INVOLVEMENT OF REGULATORY NON-CODING RNA IN MOTILITY, BIOFILM FORMATION AND ADAPTIVE RESISTANCE IN *PSEUDOMONAS AERUGINOSA*

by

Patrick Kyle Taylor

B.Sc, The University of British Columbia, 2009

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate and Postdoctoral Studies
(Microbiology and Immunology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

May, 2014

Abstract

Small, non-coding RNA (sRNA) transcripts are emerging as a major mechanism for regulating translational expression in bacteria. Since the discovery of 6S RNA acting to regulate translation of RNA polymerases in *Escherichia coli*, our understanding of sRNA regulation of translation has expanded, and sRNAs are now known to have a broad range of functions in bacteria ranging from metabolic regulation to virulence determination. The Gram-negative bacterium *Pseudomonas aeruginosa* is commonly found in natural microbiomes, and is also an opportunistic pathogen as it causes disease in immunocompromised individuals. *P. aeruginosa* displays a high level of resistance to numerous clinically relevant antibiotics, and is capable of developing biofilms on multiple surfaces in hospital environments. *P. aeruginosa* is also capable of swarming which is a complex motility involving rhamnolipid surface whetting agents, flagella and type IV pili. This work investigated the involvement of 32 sRNA species in adaptive resistance to antibiotics, swarming motility, and biofilm formation in *P. aeruginosa*. Unique expression profiles under conditions of swarming and biofilm formation for 27 previously uncharacterized sRNAs were found. It was also found that the sRNAs *prrF1*, *prrF2* and *phrS* are involved in swarming motility and/or biofilm formation. Compared to free-swimming, planktonic growth expression of the *prrF* gene loci was up-regulated 163- and 13-fold under swarming and biofilm conditions, respectively, and mutants lacking the entire locus demonstrated modest decreases in swarming while *prrF1* mutants demonstrated increased biofilm formation. A transposon insertion mutant in *phrS* in *P. aeruginosa* PA14 wildtype displayed a deficiency in swarming motility and biofilm formation. *phrS* was also found to be involved in the development of adaptive resistance to polymyxin B by impacting on the translation of a lipid A modification operon. Together this work demonstrates that sRNA regulation plays a critical role in swarming motility, biofilm formation and the development of adaptive resistance in *P. aeruginosa*.

Preface

Data from Chapter 2 is being included in a manuscript by our collaborators in Fiona Brinkman's Laboratory at Simon Fraser University for publication. I was responsible for producing and analyzing all the RT-qPCR data in Chapter 2.

Parts of Chapter 3 are being including in a manuscript in preparation in Robert E.W. Hancock's laboratory. I was responsible for carrying out all experiments and analysis of data in Chapter 3

Chapter 4 is being prepared as a manuscript for publication. Instruction in producing the complemented *phrS* strain was provided by Manjeet Bains but the work was done by me. Biofilm flow-cell assays and confocal microscopy imaging was done by César de la Fuente-Núñez and Fany Reffuveille. All other studies were done by me.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

5’ UTR	–	5’ untranslated region
AMP	–	ampicillin
BM2	–	basal medium 2
bp	–	base pairs
CAA	–	casamino acid medium
CAZ	–	ceftazidime
CF	–	Cystic Fibrosis
CRISPRs	–	clustered regularly interspaced short palindromic repeats
EPS	–	exopolysaccharide
GEN	–	gentamicin
IND	–	indolicidin
IR	–	intergenic region
KAN	–	kanamycin
LB	–	Luria broth
LPS	–	lipopolysaccharide
nt	–	nucleotides
OD	–	optical density
PIP	–	piperacillin
PXB	–	polymyxin B
QS	–	quorum sensing
RBS	–	ribosomal binding site
RT-qPCR	–	real-time, semi-quantitative polymerase chain reaction
sRNA	–	small RNA
T3SS	–	type III secretion system
T6SS	–	type VI secretion system
TCS	–	two component regulatory system
TET	–	tetracycline
TOB	–	tobramycin
UTR	–	Untranslated region
WT	–	Wildtype

Acknowledgements

I would like to thank my supervisor, Dr. Bob Hancock, for providing me the opportunity to begin a career in science. Under his mentorship I have developed the strong foundation of skills required to pursue a scientific career.

Drs. Steven Hallam and Michael Murphy, while acting as members of my committee, have given me both encouragement and insightful thought, for which I am greatly appreciative.

I would also like to thank my colleagues in the Hancock lab for their assistance while at work and their friendship both in and outside of the lab.

I would like thank my fiancée Steph Halmhofer for her never ending support. She has taken the good times and the bad with me while I ventured through graduate studies. Finally, I could not have done this without the support of family, most notably my grandfather, Laird McCallum. He is no longer here to see me pursue a career in science but he was a constant supporter of my efforts to do so and his wisdom and out-look on life that I have taken to heart, will continue to motivate me for the rest of my scientific career.

1 Introduction

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative bacterium commonly found in aqueous environments, but it also is an opportunistic pathogen of humans, causing diverse and severe infections in ranging from acute burn and lung infections, to chronic colonization of the lungs of individuals afflicted with the genetic disease Cystic Fibrosis (CF) (Lyczak et al, 2000). *P. aeruginosa* is capable of colonizing diverse environments in part due to its ability to utilize aerobic and anaerobic metabolic pathways, its mechanisms of intrinsic, acquired, and adaptive antimicrobial resistance, and its expression of a multitude of virulence factors.

P. aeruginosa has a large genome of more than 6.2 Mbp encoding >5500 genes (Stover et al, 2000). This is ~30% larger than other phylogenetically closely related pathogenic Proteobacteria, such as *Escherichia coli* and *Salmonella enterica* (Blattner et al, 1997; Holt, et al 2008; McClelland et al, 2001). Nearly a tenth of the *P. aeruginosa* genome contains genes encoding regulatory proteins (Stover et al, 2000). In addition, the *P. aeruginosa* genome has numerous large intergenic regions without annotated genes that have classically been considered to include 'junk' DNA. However, recent research revealed many genes within intergenic regions that encode regulatory RNAs (Dötsch et al, 2012; Gómez-Lozano et al, 2012; Wurtzel et al, 2012).

1.2 Swarming Motility

P. aeruginosa is capable of swarming, a highly organized form of surface motility requiring flagella, type IV pili, and rhamnolipid surfactants. Under conditions of intermediate viscosity (0.4-0.6% agar) and a weak nitrogen source (amino acids), *P. aeruginosa* will swarm outwards from a central inoculation point, aligning longitudinally and moving in a concerted fashion, to form either a dendritic (PA14 strains) or starburst (PAO1 strains) pattern (Déziel et al, 2003; Köhler et al, 2000; Overhage et al, 2007). Swarming involves the dysregulation of 417 genes including the overexpression of a large number of virulence-related genes, including genes encoding the type III secretion system and its effectors, extracellular proteases, and iron transport systems (Overhage et al, 2008). In addition, swarming is dependent on the function of more than 230 genes that when mutated alter swarming (Leung et al, 2009). Thus, swarming is a highly complex social behaviour. The level of viscosity and amino acids as a nitrogen source, both of

which are required for swarming, resembles the conditions of mucosal surfaces (i.e., list an example). Therefore, swarming acts as an analog for the virulent state of *P. aeruginosa* when it is establishing infections in human hosts.

1.3 Biofilms

Biofilms are structured multicellular consortia of bacteria embedded in a protective self-produced extracellular matrix (Friedman et al, 2004). *P. aeruginosa* readily forms biofilms on abiotic and biotic surfaces, including medical devices such as heart valves (causing endocarditis), prosthetic joint, catheters and stents, as well as patients with chronic infections such as individuals afflicted with the genetic disease of Cystic Fibrosis (Lindsay et al, 2006). The biofilm mode of growth is a major problem in hospital settings due to the exceptional (adaptive) resistance mechanisms of biofilms to antibiotics, antiseptics, disinfectants, and the immune system. Biofilms are up to one thousand-fold more resistant to antibiotics than their planktonic counterparts (Hoyle et al, 1991). Many characteristics of biofilms have been proposed to contribute to adaptive antibiotic resistance in biofilms and are discussed below. *P. aeruginosa* is a model organism for understanding biofilm development and provides a template for understanding how antibiotic resistance arises during biofilm growth.

Biofilm development on solid substrates occurs in 5 major stages that complete the cycle from colonization of surfaces to dispersal. First, in a process mediated by flagella and/or type IV pili, free-swimming (planktonic) bacteria adhere to a solid surface (e.g. on a glass, plastic, metal, or tissue substratum) (O'Toole et al, 1998; Toutain et al, 2007). The second stage consists of several rounds of cell division and growth that lead to the formation of aggregates, also known as microcolonies (Sriramulu et al, 2005). Third, as the biofilm grows and matures, independent microcolonies grow together to form a mat, which is typically visible to the naked eye. Fourth, biofilms subsequently develop colonial structures at the microscopic level, which possess subpopulations of cells with separate physiologies. Bacterial biofilms are permeated by water channels allowing for flow of nutrients through the biofilm. Finally, the subsequent dispersal of single cells or small microcolonies from the biofilm enables the bacteria to move to a new location to initiate and propagate new biofilm colonies.

A defining feature of a biofilm is the extracellular polymeric substance (EPS) matrix that provides a structural lattice interconnecting biofilm cells. EPS can be comprised of bacterial secreted components, including polysaccharides, extracellular DNA (eDNA), proteins, lipids,

and biosurfactants (Allesen-Holm et al, 2006; Barken et al, 2008; Klausen et al, 2003). The Pel and Psl polysaccharides are major contributors in *P. aeruginosa* biofilm formation (Jackson et al, 2004; Vasseur et al, 2005). Pel polysaccharide is a glucose-rich polymer while the Psl polysaccharide is a mannose-rich polymer. Both polysaccharides contribute to microcolony formation, during the initial stages of biofilm formation, subpopulation interactions, macrocolony formation in the later stages of biofilm formation, and eDNA release and distribution within the biofilm (Ma et al, 2009).

Extracellular DNA in a *P. aeruginosa* biofilm is generated by lysis of a subpopulation of the bacteria via a mechanism dependent on quorum sensing (QS), as well as flagella and type IV pili (Allesen-Holm et al, 20069). Microscopic investigations of flow chamber-grown *P. aeruginosa* biofilms stained with different DNA stains indicate that eDNA is located primarily in the stalks of mushroom-shaped multicellular structures (Allesen-Holm et al, 2006). DNAse-treatment dissolves mature biofilms formed by *P. aeruginosa* suggesting that eDNA is involved in cell-cell interconnection in young biofilms, with possible roles in initial attachment of bacterial cells (Whitchurch et al, 2002). The secretion of eDNA during early stages of growth might also be important for the survival of biofilms since it contributes to *P. aeruginosa* competition with other microbes and the development of adaptive resistance (Mulcahy et al, 2008).

The temporal and spatial development of biofilms is to some extent regulated by QS, a cell-cell communication mechanism that plays an important role in certain aspects of bacterial life, at high population densities, including virulence as well as biofilm formation (Davis et al, 1998; De Kievit et al, 2001). Prominent small molecules involved in QS of *P. aeruginosa* (and other bacteria) are acyl homoserine lactones. These molecules are freely diffusible into the environment and are very important for biofilm regulation, but are not the only quorum sensing molecules utilized by *P. aeruginosa*. In *P. aeruginosa*, QS for biofilm development involves 3 intertwined QS systems, the homoserine lactone-based LasRI and RhlRI systems and the *Pseudomonas* quinolone signal (PQS). When an appropriate bacterial density is reached, these molecules reach a threshold concentration in the cellular environment and are taken up by all cells in the vicinity, bind to their corresponding transcriptional regulators in the cell and result in coordinated multi-locus gene expression in the entire bacterial population.

1.4 Antibiotic Resistance in Biofilms

P. aeruginosa cells in a biofilm state of growth are significantly more resistant to antimicrobial agents than are planktonic cells (Hoyle et al, 1991). This has been ascribed to a range of different factors including a complex array of adaptive gene expression changes, some of which influence antibiotic susceptibility, the low metabolic state of organisms deep within the biofilm, poor antibiotic penetration into the biofilm, and the higher concentration of extracellular antibiotic degrading enzymes. Of these the most intriguing involves adaptive changes in gene expression that accompany the switch to the biofilm mode of growth, which includes a range of genes that could be involved in determining biofilm resistance since they modulate resistance to one or more antibiotics. The *Pseudomonas* efflux pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM expel multiple families of antimicrobial compounds, and have been proposed as a major cause of adaptive resistance during the biofilm mode of growth (Poole, 2001). Alterations to the permeability of the outer membrane via the PhoPQ and PmrAB pathways can be induced by divalent cation deficiency due to the high concentrations of extracellular polyanionic DNA in biofilms, resulting in tolerance to polymyxins and other cationic antimicrobial peptides (Mulcahy et al, 2008). The increasing accumulation of acquired mutations giving rise to antimicrobial resistance has been observed in the laboratory (Amini et al, 2011; Macia et al, 2011) and during the development of heterogeneous populations of *P. aeruginosa* in chronic lung infections (Wang et al, 2010; Xu et al, 1998), with the latter phenomenon being exacerbated by the development of mutants in mutator genes (Macia et al, 2011).

Although it is often discussed, the effects of antimicrobial agents cannot be explained solely by an inability of compounds to penetrate the biofilm (Anderl et al, 2000), although the extracellular matrix possesses some ability to counter the effects of antimicrobials. For example the extracellular DNA present in the matrix has an overall anionic charge and has been shown to reduce the efficacy of positively charged antimicrobial agents including polymyxins and aminoglycosides (Kumon et al, 1994). Many antimicrobial agents penetrate reasonably well into biofilms, although certain nutrients such as oxygen might be limiting in the interior part of biofilms and create an anaerobic or microaerophilic environment (Borriello et al, 2004; Xu et al, 1998). The anaerobic environment within biofilms would impact directly on aminoglycoside antibiotic activity (Kindrachuk et al, 2011) due to decreased energy-dependent uptake (Hancock,

1981), as well as triggering changes in gene expression. Due to these and other changes brought about by the dense aggregation of bacteria within biofilms, such as limiting nutrient availability, bacteria deep within biofilms likely have reduced metabolic activity and lower rates of cell division than those closer to the surface of biofilms. Such cells in biofilms that survive antibiotic treatment due to non-mutational mechanisms have been termed persister cells. Persisters are proposed to be dormant cells that can survive antimicrobial treatments that kill the majority of their genetically identical siblings and thus represent a distinct category of adaptive resistance. Persister cells are considered to have entered an extremely slow-growing or non-growing physiological state (although the basis for this is unknown), which makes them insensitive or tolerant to the action of antimicrobial drugs.

1.5 Non-coding RNA

Not all transcripts are utilized for protein translation. The roles of other so-called non-coding (i.e. non-translated) RNAs are varied, with diverse functions and unique mechanisms within the cell. The tRNAs and rRNAs have well characterized roles essential to mediating translation. There are also CRISPRs (clustered regularly interspaced short palindromic repeats) that have defensive roles in eliminating foreign gene expression through complementary binding to direct a cleavage enzyme, thus mitigating the effects of invasive phages and plasmids. A third major group of non-coding RNAs play a significant role in regulating translation within the cell and use evolutionarily distinct mechanisms.

Our view of bacteria as a simple form of life on the planet is rapidly evolving as the complex life cycles and mechanisms of cellular signalling are being further elucidated. One of the most rapidly growing areas of research is the how non-coding RNAs are involved in regulating expression changes within a bacterial cell and add redundancy and integration to expression changes. At one level bacterial cells respond to environmental stimuli by altering a gene expression. Activation of gene expression creates mRNA transcripts, which then are translated into proteins that carry out various functions by mediating enzymatic reactions, thereby impacting on cell structure, or further regulating gene expression. Non-coding RNAs have come forward as a major mechanism that bacteria utilize for transcriptional and post-transcriptional regulation (Delihas et al, 2001). Various mnemonics have been used previously to describe non-coding, regulatory RNA species in bacteria such as non-coding RNA (ncRNA), non-protein coding RNA (npcRNA), small non-messenger RNA (snmRNA), untranslated RNA (utRNA),

and small RNA (sRNA). These mnemonics are largely synonymous with one another in bacterial species and, for simplicity; sRNA will hereafter be used to refer to non-coding, regulatory RNAs.

This work specifically focused on a sub-type of sRNAs that act to regulate levels of translation of target mRNAs and thereby are an additional mechanism of regulation within the cell as well as a mechanism of signal integration between networks (Brencic et al, 2009; Sonnleitner et al, 2008; Sonnleitner et al, 2011; Wilderman et al, 2004; Olgesby et al, 2010). Of sRNAs that act within the cell to regulate translational levels there are two-major classifications according to their respective mechanisms of action, *cis*- and *trans*-encoded sRNAs.

Cis-sRNAs are encoded on the opposing genomic strand to their target genes and have a high-degree of sequence complementarity, thereby silencing translation of complementary mRNA targets and even promoting mRNA degradation (Sonnleitner et al, 2011; Storz et al, 2011). *Trans*-encoded sRNAs have a larger array of confirmed mechanisms of action than *cis*-encoded sRNAs, and are the focus of this work. The gene architecture of *trans*-RNAs is unique from that of *cis*-RNAs (Biesel et al, 2010; Sonnleitner et al, 2011; Storz et al, 2011). *Trans*-encoded RNAs often do not have identifiable promoter regions and do not commonly have any identifiable ribosome binding site (RBS), which has made the identification of sRNA genes difficult and elusive until the recent development of second generation sequencing methods as explained below (Sonnleitner et al, 2008; Gómez-Lozano et al, 2012; Wurtzel et al, 2012).

Trans-RNAs largely rely upon RNA binding proteins to directly or indirectly carry out regulatory functions. One well-characterized system involves the interactions of sRNAs RsmY and RsmZ with a small protein RsmA. In this system RsmA is an RNA binding protein with high affinity for target mRNA transcripts. RsmY and RsmZ sRNAs have higher affinity for RsmA than any target mRNA transcript (González et al, 2008; Brencic et al, 2009). When RsmY and RsmZ are expressed, RsmA is sequestered and target mRNA transcripts of RsmA are then released, the RBS is exposed, and translation can occur.

Another major mechanism of *trans*-encoded sRNAs involves an RNA binding chaperone protein, Hfq (Sonnleitner et al, 2008). Hfq is highly conserved in bacteria and has been known for a long time to contribute to RNA stability. *Trans*-sRNAs share very little complementarity with their target transcripts. Often there is only a 7-nucleotide “seeder” region of sequence complementarity with the 5’ untranslated region (UTR) of the target mRNA transcript around the

RBS (Biesel et al, 2010). This low level of sequence complementarity allows *trans*-encoded sRNAs significant flexibility in interacting with multiple different target mRNAs. In addition to interacting with the RBS of some target mRNAs to negatively regulate translation, some *trans*-encoded sRNAs act at regions of the 5'UTR of mRNAs to alter secondary structure and expose the RBS to allow translation to occur, thereby having a positive effect on regulation. Because *trans*-encoded sRNAs are often of considerable length, in the range of 50-400 nucleotides, a single sRNA can possess a large number of "seeder" regions that access a large array of different target transcripts, acting as a positive regulator for specific targets and as a negative regulator for others, consistent with the results obtained in this investigation. Because of this, sRNAs are emerging a central mechanism by which bacteria regulate intracellular signalling pathways and rapidly provide exceptionally precise responses to environmental stimuli.

Until recently, determination of novel sRNA genes and subsequent study of functional roles was highly limited and biased towards sRNAs that had protein interaction partners or had a high degree of complementarity to target mRNAs. A method of protein precipitation and northern blotting termed RNomics was previously used to elucidate sRNAs in *P. aeruginosa* (Sonnleitner et al, 2008). More recent studies of sRNAs have made use of second generation RNA-Sequencing (RNA-Seq) methods for high-throughput analysis. RNA-Seq utilizes transcripts expressed from the genome (the transcriptome) as templates for sequencing by synthesis. RNA-Seq has thus provided a high-throughput method of identifying novel sRNA genes. Several studies utilizing RNA-Seq for the study of sRNAs have found that the *P. aeruginosa* genome encodes at least 170 sRNAs in intergenic regions (IR) (Dötsch et al, 2012; Gómez-Lozano et al, 2012; Wurtzel et al, 2012).

1.6 Goals of This Study

Adaptive responses to stress in the environment, swarming motility, and biofilm formation are all complex behaviours in bacteria, and molecular and regulatory mechanisms are known to coordinate these activities. The participation of sRNA species in the regulation and coordination of these complex behaviours is poorly understood. Indeed, many studies have focused on quantifying the number of sRNAs encoded in the genome, but relatively few studies have investigated how these species impact on cells. Thus, very few sRNA species have been characterized to have roles such as in swarming motility and/or biofilm formation, and no studies have investigated the roles that sRNAs might play in adaptive resistance. Here I **hypothesized**

that trans-encoded sRNAs have a significant influence in regulating complex adaptive behaviours.

I thus aimed to to demonstrate that certain sRNAs have a central role in regulating swarming motility, biofilm resistance, and adaptive resistance to antimicrobial agents. To achieve this aim a genetic approach was taken to determine expression profiles of sRNAs and the phenotypic effects of sRNA mutants under conditions of swarming and biofilm formation. In chapter 2, I examined the dysregulation of 32 sRNAs under conditions of swarming as well as biofilm formation. In chapter 3, deletion mutants of *prrF* locus sRNAs were used to investigate the effects of these sRNAs on swarming motility and biofilm formation. In chapter 4, a transposon insertion in *phrS* was used to investigate the effects of *phrS* on swarming motility, biofilm formation, and adaptive resistance to antimicrobial agents. The purpose of this work was thus to further our understanding of how sRNAs are involved in complex behaviours, such as swarming motility, biofilm formation, and adaptive resistance.

2 Confirmation and Differential Regulation of Novel sRNA Species in *Pseudomonas aeruginosa* Under Conditions of Biofilm Formation and Swarming Motility

2.1 Introduction

A variety of studies have previously been undertaken to identify novel sRNAs and determine their functional roles within *P. aeruginosa*. The earliest studies investigating sRNAs in *P. aeruginosa* utilized pull-down co-precipitation techniques and sequencing of the single-stranded RNAs that associated with RNA-binding proteins in the cell (Brencic et al, 2009; Sonnleitner et al, 2008; Sonnleitner et al, 2009; Sonnleitner et al, 2011). The methods used in these studies built an initial understanding of the functional roles of sRNAs in *P. aeruginosa*, but required time intensive methods that were not exhaustive and were biased towards sRNAs that interacted strongly with RNA-binding proteins. More recent studies have sought to be more exhaustive in determining the identity and number of sRNAs encoded in the *P. aeruginosa* genome with minimal bias through the use of the second-generation sequencing technology, i.e., RNA-Seq.

The number of identified sRNA genes encoded in the *P. aeruginosa* genome has increased from an initial 40 to approximately 170 within a matter of years through increasingly intensive RNA-Seq analysis (Gómez-Lozano et al, 2012; González et al; 2008; Wurtzel et al, 2012). The variability between studies in the number of sRNA genes in the *P. aeruginosa* genome is based on the limitations of computational methods of analysis of whether an sRNA is indeed transcribed (given difficulties in identifying their promoters), and whether any sRNA is untranslated or instead is an mRNA expressing a small protein. An example of this can be seen in two recent studies using RNA-Seq analysis wherein Gómez-Lozano et al reported 513 novel sRNA transcripts expressed in *P. aeruginosa*, while another study by Wurtzel et al reported only 165 novel sRNA transcripts. Unfortunately, these studies performed very little follow up to confirm that the reported sRNAs are actually transcribed within cells.

In this work, it was hypothesized that sRNA transcripts would have unique expression profiles under different growth conditions of *P. aeruginosa* cultures, and that novel sRNAs could be confirmed as genuine transcripts by studying the changes in expression under different growth conditions. Recent studies have investigated the expression profiles of sRNAs in *P. aeruginosa* but only using sRNAs that were well characterized in the literature (Dötsch et al, 2012). Collaborator Fiona Brinkman's group at Simon Fraser University undertook a study to analyze the *P. aeruginosa* transcriptome in a PAO1 strain. At the time, Erin Gill of the Brinkman group

utilized a conservative manual curation of RNA-Seq data to initially identify 31 sRNA transcripts in *P. aeruginosa*, in addition to the 39 well-characterized sRNAs. The general method of RNA-Seq sequences short stretches (50 or 75 bp) derived as cDNAs from RNAs transcribed from the genome, to generate sequence-reads which are then mapped back onto the genome to determine the sequence of genome-wide transcripts at single-nucleotide resolution (Croucher et al, 2010). Determination of the threshold that represents genuine transcript expression from the genome vs. noise in the data was calculated and genes with read counts below this level were eliminated. Automated curation methods utilize software to quantitatively determine any reads that surpass the cut-off within a single dataset and were not previously described as genes based on a lack of an identifiable promoter. Manual curation involves looking manually for features consistent with non-coding RNAs especially the lack of a recognizable ribosomal binding site in sequences identified by RNA-Seq. For this work transcripts were considered to be putative sRNAs were determined on the basis of being between 50-500 nt in length and lacking a ribosomal binding site and often an identifiable promoter. There have been no characterized sRNAs in prokaryotes that fall outside these definitions. Here we tested the expression of these sRNAs under swarming and biofilm formation conditions *P. aeruginosa* strain PAO1. Multicellular biofilms and cultures moving in a coordinated fashion in swarming motility require rapid and tightly controlled responses. The regulatory actions of sRNAs would provide one possible mechanism to enable cells to rapidly respond under conditions of biofilm formation and swarming motility. Here I confirmed the expression of 28 of the sRNAs found by RNA-Seq methods and demonstrated that, for most of these, sRNA expression varied under conditions of swarming and biofilm formation in *P. aeruginosa*.

2.2 Materials and Methods

2.2.1 Bacterial Strains and Growth Conditions

Control growth conditions used as a basis of comparison with biofilm and swarming motility used *P. aeruginosa* strain PAO1 from the UBC mini-Tn5-*lux* library grown overnight in Luria broth (LB) liquid media at 37 ºC. Cultures were sub-cultured 1/100 into Basal Medium 2 (BM2, 7 mM $(NH_4)_2SO_4$, 40 mM K_2HPO_4, 22 mM KH_2PO_4, 0.5 mM $MgSO_4$, 10 µM $FeSO_4$, and 22 mM glucose at pH 7), and grown to the mid-logarithmic growth phase at 37 ºC with shaking. Biofilms were grown by sub-culturing overnight cultures into fresh BM2-glucose liquid medium

and grown at room temperature, ~23ºC, for 48 hrs without shaking. Cells growing as biofilms at the air-liquid interface were collected into fresh BM2 medium without glucose and biofilms were physically disrupted. Swarming was performed by inoculating 1 µl of mid-logarithmic phase culture grown in BM2-glucose onto swarming agar plates. Swarming agar plates consisted of 0.5% agar with BM2-glucose, except that 0.5 % casamino acids were used as a complex nitrogen source in place of $(NH_4)_2SO_4$. Swarming cultures were grown for 18 hrs and only colony growth at the ends of swarm "tendrils" were used for analysis.

2.2.2 RNA ISOLATION AND CDNA SYNTHESIS

Whole cell RNA was isolated from *P. aeruginosa* strain PAO1 using Qiagen QIAprep® Spin Miniprep Kit (27106) according to the manufacturer's instructions. Isolated RNA preparations were treated with Ambion® Turbo DNA-free™ (AM1907) by adding one tenth volume of 10X Turbo DNase™ buffer, 0.5 µl Turbo DNase™, and 1 µl Ambion® SUPERase™ inhibitor (AM2694) to isolated RNA and incubating for 1 hour. Another 0.5 µl of Turbo DNase™ was then added and the mixture incubated for a further hour at 37ºC. Inactivation reagent was added to one tenth the volume of the reaction mixture, and incubated for 20 min at 37ºC before centrifuging the mixture at 10 000 x g in a microfuge. Treated RNA was extracted in the supernatant and checked for purity by spectrophotometric $OD_{260/280}$ ratios as well as by PCR using primers for the house-keeping gene, *rpsL*. RNA was stored at -80ºC. Synthesis of cDNA for use in real-time semi-quantitative PCR (RT-qPCR) was performed by using 1 µg RNA added to a final volume of 15 µl reaction mixture containing final concentrations of 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 µM dithiothreitol (DTT), 500 µM triphosphate deoxyribonucleotides, with 0.75 µl Invitrogen™ SuperScript™ II reverse transcriptase (18064-022), and 0.375 µl Ambion® SUPERase™ inhibitor placed in a thermocycler programmed to run 1 hr at 37ºC, 3 hrs at 42ºC, and 10 min at 72ºC. Yield was calculated by spectrophotometric $A_{260/230}$ ratios and cDNA was stored at -20ºC.

2.2.3 PRIMER DESIGN AND SEMI-QUANTITATIVE PCR

For detection of novel sRNAs previously characterized by Gill et al, primers for RT-qPCR were designed with the NCBI primer-BLAST server using default parameters and ordered from Invitrogen (Table 2.1).

Table 2.1 Primers used in this study (F: forward; R: reverse).

Primer target	Direction	Sequence
PA0123.1	F	CGTCGGGTTTCGGAAAAA
	R	CCTGATTAGTTCTTTGGCTGACTCA
PA0290.1	F	CGCCAGAAAGGAAGCTGTAATAG
	R	CTCCCGGCTGACGGG
PA0296.1	F	GGCCGTTTTCAGGGCAT
	R	CCTTCGACGCGAGGTTTTT
PA0314.1	F	CGGGCTTCGCAGTGGA
	R	TGCCTTCCGAATCAGGGA
rsmY	F	CAGGAAGCGCCAAAGACAAT
	R	TCCGTGCTACGCCACCA
PA0667.1	F	CGCTGCAACACCGCTG
	R	AGAAAGCGCCGCCGTATTA
PA0730.1	F	AAATAGAGAGCGTCCGAAATCCT
	R	TTCCTGCCCGGCCAAT
PA0805.1	F	TGGTATTGCGGGACGCC
	R	ACTCTTCTGAAGCAATCCCCTG
PA0958.1	F	TCTTGTTGAGGTCGCTTCTCAA
	R	CGGAACATGACATTTTTATTACAAGG
PA1091.1	F	AAAGCTCCGCCGGGAA
	R	GCTCAGGTGCCCCAAGAAT
PA1156.1	F	GACTGTGAGTGCCTCCCTGG
	R	AGGTATTGTGTTCGACGGCAA
PA2461.1	F	TGAACCACGTGAAGCGGATA
	R	AGGGAGGCTCCGCGAG
PA2461.2	F	ACAGAACTTCAAAAGCCAGACTTTC
	R	GGGCGGCTAGAGTCTACGC
PA2461.3	F	CCCCTTCGTCCTCGTGC
	R	CCTAGCCAGATTCGACTAACATTCA
PA2633.1	F	CCTCGGCCTCCACCGT
	R	TTCCAGTCGCAATCTCGTCA
PA2952.1	F	CAATACGGCAAAAGGGTGGT
	R	TGAATTCTTTGGAAGCCTGATAGA
PA3159.1	F	CCGAGCTTCGAATACGGCT
	R	TGTGCGAGAAGATGCCAAGT
PA3299.1	F	ACCGCTCATGGCGGC
	R	GCGCCTAATAGCCCTGGG
PA3514.1	F	CGCGGAGAATTACCGAGGA
	R	ACCGCGTGAAAACCGCT
PA3580.1	F	AAACCGGAGGGTCGTTTTT

Primer target	Direction	Sequence
	R	TTCACAAAGGAATGCTGTCAA
PA4055.1	F	GGATCTTGCGGGCGC
	R	TCCGGATAAAGAGAGAACGGG
PA4539.1	F	TTCTCCGCCTTGAAACCG
	R	GCAGGGAAAAGAAGCCGATA
PA4639.1	F	ATTAGCGCTTGAAACAGCCC
	R	AGGCTCTGGTCATGAGGTATCC
PA4656.1	F	CGTTTTCGACTCAGCCAAGG
	R	GCTGGCGCCGTTCACTAA
PA4726.3	F	CGCCCGAGAGGTCCTGATA
	R	GCGTTGCTCAAACAGGACG
PA5078.1	F	AAAAGAATGCCTGTTTCCAGTCA
	R	TGCCCCCTGGTCTTCCA
PA5304.1	F	TAGGAAGAGGCAGGCAGAAA
	R	CCCCTAATTGTCCGGTTTTT
PA5304.1	F	ACCCGCTGCATCCCG
	R	TTCTGATATAAAGCTGCGCTCTTTT

Samples of cDNA were diluted 1/100 for RT-qPCR on an Applied Biosystems® 7300 Real Time PCR System programmed for a dissociation stage of 95ºC for 15 s and an elongation stage of 60ºC for 30 s repeated for 40 cycles. Expression changes were analysed using the comparative Ct method as per the following equations: $\Delta Ct_{test}=Ct_{test}-Ct_{housekeeping\ gene,\ rpsL}$); $\Delta Ct_{control}=Ct_{control}-Ct_{rpsL}$; $\Delta\Delta Ct=\Delta Ct_{test}-\Delta Ct_{control}$; $FC=2^{-\Delta\Delta Ct}$, where *Ct* indicates the cycle number threshold at which PCR amplification was exponential for all detectable samples, ΔCt_{test} was for cells grown under the conditions of biofilm or swarming, while $\Delta Ct_{control}$ was determined for planktonic mid-logarithmic phase growth of *P. aeruginosa* PAO1 in rich media, and *FC* represented positive fold changes in expression of ΔCt_{test} compared to $\Delta Ct_{control}$. Negative fold changes or down-regulation was represented on a linear scale by taking the negative inverse of *FC*.

2.3 Results

Previous studies have utilized predominately computational methods to automate searches for sRNAs within the genome of *P. aeruginosa* (Gómez-Lozano et al, 2012). Unfortunately, these searches are only as good as the underlying assumptions guiding them, and as sRNAs have limited sequence features to enable recognition, it is difficult to definitively assign function. Here a conservative approach for confirmation of novel sRNAs by RT-qPCR was taken by studying differential expression of novel sRNAs found by manual analysis of RNA-Seq sequencing data from *P. aeruginosa* strain PAO1. Despite the high stringency cutoffs used by Dr. Gill, four of the sRNAs studied here, PA0296.1, PA0667.1, PA2952.1, and PA3299.1 were not previously identified by Gomez-Lozano et al (2012), despite their low stringency analysis. Conversely Wurtzel et al (2012) who used intermediate stringency did not observe 15 of these sRNAs (although 2 of these have no homologs in strain PA14 in which they performed their studies).

Here I examined the expression of a list of 31 sRNAs identified by manual curation of RNA-Seq data by Erin Gill. Four sRNAs found by the Brinkman group's analysis of RNA-Seq data could not be amplified by RT-qPCR. RsmY acted as positive control since its expression profile has been previously characterized under biofilm conditions in *P. aeruginosa* strain PA14 (Dötsch et al, 2012), and the observed 4-fold upregulation agreed with the literature data (Table 2.2).

Table 2.2 Small RNA species detected by RT-qPCR to have differential expression in biofilms and during swarming motility.

Name	Complementarity	Identity Gomez-Lozano et al, 2012	Identity Wurtzel et al, 2012	Fold change in biofilms	Fold change in swarming motility
PA0123.1	None	pant15	Not identified	1.0±0.1	10.6±4.4
PA0290.1	*pilW*, *plcH*	pant37	PA14sr_012	-8.5±-2.2	1.1±2.1
PA0296.1	*rne*	Not identified	P1	-2.5±-0.8	1.0±1.2
PA0314.1	None	pant42	Not identified	-4.5±-5.6	1.0±1.3
rsmY	None	*rsmY*	*rsmY*	4.1±2.3	10.6±7.8
PA0667.1	PA3505, PA2897, PA0690	Not identified	Not identified	-3.4±-1.9	1.0±1.4
PA0730.1	None	pant80	PA14sr_122	-3.0±-1.4	44.0±2.5
PA0805.1	None	pant89	PA14sr_119/ PA14sr_120	-4.8±-3.8	-5.0±-3.1
PA0958.1	None	pant103	PA14sr_112	-6.1±-1.7	2.4±1.5
PA1091.1	PA0588	pant119	Not identified	2.8±0.7	4.5±3.2
PA1156.1	PA1123, *phuR*	pant125	PA14sr_105	1.1±0.1	1.3±0.1
PA2461.1	PA2460, PA2458	pant225	PA14sr_076	-8.0±-1.2	-3.4±-1.0
PA2461.2	PA2460, PA2458	pant226	PA14sr_077	-5.4±-1.6	1.3±0.1
PA2461.3	PA5134	pant233	Not identified	-2.0±-0.1	3.4±1.0
PA2633.1	PA3672, *recJ*, *nrdG*, PA3522, PA3949, PA5325	pant235	PA14sr_067	4.9±0.8	7.6±5.5
PA2952.1	PA4629	Not identified	PA14sr_061	-2.0±-0.1	2.3±1.7
PA3159.1	None	pant292	no ortholog in PA14	-1.8±-0.5	2.7±1.1
PA3299.1	PA0690, *cyoB*	Not identified	Not identified	2.0±4.5	1.3±2.3
PA3514.1	*tagF1*	pant326	no ortholog in PA14	10.2±4.7	1.0±0.4
PA3580.1	None	pant337	no reads	-4.0±-0.2	1.2±1.7
PA4055.1	PA2728, *mfd*, *chpA*, PA3641	pant373	Not identified	-2.9±-1.4	-2.1±-2.9
PA4539.1	*wzz*	pant415	Not identified	1.5±2.5	10.4±3.3
PA4639.1	PA5156, PA2502, PA4510, *aruI*, PA0475, PA0558, PA1025	pant428	PA14sr_139	4.7±5.2	n/a
PA4656.1	PA2038, PA3517, PA2152, *pslE*, PA2472, PA2750	pant430	Not identified	-3.6±1.9	3.5±1.1
PA4726.3	*ispA*, *hepA*, PA2018, PA3461	pant439	PA14sr_141	-5.3±-3.4	1.0±3.4
PA5078.1	PA0312, *kds*	pant465	Not identified	-5.7±-4.6	2.9±1.5
PA5304.1	None	pant487	Not identified	-3.5±-3.4	4.8±0.5
PA5316.2	PA1302, PA2933, *gcp*, PA0241, PA0364, *pilJ*, *hsiC2*, PA2325, PA3037, *rnhB*, *pchF*, *recD*, *algP*	pant488	PA14sr_154	-6.8±-3.8	-1.5±-3.2

In total, 27 of the 28 sRNAs tested by RT-qPCR were modestly to considerably dysregulated under biofilm growth and/or swarming motility conditions. There were 25 sRNAs dysregulated in biofilms and 15 sRNAs were significantly dysregulated under swarming conditions (Table 2.2). Various patterns of regulation were observed including inverse relationships between sRNA expression under swarming and biofilm conditions and coordinate sRNA expression profiles consistent with other studies demonstrating regulators that coordinately or oppositely regulate swarming and biofilm formation (Overhage et al, 2008; Yeung et al, 2009).

In this work the sRNA RsmY was used as a positive control as it is among the best-studied sRNA species in *P. aeruginosa* and its expression has previously been studied under biofilm conditions. In comparison to planktonic PA14, RsmY expression was upregulated 4.5-fold, on average, during biofilm growth (Dötsch et al, 2012). In this regard, I observed that RsmY was upregulated by 4-fold in PAO1 biofilms compared to planktonic growth, which is consistent with published results. Neither RsmY, nor any other sRNA, has been previously investigated in the context of swarming motility. It was found here that RsmY is also highly upregulated under swarming conditions with a 10-fold increase in expression compared to planktonic cultures of PAO1.

Under biofilm formation and swarming conditions, significant differences in expression were observed for all but one, PA1156.1, of the novel sRNA genes tested. Four of the sRNAs found in the initial RNA-Seq analysis by the Brinkman group failed to be amplified by PCR, suggesting that either these were incorrectly identified and are not expressed as transcripts, they had expression profiles too small to be detected under the growth conditions used, or that the selected primers were ineffective in amplifying the transcripts. The regions, which could not be amplified through PCR, mapped to the coordinates of 68836-69271, 99801-100048, 707395-707685, and 830970-831031. There was no consistent pattern of length or sequence homologies that helped to explain why other sRNA genes could readily be amplified while these four could not. One sRNA species, PA4639.1, was unable to be amplified in any of 3 biological repeats under conditions of swarming motility, but was readily detectable and was significantly upregulated in biofilms. There were nine sRNA genes (PA0290.1, PA0296.1, PA0314.1, PA0667.1, PA2461.2, PA3299.1, PA3514.1, PA3580.1, and PA4726.3) that were dysregulated only under biofilm conditions, while only PA0123.1 was dysregulated uniquely under swarming motility conditions. Seven sRNAs (PA0730.1, PA0958.1, PA2461.3, PA2952.1, PA3159.1, PA4656.1, and

PA5078.1) were reciprocally regulated, being upregulated under swarming conditions, and 8 (*rsmY*, PA0805.1, PA1091.1, PA2461.1, PA2633.1, PA4055.1, PA4539.1, and PA5316.2) were coordinately regulated. These data are thus consistent with sRNAs being involved in the fine control of complex adaptations.

2.4 Discussion

This work demonstrated that nearly all of the tested sRNAs were expressed under normal lab growth conditions. In comparison to planktonic PAO1 cultures in log phase growth, most sRNAs demonstrated significantly altered expression profiles during biofilm formation, swarming motility, or both. This implies that sRNAs are intimately involved in the fine regulation of complex adaptations. However, it is entirely possible that no novel targets would be found by proteomic analysis due to the moderate effects that an sRNA may have on its targets as well as the limit of resolution of proteomics for poorly expressed targets. In addition, determination of direct RNA-binding interactions of mRNA targets by sRNAs could be performed by electro-mobility shift assays after incubating RNA *in vitro* for novel interactions but require knowledge of the target RNA. To fully study mechanisms of action and interaction partners of sRNAs interdisciplinary approaches will be required. Knockout and overexpression of the sRNA combined with proteomic and transcriptomic investigations will aid in enhancing our understanding of the impact, mechanisms of action and regulomes of sRNAs. Other studies have recently been undertaken to identify the sRNAs expressed in *P. aeruginosa*, usually by RNA-Seq combined with bioinformatic analysis (Dötsch et al 2012; Gómez-Lozano et al, 2012; González et al, 2008; Wurtzel et al, 2012). The advantages of using RNA-Seq have allowed for high-throughput data generation under different bacterial growth conditions. However, the quality of these analyses depend on the depth of sequencing and minimal read count used to determine if a sequence is actually expressed, the accuracy of algorithms used to determine if the expressed sequence is actually translated, and particularly the growth conditions utilized since not all sRNAs would be expected to be transcribed under each growth condition utilized. The expression of putative sRNAs has been rarely confirmed using PCR.

Finding the target genes of sRNAs is not simple since only a small portion of any given sRNA is actually involved in binding to targets, and even then chaperone proteins often enhance binding affinity and/or presentation to target mRNAs. Complementarity of the sRNAs to genes in the PAO1 genome was used to determine potential target mRNAs upon which the novel

sRNAs might act. It was considered that any observed similarity in the functional roles of genes that contained sequence homologies to a given sRNA might inform as to the functions of these sRNAs. In addition, complementarity to other genes within the genome was used to determine whether there were any specific sequences that might emerge as conserved target interaction regions.

Because relatively short stretches of nucleotides are used as interaction regions with target sequences, complementarity was decided by any stretch of homology larger than 7 nt that had an E-value of less than 1 when using BLAST. Discontiguous MegaBLAST was used to allow for more divergent short stretches of homology, and it was initially considered that an indecipherable number of return hits would be found using such broad search strategies. However, most sRNAs returned only small numbers of potential hits and only sRNAs PA2633.1, PA4639.1, PA4656.1, and PA5316.2 had more than 4 hits within in the genome. Overall the majority of novel sRNAs (19 of 27) had some complementarity in the genome. Of the 61 genes found that revealed some complementarity with an sRNA, only 23 had known functions with the remaining genes all coding for hypothetical proteins. For any single sRNA there was no common function for the genes such as all being part of the same metabolic or signalling pathway, or involved in particular biological functions such as motility. Taken together, these findings indicate that using sequence complementarity alone is insufficient to enable accurate elucidation of the targets of sRNAs. Indeed it seems likely that advanced proteomic methods or pulldowns with different combinations of sRNAs and chaperone proteins is needed to elucidate targets. However, it is clear from this work that sRNAs are themselves regulated and possess distinct expression patterns between different modes of growth such as swarming motility and biofilms. This is consistent with the suggestion that sRNAs might themselves have specific regulatory roles.

3 The Role of the *prrF* Locus in *Pseudomonas aeruginosa*

3.1 Introduction

Iron is an essential nutrient for bacteria. However, the availability of biologically useful ferrous (Fe^{2+}) sources is highly limited in the environment. Because of this, bacteria have tightly regulated mechanisms for iron uptake and metabolism in the cell and have sophisticated uptake systems for the acquisition of iron, e.g. using siderophores. Pyoverdin is one of the major iron siderophores of *Pseudomonas aeruginosa*. Iron homeostasis within the cell and siderophore biosynthesis is largely controlled by the transcriptional regulator Fur (Ferric uptake regulator). Recent work has found that Fur also exerts a regulatory effect by controlling the expression of sRNA species (Davis et al, 2005; Massé et al, 2005; Mey et al, 2002; Wilderman et al, 2004).

Fur is in an active repressor conformation when complexed with divalent Fe^{2+} ions under conditions of excess iron within the cell. In the active state, Fur binds target promoters and represses the transcription of iron acquisition genes. When iron becomes limiting within the cell, Fe^{2+} ions will dissociate from Fur rendering it inactive (Leoni et al, 1996; Prince, 1991; Vasil et al, 1999). While the mechanism of Fur regulation has been well characterized, it has also been observed that Fur can both positively and negatively regulate certain target genes (Ochsner et al, 2002). This dual activity of Fur was resolved by characterization of sRNA species regulated by Fur (Davis et al, 2005; Massé et al, 2005; Mey et al, 2002; Wilderman et al, 2004).

In *P. aeruginosa*, Fur has been confirmed to regulate two tandem sRNA genes, *prrF1* and *prrF2* [*Pseudomonas* regulatory RNA involving iron (Fe)] (Wilderman et al, 2004). Evidence suggests that within the *prrF* locus there is potentially a third sRNA, prrH containing both individual sRNAs, which is also regulated by Fur (Oglesby-Sherrouse, 2010). The *prrF1* and *prrF2* sRNAs are nearly identical in sequence and regulation. These sRNAs are considered to be largely redundant with regard to functional roles within the cell and proposed to act as repressors of translation of their target transcripts. The *prrF* sRNAs have previously been found to be involved in the production of *Pseudomonas* Quinolone Signal (PQS) for quorum sensing under iron-limiting conditions, providing a link between pathways for regulation of iron homeostasis and quorum sensing in *P. aeruginosa* (Oglesby et al, 2008).

In *Escherichia coli* and *Vibrio cholerae* there is an sRNA, *ryhB*, that is regulated by Fur similar to the *prrF* sRNAs in *P. aeruginosa*, and is utilized to regulate iron homeostasis (Davis et al, 2005; Massé et al, 2005; Mey et al, 2002). The sRNA *ryhB* also has a role in regulating

virulence (Davis et al, 2005). Because RyhB has this role beyond strict maintenance of iron homeostasis, I hypothesized that *prrF1* and *prrF2* might also be involved in regulating translational expression of targets for complex biological phenomena such as coordinated motility in swarm colonies and biofilm formation in *P. aeruginosa*.

3.2 Materials and Methods

3.2.1 Bacterial Strains and Growth Conditions

For all experimental conditions, the *P. aeruginosa* strains indicated in Table 3.1 were grown overnight in LB liquid media at 37 ºC then sub-cultured by 1/100 dilution into BM2 glucose medium and grown to logarithmic growth phase. For sRNA gene expression changes under biofilm conditions, *P. aeruginosa* strains were inoculated in BM2-glucose medium and were incubated at 23ºC for 48 hrs. For crystal violet staining of simple biofilms, bacteria were inoculated into BM2-glucose medium in 96-well microtitre plates and incubated at 37ºC without shaking for 24 hrs. Anaerobic biofilm growth conditions were set up similarly to aerobic conditions but within a sealed chamber that had atmospheric oxygen chemically removed using a BD BBL™ GasPak™ anaerobic system. Crystal violet staining was done by washing plates with de-ionized water and incubated with 0.1% [w/v] crystal violet for 20 minutes to stain adhered biofilm growth before rinsing again and solubilizing with 70% ethanol. The absorbance at 595 nm was recorded on a Powerwave™ X340 Bio-tek Instruments®, Inc. for biofilm development. Agar plates for swarming motility studies consisted of BM2-glucose agar (0.5% [w/v]) lacking NH_2SO_4 and supplemented with 0.1% [w/v] casamino acids as a weak nitrogen source. Iron depleted swarming plates contained a final concentration of 150 µM of the iron chelator 2,2-dipyridyl in place of adding $FeSO_4$. Swarming cultures were grown for 18 hrs and only colony growth at the ends of swarm "tendrils" were used for transcriptional analyses. Pyocyanin secretion studies were done in LB liquid media and Pyoverdin secretion studies were performed in casamino acid medium (CAA, 0.5 % casamino acids, 7 mM K_2HPO_4, and 0.1 mM $MgSO_4$ at pH 7.2) (Mirleau, 2000; Baysse, 2002).

Table.3.1 PAO1 strains and plasmids used

Strain or plasmid	Genotype or characteristics	Reference
P. aeruginosa		
WT	Wild-type *P. aeruginosa* PAO1 strain H103	Stover et al, 2000
Δ*prrF1*	PAO1 deletion mutant of *prrF1*; GEN^R	Wilderman et al, 2004
Δ*prrF2*	PAO1 deletion mutant of *prrF2*; GEN^R	Wilderman et al, 2004
Δ*prrF1-F2* (Δ*prrH*)	PAO1 deletion mutant of entire *prrF* locus; GEN^R	Wilderman et al, 2004; Olgesby-Sherrouse et al, 2010
$prrF1^+$	Δ*prrF1* pVLT31::*prrF1*; GEN^R, TET^R; a complemented isolate	Wilderman et al, 2004
$prrF2^+$	Δ*prrF2* pVLT31::*prrF2*; GEN^R, TET^R; a complemented isolate	Wilderman et al, 2004
$prrF1\text{-}F2^+/prrH^+$	Δ*prrF1-F2* pVLT31::*prrF1-F2*; GEN^R, TET^R; a complemented isolate	Wilderman et al, 2004; Olgesby-Sherrouse et al, 2010
Plasmid		
pVLT31	Parent pMMB207 with TET^R	de Lorenzo et al, 1993

3.2.2 Primer Design and Semi-Quantitative PCR

Primers for the detection of *prrF* gene loci expression (Table 3.2) were designed using the NCBI primer-BLAST server at default settings. For the detection of the PrrH sRNA the forward primer of PrrF1 was used with the reverse primer of PrrF2. Whole cell RNA was isolated using a Qiagen QIAprep® Spin Miniprep Kit (27106) and used to produce cDNA for use in RT-qPCR. RT-qPCR was performed on an Applied BioSystems® 7300 Real Time PCR System under conditions of biofilm growth and swarming motility.

Table 3.2 Primers used in this study (Forward: F; Reverse: R)

Primer name/ target gene	Primer direction	Sequence
prrF1	F	TCGCGAGATCAGCCGG
	R	GCCTGATGAGGAGATAATCTGAAGA
prrF2	F	ACTGGTCGCGAGGCCA
	R	GCCTGATGAGGAGATAATCTGAAGA

3.2.3 Pyocyanin and Pyoverdin Secretion

For the measurement of pyocyanin secretion, overnight growth cultures were centrifuged and supernatant was collected in new sterile microfuge tubes. One volume of $CHCl_3$ was added to the supernatant and vigorously shaken to extract pyocyanin from the aqueous layer. Transferring the $CHCl_3$ phase to 0.2 N HCl and again shaking vigorously was performed to remove pyocyanin from the hydrophobic layer. The absorbance of pyocyanin in 0.2 N HCl was measured at 520 nm (Whooley, 1982; Mavordi, 2001). For the measurement of pyoverdin secretion, overnight cultures were centrifuged and pellets were discarded and supernatant was diluted 1/200 in 10 mM Tris-HCl. Dilutions were excited at 405 nm for fluorescence emission detection of pyoverdin at 460 nm on a Perkin Elmer® Fluorescence Spectrometer LS 50B (Mirleau, 2000; Baysse, 2002).

3.3 Results

3.3.1 Deletion Mutants of the *prrF* Locus had a Distinct Pyoverdin and Pyocyanin Secretion Phenotype from PAO1 Wildtype.

The *prrF1* and *prrF2* sRNAs have previously been characterized as being regulated by Fur in *P. aeruginosa* (Wilderman et al, 2004; Oglesby-Sherrouse, 2010). However, it has not been investigated whether regulation by the *prrF* sRNAs has any observable effect on the secretion of siderophores for iron acquisition. Pyoverdin is a major iron siderophore in *P. aeruginosa* and is readily detectable due to its ability to fluoresce when excited with ultraviolet light. Pyoverdin reproducibly emits between wavelengths of 420–540 nm when excited with ultraviolet radiation, with peak emission occurring at 460 nm. Relative differences between PAO1 strains were calculated, wherein emission intensity is positively correlated with the concentration of pyoverdin present. For analysis of pyoverdin secretion, cultures were incubated in BM2 minimal medium without added any added ferric iron in the form of $FeSO_4$ and therefore, only trace amounts iron were possibly available, to maximize pyoverdin secretions. The data show that deletion of the entire *prrF* gene locus in the PAO1 strain Δ*prrF1-2* consistently upregulated pyoverdin secretion compared to PAO1 wildtype (Figure 3.1).

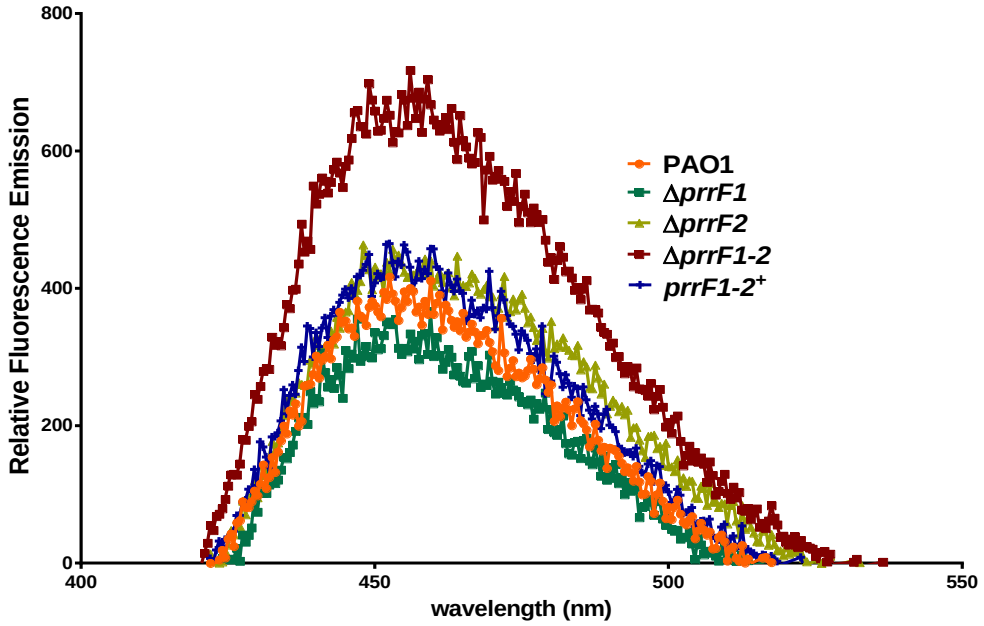

Figure 3.1. Pyoverdin secretion by *prrF* locus mutant strains. The excitation spectra and relative intensity of pyoverdin fluorescence after excitation at 400 nm are shown. PAO1 is the wild type parent strain of the *prrF* deletion mutants. Strains Δ*prrF1* and Δ*prrF2* are deletion strains of PrrF1 and PrrF2, respectively. The Δ*prrF1-2* strain is a deletion of the entire *prrF* locus and Δ*prrF1-2* pVLT31::*prrF1-2* is a complementation strain restoring *prrF1-2* expression on a plasmid construct.

Re-introducing PrrF expression on a plasmid construct with native promoters in the complemented strain Δ*prrF1-2* pVLT31::*prrF1-2* restored pyoverdin secretion to wild type levels. In deletion strains lacking the individual sRNAs *prrF1* or *prrF2* alone, there was no observable difference in pyoverdin secretion compared to the PAO1 parent strain (Figure 3.1).

In addition, secretion of the phenazine pyocyanin was also tested for unique phenotypes in *prrF* sRNA deletion strains. Pyocyanin creates a blue tint in the supernatants of *P. aeruginosa* cultures. Pyocyanin secretion was not significantly reduced in the Δ*prrF1-2* mutant strain of PAO1 as determined by one-way ANOVA, however, the difference was nearly significant when using a Bonferronni multiple comparison analysis with a p-value equal to 0.09 (Figure 3.2).

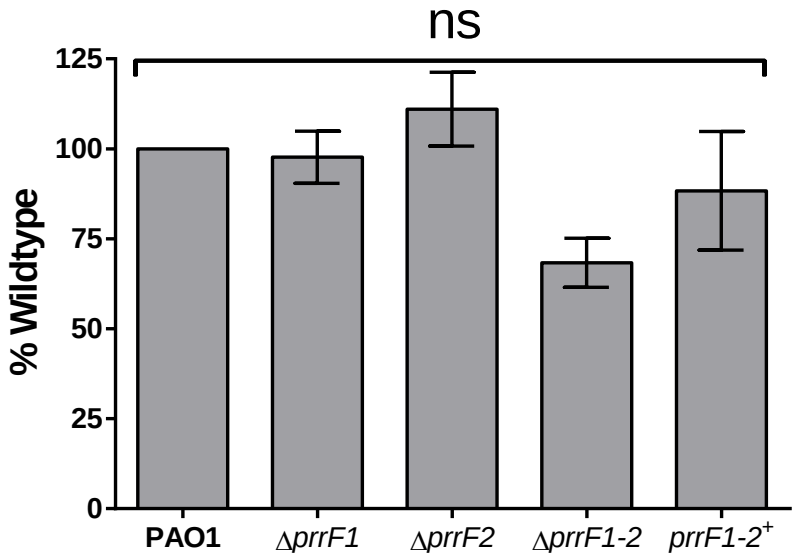

Figure 3.2 Pyocyanin secretion of *prrF* locus mutant strains compared to PAO1 WT. Analysis by one-way ANOVA found no significant difference between any of the strains where $p < 0.05$.

Restoring *prrF* sRNA expression in the complemented strain (Δ*prrF1-2* pVLT31::*prrF1-2*) restored wildtype levels of pyocyanin secretion. Deletion of the individual sRNAs in mutant strains Δ*prrF1* and Δ*prrF2*, respectively, had no significant effect on pyocyanin secretion relative to PAO1 wildtype.

3.3.2 Deletion Mutants in *prrF* Showed Unique Swarming Phenotypes in PAO1.

To further study the biological roles of the *prrF* sRNAs distinct from iron metabolism, the *prrF* deletion strains were investigated for unique phenotypes in swarming motility and biofilm formation. Previous studies utilizing RNA-Seq demonstrated that the *prrF* genes are significantly increased under conditions of biofilm growth when compared to planktonic, free-swimming growth (Dötsch et al, 2012). Here semi-quantitative PCR (qPCR) was used to determine the expression profiles of *prrF* sRNAs during swarming motility and biofilm formation. In this regard, *prrF1* and *prrF2* were highly upregulated in swarming colonies and during biofilm formation. Relative to planktonic PAO1 cultures, biofilm cultures upregulated *prrF1* and *prrF2* 12- and 20-fold, respectively. Expression of the putative third sRNA, *prrH*, which consists of both *prrF1* and *prrF2* in a single transcript, also showed a 13-fold increase under biofilm conditions. (Table 3.3).

Table 3.3 Fold change in *prrF* locus expression during swarming motility and biofilm formation.*

Name	Genomic Coordinates	Size (bp)	Complementarity	Fold increase in biofilms	Fold increase in swarming motility
prrF1	5283960 - 5284110	151	*prrF2*	12.1±2.1	217±116
prrF2	5284172 - 5284319	148	*prrF1*	20.1±4.2	141±66.6
prrF1-2 (*prrH*)	5283960 - 5284319	360	*prrF1, prrF2*	12.5±1.7	163±54.0

* Fold change reported is the mean value for 3 biological replicates.

Under swarming motility conditions, the *prrF* locus was even more highly up-regulated than it was under biofilm cultures. The *prrF1* and *prrF2* sRNAs showed an up-regulation of 217-fold and 141-fold, respectively. Moreover, compared to free-swimming cultures, *prrH* was up-regulated 163-fold in swarming colonies.

It was then further studied whether *prrF* sRNA deletion mutants had observable phenotypes in swarming motility and biofilm formation. To test swarming, *prrF* deletion mutants were inoculated on swarm plates and compared with wildtype PAO1. The swarming colonies in the complete *prrF* locus deletion strain (Δ*prrF1-2*) were noticeably (46%) reduced in size, whereas deletions of only *prrF1* and *prrF2* (Δ*prrF1* and Δ*prrF2*, respectively) maintained wildtype swarming levels. The complemented strain expressing *prrF* sRNAs from a plasmid constructed in a complete *prrF* deletion background was unable to restore wildtype levels of swarming (Figure 3.3), which was possibly due to gene dosage effects. Swarming was performed under iron rich (Figure 3.3A) as well as iron depleted conditions, the latter of which was accomplished by the inclusion of 2,2-dipyridyl (Figure 3.3B). Limited iron reduced the swarming ability of all PAO1 strains, but that the relative differences between PAO1 WT and mutant strains were maintained regardless of iron availability.

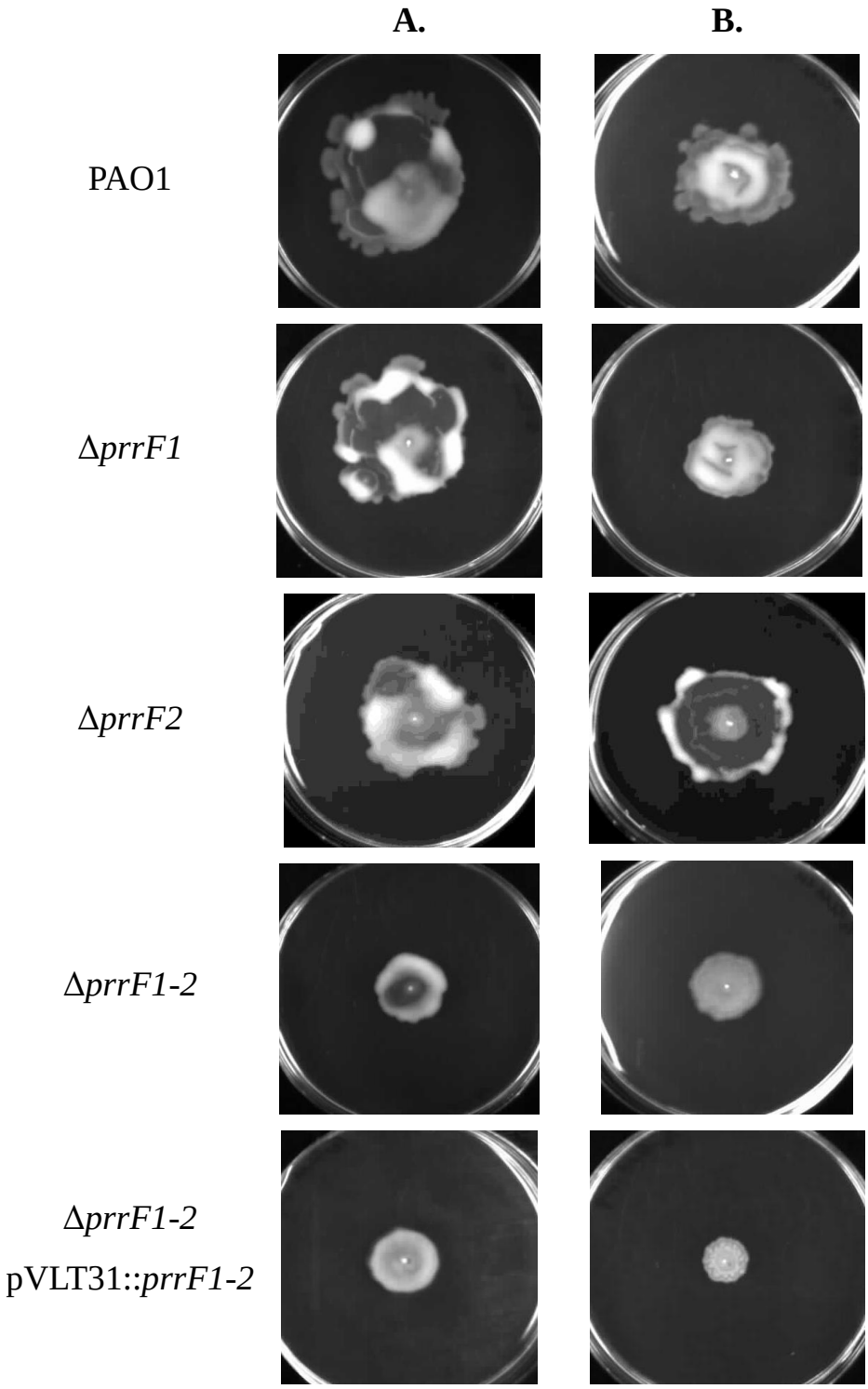

Figure 3.3 Swarming phenotype of *prrF* locus mutants compared to PAO1 WT (H103). (A) Swarming on regular swarming minimal medium with glucose as a carbon source and 10 µM $FeSO_4$. (B) Swarming under iron depleted conditions created by including 150 µM 2,2 dipyridyl.

To investigate biofilm formation in the *prrF* sRNA mutants, crystal violet staining was used to assess plastic-adherent biofilms. In contrast to the results observed for swarming, the deletion strains in *prrF2* (Δ*prrF2*) and the whole *prrF* gene locus (Δ*prrF1-2*) were not significantly different from PAO1 wildtype under iron replete or iron depleted conditions. However, the *prrF1* deletion strain, Δ*prrF1*, formed biofilms on surfaces ~250% better than PAO1 under conditions of excess iron ,and nearly 150% greater under iron depleted growth condtions ($p<0.0001$) (Figure 3.4).

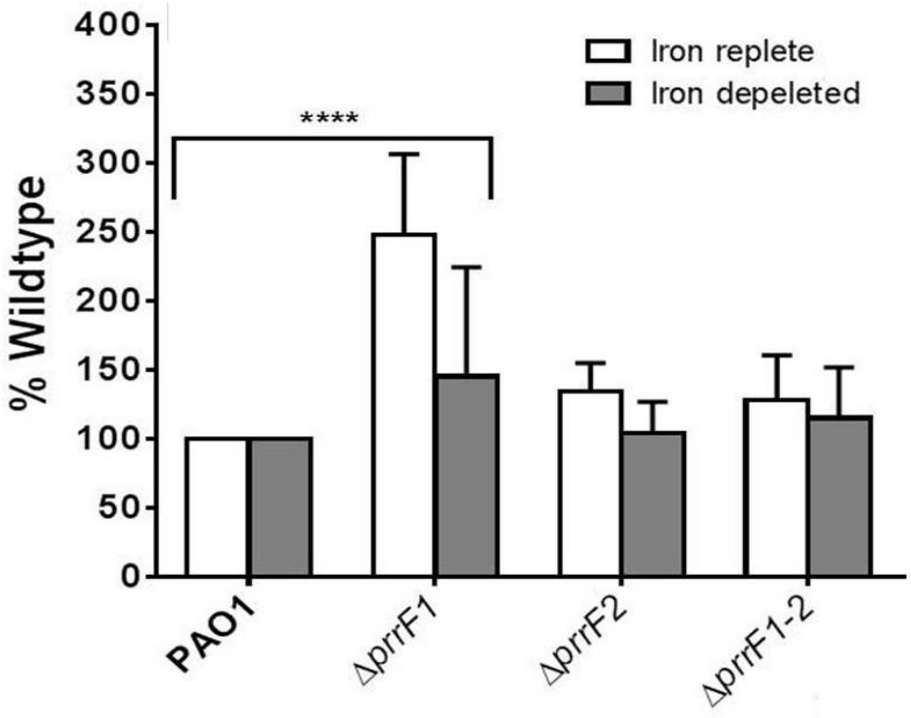

Figure 3.4 Crystal violet staining of *prrF* mutant strains. Iron replete conditions consisted of 10 µM $FeSO_4$ and iron depleted conditions included the addition of the iron chelator 150 µM 2, 2 dipyridyl. Statistical analysis was performed by the unpaired Student's t test where significance is indicated by $p<0.0001$ (****).

3.4 Discussion

The *prrF* locus sRNAs were previously characterized as translational regulators of enzymes involved in iron utilization and act as a mechanism to enhance effective use of iron under conditions of limited availability. The aim of this work was to investigate novel phenotypes in deletion mutants of the *prrF* sRNAs that might give insight into broader functions regulated by the *prrF* sRNAs. Here I found that the deletion of the *prrF* locus in PAO1 affects the secretion of both the iron siderophore pyoverdin, and the phenazine pyocyanin, as well as affects the complex

social behaviour of *P. aeruginosa*, namely swarming motility. The molecular structure of pyoverdine includes several aromatic rings and readily fluoresces in the range of 400-500nm when excited with 405 nm ultraviolet light. Using this as an indicator of levels of pyoverdin synthesized and secreted demonstrated that the deletion mutant of the entire *prrF* locus in the Δ*prrF1-2* strain was heightened compared to PAO1 WT (Figure 3.1). Complementation of the *prrF* locus in the Δ*prrF1-2* pVLT31::*prrF1-2* strain restored WT levels of pyoverdin synthesis. Previous work showed that *prrF1* and *prrF2* are redundant in their roles within the cell, and pyoverdin secretion in the Δ*prrF1* and Δ*prrF2* strains support this conclusion as both have the same level of secretion as PAO1 WT (Figure 3.1).

I also investigated whether the *prrF* sRNAs regulate levels of another large secreted molecule, namely the phenazine compound pyocyanin. *P. aeruginosa* secretes many different pigmented compounds of which pyocyanin is one of the most predominant. Pyocyanin gives *P. aeruginosa* colonies their characteristic blue hue. Interestingly, compared to PAO1 WT, pyocyanin secretion in Δ*prrF1-2*, in which the *prrF* locus is completely deleted, was significantly reduced, whereas complementation of the full deletion of *prrF1-2* partially restored wildtype levels of secretion (Figure 3.2). Redundant roles of *prrF1* and *prrF2* was revealed by the fact that deletion strains of only a single *prrF* sRNA gene had no significant effect on pyocyanin secretion compared to wildtype. These data demonstrated that the *prrF* sRNAs had functional roles other than regulation of iron usage within the cell.

Previous work in *V. cholerae* has indicated that RyhB, a functional homolog of the *prrF* sRNAs, has a regulatory role in biofilm formation (Mey et al, 2005). Here, analysis by crystal violet staining was used to study any effects the *prrF* sRNAs had on biofilm formation. I demonstrated that the deletion mutant of *prrF1*, under conditions of both excess iron and trace iron, led to biofilm formation of more than twice that of PAO1 WT. The effect of deleting a single *prrF* gene was unique to this assay system since in all other assays here, e.g. swarming, no significant differences were observed when either one of the single *prrF* sRNAs were deleted (Figure 3.3 and Figure 3.4). Both the *prrF2* deletion strain and deletion of the whole of *prrF* locus (Δ*prrF1-2*) maintained the same level of biofilm formation as the PAO1 WT parent strain (Figure 3.4). These results are counter-intuitive when taking into consideration previous results in the literature, and those described here, which demonstrate that *prrF1* and *prrF2* possess nearly identical nucleotide sequences, promoters (Wilderman, 2004; Oglesby-Sherrouse, 2010)

and functions, as shown above. Crystal violet staining is used as an indicator of levels of biofilm formation due to the fact that it measures the level of cell adhesion to solid surfaces, which is a requirement for mature biofilms to develop. However, qPCR data showed *prrF1* to be expressed at levels nearly half that of *prrF2* from biofilm colonies (Table 3.3). Overall this data suggests that *prrF1* has a negative regulatory role on biofilm formation although similar removal of *prrF1* expression in the Δ*prrF1-2* showed no significant difference compared to PAO1 WT; this might indicate that the full regulatory effects of the *prrF* sRNAs require an interplay of both sRNA transcripts, with specific roles for each.

Under swarming conditions, *prrF1* was somewhat more highly upregulated (217±116-fold change in expression) than *prrF2* and *prrF1-2* (141±66- and 163±54.0-fold change, respectively), The *prrF1* and *prrF2* sRNAs appear to have largely redundant roles in swarming since deleting either *prrF1* or *prrF2* alone had no effect on the ability to swarm. A reduced swarming phenotype was observed in both excess iron and iron depleted conditions when the complete *prrF1-2* region is deleted in the Δ*prrF1-2* strain (Figure 3.3). Complementation of the *prrF1-2* deletion was however unsuccessful in restoring swarming, which I ascribed to gene dosage effects. Overall swarming appeared to be reduced under iron depleted conditions (Figure 3.3B). The ability and morphology of swarming was variable in single deletion mutants of *prrF1* or *prrF2*, but deletion of the whole *prrF1-2* region consistently resulted in a significantly reduced ability to swarm. Due to the fact that swarming phenotypes appear to be independent of the availability of iron, this suggests that *prrF* sRNAs have regulatory roles than are not limited to the regulation of iron usage by the cell. The targets that the *prrF* sRNAs act upon are still not well understood. Previous studies have only investigated dysregulation of gene expression in *prrF* locus deletion mutants, which cannot indicate direct targets of sRNAs. Only general conclusions on downstream effects can be made when analyzing gene expression profiles in sRNA mutants. Searching for targets of the *prrF* sRNAs by sequence homology yielded no results as sequences from the *prrF* locus only had homology to themselves (Table 3.3). In conclusion, the *prrF* sRNAs likely have greater regulatory roles than previously concluded with regards to dysregulation of pyocyanin production, being highly upregulated during biofilm development and in swarming colonies, and participating to some extent in both complex processes. In addition, this work indicates that the *prrF* sRNAs might not be entirely redundant although they appear to usually work in concert to effectively regulate targets.

4 Role of the *phrS* sRNA in *Pseudomonas aeruginosa*

4.1 Introduction

In a clinical setting *P. aeruginosa* is able to develop resistance when exposed to low-levels of antibiotics and this is termed adaptive resistance. The phenomenon of adaptive resistance results from changes in gene expression rather than heritable changes. One of the best-studied mechanisms of adaptive resistance in *P. aeruginosa* is by reduction of the permeability of the outer membrane to polycationic antimicrobials through modification of the lipid A portion of lipopolysaccharide (LPS) molecules that make up the outer leaflet (Briedenstein et al, 2011; Moskowitz et al, 2004). Lipid A can be altered by capping the negatively charged phosphate molecules with positively-charged arabinosamine, thus reducing the ability of cationic antimicrobial agents such as polymyxin B to destabilize the integrity of the outer membrane, which leads to uptake across the outer membrane and cell death. The *arn* operon of *P. aeruginosa* encodes enzymes responsible for the arabinosaminylation of the lipid A portion of lipopolysaccharide (LPS) and multiple transcriptional regulators, which are to some extent redundant, that have been shown to regulate the *arn* operon. The role of sRNAs in regulating expression of *arn* operon proteins has not been proposed.

One sRNA, *phrS* was previously characterized to post-transcriptionally regulate the production of a transcriptional regulator, PqsR (MvfR), which is a key regulator for the synthesis of the *Pseudomonas* Quinolone Signal (PQS) that is involved in one type of *P. aeruginosa* quorum sensing. Sonnleitner et al (2011) found that *phrS* was positively regulated by the transcriptional regulator Anr. Moreover, *phrS* positively regulates translational levels of PqsR by interacting with the 5'UTR of the *pqsR* mRNA transcript, alleviating secondary structures blocking access of the ribosome to the RBS. Previous deep-sequencing of *P. aeruginosa* using RNA-Seq found that *phrS* is significantly differentially expressed under conditions of biofilm formation compared to planktonic growth. Here, other functions of *phrS* were considered including roles in antibiotic susceptibility, biofilm formation and swarming motility.

4.2 Materials and Methods

4.2.1 Bacterial Strains and Growth Conditions

Wildtype PA14, as well as *phrS*, *anr*, and *pqsR* mutant strains of *P. aeruginosa* were obtained from the Harvard Transposon mutant library (Table 4.1).

Table 4.1 Strains and plasmids used

Strain or plasmid	Genotype or characteristics	Reference
P. aeruginosa		
WT	Wild-type *P. aeruginosa* PA14	Liberati et al, 2006
anr	PA14 *anr*::MrT7; GEN^R	Liberati et al, 2006
pqsR/MvfR	PA14 *pqsR*::MrT7; GEN^R	Liberati et al, 2006
phrS	PA14 *phrS*::MrT7; GEN^R	Liberati et al, 2006
$phrS^+$	*phrS*/pUCP18::*phrS*; GEN^R, AMP^R	this study
E. coli TOP10	DH5α parent; F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*_M15 Δ*lacX74 recA1 ara*Δ*139* Δ(*ara-leu*)*7697 galU galK rpsL* (STR^R) *endA1 nupG*	Invitrogen
Plasmids		
pCR-BLUNT II-TOPO	PCR cloning vector; KAN^R	Invitrogen
pUCP18	*E. coli* derived plasmid containing AMP^R marker	Schweizer, 1991
pUCP*lux*::PA3552	pUCP23 containing intergenic region between PA3551 and PA3552 immediately upstream of *luxCDABE*	McPhee et al, 2003

Assessment of the minimal inhibitory concentrations (MICs) of antibiotics was performed in 96-well microtitre plates in LB media using 2-fold dilutions of antibiotics (Wiegand et al, 2008). MIC measurements under aerobic conditions involved shaking at 250 rpm to encourage aeration of cultures. Microaerobic conditions were created by supplementing LB media with 15 mM KNO_3 and sealing the plates with parafilm before incubation. Anaerobic MIC conditions utilized LB media supplemented with 15 mM KNO_3, and microtitre plates were placed in an air-tight chamber and atmospheric oxygen was removed by a BD BBL™ GasPak™ aneraobic system.

Kill curves for PA14 strains were performed by growing strains overnight in LB medium, subculturing them into BM2-glucose medium and growing them to the mid logarithmic phase of growth. Log phase cultures were washed once with BM2 buffer salts, and were diluted 10-fold

into BM2 buffer salts. Cultures were sampled and plated on LB agar plates at 0 min to obtain a total colony count. They were then challenged with 2 µg/ml polymyxin B and incubated at room temperature. Cultures were sampled and plated for colony forming unit counts at time intervals of 5, 10, and 20 min.

4.2.2 BIOFILM DEVELOPMENT

Biofilm flow-cell analysis of PA14 strains was performed by inoculating overnight cultures into flow-cells and letting cultures grow undisturbed for 3 hours before activating pumps and initiating flow. Biofilms were then grown for 3 days before flow-cell cultures were stained with syto9 (live cell stain) and propidium iodide (damaged and dying cells) to be analyzed by scanning-LASER confocal microscopy.

4.2.3 SWARMING MOTILITY

For swarming growth analysis, overnight cultures of PA14 strains were sub-cultured 1/100 into fresh BM2-glucose media and grown to the mid logarithmic phase of growth. Swarming agar plates were made with BM2-glucose media supplemented with 0.5% casamino acids as a nitrogen source with 0.5% agar. They were inoculated with 1 µl log phase cultures and incubated at 37ºC for 18 hrs.

4.2.4 TRANSFORMATION OF *PSEUDOMONAS AERUGINOSA*

Complementation of *phrS* was performed by ligating the PCR-amplifying *phrS* and its native promoter (using the following primers: forward, 5'CTTGATGGCGAACTTGAGCG and reverse, 5'TTTGAACCTGACCTTCCGCC), which was then ligated into the pCR-BLUNT II-TOPO vector. The resultant clone was transfected by the heat-shock technique into competent *E. coli* TOP10 (Table 4.1). The *phrS* construct was removed from the TOPO vector by endonuclease digestion with enzymes *XbaI* and *KpnI*. The *phrS* construct was then ligated into the similarly-cut pUCP18 *P. aeruginosa* expression plasmid, using T4 DNA ligase (New England Biolabs Cat. # M0202L) for 18 hrs at 37ºC. Successful insertion of the *phrS* construct was detected by endonuclease digestion with *PstI*. For electroporation of the pUCP18::*phrS* and pUCP*lux*::PA3552 vectors into *P. aeruginosa*, cells were washed and suspended in 100 µl of sterile 10% glycerol, 6 µl of purified plasmid added, and cells were pulsed at 200 Ω, 25 µFD and 2.5 V using a BioRad MicroPulser™ electroporator (165-2100). Cultures were allowed to re-cover for 1 hour in LB media with shaking at 37ºC before being plated on selective ampicillin plates.

4.2.5 Semi-Quantitative Analysis of *phrS* Effects on Two-Component Systems and the Lipid A Modification Operon in *Pseudomonas aeruginosa*

To study the effects of *phrS* on the Lipid A arabinosaminylation operon of *P. aeruginosa* PA14, strains were transformed with the pUC*lux*::PA3552 expression vector, which contains the *luxCDABE* luciferase operon fused to the *arnBCADTEF* promoter and 5' untranslated region (5'UTR) of the *arn* mRNA. Detection of luminescence under the regulation of the *arn* promoter and 5'UTR and assessing growth of cultures by absorbance at 620 nm (A_{620}) was performed on a TECAN SPECTRAFluor Plus with or without induction with 2 µg/ml indolicidin. RT-qPCR, using the appropriate primers described in Table 4.2 was performed on an Applied BioSystems® 7300 Real Time PCR System in cells induced or not with indolicidin.

Table 4.2 Primers used in this study (Forward: F; Reverse: R)

Primer target	Direction	Sequence
RT-qPCR		
phrS	F	GTGCTCTGTGTATCCGGGAG
	R	GTAGGCCTCATGGTCGCTTT
cprR	F	GCATATCCACGTACTCGTCGTC
	R	GTGATCGCAGACCACCCC
Cloning		
phrS	F	CTTGATGGCGAACTTGAGCG
	R	TTTGAACCTGACCTTCCGCC

4.3 Results

4.3.1 The sRNA *phrS* had a Polymyxin B Resistance Phenotype Distinct from Its Known Transcriptional Regulator and a Known Downstream Effector Gene

Antimicrobial compounds commonly used in the clinic to treat *P. aeruginosa* infections were selected to determine if a *phrS* mutant strain had any unique antibiotic resistance phenotypes. Since previous studies demonstrated that Anr is a transcriptional regulator of *phrS* expression and is itself up-regulated in decreasing levels of available oxygen, mutants in this gene were also tested in addition to utilizing microaerobic and anaerobic conditions (which activate Anr) in addition to aerobic growth. In addition a mutant in the gene for PqsR, which has been shown to be downstream of *phrS*, was tested. Under aerobic conditions, the *phrS* mutant demonstrated a 4-fold increase in resistance to polymyxin B compared to its parent PA14 WT, *anr* mutant, and *pqsR* mutant strains (modal MICs of 5 biological repeats; Table 4.3). All other changes were less

than 2-fold (which is considered by convention within the margin of error for the MIC assay), and none of the observed changes were specific to the *phrS* mutant.

Table 4.3 MICs of the *phrS* mutant compared to WT, and mutants in an upstream regulator *anr* and a downstream target *pqsR* under conditions of differing oxygen availability.

Strain and growth condition	MICs (μg/mL)				
	Ceftazidime	Ciprofloxacin	Piperacillin	Polymyxin B	Tobramycin
Aerobic					
PA14 WT	1	0.2	8	0.25	0.5
phrS	1	0.2	4	1	0.5
anr	1	0.1	4	0.25	0.5
pqsR	2	0.1	4	0.25	0.5
Microaerobic					
PA14 WT	8	0.8	8	0.5	2
phrS	4	0.4	8	1	2
anr	4	0.2	8	1	2
pqsR	4	0.8	4	1	2
Anaerobic					
PA14	8	0.2	>64	0.5	16
phrS	8	0.2	>64	1	32
anr	4	0.1	>64	0.5	32
pqsR	4	0.1	>64	0.5	8

With decreasing levels of available oxygen, decreased antibiotic susceptibility was generally observed for all strains and antibiotics used. The only *phrS* specific phenotype observed was a 2-fold increase in resistance to polymyxin under anaerobic, which is not considered significant as mentioned above (Table 4.3). Under microaerobic conditions, all 3 mutant strains showed the same 2-fold resistance. Other than a 4-fold increase in susceptibility to ciprofloxacin in the *anr* mutant under microaerobic conditions, no other significant changes in susceptibility were observed.

Kill curves with polymyxin B were used to confirm the greater resistance of the *phrS* mutant to killing by polymyxin B. The effects of polymyxin B in cell killing is rapid and can be measured by plating cultures and counting viable cells. The PA14 wildtype and *phrS* mutant were incubated with a bactericidal concentration of 2 μg/ml polymyxin B over a time course of up to 20 min. Figure 4.1 shows that the WT PA14 demonstrated a rapid decrease in viable cells by ~100-fold within 5 minutes, while the *phrS* mutant showed only a mild (~2-fold) decrease at this time. This difference in killing was maintained over the entire 20 minute time course.

Complementation with the *phrS* gene behind its own promoter (*phrS*$^{+}$) restored antibiotic susceptibility.

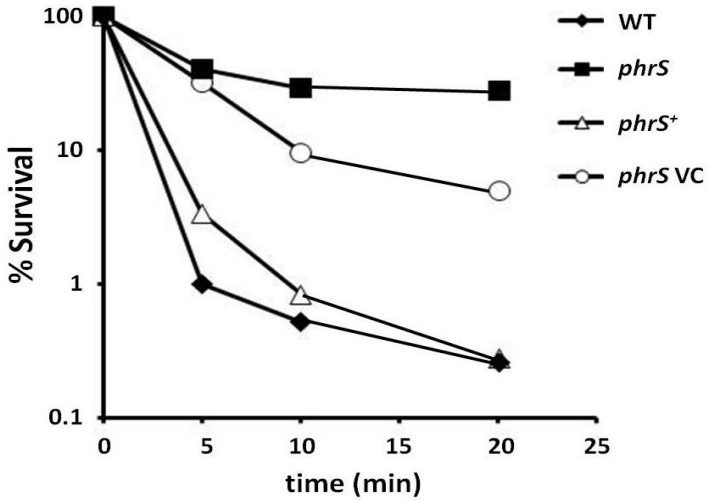

Figure 4.1 Increased resistance of the *phrS* mutant, cf. the WT, to polymyxin B (2 µg/ml). Results shown are representative of 3 biological repeats. Complementation with *phrS* under the regulation of its native promoter in the *phrS*$^{+}$ strain restored strain PA14 WT susceptibility. The *phrS* VC is an empty vector control strain in a *phrS* mutant background.

Together Table 4.3 and Figure 4.1 demonstrate that the *phrS* transposon mutant has a greater resistance to polymyxin B. *P. aeruginosa* has been previously shown to undergo adaptive resistance to cationic antimicrobial compounds, like polymyxin B, through alterations of the lipid A portion of lipopolysaccharides (LPS) in the outer membrane. The *arnBCADTEF* operon mediates the addition of arabinosamine to LPS. To determine whether *phrS* regulated this operon transcriptionally or post-transcriptionally, a combination of semi-quantitative RT-qPCR and assessment of luminescence to assess effects on protein expression was utilized. Analysis by RT-qPCR demonstrated that *arn* expression was not significantly different between the *phrS* mutant and PA14 wildtype indicating that the *phrS* sRNA species did not act through upstream regulators of the *arn* operon (Table 4.4).

Table 4.4 Lack of change in peptide-induced *arnBCADTEF* operon gene expression in a *phrS* mutant relative to that in PA14 WT. Also shown is the decreased induced expression of the *arn* operon in a *cprR* mutant used as a positive control (Fernández et al, 2012) for transcriptional regulation of the *arn* operon.

Strain	Relative fold change in *arnB* gene expression (cf. WT) after treatment with 4 μg/ml indolicidin
phrS	1.0±0.2
cprR	-4.7±-1.6

To assess the impact on translation, wildtype and *phrS* mutant strains were transformed with a pUCP23 plasmid containing a transcriptional fusion construct of the 5' untranslated region (UTR) and promoter region of the *arnBCADTEF* operon fused to the open reading frame of the *luxCDABE* operon which codes for the expression of the luminescent protein luciferase. This *lux* fusion construct enabled levels of luminescence through luciferase expression by *arnBCADTEF* regulatory pathways. While this construct assessed effects on regulation, transcription, and translation of the *arnBCADTEF* operon in the face of the data in Table 4.4, any effects would reflect the interaction of *phrS* with the upstream region of the *arn* operon mRNA, impacting on translation of the fused *lux* genes. The *phrS* mutant and WT were transformed with a plasmid construct containing the promoter and 5' UTR of the *arn* operon upstream of a promoterless *luxCDABE* cassette. When the *arn* operon inducer, cationic antimicrobial compound indolicidin, was applied, as well as when unchallenged, the *phrS* mutant increased both basal and inducer-enhanced expression by about 2-fold when compared to that for the wildtype strain PA14 (Figure 4.2).

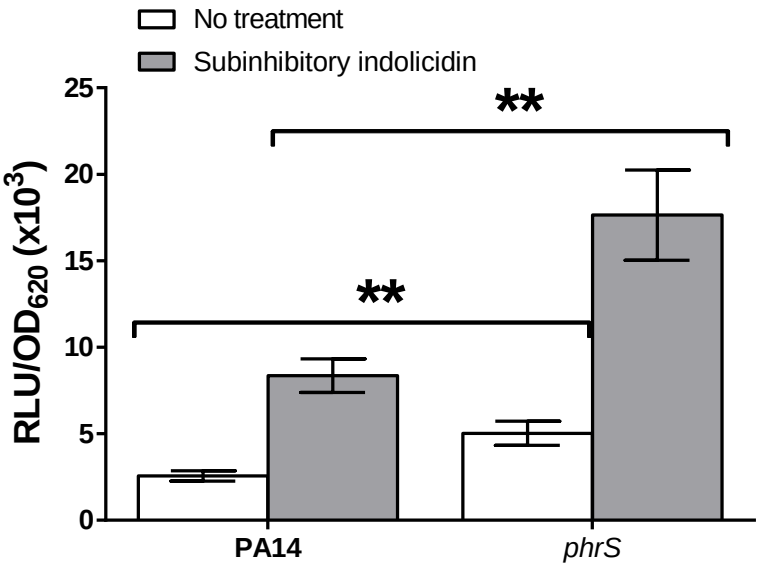

Figure 4.2 Effect of the phrS mutant on expression of a *luxCDABE* cassette assessed as relative luminescence of a *lux* reporter linked to the promoter and 5' untranslated region of the *arn* operon upstream. Unpaired Student's t-tests were performed for statistical analysis and significance was found to confidence interval of $p<0.01$ (**).

Taken together with the qPCR data showing that the lack of *phrS* had no effect on gene expression, these luminescence data showed that *phrS* likely acts directly on the *arnBCADTEF* operon mRNA to negatively impact translation.

4.3.2 The sRNA *phrS* Displayed Reduced Swarming Motility and Increased Biofilm Formation Compared to PA14 Wildtype

Since *phrS* has dual roles in PQS synthesis and polymyxin susceptibility, I examined whether it might have additional roles in social behaviours such as swarming motility and biofilm formation. The *phrS* mutant demonstrated similar twitching and swimming motility when compared to WT (Figure 4.3), indicating that it had functional pili and flagella, respectively. In contrast, *phrS* showed a strongly diminished ability to swarm compared to WT. Wildtype swarming was partly restored in the *phrS* mutant when transfected with a plasmid encoding the *phrS* gene and its native promoter (Figure 4.4A).

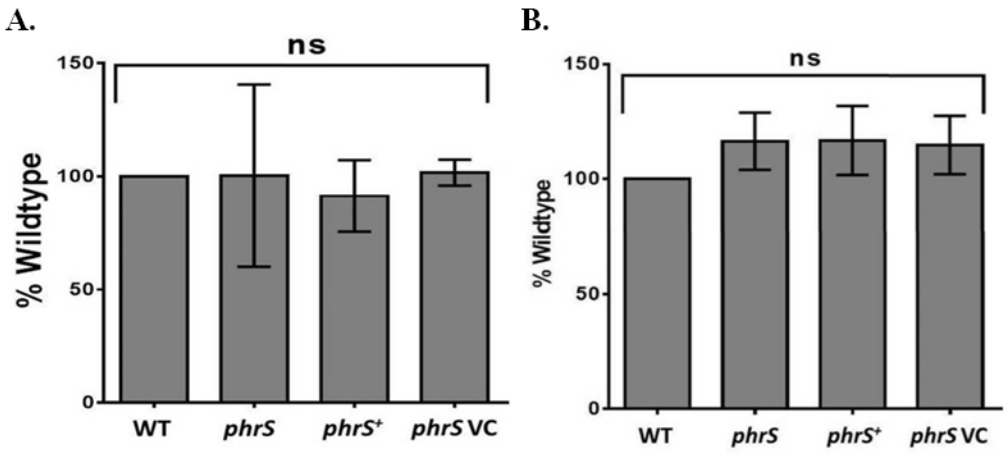

Figure 4.3 Lack of impact of a *phrS* mutant on twitching (A) and swimming (B) motility. A complemented strain (*phrS*$^+$), an empty vector control strain (*phrS* VC), and the wildtype PA14 parent strain (WT) were also tested. No significance (ns) was found when performing one-way ANOVA with a confidence interval of $p < 0.05$.

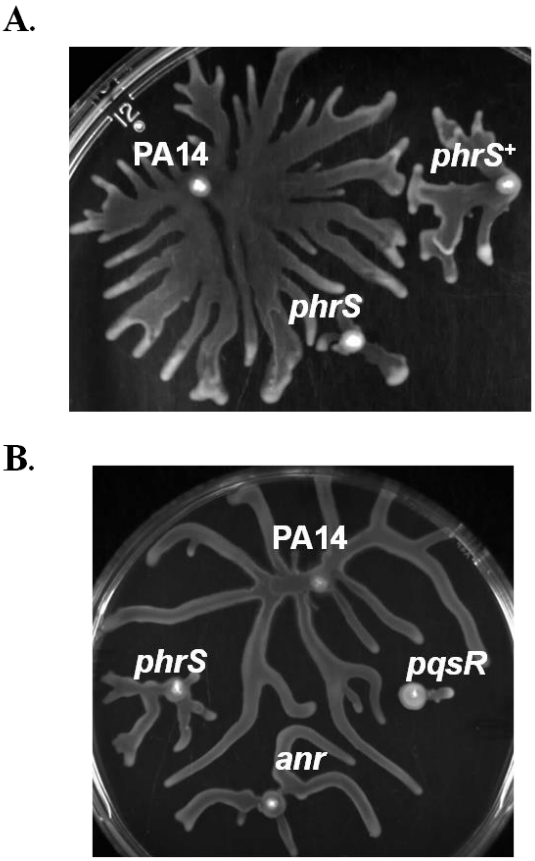

Figure 4.4 Inhibition of swarming motility by the *phrS* mutant. (A) Swarming motility was partially restored by complementation with *phrS* expressed on a pUCP18 vector (*phrS*$^+$). (B) The swarming motility of the *pqsR* mutant was highly reduced compared to wildtype PA14 and *phrS* while the *anr* mutant was moderately reduced in its ability to swarm.

The swarming motility of a transposon insertion mutant in *pqsR* found it to be highly reduced in its ability to swarm, while an *anr* mutant displayed only a moderate reduction in its ability to swarm (Figure 4.4B). Previous work on the *phrS* sRNA demonstrated that it is regulated by Anr and that the *phrS* sRNA regulates the translational levels of the PqsR transcriptional regulator in a positive manner (Sonnleitner et al, 2011). To assess biofilm formation, flow cell methods were used combined with Syto-9 staining and confocal microscopy (Figure 4.5).

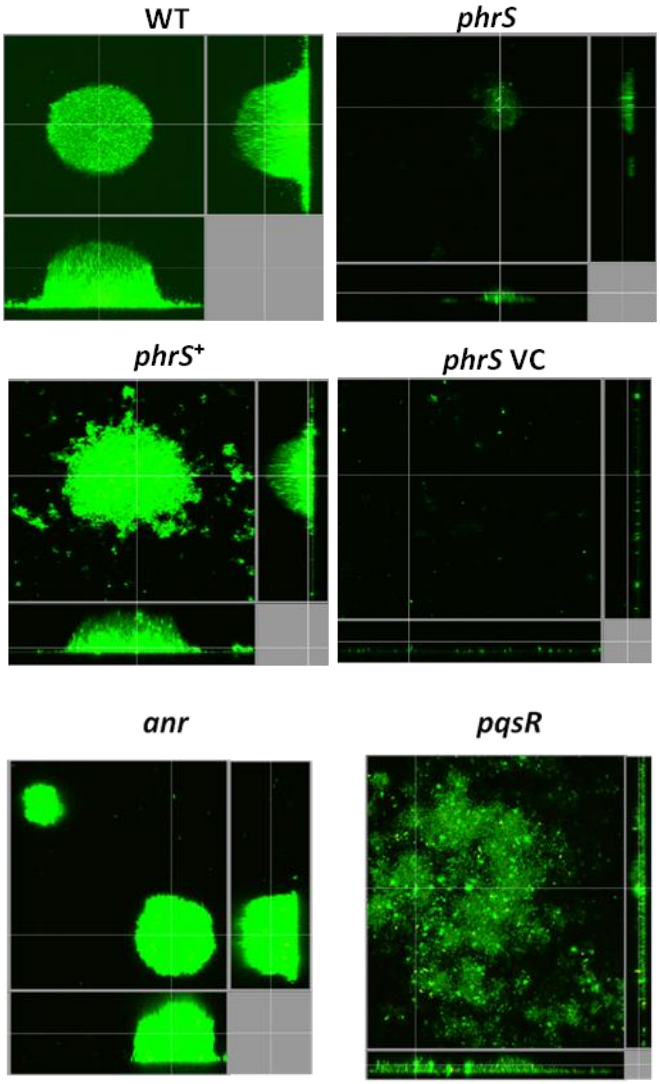

Figure 4.5 Flow-cell analysis of the impact of the *phrS* mutant and its complemented strain (*phrS*$^{+}$) on biofilm formation. Other strains tested were a *phrS* vector control (*phrS* VC), *anr* and *pqsR* transposon insertion mutants, and PA14 parent strain (WT). After 3 days, bacteria were stained green with the all bacteria stain Syto-9, and red with the damaged cell wall/dead-bacteria stain propidium iodide prior to confocal imaging. Each panel shows reconstructions from the top in the large panel and sides in the right and bottom panels (xy, yz and xz dimensions).

The *phrS* mutant demonstrated significantly reduced micro-colony formation compared to the PA14 wildtype and near-normal biofilm formation was restored by complementation. This indicated that the *phrS* mutant had a tremendously reduced ability to form biofilms. Under conditions of biofilm formation the *pqsR* mutant displayed a reduced ability to form biofilms in a flow-cell apparatus. The *anr* mutant displayed no reduction in ability to form biofilms (Figure 4.5) The reduced swarming motility and biofilm formation observed in the *phrS* mutant is therefore likely due to the loss of the positive regulatory effect the *phrS* sRNA has on translation of *pqsR* and the phenotypes observed are due to dysregulation from PqsR dependent pathways.

4.4 Discussion

Analysis of swarming motility and biofilm formation indicated that, relative to the PA14 parent strain, the *phrS* mutant was highly reduced in its ability to swarm, and was deficient in its ability to form biofilms. A transposon mutant in *anr*, a transcriptional regulator of *phrS*, had no observable defect in swarming motility and biofilm formation compared to wildtype. It was also observed that a transposon insertion mutant in *pqsR*, of which *phrS* was previously found to have a positive regulatory effect on its translation (Sonnleitner et al, 2011), had a reduced swarming, as well as biofilm phenotype similar to the *phrS* mutant (Figure 4.4 and Figure 4.5). It is likely that the effects on swarming motility and biofilm formation observed are in part due to dysregulation of PqsR dependent regulatory pathways for the synthesis of the cellular communication molecule PQS. However, because clear observable differences in the phenotypes of the *pqsR* and *phrS* mutants exist it is also likely that *phrS* exerts its regulatory effects on a large number of mRNA targets. This indicates that *phrS* has important roles in promoting complex social behaviours. Predictions of mRNA targets of *phrS*, determined by sequence complementarity, indicated that the proteases *clpV1*, PA2371, and *clpB* are potential targets of *phrS* (Table 4.5).

Table 4.5 Genes containing complementarity with *phrS*.

PA gene number	Gene Name	Description
PA0090	*clpV1*	ATP-binding subunits of Clp protease and DnaK/DnaJ chaperones
PA0459		Probable ClpA/B protease ATP binding subunit
PA1555.1	*ccoQ2*	Cytochrome c oxidase, cbb3-type, CcoQ subunit
PA1784		Hypothetical, unclassified, unknown
PA2333		probable sulfatase
PA2371		Probable ClpA/B-type protease
PA2492	*mexT*	transcriptional regulator MexT
PA2525	*opmB*	Membrane proteins, transport of small molecules, antibiotic resistance
PA2544		Hypothetical, unclassified, unknown
PA2830	*hptX*	heat shock protein HtpX
PA3100	*xcpU*	General secretion pathway outer membrane protein H precursor
PA3871	*nifM*	probable peptidyl-prolyl cis-trans isomerase, PpiC-type
PA4282	*sbcC*	probable exonuclease
PA4542	*clpB*	ATP-binding subunits of Clp protease and DnaK/DnaJ chaperones
PA4560	*ileS*	isoleucyl-tRNA synthetase
PA5176		Hypothetical, unclassified, unknown
PA5378		Hypothetical, unclassified, unknown

Previous research demonstrated that ClpP and ClpS proteases affect swarming motility, biofilm formation and antibiotic resistance (Fernández et al, 2012). It is possible that *phrS* might be involved in the regulation of ClpP and ClpS proteases.

The work here showed that *phrS* is involved in the translation of the *arn* operon of *P. aeruginosa* (Table 4.4 and Figure 4.2). Taken together, the qPCR (Table 4.4) and luciferase expression experiments demonstrated that *phrS* was exerting a direct negative regulatory role on translational expression of the *arnBCADTEF* operon. Given the lack of impact on transcription of this operon, measuring luciferase expression through detection of luminescence under the control of the *arn* 5' UTR revealed that *phrS* exerted a repressive role on expression, as revealed by stimulation in the mutant (Figure 4.2). Consistent with this, in the *phrS* mutant luciferase

expression was significantly increased, cf. WT, both in the absence of the inducer indolicidin as well as in its presence, despite clear differences in overall expression in WT, as expected due to increased transcription in the presence of inducer (Figure 4.2). From this evidence it can be concluded that *phrS* has a role in regulating the protein expression of the *arn* operon, by acting directly on the 5' UTR region of the *arn* operon transcript. Since the *phrS* mutant caused no change in the expression of the transcriptional regulators previously characterized to be involved in controlling adaptive polymyxin resistance, this further supports that *phrS* is a novel and independent pathway regulating lipid A modifications involved in the development of adaptive resistance, and in fact suppresses this phenotype.

Sonnleitner et al (2011) previously demonstrated that *phrS* has a positive regulatory effect on the translation of *pqsR* transcripts by altering mRNA folding upon *phrS* interactions exposing the RBS of *pqsR*. In addition the work in this thesis demonstrated that *phrS* is also able to negatively impact on the translation of the *arn* operon. The activity of sRNA species in the literature currently attributes either positive or negative regulatory roles to a specific sRNA. In theory sRNAs can have multiple interaction sites and exert both positive and negative regulatory effects depending on target mRNAs. The work here is the first to provide evidence suggesting this.

5 Concluding Remarks

5.1 Introduction

Our current understanding of the regulation of translation by sRNAs in bacteria is at present modest, in part due to the difficulties that exist in studying the functions of non-coding RNAs. The use of RNA-Seq has been instrumental in revealing that the number of transcribed sRNAs encoded in genomes is much higher than initially thought. This is consistent with the suggestion that they might represent an important mechanism by which bacteria modulate and integrate intracellular signalling. Future work will require a detailed understanding of the breadth of targets that each single sRNA can act on within the cell, while developing a better understanding of the mechanisms by which sRNAs regulate targets. The use of high-throughput proteomics approaches and more accurate bioinformatic analyses is required to continue promoting an understanding of these elements. However, proteomic studies on such a large scale are still quite costly. Also, current bioinformatic studies to determine targets and the extent of involvement of sRNAs in signalling networks are not very accurate, and inevitably better strategies will rely on developing more information regarding the mechanisms and targets of sRNA. The current research studying sRNAs in *P. aeruginosa* aimed to provide greater knowledge of the biological roles of sRNAs in this bacterium. Here it is demonstrated that sRNAs such as *prrF1, prrF2,* and *phrS* have important roles in complex biological behaviours such as swarming motility and biofilm formation of *P. aeruginosa*, in addition to showing that *phr*S has a role in adaptive resistance to polymyxin B. This work thus demonstrates that sRNAs have diverse roles within the cell that are far reaching. Unfortunately, it still remains difficult to predict the targets of sRNAs based just on complementary sequence analyses.

5.2 Expression of Novel sRNA Species

In the literature, confirmation of novel sRNA species is not as frequent as primary studies, meaning that it is difficult to conclude with certainty whether the identified sRNAs are truly expressed transcripts or artefacts of the data analysis procedure. Two studies have been published suggesting that the number of sRNA genes in *P. aeruginosa* lies between 165 and 513 (González et al, 2012; Wurtzel et al, 2012). None of these studies can claim to be sufficiently accurate to identify the actual number of sRNAs in the *P. aeruginosa* genome. The deeper that one sequences the more transcripts one will find, but deciding the cut-off between noise and actual transcripts can be difficult. Furthermore, numerous assumptions are made with regards to

gene architecture, length, potential for translation, and levels of expression that might influence the determination of the possibility of an sRNA gene. All sRNAs likely have specific conditions under which they are more abundantly expressed (see e.g. Table 2.2), and multiple different growth conditions would have to be considered to enable exhaustive discovery of novel sRNA genes and gain an appreciation for possible biological roles. Here we were able to demonstrate that the study of sRNA expression under different growth conditions of biofilm formation and swarming motility resulted in unique expression profiles for 26 of the 31 novel sRNAs identified by our collaborators Dr. Gill and Dr. Brinkman from SFU, and this provided insights into the prospective biological roles these sRNAs might have in the cell.

5.3 The *prrF* Locus sRNAs

The *prrF* locus is noteworthy for encoding sRNAs that are regulated by Fur. Also the two sRNA genes, *prrF1* and *prrF2* are to be redundant in their roles (Wilderman et al, 2004), while there is potentially a third transcript of *prrH* consisting of the entirety of the *prrF1-2* sequences with potentially unique roles (Olgesby-Sherrouse et al, 2010). Previous work has shown that regulation of the *prrF* sRNAs is dependent on the availability of iron (Wilderman et al, 2004) and is likely involved in iron homeostasis within the cell. Consistent with this possibility, I showed that *prrF* negatively regulated the production of the iron chelator pyoverdin. In addition to this role, the work described here showed that the *prrF* sRNAs are also redundantly involved in regulating pyocyanin production and swarming motility, since deletion of the entire locus, but not the individual sRNA genes resulted in reduced pyocyanin production and swarming. The availability of iron under swarming conditions had no visible effect on the relative extent of swarming in the PAO1 wildtype and any *prrF* mutants, indicating that the swarming phenotype was independent of any role in iron utilization.

In addition, the *prrF* sRNAs were found to be highly up-regulated under conditions of biofilm formation and swarming motility which seems unlikely to be mediated by Fur regulation. Iron availability can have an effect on biofilm formation (Banin et al, 2005), but the availability of iron, as shown here, did not appear to have an effect on swarming in *P. aeruginosa*. Whether the effects on regulation by *prrF* sRNAs are direct or indirect, this thesis demonstrated that the *prrF* sRNAs are substantially dysregulated during the complex social behaviours of biofilm formation and swarming motility in *P. aeruginosa*, and in particular play a role in the latter.

5.4 The *phrS* sRNA

The sRNA *phrS* affects complex social behaviours of *P. aerugnisa*, including biofilm formation and swarming motility; indeed they appear to be required for such social behaviours. It is still not understood to what extent, and which signalling pathways or essential events are regulated by *phrS* for these effects to be manifested. It seems possible that the effects of *phrS* on biofilm formation might be mediated by an effect on matrix polysaccharide synthesis as well as the known influence of downstream target *pqsR* on biofilm formation (Guo et al, 2013). The effects on swarming might be due to the influence of *pqsR* on swarming motility as well as its possible regulation of the ClpP intracellular protease, which is known to be essential for swarming and important in biofilm formation (Fernández et al 2012). Here it was confirmed that *phrS* regulates also pathways outside of the PQS quorum sensing pathway, since *phrS* had a role in regulating protein expression of the *arnBCADTEF* operon that mediates lipid A modifications leading to adaptive resistance upon exposure to polymyxin. This work has thus shown that a single sRNA species, *phrS*, has diverse and far-reaching roles in both complex growth states and responses to antibiotic stress, in addition to its known effect on PQS synthesis.

5.5 Future Research Directions

The expression profiles of novel sRNAs during biofilm formation and swarming motility, compared to planktonic growth used for the majority of previous studies, showed substantial changes in expression under the different growth conditions. To further study the roles of these novel sRNAs, mutational and gene overexpression analysis of the sRNA genes would provide an easy way to observe mutant phenotypes and further attribute biological functions to the given sRNAs. Determination of targets could also be performed by proteomic analysis under the different growth conditions, utilizing mutant and overexpressing strains to narrow down the pathways that given sRNAs might be regulating. Novel sRNA transcripts are continually being reported based on second-generation RNA-Seq methods. However, confirmation of these sRNAs as real transcripts rather than artefacts of deep sequencing or limited informatic methods is somewhat lacking in the literature. In part, this is due to a lack of understanding of the architecture of sRNAs. The range of gene architectures that exist for sRNAs is currently being studied by others; they appear to able to lack obvious promoters, may possess a prospective RBS despite not coding for functional proteins, and may or may not have terminator regions characteristic of protein coding genes. Attempts to refine the bioinformatics underlying searches

for sRNAs are already being undertaken (e.g. Gómez-Lozano et al, 2012), but automatic assignment has not yet been proven to be accurate as the current study found sRNAs, not included in the Gómez-Lozano study, which were confirmed to be expressed by PCR.

Confirmation of active, novel sRNAs requires quantitative PCR to determine protein expression using high-throughput proteomic analyses under varied biological growth conditions to elucidate specific effects on translation and gain insight into the functional roles of sRNAs. Unfortunately, high-throughput proteomic studies require considerable investment to achieve a genome level understanding of how single sRNA species regulate the protein expression of their targets. However, there is great potential in future studies utilizing high-throughput proteomic methods. Methods enabling the study of thousands of proteins at once are rapidly progressing to enable investigators to handle the requisite workflow and data analysis through use of improved digestion methods and computational analyses (Covert et al, 2004; Vaezzadeh et al, 2010). The greatest potential of high-throughput proteomics for studying the regulomes of sRNAs would be achieved by utilitizing proteomic analysis in conjunction with genetic and mutational studies of sRNAs. Comparison of the most highly expressed 1000 to 2000 proteins in sRNA deletion mutants cf. wildtype would provide a powerful, unbiased method for observing the regulome for any sRNA species. This would be highly beneficial to advance our understanding of how far reaching is the regulation of translation by sRNAs. One shortfall of proteomic analysis would be that nothing would be determined about direct interactions of sRNAs with targets, however this would enable gel shift studies with targeted mRNAs.

Gaining an understanding of direct mRNA targets in sRNA interactions will help in understanding the mechanisms of sRNAs and determine sequence motifs used by sRNAs to specify targets. This would also help to improve the ability to predict sRNA targets.

In the case of regulation by *phrS*, a investigation of both gene expression and post-transcriptional regulation of the putative target *arnBCADTEF* found that *phrS* is likely directly interacting with the 5' UTR of the *arnBCADTEF* transcript and negatively regulating its expression. It is interesting to consider that while *phrS* appears to have an effect on the translation of the *arn* operon transcript, it cannot be ruled out that there might in fact be another factor, regulated by *phrS*, which is actually the direct interaction partner that regulates the *arn* operon. Similarly although *phrS* works through the chaperone RNA-binding protein Hfq in *pqrS* regulation (Sonnleitner et al, 2011), it is not clear that this is true for the other events, shown

here, that are regulated by *phrS*. This highlights the need for highly detailed analysis of even a single sRNA species to understand its role and direct targets. To confirm whether *phrS* directly interacts with the *arn* operon, interaction assays with purified transcripts would be required.

The use of proteomic analysis with *phrS* mutants would provide the best understanding as to the targets that are affected during swarming motility and biofilm development. While this would not inform regarding direct interaction partners, proteomic analysis to determine dysregulated protein expression in *phrS* mutants compared to wildtype and would narrow down potential targets. Bioinformatic analysis would then be helpful in determining signalling networks that exist within the pool of potential targets of *phrS*. It is anticipated that there would be a sufficiently small enough number of potential targets that direct interaction studies through pull-down or gel band shift assays could be performed to confirm which are direct interaction partners of *phrS*. Likewise, this same progression of analysis could be utilized to better understand the targets of the *prrF* sRNAs as well as any novel sRNA species found by RNA-Seq, allowing us to better refine our understanding of sRNA interactions with targets, the mechanisms of action of sRNAs, and iteratively enhancing future bioinformatic analysis for unexplored sRNA species.

References

Allesen-Holm, M., Barken, K.B., Yang, L., Klausen, M., Webb, J.S., Kjelleberg, S., Molin, S., Givskov, M., and T. Tolker-Nielsen. 2006. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. Mol. Microbiol. **59**:1114-28.

Amini, S., Hottes, A.K., Smith, L.E., and S. Tavazoie. 2011. Fitness landscape of antibiotic tolerance in *Pseudomonas aeruginosa* biofilms. PLoS Pathog. **7**:e1002298.

Anderl, J.N., Franklin, M.J. and P.S. Stewart. 2000. Role of antibiotic penetration limitation in klebsiella pneumoniae biofilm resistance to ampicillin and ciprofloxacin. Antimicrob. Agents Chemother. **44**:1818-24.

Banin, E., Vasil, M.L., and P. Greenberg. 2005. Iron and *Pseudomonas aeruginosa* biofilm formation. Proc. Natl. Acad. Sci. USA **102**:11076-81.

Barken, K.B., Pamp, S.J., Yang L., Gjermansen, M., Bertrand, J.J., Klausen, M., Givskov, M., Whitchurch, C.B., Engel, J.N., and T. Tolker-Nielsen. 2008. Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. Environ. Microbiol. **10**:2331-43.

Baysse, C., Budzikiewicz, H., Fernandez, D.U., and P. Cornelis. 2002. Impaired maturation of the siderophore pyoverdin chromophere in *Pseudomonas fluorescens* ATCC 17400 deficient for the cytochrome c biogenesis protein CcmC. FEMS Microbiol. Lett. **523**:23-28.

Beisel, C.L. and G. Storz. Base pairing small RNAs and their roles in global regulatory networks. FEMS Microbiol. Rev. **34**:866-82.

Blattner, F.R., Plunkett III, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B., and Y. Shao. 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277**:1453-62.

Borriello, G., Werner, E., Roe, F., Kim, A.M., Ehrlich, G.D. and P.S. Stewart. 2004. Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. Antimicrob. Agents Chemother. **48**:2659-64.

Briedenstein, E.B.M., de la Fuente-Núñez, C., and R.E.W. Hancock. 2011. *Pseudomonas aeruginosa*: All roads lead to resistance. Trends Microbiol. **19**:419-26.

Brencic, A., McFarland, K.A., McManus, H.R., Castang, S., Mogno, I., Dove, S.L., and S. Lory. 2009. The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts

exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs. Mol. Microbiol. **73**:434-45.

Covert, M.W., Knight, E.M., Reed, J.L., Herrgard, M.J., and B.O. Palsson. 2004. Integrating high-throughput and computational data elucidates bacterial networks. Nature **429**:92-6.

Croucher, N.J., and N.R. Thomson. 2010. Studying bacterial transcriptomes using RNA-seq. Curr. Opin. Microbiol. **13**:619-24.

Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W. and E.P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science. **280**:295-8.

Davis, B.M., Quinones, M., Pratt, J., Ding, Y., and M.K. Waldor. 2005. Characterization of the Small Untranslated RNA RyhB and its regulon in *Virbio cholerae*. J Bacteriol. **187**:4005-14.

De Kievit, T.R., Gillis, R., Marx, S., Brown, C. and B.H. Iglewski. 2001. Quorum-sensing genes in *Pseudomonas aeruginosa* biofilms: Their role and expression patterns. Appl. Environ. Microbiol. **67**:1865-73.

de Lorenzo, V., Eltis, L., Kessler, B., and K.N. Timmis. 1993. Analysis of *Pseudomonas* gene products using *lacI*^{q}/*Ptrp-lac* plasmids and transposons that confer conditional phenotypes. Gene **123**:17-24.

Delihas, N. and S. Frost. 2001. *MicF*: an antisense RNA gene involved in response of *Escherichia coli* to global stress factors. J. Mol. Biol. **313**:1-12.

Déziel, E., Lépine, F., Milot S., and R. Villemur. 2003. *rhlA* is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*: 3-(3-hydroxylakanoyloxy)alkonoic acids (HAAs), the precursors of rhamnolipids. Microbiology **49** :2005-13.

Dötsch, A., Eckweiler, D., Schniederjans, M., Zimmermann, A., Jensen, V., Scharfe, M., Geffers, R., and S. Häussler. 2012. The *Pseudomonas aeruginosa* transcriptome in planktonic cultures and static biofilms using RNA Sequencing. PLoS ONE. **7**:e31092.

Fernández, L., Breidenstein, E.B.M., Song, D., and R.E.W. Hancock. 2012. Role of intracellular proteases in the antibiotic resistance, motility, and biofilm formation of *Pseudmonas aeruginosa*. Antimicrob. Agents Chemother. **56**:1128-32.

Fernández, L., Jenssen H., Bains M., Wiegand, I., Gooderham, W.J., and R.E.W. Hancock. 2012. The two-component system CprRS senses cationic peptides and triggers adaptive resistance in *Pseudomonas aeruginosa* independently of ParRS. Antimicrob. Agents Chemother. **56**:6212-22.

Friedman, L. and R. Kolter. 2004. Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. Mol. Microbiol. **51**:675-90.

Gómez-Lozano, M., Marvig, R.L., Molin, S., and K.S. Long. 2012. Genome-wide identification of novel small RNAs in *Pseudomonas aeruginosa*. Environ. Microbiol. **14**:2006-16.

González, N., Heeb, S., Valverde, C., Kay, E., Reimann, C., Junier, T., and D. Haas. 2008. Genome-wide search reveals a novel GacA-regulated small RNA in *Pseudomonas* species. BMC Genomics **9**:167-81.

Guo, Q., Kong, W., Jin, S., Chen, L., Xu, Y. and K. Duan. 2013. PqsR-dependent and PqsR-independent regulation of motility and biofilm formation by PQS in *Pseudomonas aeruginosa* PAO1. J. Basic Microbiol. (Epub ahead of print).

Hancock, R.E.W. 1981. Aminoglycoside uptake and mode of action-with special reference to streptomycin and gentamicin. II. effects of aminoglycosides on cells. J. Antimicrob. Chemother. **8**:429-45.

Holt, K.E., Parkhill, J., Mazzoni, C.J., Roumagnac, P., Weill, F.-X., Goodhead, I., Rance, R., Baker, S., Maskell, D.J., Wain, J., Dolecek, C., Achtman, M., and G. Dougan. High-throughput sequencing provides insights into genome variation and evolution in *Salmonella* Typhi. Nat. Genet. **40**:987-93.

Hoyle, B.D. and J.W. Costerton. 1991. Bacterial resistance to antibiotics: The role of biofilms. Prog. Drug Res. **37**:91-105.

Jackson, K.D., Starkey, M., Kremer, S., Parsek, M.R. and D.J. Wozniak. 2004. Identification of psl, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. J. Bacteriol. **186**:4466-75.

Kindrachuk, K.N., Fernandez, L., Bains, M. and R.E.W. Hancock. 2011. Involvement of an ATP-dependent protease, PA0779/AsrA, in inducing heat shock in response to tobramycin in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **55**:1874-82.

Klausen, M., Heydorn, A., Ragas, P., Lambertsen, L., Aaes-Jorgensen, A., Molin, S. and T. Tolker-Nielsen. 2003. Biofilm formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants. Mol. Microbiol. **48**:1511-24.

Köhler, T., Curty, L.K., Barja, F., van Deiden, C., and J.-C. Pechére. 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J. Bacteriol. **182**:5990-6.

Kumon, H., Tomochika, K., Matunaga, T., Ogawa, M. and H. Ohmori. 1994. A sandwich cup method for the penetration assay of antimicrobial agents through *Pseudomonas* exopolysaccharides. Microbiol. Immunol. **38**:615-9.

Leoni, L., Ciervo, A., Orsi, N., and P. Visca. 1996. Iron-regulated transcription of the *pvdA* gene in *Pseudomonas aeruginosa*: Effect of Fur and PvdS on promoter activity. J. Bacteriol. **178**:2299-313.

Liberati, N.T., Urbach, J.M., Miyata, S., Lee, D.G., Drenkard, E., Wu, G., Villanueva, J., Wei, T., and F.M. Ausubel. 2006. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc. Natl. Acad. Sci. USA **103**:2833-8.

Lindsay, D. and A. von Holy. 2006. Bacterial biofilms within the clinical setting: What healthcare professionals should know. J. Hosp. Infect. **64**:313-25.

Lyczak, J.B., Cannon, C.L., and G.B. Pier. 2000. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microb. Infect. **2**:1051-60.

Ma, L., M., Conover, H. Lu, Parsek, M.R., Bayles, K. and D.J. Wozniak. 2009. Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. PLoS Pathog. **5**:e1000354.

Macia, M.D., Perez, J.L., Molin, S. and A. Oliver. 2011. Dynamics of mutator and antibiotic-resistant populations in a pharmacokinetic/pharmacodynamic model of Pseudomonas aeruginosa biofilm treatment. Antimicrob. Agents Chemother. **55**:5230-7.

Marvrodi, D.V., Bonsall, R.F., Delaney, S.M., Soule, M.J., Phillips, G., and L.S. Thomashow. 2001. Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **183**:6454-64.

Massé, E. and S. Gottesman. 2002. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **99**:4620-5.

McClelland, M., Sanderson, K.E., Spieth, J., Clifton, S.W., Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F., Hou, S., Layman, D., Leonard, S., Nguyen, C.,

Scott, K., Holmes, A., Grewal, N., Mulvaney, E., Ryan, E., Sun, H., Florea, L., Miller, W., Stoneking, T., Nhan, M., Waterson, R., and R.K. Wilson. 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature **413**:852-6.

McPhee, J.B., Lewenza, S., and R.E.W. Hancock. 2003. Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. Mol. Microbiol. **50**:205-17.

Mey, A.R., Craig, S.A., and S.M. Payne. 2005. Characterization of *Vibrio cholerae* RyhB: The RyhB regulon and role of *ryhB* in biofilm formation. Infect. Immun. **73**:5706-19.

Mirleau, P., Delorme, S., Philippot, L., Meyer, J-M., Mazurier, S., and P. Lemanceau. 2000. Fitness in soil and rhizosphere of *Pseudomonas fluorescens* C7R12 compared with a C7R12 mutant affected in pyoverdin synthesis and uptake. FEMS Microbiol. Ecol. **34**:35-44.

Moskowitz, S.M., Ernst, R.K., and S.I. Miller. 2004. PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. J. Bacteriol. **186**:575-9.

Mulcahy, H., Charron-Mazenod, L. and S. Lewenza. 2008. Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginos*a biofilms. PLoS Pathog. **4**:e1000213.

O'Toole, G.A. and R. Kolter. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. **30**:295-304.

Ochsner, U.A., Wilderman, P.J., Vasil, A.I., and M.L. Vasil. 2002. GeneChip® expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: Identification of novel pyoverdin biosynthesis genes. Mol. Microbiol. **45**:1277-87.

Oglesby, A., Farrow III, J.M., Lee, J.-H., Tomaras, A.P., Greenberg, E.P., Pesci, E.C., and M.L. Vasil. 2008. The influence of iron on *Pseudomonas aeruginosa* physiology: A regulatory link between iron and quorum sensing. J. Biol. Chem. **283**:15558-67.

Oglesby-Sherrouse, A.G., and M.L. Vasil. 2010. Characterization of a Heme-regulated non-coding RNA encoded by the *prrF* locus of *Pseudomonas aeruginosa*. PLoS ONE. **5**:e9930.

Overhage, J., Lewenza, S., Marr, A.K., and R.E.W. Hancock. 2007. Identification of genes involved in swarming motility using a *Pseudomonas aeruginosa* PAO1 mini-Tn5-*lux* mutant library. J. Bacteriol. **189**:2164-2169.

Overhage, J., Bains, M., Brazas, M.D., and R.E.W. Hancock. 2008. Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. J. Bacteriol. **190**:2671-9.

Poole, K. 2001. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. J. Mol. Microbiol. Biotechnol. **3**:255-64.

Prince, R.W., Storey, D.G., Vasil, A.I., and M.L. Vasil. 1991. Regulation of *toxA* and *regA* by the *Escherichia coli_fur* gene and identification of a Fur homologue in *Pseudomonas aeruginosa* PA103 and PAO1. Mol. Microbiol. **5**:2823-31.

Schweizer, H.P. 1991. Improved broad-host-range *lac*-based plasmid vectors for the isolation and characterization of protein fusions in *Pseudomonas aeruginosa*. Gene **103**:87-92.

Sonnleitner, E., Sorger-Domenigg, T., Madej, M.J., Findeiss, S., Hackermüller, J., Hüttenhofer, A., Stadler, P.F., Bläsi, U., and I. Moll. 2008. Detection of small RNAs in *Pseudomonas aeruginosa* by RNomics and structure-based bioinformatic tools. Microbiology **154**:3175-87.

Sonnleitner, E., Abdou, L, and D. Haas. 2009. Small RNA as global regulator of carbon catabolite repression in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U.S.A. **106**:21866-71.

Sonnleitner, E. and D. Haas. 2011. Small RNAs as regulators of primary and secondary metabolism in *Pseudomonas aeruginosa.* Appl. Microbiol. Biotechnol. **91**:63-79.

Sonnleitner, E., González, N., Sorger-Domenigg, T., Heeb, S., Richter, A.S., Backofen, R., Williams, P., Hüttenhofer, A., Haas, D., and U. Bläsi. 2011. The small RNA PhrS stimulates synthesis of the *Pseudomonas aeruginosa* quinolone signal. Mol. Microbiol. **80**:868-85.

Sriramulu, D.D., H. Lunsdorf, J.S. Lam, and U. Romling. 2005. Microcolony formation: A novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung. J. Med. Microbiol. **54**:667-76.

Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, F.R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G.K., Wu, Z.P., Reizer,

J., Saier, M.H., Hancock, R.E.W., Lory, S., and M.V. Olson. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature. **406**:959-64.

Storz, G., Vogel, J., and K.M. Wasserman. 2011. Regulation of Small RNAs in Bacteria: Expanding Frontiers. Mol. Cell **43**:880-91.

Toutain, C.M., N.C. Caizza, M.E. Zegans and G.A. O'Toole. 2007. Roles for flagellar stators in biofilm formation by *Pseudomonas aeruginosa*. Res. Microbiol. **158**:471-7.

Vaezzadeh, A.R., Deshusses, J.M.P., Wariel, P, François, Zimmermann-Ivol, C.G., Lescuyer, P, Schrenzel, J., and D.F. Hochstrasser. 2010. Accelerated digestion for high-throughput proteomic analysis of whole bacterial proteomes. J. Microbiol. Methods **80**:56-62.

Vasil, M.L. and U.A. Ochsner. 1999. The response of *Pseudomonas aeruginosa* to iron: Genetics, biochemistry and virulence. Mol. Microbiol. **34**:399-13.

Vasseur, P., Vallet-Gely, I., Soscia, C., Genin, S. and A. Filloux. 2005. The pel genes of the *Pseudomonas aeruginosa* PAK strain are involved at early and late stages of biofilm formation. Microbiology. **151**:985-97.

Wang, J., Zhou, J.Y., Qu, T.T., Shen, P., Wei, Z.Q., Yu, Y.S. and L.J. Li. 2010. Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from chinese hospitals. Int. J. Antimicrob. Agents **35**:486-91.

Wiegand, I., Hilpert, K., and R.E.W. Hancock. 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature Protocols **3**:163-175.

Wilderman, P.J., Sowa, N.A., FitzGerald D.J., FitzGerald, P.C., Gottesman, S., Ochsner, U.A., and M.L. Vasil. 2004. Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. Proc. Natl. Acad. Sci. U.S.A. **101**:9792-7.

Whitchurch, C.B., Tolker-Nielsen, T., Ragas, P.C. and J.S. Mattick. Extracellular DNA required for bacterial biofilm formation. Science. 2002; 29:1487.

Whooley, M.A. and A.J. McLoughlin. 1982. The regulation of pyocyanin production in *Pseudomonas aeruginosa*. European J Appl Microbiol Biotechnol. **15**:161-6.

Wurtzel, O., Yoder-Himes, D.R., Han, K., Dandekar, A.A., Edelheit, E., Greenberg, P., Sorek, R., and S. Lory. 2012. The single-nucleotide resolution transcriptome of *Pseudomonas aeruginosa* grown in body temperature. PLoS Pathog. **8**:e1002945.

Yeung, A.T.Y., Torfs, E.C.W., Jamshidi, F., Bains, M., Wiegand, I., Hancock, R.E.W., and J. Overhage. 2009. Swarming of *Pseudomonas aeruginosa* is controlled by a broad spectrum of transcriptional regulators, including MetR. J. Bacteriol. **191**:5592-602.

Xu, K.D., Stewart, P.S., Xia, F., Huang, C.T. and G.A. McFeters. 1998. Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. Appl. Environ. Microbiol. **64**:4035-9.