable of infecting plant, insect, nematode, and animal tissues (Rahme et al. 2000). This remarkable versatility and adaptability is governed by a relatively large and complex genome permitting nutritional and metabolic adaptation driven by sophisticated and coordinated regulation of gene expression by a large proportion of regulatory genes (Stover et al. 2000).

P. aeruginosa is a major opportunistic pathogen of immunocompromised patients. It is the third most common hospital-acquired pathogen in North America, the most common cause of ventilator-associated pneumonia, and a leading cause of infection and sepsis in burn patients (Fridkin et al. 1999). Multi-determinant virulence and high intrinsic antibiotic resistance are two noteworthy hallmarks of *P. aeruginosa* infections (Hancock and Speert 2000). *P. aeruginosa* is also well known as a dominant cause of chronic lung infections and resulting morbidity and mortality in cystic fibrosis, the most common genetic disease in North America (Gibson et al. 2000). Adaptive and mutational resistance is increasingly impacting on treatment success and new antimicrobial therapeutic options are needed for resistant strains, some of which have developed resistance to virtually every antibiotic and have thus become hospital "Superbugs" (Falagas and Bliziotis 2007; Mesaros et al. 2007).

***P. aeruginosa* virulence**

P. aeruginosa is capable of causing both chronic and acute infections and appropriately regulates the virulence determinants required for each type of infection

(Furukawa et al. 2006). Often refractory to antimicrobial treatment, chronic infections can be microcolony or biofilm-like in nature, such as those present in cystic fibrosis lung infections and on urinary catheters and other indwelling medical devices (Parsek and Singh 2003). Conversely, acute *P. aeruginosa* infections are typified by bacterial penetration of the host epithelium and systemic spread, such as in severe burn wound infections.

An arsenal of virulence factors impacts on the pathogenicity of *P. aeruginosa* (Lyczak et al. 2000). Included among these are cell-associated factors such as lipopolysaccharide (LPS), flagella and type IV pili, exopolysaccharides such as alginate, and secreted factors including toxins, elastase and other proteases, phospholipases and small molecules such as phenazines, rhamnolipids and cyanide. The single polar flagellum is an important and highly regulated virulence factor for motility and attachment to surfaces. *P. aeruginosa* isolates from long-term chronic cystic fibrosis infections often lack flagella and it is thought that these non-motile cells are at some stage of a biofilm-like adaptation (Singh et al. 2000). In addition, quorum-sensing, secretion systems and other regulatory systems are important for virulence (Kirisits and Parsek 2006; Venturi 2006).

Biofilms are bacterial communities surrounded and infiltrated by an extracellular matrix containing exopolysaccharide, DNA and proteins. *P. aeruginosa* biofilms are readily found both in natural environments (e.g. attached to rocks in streams and to particulate matter in soil) and health-care settings (e.g. attached to indwelling medical devices). Regardless, whether growing as true biofilms or as microcolonies, *P. aeruginosa* infections in cystic fibrosis are highly resistant to antibiotics, making eradication impossible after establishment of mature infections (Gibson et al. 2003). *P. aeruginosa* biofilm formation *in vitro* is thought to follow three distinct stages: surface attachment, microcolony formation,

and differentiation into mature biofilm communities encased in an extracellular matrix (Kirisits and Parsek 2006). Type IV pili-dependent twitching and flagellar-based swimming motility are important for initial surface attachment and niche colonization (O'Toole and Kolter 1998), and many genes associated with these motility appendages are necessary for proper biofilm formation (Kirisits and Parsek 2006). In addition, there is a strong but not obligate correspondence in gene requirements between biofilm formation and swarming motility. In *P. aeruginosa*, swarming motility (distinct from swimming and twitching motility), is dependent on both flagella and type IV pili appendages and is thought to represent a complex social adaptation to viscous environments (Kohler et al. 2000; Overhage et al. 2008).

Type III secretion system-mediated cytotoxicity is a key virulence property of *P. aeruginosa* (Yahr and Wolfgang 2006). Gene expression of the five type III secretion system operons is modulated by a complex set of regulatory mechanisms and the expression of this secretion machinery is coupled to the secretory activity of three effectors, ExoS, ExoT and ExoY, which possess anti-phagocytic and cytotoxic properties (Yahr and Wolfgang 2006). The two *P. aeruginosa* type II secretion systems (*xcp* and *hxc*) mediate the secretion of a broad range of toxins and other virulence related enzymes including exotoxin A, lipase, and phospholipase C.

P. aeruginosa LPS is a prominent virulence factor that mediates virulence, resistance to antibiotics, and induction of host inflammatory and innate immune responses (Pier 2007). *P. aeruginosa* contains a typical Gram-negative LPS structure composed of lipid A, core, and O-antigen. *P. aeruginosa* produces two types of LPS, B-band (O-antigen) and A-band (polymer of D-rhamnose), each attached to separate core/lipid A molecules (Pier 2007). The

lipid A (contains an *N*- and *O*-acylated diglucosamine bisphosphate backbone) can be modified with additional chemical groups and fatty acids. Environmental growth conditions (e.g. Mg^{2+} levels) are known to affect the degree of acylation and presence of lipid A modifications. For example, LPS of isolates from cystic fibrosis patients predominantly contains lipid A with aminoarabinose modifications (Ernst et al. 1999) and lacks O-antigen moieties (Pier 2007). Lipid A plays an important role in the pathogenesis of many Gram-negative bacterial infections by acting as a signature of such infections, activating the innate immune system, triggering the synthesis of host defense peptides, cytokines, clotting factors, and other immunostimulatory molecules (Pier 2007).

***P. aeruginosa* regulators**

Bacterial survival in dynamic environmental conditions requires the capability to sense and quickly respond to many different stimuli. Often, these physiological responses are at the level of gene expression and are accomplished by regulatory proteins. One class of regulators, the bacterial two-component regulatory systems, are designed to sense diverse stimuli and enact a rapid and precise adaptive physiological response that can involve altered transcription of a substantial number of genes. The *P. aeruginosa* genome encodes one of the largest complements of regulatory proteins at just under 10% of all genes and a major subset are genes encoding 64 sensor kinases and 72 response regulators (Stover et al. 2000; Rodrigue et al. 2000).

Two-component regulatory systems classically comprise an inner membrane-spanning sensor histidine kinase and a cytoplasmic response regulator (Stock et al. 2000). The functional mode of both sensor kinase and response regulator is determined by reversible phosphotransfer reactions and ensuing protein conformational changes (Stock et

al. 2000). In the archetypical system, the sensor kinase contains an N-terminal periplasmic input domain that detects a specific stimulus and a C-terminal cytoplasmic transmitter domain that binds ATP and has histidine kinase activity. The cognate response regulator contains a conserved receiver domain and a variable output domain that often binds DNA. Classically, a membrane-bound dimeric sensor kinase detects an environmental stimulus/ligand in the periplasm via its input domain, and then undergoes trans-autophosphorylation at a conserved histidine residue in its transmitter domain. This phosphoryl group is then transferred to (and catalyzed by) the conserved aspartate-containing receiver domain of the response regulator. Phosphorylation of the response regulator receiver domain often modifies the activity of the output domain, of which there are many types. Frequently, the output domain is a helix-turn-helix DNA binding domain and so phosphorylation of the receiver domain changes the response regulator's affinity for specific DNA elements so as to modify gene expression and initiate the corresponding cellular response. Dephosphorylation of the response regulator by the sensor kinase serves to return the system to its pre-activation state.

Cationic antimicrobial peptides (host defense peptides)

The last 40 years have seen only three new classes of antibiotics enter medicine (lipopeptides, oxazolidinones, and streptogramins), all geared towards Gram-positive bacterial infections. A lack of new antibiotics, particularly for treatment of Gram-negative infections, combined with emerging multi-drug resistance issues demands that new antimicrobial strategies be explored for treating these infections (Chopra et al. 2008). Cationic antimicrobial peptides (host defense peptides) are presently forming the foundation of a new class of antimicrobial compounds for clinical use (Marr et al. 2006).

Cationic antimicrobial peptides have a pivotal role in preventing infections by microbial pathogens in many organisms (Zasloff 2002). To date more than 600 peptides in virtually all species of life have been described that not only kill pathogenic microorganisms, including Gram–positive and Gram–negative bacteria, viruses, protozoa, and fungi, but also to play a central role in recruiting and promoting elements of the innate immune system (Finlay and Hancock 2004; Brown and Hancock 2006). This enormous peptide diversity is achieved through several structural classes, whereby all peptides, regardless of class, share a net positive charge and around 50% hydrophobic residues, which confers the ability to fold into an amphiphilic conformation upon interaction with bacterial membranes (Jenssen et al. 2006). Although interaction with the cytoplasmic membrane is obligatory and some peptides are able to perforate membranes at their minimal effective concentrations, a number of peptides have been shown to translocate across the membrane and act on gene expression and on cytoplasmic targets including macromolecular synthesis, enzyme inhibition, stimulation of autolysis, and cell division (Brogden 2006; Hale and Hancock 2007).

Indeed, many severe bacterial infections require systemic antibacterial drug administration to quickly halt and limit the spread of infection; in this case endotoxaemia/sepsis is a common and dangerous complication of systemic therapy in individuals with bacteremia. One substantial advantage of peptides over conventional antibiotics is that they have dual ability to neutralize sepsis/endotoxemia and to participate in diverse roles in modulating mammalian innate immunity (Brown and Hancock 2006). Indeed, one of the most important roles described is an ability to stimulate the innate immune response while simultaneously dampening the potentially harmful inflammatory

response (Finlay and Hancock 2004). Other motivations for therapeutic antimicrobial use include a diversity of potential applications that could include use as a single antimicrobial or in combination with other antibiotics for synergistic effects in order to overcome barriers resistant bacteria have created against currently used antibiotics (Zasloff 2002). Indeed, peptides are not hindered by some of the resistance mechanisms that are placing currently used antibiotics in jeopardy, as excellent activity is seen against methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *P. aeruginosa*. The most potent antimicrobial peptides can have an unusually broad spectrum of activity, killing most Gram-negative and Gram-positive bacteria, while this spectrum can extend to fungi and even viruses (Jenssen et al. 2006). Peptide killing of bacteria is extremely rapid and can involve multiple bacterial cellular targets and often minimal inhibitory concentrations and minimal bactericidal concentrations coincide within 2-fold, indicating that killing is generally bactericidal (a highly desirable mode of action).

***P. aeruginosa* resistance to antimicrobial peptides**

The major reasons for the high intrinsic resistance of *P. aeruginosa* to multiple classes of antibiotics are its low outer membrane permeability coupled with active antibiotic efflux systems (Hancock and Speert 2000). Furthermore, in addition to classical mutational or acquired antibiotic resistance, *P. aeruginosa* resistance can be triggered by environmental factors and is therefore a subset of adaptive resistance. For example, sub-inhibitory concentrations of antibiotics have a variety of effects on bacteria and can themselves induce resistance to subsequent exposure to otherwise lethal concentrations of antibiotics (Linares et al. 2006; Davies et al. 2006). As discussed below, *P. aeruginosa* is known to regulate resistance to antimicrobial peptides through adaptive resistance (Gilleland and Farley 1982).

During infections in health-care settings, *P. aeruginosa* is likely exposed to endogenous β-defensin and cathelicidin antimicrobial (host defense) peptides at epithelial surfaces and also to polymyxins (peptide antibiotics) when therapeutically administered. Owing to a severe limitation of therapeutic options for multidrug-resistant *P. aeruginosa* infections, polymyxin peptides have re-emerged as effective drugs for combating these formidable infections (Li et al. 2006; Zavascki et al. 2007). Adaptive resistance to cationic antimicrobial peptides and polymyxin B is known to occur in response to limiting extracellular concentrations of divalent Mg^{2+} and Ca^{2+} cations, but this mechanism is not likely a clinically meaningful observation as the body contains 1-2 mM divalent cations. However as noted below peptides themselves are able to induce peptide resistance mechanisms through two-component systems.

The PhoP-PhoQ two-component system mediates in part the adaptive response to limiting (μM) extracellular Mg^{2+} concentrations and concurrent resistance to polymyxin B and antimicrobial peptides (Fig. 1.1; Macfarlane et al. 1999; Ernst et al. 1999; Macfarlane et al. 2000). Another two-component system, PmrA-PmrB, also responds to low Mg^{2+} signals and regulates resistance to polymyxin B and antimicrobial peptides (Fig. 1.1; McPhee et al., 2003; Moskowitz et al. 2004). In response to low Mg^{2+}, both PhoP and PmrA response regulators positively regulate the *arnBCADTEF* operon which encodes a pathway for addition of 4-aminoarabinose (positively charged amino sugar residue) to lipid A (Moskowitz et al. 2004; McPhee et al. 2003), causing resistance by reducing the net negative charge of LPS and limiting its interaction with polycationic antibiotics such as polymyxin B and other cationic peptides. Moreover, the addition of aminoarabinaose to lipid A appears to be specific for *P. aeruginosa* cystic fibrosis isolates (Ernst et al. 2007).

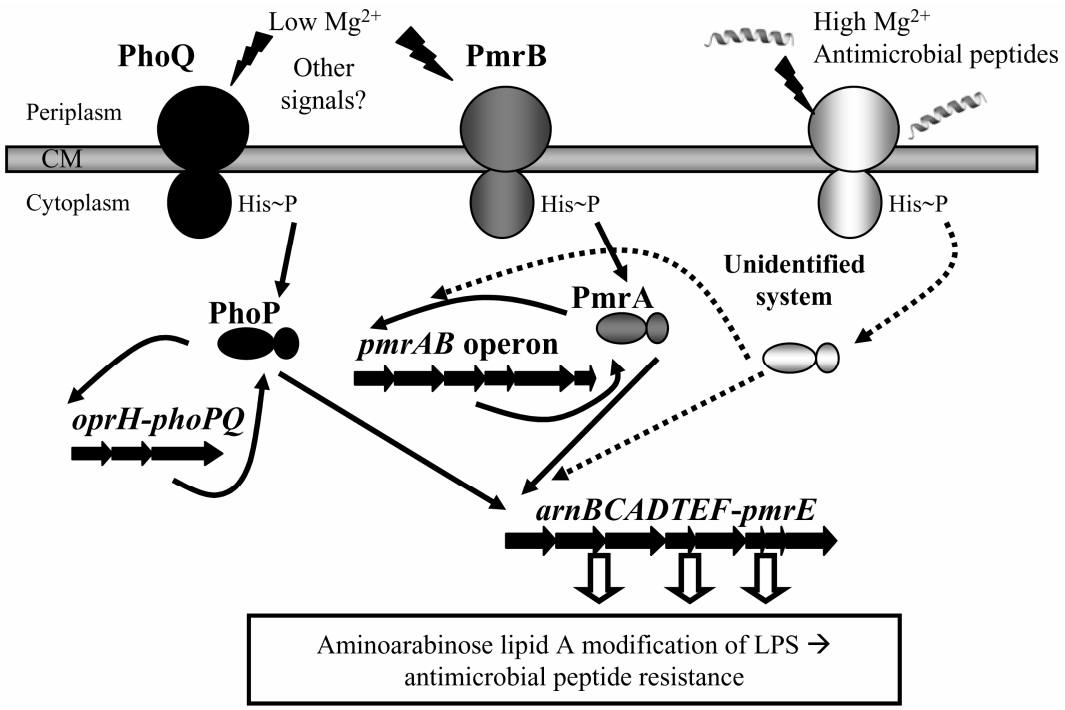

Figure 1.1. **A model for the *P. aeruginosa* PhoP-PhoQ and PmrA-PmrB regulatory networks in resistance to cationic antimicrobial peptides.** Mg^{2+} limitation leads to the activation (phosphorylation) of the PhoP and PmrA response regulators which positively autoregulate the transcription of their respective operons, as well as the *arnBCADTEF* operon. The ArnBCADTEF pathway modifies Lipid A with aminoarabinaose, reducing the net negative charge on LPS, and consequently decreasing self promoted uptake across the outer membrane, increasing resistance to polymyxins and cationic antimicrobial peptides. Conversely, under high Mg^{2+} conditions, PhoP and PmrA are dephosphorylated and presumed inactive (not shown). However, during growth in high Mg^{2+} plus subinhibitory antimicrobial peptides, an unidentified regulatory system is proposed to promote activation of *arnBCADTEF* and *pmrAB* operons, consequently increasing resistance to antimicrobial peptides. Other genes regulated by PmrA and PhoP are not shown.

Interestingly, both *pmrAB* and *arnBCADTEF* operons were shown to be strongly induced by a variety of cationic antimicrobial peptides, including polymyxins, indolicidin, human LL-37, and other synthetic peptides (McPhee et al. 2003), in stark contrast to *Salmonella* where direct binding of peptides to PhoQ mediates peptide resistance (Bader et al. 2005). In *P. aeruginosa* the induction of *arnBCADTEF* was partially dependent on PmrA-PmrB (McPhee et al. 2003). Transposon mutations in both *pmrAB* and *arnBCADTEF* operons result in super-susceptibility to polymyxin B and antimicrobial peptides such as

improved variants of indolicidin, an endogenous bovine neutrophil host defense (antimicrobial) peptide (Lewenza et al. 2005). Peptide-induced activation of the *pmrAB* operon appeared to be independent of both the PhoP-PhoQ and PmrA-PmrB systems, which likely indicates that another protein regulator responds to cationic peptides to promote resistance (Fig. 1.1).

Interestingly, a *phoQ* mutant displays super-resistance to polymyxin B and antimicrobial peptides when grown in high Mg^{2+}, a constitutive resistance phenotype, in contrast to wild-type cells which are normally sensitive to antimicrobial peptides in high Mg^{2+} (Macfarlane et al. 1999). The role of PhoP in this resistance appears to be significant as overexpression of the PhoP response regulator in a *phoP* mutant gives *phoQ*-like constitutive polymyxin B resistance (Macfarlane et al. 1999). These findings indicate that in non-inducing high Mg^{2+} conditions, PhoQ dephosphorylates PhoP (inactivating it), and conversely in the *phoQ* mutant this activity is lost and possibly some other protein/mechanism allows PhoP to be activated through phosphorylation. To note, a *phoP* mutant displays wild-type polymyxin B resistance in low Mg^{2+} media (due to the activity overlap of PmrAB) and remains sensitive when grown under high Mg^{2+} conditions (in contrast to *phoQ*; Macfarlane et al. 1999; McPhee et al. 2003).

Goals of this study

The overall theme of these studies encompassed *P. aeruginosa* regulation of antimicrobial peptide resistance and virulence. A starting point was to examine how *P. aeruginosa* regulates resistance to polymyxin B and antimicrobial peptides under non-resistance-inducing high (mM) Mg^{2+} conditions. Preliminary microarray experiments were used to gain insight into how *P. aeruginosa* regulates its transcriptome in response to sub-

inhibitory concentrations of antimicrobial peptides. These results showed the *psrA* gene (encoding a transcriptional regulator) was transcriptionally up-regulated in response to peptides. Second, with collaborators, we found that the *phoQ* mutant is highly attenuated for virulence in a rat model of chronic lung infection.

Therefore, the major hypotheses and goals included:

1. PsrA regulates resistance to antimicrobial peptides and other virulence processes. Goals were to phenotypically characterize these processes and the corresponding gene expression governing them.
2. PhoQ is a regulator of virulence and the PhoQ regulon contributes to virulence and antimicrobial peptide resistance. The goal here was to investigate this phenotype by making a new *phoQ* mutant, performing microarray analysis, and further characterize other phenotypes of this mutant.

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CHAPTER 2 – PsrA of *Pseudomonas aeruginosa*[*]

INTRODUCTION

The opportunistic Gram-negative bacterium *Pseudomonas aeruginosa* is a dominant pathogen in the lungs of cystic fibrosis patients (Gibson et al. 2003; Rowe et al. 2005) and the third leading cause of severe hospital-acquired infections (Kielhofner et al. 1992; Lyczak et al. 2000). *P. aeruginosa* infections can be extremely difficult to combat owing to high intrinsic antibiotic resistance and a wide repertoire of virulence factors (Hancock and Speert 2000). Exacerbating these problems is the emergence of multi-drug resistant *P. aeruginosa* clinical isolates resistant to virtually all antibiotics, infamously designating these strains as 'superbugs' (Falagas and Bliziotis 2007).

Current treatment of *P. aeruginosa* infections often involves potent β-lactam antibiotics, aminoglycosides or fluoroquinolones, or a combination thereof, however resistance can nevertheless arise (Mesaros et al. 2007). When multiple drug resistance occurs, such as often found in the late stages of cystic fibrosis lung disease, polymyxins have become a drug of last resort (Li et al. 2006; Zavaski et al. 2007). Thus it is important to understand the basis for resistance in this organism and its interrelationship with pathogenesis. For example, there is a well-known discrepancy discrepancy between *in vitro* antibiotic susceptibility and the clinical success of particular antibiotics against *P. aeruginosa* (Flick et al. 1972; Hanccok and Speert 2000; Karlowsky et al. 1997). One basis for this is the induction of resistance mechanisms due to environmental factors, a process termed adaptive resistance

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that is differentiated from acquired or mutational resistance since it reverts upon removal of the antibiotic or selective pressure.

Structurally diverse cationic antimicrobial peptides are part of the innate immune system of complex organisms, and can possess direct antimicrobial activity and/or a profound ability to modulate innate immunity (Jenssen et al. 2006). Directly antimicrobial peptides demonstrate considerable promise against infections by multidrug resistant bacteria (Marr et al. 2006; Hancock and Sahl 2006). However, *P. aeruginosa* is able to sense the presence of peptide and become adaptively resistant (Glilleland and Farley 1982), for example through peptide-mediated regulation of the *arnBCADTEF* (*pmrHFIKLM*; PA3552-9) LPS modification operon, independently of the PmrA-PmrB or PhoP-PhoQ two-component regulatory systems (McPhee et al. 2003; Moskowitz et al. 2004).

Virulence is similarly complex, representing a series of complex adaptations to growth in a host organism, including biofilm formation, swarming motility, and quorum sensing. For example, *P. aeruginosa* motility is important for biofilm formation, virulence, and colonization of different niches (Klausen et al. 2003; O'Toole and Kolter 1998; Parsek and Greenberg 2005). Type IV pili extend and retract to promote twitching motility on solid surfaces, whereas flagella power swimming motility. On the other hand, swarming motility appears to be a coordinated and complex adaptation to low viscosity surfaces, and involves a number of factors that include flagella, type IV pili, quorum sensing, and rhamnolipids etc (Overhage et al. 2007; Overhage et al. 2008). There is considerable overlap in the genes utilized in swarming motility and biofilm formation (Caiazza et al. 2007; Shrout et al. 2006), both of which have been proposed to contribute to disease pathogenesis (Parsek and Singh 2003) and lead to increased resistance to several antibiotics (Overhage et al. 2008).

In this study, it was demonstrated that antimicrobial peptides transcriptionally upregulated the expression of *psrA*, a previously documented *Pseudomonas* regulator of RpoS and the type III secretion system, but one for which the activating signals were unknown (Kojic and Venturi 2001; Kojic et al. 2002; Shen et al. 2006). Detailed phenotypic studies indicate that PsrA regulated polymyxin and antimicrobial peptide resistance, motility, and biofilm formation. Microarray analysis of the *psrA* mutant provided insight into the basis for these observed phenotypes.

MATERIALS AND METHODS

Bacterial strains 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 (LB) broth containing 1.8% (wt/vol) Difco agar (Becton-Dickinson Co.) when appropriate. The defined medium used was BM2-glucose minimal medium [62 mM potassium phosphate buffer (pH 7), 7 mM $(NH_4)_2SO_4$, 10 µM FeSO4, 0.4% (w/v) glucose] containing 2 mM (high) $MgSO_4$ concentrations. Antibiotics for selection were used at the following concentrations: tetracycline, 50-100 µg/ml for *P. aeruginosa*; ampicillin 100 µg/ml for *E. coli*; carbenicillin, 500 µg/ml for *P. aeruginosa*; gentamicin 30 µg/ml for *P. aeruginosa* and 15 µg/ml for *E. coli*.

Genetic manipulations.

Routine molecular biology techniques were performed according to standard protocols (Ausubel 1987). Primers were synthesized by AlphaDNA Inc. (Montreal, QC, Canada). Plasmid DNA was isolated using QIAprep spin miniprep kits (Qiagen Inc., Canada) and agarose gel fragments were purified using a QIAquick gel extraction kit

(Qiagen Inc., Canada). T4 DNA ligase was from Invitrogen and restriction endonucleases were from New England Biolabs.

Mobilizing the UW-*psrA* transposon mutation into a new PAO1 background.

The UW-*psrA* mutation (confirmed to be correct by PCR and sequencing the junctions of the transposon mutation) was first transferred into our laboratory wild type *P. aeruginosa* PAO1 strain H103 as described previously (Choi et al. 2006). Genomic DNA was isolated from the UW-*psrA* mutant using the hexadecyltrimethyl ammonium bromide (CTAB) method (Ausubel 1987). Approximately one microgram of this DNA (which contained the tetracycline resistance-encoding transposon IS*lacZ*/*hah*-Tc insertion in *psrA*) was electroporated into WT H103. Cells recovered for one hour at 37°C, and then were plated onto LB agar plates containing 100 µg/ml tetracycline. After 18 hours of growth at 37°C, tetracycline resistant transformants were then analyzed by colony PCR using a transposon-specific primer and a custom gene primer together with Taq polymerase (Invitrogen Canada Inc.) to verify that the transposon was correctly inserted into *psrA*. This new *psrA* mutant allowed better analysis of motility-related phenotypes (H103 is swarm$^+$ under our conditions, see below) and was therefore used for all experiments reported in this study.

Genetic complementation of *psrA*.

Forward and reverse primers for *psrA* were designed from the *P. aeruginosa* PA01 genome sequence (www.pseudomonas.com) using Primer3 [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi; (Rozen and Skaletsky 2000)] to clone *psrA*$^+$ together with 347 bp of upstream DNA with the native promoter and 67 bp downstream DNA. Amplification of *psrA* from *P. aeruginosa* wild-type H103 genomic DNA was carried out

using high-fidelity Platinum *Pfx* DNA polymerase (Invitrogen Canada Inc.) with primers PsrA-L 5'-CGGAGCACAGAGAAAGGAGA-3' and PsrA-R 5'-GACTTGAAGCCGAGTTCCTG-3'. The resulting PCR product was then cleaned (Qiagen PCR Purification Kit) and the amplicons were cloned into pCR-Blunt II-TOPO using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen Inc.) and transformed into One Shot TOP10 cells (Invitrogen Inc.), creating pCR-*psrA*$^+$. A Nsi1 fragment containing *psrA* was excised from pCR-*psrA*$^+$ and subcloned into pUC18mini-Tn7T-Gm, generating pUC18-mini-Tn7T-Gm-*psrA*$^+$. pUC18mini-Tn7T-Gm-*psrA*$^+$ was co-electroporated with pTNS2 into the *psrA* mutant using sucrose electroporation (Choi et al. 2005). Gentamicin resistant transformants were analyzed by colony PCR using primers PglmS-up and PTn7L to determine correct transposon integration of mini-Tn7 into the chromosome as previously described (Choi et al. 2005).

Killing curves.

Overnight *P. aeruginosa* cultures were diluted 1/100 into fresh BM2-glucose minimal medium containing 2 mM Mg^{2+}. Upon reaching the mid-logarithmic phase of growth (~0.5 OD_{600}), 1 ml of each culture was pelletted and resuspended in 1 ml 1× BM2 salts (buffer), and diluted 1/10 into pre-warmed 1× BM2 salts. Killing was then initiated by the addition of 1 µg/ml polymyxin B sulphate (Sigma Inc., Oakville, Canada) or 64 µg/ml indolicidin for analysis of intrinsic resistance. Flasks were shaken at 37°C and aliquots were withdrawn at the designated times for assaying survivors by plating diluted 100 µl aliquots onto LB agar plates.

Outer membrane permeabilization assays.

P. aeruginosa outer membrane barrier function and the efficiency of the self promoted uptake route was determined by the 1-N-phenylnapthylamine (NPN) assay (Loh et al. 1984). Cultures were grown to mid-logarithmic phase in BM2-glucose minimal medium containing 2 mM $MgSO_4$. The cells were then harvested, washed, and resuspended to an OD_{600} of 0.5 in 5 mM HEPES (pH 7.0), 5 mM glucose, and 5 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Two ml of these bacterial suspensions were placed in a quartz cuvette with a magnetic stir bar. NPN (Sigma Inc., Oakville, Canada) was then added to the cuvette at a concentration of 10 μM and the fluorescence (baseline) was measured using a LS-50B fluorescence spectrophotometer (PerkinElmer, Inc.; excitation and emission wavelengths of 350 nm and 420 nm, respectively). Indolicidin peptide was then added to initiate the assay and the increase in fluorescence due to peptide-mediated entry of NPN into the membrane was measured until a stable signal was observed (indicating that additional partitioning of NPN into the membrane had stopped).

Biofilm and attachment assays.

Static microtitre biofilm assays were generally performed as those previously described (O'Toole and Kolter 1998). Overnight LB cultures were diluted 1/1000 into fresh LB broth, and 100 μl was inoculated into wells of a 96-well polystyrene round bottom microtitre plate (Falcon). For PA14 strains, overnight cultures were diluted 1:500 into BM2-glucose medium containing 2 mM $MgSO_4$ and 0.5% Casamino acids. Plates were then incubated at 37°C without shaking. At the specified time point, media and planktonic cells were dumped and the wells were washed three times with dH_2O. Surfaced attached bacteria were then stained

with 0.1% (w/v) crystal violet for 20 min, followed by ethanol solubilization of crystal violet for quantification at A_{600}.

Rapid attachment was assayed as described previously with slight modification (Ma et al. 2006). Overnight cultures were first diluted 1/100 into fresh LB medium, grown to an OD_{600} of ~0.5, and 100 µl was added to each well of a 96-well polystyrene microtitre plate (Falcon). Cells were allowed to attach for 30 min at room temperature prior to staining with crystal violet as decribed above.

Motility assays.

Swimming motility was assayed on BM2-glucose plates containing 0.3% (wt/vol) agar. Swarming was assayed on modified BM2 glucose plates containing 0.5% (wt/vol) agar and with 0.5% (wt/vol) Casamino acids (or 0.1% for PA14 strains) substituted for 7 mM $(NH_4)_2SO_4$ (Overhage et al. 2007). Swimming and swarming motility were assayed by inoculating 1 µl of mid-logarithmic growth phase liquid cultures grown in BM2-glucose containing 2 mM Mg^{2+} onto the motility plate, incubating for 16-18 hrs at 37°C, and measuring motility zone diameters. Twitching motility was assessed by toothpick inoculating cells from agar plates into thin LB agar (1%) plates, down to the agar-plastic interface, and measuring the twitch-zone diameter after 24 and 48 h incubation at 37°C.

Growth curves.

Overnight cultures were grown in BM2-glucose containing 2 mM Mg^{2+} and 0.1 ml was diluted into 10 ml fresh medium. Flasks were shaken at 37°C and aliquots were withdrawn periodically to determine the cell density as OD_{600}. Similarly, determination of planktonic growth at 37°C in static 96-well polystyrene microtitre plates (simple biofilm conditions) was assayed by monitoring OD_{600}.

Microarray analysis.

Detailed technical descriptions of microarray analyses were provided previously (McPhee et al. 2006). In overview, for each strain, microarray analysis involved five independent cultures. *P. aeruginosa* WT and *psrA* mutant cultures were grown with shaking in BM2-glucose medium plus 2 mM $MgSO_4$ at 37°C for 18 hrs, and then diluted 1 in 100 into fresh medium. Cultures were grown at 37°C with shaking (250 rpm) to the mid-logarithmic phase of growth (OD_{600}=0.5) and then total RNA was isolated using RNeasy midi columns (Qiagen). Contaminating genomic DNA was removed by treatment with a DNA-free kit (Ambion Inc., Austin, TX). RNA was stored at −80°C with 0.2 U/µl of SUPERase-In RNase inhibitor (Ambion Inc., Austin, TX). RNA quality was assessed by agarose gel electrophoresis and spectrophotometrically. RNA was converted to cDNA, hybridized, and analyzed as previously described (McPhee et al. 2006). *P. aeruginosa* PAO1 microarray slides were provided by The Institute for Genomic Research (TIGR) Pathogenic Functional Genomics Resource Center (http://pfgrc.tigr.org/). Images of slides were quantified using ImaGene 6.0 Standard Edition software (BioDiscovery, Inc., El Segundo, CA). ArrayPipe version 1.7 was used for assessment of slide quality, normalization, detection of differential gene expression, and statistical analysis using available genome annotation from www.pseudomonas.com. Data analysis of DNA microarrays was carried out as previously described (McPhee et al. 2006). The averaging of the five biological replicates were performed to obtain overall fold changes for *psrA* mutant relative to wild-type and a two-sided one-sample Student's *t* test was applied to determine significant changes in gene expression. Fold changes greater than or equal to 2.0 with a Student's *t* test *P* value of <0.05 were used as the cut-offs for reporting expression changes.

Real-time qPCR.

Total RNA was isolated from *P. aeruginosa* grown in BM2-glucose minimal media containing 2 mM Mg^{2+} using RNeasy midi columns (Qiagen), with or without 2 µg/ml indolicidin, to the mid-logarithmic phase of growth. DNase treatment of RNA samples, cDNA synthesis, and real-time qPCR was carried out as described previously (McPhee et al. 2006): cDNA was diluted 1/1000, and 1 µl was used as template for real-time PCR using 1× SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA) in an ABI Prism 7000 (Applied Biosystems, Foster, CA). Forward and reverse primers were designed internal to *psrA* using PrimerExpress (Applied Biosystems, Foster, CA). All reactions were normalized to the *rpsL* gene encoding the 30S ribosomal protein S12.

RESULTS

Activation of *psrA* transcription in response to antimicrobial peptides.

Preliminary microarray analysis of the *P. aeruginosa* response to sub-inhibitory indolicidin antimicrobial peptides (2 µg/ml) indicated that the *psrA* (PA3006) gene was 2.5-fold induced ($P < 0.05$) during growth in the presence of this peptide. This preliminary result utilized cultures grown to mid-logarithmic phase under high Mg^{2+} (2 mM $MgSO_4$) conditions with or without sub-inhibitory indolicidin, a 13-amino acid endogenous cationic host defense (antimicrobial) peptide from bovine neutrophils (Falla et al. 1996; Selsted et al. 1992). Independent real-time qPCR experiments confirmed that the transcription of *psrA* was 3.0 ± 0.3 fold up-regulated in the presence of indolicidin, with similar induction by the indolicidin-variant peptide CP11CN (data not shown).

Indolicidin was chosen as it is capable of causing strong induction of *pmrA-pmrB* and *arnBCADTEF* (aminoarabinaose LPS modification) operons, stronger induction than

polymyxin B is capable of (McPhee et al. 2003), and the concentration used (2 μg/ml = one eighth MIC) was sub-inhibitory and caused no growth impairment towards wild-type or *psrA* mutant cells (data not shown). In addition, growth of cells in high Mg^{2+} with peptide was chosen to prevent low Mg^{2+}-mediated induction of resistance, which is believed to involve different mechanisms than that of peptide-induced resistance (McPhee et al. 2003). As a positive control, in agreement with previous studies, *arnB* (first gene of the *arnBCADTEF* operon), was confirmed here by RT-qPCR to be 54 ± 8 up-regulated under these conditions, and microarray data confirmed that the indolicidin-regulated *pmrA-pmrB* operon was also upregulated in the presence of sub-inhibitory indolicidin, but the Mg^{2+}-regulated *oprH-phoP-phoQ* operon was not.

Contribution of *psrA* to intrinsic antimicrobial peptide and polymyxin B resistance.

The influence of the *psrA* gene on intrinsic resistance to peptides was examined. Intrinsic resistance was assayed by growing cells under high (2 mM) Mg^{2+} conditions to suppress the possibility of induction by limiting Mg^{2+}. The *psrA* mutant exhibited an intrinsic increased susceptibility to the polycationic lipopeptide polymyxin B as shown by killing curves (Fig. 2.1A). This super-susceptibility phenotype could be complemented to normal wild-type polymyxin B susceptibility by introducing a single wild-type $psrA^{+}$ allele into the chromosome of the mutant by mini-Tn7 integration technology (Fig. 2.1A). Similarly, the *psrA* mutant demonstrated super-susceptibility to the cationic antimicrobial peptide indolicidin, which could be complemented back to wild-type susceptibility (Fig. 2.1B). Thus the *psrA* gene product appeared to be essential for normal expression of intrinsic peptide resistance.

The *psrA* mutation affected the permeabilization of the outer membrane.

Polycationic molecules like polymyxin B and antimicrobial peptides pass across the outer membrane by self-promoted uptake. The first stage of self promoted uptake involves the interaction of the polycation with divalent cation binding sites on surface polyanionic LPS causing a disruption of the permeability barrier and subsequent uptake of the permeabilizing polycationic antibiotic. To address the possibility that altered outer membrane permeability was the basis for peptide super-susceptibility in the *psrA* mutant, NPN was used as probe for outer membrane permeabilization by indolicidin (Fig. 2.2). The hydrophobic fluorophore NPN is normally excluded from entering cells due to its inability to penetrate the outer membrane. Upon permeabilization of the outer membrane (as occurs during self promoted uptake) NPN is taken up and becomes strongly fluorescent in the nonpolar/hydrophobic environment of cell membranes. There was no obvious difference in the ability of *psrA* and wild type to exclude NPN. However, indolicidin at concentrations of 1.5 and 3.0 µg/ml was able to permeabilize the outer membranes of the *psrA* mutant to a greater extent than those of wild-type cells (Fig. 2.2A). Thus the super-susceptibility of the *psrA* mutant to indolicidin correlated with an outer membrane that was more easily permeabilized by this antimicrobial peptide. Similarly, polymyxin B also preferentially permeabilized the *psrA* mutant (Figure 2.2B).

Contribution of *psrA* to biofilm formation and attachment.

Other genes such as PhoQ, that regulate antimicrobial peptide resistance, also regulate biofilm formation and motility (Ramsey and Whiteley 2004). To assess the ability of the *psrA* mutant to form simple biofilms, static microtitre biofilm assays were employed. These experiments demonstrated that the *psrA* mutant displayed significant ($P < 0.001$ by Student's

t test) impairment in biofilm formation at 18 h, by more than 4-fold (Fig. 2.3A). Biofilm impairment could be successfully complemented by introducing the wild-type *psrA* allele into the mutant (Fig. 2.3A). No observable growth differences were observed when the OD_{600} of planktonic cells was measured as a function of time during the period of growth in the microtitre wells (Fig. 2.3B). Similarly, assessment of growth in defined medium in shaking flasks revealed no differences between mutant and wild type (data not shown), indicating no primary growth defect.

To determine whether this biofilm formation phenotype occurred during initial attachment stage or later during biofilm development, a rapid (30 min) attachment assay was performed. The *psrA* mutant displayed more than two-fold ($P < 0.001$ by Student's *t* test) impaired attachment, and this defect could be complemented with the $psrA^+$ gene (Fig. 2.3C). Light microscopic observations of both *psrA* mutant and wild type cultures showed no obvious morphological differences under these conditions that might contribute to this phenotype.

Requirement for PsrA for normal swarming.

Mutant studies have revealed an intricate relationship between motility and biofilm formation in *P. aeruginosa* (Klausen et al. 2003; O'Toole and Kolter 1998). Therefore, the *psrA* mutant was assessed for its ability to undergo swimming, twitching, and swarming motility. Neither flagella-mediated swimming nor type IV-pilus-mediated twitching motility were significantly affected in the *psrA* mutant (data not shown). However, the *psrA* mutant demonstrated a severe impairment in swarming motility with a more than 2.5-fold ($P < 0.01$ by Student's *t* test) decrease in swarming zone size (Fig. 2.4). Introducing the wild-type

psrA allele restored wild-type swarming in the mutant, demonstrating that *psrA* is necessary for normal swarming motility (Fig. 2.4).

Microarray analysis.

The above-described complexity of phenotype indicated that PsrA might control the expression of a substantial regulon. To assess this and to identify candidate genes that might explain the observed *psrA* mutant phenotypes, microarray analysis was performed comparing the *psrA* mutant to wild type after growth to mid-logarithmic phase in BM2-glucose minimal medium containing 2 mM Mg^{2+}. There were a total of 178 genes that were significantly ($P \leq 0.05$) dysregulated ≥2-fold of which 70 were up-regulated and 108 down-regulated in the mutant relative to the wild-type. A selection of these genes (not including hypothetical and unclassified ORFs) is shown in Tables 2.2-2.4. Most previously identified genes with predicted PsrA binding sites in their promoters (Kojic et al. 2005) were identified by this microarray analysis (Table 2.2).

In addition we observed dysregulation of the entire type III secretion apparatus and its effectors, certain adhesion and motility genes, 17 regulators (*rpoS*, *pcrH*, *mdcR*, *toxR*, *arsA*, PA0513, PA1399, PA1976, PA1978, PA2432, PA2469, PA2551, PA3077, PA3409, PA3630, PA4135, and PA4296), and a variety of metabolic and energy metabolism genes. Independent RT-qPCR analysis confirmed the regulation of 6 of these genes (indicated by * in Tables 2.2-2.4) and thus provided validation for our *psrA* microarray data.

Additional mutant phenotypic analyses.

The list of genes dysregulated in the *psrA* mutant provided a useful starting point towards understanding the basis for the observed *psrA* mutant phenotypes. To understand

the phenotypes associated with selected dysregulated genes, transposon mutants from the PA14 comprehensive non-redundant library were utilized (Table 2.1; Liberati et al. 2005).

The microarray was examined to find genes that might influence peptide susceptibility (since the microarray and time kill experiments used similar growth conditions). The dysregulation of several genes of the *wbp* gene cluster (Table 2.4), which is involved in the biosynthesis of B-band (serotype O antigen) LPS (Burrows et al. 1996), suggested a possible role for B-band LPS in the observed supersuceptibility of the *psrA* mutant. Therefore, in order to look at the requirement of B-band LPS, we analyzed mutants in *wbpI* and *wbpL*, as well as a mutant in *wbpM* that had been previously shown to lack B-band LPS (Burrows et al. 1996; Burrows et al. 2000). In addition, a small panel of PA14 mutants including others related to energy metabolism was tested, since our preliminary unpublished observations have indicated a role for energy metabolism in resistance to antimicrobial peptides. It was hypothesized that one or more of these mutants would show peptide super-susceptibility, thereby indicating a putative contribution of the dysregulated genes in the intrinsic super-susceptibility of the *psrA* mutant.

As shown in Fig. 2.5A, both *wbpM* and *coxA* (cytochrome C oxidase subunit 1) mutants showed modest super-susceptibility to indolicidin relative to wild-type at 25 min. No differences were seen for mutants in *fhp*, PA1883, *mexC* and *wzz* (data not shown). Unfortunately, an *etfA* (energy metabolism) mutant clumped during growth which made performing killing curves on this mutant difficult.

No differences in O antigen chain length expression were seen when LPS was isolated from WT and *psrA* mutant and analyzed by SDS-PAGE and silver staining (data not shown). However, as small changes in LPS that influence functionality (such as substitution by

sugars, phosphates and fatty acids) could be affected by *psrA* mutation, we analyzed, mutants in *wbpI*, *wbpL* (both downregulated in the *psrA* mutant) and *wbpM* for possible outer membrane permeability phenotypes. Like the *psrA* mutant, all three of these mutants in LPS B-band biosynthetic genes showed increased outer membrane permeabilization by peptides relative to wild-type (Fig. 2.6A, B), although the effect observed with the *wbpM* mutant was more prominent (Fig. 2.6B).

A panel of PA14 mutants were also analyzed for possible roles in swarming motility and biofilm formation. The *wbpM* mutant showed a significant swarming impairment phenotype, as did an *etfA* mutant, although in the latter mutant this might be related to the tendency of this mutant to clump (Fig. 2.5B and 2.5C). No swarming differences were seen for *coxA* and *wzz* (Fig. 2.5C) or *mexC* and PA1883 mutants (data not shown).

The downregulation of certain genes of the type IVb pilus-encoding *tad* cluster (de Bentzmannn et al. 2006) led us to analyze mutants in these genes for possible biofilm formation phenotypes. In simple biofilm growth conditions, none of the *tad* mutants analyzed displayed biofilm impairment (Fig. 2.5D), confirming previously reported results (de Bentzmann et al. 2006). However, a *pprB* (PA4296) mutant, encoding a two-component response regulator located adjacent to the *tad* cluster and substantially down-regulated on the *psrA* arrays (Table 2.3) did demonstrate a significant (P <0.001 by Student's t test) biofilm impairment phenotype (3-fold) at the time point analyzed (Fig. 2.5D).

DISCUSSION

The *psrA* gene of *P. aeruginosa* is an important regulator of both resistance to cationic antimicrobials and virulence features. It was up-regulated in response to the cationic antimicrobial peptide indolicidin and mediates intrinsic cationic peptide resistance and

certain virulence-related processes such as biofilm formation, rapid attachment, swarming motility. The involvement of PsrA in these phenotypes was supported by studies of a *psrA* mutant and single-copy complementation of the *psrA* defect.

PsrA was previously characterized by the Venturi group as a positive regulator of transcription of the alternative sigma factor RpoS (Kojic and Venturi 2001; Kojic et al. 2002), as also confirmed here. In other *Pseudomonas* spp, PsrA is known to be involved in antifungal metabolite production (Chin et al. 2005) and the regulation of quorum sensing (Chatterjee et al. 2007). However, the direct signals that activate *psrA* were unknown, and the data here now demonstrates that the cationic antimicrobial peptide indolicidin is an activating signal for transcription, consistent with other studies demonstrating that peptides are key regulators of bacterial virulence and resistance processes (Bader et al. 2005).

The demonstration that *psrA* contributes to cationic peptide resistance adds another regulator to the increasingly complex regulatory network influencing resistance, which already includes PhoP-PhoQ and PmrA-PmrB (Ernst et al. 1999; Macfarlane et al. 1999; McPhee et al. 2003; Moskowitz et al. 2004). However unlike these two-component regulators which mediate an increase in resistance to peptides in limiting Mg^{2+} growth conditions, PsrA mediates intrinsic resistance. Thus unlike *psrA*, *pmrA* and *phoP* mutants do not demonstrate supersusceptibility under non-inducing conditions and there appears to be no obvious regulatory hierarchy since *psrA* was not apparently transcriptionally regulated by PmrA or PhoP (or vice versa), nor was there any substantial overlap in dysregulated genes (McPhee et al. 2006). Polymyxin B and cationic antimicrobial peptides passage into cells via the self-promoted uptake pathway which involves interaction with divalent cation binding sites on LPS and subsequent distortion of the outer membrane permeability barrier (Falla et

al. 1996; Gilleland and Farley 1982; Jenssen et al. 2006). All three systems however appear to mediate resistance by influencing the ability of cationic agents to permeabilize outer membranes and we were able to demonstrate for PsrA that the increase in permeabilization by cationic agents (Fig. 2.2) correlated with the supersusceptibility of the *psrA* mutant to polymyxin B and indolicidin (Fig. 2.1).

There were strong candidate genes that were dysregulated in the *psrA* mutant that probably contributed to the *psrA* peptide super-susceptibility phenotype through changes in outer membrane permeability. Three genes (*wbpG*, *wbpI*, *wbpL*; Table 2.4) involved in LPS B-band (serotype O antigen) biosynthesis were down-regulated by 2.0-to-4.4 fold suggesting that the entire *wbpGHIJKLM* locus would be transcriptionally repressed (consistent with the intrinsic limitations of microarray experiments; Table 2.4). Supporting this link between peptide super-susceptibility and outer membrane permeability of the *psrA* mutant, mutants in two genes (*wbpI* and *wbpL*) downregulated on the *psrA* array displayed increased outer membrane permeability (Fig. 2.6A). Further, a *wbpM* mutant, which has been previously demonstrated to be B-band deficient and necessary for LPS O antigen biosynthesis (Burrows et al. 1996; Burrows et al. 2000) was shown here to be peptide supersusceptible (Fig. 2.5A), and swarming deficient (Fig. 2.5B), and display substantial increased outer membrane permeability (Fig. 2.6B). This correlates with previous findings in *Proteus mirabilis* that LPS O antigen contributes to both antimicrobial peptide resistance and swarming motility (McCoy et al. 2001). Other possible candidates to explain peptide supersusceptibility would be gene products involved in energy generation and thus potentially in interaction of cationic peptides with the cytoplasmic membrane. One of the tested genes, *coxA*, encoding a subunit of cytochrome C oxidase, was 3.4-fold down-regulated in the arrays (Table 2.4) and a

mutant in this gene led to a modest supersusceptibilty phenotype relative to wild-type (Fig. 2.5A).

The substantial swarming motility impairment displayed by the *psrA* mutant indicated that PsrA is involved in the complex regulatory mechanisms controlling this complex adaptation (Overhage et al. 2008). Although swarming requires both flagella and pili (Overhage et al. 2007), the *psrA* mutant did not exhibit a defect in either flagella-mediated swimming or type IV-pilus-mediated twitching motility, indicating that it did not control a primary motility organelle. PsrA regulation of swarming might reflect the down-regulation of both Lon protease (Table 2.3), which is required for normal swarming (Marr et al. 2007), and the LPS O antigen B-band biosynthetic gene cluster, since the *wbpM* mutant was also swarming deficient (Fig. 2.5B). Our observation that LPS O antigen biosynthetic genes (*wbpG*, *wbpI*, *wbpL*; Table 2.4) were down-regulated in the *psrA* mutant also may help explain the swarming impairment as LPS O antigen is required for swarming motility in other bacteria (Belas et al. 1995; McCoy et al. 2001; Toguchi et al. 2000).

Biofilm formation in *P. aeruginosa* is initiated by initial attachment of cells to a surface, followed by complex steps leading to development of mature biofilms (Parsek and Greenberg 2005). The *psrA* biofilm impairment phenotype was likely related in part to early stages, as the *psrA* mutant displayed a significant impairment in rapid attachment to the polystyrene surface used for the simple biofilm experiments described here (Fig. 2.3C). Although *psrA* mutants were able to attach and form simple biofilms, they did this at significantly reduced levels comparable to wild-type. Two possible genes that might influence the regulation of biofilm formation by PsrA are *lon* (Marr et al. 2007) and the *pprB* response regulator gene (Fig. 2.5D; found directly adjacent to the *tad* gene cluster) in

the *psrA* mutant microarray since mutants in both displayed impaired biofilm formation. The finding that the *psrA* mutant displays both impaired biofilm formation and swarming motility suggests that PsrA is an integral component of the regulatory network that controls these two separate complex adaptations, and is consistent with observations that other regulators control both processes (Shrout et al. 2006; Caiazza et al. 2007; Overhage et al. 2007).

Our results are consistent with previous observations that RpoS is a negative regulator of the type III secretion system, since *rpoS* is positively regulated by PsrA (Table 2.2; Kojic and Venturi 2002; Kojic et al. 2005). PsrA was previously shown to be a positive regulator of the type III secretion system in a mucoid strain of *P. aeruginosa* grown in complex medium (Shen et al. 2006). In contrast, the data presented here favor negative regulation of this secretion system by PsrA in the non-mucoid *P. aeruginosa* strain PAO1 grown in defined medium. We presume this is because of other underlying regulatory mutations that are known to occur in mucoid isolates of *P. aeruginosa*. Consistent with these observations, the *psrA* mutant presented here had no effect on cytotoxicity towards epithelial cells which is partially dependent on type III secretion (data not shown).

PA0506, an acyl-CoA dehydrogenase, was highly up-regulated in the *psrA* mutant (43-fold according to qRT-PCR confirmation experiments). This gene was also a previously characterized target of PsrA (Kojic et al. 2005) and our microarray analysis confirmed PsrA as a negative regulator of this gene (Table 2.2). Noteworthy, PA0506 has previously been shown to be mutated in cystic fibrosis *P. aeruginosa* isolates, possibly indicating that mutation of this gene might favor chronic infection and that this gene might be involved in adaptation to the cystic fibrosis lung (Smith et al. 2006).

Our microarray gene lists uncovered many other interesting genes as part of the PsrA regulon. The downregulation of genes of the *tad* (tight adherence) cluster (Table 2.3), involved in the assembly of extracellular cell-surface Flp pili appendages (de Bentzmann et al. 2006; Tomich et al. 2007), was consistent with the attachment defect in *psrA* mutants in the face of normal piliation and twitching motility. However, no differences were seen in biofim formation by mutants in key components of the *tad* cluster (Fig. 2.5D). Probable type II secretion system genes (PA0683, PA2672, PA2673) also showed modest to strong repression (Table 2.2 and 2.3) and could encode adhesion-associated products (based on the similarity of pili to the components of the type II secretion system), and thus might contribute to the attachment and biofilm phenotype observed for the *psrA* mutant.

Biofilm formation, attachment, and swarming motility appear to be very important in *P. aeruginosa* colonization and virulence, while it has been strongly suggested that *Pseudomonas* is exposed to cationic antimicrobial peptides during infections and occasionally polymyxin B during therapy. The involvement of PsrA in these processes, together with its inducibility by a cationic antimicrobial peptide, highlights the likely importance of this gene in adaptation to the lung environment through regulation of virulence and antimicrobial peptide resistance. The results presented here are consistent with the massive complexity of the regulatory network influencing these processes.

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

Strain or plasmid	Genotype or characteristics[a]	Source or reference
WT	Wild-type *P. aeruginosa* PAO1; H103	Lab collection
UW WT	UW wild-type *P. aeruginosa* PAO1	Jacobs et al. 2003
UW-*psrA*	*psrA*::IS*lacZ*/hah-Tc^R; insertion at 46(702 bp) in *psrA*; derived from UW WT	Jacobs et al. 2003
psrA mutant	*psrA*::IS*lacZ*/hah-Tc^R, H103 background; Tc^R	This study
psrA (Tn7-*psrA*$^+$)	*psrA* mutant with Tn7-*psrA*$^+$ integrated; Tc^R, Gm^R	This study
PA14	Wild-type *P. aeruginosa* PA14	Liberati et al. 2006
coxA	05_2:A11; derived from PA14	Liberati et al. 2006
etfA	04_4:A12; derived from PA14	Liberati et al. 2006
fhp	09_1:F11; derived from PA14	Liberati et al. 2006
mexC	01_4:H2; derived from PA14	Liberati et al. 2006
pprB	08_3:C3; derived from PA14	Liberati et al. 2006
rhlG	05_3:A8; derived from PA14	Liberati et al. 2006
flp	14_1:F4; derived from PA14	Libertai et al. 2006
rcpA	01_2:B12; derived from PA14	Liberati et al. 2006
tadA	01_2:A7; derived from PA14	Liberati et al. 2006
tadB	04_2:H5; derived from PA14	Liberati et al. 2006
wbpM	03_4:E4; derived from PA14	Liberati et al. 2006
wzz	06_1:F2l; derived from PA14	Liberati et al. 2006
PA1883 (homolog)	12_1:A7; derived from PA14	Liberati et al. 2006
wbpI	*wbpI*:: IS*lacZ*/hah-Tc^R; insertion 807(1065 bp)	Jacobs et al. 2003
wbpL	*wbpL*:: IS*lacZ*/hah-Tc^R; insertion 302(1020 bp)	Jacobs et al. 2003
E .coli TOP10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen Inc.
pCR-Blunt II-TOPO	PCR cloning vector, Kan^R	Invitrogen Inc.
pCR-*psrA*$^+$	pCR-BluntII-TOPO harboring 1.12 kb *psrA* amplicon	This study
pUC18-mini-Tn7T-Gm	Suicide plasmid; Gm^R, Amp^R	Choi et al. 2005
pUC-Tn7-*psrA*$^+$	pUC18-mini-Tn7T-Gm with 1.12 kb *psrA* fragment from pCR-*psrA*	This study
pTNS2	Transposition helper plasmid; Amp^R	Choi et al. 2005

[a] Antibiotic resistance phenotypes: Amp^R, ampicillin for *E. coli* and carbenicillin for *P. aeruginosa*; Gm^R, gentamicin; Kan^R, kanamycin; Tc^R, tetracycline.

Table 2.2 Known PsrA targets significantly dysregulated in *psrA* mutants as determined using microarray. Only genes showing ≥2-fold change in the *psrA* mutant are depicted. * indicates confirmation of gene regulation by qRT-PCR.

Gene ID[a]	Name	Fold change[b]	*P* value	Description[a]
PA0506		16.2*	<0.0001	probable acyl-CoA dehydrogenase
PA2673	*hplV*	−27.9	<0.0001	probable type II secretion system protein
PA2951	*etfA*	3.3	<0.0001	electron transfer flavoprotein alpha-subunit
PA2952	*etfB*	3.8	<0.0001	electron transfer flavoprotein beta-subunit
PA2953		6.4*	<0.0001	electron transfer flavoprotein-ubiquinone oxidoreductase
PA3013	*foaB*	15.2*	<0.0001	fatty-acid oxidation complex beta-subunit
PA3014	*faoA*	11.6	<0.0001	fatty-acid oxidation complex alpha-subunit
PA3622	*rpoS*	−2.5*	<0.0001	alternative sigma factor RpoS

[a] Information according to the *P. aeruginosa* genome website (www.pseudomonas.com/)
[b] Fold regulation of genes differentially expressed in the *psrA* mutant relative to WT. A positive number indicates transcript up-regulation in the *psrA* mutant.

Table 2.3 Type III secretion, adhesion (*tad*), motility, and type II secretion genes significantly dysregulated in *psrA* mutants as determined using microarray. * indicates confirmation of gene regulation by qRT-PCR.

Gene ID[a]	Name	Fold change[b]	*P* value	Description[a]
Type III secretion				
PA0044	*exoT*	4.6	0.0005	exoenzyme T; Type III secretion system effector
PA1695	*pscP*	3.0	<0.0001	translocation protein in type III secretion
PA1697	*pscN*	2.1	0.004	ATP synthase in type III secretion system
PA1698	*popN*	2.6	0.004	outer membrane protein PopN
PA1699		2.3	0.003	conserved protein in type III secretion
PA1700		2.0	0.0009	conserved protein in type III secretion
PA1701		4.5	0.0003	conserved protein in type III secretion
PA1702		2.0	0.0002	conserved protein in type III secretion
PA1703	*pcrD*	2.0	0.01	type III secretory apparatus protein PcrD
PA1705	*pcrG*	3.8	0.002	regulator in type III secretion
PA1706		2.1	0.001	type III secretion protein PcrV
PA1707	*pcrH*	2.4	0.0002	regulatory protein PcrH
PA1708	*popB*	3.1	0.005	translocator protein PopB
PA1709	*popD*	2.7	0.002	translocator protein PopD
PA1710	*exsC*	2.1	<0.0001	exoenzyme S synthesis protein C
PA1712	*exsB*	2.0	0.001	exoenzyme S synthesis protein B
PA1715	*pscB*	2.5	0.001	type III export apparatus protein
PA1717	*pscD*	2.8	0.01	type III export protein PscD
PA1721	*pscH*	2.0	0.01	type III export protein PscH
PA1723	*pscJ*	2.1	0.01	type III export protein PscJ
PA2191	*exoY*	2.1	0.0004	adenylate cyclase ExoY
PA3841	*exoS*	2.6	0.001	exoenzyme S; Type III secretion effector
PA3842	*orf1*	3.5	0.001	chaperone for ExoS secretion
Adhesion and motility				
PA0176	*aer2*	–4.2	0.04	aerotaxis methyl-accepting chemotaxis protein
PA1803	*lon*	–2.0	0.05	ATP-dependent Lon protease
PA4296	*pprB*	–4.9*	0.05	PprB two-component response regulator
PA4300	*tadC*	–2.2	0.04	Flp pilus assembly protein, PilC-like
PA4302	*tadA*	–4.1*	0.01	TadA traffic ATPase in Flp pilus assembly
PA4303	*tadZ*	–2.7	0.02	Flp pilus assembly protein
PA4305	*rcpC*	–2.1	0.05	Flp pilus assembly protein
Type II secretion				
PA0683	*hxcY*	–4.4	0.008	Hxc type II secretion system membrane protein
PA1871	*lasA*	10.5	0.002	LasA protease
PA2672	*hplW*	–2.5	0.003	type II secretion prepilin peptidase substrate

[a] Information according to the *P. aeruginosa* genome website (www.pseudomonas.com/)
[b] Fold regulation of genes differentially expressed in the *psrA* mutant relative to WT. A positive number indicates transcript up-regulation in the *psrA* mutant.

Table 2.4 Other known genes significantly dysregulated in *psrA* mutants as determined using microarray. Dysregulated hypothetical or unclassified ORFs are not included. Only genes showing ≥2-fold change in the *psrA* mutant are depicted.

Gene ID[a]	Name	Fold change[b]	*P* value	Description[a]
PA0106	*coxA*	−3.4	0.01	cytochrome c oxidase, subunit I
PA0217	*mdcR*	−4.8	0.007	transcriptional regulator
PA0459	*clpC*	−3.5	0.05	ClpA/B protease ATP binding subunit
PA0507		−3.2	0.001	probable acyl-CoA dehydrogenase
PA0511	*nirJ*	4.8	0.007	heme d1 biosynthesis protein
PA0512	*nirH*	2.3	0.04	conserved hypothetical protein
PA0513	*nirG*	4.5	0.008	probable transcriptional regulator
PA0517	*nirC*	−2.6	0.001	c-type cytochrome
PA0530		−4.3	0.04	pyridoxal phosphate-dependent aminotransferase
PA0588	*yeaG*	−3.3	0.001	conserved hypothetical protein
PA0707	*toxR*	−4.2	0.009	ToxR/RegA transcriptional regulator
PA0719		−4.1	0.009	bacteriophage Pf1 protein
PA0724	*coaA*	2.8	0.02	coat protein A of bacteriophage Pf1
PA0840		−8.9	0.006	probable oxidoreductase
PA0852	*cbpD*	2.2	0.04	chitin-binding protein CbpD
PA1041		−12.9	0.005	OmpA-family outer membrane protein
PA1173	*napB*	−2.6	0.02	cytochrome c-type protein NapB
PA1187	*lcaD*	−2.1	0.04	acyl-CoA dehydrogenase
PA1399		−2.0	0.004	Probable LysR-family transcriptional regulator
PA1648		−3.0	0.002	probable oxidoreductase
PA1649		−6.3	0.0003	probable short-chain dehydrogenase
PA1650		−2.0	0.008	probable transporter
PA1828		−3.1	0.006	probable short-chain dehydrogenase
PA1881		−2.2	0.03	probable oxidoreductase
PA1883		−10.2	0.002	NADH-ubiquinone/plastoquinone oxidoreductase
PA1927	*metE*	2.1	0.007	methionine synthase
PA1976		−2.2	0.0009	two-component sensor kinase
PA1978	*agmR*	−2.9	0.003	two-component response regulator
PA1982	*exaA*	−2.7	0.03	quinoprotein ethanol dehydrogenase
PA1983	*exaB*	−2.7	0.02	cytochrome c550
PA1984	*exaC*	−3.6	0.05	aldehyde dehydrogenase
PA1985	*pqqA*	−4.5	0.002	pyrroloquinoline quinone biosynthesis protein A
PA2124		3.2	0.01	probable dehydrogenase
PA2277	*arsR*		0.05	transcriptional regulator
PA2278	*arsB*		0.02	ion transport membrane protein
PA2339	*mtlF*	−2.1	0.05	maltose/mannitol transport protein
PA2350		3.1	0.02	probable ATP-binding component of ABC transporter
PA2352		2.4	0.03	probable glycerophosphoryl diester phosphodiesterase
PA2371	*clpV3*	−3.0	0.02	probable ClpA/B-type protease
PA2396	*pvdF*	2.1	0.05	pyoverdine synthetase F

Gene ID[a]	Name	Fold change[b]	*P* value	Description[a]
PA2398	*fpvA*	2.5	0.03	ferripyoverdine outer membrane receptor
PA2432		5.8	0.03	probable transcriptional regulator
PA2469		–4.1	0.01	probable transcriptional regulator
PA2522	*czcC*	–3.7	0.01	outer membrane efflux protein
PA2535		–2.3	0.05	probable oxidoreductase
PA2536	*ynbB*	2.2	0.03	phosphatidate cytidylyltransferase
PA2550		–3.7	0.001	probable acyl-CoA dehydrogenase
PA2551		–2.3	0.001	probable transcriptional regulator
PA2573		–2.9	0.02	probable chemotaxis transducer
PA2664	*fhp*	–83.1	0.0004	flavohemoprotein
PA2892		–2.2	0.04	probable short-chain dehydrogenase
PA2893		–2.4	0.03	probable very-long-chain acyl-CoA synthetase
PA2939	*pepB*	–4.9	0.007	secreted aminopeptidase
PA3077		2.5	0.03	two-component response regulator
PA3145	*wbpL*	–2.5	0.03	WbpL rhamnosyltransferase in LPS biosynthesis
PA3148	*wbpI*	–4.4	0.04	UDP-N-acetylglucosamine 2-epimerase WbpI
PA3150	*wbpG*	–2.0	0.05	LPS biosynthesis protein WbpG
PA3152	*hisH2*	–2.3	0.03	glutamine amidotransferase
PA3277		–4.2	0.0003	probable short-chain dehydrogenase
PA3327		–2.7	0.02	probable non-ribosomal peptide synthetase
PA3387	*rhlG*	–2.7	0.0002	beta-ketoacyl reductase
PA3409		–2.1	0.05	probable transmembrane sensor
PA3418	*ldh*	–3.0	<0.0001	leucine dehydrogenase
PA3427		–2.6	0.002	probable short-chain dehydrogenases
PA3454		–2.1	0.002	probable acyl-CoA thiolase
PA3630		2.2	0.05	probable transcriptional regulator
PA3723	*yqiM*	–3.0	0.005	FMN oxidoreductase
PA3877	*narK1*	–3.8	0.0008	nitrite extrusion protein 1
PA3957		3.7	0.01	probable short-chain dehydrogenase
PA4135		–2.9	0.02	probable transcriptional regulator
PA4497		–4.1	0.01	binding protein component of ABC transporter
PA4599	*mexC*	–2.0	0.008	RND multidrug efflux membrane fusion protein
PA4654		–5.6	0.02	major facilitator superfamily (MFS) transporter
PA4911		–6.6	0.004	probable permease of ABC amino acid transporter
PA5020		–3.7	0.003	probable acyl-CoA dehydrogenase
PA5097	*hutT*	2.7	0.02	amino acid permease
PA5141	*hisA*	2.8	0.02	histidine biosynthesis protein
PA5187		–3.2	0.0004	probable acyl-CoA dehydrogenase
PA5188		–2.0	0.0004	probable 3-hydroxyacyl-CoA dehydrogenase
PA5234		–2.2	0.005	probable oxidoreductase
PA5302	*dadX*	–3.3	0.02	catabolic alanine racemase

[a] Information according to the *P. aeruginosa* genome website (www.pseudomonas.com/)
[b] Fold regulation of genes differentially expressed in *psrA* mutant relative to WT. A positive number indicates transcript up-regulation in the *psrA* mutant.

Figure 2.1

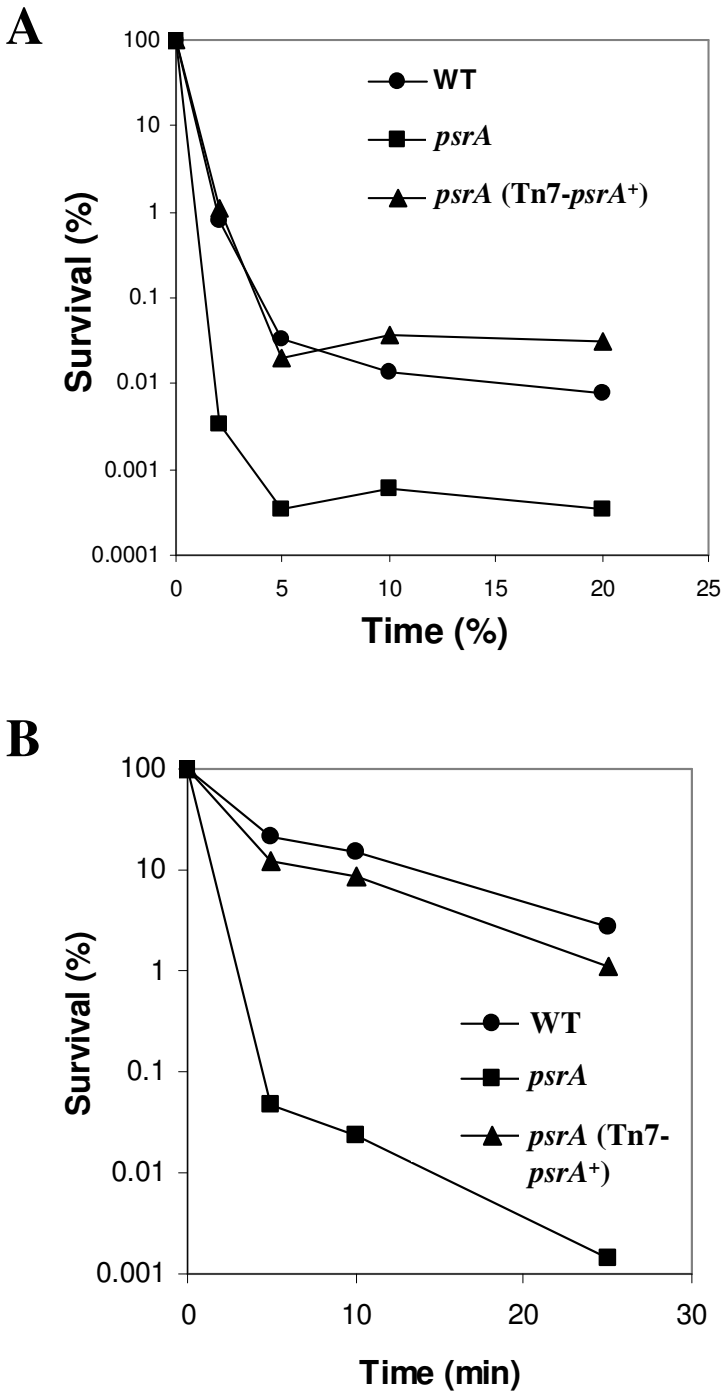


Figure 2.1 Intrinsic polymyxin B and antimicrobial peptide super-susceptibility in *psrA* mutants. Intrinsic sensitivity was analyzed by first growing cells to mid-log in BM2-glucose with 2 mM Mg^{2+}, then exposing them to 1 µg/ml polymyxin B (A) or 64 µg/ml indolicidin (B), and plating diluted aliquots for survivors. For each condition, one representative experiment is shown of 4 independent experiments that produced identical trends.

Figure 2.2

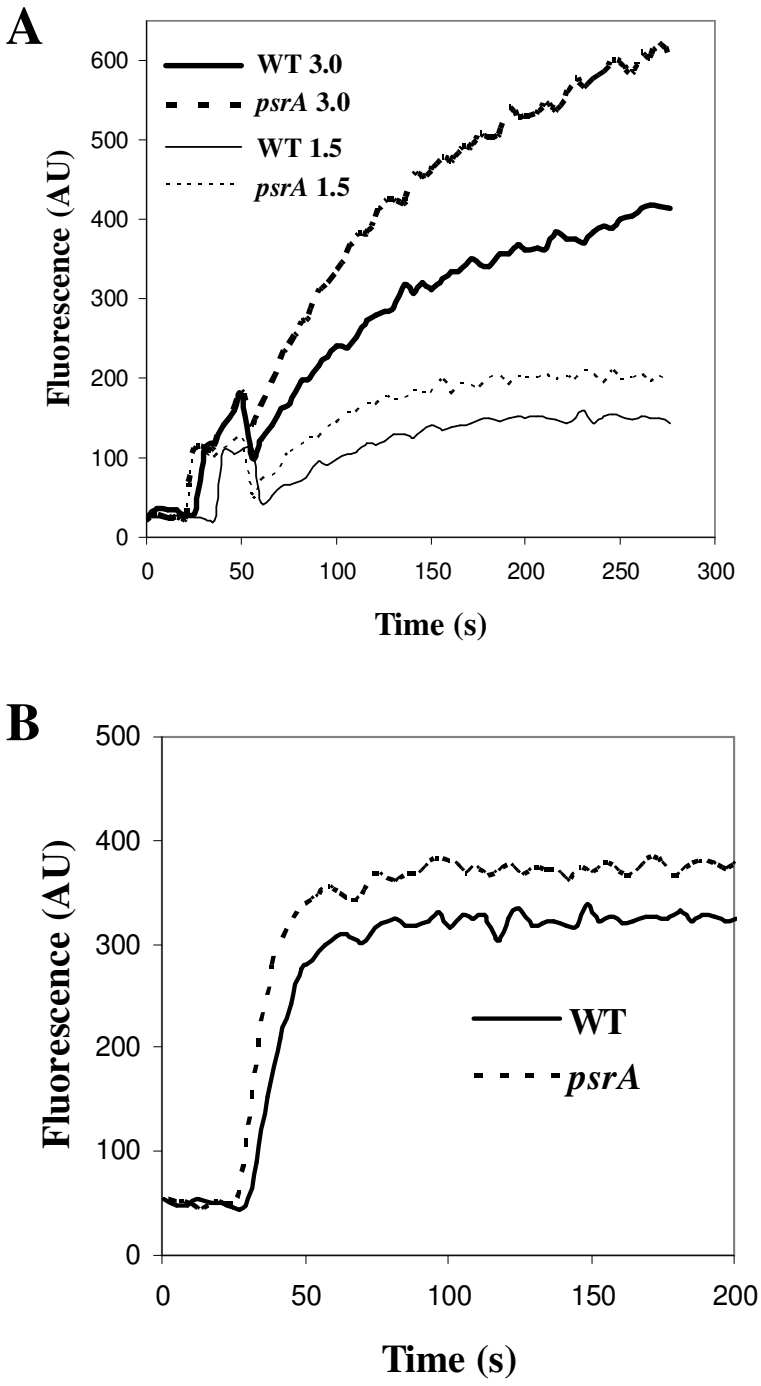
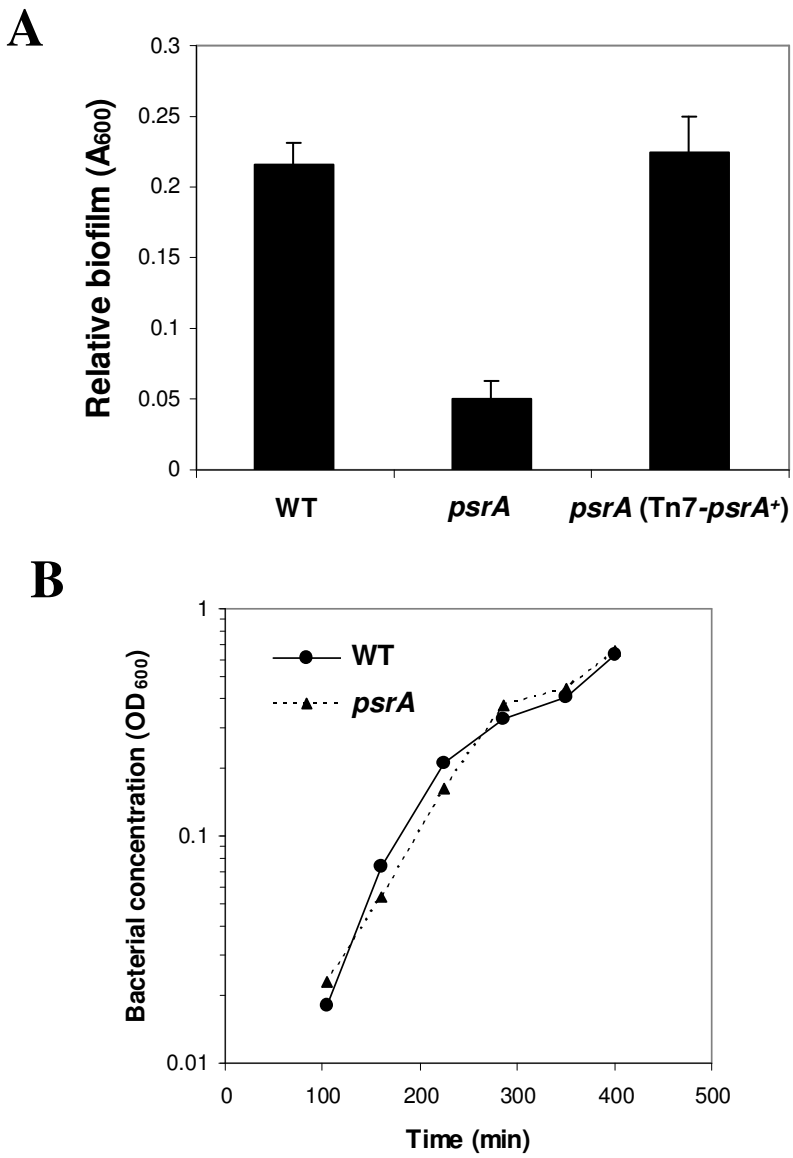


Figure 2.2 PsrA mutation effect on outer membrane permeabilization to peptides. Cells from mid-logarithmic phase cultures of wild-type and *psrA* mutant were exposed to 1.5 or 3 µg/ml of indolicidin (A) or 0.2 µg/ml of polymyxin B (B) and the increase in fluorescence due to peptide-stimulated partitioning of NPN into the outer membrane was measured. NPN experiments were repeated independently three times, each of which produced reproducible observed trends, and one representative experiment is shown.

Figure 2.3


A
Relative biofilm (A600)
0.3
0.25
0.2
0.15
0.1
0.05
0
WT
psrA
psrA (Tn7-psrA+)
B
Bacterial concentration (OD600)
1
0.1
0.01
WT
psrA
0
100
200
300
400
500
Time (min)

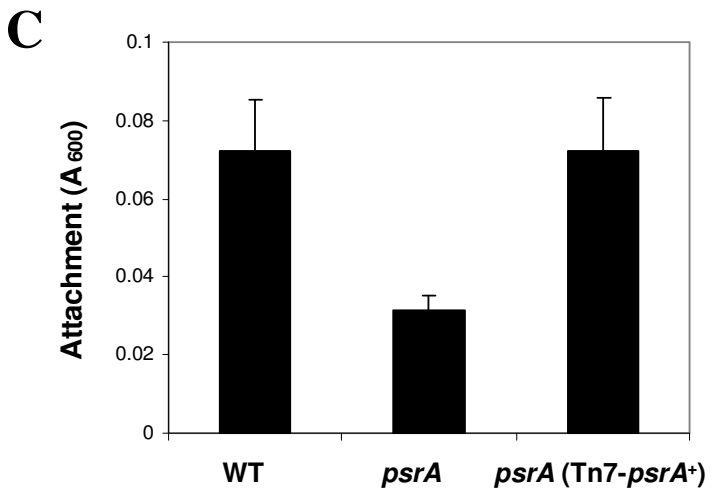


Figure 2.3 Defects in biofilm formation and attachment in *psrA* mutants. (A) Requirement for *psrA* in static biofilm formation. Cells were grown at 37°C for 18 h in polystyrene microtitre plates containing LB. Adherent biofilm cells were stained with crystal violet followed by ethanol solubilization of the crystal violet and quantification (A_{600}) of stained wells. (B) Planktonic growth of *psrA* mutant under these biofilm conditions was unaffected. Planktonic cells were grown as in biofilm microtitre assays and turbidity was measured (OD_{600}). (C) Requirement of *psrA* for rapid attachment. Rapid attachment was assayed using mid-log cells for 30 min. Attached cells were stained with crystal violet followed by ethanol extraction of the crystal violet for quantification at A_{600}. Results are shown as averages ± standard deviation for three biological experiments, each with eight technical repeats.

Figure 2.4

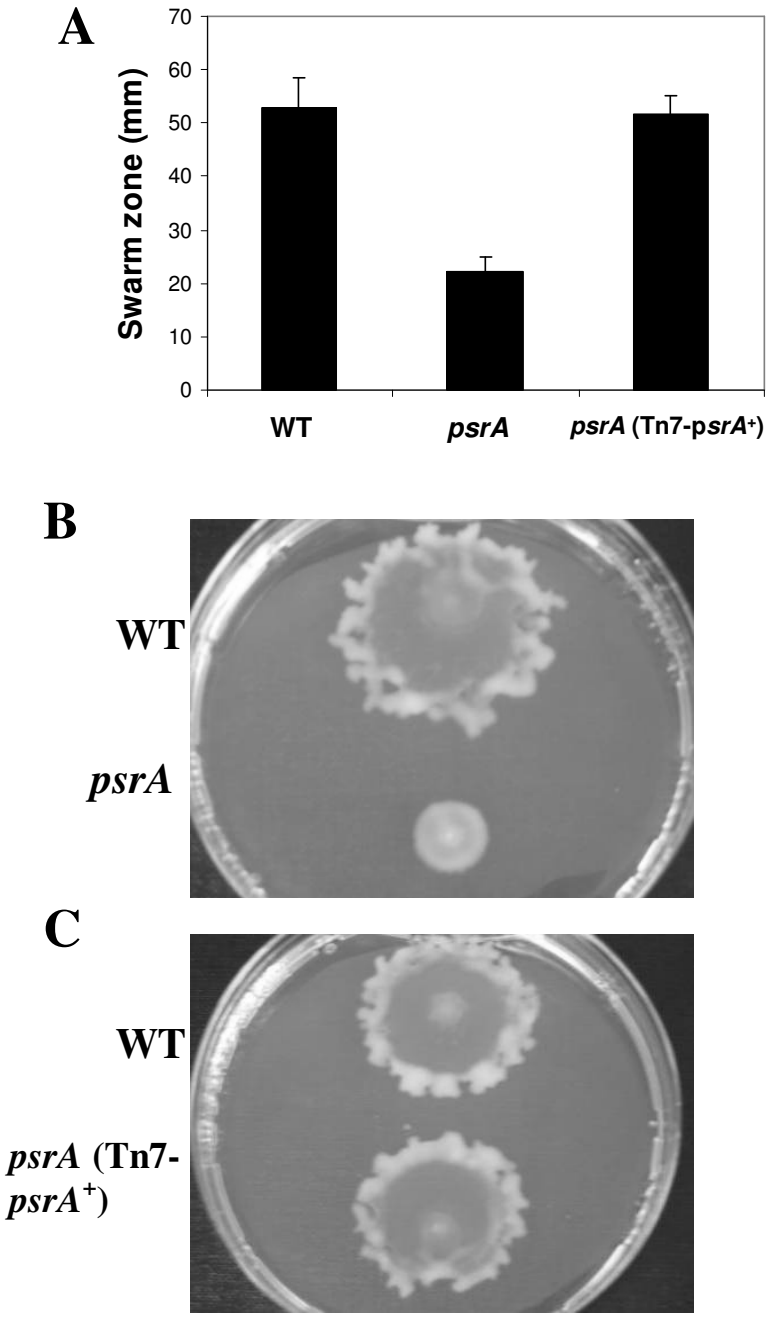
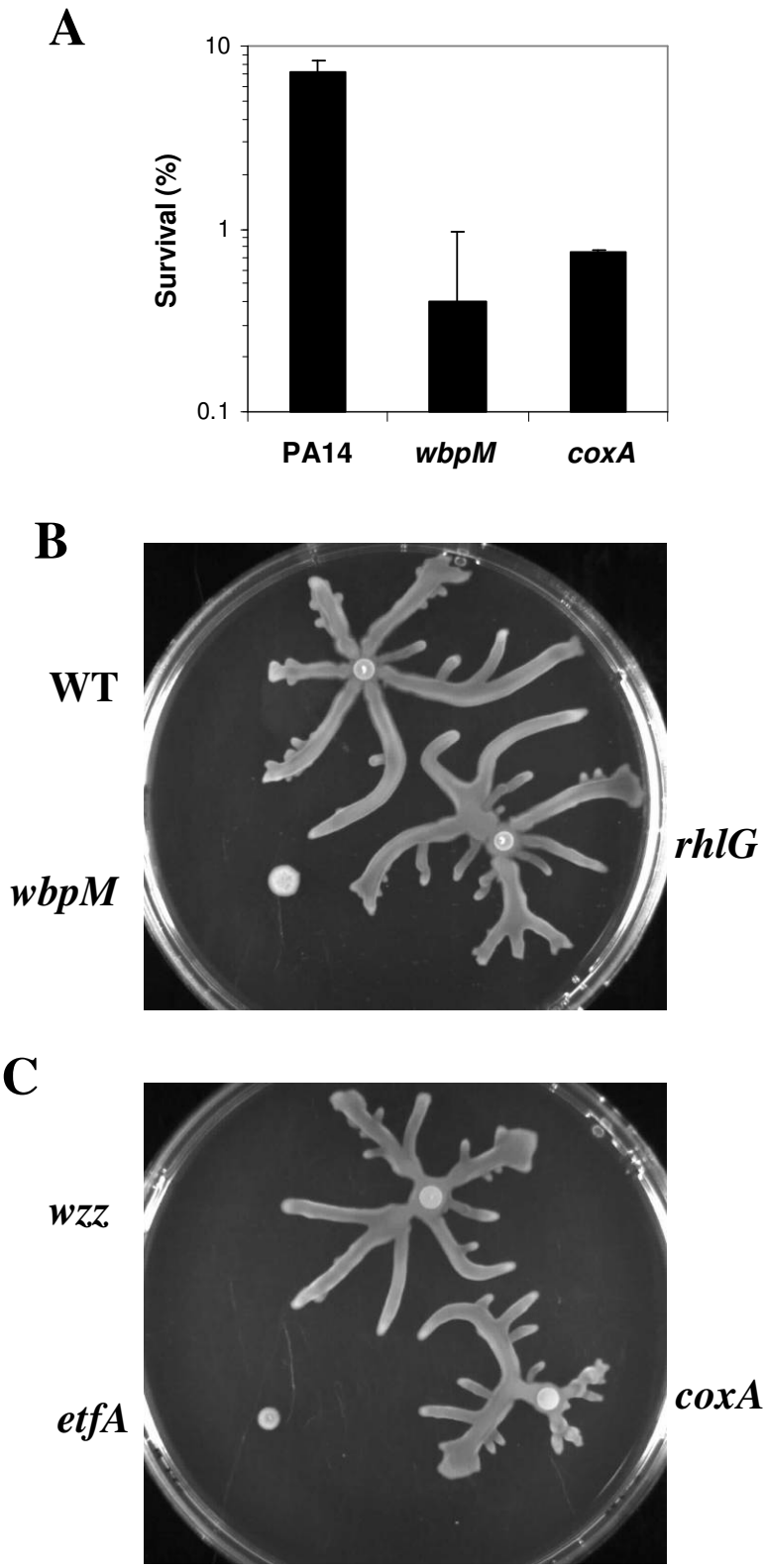


Figure 2.4 Swarming motility defect in *psrA* mutants. (A) Swarming motility was evaluated by spot inoculating cells onto BM2 swarm plates containing 0.5% agar at 37°C for 18 h. Diameters of the characteristic circular PAO1 swarm zones were measured and averages ± standard deviation are reported for three biological repeats each with three technical repeats. (B) Representative WT (top) and *psrA* mutant (bottom) swarming morphology. (C) Complemented *psrA* mutant swarming morphology: WT (top) and *psrA* (Tn7-*psrA*$^+$; bottom).

Figure 2.5



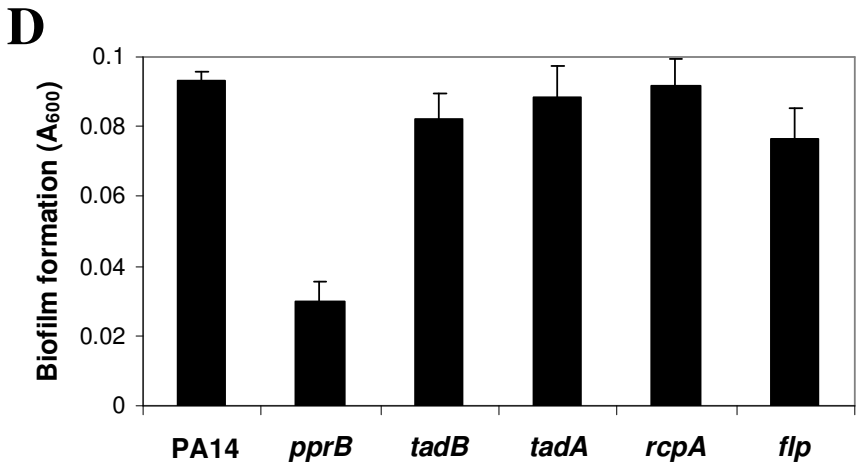


Figure 2.5 Peptide susceptibility, swarming and biofilm analysis of PA14 mutants in selected genes transcriptionally downregulated in *psrA* mutants. (A) Intrinsic indolicidin super-susceptibility time-course killing curve analysis of PA14 *coxA* and *wbpM* mutants compared to wild-type. Cells were grown to mid-logarithmic phase and exposed to 64 µg/ml indolicidin and survival was assessed after 25 min. Shown is the means ± standard deviation of three independent experiments are shown. (B and C) PA14 mutants in *wbpM* and *etfA* cannot undergo normal swarming motility. Mid-logarithmic phase cultures were spot inoculated onto PA14-type swarm agar plates and incubated for 18 h at 37°C. Swarming assays were performed with three independent cultures of each strain and a representative swarm morphology was photographed. (D) Biofilm impairment of the *pprB* mutant. Overnight cultures were diluted 1:500 and then grown for 18 h at 37°C in microtitre wells, followed by washing with dH_20 and staining with crystal violet. Biofilm formation was repeated three times, each time with six technical replicates, and shown are the averages ± standard deviations of one experiment.

Figure 2.6

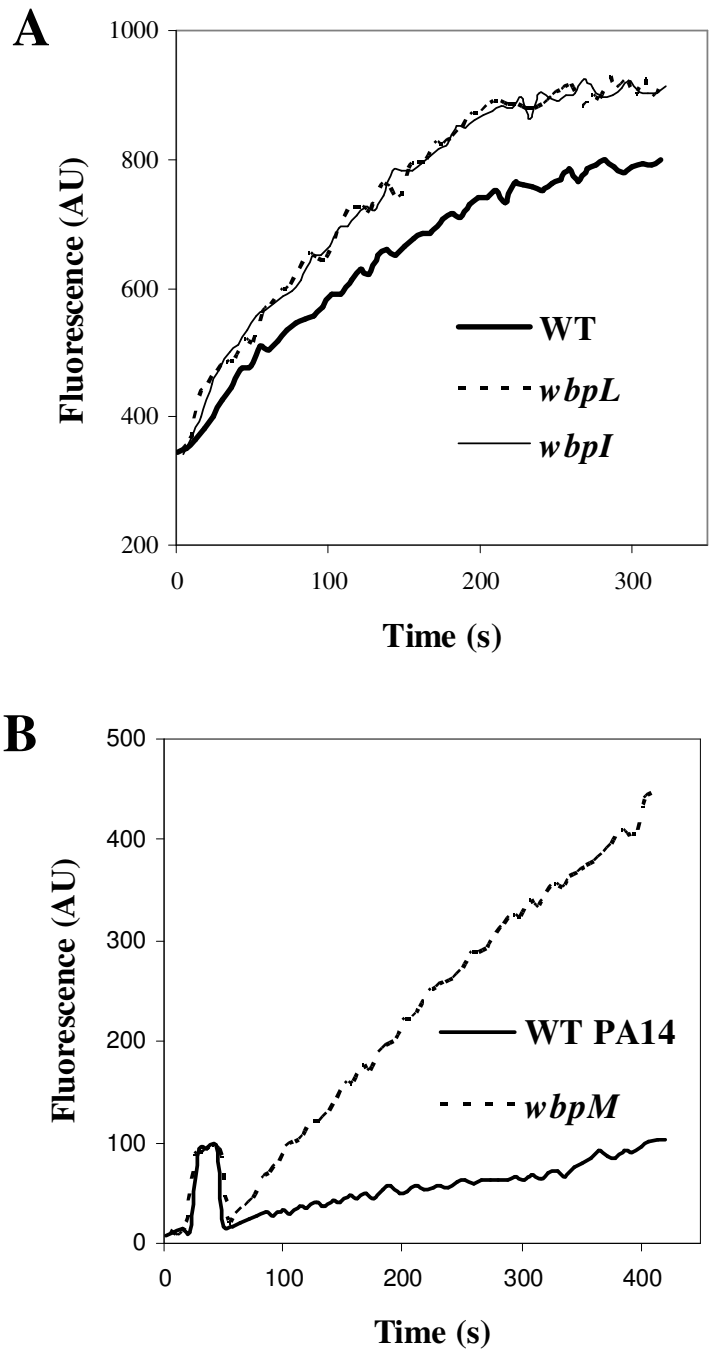


Figure 2.6 Mutants in the B-band O antigen biosynthetic operon demonstrating altered outer membrane permeabilization by indolicidin antimicrobial peptides. (A) Cells from mid-logarithmic phase cultures of wild-type PAO1 and *wbpI* and *wbpL* mutants were exposed to 3.0 µg/ml indolicidin and the increase in fluorescence due to peptide-stimulated partitioning of NPN into the outer membrane was measured. (B) Cultures of wild-type PA14 and *wbpM* mutant were exposed to 1.5 µg/ml indolicidin. One representative experiment is shown from at least three independent trials, each of which produced the same trends

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CHAPTER 3 – PhoQ of *Pseudomonas aeruginosa*[*]

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous environmental Gram-negative bacterium and is also an opportunistic pathogen capable of causing both acute and chronic infections (Stover et al. 2000). *P. aeruginosa* acute infections are often associated with the immunocompromised, especially burn victims and patients requiring continuous mechanical ventilation (El Solh et al. 2008), and it is the third most predominant nosocomial pathogen in North America (Fridkin et al. 1999). *P. aeruginosa* also is well known as a dominant pathogen in chronic lung infections in individuals with cystic fibrosis, the most prevalent inherited disorder of Caucasian populations (Lyczak et al. 2002; Gibson et al. 2003). Both *P. aeruginosa* infection types are noted for being intrinsically refractory to antimicrobial treatment, which contributes to the high degree of morbidity and mortality associated with *P. aeruginosa* infections (Hancock and Speert 2000; Mesaros et al. 2007).

A number of genetic differences have been observed between *P. aeruginosa* strains causing chronic or acute infections (Furukawa et al. 2006). Acute *P. aeruginosa* infections have been fairly well-characterized and are broad range, affecting humans, animals, plants, nematodes and amoebae (Rahme et al. 2000). In contrast to acute infections, chronic infections differ most markedly because of their narrow host range, being observed primarily in the lungs of humans with cystic fibrosis and also on catheters and other indwelling medical devices.

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[*] A version of this chaper will be submitted for publication.
Gooderham, W. J., J. B. McPhee, M. Bains, S. Gellatly, F. Sanschagrin, C. Cosseau, R. C. Levesque, and R. E.W. Hancock. Regulation of virulence by the PhoQ sensor kinase in *Pseudomonas aeruginosa*.

The PhoP-PhoQ two-component regulatory system has been well studied in a number of organisms, including *Salmonella* (Miller et al. 1989; Bader et al. 2005) and *P. aeruginosa* (Macfarlane et al. 1999; Macfarlane et al. 2000). This classical-type two-component system comprises a membrane-bound sensor histidine kinase PhoQ which recognizes a stimulus (limiting Mg^{2+} and in *Salmonella* but not *Pseudomonas* cationic antimicrobial peptides) and then acts to auto-phosphorylate and phosphorylate and/or dephosphorylate its cognate response regulator PhoP, thus influencing this regulator's ability to bind DNA and influence gene expression (Groisman 2001). In *S.* Typhimurium, both PhoP and PhoQ are required for full virulence (Miller et al. 1989). In contrast, in *P. aeruginosa* a mutant in *phoQ*, but not *phoP*, was attenuated for virulence in the neutropenic mouse model of acute infection (Macfarlane et al. 1999). In response to limiting concentrations of extracellular Mg^{2+}, *P. aeruginosa* PhoPQ is involved in resistance to polymyxin B and antimicrobial peptides (Macfarlane et al. 1999). Polymyxins have recently re-emerged as systemic therapeutic agents due to their good activity against multi-drug resistant Gram-negatives including *P. aeruginosa* (Li et al. 2006; Zavascki et al. 2007).

Here, we demonstrate that a *P. aeruginosa* PAO1 strain containing a mutation in *phoQ* was highly attenuated for persistence in a chronic lung infection model. In addition, the *phoQ* mutant displayed reduced twitching motility and was less cytotoxic towards human bronchial epithelial cells, suggesting that the *in vivo* virulence defect observed may be partly due to these phenotypes. Through the use of microarrays it was shown that the loss of PhoQ led to up-regulation of PhoP- and PmrA-regulated genes and that the PhoQ regulon additionally includes non-Mg^{2+} regulated genes. The potential of all of these regulatory alterations to contribute to virulence are discussed.

MATERIALS AND METHODS

Tissue culture, 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, Canada). Cultures were routinely grown in Luria-Bertani (LB) 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, 50 μg/ml, carbenicillin, 300 μg/ml, and gentamicin, 30 μg/ml. Routine genetic manipulations were carried out according to standard molecular biology procedures (Ausubel 1987).

The SV40-transformed, immortalized 16HBE4o- cell line (human bronchial epithelial cells) was a gift from Dr. D. Gruenert (University of California, San Francisco, CA). It was grown in cell culture flasks (Costar, Cambridge, MA) at 37°C in a 5% CO_2 atmosphere in MEM with Earle's salts (Invitrogen Inc., Canada) containing 10% FBS and 2 mM L-glutamine. Cells were passaged by treating the monolayer with Trypsin-EDTA (Invitrogen Inc., Canada) at 37°C for 5 min to dissociate the cells from the flask. The detached cells were transferred to a 50 ml centrifuge tube containing 20 ml complete MEM medium and then centrifuged for 5 min at 1000 × g. The supernatant was discarded and the cells were resuspended in MEM complete medium.

Competitive index (CI) determination.

For *in vivo* assays, bacteria were enmeshed into agar beads and the rat model of chronic lung infection was used (Cash et al. 1979). Bacterial cells embedded in agarose beads were prepared as described (van Heeckeren and Schluchter 2002). Briefly, cultures were grown in tryptic soy broth (TSB). A culture from the wild-type strain PAO1 containing

the pUCP19 plasmid and a culture from each mutant strain were grown overnight at 37°C. A 200 µl aliquot of an overnight culture was diluted 1:5 into fresh TSB to give a final concentration of approximately 1×10^{10} CFU/ml. 500 µl of a 1:1 mixture of wild-type and mutant bacteria was added to 4.5 ml of TSB. This culture was mixed with 50 ml of 2% sterile agarose (Nusieve GTG, FMC, Rockland, Maine) in phosphate-buffered saline (PBS), pH 7.2 at 48°C. The agarose-broth mixture was added to a 250 ml Erlenmeyer flask containing 200 ml of heavy mineral oil at 48°C and rapidly stirred on a magnetic stirrer in a water bath (setting 500-600 rpm on a Hotplate Stirrer, Model M13, Staufen, Germany). The mixture was cooled gradually with ice chips to 0°C over a period of 5 min. The agarose beads were transferred to a sterile 500 ml Squibb separator funnel and washed once with 200 ml of 0.5% deoxycholic acid sodium salt (SDC) in PBS, once with 200 ml of 0.25% SDC in PBS, and 3 times with 200 ml of PBS. Agarose beads were incubated on ice and the remaining PBS was removed. Prepared agarose beads were stored at 4°C; bacterial counts were stable for up to one month.

To determine the competitive index (CI), 1 ml of bead slurry was diluted in 9 ml PBS, homogenized and serial dilutions were plated on *Pseudomonas* isolation agar (PIA) and on Mueller Hinton agar (MHA, Difco) supplemented with appropriate antibiotics. CFUs were determined after 18h at 37°C and were used to calculate the input ratio of mutant to wild-type bacterial cells. Male Sprague-Dawley rats of 500 g in weight were used according to the recommendations of the ethics committee for animal treatment. The animals were anaesthetized using Isofluorane; inoculation into the lungs was done by intubation with ~120 µl of an agarose bead suspension containing a total of 10^5 bacterial cells. At 7 days post-infection, animals were sacrificed, lungs were removed and homogenized tissues were

plated on Mueller Hinton agar supplemented with appropriate antibiotics. After the *in vivo* passage, CFUs on plates represented the total number of bacteria present in the rat lungs. The CI is defined as the CFU output ratio of mutant when compared to wild-type strain, divided by the CFU input ratio of mutant to wild-type strain (Lehoux et al. 2000). The final CI was calculated as the geometric mean for animals in the same group and experiments were done at least in triplicate (Hava and Camilli 2002). Each *in vivo* competition was tested for statistical significance by Student's two-tailed *t* test (Hava and Camilli 2002).

RNA extraction, cDNA synthesis and hybridization to DNA microarrays.

P. aeruginosa PAO1 microarray slides were provided by The Institute of Genomic Research-Pathogenic Functional Genomics Resource Center (http://pfgrc.tigr.org/). Microarrays were performed using five biologically independent experiments (for each strain, five independent cultures). Cultures were grown for 18 hr in BM2-glucose medium supplemented with 2 mM $MgSO_4$. Cultures were then diluted 1/100 into the fresh media and cells were harvested at mid-logarithmic phase (high Mg^{2+} OD_{600} 0.5) after growth at 37°C with shaking (250 rpm). RNA was isolated using the Qiagen RNeasy Midi RNA isolation kit according to the manufacturer's protocols (Qiagen Inc., Canada). Contaminating genomic DNA was removed by treatment with the DNA-free kit (Ambion Inc., Austin, TX, USA). RNA was stored at -80°C with 0.2 units/µl of SUPERase-In RNase Inhibitor (Ambion Inc., Austin, TX, USA). RNA quality was assessed spectrophotometrically and by agarose gel electrophoresis. RNA was converted to cDNA and hybridized as previously described (McPhee et al. 2006).

Analysis of DNA Microarrays.

Data analysis of DNA microarrays was carried out as previously described (McPhee et al. 2006). Slide images from the 5 biologically independent experiments were quantified using ImaGene 6.0 Standard Edition software (BioDiscovery, Inc., El Segundo, CA, USA). Assessment of slide quality, normalization, detection of differential gene expression and statistical analysis was carried out with ArrayPipe (version 1.7) a web-based, semi-automated software specifically designed for processing of microarray data (http://koch.pathogenomics.ca/cgi-bin/pub/arraypipe.pl; Hokamp et al. 2004) using genome annotation from the *Pseudomonas* genome database (www.pseudomonas.com; Winsor et al. 2005). The following processing steps were applied: (1) flagging of markers and control spots, (2) subgrid-wise background correction, using the median of the lower 10% foreground intensity as foreground intensity as an estimate for the background noise, (3) data-shifting, to rescue most of the negative spots, (4) printTip LOESS normalization, (5) merging of replicate spots, (6) two-sided, one-sample Student's t-test on the $\log_2$-ratios within each experiment, and (7) averaging of biological replicates to yield overall fold-changes for each treatment group. Two-sided one-sample Student's *t* test for the $\log_2$ ratios within each experiment and averaging of the five biological replicates to obtain overall fold changes for *psrA* mutant relative to wild-type. Greater than or equal to 2-fold changes and Student's *t* test *P* value of ≤ 0.05 were used as the cut-offs for reporting expression changes.

Quantitative Real-Time PCR (RT-qPCR).

Total RNA was isolated, using RNeasy mini columns (Qiagen Inc., Mississauga, ON), from mid-logarithmic phase *P. aeruginosa* grown in BM2-glucose minimal media with 2 mM Mg^{2+}. RNA samples were treated with DNase I (Invitrogen Inc., 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 1 × 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 Inc., Carlsbad, CA). The RNA was subsequently destroyed by the addition of 170 mM NaOH and incubation at 65°C for 10 min. The reaction was then neutralized by the addition of HCl, and the cDNA was used as a template for Q-PCR. Analysis was carried out in the ABI Prism 7000 sequence detection system (Applied Biosystems) using the two step qRT-PCR kit with SYBR Green detection (Invitrogen). Melting curve analysis was performed to ensure specificity. Fold-change was determined using the comparative Ct method by comparison to the *rpsL* gene, encoding the 30S ribosomal protein S12. Experiments were repeated with three independent cultures, each assayed in duplicate, with the average ± standard deviation reported.

Cytotoxicity assays.

For the interaction assay, 16HBE4o- cells were seeded in 96-well tissue culture plates (Corning Life science, New York, USA) at 1×10^4 cells/well in complete MEM containing 10% FBS and 2 mM L-glutamine. The cells were incubated at 37°C and 5% CO_2 for approximately 2 days to achieve >90% confluency. Bacteria were grown in LB media to logarithmic phase, washed with PBS, and resuspended and diluted in serum-free MEM. The interaction assay was performed at a multiplicity of infection (MOI) of 50 bacteria per epithelial cell in serum-free MEM with 2 mM L-glutamine at 37°C and 5% CO_2. At the post-infection time point, cell supernatants were removed, placed in microfuge tubes and spun for five minutes at maximum speed to pellet the bacteria. The level of LDH in the supernatant was then assayed in triplicate using a colorimetric Cytotoxicity Detection Kit

(Roche Inc., Mannheim, Germany). As a positive control for maximum LDH release, cells were treated with 2% Triton X-100 (Sigma Inc., Oakville, Canada), resulting in complete cell lysis, while untreated cells were used to assess background (0%) LDH release.

Minimal inhibitory concentration determination (MICs).

MICs were assessed using standard broth microdilution procedures in BM2-glucose minimal medium containing 2 mM Mg^{2+} (Macfarlane 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 (Wu and Hancock 1999).

Twitching motility analysis.

Twitch motility plates were made with 1.5 % (wt/vol) LB and 1 % (wt/vol) agar. Bacteria were grown overnight in LB media, diluted 1:100 into fresh LB media and grown to mid-logarithmic phase, where 1 µl was used to inoculate twitch motility plates by stabbing down to the agar-plastic interface. The diameter of the twitch zone, visible at the interface between the agar and plastic bottom, was measured after 24 h incubation at 37°C.

Lettuce leaf model of infection.

We followed a protocol previously described (Rahme et al. 1997; Filiatrault et al. 2006). Briefly, lettuce leaves from Romaine hearts were washed with dH_20 and 0.1% bleach. Mid-logarithmic phase cultures of *P. aeruginosa* were washed twice with 10 mM $MgSO_4$. Lettuce leaf midribs were then inoculated with 10 µL of *P. aeruginosa* at a density of 1×10^8 cells/ml (~1×10^6 cells). Leaves were then placed in humid plastic containers at 37°C and symptoms were monitored for several days. Experiments were repeated three times on different days.

RESULTS

PhoQ mutants were highly attenuated for virulence in a model of chronic lung infection.

In a previous study, we examined the contribution of the PhoPQ and PmrAB two-component systems to Mg^{2+} regulation in *P. aeruginosa* (McPhee et al. 2006). Given the importance of the PhoPQ and PmrAB systems to virulence in *S*. Typhimurium, we examined *P. aeruginosa phoPQ* and *pmrAB* mutants in a rat model of chronic lung infection. These experiments indicated that the *phoQ* mutant was highly attenuated for maintenance in this model system (Table 3.2). Somewhat surprisingly, mutants in the cognate response regulator PhoP and in genes encoding the PmrA response regulator and the PmrB sensor kinase were not attenuated for virulence in this model of infection and were as capable of surviving as the wild-type parental strain (Table 3.2). It therefore appeared that the PhoQ sensor kinase was required for full virulence in this model of chronic lung infection.

Mutants in PA4773 (first gene in *pmrAB* operon) and *pmrE* (last gene in *arnBCADTEF-pmrE* aminoarabinose LPS modification operon) genes, which are part of operons important for resistance to polymyxin B and cationic antimicrobial peptides (McPhee et al. 2003; Lewenza et al. 2005), were similarly un-attenuated for maintenance in this model of infection. Also, mutants in the *feoAB* (PA4358/9) ferrous iron transport system and the *putP* (PA0783) sodium/proline symporter were similarly un-attenuated for virulence in this model (Table 3.2).

PhoQ mutants demonstrated reduced cytotoxicity toward human bronchial epithelial cells.

Human airway epithelial cells (16HBE4o-) were used to monitor the *in vitro* cytotoxicity of the *phoQ* mutant compared to its isogenic wild-type parent. This cell line maintains many of the properties of primary airway epithelial cells including the ability to form tight junctions and differentiation to produce microvilli and cilia (Cozens et al. 1994). In control experiments, wild-type and *phoQ* mutant cells cultured on the epithelial cells were shown to display the same growth properties (data not shown). The cytotoxic effects of both strains were monitored via lactate dehydrogenase (LDH) activity released from the dying cells. Both *phoQ* and wild-type strains induced LDH release on 16HBE4o- cells showing that they both displayed some cytotoxicity toward this cell line. However, wild-type *P. aeruginosa* displayed a 2.2-fold ($P < 0.05$) greater cytotoxicity than the *phoQ* mutant after 8.5 hours of interaction (Fig. 3.1). This difference remained at 16 hours with the wild-type strain always showing greater cytotoxicity than the *phoQ* mutant (Fig. 3.1). Introducing the wild-type $phoQ^+$ allele into the *phoQ* mutant *in trans* restored cytotoxicity to wild-type levels at both time points (Fig. 3.1). These results show that PhoQ is necessary for wild-type levels of cytotoxicity towards human bronchial epithelial cells *in vitro*.

PhoQ mutants were impaired in twitching motility.

Twitching of *P. aeruginosa* is an important form of motility dependent on type IV pili. These motility appendages have been associated with colonization, attachment and biofilm formation (O'Toole and Kolter 1998). As the *phoQ* mutant has been previously shown to have reduced swarming motility (Brinkman et al. 2001) and also a defect in biofilm formation (Ramsey and Whiteley 2004), we chose to analyze twitching motility in

this mutant due to the interrelatedness of these three processes. The *phoQ* mutant displayed a significant (P <0.001) impairment in, though not complete abolishment of, twitching motility (Fig. 3.2). This impaired twitching phenotype could be successfully restored back to wild-type twitching levels by providing a wild-type $phoQ^+$ allele *in trans* on a plasmid (Fig. 3.2). This demonstrates that PhoQ is necessary for normal wild-type twitching motility.

Reduced lettuce virulence of *phoQ* mutants.

Like other phytopathogenic *Pseudomonas* spp, *P. aeruginosa* can infect an assortment of plants. The lettuce leaf model, originally developed using whole *Arabidopsis* plants, is a simple model geared towards analyzing large differences in virulence phenotypes between different *P. aeruginosa* strains (Rahme et al. 1997). Here, *P. aeruginosa* cells are inoculated into the Romaine lettuce leaf stem midrib and macroscopic symptoms elicited by infection are followed over several days. Relative to wild-type PAO1, the *phoQ* mutant was consistently found to have an impairment in its ability to cause spreading soft-rot destruction inside the leaf midrib outwards from the point of inoculation (Fig. 3.3). Thus it appears PhoQ was necessary for full virulence expression in this model of plant infection.

Analysis of the altered transcriptome in the *phoQ* mutant.

To investigate how *phoQ* mutation contributes to gene expression in *Pseudomonas* and possibly to the virulence phenotypes observed, microarray studies were performed to compare the *phoQ* mutant to wild-type. Of note, we used a freshly constructed *phoQ* mutant based on the cloning protocol originally described, since our original mutant underwent phenotypic dilution over time. For the microarray, cells were grown in BM2-glucose containing 2 mM Mg^{2+}, a condition under which PhoP is phosphorylated and constitutively active (Macfarlane et al. 1999; Groisman 2000; McPhee et al. 2003). Using these conditions

we observed 474 genes that were differentially regulated ($P \leq 0.05$) by more than 1.5-fold under high Mg^{2+} conditions, with 296 transcriptionally up-regulated and 178 down-regulated. A selection of these genes is presented in Table 3.3.

The *phoQ* mutation affected the regulation of many genes that were previously shown to be regulated by PhoP and/or PmrA in an Mg^{2+} dependent manner (McPhee et al. 2006). Many previously-identified genes positively dependent on PhoP and Mg^{2+} limitation appeared dysregulated on our *phoQ* array and included PA0921, *oprH-phoP*, the LPS-modification operon *arnBCADTEF*, PA3885, PA4010-11, PA4453-5 and PA1343. PmrA-dependent genes shown to be up-regulated included *feoAB*, PA1559-60, PA4357-9, PA4773-8 (includes *pmrAB*), *arnBCADTEF*, and PA4286 (Table 3.3). The up-regulation of *pmrA* was confirmed using qRT-PCR, demonstrating that this gene was induced 4.2 ± 1.7 fold in the *phoQ* mutant (Table 3.4). Similarly the up-regulation of *arnB* was confirmed by qRT-PCR and shown to be 505 ± 21 fold up-regulated (Table 3.4). The massive up-regulation of *arnB* together with the rest of the genes of this LPS modification operon directly correlated with the high-level resistance to antimicrobial peptides such as polymyxin B in the *phoQ* mutant. Indeed, the freshly made *phoQ* mutant was >64-fold more resistant to polymyxin B than wild-type cells as assayed by broth dilution MIC in high Mg^{2+} BM2-glucose medium.

The switch to the mucoid (alginate producing) phenotype in most cystic fibrosis isolates is caused by mutations in the *mucA* gene (encoding an anti-sigma factor) and the resulting block in sequestration and activation of AlgU, an alternative sigma factor. The *phoQ* microarray revealed transcriptional dysregulation of the *algU-mucABCD* alginate regulatory gene cluster, namely up-regulation of *algU* and the corresponding AlgU-

dependent (mucoidy) genes (Firoved and Deretic 2003) including *slyB*, encoding a putative porin, the *osmC* and *osmE* osmotically induced lipoproteins, and the *pfpI* protease (Table 3.3). These three genes are among the strongest AlgU-dependent genes (Firoved and Deretic 2003) and were the only members of the AlgU stimulon that appeared on our microarray. This is not entirely unsurprising, as the *phoQ* mutant possessed a wild-type copy of *mucA* (which was similarly induced) and so the anti-AlgU sigma factor activity of MucA would likely therefore limit the degree to which less robust AlgU targets are induced. Of note, the alginate biosynthetic cluster (*algABCD*) did not appear to be significantly dysregulated in *phoQ* mutants, and *phoQ* mutants did not possess a mucoid phenotype. The *algU-mucAB* genes are also positively regulated by low Mg^{2+} (McPhee et al. 2006) and this is relevant as cells grown in low Mg^{2+} media have LPS analogous to that from cells isolated from CF lungs which become colonized with mucoid *P. aeruginosa* (Ernst et al. 1999).

Microarray analysis also indicated many interesting genes that while Mg^{2+} regulated, were strongly affected by mutation of the *phoQ* gene. These included *norC* (PA0523; nitric-oxide reductase subunit C) which was strongly down-regulated and two type II secretion genes, *hxcQ* (PA0685; probable type II secretion system protein) and *xqhA* (PA1868; type II secretion protein) that were down-regulated. Since down-regulation of two type II secretion genes (*hxcQ* and *xqhA*) was observed, we chose to analyze the regulation of the type II secretion-dependent lipase major virulence factor, *lipA*, which was demonstrated to be 3.2 ± 0.6-fold down-regulated in the *phoQ* mutant (Table 3.4). Interestingly, the *Pseudomonas* quinolone signal (PQS) biosynthetic genes *pqsBCD* were up-regulated. Also induced were four iron scavenging-related genes: pyoverdine genes *pvcA* (PA2254; pyoverdine biosynthesis protein), *pvdA (*PA2386; ornithine oxygenase in pyoverdine biosynthesis) and

fpvA (PA2398; ferripyoverdine receptor) and another siderophore gene *pchC* (PA4229; pyochelin biosynthesis thioesterase). The up-regulation of these green pigmented siderophore genes was supported by the observation that *phoQ* mutants were a darker green colour on plates and in broth culture (Fig. 3.4).

Additional regulation of genes associated with virulence was observed in *phoQ* mutants. The AlgR response regulator regulates a diverse assortment of virulence determinants including twitching and swarming motility and biofilm formation (Lizewski et al. 2002; Belete and Wozniak 2008). The transcriptional up-regulation of *algR* was confirmed by qPCR and showed that this gene was actually more strongly induced at 4.6 ± 2.6-fold in the *phoQ* mutant (Table 3.4). Of note, the major transcriptional regulator of the type III secretion system, *exsA*, was very modestly down-regulated on our *phoQ* microarray (−1.6, P <0.01; Appendix II). We also analyzed the level of regulation of the GacS/GacA/*rsmZ* pathway which operates at the level of post-translational regulation, since previous work indicated some overlap in phenotypes between GacA and PhoPQ. RsmZ is a small non-coding RNA that post-translationally sequesters the RNA-binding protein RsmA and thus indirectly regulates a broad range of virulence properties including liapse production (Heurlier et al. 2004). However, we were unable to demonstrate a significant level of regulation of *gacA* or *rsmZ*, thus indicating that this pathway may not play a role in the *phoQ* virulence defects.

DISCUSSION

In this study it is demonstrated that the PhoQ sensor kinase of *P. aeruginosa* was capable of regulating virulence in a rat model of chronic lung infection. Consistent with this, the *phoQ* mutant displayed reduced cytotoxicity towards polarized human bronchial

epithelial cells, reduced twitching motility, and impaired virulence in lettuce leaves relative to wild-type. Microarray analysis of the *phoQ* mutant produced a large list of dysregulated genes and uncovered new genes that are likely to form part of the PhoQ regulon.

The PhoP-PhoQ and PmrA-PmrB two-component systems of *P. aeruginosa* are responsible for regulating the adaptive response of *P. aeruginosa* to limiting concentrations of cations (e.g. Mg^{2+}; McPhee et al. 2006). They are also of critical importance in controlling resistance to cationic antimicrobial peptides in response to low Mg^{2+} conditions (Ernst et al. 1999; Macfarlane et al. 1999; McPhee et al. 2003; Moskowitz et al. 2004). Somewhat surprisingly, mutants in the PhoP and PmrA response regulators, and the PmrB sensor kinase were not attenuated for virulence in this model of infection and were fully capable of competing with the wild-type parental strain *in vivo* (Table 3.2). Furthermore, a mutant in *pmrE* (PA3559), a component of the aminoarabinaose lipid A LPS modification operon (*arnBCADTEF-pmrE*) was not impaired in virulence in this model (Table 3.2). As these operons are major mediators of intrinsic and mutational resistance to cationic antimicrobial peptides (McPhee et al. 2003; Moskowitz et al. 2004; Lewenza et al. 2005), this would seem to indicate that an increased susceptibility to such peptides did not impact on fitness of *P. aeruginosa*. Similarly, the increased antimicrobial peptide resistance of the PhoQ mutant was associated with decreased fitness in this model rather than increased resistance to host defence mechanisms, suggesting that altered virulence and polymyxin B/antimicrobial peptide resistance are independent phenotypes of the PhoQ mutant.

The PhoP-PhoQ system of *Salmonella* is a master regulator of virulence (Miller et al. 1989). We have previously compared the extensive differences in the architecture of these systems between *P. aeruginosa* and *S.* Typhimurium (McPhee et al. 2003), a theme that has

been mirrored in several other studies on *Yersinia* (Marceau et al. 2004; Winfield and Groisman, 2004; Winfield et al. 2004). The *Yersinia* PhoP-PhoQ system is important for regulating virulence gene expression since *phoP* mutants were less virulent during *Yersinia pseudotuberculosis* and *Y. pestis* intra-macrophage survival (Grabenstein et al. 2004). However, a *Salmonella* strain constitutively expressing PhoP was attenuated for virulence in mice and this was linked to a requirement to initiate an initial large induction of positive autoregulation (Shin et al. 2006). It is possible that this constitutive PhoP phenotype in *Salmonella* is similar to that which is occurring in the attenuated virulence phenotype displayed by our *P. aeruginosa phoQ* mutant which also has a constitutive PhoP phenotype. One of the most fundamental differences between the *Salmonella* and *Pseudomonas* systems is that *Salmonella* PhoQ appears able to both phosphorylate and dephosphorylate PhoP whereas in *Pseudomonas* the primary role of PhoQ appears to be in the dephosphorylation of PhoP and we suspect that another kinase may activate *Pseudomonas* PhoP (Macfarlane et al. 1999; Macfarlane et al. 2000).

In contrast to the situation in enteric *S.* Typhimurium and insect-pathogenic *Photorhabdus luminescens* (Bennett and Clarke 2005; Gunn et al. 2000), mutation of the *pmrE* (PA3559) gene of the *arnBCADTEF-pmrE* LPS modification operon in *P. aeruginosa* did not result in decreased survival in the chronic infection mouse model (Table 3.2). PmrE, a UDP-glucose dehydrogenase that has been referred to as *ugd* (McPhee et al. 2006), is required for *P. aeruginosa* resistance to antimicrobial peptides (Lewenza et al. 2005). However, it is possible that because upstream genes are not affected by the *pmrE* transposon mutation, the other *arn* genes in this operon could still have been required for virulence in this chronic model. Mice inoculated with *S. enterica* serovar *typhimurium pmrA* and

pmrHFIJKLM (*arnBCADTEF*) mutant strains demonstrated virulence attenuation when administered orally but not when administered intraperitoneally, indicating that aminoarabinose addition to LPS may be important for resistance to host innate defences within certain specific tissues (Gunn et al. 2000). This observation is similar to that which has been made for *Y. pseudotuberculosis* (a soil and waterborne enteropathogen), in which the *pmrF*-containing operon was dispensable for full virulence in mice, even though it was required for resistance to cationic antimicrobial peptides and polymyxin B (Marceau et al. 2004). It would be interesting to analyze *P. aeruginosa* mutants in the first genes of the *arnB* operon to see if these genes had virulence attenuation phenotypes.

In this study we have determined the dysregulated *in vitro* transcriptome of a *phoQ* mutant and used this gene list to attempt to explain our findings regarding the *in vivo* chronic rat lung infection attenuation phenotype. We are aware of a number of problems with this sort of comparison. The growth condition (BM2-glucose with high Mg^{2+}) used for our *in vitro* microarray experiment is expected to be quite different from the growth conditions found in the rat lung and as a result, the gene expression patterns might be quite different. Of note, a defined synthetic artificial cystic fibrosis sputum medium (based on analysis of cystic fibrosis sputum) for growth of *P. aeruginosa* contained a variety of carbon sources, with glucose having the largest concentration of any carbon source (Palmer et al. 2007). Nevertheless, a *phoQ* mutation results in a PhoP response regulator that would be relatively insensitive to additional stimuli (i.e. constitutively active) and might therefore be expected to have some of the same direct effects on gene expression regardless of growth conditions. Also, these microarrays are directly comparable to those utilized for previous

studies of the adaptive response to Mg^{2+} limitation, and the effects observed with *phoP* and *pmrA* mutants *in vitro*.

A *P. aeruginosa phoQ* mutant (in strain PA14) was shown to be impaired in the formation of both microtitre plate and mature flow-cell mushroom-shaped biofilms (Ramsey and Whiteley 2004). It was suggested in this study that the biofilm defect was multi-factorial and potentially involved changes in LPS, as LPS was required for biofilm formation in *P. aeruginosa* and *P. fluorescens* (de Lima Pimenta et al. 2003; Sabra et al. 2003). While in agreement with a potential role for LPS due to the strong upregulation of the *arnBCADTEF* operon, our data is consistent with an influential role for the observed impaired twitching motility of the *phoQ* mutant (Fig. 3.2), since twitching motility has been implicitly linked to biofilm formation (O'Toole and Kolter 1998). How the transcriptional dysregulation of this type IV-pili-dependent form of motility occurs in the *phoQ* mutant is not immediately apparent, given that the array and twitching motility experiments were done under different conditions. However, the *phoQ* microarray list did show down-regulation of some type IV-pili genes (data not shown as fold changes were significant but less than two-fold; Appendix II). It is also possible that the biofilm defect in this strain was indeed multi-factorial given the large number of genes differentially regulated in the *phoQ* mutant.

Mutation of *phoQ* affects a large number of genes outside of the PhoP regulon (McPhee et al. 2006), as indicated by comparison of microarray analyses. At present it is not known how the membrane-bound sensor kinase PhoQ accomplishes this. As PhoQ itself is not directly regulating these genes, it presumably is capable of modifying the phosphorylation state of another as yet unknown regulatory protein. If so, in the *phoQ*

mutant this activity would be lost and consequently gene expression would be modified. Experiments attempting to uncover such a biochemical target of PhoQ are ongoing.

Despite the *phoQ* mutant being highly antimicrobial peptide resistant *in vitro*, data are presented here that demonstrate that this mutant is attenuated for survival in a chronic lung infection model. In reflection, two considerations are relevant to this phenotype. First, other *phoQ* phenotypes such as reduced twitching motility and cytotoxicity may mask or contribute more than the hyper-resistance phenotype to the final *in vivo* rat lung phenotype. Second, these seemingly contradictory findings are perhaps not surprising given the growing body of knowledge regarding the indirect roles of host defense (antimicrobial) peptides as important regulators of the immune system, rather than direct antimicrobial compounds (Jenssen et al. 2006) and the complete lack of α-defensins in mouse phagocytes, in contrast to human neutrophils where they are a major mediator of direct microbicidal killing of pathogens.

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

Strain	Genotype or characteristics[a]	Source or reference
WT	Wild-type *P. aeruginosa* PAO1; strain H103	Lab collection
phoQ	*phoQ*::*xylE*-Gm^R; PAO1 background	Macfarlane et al. 1999
phoQ (pUC-*phoQ*$^+$)	*phoQ* mutant with pUCP22-*phoQ*$^+$; Gm^R, Cb^R	Macfarlane et al. 1999
phoP	*phoP*::*xylE*:*aacC1*; Gm^R derivative of WT	Macfarlane et al. 1999
pmrA	*pmrA*::*xylE*:*aacC1*; Gm^R derivative of WT	McPhee et al. 2003
pmrB	*pmrB*::*xylE*:*aacC1*; Gm^R derivative of WT	McPhee et al. 2003
putP	PA0783::*luxCDABE*-Tc^R; derivative of WT	Lewenza et al. 2005
PA4773	PA4773::*luxCDABE*-Tc^R; inserted between PA4773 and PA4774; derivative of WT	Lewenza et al. 2005
pmrE	PA3559::*luxCDABE*-Tc^R; derivative of WT	Lewenza et al. 2005
feoA	PA4359::*luxCDABE*-Tc^R; derivative of WT	Lewenza et al. 2005
Plasmid		
pUCP19, 22	*Escherichia-Pseudomonas* shuttle vectors	West et al. 1994

[a] Antibiotic resistance: Amp^R, ampicillin for *E. coli*, carbenicillin for *P. aeruginosa*; Gm^R, gentamicin; Tc^R, tetracycline.

Table 3.2 Competitive index (CI) analysis of *P. aeruginosa* mutant strains grown with the wild type PAO1 strain after 7 days of *in vivo* passage in the rat lung. The ability of wild-type PAO1 and mutant strains to compete with each other was determined in the rat lung model of chronic infection.

Gene disrupted	Geometric mean of CI[a]	No. of animals
phoP	0.52	3
phoQ	<0.00019 (P <0.02)	6
pmrA	1.27	3
pmrB	0.70	2
putP	0.54	2
PA4773	1.06	2
pmrE	1.51	2
feoA	0.80	3

[a] Competitive index (CI) analysis of mutant relative versus wild-type. The CI is defined as the CFU output ratio of mutant when compared to wild-type strain, divided by the CFU input ratio of mutant to wild-type strain. A CI <1 theoretically repesents a competitive disadvantage of the mutant strain compared to the wild-type parental strain.

Table 3.3 Microarray analysis of genes significantly dysregulated in the *phoQ* mutant relative to wild-type. Dysregulated hypothetical and/or unclassified ORFs are not included and only genes showing ≥2 fold change in the *phoQ* mutant are depicted.

Gene ID[a]	Name[a]	Fold change[b]	*P* value	Description[a]
PA0048		−19.1	0.002	probable transcriptional regulator
PA0059	*osmC*	2.1	0.003	osmotically inducible protein OsmC
PA0062		3.0	0.0001	hypothetical protein
PA0082		2.0	0.04	hypothetical protein
PA0135		−5.5	0.008	hypothetical protein
PA0161		−3.7	0.001	hypothetical protein
PA0165		−2.5	0.01	hypothetical protein
PA0198	*exbB1*	−2.1	0.01	transport protein ExbB
PA0199	*exbD1*	−2.0	0.02	transport protein ExbD
PA0224		4.0	0.01	probable aldolase
PA0307		−2.1	0.005	hypothetical protein
PA0327		2.3	<0.0001	hypothetical protein
PA0329		2.6	<0.0001	conserved hypothetical protein
PA0355	*pfpI*	2.4	0.05	intracellular protease PfpI
PA0459	*clpC*	−2.1	0.02	probable ClpA/B protease ATP binding subunit
PA0460		5.0	<0.0001	hypothetical protein
PA0490		3.0	<0.0001	hypothetical protein
PA0521	*nirO*	−2.2	0.02	probable cytochrome c oxidase subunit
PA0523	*norC*	−13.2	0.002	nitric-oxide reductase subunit C
PA0529		5.4	0.02	conserved hypothetical protein
PA0537		2.2	<0.0001	conserved hypothetical protein
PA0567	*yqaE*	2.0	0.01	conserved hypothetical protein
PA0569		−2.8	0.0004	hypothetical protein
PA0623		2.1	0.0008	probable bacteriophage protein
PA0631		2.0	0.003	hypothetical protein
PA0637		2.3	0.0004	conserved hypothetical protein
PA0685	*hxcQ*	−3.0	0.03	probable type II secretion system protein
PA0715		−2.5	0.04	hypothetical protein
PA0718		2.1	0.0004	hypothetical protein of bacteriophage Pf1
PA0737		2.2	0.01	hypothetical protein
PA0739		3.1	0.02	probable transcriptional regulator
PA0762	*algU*	6.4	<0.0001	sigma factor AlgU
PA0763	*mucA*	6.5	<0.0001	anti-sigma factor MucA
PA0764	*mucB*	2.7	<0.0001	negative regulator for alginate biosynthesis
PA0765	*mucC*	2.2	0.001	positive regulator for alginate biosynthesis
PA0766	*mucD*	2.7	0.005	serine protease MucD precursor
PA0801		2.2	0.004	hypothetical protein
PA0802		2.0	0.004	hypothetical protein
PA0806		−4.5	0.01	hypothetical protein
PA0814		2.8	0.03	conserved hypothetical protein

Gene ID[a]	Name[a]	Fold change[b]	*P* value	Description[a]
PA0833		8.2	<0.0001	hypothetical protein
PA0853		3.4	<0.0001	probable oxidoreductase
PA0854	*fumC2*	3.1	0.0001	fumarate hydratase
PA0874		–2.1	0.02	hypothetical protein
PA0879		–3.7	0.02	probable acyl-CoA dehydrogenase
PA0885	*dctQ*	–2.4	0.05	probable C4-dicarboxylate transporter
PA0910		3.3	0.02	hypothetical protein
PA0911		2.4	<0.0001	hypothetical protein
PA0914		–4.5	0.01	hypothetical protein
PA0919		2.3	<0.0001	hypothetical protein
PA0920		3.2	0.0001	hypothetical protein
PA0921		14.5	<0.0001	hypothetical protein
PA0929	*pirR*	2.3	0.0004	PirR two-component response regulator
PA0939		–5.8	0.008	hypothetical protein
PA0949	*wrbA*	4.5	0.003	Trp repressor binding protein WrbA
PA0984		–2.1	0.05	colicin immunity protein
PA0997	*pqsB*	2.5	0.0001	quinolone signal (PQS) biosynthesis
PA0998	*pqsC*	2.4	0.0007	quinolone signal (PQS) biosynthesis
PA0999	*pqsD*	2.3	0.0007	quinolone signal (PQS) biosynthesis
PA1001	*phnA*	2.6	0.0002	pyocyanin biosynthesis
PA1041		2.5	0.03	probable outer membrane protein
PA1053	*slyB*	4.1	<0.0001	outer membrane lipoprotein
PA1149		–2.6	0.04	hypothetical protein
PA1178	*oprH*	33.1	<0.0001	outer membrane protein OprH
PA1179	*phoP*	82.6	<0.0001	two-component response regulator PhoP
PA1317	*cyoA*	–2.8	0.005	cytochrome o ubiquinol oxidase subunit II
PA1318	*cyoB*	–2.7	0.002	cytochrome o ubiquinol oxidase subunit I
PA1319	*cyoC*	–2.4	0.01	cytochrome o ubiquinol oxidase subunit III
PA1321	*cyoE*	–3.5	0.007	cytochrome o ubiquinol oxidase protein CyoE
PA1323		6.6	<0.0001	hypothetical protein
PA1324		3.4	0.0008	hypothetical protein
PA1325	*yybH*	–2.6	0.0007	conserved hypothetical protein
PA1326	*ilvA2*	–2.9	0.0003	threonine dehydratase
PA1343		16.6	<0.0001	hypothetical protein
PA1344	*yvaG*	9.1	<0.0001	probable short-chain dehydrogenase
PA1346		–2.7	0.04	hypothetical protein
PA1385		–3.4	0.02	probable glycosyl transferase
PA1403		–2.6	0.03	probable transcriptional regulator
PA1471		7.1	<0.0001	hypothetical protein
PA1494		3.3	<0.0001	conserved hypothetical protein
PA1498	*pykF*	3.7	0.02	pyruvate kinase I
PA1506		–2.5	0.008	hypothetical protein
PA1547		–2.4	0.0007	hypothetical protein
PA1549	*fixI*	–2.4	0.006	probable cation-transporting P-type ATPase

Gene ID[a]	Name[a]	Fold change[b]	*P* value	Description[a]
PA1559		5.7	0.002	hypothetical protein
PA1560		6.0	0.001	hypothetical protein
PA1562	*acnA*	2.0	0.0007	aconitate hydratase 1
PA1579		2.4	<0.0001	hypothetical protein
PA1592		14.6	<0.0001	hypothetical protein
PA1596	*htpG*	−3.2	0.03	heat shock protein HtpG
PA1631		6.3	0.007	probable acyl-CoA dehydrogenase
PA1656		−2.0	0.001	hypothetical protein
PA1660		10.7	0.003	hypothetical protein
PA1680		2.0	0.05	hypothetical protein
PA1688		2.6	<0.0001	hypothetical protein
PA1689		3.3	<0.0001	conserved hypothetical protein
PA1715	*pscB*	−2.4	0.0004	type III export apparatus protein
PA1797		4.0	0.0002	hypothetical protein
PA1824		−3.8	0.02	conserved hypothetical protein
PA1868	*xqhA*	−3.9	0.02	type II secretion protein XqhA
PA1917		2.4	0.05	hypothetical protein
PA1920	*nrdD*	2.7	0.03	ribonucleotide reductase
PA1975		−2.5	0.04	hypothetical protein
PA1977		−2.4	0.005	hypothetical protein
PA1978	*agmR*	−2.4	0.05	transcriptional regulator AgmR
PA1979	*exaD*	−3.3	0.02	two-component sensor kinase ExaD
PA1981		−8.6	0.004	hypothetical protein
PA1982	*exaA*	−10.9	0.003	quinoprotein alcohol dehydrogenase
PA1983	*exaB*	−9.6	0.004	cytochrome c550
PA1984	*exaC*	−9.6	0.02	aldehyde dehydrogenase
PA2011	*gnyL*	2.6	0.04	hydroxymethylglutaryl-CoA lyase
PA2019	*amrA*	2.7	0.02	RND multidrug efflux membrane fusion protein
PA2021		2.5	0.007	hypothetical protein
PA2023	*galU*	2.2	0.001	UTP--glucose-1-phosphate uridylyltransferase
PA2078		−2.9	0.03	hypothetical protein
PA2106		2.2	0.009	hypothetical protein
PA2121		2.0	0.02	probable transcriptional regulator
PA2147	*katE*	−3.4	0.02	catalase HPII
PA2186		−3.4	0.02	hypothetical protein
PA2194	*hcnB*	2.1	0.02	hydrogen cyanide synthase HcnB
PA2252		2.1	0.002	AGCS sodium/alanine/glycine symporter
PA2254	*pvcA*	5.1	0.009	pyoverdine biosynthesis protein PvcA
PA2258	*ptxR*	2.6	0.02	transcriptional regulator PtxR
PA2280	*arsH*	−4.6	0.01	conserved protein in arsenic resistance
PA2298		−2.9	0.005	probable oxidoreductase
PA2353		2.5	0.04	conserved hypothetical protein
PA2358		7.9	<0.0001	hypothetical protein
PA2373		3.1	0.02	conserved hypothetical protein

Gene ID[a]	Name[a]	Fold change[b]	*P* value	Description[a]
PA2384		2.0	0.01	hypothetical protein
PA2386	*pvdA*	4.3	0.01	Pyoverdine biosyntheis, ornithine oxygenase
PA2398	*fpvA*	4.0	0.0005	ferripyoverdine receptor
PA2403		3.1	0.04	hypothetical protein
PA2404		4.0	0.003	hypothetical protein
PA2405		5.2	0.0002	hypothetical protein
PA2406		2.8	0.0009	hypothetical protein
PA2407		2.2	0.003	probable adhesion protein
PA2409		2.7	0.0004	probable permease of ABC transporter
PA2433		2.3	0.001	hypothetical protein
PA2434		3.2	0.05	hypothetical protein
PA2435		4.2	0.02	probable cation-transporting P-type ATPase
PA2436		2.5	0.04	hypothetical protein
PA2460		−2.0	0.002	hypothetical protein
PA2467		2.5	0.04	probable transmembrane sensor
PA2470	*gtdA*	2.4	0.05	gentisate 1,2-dioxygenase
PA2485		5.5	<0.0001	hypothetical protein
PA2486		3.4	<0.0001	hypothetical protein
PA2506		−2.9	0.03	hypothetical protein
PA2548		−3.1	0.02	hypothetical protein
PA2562		3.2	<0.0001	hypothetical protein
PA2569		2.3	0.0008	hypothetical protein
PA2650	*ybaJ*	−2.5	0.04	conserved methylase protein
PA2653	*yuiF*	−4.8	0.01	probable transporter
PA2658		2.0	0.0002	hypothetical protein
PA2659		2.3	0.0002	hypothetical protein
PA2662		−10.1	<0.0001	conserved hypothetical protein
PA2663		−14.9	0.008	hypothetical protein
PA2664	*fhp*	−30.5	0.02	flavohemoprotein
PA2779		2.9	0.0004	hypothetical protein
PA2787	*cpg2*	2.7	0.03	carboxypeptidase G2 precursor
PA2815	*yafH*	2.0	0.004	probable acyl-CoA dehydrogenase
PA2823		2.4	0.05	conserved hypothetical protein
PA2929		−2.8	0.03	hypothetical protein
PA2987	*ycfV*	2.1	0.01	ATP-binding component of ABC transporter
PA3001		−6.1	0.007	glyceraldehyde-3-phosphate dehydrogenase
PA3031		2.6	0.0001	hypothetical protein
PA3069		3.6	<0.0001	hypothetical protein
PA3189	*gltF*	2.4	0.02	probable permease of ABC sugar transporter
PA3239		2.0	0.003	conserved hypothetical protein
PA3277		−2.1	0.03	probable short-chain dehydrogenase
PA3391	*nosR*	−3.0	0.004	regulatory protein NosR
PA3404	*opmM*	13.0	0.003	outer membrane protein
PA3405	*hasE*	−4.6	0.01	metalloprotease secretion protein

Gene ID[a]	Name[a]	Fold change[b]	*P* value	Description[a]
PA3425		−2.6	0.04	hypothetical protein
PA3436		−2.7	0.003	hypothetical protein
PA3474	*yigM*	−2.1	0.02	conserved membrane protein
PA3475	*pheC*	−2.4	0.003	cyclohexadienyl dehy. (phenylalanine synthesis)
PA3506		3.4	0.02	probable decarboxylase
PA3515		2.9	0.02	hypothetical protein
PA3516		3.8	0.02	probable lyase
PA3517		3.7	0.04	probable lyase
PA3530	*bfd*	3.4	0.02	bacterioferritin-associated ferredoxin
PA3537	*argF*	2.5	0.04	ornithine carbamoyltransferase, Arg biosynthesis
PA3552	*arnB*	44.9	<0.0001	Aminotransferase in L-Ara4N biosynthesis
PA3553	*arnC*	75.4	<0.0001	Glycosyltransferase in L-Ara4N biosynthesis
PA3554	*arnA*	77.8	<0.0001	Dual function enzyme in L-Ara4N biosynthesis
PA3556	*arnT*	45.4	<0.0001	Transferase in L-Ara4N biosynthesis
PA3557	*arnE*	45.6	<0.0001	Transport system for L-Ara4N
PA3558	*arnF*	69.0	<0.0001	Transport system for L-Ara4N
PA3559	*pmrE*	3.4	0.004	UDP-glucose dehydrogenase
PA3560	*fruA*	2.0	0.04	fructose IIBC phosphotransferase system
PA3598	*ypqQ*	4.6	0.03	conserved hypothetical protein
PA3607	*potA*	−2.5	0.04	polyamine transport protein PotA
PA3610	*potD*	−3.2	0.02	polyamine transport protein PotD
PA3691		6.5	<0.0001	hypothetical protein
PA3692		6.2	<0.0001	probable outer membrane protein
PA3713		−2.8	0.02	hypothetical protein
PA3788		2.0	0.005	hypothetical protein
PA3819	*ycfJ*	12.0	<0.0001	putative porin
PA3840	*ybiN*	−2.3	0.0002	conserved protein (methylase)
PA3867		−2.5	0.04	probable DNA invertase
PA3885		3.2	<0.0001	hypothetical protein
PA3889		2.2	0.04	binding protein component of ABC transporter
PA3890		2.4	0.0002	probable permease of ABC transporter
PA3891		2.5	0.04	ATP-binding component of ABC transporter
PA3922		2.6	0.002	conserved hypothetical protein
PA3940		3.1	0.005	probable DNA binding protein
PA4010		5.0	<0.0001	hypothetical protein
PA4011		9.2	<0.0001	hypothetical protein
PA4040		−2.5	0.04	hypothetical protein
PA4092	*hpaC*	−6.2	0.007	4-hydroxyphenylacetate 3-monooxygenase
PA4129		−3.5	0.008	hypothetical protein
PA4154	*ygiM*	2.6	0.001	conserved hypothetical protein
PA4204		3.2	<0.0001	conserved hypothetical protein
PA4229	*pchC*	10.7	0.003	thioesterase PchC, pyochelin biosynthesis
PA4338		4.4	0.01	hypothetical protein
PA4357	*yhgG*	3.2	0.009	conserved hypothetical protein

Gene ID[a]	Name[a]	Fold change[b]	*P* value	Description[a]
PA4358	*feoB*	3.0	0.003	ferrous iron transport protein
PA4359	*feoA*	3.7	<0.0001	ferrous iron transport protein
PA4370	*icmP*	2.2	0.003	outer-membrane insulin-cleaving protease
PA4452		5.2	<0.0001	conserved hypothetical protein
PA4453		5.9	<0.0001	conserved hypothetical protein
PA4454	*yrbD*	6.3	<0.0001	conserved hypothetical protein
PA4455	*yrbE*	5.3	<0.0001	probable permease of ABC transporter
PA4456	*yrbF*	4.1	<0.0001	ATP-binding component of ABC transporter
PA4469		3.6	0.02	hypothetical protein
PA4479	*mreD*	3.2	0.05	rod shape-determining protein MreD
PA4495		3.7	<0.0001	hypothetical protein
PA4514	*piuA*	2.9	0.03	outer membrane receptor for iron transport
PA4517		6.8	<0.0001	conserved hypothetical protein
PA4621		–2.0	0.002	probable oxidoreductase
PA4635	*mgtC*	3.1	<0.0001	conserved membrane protein MgtC
PA4644		–4.0	0.01	hypothetical protein
PA4675	*iutA*	2.0	0.007	probable TonB-dependent receptor
PA4713		2.0	0.02	hypothetical protein
PA4762	*grpE*	–2.7	0.03	heat shock protein GrpE
PA4773		7.2	0.0003	hypothetical protein
PA4774		8.0	0.0002	hypothetical protein
PA4776	*pmrA*	4.0	0.0003	two-component response regulator
PA4777	*pmrB*	3.2	0.005	two-component sensor
PA4800		–2.5	0.04	hypothetical protein
PA4817		–2.0	0.01	hypothetical protein
PA4826		2.6	<0.0001	hypothetical protein
PA4873		–3.0	0.03	probable heat-shock protein
PA4876	*osmE*	4.5	<0.0001	osmotically inducible lipoprotein OsmE
PA4880		6.6	<0.0001	probable bacterioferritin
PA4892	*ureF*	–2.0	0.002	urease accessory protein UreF
PA5020		–2.3	0.05	probable acyl-CoA dehydrogenase
PA5030	*ynfM*	–2.5	0.0006	probable MFS transporter
PA5053	*hslV*	–2.0	0.002	heat shock protein HslV
PA5061	*phaI*	4.4	<0.0001	polyhydroxyalkanoic acid biosynthesis
PA5107	*blc*	2.6	0.005	outer membrane lipoprotein Blc
PA5144		–2.5	0.04	hypothetical protein
PA5157	*marR*	–2.0	0.003	transcriptional regulator MarR
PA5158	*opmG*	–2.8	0.001	outer membrane protein
PA5169		2.5	0.04	probable C4-dicarboxylate transporter
PA5172	*arcB*	–2.7	0.0005	ornithine carbamoyltransferase, catabolic
PA5178		3.6	0.0004	conserved hypothetical protein
PA5182		4.6	<0.0001	hypothetical protein
PA5183		2.4	0.02	hypothetical protein
PA5188		-2.2	0.01	probable 3-hydroxyacyl-CoA dehydrogenase

Gene ID[a]	**Name**[a]	**Fold change**[b]	***P* value**	**Description**[a]
PA5212		8.3	<0.0001	hypothetical protein
PA5231	*yhiH*	−3.4	0.03	ATP-binding/permease fusion ABC transporter
PA5302	*dadX*	−2.9	0.004	catabolic alanine racemase
PA5390		−2.6	0.04	probable peptidic bond hydrolase
PA5424	*yeaQ*	3.4	0.0002	conserved hypothetical protein
PA5473	*yjbB*	2.6	0.001	conserved hypothetical protein
PA5493	*polA*	3.4	<0.0001	DNA polymerase I
PA5517		−2.2	0.004	conserved hypothetical protein
PA5526		7.0	<0.0001	hypothetical protein
PA5531	*tonB*	2.5	0.009	TonB protein
PA5537		2.0	0.008	hypothetical protein

[a] Information according to the *P. aeruginosa* genome website (www.pseudomonas.com/)
[b] Fold regulation of genes differentially expressed in the *phoQ* mutant relative to WT. A positive number indicates transcript up-regulation in the *phoQ* mutant.

Table 3.4 qPCR gene expression analysis of select genes in *phoQ* mutants relative to wild-type. Experiments were performed using three independent biological samples each with two technical replicates and the average ± standard deviation is reported.

Gene ID[a]	Name[a]	Fold change[b]	Description[a]
PA0762	*algU*	5.1 ± 0.9	Sigma factor AlgU
PA2862	*lipA*	3.2 ± 0.6	LipA lactonizing lipase precursor
PA3552	*arnB*	505 ± 21	Aminotransferase in L-Ara4N biosynthesis
PA4776	*pmrA*	4.1 ± 1.7	PmrA response regulator
PA5261	*algR*	4.6 ± 2.6	AlgR response regulator

[a] Information according to the *P. aeruginosa* genome website (www.pseudomonas.com/)
[b] Fold regulation of genes differentially expressed in the *phoQ* mutant relative to WT. A positive number indicates transcript up-regulation in the *phoQ* mutant.

Figure 3.1

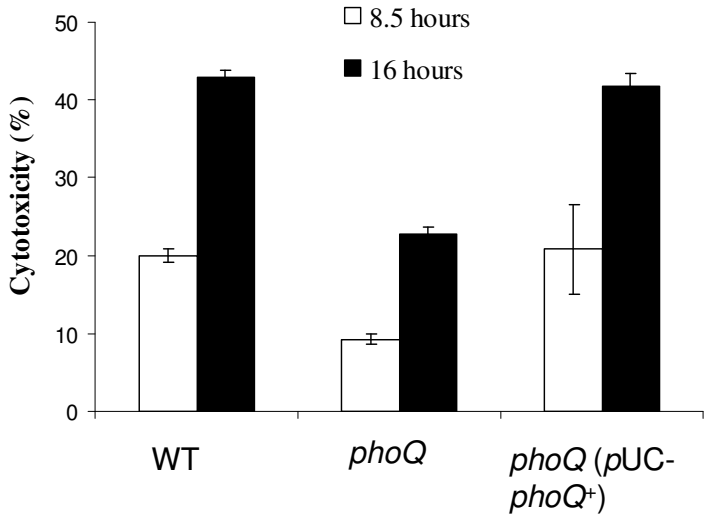

Figure 3.1 PhoQ mutants displayed reduced *in vitro* cytotoxicity towards human bronchial epithelial cells. The ability of the wild-type PAO1 and *phoQ* mutant strains to induce cell damage was determined by monitoring the release of intracellular lactate dehydrogenase (LDH) into the supernatant from human bronchial epithelial cells. Bacteria were co-cultured with the cells and LDH release was monitored at the time point indicated. Data represent the mean of 3 biological repeats, each assayed in triplicate, with the data reported as averages ± standard deviation.

Figure 3.2

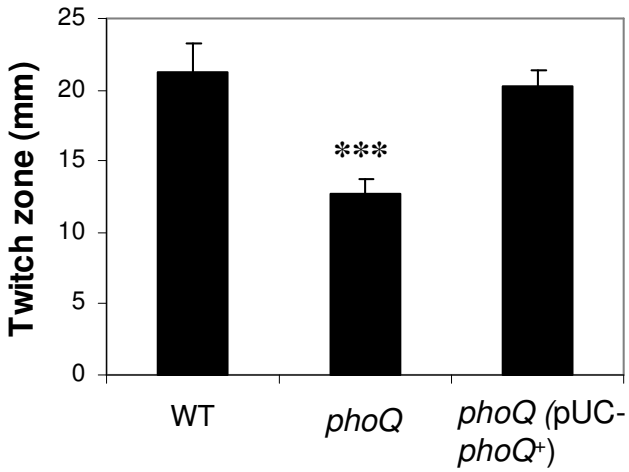

Figure 3.2 PhoQ mutants displayed reduced twitching motility. Twitching motility was assessed by inoculating cells from mid-logarithmic phase cultures into thin LB agar (1% wt/vol) plates, down to the agar-plastic interface, and measuring colony diameter after 24 hr incubation at 37°C. Results shown are averages of several independent biological replicates for each strain. *** represents a statistically significant difference (P <0.001) between *phoQ* mutant and wild-type as determined by Student's *t* test.

Figure 3.3

WT ***phoQ***

Figure 3.3 PhoQ mutants were attenuated for virulence in lettuce leaves. Day 4 symptoms of Romaine leaf infections after midribs were inoculated with 1×10^6 CFU of *P. aeruginosa*. Leaves were incubated at 37°C in small containers with moistened paper towels. Shown is one representative leaf of several that produced similar symptoms.

Figure 3.4

phoQ **WT**

Figure 3.4 Pigmentation differences of mid-logarithmic phase *phoQ* mutant and wild-type liquid cultures. Photograph of cultures grown in BM2-glucose minimal medium containing 2 mM $MgSO_4$.

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CHAPTER 4 – Concluding remarks

INTRODUCTION

The opportunistic pathogen *P. aeruginosa* causes serious infections including those associated with burn wounds, ventillator-associated pneumonia, and inside the lungs of cystic fibrosis patients. Without the development of new drugs active against these and other hospital-acquired Gram-negative bacterial infections, a foundation of modern medicine faces an ominous future (Falagas and Bliziotis 2007).

The studies on PsrA and PhoQ presented in this thesis highlight the complex nature of *P. aeruginosa* regulation of virulence and antimicrobial peptide resistance. Mutation of either gene is associated with several phenotypic differences relative to wild-type and accompanied by the transcriptional dysregulation of a relatively large number of genes. The ability of PsrA and PhoQ to affect a wide-range of important overlapping processes in virulence is noteworthy. Further, the inclusion of antimicrobial peptide resistance with these virulence phenotypes highlights the significance of these findings.

PsrA

As described in Chapter 2, the PsrA transcriptional regulator appears capable of regulating intrinsic resistance to antimicrobial peptides and polymyxin B, swarming motility and biofilm formation. The finding that *psrA* was one of the few regulatory protein-encoding genes induced by antimicrobial peptides is significant given that peptides are known to regulate an assortment of resistance and virulence genes in other bacteria.

The downregulation of the *pprB* gene on the PsrA microarray was possibly partly responsible for the *psrA* mutant's biofilm formation phenotype as a *pprB* mutant was defective in biofilm formation. PprB is a response regulator and forms a two-component

regulatory system with its cognate sensor kinase PprA. Interestingly, this system was found to have a role in membrane permeability, antibiotic resistance, virulence factor production, and modulation of quorum sensing (Dong et al. 2005; Wang et al. 2003), similar phenotypes to those of PsrA.

Determining whether PsrA could have a clinically significant role is an interesting subject. PsrA does regulate processes that are necessary for virulence, such as biofilm formation and motility. Further, *P. aeruginosa* would be exposed to antimicrobial peptides during both therapeutic administration (e.g inhaled polymyxins) and during meetings on epithelial or mucosal surfaces (e.g. host defensins). For this reason, it would be of interest to examine whether sub-inhibitory concentrations of peptides modulate the observed *psrA* phenotypes in wild-type *P. aeruginosa* (e.g. swarming or biofilms) and then see if this modulation is lost in the *psrA* mutant. Swarming motility itself is a complex motility adaptation where cells become more antibiotic resistant and increase production of virulence factors (Overhage et al. 2008). Further, biofilm formation is widely recognized now to be a leading determinant in the antibiotic resistance of some chronic infections. As such, the finding that PsrA can regulate swarming and biofilms adds more insight into the regulation of these virulence determinants in *P. aeruginosa*. Indeed, the inverse regulation of type III secretion and biofilm formation of the *psrA* mutant is reminiscent of the two main phenotypes of the *ladS* mutant in *P. aeruginosa* (Ventre et al. 2006). LadS is a hybrid-type sensor kinase and a master regulator capable of inversely controlling chronic (e.g. biofilm) and acute (e.g. type III secretion) virulence determinants (Ventre et al. 2006).

PhoQ

The *phoQ* work documents an interesting dilemma between antimicrobial peptides and virulence (Chapter 3). That is, although the *phoQ* mutant possesses the most antimicrobial peptide super-resistant phenotype known in *P. aeruginosa*, this mutant was nevertheless highly attenuated for virulence. Further, from our laboratory's recent screens of *P. aeruginosa* transposon mutant libraries for increased peptide resistance, the *phoQ* mutation represents one of the few single gene mutations which increases antimicrobial peptide resistance. Conversely, screens for mutants resistant to conventional antibiotics such as the aminoglycoside tobramycin or the fluoroquinolone ciprofloxacin have uncovered large numbers of genes conferring resistance upon knockout mutation. This inherent difficulty in developing mutational antimicrobial peptide resistance is significant and lends added support for the continued development of antimicrobial peptides as therapeutics.

FUTURE RESEARCH DIRECTIONS

Although cationic antimicrobial peptides are being pursued as novel antimicrobials against these infections, some antimicrobial resistant determinants have already been described. As mentioned, sub-inhibitory concentrations of these peptides have been shown to have a variety of effects on bacteria independent of their lethal effects at higher concentrations. A central goal has been to find the *P. aeruginosa* regulator of adaptive peptide resistance. That is, finding the regulatory system that senses sub-inhibitory concentrations of peptides and subsequently promotes resistance to normally lethal concentrations of peptides. In *Salmonella*, it is PhoQ that directly senses peptides and activates its cognate regulator PhoP to promote resistance, but *P. aeruginosa* PhoQ does not perform this task as the periplasmic sensing domains of these proteins are quite dissimilar

(Bader et al. 2005). PsrA is also not the regulator that responds to peptides (even though *psrA* was transcriptionally induced by peptides), as growing *P. aeruginosa* with sub-lethal levels of peptides (to promote resistance) and then killing with high concentrations showed that the *psrA* mutant was not super-sensitive relative to wild-type. It is possible that one of the many uncharacterized two-component regulatory systems in *P. aeruginosa* functions as this elusive peptide sensor/regulator. Further, this regulator is likely a homolog of PmrA due to the fact that the mystery regulator must recognize similar PmrA target promoters (PA4773-*pmrAB* and *arnBCADTEF*) to promote resistance through LPS modification.

Although no differences were seen in the lettuce model for the *psrA* mutant, it is interesting nevertheless to speculate on the nature of the virulence phenotype for the *psrA* mutant in an *in vivo* model (e.g. the rat model of chronic lung infections). The capacity to form biofilms is generally associated with a chronic infection state, so this mutant might be predicted to be attenuated for virulence. Indeed, the other *psrA* mutant phenotypes such as impaired swarming and antimicrobial peptide susceptibility would lend support to this prediction. However, a mutant possessing multiple phenotypes makes predicting such an outcome difficult (as was described for PhoQ).

It is interesting to speculate how antimicrobial peptides are capable of inducing *psrA* transcription. The first step would be to determine whether this peptide-mediated regulation is direct (peptides interacting with PsrA) or indirect (peptides affecting another protein which then effects *psrA* transcription). Recently, it was shown that both sub-inhibitory concentrations of both indolicidin and human LL-37 cationic peptides are able to inhibit *P. aeruginosa* biofilm formation while not killing the bacteria (Overhage and Hancock, unpublished). Due to the similar overlap of these peptide and biofilm associated phenotypes

in the *psrA* mutant, it would be interesting to test this peptide-influenced biofilm formation in the *psrA* mutant to see if PsrA modulates this effect.

Mutation of *phoQ* was shown to affect a large number of genes outside of the PhoP regulon. However, at present it is not known how the membrane-bound sensor kinase PhoQ accomplishes this. As membrane-bound PhoQ cannot be directly regulating these genes, presumably it is capable of modifying the phosphorylation state of an unknown intermediate regulatory protein. In the *phoQ* mutant this activity is lost and consequently gene expression is modified. Finding such a protein would greatly add an important mechanistic aspect to a presently quite descriptive PhoQ story.

The future study on *P. aeruginosa* regulatory genes (known and uncharacterized) involved in virulence and antibiotic resistance is anticipated to shed more light on these important processes and may ultimately help establish connections and hierarchy between these regulatory systems in this important opportunistic human pathogen.

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APPENDIX I.

Other genes significantly dysregulated in *psrA* mutants and displaying ≥ 1.5-fold change as determined using microarray. This list includes hypothetical genes and other genes not included in Table 2.4.

Gene ID[a]	Gene name[a]	Fold change[b]	*P* value	Description[a]
PA0050		−1.7	0.02	hypothetical protein
PA0095	*vgr*	−1.7	0.004	conserved hypothetical protein
PA0109		−1.5	0.04	hypothetical protein
PA0172		1.5	0.008	hypothetical protein
PA0177		−1.5	0.03	purine-binding chemotaxis protein
PA0178		−1.8	0.04	probable two-component sensor
PA0179		−1.8	0.008	two-component response regulator
PA0321		−1.6	0.01	probable acetylpolyamine aminohydrolase
PA0460		−1.8	0.03	hypothetical protein
PA0484		−1.6	0.02	conserved hypothetical protein
PA0524	*norB*	−1.9	0.0008	nitric-oxide reductase subunit B
PA0586	*ycgB*	−1.6	0.01	conserved hypothetical protein
PA0587	*yeaH*	−1.9	0.02	conserved hypothetical protein
PA0617		−1.5	0.02	probable bacteriophage protein
PA0645		−1.7	0.008	hypothetical protein
PA0803		1.8	0.01	hypothetical protein
PA0805		−1.8	0.05	hypothetical protein
PA0808		−2.0	0.06	hypothetical protein
PA0816		1.6	0.01	probable transcriptional regulator
PA0841		−1.6	0.01	hypothetical protein
PA0861		−1.5	0.007	hypothetical protein
PA0873	*phhR*	−1.5	0.02	transcriptional regulator PhhR
PA0912		−1.6	0.06	hypothetical protein
PA0997	*pqsB*	−1.7	0.02	hypothetical protein
PA1065		−2.1	0.01	conserved hypothetical protein
PA1090		−1.5	0.01	hypothetical protein
PA1107		−1.7	0.004	conserved hypothetical protein
PA1124	*dgt*	−1.6	0.01	triphosphohydrolase
PA1133		−1.6	0.04	hypothetical protein
PA1150	*pys2*	−1.5	0.02	pyocin S2
PA1175	*napD*	−1.9	0.003	NapD of periplasmic nitrate reductase
PA1311	*phnX*	−1.6	0.006	2-phosphonoacetaldehyde hydrolase
PA1366		−1.6	0.01	hypothetical protein
PA1370		−1.7	0.02	hypothetical protein
PA1373	*fabF2*	1.7	0.02	3-oxoacyl-acyl carrier protein synthase II
PA1436		−1.6	0.02	probable RND efflux transporter
PA1437		−1.7	0.05	two-component response regulator
PA1450		−2.0	0.02	conserved hypothetical protein

Gene ID[a]	Gene name[a]	Fold change[b]	*P* value	Description[a]
PA1469		1.7	0.005	hypothetical protein
PA1577		−1.6	0.06	hypothetical protein
PA1692	*pscS*	1.6	0.002	translocation protein in type III secretion
PA1694	*pscQ*	1.6	0.004	translocation protein in type III secretion
PA1696	*pscO*	1.6	0.0003	translocation protein in type III secretion
PA1704	*pcrR*	1.7	0.02	transcriptional regulator protein PcrR
PA1711		1.7	0.001	hypothetical protein
PA1714	*exsD*	1.8	0.02	hypothetical protein
PA1718	*pscE*	1.8	0.03	type III export protein PscE
PA1720	*pscG*	1.7	0.01	type III export protein PscG
PA1829		−1.6	0.005	hypothetical protein
PA1875		−1.5	0.03	hypothetical protein
PA1876		−1.6	0.03	ATP-binding fusion ABC transporter
PA1891		1.7	0.03	hypothetical protein
PA1903	*phzE2*	−1.7	0.02	phenazine biosynthesis protein PhzE
PA1988	*pqqD*	−1.9	0.009	pyrroloquinoline quinone biosynthesis
PA2231	*pslA*	−1.6	0.05	probable glycosyl transferase
PA2313		−2.0	0.06	hypothetical protein
PA2318		2.2	0.04	hypothetical protein
PA2324		−2.2	0.04	hypothetical protein
PA2358		1.8	0.01	hypothetical protein
PA2363		−1.9	0.02	hypothetical protein
PA2365		−4.1	0.01	conserved hypothetical protein
PA2367		−5.4	0.006	hypothetical protein
PA2369		−4.6	0.008	hypothetical protein
PA2373		−2.5	0.03	conserved hypothetical protein
PA2412		−2.7	0.02	conserved hypothetical protein
PA2428		3.6	0.002	hypothetical protein
PA2430		2.6	0.03	conserved hypothetical protein
PA2439		2.30	0.04	hypothetical protein
PA2462		−2.2	0.005	hypothetical protein
PA2463		1.5	0.009	hypothetical protein
PA2504		2.0	0.06	hypothetical protein
PA2539	*ynbD*	1.6	0.02	conserved hypothetical protein
PA2546		−1.6	0.01	probable ring-cleaving dioxygenase
PA2562		−2.0	0.004	hypothetical protein
PA2565		−2.3	0.04	hypothetical protein
PA2597		1.7	0.03	hypothetical protein
PA2622	*cspD*	−1.5	0.002	cold-shock protein CspD
PA2655		−1.5	0.003	hypothetical protein
PA2662		−7.5	0.004	conserved hypothetical protein
PA2668		−1.51	0.008	hypothetical protein
PA2706		−1.6	0.004	hypothetical protein
PA2729		−1.6	0.01	hypothetical protein

Gene ID[a]	Gene name[a]	Fold change[b]	*P* value	Description[a]
PA2731		−2.1	0.02	hypothetical protein
PA2732		−1.7	0.002	hypothetical protein
PA2750		1.5	0.04	hypothetical protein
PA2790		−1.6	0.03	hypothetical protein
PA2833		−2.0	0.0005	conserved hypothetical protein
PA3012		3.5	<0.001	hypothetical protein
PA3097	*xcpX*	−1.5	0.006	general secretion pathway protein K
PA3125		3.9	0.01	hypothetical protein
PA3136		−1.6	0.02	probable secretion protein
PA3261		−1.5	0.004	hypothetical protein
PA3300	*fadD2*	−1.8	0.02	long-chain-fatty-acid--CoA ligase
PA3314		1.5	0.03	ATP-binding cmpt of ABC transporter
PA3320		−2.1	0.006	hypothetical protein
PA3327		−2.7	0.02	probable non-ribosomal peptide synthetase
PA3335		−3.5	0.01	hypothetical protein
PA3360		2.0	0.009	probable secretion protein
PA3394	*nosF*	1.9	0.03	NosF protein
PA3424		1.5	0.04	hypothetical protein
PA3426		−1.6	0.009	probable enoyl CoA-hydratase/isomerase
PA3448	*ygaM*	−1.7	0.01	probable permease of ABC transporter
PA3451		−2.1	0.05	hypothetical protein
PA3452	*mqoA*	−1.6	0.004	malate:quinone oxidoreductase
PA3533	*ydhD*	−1.50	0.02	conserved hypothetical protein
PA3534		−1.9	0.0007	probable oxidoreductase
PA3575		1.7	0.01	hypothetical protein
PA3592	*baiF*	−2.1	0.05	conserved hypothetical protein
PA3667		1.52	0.02	pyridoxal-phosphate dependent enzyme
PA3720		10.4	0.002	hypothetical protein
PA3773		−6.4	0.004	hypothetical protein
PA3789		18.4	0.001	hypothetical protein
PA3843		2.7	0.0007	hypothetical protein
PA3852		−1.8	0.009	hypothetical protein
PA3855		−1.9	0.01	hypothetical protein
PA3858	*aapJ*	−1.6	0.007	probable amino acid-binding protein
PA3945		−1.7	0.002	conserved hypothetical protein
PA3972	*aidB*	−1.8	0.02	probable acyl-CoA dehydrogenase
PA3973		−1.8	0.03	probable transcriptional regulator
PA3986		−2.5	0.01	hypothetical protein
PA4015		−2.0	0.003	conserved hypothetical protein
PA4096	*phlE*	2.0	0.05	probable MFS transporter
PA4242	*rpmJ*	−1.9	0.01	50S ribosomal protein L36
PA4245	*rpmD*	−1.7	0.02	50S ribosomal protein L30
PA4296		−2.9	0.06	two-component response regulator
PA4298		−3.7	0.01	hypothetical protein

Gene ID[a]	Gene name[a]	Fold change[b]	*P* value	Description[a]
PA4300		−2.2	0.04	hypothetical protein
PA4303		−2.7	0.02	hypothetical protein
PA4305		−2.1	0.05	hypothetical protein
PA4364		−1.8	0.01	hypothetical protein
PA4479	*mreD*	1.7	0.04	rod shape-determining protein MreD
PA4487		1.5	0.03	conserved hypothetical protein
PA4507		5.7	0.005	hypothetical protein
PA4531		−4.5	0.02	hypothetical protein
PA4586		1.5	0.01	hypothetical protein
PA4591		−2.0	0.007	hypothetical protein
PA4624		−1.6	0.03	hypothetical protein
PA4625		−3.7	0.006	hypothetical protein
PA4632		1.5	0.04	hypothetical protein
PA4658		1.6	0.02	hypothetical protein
PA4691		−1.7	0.03	hypothetical protein
PA4738	*yjbJ*	−1.8	0.0007	conserved hypothetical protein
PA4739		−1.6	0.02	conserved hypothetical protein
PA4783	*yedA*	1.7	0.02	conserved hypothetical protein
PA4822		−2.4	0.03	hypothetical protein
PA4889		1.5	0.03	probable oxidoreductase
PA4908		−6.3	0.005	hypothetical protein
PA5178		−2.7	0.02	conserved hypothetical protein
PA5185		−1.5	0.04	conserved hypothetical protein
PA5255	*algQ*	−1.52	0.02	Alginate regulatory protein AlgQ
PA5348		1.7	0.03	probable DNA-binding protein
PA5479	*gltP*	1.5	0.04	proton-glutamate symporter

[a] Information according to the *P. aeruginosa* genome website (www.pseudomonas.com/)
[b] Fold regulation of genes differentially expressed in *psrA* mutant relative to WT. A positive number indicates transcript up-regulation in the *psrA* mutant.

APPENDIX II.

Significantly dysregulated genes showing ≥ 1.5-fold change in *phoQ* mutants as determined using microarray. Genes represent those not included in Table 3.3.

Gene ID[a]	Name	Fold change[b]	*P* value	Description[a]
PA0034		1.8	0.05	probable two-component response regulator
PA0038		1.7	0.003	hypothetical protein
PA0060		1.6	0.01	conserved hypothetical protein
PA0069		−1.7	0.05	conserved hypothetical protein
PA0084		1.6	0.004	conserved hypothetical protein
PA0120		−1.8	0.01	probable transcriptional regulator
PA0125		−1.8	0.03	hypothetical protein
PA0266	*gabT*	1.5	0.008	4-aminobutyrate aminotransferase
PA0360		−1.5	0.09	hypothetical protein
PA0371		1.7	0.005	hypothetical protein
PA0372		1.5	0.01	probable zinc protease
PA0423	*yceI*	1.7	0.02	conserved hypothetical protein
PA0426	*mexB*	1.6	0.001	RND multidrug efflux transporter MexB
PA0432	*sahH*	−1.5	0.01	S-adenosyl-L-homocysteine hydrolase
PA0454	*yccS*	−1.6	0.05	conserved hypothetical protein
PA0471	*fiuR*	1.7	0.02	probable transmembrane sensor
PA0481		−1.5	0.023	hypothetical protein
PA0500	*bioB*	−1.6	0.004	biotin synthase
PA0536		1.6	0.0007	hypothetical protein
PA0552	*pgk*	1.5	0.02	phosphoglycerate kinase
PA0565		−1.6	0.01	conserved hypothetical protein
PA0568		−1.6	0.007	hypothetical protein
PA0570		−1.8	0.004	hypothetical protein
PA0571		−1.7	0.001	hypothetical protein
PA0604		1.7	0.03	binding protein component of ABC transporter
PA0605		1.9	0.01	probable permease of ABC transporter
PA0613		1.6	0.0006	hypothetical protein
PA0616		1.5	0.008	hypothetical protein
PA0617		1.7	0.001	probable bacteriophage protein
PA0618		1.7	0.0008	probable bacteriophage protein
PA0624		1.5	0.002	hypothetical protein
PA0625		1.6	0.001	hypothetical protein
PA0626		1.6	0.01	hypothetical protein
PA0627		1.6	0.002	conserved hypothetical protein
PA0628		1.6	0.001	conserved hypothetical protein
PA0632		1.9	0.007	hypothetical protein
PA0634		1.9	0.0002	hypothetical protein
PA0635		1.9	0.0002	hypothetical protein
PA0638		1.9	0.03	probable bacteriophage protein

Gene ID[a]	Name	Fold change[b]	*P* value	Description[a]
PA0639		1.8	0.002	conserved hypothetical protein
PA0643		1.7	0.004	hypothetical protein
PA0667	*yebA*	1.5	0.02	conserved hypothetical protein
PA0792	*prpD*	−1.6	0.008	propionate catabolic protein PrpD
PA0797		−1.5	0.002	probable transcriptional regulator
PA0834		−1.7	0.03	conserved hypothetical protein
PA0840		−1.6	0.02	probable oxidoreductase
PA0852	*cbpD*	1.7	0.03	chitin-binding protein CbpD precursor
PA0876		−1.7	0.02	probable transcriptional regulator
PA0887	*acsA*	1.6	0.01	acetyl-coenzyme A synthetase
PA0924		1.9	0.03	hypothetical protein
PA0943		1.5	0.01	hypothetical protein
PA0958	*oprD*	−1.5	0.01	outer membrane porin protein OprD precursor
PA0968	*ybgC*	1.5	0.005	conserved hypothetical protein
PA1002	*phnB*	1.6	0.002	anthranilate synthase component II
PA1048		1.8	0.002	probable outer membrane protein
PA1106		1.5	0.02	hypothetical protein
PA1159		1.7	0.008	probable cold-shock protein
PA1175	*napD*	1.5	0.01	NapD protein of periplasmic nitrate reductase
PA1249	*aprA*	1.9	0.001	alkaline metalloproteinase precursor
PA1250	*aprI*	1.6	0.001	alkaline proteinase inhibitor AprI
PA1282		−1.6	0.04	probable MFS transporter
PA1296		1.9	0.0008	probable 2-hydroxyacid dehydrogenase
PA1320	*cyoD*	−1.8	0.01	cytochrome o ubiquinol oxidase subunit IV
PA1327		−1.9	0.04	probable protease
PA1389		1.7	0.02	probable glycosyl transferase
PA1402		−1.8	0.03	hypothetical protein
PA1437		1.6	0.01	probable two-component response regulator
PA1535		−1.6	0.01	probable acyl-CoA dehydrogenase
PA1542		−1.8	0.05	hypothetical protein
PA1696	*pscO*	−1.7	0.03	translocation protein in type III secretion
PA1707	*pcrH*	−1.6	0.05	regulatory protein PcrH
PA1713	*exsA*	−1.6	0.005	transcriptional regulator ExsA
PA1726	*bglX*	1.5	0.03	periplasmic beta-glucosidase
PA1800	*tig*	1.8	0.05	trigger factor
PA1804	*hupB*	1.5	0.05	DNA-binding protein HU
PA1882		1.8	0.02	probable transporter
PA1912		−1.8	0.03	probable sigma-70 factor, ECF subfamily
PA1930		1.5	0.005	probable chemotaxis transducer
PA2071	*fusA2*	1.9	0.004	elongation factor G
PA2177		1.8	0.002	probable sensor/response regulator hybrid
PA2186		−3.4	0.02	hypothetical protein
PA2193	*hcnA*	1.7	0.0007	hydrogen cyanide synthase HcnA
PA2232	*pslB*	−1.8	0.002	probable phosphomannose isomerase/GDP-

Gene ID[a]	Name	Fold change[b]	*P* value	Description[a]
				mannose pyrophosphorylase
PA2234	*pslD*	−1.5	0.005	probable exopolysaccharide transporter
PA2236	*pslF*	−1.7	0.005	hypothetical protein
PA2237	*pslG*	−1.7	0.0003	probable glycosyl hydrolase
PA2238	*pslH*	−1.7	0.005	hypothetical protein
PA2299		−1.9	0.04	probable transcriptional regulator
PA2302		1.8	0.002	probable non-ribosomal peptide synthetase
PA2303		1.8	0.0002	hypothetical protein
PA2307		−1.9	0.02	probable permease of ABC transporter
PA2344	*mtlZ*	−1.6	0.04	fructokinase
PA2364		−1.8	0.02	hypothetical protein
PA2365		−1.5	0.04	conserved hypothetical protein
PA2366		−1.6	0.04	conserved hypothetical protein
PA2367		−1.7	0.03	hypothetical protein
PA2396	*pvdF*	1.6	0.05	hypothetical protein
PA2444	*glyA2*	−1.8	0.003	serine hydroxymethyltransferase
PA2461		−1.6	0.02	hypothetical protein
PA2482		−1.6	0.02	probable cytochrome c
PA2527	*yegN*	1.6	0.007	RND efflux transporter
PA2528	*yegM*	1.7	0.0005	RND efflux membrane fusion protein precursor
PA2529		1.6	0.01	hypothetical protein
PA2539		−1.8	0.01	conserved hypothetical protein
PA2630		−1.5	0.02	conserved hypothetical protein
PA2650		−2.5	0.04	conserved hypothetical protein
PA2656		1.9	0.0006	probable two-component sensor
PA2657		1.9	0.001	probable two-component response regulator
PA2728		1.7	0.002	hypothetical protein
PA2771		1.5	0.02	conserved hypothetical protein
PA2777		1.6	0.04	conserved hypothetical protein
PA2778		1.6	0.03	hypothetical protein
PA2847		−1.9	0.002	conserved hypothetical protein
PA2850	*ohr*	−1.5	0.02	organic hydroperoxide resistance protein
PA2895		1.9	0.0002	hypothetical protein
PA2896		1.5	0.01	probable sigma-70 factor, ECF subfamily
PA2958		−1.7	0.02	hypothetical protein
PA2972	*yceF*	−1.7	0.05	conserved hypothetical protein
PA3038	*opdQ*	1.7	0.003	porin
PA3040	*yqjD*	1.8	0.004	conserved hypothetical protein
PA3041	*yqjE*	1.6	0.0007	hypothetical protein
PA3042		1.7	0.008	hypothetical protein
PA3119	*yafE*	−1.5	0.004	conserved protein
PA3136		−1.7	0.002	probable secretion protein
PA3160	*wzz*	1.9	0.0002	O-antigen chain length regulator
PA3180		1.8	0.05	hypothetical protein

Gene ID[a]	Name	Fold change[b]	*P* value	Description[a]
PA3188	*gltG*	1.8	0.009	permease of ABC sugar transporter
PA3197		−1.6	0.03	hypothetical protein
PA3234	*yjcG*	1.8	0.001	sodium:solute symporter
PA3235	*yjcH*	1.8	0.007	conserved protein
PA3243	*minC*	1.8	0.003	cell division inhibitor MinC
PA3268		1.7	0.04	probable TonB-dependent receptor
PA3304		−1.5	0.003	conserved hypothetical protein
PA3310		1.9	0.004	conserved hypothetical protein
PA3385		1.8	0.0005	hypothetical protein
PA3441	*ssuF*	−1.6	0.009	molybdopterin-binding protein
PA3459	*asnB*	1.7	0.005	probable amidotransferase
PA3461	*yhfE*	1.5	0.006	conserved protein
PA3532		−1.9	0.02	hypothetical protein
PA3581	*glpF*	−1.8	0.0005	glycerol uptake facilitator protein
PA3584	*glpD*	−1.9	0.007	glycerol-3-phosphate dehydrogenase
PA3603	*dgkA*	1.5	0.02	diacylglycerol kinase
PA3677		1.5	0.05	RND efflux membrane fusion protein precursor
PA3760	*nagF*	1.9	0.02	phosphotransferase protein
PA3762		1.6	0.01	hypothetical protein
PA3770	*guaB*	−1.6	0.03	inosine-5-monophosphate dehydrogenase
PA3795		1.6	0.002	probable oxidoreductase
PA3815		−1.8	0.002	conserved hypothetical protein
PA3817		−1.5	0.02	probable methyltransferase
PA3857	*pcs*	1.5	0.005	conserved hypothetical protein
PA3902		1.9	0.0008	hypothetical protein
PA3962		1.6	0.001	hypothetical protein
PA3979		−1.5	0.04	hypothetical protein
PA3990		−1.5	0.05	conserved hypothetical protein
PA4190	*pqsL*	1.5	0.009	probable FAD-dependent monooxygenase
PA4297		−1.7	0.03	hypothetical protein
PA4312		1.9	0.03	conserved hypothetical protein
PA4345		1.6	0.03	hypothetical protein
PA4366	*sodB*	1.6	0.02	superoxide dismutase
PA4372		1.7	0.003	hypothetical protein
PA4374		1.5	0.02	RND efflux membrane fusion protein precursor
PA4377		1.8	0.0003	hypothetical protein
PA4378	*inaA*	1.8	0.008	InaA protein
PA4379		1.9	0.001	conserved hypothetical protein
PA4391		−1.5	0.03	hypothetical protein
PA4448	*hisD*	−1.6	0.02	histidinol dehydrogenase
PA4496		1.8	0.003	binding protein component of ABC transporter
PA4550	*fimU*	1.8	0.001	type 4 fimbrial biogenesis protein FimU
PA4552	*pilW*	1.8	0.0003	type 4 fimbrial biogenesis protein PilW
PA4582		−1.6	0.007	conserved hypothetical protein

Gene ID[a]	Name	Fold change[b]	*P* value	Description[a]
PA4606	*cstA*	1.6	0.002	conserved protein
PA4620		–1.9	0.002	hypothetical protein
PA4638		1.6	0.013	hypothetical protein
PA4717		1.7	0.009	conserved hypothetical protein
PA4735		1.6	0.004	hypothetical protein
PA4781		1.9	0.004	probable two-component response regulator
PA4782		1.6	0.005	hypothetical protein
PA4785		1.7	0.004	probable acyl-CoA thiolase
PA4786		1.7	0.01	probable short-chain dehydrogenase
PA4874		1.6	0.02	conserved hypothetical protein
PA4885	*irlR*	-1.7	0.0004	two-component response regulator
PA4891	*ureE*	-1.6	0.01	urease accessory protein UreE
PA4917		-1.9	0.004	hypothetical protein
PA4919	*pncB1*	-1.8	0.03	nicotinate phosphoribosyltransferase
PA4983	*dmsR*	-1.6	0.01	two-component response regulator
PA4995		-1.6	0.004	probable acyl-CoA dehydrogenase
PA5026		-1.9	0.01	hypothetical protein
PA5041	*pilP*	-1.5	0.007	type 4 fimbrial biogenesis protein PilP
PA5042	*pilO*	-1.7	0.02	type 4 fimbrial biogenesis protein PilO
PA5043	*pilN*	-1.7	0.004	type 4 fimbrial biogenesis protein PilN
PA5054	*hslU*	-1.8	0.02	heat shock protein HslU
PA5108		1.9	0.0001	hypothetical protein
PA5116		1.8	0.008	probable transcriptional regulator
PA5139		-1.7	0.04	hypothetical protein
PA5173	*arcC*	-1.7	0.05	carbamate kinase
PA5179		-1.5	0.04	probable transcriptional regulator
PA5217		1.9	0.01	binding protein of ABC iron transporter
PA5235	*glpT*	-1.7	0.008	glycerol-3-phosphate transporter
PA5248		1.5	0.04	hypothetical protein
PA5261	*algR*	1.8	0.007	alginate biosynthesis regulatory protein AlgR
PA5367	*pstA*	-1.8	0.01	membrane protein of phosphate transporter
PA5369		-1.7	0.001	hypothetical protein
PA5450	*wzt*	1.8	0.002	ABC subunit of A-band LPS efflux transporter
PA5451	*wzm*	1.5	0.01	subunit of A-band LPS efflux transporter
PA5452	*wbpW*	1.6	0.003	phosphomannose isomerase/ WbpW
PA5483	*algB*	1.8	0.005	two-component response regulator AlgB
PA5542		1.5	0.02	hypothetical protein

[a] Information according to the *P. aeruginosa* genome website (www.pseudomonas.com/)
[b] Fold regulation of genes differentially expressed in *phoQ* mutant relative to WT. A positive number indicates transcript up-regulation in the *phoQ* mutant.