A sigma factor and anti-sigma factor that control swarming motility and biofilm formation in Pseudomonas aeruginosa

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A σ factor and anti-σ factor that control swarming motility
and biofilm formation in *Pseudomonas aeruginosa*

A dissertation presented

by

Bryan Alexander McGuffie

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Microbiology and Molecular Genetics

Harvard University

Cambridge, Massachusetts

May 2015
A σ factor and anti-σ factor that control swarming motility 
and biofilm formation in \textit{P. aeruginosa}

Abstract

\textit{Pseudomonas aeruginosa} is an environmental bacterium and opportunistic human pathogen of major clinical significance. It is the principal cause of morbidity and mortality in patients with cystic fibrosis (CF) and a leading cause of nosocomial infections. Although the organism is unicellular, \textit{P. aeruginosa} exhibits two forms of multicellular behaviors when associated with a surface under the right conditions: swarming motility and biofilm formation. Swarming motility is a multicellular cooperative form of flagella-dependent surface motility, while biofilm formation produces a sessile community of bacteria enclosed by a self-produced extracellular polymeric matrix. \textit{P. aeruginosa} is thought to grow as a biofilm in the lungs of CF patients and the growth of \textit{P. aeruginosa} biofilms on indwelling medical devices, such as endotracheal tubes and catheters, is a significant source of nosocomial infection. By growing as a biofilm, \textit{P. aeruginosa} resists clearance by the immune system and increases its resistance to antimicrobial therapy. In this thesis, I describe the characterization of a σ factor and anti-σ factor implicated in \textit{P. aeruginosa} virulence and cell envelope stress that control the expression of a novel regulator of swarming motility and biofilm formation. In addition, I describe work done to investigate the role of a post-translational regulator of flagellar motility that has been described in other bacteria, but has not been studied extensively \textit{P. aeruginosa}. 

iii
In bacteria, RNA polymerase (RNAP) requires σ factors for promoter-specific transcription initiation. σ factors guide RNAP to promoters by recognizing conserved DNA sequences within the promoter called the -10 and -35 elements. Most bacteria encode a primary σ factor and several alternative σ factors, each of which recognizes different promoter -10 and -35 sequences. By modulating the activity of alternative σ factors, bacteria can rapidly alter their transcriptional program in response to changes in growth, morphological development, and environmental conditions. The extracytoplasmic function (ECF) σ factors are the largest and most diverse group of bacterial σ factors. The gene encoding an ECF σ factor is often cotranscribed with its own negative regulator, called an anti-σ factor, which directly binds to and inhibits its partner σ factor until the appropriate extracytoplasmic signal stimulates σ factor release and expression of the σ factor’s regulon.

In this thesis, I describe the characterization of the *P. aeruginosa* ECF σ factor PA2896 and its cognate anti-σ factor PA2895. Using immunoprecipitation, we show that the ECF σ factor PA2896 and RNAP co-purify *in vivo*. Utilizing DNA microarrays, we identify the genes that constitute the PA2895 and PA2896 regulon and infer the putative -10 and -35 consensus sequences recognized by PA2896. Genetic analysis revealed a subset of genes within the PA2896 regulon that share the putative promoter consensus sequence are positively regulated by the ECF σ factor PA2896 and negatively regulated by the anti-σ factor PA2895. Using a bacterial two-hybrid assay, we show that PA2895 directly interacts with PA2896. We present evidence that increased expression of the PA2896 regulon in ΔPA2895 mutants cells leads to the inhibition of swarming motility and enhanced biofilm formation. We further show that one gene in the PA2896 regulon, PA1494, is necessary and sufficient for the inhibition of swarming motility and promotion of biofilm formation. Thus we report the discovery of a system that may respond to a
stress signal by activating PA2896-dependent expression of PA1494 to inhibit swarming motility and promote the formation of a protective biofilm.

In many bacteria, swarming motility and biofilm formation are controlled by the second messenger c-di-GMP. In *P. aeruginosa*, elevated intracellular c-di-GMP generally results in the inhibition of flagellar-dependent swarming motility and enhanced biofilm formation. In some species of bacteria, c-di-GMP-mediated repression of flagellar motility is achieved by repressing the expression of the flagellar genes. However, flagellar gene expression in *P. aeruginosa* does not appear to be influenced by elevated c-di-GMP, suggesting c-di-GMP controls flagellar motility post transcriptionally in this bacterium. Mechanisms by which c-di-GMP controls flagellar function post translationally have been described in both Gram-negative and Gram-positive bacteria, however the mechanism in *P. aeruginosa* remains unclear. Gram-negative bacteria appear to utilize a “flagellar brake” to control flagellar function in response to c-di-GMP. *P. aeruginosa* encodes a homolog of this brake and in this thesis I present evidence that this homolog is involved in controlling flagellar function in *P. aeruginosa*.

Together, the characterization of PA2895 and PA2896, the identification of PA1494 as a novel regulator of swarming motility and biofilm formation, and evidence of a functional flagellar brake in *P. aeruginosa* advance our understanding of how this bacterium controls the transition from motile cell to sessile biofilm.
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Acknowledgements

Many people contributed to the completion of this dissertation: the educators and scientists that fostered my interest in microbiology, my coworkers, my friends, and my family. First, I would like to thank Simon. I hope that I have absorbed even a fraction of his knowledge and his ability to thoughtfully analyze complex scientific problems. His guidance and support helped me through challenges both in and out of the lab that at times made the completion of this dissertation seem impossible. His kindness, openness, intelligence, and sense of humor make him a wonderful mentor and scientist and I feel truly lucky to have had the opportunity to be a member of Simon’s lab.

I would also like to thank the other members of the Dove lab, past and present: Isabelle, Josh, Sandra, Heather, Keith, James, Kirsty, Amy, Kathryn, Tracy, Melisa, Ngat, and Jenny. Isabelle, who left before I joined the lab, contributed experimental data to Chapter 2 of this dissertation. Amy and Kathryn were my baymates in the “Back Bay” for the majority of my time in graduate school and I cannot imagine this journey without them. I have probably spent more time with Amy than any other person over the last six years and she has become a great friend. The members of the Dove lab made coming in to work everyday fun and it has been a privilege to work with such wonderful people.

I would not have started down the path that led me to this point were it not for Matt Chapman, my undergraduate thesis advisor, who introduced me to microbial genetics and showed me how fun and exciting science can be. I would also like to thank my Dissertation Advisory Committee, Ann Hochschild, Tom Bernhardt, and Steve Lory for their guidance during my thesis work.

I would like to thank the friends I’ve made during graduate school who made Boston feel like home. I am very grateful for the friendship and support of Allison, Alex, Scott, Sarah, Allegra, Matt, Sky, Rob, Tim, and many more who have been such good
friends. I want to thank my girlfriend Alyssa, for trusting me enough to go camping with me on our third date and for her love, companionship, and support.

Finally, I would like to thank my family: my extended family, my brother Matthew, my father James, and my mother Christine for their love and unwavering support. I especially want to thank my parents, who nurtured my interest in science from a young age. They have loved and supported me, always.
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For my parents
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Chapter 1

Introduction
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**Pseudomonas aeruginosa**

*Pseudomonas aeruginosa* is an environmental Gram-negative bacterium capable of growing in a wide range of environmental conditions, including in soil, water, and on plants and animals [1] [2]. While *P. aeruginosa* is found ubiquitously in the environment, the ability to cause human infection led to its discovery and isolation in 1882 from infected linen bandages [3]. *P. aeruginosa* is capable of causing a variety of opportunistic infections, particularly among immunocompromised patients with cystic fibrosis (CF), burn wounds, or neutropenia, as well as in patients with indwelling medical devices such as endotracheal tubes and urinary catheters [2], [4], [5].

In patients with CF, *P. aeruginosa* is the major cause of morbidity and mortality [5]. CF is the most common inherited lethal disease in the Caucasian population, occurring in approximately 1 in 3000 births [6]. The disease is caused by mutations that disrupt the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a protein which functions as a chloride ion channel [7]. The disruption of CFTR results in abnormal ion and fluid transport across the epithelia in the lung, pancreas, intestines, and other organs. In the airways of the lung, this results in the accumulation of thick, dehydrated mucus. This thickened mucus is thought to prevent the normal clearance of pathogenic microbes trapped in mucus secretions that would otherwise be carried out of the lung by ciliated epithelial cells [5], [6], [8]. The abnormal conditions present in the CF lung predispose patients to colonization by *P. aeruginosa*, which is often difficult or impossible to resolve once an infection has been established [8], [9]. The resulting persistent infection causes chronic inflammation and the progressive loss of lung function over time. Ultimately 80 to 95% of CF patients succumb to respiratory failure as a result of *P. aeruginosa* infection [8].
P. aeruginosa clinical isolates recovered from CF patients often exhibit a mucoid phenotype, overproducing the exopolysaccharide alginate [5], [8]. In lung sections and sputum collected from CF patients, P. aeruginosa has been observed growing in microcolonies surrounded by a dense extracellular matrix thought to be composed of alginate, reminiscent of structured, matrix-enclosed bacterial communities called biofilms [10], [11]. Measurements of the levels of quorum sensing molecules, produced and sensed by bacteria to coordinate multicellular behavior, in CF sputum were found to be similar to those produced by P. aeruginosa grown as a biofilm in vitro [11], further suggesting P. aeruginosa exists in a biofilm-like state in the CF lung. Growth as a biofilm in vitro increases the resistance of P. aeruginosa to antibiotics and it is thought that growth as a biofilm in the CF lung may explain the ability of the organism to resist antimicrobial therapy during chronic infection [6], [8], [9], [12].

P. aeruginosa is also a major cause of healthcare acquired infections. In the United States, nosocomial pathogens have been estimated to cause 1.7 million infections and 99,000 deaths per year, resulting in annual costs of $28.4 to $45 billion to the US healthcare system [13], [14]. In the US, P. aeruginosa is the fifth most common healthcare acquired infection overall and the second most common cause of ventilator-associated pneumonia and catheter-associated urinary tract infections [15], [16]. In cases of ventilator-associated pneumonia, P. aeruginosa has been found to grow as a biofilm on the surface of the plastic endotracheal tubes used for intubation [9]. Similarly, in cases of catheter associated urinary tract infections, P. aeruginosa has been found growing as a biofilm on the surface of the plastic catheter [2], [17]. Thus our clinical understanding of P. aeruginosa infection requires an examination of the mechanisms and pathways controlling biofilm in this bacterium.
**P. aeruginosa** biofilm development

Biofilms are a structured multicellular community of bacterial cells enclosed in a self-produced polymeric matrix that adhere to an inert or living surface [12]. As noted above, growth as a biofilm imbues *P. aeruginosa* with increased resistance to antimicrobials, but also increases its resistance to phagocytic uptake and clearance by the immune system [12]. A model has been proposed for describing biofilm development as a series of distinct developmental stages (depicted in Figure 1.1) comprising (i) reversible attachment, (ii) irreversible attachment, (iii) formation of microcolonies, (iv) formation of macrocolonies, and finally (v) dispersal [12], [18].

In the first stage of biofilm development, the *P. aeruginosa* utilizes flagellar-mediated swimming motility to overcome repulsive forces as cells near a surface [19]. *P. aeruginosa* mutants that are unable to produce flagella are defective for attachment (i) [18], [20]. Once associated with a surface, *P. aeruginosa* becomes irreversibly attached (ii) and begins migrating across the surface via type IV pili-dependent twitching motility to form aggregate clusters of cells called microcolonies (iii) [20]. As the cell density within the maturing biofilm increases, cells begin quorum sensing, triggering the next stages of development [18], [21]. Cells begin to produce and surround themselves with an extracellular biofilm matrix composed of exopolysaccharides, proteins, and DNA [18], [22]-[29]. This leads to the development of mature macrocolonies (iv) that appear as mushroom or columnar structures in biofilms grown in flow cells. This architecture results in the formation of cell-free water channels that allow for the flow of water and nutrients through the biofilm [30], potentially allowing denser bacterial growth. Eventually, cells begin to release from the biofilm in a process called dispersion (v) and begin the developmental cycle again on a new surface [12], [18].
Figure 1.1. Model depicting the development of a mature surface associated *P. aeruginosa* biofilm. (i) Flagellar motility is required for approaching and reversibly attaching to a surface. (ii) Cells transition to irreversible attachment. (iii) Type IV pil-mediated twitching is required for the formation of microcolonies. The biofilm matrix begins to form and increased cell density activates quorum sensing. (iv) Maturation of microcolonies into matrix-enclosed macrocolonies. (v) In the dispersal stage, swimming planktonic cells are released from the interior of mature macrocolonies. These planktonic cells disperse and begin the process anew.

**The *P. aeruginosa* biofilm matrix**

*Matrix exopolysaccharides*

*P. aeruginosa* is known to produce three secreted exopolysaccharides (EPS): alginate, Psl, and Pel. The mucoid phenotype of CF clinical isolates is due to the overproduction of alginate, which results in reduced phagocytic uptake by immune cells, resistance to killing by macrophages, and increased antibiotic resistance [5], [31], [32]. However, despite the importance of alginate in mucoid strains it has not been found to be an important biofilm matrix EPS in non-mucoid environmental isolates or laboratory
strains of *P. aeruginosa* [23], [33]. In non-mucoid strains, Psl and Pel are the primary biofilm matrix EPSs [22], [23], [34]-[36].

Psl is composed of a repeating pentamer containing D-mannose, L-rhamnose, and D-glucose [37] that contributes to surface adhesion and the structure of mature macrocolonies [38]. Psl surrounds mature macrocolonies [39] and results in increased antibiotic resistance [40], resistance to phagocytosis [41], and acts as a self-produced signal to stimulate further biofilm formation [42]. Psl is the primary matrix EPS in the commonly used *P. aeruginosa* strain PAO1, however it is not produced by *P. aeruginosa* strain PA14 due to a partial deletion of the *psl* locus in that strain [22], [36].

Pel is the primary matrix EPS in PA14, however both PAO1 and PA14 are capable of producing Pel [36], [43]. The structure of Pel has not been characterized, but it is thought to be glucose rich [23]. Pel has been shown to contribute to surface adhesion and the structure of mature biofilms, and to increase antibiotic resistance [44].

**Matrix proteins**

In addition to flagella and type-IV pili, several other proteins have been shown to contribute to *P. aeruginosa* biofilm formation. These include chaperone-usher pili, carbohydrate-binding proteins, and amyloid fibers.

*P. aeruginosa* encodes several chaperone-usher pili (Cup) that have been shown to be involved in biofilm formation. PAO1 encodes three Cup fimbriae, CupA, CupB, and CupC. CupA fimbriae were the first to be discovered in a screen to identify novel adhesins that promoted biofilm formation in a strain lacking type-IV pili [45]. While this study did not find a role for CupB or CupC in biofilm formation, a later study found that *cupB* and *cupC* were not expressed under the conditions used in the first study [27]. When *cupB* and *cupC* were ectopically expressed, they were both found to promote
biofilm formation [27]. A fourth Cup fimbriae, CupD, is encoded on the PAPI-1 pathogenicity island in PA14 and also promotes biofilm formation [28].

In addition to Cup fimbriae, *P. aeruginosa* produces several carbohydrate binding proteins that have been shown to enhance biofilm formation. The secreted adhesin CdrA has been shown to bind Psl, strengthening biofilms [25], and the secretion of carbohydrate-binding lectins LecA and LecB are thought to contribute to biofilm formation by attaching cells to EPS or by crosslinking EPS polymers to strengthen the biofilm matrix [46], [47].

The biofilm matrix of *P. aeruginosa* may also contain extracellular amyloid fibers. The *P. aeruginosa* fap genes encode a functional amyloid system that has been shown to contribute to cell aggregation and biofilm formation [29], [48]. The extracellular fibers produced by the fap genes in *P. aeruginosa* are thought to be analogous to curli fibers produced by *E. coli* and *Salmonella* [49], [50], and to TasA amyloid fibers produced by *B. subtilis* [51].

**Matrix DNA**

Extracellular DNA (eDNA) is also a major component of the *P. aeruginosa* biofilm matrix. The first evidence of this came from work that showed *P. aeruginosa* biofilms could be dispersed by DNase treatment, suggesting eDNA plays a structural role in biofilms [24]. Subsequent work revealed eDNA is three to four-fold more abundant than carbohydrates and five-fold more abundant than proteins in the biofilm matrix [35]. The release of eDNA is thought to occur via the lysis of a subset of cells within the biofilm [52]. A recent study reports the discovery that Psl is capable of binding to eDNA and that the two molecules co-localize in biofilms to form what appears to be a web of eDNA-Psl fibers [53].
**P. aeruginosa swarming motility**

*P. aeruginosa* is capable of several forms of motility, including twitching, swimming, and swarming motility. Twitching motility is a form of surface motility powered by the extension and ATP-dependent retraction of type IV pili (T4P). Swimming and swarming are both flagellar-dependent forms of motility. Swimming is a single celled form of motility powered by rotating flagella that occurs in liquid environments and relies on the chemotaxis system to direct cells along chemical gradients. In contrast, multicellular, flagellar-dependent swarming motility does not require chemotaxis (reviewed in [54],[55]. In addition to the flagellum, swarming motility requires molecules called surfactants, which reduce surface tension and are thought to reduce friction between cells and the surface they move on. Swarming motility occurs on semisolid surfaces and in viscous environments, and is generally antagonistic to biofilm formation. This relationship results in the inverse regulation of these two behaviors. This regulatory phenomenon is discussed later in this chapter in the context of *P. aeruginosa*.

Swarming motility by *P. aeruginosa* occurs under a narrow range of conditions in the laboratory. Agar plates used to observe swarming motility must have an agar concentration high enough to prevent swimming (>0.3%), but not high enough that swarming is inhibited (<1%) [54]. Most *P. aeruginosa* swarming experiments are carried out on 0.5% agar. In addition, a defined protocol for pouring and preparing the plates is required to ensure the plates have the proper moisture content for swarming motility, and attention must be paid to humidity levels within the incubator during overnight growth [56], [57].

Swarming motility in several bacterial species, such as *E. coli*, *B. subtilis*, and *V. parahaemolyticus* requires that cells increase the number of flagella on their surface, resulting in “hyperflagellated” swarmer cells [54]. In contrast, *P. aeruginosa* is capable of
swarming with a single polar flagellum, although cells with two flagella have been observed in swarms [58], [59]. In addition to the flagellum, PAO1 may also require T4P for swarming motility [58], [60]. However, in strain PA14, T4P mutants are capable of swarming, suggesting that functional T4P may not be required for this strain to engage in swarming motility [61], [62].

*P. aeruginosa* swarming motility is also dependent upon the secreted surfactants 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA), mono-rhamnolipids, and di-rhamnolipid [58], [59], [63]. The production of HAA and rhamnolipids is controlled by the Rhl quorum sensing system (discussed later in this chapter). Interestingly, in addition to surfactant activity, HAA is thought to act as a repellent while di-rhamnolipids are thought to act as attractants [64]. It is thought that these opposing signals give rise to the characteristic dendrites formed by *P. aeruginosa* swarming motility in the laboratory, an example of which can be seen in Figure 1.2A [64].

Rhamnolipids can act as secreted virulence factors [65] and several studies suggest that swarming motility in *P. aeruginosa* may be correlated with increased virulence. In a study that investigated the changes in gene expression that occur in swarming cells relative to swimming cells, swarming cells upregulated the expression of genes encoding the type III secretion system and secreted virulence factors, including exoenzymes ExoT, ExoY, and ExoS; secreted proteases and elastases; and the biosynthetic genes for the production of pyocyanin and the siderophores pyoverdine and pyochelin [66]. In addition, the GacS/GacA regulatory system (discussed later in this chapter) coordinately regulates swarming motility, type II secretion, type III secretion, and the virulence factors ToxA and LipA secreted by the type II secretion system [67], [68].
Figure 1.2. Swarming motility and the flagellar basal body. (A) Example of swarming motility by 
P. aeruginosa PA14. (B) The flagellar basal body. The MotAB and MotCD stator complexes are 
anchored to the peptidoglycan and contact the C-ring in the cytoplasm. YcgR is a PilZ-domain 
protein that binds c-di-GMP and interacts with the C-ring and the stator complexes to slow 
flagellar rotation in response to elevated intracellular c-di-GMP. Labels: L-ring (L), P-ring (P), MS-
ring (MS), FliG C-ring protein (G), FliM C-ring protein (M), FliN C-ring protein (N).

The Flagellum

The flagellum is a complex molecular machine composed of over 25 different 
proteins that generates propulsive motile force by rotating a helical “tail” filament like a 
propeller (reviewed in [69] and [70]). The flagellar tail is connected by a flexible “hook” to 
a structure called the basal body, which utilizes the electrochemical gradient across the 
inner membrane to generate torque and rotate the flagella. Within the basal body, the 
hook is connected to a “rod”-like structure that rotates within the L-ring, which spans the 
outer membrane, and the P-ring, which spans the peptidoglycan (the L- and P-rings are 
not present in Gram-positive bacteria) (Fig. 1.2B). The base of the rod is connected to 
the MS-ring, which spans the inner membrane and connects to the C-ring located in the 
cytoplasm (Fig. 1.2B). The C-ring is where rotational energy is imparted to the flagella 
and where chemotactic inputs governing the direction of flagellar rotation are received.
Surrounding this central structure are the MotAB stator complexes, which are anchored to the peptidoglycan and act as ion channels (Fig. 1.2B). By harnessing the flow of cations across the cytoplasmic membrane, the stators convert the potential energy stored in the electrochemical gradient into kinetic energy, imparting torque on the C-ring to generate rotational energy and thus rotating the flagella and propelling cells. \textit{P. aeruginosa} encodes two stator complexes, MotAB and MotCD \cite{71}, \cite{72}. ΔmotCD mutant cells exhibit lower average velocities and are unable to swarm on 0.5% agar, while ΔmotAB retain the ability to swarm on 0.5% agar \cite{71}, \cite{72}. These observations suggest the MotCD stator is capable of generating more torque than the MotAB stator.

The entire structure of the flagella is hollow and a type III-like secretion system located within the C-ring (not shown in Fig. 1.2B) secretes the structural components of the flagella once the secretion apparatus is assembled. An intricate hierarchy of σ factors and transcription regulators orchestrate the expression of the flagellar genes, such that genes required for later stages of flagellar construction are not expressed until construction of the secretion apparatus has been completed \cite{73}, \cite{74}. Once constructed, flagellar function is controlled post-translationally by the chemotaxis system and by c-di-GMP. Post-translational regulation of the flagella in response to c-di-GMP will be discussed later in this chapter.

\textbf{Coregulation of swarming motility and biofilm formation in \textit{P. aeruginosa}}

\textit{Multicellular behavior and quorum sensing}

The cooperative behaviors of biofilm formation and swarming motility are dependent upon quorum sensing, which allows individual cells to assess the density of neighboring cells by detecting the concentrations of secreted signals. When the concentration of these secreted so-called auto-inducers crosses a certain threshold,
quorum sensing is activated and the cells alter gene expression to begin group behavior. The most well studied quorum-sensing systems in *P. aeruginosa* signal with acylhomoserine lactones (AHL) and quinolones as their signaling molecules. The interconnected quorum sensing network in *P. aeruginosa* is depicted in Figure 1.3.

![Figure 1.3](image)

**Figure 1.3.** Quorum sensing in *P. aeruginosa*. (A) The Las system. LasI produces 3-oxo-C12-HSL, which activates LasR to positively control the production of elastase, alkaline protease, exotoxin A (ToxA), and the Rhl quorum sensing system. (B) The Rhl system. RhlI produces C4-HSL, which activates RhlR to positively control the production of the surfactants HAA and rhamnolipid (required for swarming motility), pyocyanin, and lectins. The Rhl system negatively regulates PQS quorum sensing. (C) The PQS system. HHQ and PQS quinolone signaling molecules positively control pyocyanin production, lectin expression, and biofilm formation. HHQ and PQS negatively regulate swarming motility. Green lines indicate positive regulation. Red lines indicate negative regulation. Adapted from Dubern *et al.*, 2008.

The Las and Rhl quorum sensing systems in *P. aeruginosa* encode homologs of the LuxR-LuxI quorum sensing system that controls *Vibrio fischeri* bioluminescence in the light organ of the Hawaiian bobtail squid (reviewed in [75]). In these systems, LuxR-type proteins function as transcription activators when they bind an AHL auto-inducer.
produced by a partner LuxI-type AHL synthase. In the Las system, the LuxR homolog LasR binds the AHL auto-inducer N-(3-oxododecanoyl)-l-homoserine lactone (3-oxo-C12-HSL) and controls the expression of secreted virulence factors, including the elastases LasA and LasB, alkaline protease, and exotoxin A (Fig. 1.3) (ToxA) [76]-[79].

The LuxI homolog LasI acts as the 3-oxo-C12-HSL synthase. Las quorum sensing appears to be activated at an early stage in biofilm formation [18] and lasI mutants exhibit reduced Pel production and develop biofilms that fail to develop water channels and macrocolonies [21], [80]. In lasI and lasR mutants, swarming motility is reduced relative to wild type [58]. Las also controls P. aeruginosa’s second AHL quorum sensing system Rhl (Fig. 1.3) [81], [82].

The Rhl quorum sensing system is also homologous to the LuxR-LuxI system. The LuxI-type AHL synthase RhlI produces the autoinducer N-butyryl-l-homoserine lactone (C4-HSL), which is sensed by the LuxR-type protein RhlR (Fig. 1.3) [75]. RhlR controls the expression of the rhlAB operon, which encodes the biosynthetic machinery to produce the surfactants 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA) and rhamnolipid[75]. Rhl mutants that cannot produce these surfactants are unable to swarm [58], [62], [83] and are unable to form water channels or macrocolonies during biofilm development, instead producing flat, featureless biofilms [84]. In addition, rhamnolipids have been shown to mediate the dispersal step of the biofilm developmental pathway [85].

The Rhl system also regulates the Pseudomonas quinolone signal (PQS) quorum sensing system (Fig. 1.3) (reviewed in [86]). P. aeruginosa produces multiple quinolone signaling molecules, but the most studied are 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS) and its precursor 2-heptyl-4-quinolone (HHQ). HHQ is produced by the pqsABCDE operon and is converted into PQS by PqsH (Figure 1.3). These molecules are sensed by
PqsE and PqsR, which regulate the expression of genes encoding elastase, lectins, and the phenazine biosynthetic genes that produce the redox-active small molecule pyocyanin [86], [87]. During biofilm development, PQS has been shown to promote autolysis and the release of eDNA [52], [88] and the addition of exogenous PQS to cultures results in increased biofilm formation and the inhibition of swarming motility without affecting swimming or twitching motility [87], [89]. The precursor to PQS, HHQ, has also been shown to inhibit swarming motility under certain conditions [90]. Interestingly, PQS stimulates the formation of membrane vesicles and is itself packaged into membrane vesicles, which bleb off from cells and fuse with both prokaryotic and eukaryotic membranes to deliver their cargo [91].

The GacS/GacA two component system

In *P. aeruginosa*, the GacS/GacA two-component system controls a master switch between two growth states, one characterized by virulence factor production and swarming motility, the other defined by sessile growth as a biofilm [67], [68], [92], [93]. GacS/GacA positively controls the expression of two small RNAs, *rsmY* and *rsmZ*, which bind to the RNA-binding protein RsmA (Fig. 1.4) [67], [68], [94]. In the absence of RsmY and RsmZ, RsmA binds to target mRNA transcripts, preventing their translation and promoting degradation of the transcript [94]. When the GacS histidine sensor kinase is stimulated, it triggers the phosphorylation and activation of the response regulator GacA [93], [95]. Phosphorylated GacA activates the expression of *rsmY* and *rsmZ*, which bind to RsmA and prevent it from binding to target mRNA transcripts [94]. By titrating levels of free RsmA, RsmY and RsmZ de-repress the translation of transcripts targeted by RsmA. Activation of this signaling cascade downregulates swarming, type II secretion, type III secretion, T4P, exotoxin A, and lipase expression and upregulates Pel and Psl.
expression, promoting biofilm formation (Fig. 1.4) [67]. Thus the GacS/GacA system reciprocally regulates swarming motility and biofilm formation.

**Figure 1.4.** The GacS/GacA two-component system controls swarming motility and biofilm formation. The GacS sensor kinase phosphorylates the GacA response regulator. Phosphorylated GacA induces expression of the small RNAs RsmY and RsmZ. RsmY and RsmZ bind to Free RsmA to form the RsmA\(^{RsmY/RsmZ}\) complex. Expression of RsmY and RsmZ positively affects Pel and Psl expression, resulting in enhanced biofilm formation and the reduced production of virulence factors and swarming motility. In the absence of RsmY and RsmZ, RsmA has the opposite effect on gene expression. Green type indicates positive regulation. Red type indicates negative regulation. HSL: homoserine lactone quorum sensing systems. T2S: type II secretion. T3S: type III secretion. T4P: type IV pili. ToxA: exotoxin A. LipA: lipase A. Adapted from Goodman et al., 2004.
**c-di-GMP signaling**

Bis-(3’-5’) cyclic diguanylic acid (c-di-GMP) is a second messenger used by many bacterial species to control biofilm formation, motility, differentiation, and virulence (reviewed in [96]). High intracellular c-di-GMP typically results in increased production of biofilm matrix components and the inhibition of motility, while low intracellular c-di-GMP inhibits synthesis of the biofilm matrix and promotes motility (Fig. 1.5). *P. aeruginosa* encodes 41 proteins with domains that are implicated in c-di-GMP synthesis or degradation[97], [98]. c-di-GMP is produced by proteins called diguanylate cyclases (DGCs), which share a conserved “GGDEF” domain, and is degraded by phosphodiesterases (PDEs), which contain either a conserved EAL domain or a HD-GYP domain (Fig. 1.5). In a study examining the effects of c-di-GMP in *P. aeruginosa*, Kulasekara at al. reported that the expression of c-di-GMP producing DGCs enhanced biofilm formation, while PDEs had the opposite effect [97]. Additional work suggests that expression of the *pel, psl*, and *cupA* genes is increased in response to elevated levels of c-di-GMP, resulting in enhanced biofilm formation (Fig. 1.5) [99], [100]. The production of alginate and Pel exopolysaccharides is dependent upon c-di-GMP [101], [102], and secreted Psl has been shown to stimulate c-di-GMP synthesis to further enhance the production of biofilm matrix constituents [42]. Thus c-di-GMP promotes biofilm formation by upregulating the production of biofilm matrix components.
Figure 1.5. c-di-GMP inversely regulates biofilm formation and flagellar motility. c-di-GMP is produced by diguanylate cyclases (DGC) with the conserved GGDEF domain and degraded by phosphodiesterases (PDE) with either the conserved EAL or HD-GYP domain. c-di-GMP is promotes exopolysaccharide production and CupA expression and inhibits flagellar motility. Green indicate a positive regulatory effect, red indicates a negative regulatory effect.

High intracellular concentrations of c-di-GMP also reduce or inhibit flagella- and T4P-mediated motility in many bacteria. In *P. aeruginosa*, T4P-mediated twitching motility is impaired in *fimX* and *wspF* mutants due to the elevated levels of intracellular c-di-GMP in these strains, however the mechanism by which c-di-GMP influences T4P-mediated motility remains elusive [88], [103], [104]. More is understood about the effects of c-di-GMP on flagellar-mediated motility. In some bacteria, including *Vibrio cholerae*, *Bacillus subtilis*, and *Pseudomonas putida*, c-di-GMP inhibits swimming and swarming motility by reducing the expression of flagellar genes (reviewed in [105] and [106]). While the expression of flagellar genes is reduced in *P. aeruginosa* biofilms [107] and elevated intracellular c-di-GMP has been shown to increase biofilm formation and inhibit flagellar motility in *P. aeruginosa* [61], [88], [108]-[110], c-di-GMP does not appear to influence flagellar gene expression in *P. aeruginosa* [99]. Instead, mounting evidence suggests that c-di-GMP controls flagellar function post-translationally in this organism. While the
mechanism has not been determined, examples of c-di-GMP-mediated repression of flagellar motility in other bacteria suggest a possible model for the inhibition of flagellar motility in *P. aeruginosa*. For example, in *E. coli* and *Salmonella* c-di-GMP-mediated inhibition of flagellar motility is controlled post-translationally by YcgR, which contains a c-di-GMP-binding PilZ domain and is thought to interact with the flagellar rotor and stator complex to reduce torque and flagellar reversals when bound to c-di-GMP (Fig. 1.2B) [111]-[113]. In *Pseudomonas fluorescens*, a recent study reported that ectopic expression of the *Pseudomonas* YcgR homolog FlgZ reduced swimming motility, however the effect on motility was not dependent upon levels of intracellular c-di-GMP [114]. This led the authors to conclude that c-di-GMP inhibits swimming motility independently of FlgZ in *P. fluorescens* [114]. In *P. aeruginosa*, the YcgR homolog PA3353 has been shown to bind c-di-GMP [101], however Kulasekara et al. concluded PA3353 does not control flagellar rotation in response to c-di-GMP after ∆PA3353 mutant cells and WT cells exhibited similar swimming speeds and rates of flagellar reversal [115]. In this study the authors do not appear to have taken measures to increase intracellular c-di-GMP when testing for the effects of PA3353 on flagellar motility. In Chapter 3 of this thesis, I will discuss experiments that suggest PA3353 does regulate flagellar-mediated motility in a *P. aeruginosa* when intracellular c-di-GMP is elevated.

**Transcription and σ factors**

The first step in gene expression is the transcription of DNA into RNA by the multisubunit enzyme RNA polymerase (RNAP). In bacteria, two α subunits, one β, one β’, and one ω subunit (αββ’ω) join together to form core RNAP (reviewed in [116]). Core RNAP contains all of the catalytic machinery required for transcription, but it requires an
additional subunit called the $\sigma$ factor in order to be able to initiate transcription specifically at promoters (reviewed in [117]) [116]). $\sigma$ factors reversibly associate with core RNAP to form the RNAP holoenzyme, which is capable of promoter recognition and transcription initiation. Bacteria typically encode multiple $\sigma$ factors in addition to a primary “housekeeping” $\sigma$ factor related to the primary $\sigma$ factor from *E. coli* called $\sigma^{70}$ (reviewed in [118] and [119]) [117]. For example, *E. coli* has 1 primary $\sigma$ factor and 7 alternative $\sigma$ factors, whereas *Streptomyces coelicolor* is predicted to encode 1 primary $\sigma$ factor and 63 alternative $\sigma$ factors, and *P. aeruginosa* strain PAO1 is predicted to contain 1 primary $\sigma$ factor and 23 alternative $\sigma$ factors [118], [120]. During exponential growth, the transcription of most genes is dependent upon the primary $\sigma$ factor [116], [119]. However, bacteria can respond to specific stress conditions, growth transitions, and morphological changes through the use of alternative $\sigma$ factors to rapidly alter the transcriptional program of the cell [118], [119]. Alternative $\sigma$ factors achieve this by recognizing different conserved sequences in promoters, which allows for $\sigma$ factor specific expression of regulons controlling, for example, cellular responses to heat shock, flagellar biosynthesis, and iron acquisition [117], [119], [120].

There are two divergent families of $\sigma$ factors: the $\sigma^{70}$ family, whose members are related to the *E. coli* primary $\sigma$ factor, and the $\sigma^{54}$ family. Members of the $\sigma^{54}$ family have no homology to members of the $\sigma^{70}$ family and most bacteria encode only one $\sigma^{54}$ family member [118], [121]. Members of the larger $\sigma^{70}$ family share homology to *E. coli* $\sigma^{70}$ in sequence, structure, and function, and most bacteria encode several members from this family [117], [121]. In Chapter 2, I will describe the characterization of a *P. aeruginosa* $\sigma^{70}$ family $\sigma$ factor that controls swarming motility and biofilm formation.
The $\sigma^{70}$ family

When associated with RNAP in holoenzyme, $\sigma$ factors allow for sequence-specific-promoter recognition and transcription initiation [116], [117]. $\sigma^{70}$ family members typically recognize two conserved hexameric sequences in the promoter DNA called the -10 and -35 elements (Fig. 1.6A). The -10 element is centered approximately 10 base pairs upstream of the transcription start. The -35 element begins between 16 and 18 base pairs upstream of the -10 element, with 17 base pairs being the optimal distance (Fig. 1.6A).

$\sigma^{70}$ family members share a common architecture, with up to four conserved domains, numbered 1 through 4, connected to one another by flexible linkers (depicted in Figure 1.6B) [116]-[118], [121]. Region 1 is the least conserved and is found only in primary $\sigma$ factors, where it has an autoinhibitory function to prevent free $\sigma$ from binding to DNA in the absence of RNAP. Region 2 is highly conserved and found in all $\sigma^{70}$ family members. This region can be further subdivided into subregions 1.2 (classified as part of region 1 for historical reasons), 2.1, 2.2, 2.3, and 2.4. In addition, primary $\sigma$ factors have a non-conserved region (NCR) located between regions 1.2 and 2.1. Regions 2.1 and 2.2 have been implicated in binding to core RNAP. Region 2.3 is involved in melting the DNA duplex to expose the template strand for transcription initiation. Lastly, region 2.4 has been shown to recognize the -10 element (Fig. 1.6B). Region 3 is poorly conserved in alternate $\sigma$ factors [119], [122], but when present is responsible for recognizing an extended -10 promoter sequence [116]. Region 3 is linked to region 4 by the $\sigma_{3.2}$ loop, which loops into the RNAP active site channel and out through the RNA exit channel [116]. Region 4 is highly conserved in all $\sigma^{70}$ family members [121], [122] and is subdivided into two subregions, 4.1 and 4.2. Region 4.2 contains a helix-turn-helix DNA binding motif that is responsible for recognizing the -35 element of the promoter (Fig.
Region 4.1 interacts with a movable flap domain on the β subunit of RNAP, which positions region 4 the appropriate distance from region 2 to allow -10 and -35 element recognition[116]. While all primary σ factors contain all four regions, alternative σ factors often lack region 1 and occasionally lack region 3 [121], [122].

Within the σ70 family tree there are several evolutionary branches that divide the family into four phylogenetic groups [119], [122]. Chapter 2 of this thesis characterizes a member of the largest and most diverse group, the extracytoplasmic function group.

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**Figure 1.6.** Bacterial promoter architecture and σ70 family σ factors. (A) Promoter architecture showing the -10 and -35 elements with respect to the transcription start site (+1) and the downstream gene. When associated with RNAP to form RNAP holoenzyme, σ4 recognizes the conserved -35 element and σ2 recognizes the conserved -10 element. (B) The regions and subregions of a σ70 family σ factor. NCR: nonconserved region. Adapted from Murakami and Darst, 2003. (C) Comparison of a primary σ factor and an ECF σ factor. Adapted from Fekslitov et al., 2014.
**Extractoplasmic function σ factors**

Extractoplasmic function (ECF) σ factors are the largest and most diverse group within the σ⁷⁰ family. Their name comes from their initial discovery as a group of σ factors that respond to extractoplasmic signals and to control regulons associated with the cell surface [122]. They are the most abundant σ⁷⁰ group encoded by bacteria with multiple alternative σ factors [119]. For example, *B. subtilis* encodes 7 ECF σ factors, *Mycobacterium tuberculosis* encodes 10, *Caulobacter crescentus* encodes 15, *P. aeruginosa* PAO1 encodes 19, *P. aeruginosa* PA14 encodes 21, and *Streptomyces coelicolor* encodes 51 ECF σ factors. As a group, ECF σ factors lack regions 1 and 3 [119], [122], but retain the highly conserved regions 2 and 4 connected by a somewhat conserved σ₃₂ loop (Fig. 1.6C) [118], [122]. ECF σ factors are frequently found in operons with their own negative regulator, called an anti-σ factor, and the consensus -35 elements of promoters recognized by ECF σ factors often include a “CAA” motif [119]. They are frequently positively autoregulated and their regulons are typically small [119], [122]. This is thought to be the result of increased stringency in promoter recognition by ECF σ factors due to a reduced capacity to melt promoter DNA [123]. The lower efficiency of promoter melting by ECF σ factors was recently shown in the *E. coli* ECF σ factor σ⁵ to be the result of an alternative promoter melting mechanism that may be conserved amongst ECF σ factors, thus ensuring a high degree of promoter specificity and discrete control of individual regulons [124].

**ECF anti-σ factors**

The utilization of multiple σ factors results in competition between σ factors for a limited pool of core RNAP [118]. One way bacteria selectively control which σ factors are “active” and able to associate with core RNAP is by reversibly inactivating σ factors with
proteins called anti-σ factors (reviewed in [125]). Anti-σ factors allow bacteria to maintain multiple σ factors that control discrete regulons in an inactive state, such that normal transcription is unaffected. Individual σ factors can then be activated by specific signals that release them from their σ factor. This allows for the rapid expression of specific regulons without the delay of σ factor protein synthesis, facilitating a rapid response to a specific stimulus. Anti-σ factors can function by diverse mechanisms to control their cognate σ factors. Some examples include: (i) FlgM, which is secreted by the flagellar basal body and hook complex to activate the expression of flagellar tail genes [73], (ii) DnaK, a molecular chaperone which disassociates from its cognate σ factor in response to heat shock [118], [125], and (iii) AlgQ, which is thought to bind to and inhibit the primary σ factor in *P. aeruginosa* to increase the availability of core RNAP for association with the alternative σ factor AlgU [126]. However, most ECF σ factors are negatively regulated by a cognate transmembrane anti-σ factor that binds to the σ factor, sequestering it at the membrane and preventing its association with RNAP [119], [125].

ECF anti-σ factors share a conserved architecture consisting of a cytoplasmic N-terminal domain, which binds to the cognate σ factor, a transmembrane domain that spans the inner membrane, and a periplasmic C-terminal domain that acts as a receiver for signals that activate the σ factor [119], [125]. Upon activation by the appropriate signal, the σ factor is released from the anti-σ factor, which allows the σ factor to associate with RNAP and activate expression of the corresponding regulon. In the case of the ECF σ/anti-σ pair σE/RseA in *E. coli* and the orthologous AlgU/MucA pair in *P. aeruginosa*, the anti-σ factor MucA is degraded by a series of proteases in response to misfolded and mislocalized proteins in the periplasm (depicted in Figure 1.7) (reviewed in [127] and [128]). When activating peptides in the periplasm are detected by the protease AlgW (*E. coli* DegS), it cleaves the periplasmic domain of MucA (*E. coli* RseA).
After this initial proteolytic event, MucA is further degraded by the membrane protease MucP (*E. coli* RseP). 87% of mucoid clinical isolates from CF patients have mutations in *mucA*, resulting in truncated MucA that is degraded by another periplasmic protease called Prc [129], [130]. These proteolytic events result in the release of a cytoplasmic complex comprising AlgU and the N-terminal cytoplasmic domain of MucA. The MucA N-terminal domain is then degraded by ClpXP, releasing AlgU and activating expression of the AlgU regulon. The AlgU regulon includes, among other targets, the *algU-mucABCD* operon and the *algD* operon, which encodes the biosynthetic enzymes responsible for alginate production [131]. In the *algU* operon, MucB (*E. coli* RseB) is a periplasmic protein that binds to MucA, the function of MucC has not been established, and MucD (*E. coli* DegP) is a periplasmic protease that is thought to degrade AlgW activating peptides [130].

**Figure 1.7.** Comparison of the $\sigma^E$/RseA system from *E. coli* and the AlgU/MucA system from *P. aeruginosa*. $\sigma^E$ and AlgU are ECF $\sigma$ factors. RseA and MucA are anti-$\sigma$ factors. RseB and MucB bind to their respective anti-$\sigma$ factors in the periplasm. DegS and AlgW are PDZ domain proteases that cleave their respective anti-$\sigma$ factors in the periplasm in response to misfolded and mislocalized proteins in the periplasm. RseP and MucP are PDZ domain proteases that cleave their respective anti-$\sigma$ factors following initial cleavage by DegS/AlgW. DegP and MucD are periplasmic PDZ domain proteases.
ECF σ factors also respond to extracellular signals. This is exemplified by the iron starvation response ECF σ factor systems. In the most studied of these systems, the Fe(III)-citrate uptake system in *E. coli*, the σ factor FecI is activated by the binding of ferric citrate to the FecA outer membrane receptor (depicted in Figure 1.8) (reviewed in [132] and [133]). Upon binding to FecA, a multiprotein complex composed of TonB, ExbB, and ExbD harnesses the electrochemical potential of the cytoplasmic membrane to import ferric citrate into the periplasm. FecA and homologous TonB-dependent receptors are characterized by a β-barrel architecture and behave as ligand-gated channels. The binding of ferric citrate and TonB to FecA results in a conformational change in FecA that allows it to interact with the anti-σ factor FecR. The FecA-FecR interaction is thought to somehow lead to cleavage of FecR by RseP (*E. coli* MucP homolog) to facilitate the release and activation of the σ factor FecI in response to ferric citrate in the environment. Indeed, activation of the *P. aeruginosa* iron starvation response ECF σ factors FiuI and FoxI requires MucP (*E. coli* RseP) for activation in response to their cognate siderophores ferrichrome and ferrioxamine, respectively [134], [135]. Once activated by their specific ligand, iron starvation response ECF σ factors upregulate the expression of the receptor and transport system for that ligand. Thus bacteria can increase the expression of specific iron acquisition systems only when iron is present in the environment in the form recognized by the system in question.
Figure 1.8. The FecIRA system from *E. coli*. FecA is an outer membrane TonB-dependent receptor that senses and transports ferric citrate into the periplasm. Upon binding ferric citrate, FecA undergoes a conformational change that allows it to interact with TonB and FecR. Following contact between FecA and FecR, RseP is thought to cleave FecR, resulting in the release and activation of the σ factor FecI. Adapted from Koebnik, 2005.

Of the 19 ECF σ factors encoded by *P. aeruginosa* PAO1, 12 have been implicated as members of putative iron starvation response systems (Table 1.1) (reviewed in [120],[136], [137]. 10 of these 12 have identifiable Fur boxes in their promoter regions, suggesting they are subject to transcription regulation by the iron-responsive Fur repressor [120]. Conveniently, in iron starvation systems the gene encoding the system’s TonB-dependent receptor is usually found directly adjacent, or in close proximity to, the ECF σ factor/anti-σ factor operon on the chromosome [136]. 11 of the 12 putative iron starvation ECF σ/anti-σ operons in *P. aeruginosa* encode an adjacent or nearby TonB-dependent receptor [136]. The one iron-responsive σ factor that does not have a TonB-dependent receptor located nearby on the chromosome, PvdS, works in conjunction with the σ factor FpvI to produce and import the siderphore pyoverdine via the FpvA TonB-dependent receptor [120].

Of the remaining seven ECFs in *P. aeruginosa*, only three have been characterized. The *P. aeruginosa* σ^E^ homolog AlgU is the most studied because of the
role it plays in regulating the production of alginate and conferring a mucoid phenotype to colonies of *P. aeruginosa* clinical isolates. SigX, which does not have a cotranscribed anti-σ factor, has been shown to control the expression of the major outer membrane porin OprF [120]. Recent work from the Häussler lab has expanded the SigX regulon to include virulence factors, efflux pumps, and metabolic genes [138]. The last ECF σ factor in *P. aeruginosa* that has been characterized is VreI, which has been shown to control the expression of a type II secretion system, alkaline phosphatases, and a two-partner secretion system [139]. The remaining uncharacterized ECF σ factors in PAO1 that do not appear to be related to iron acquisition are PA1351, PA2050, PA2896, and PA3285 (Table 1.1). In Chapter 2 of this thesis, I will describe our efforts to understand the role of the uncharacterized ECF σ factor encoded by the PA2896 gene and its corresponding anti-σ factor encoded by PA2895. When work began on this project, the only published results regarding the system suggested that PA2896 was expressed in response to osmotic or cell wall stress and that ΔPA2895 mutant cells exhibited a protease secretion defect [140]-[142]. In Chapter 2 of this thesis, I present evidence that PA2895 is an anti-σ factor that inhibits the activity of its cognate σ factor, PA2896. I also describe the identification of the putative PA2896 regulon, which includes a novel regulator of swarming motility and biofilm formation in *P. aeruginosa* PA14.
Table 1.1. $\sigma$ factors in *P. aeruginosa*. Non-iron-related uncharacterized $\sigma$ factors and their putative anti-$\sigma$ factors are bolded.

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<th>Gene number</th>
<th>Gene name</th>
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<td><em>rpoD/$\sigma^{70}$</em></td>
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<td><strong>ECF anti-(\sigma)</strong></td>
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References


Chapter 2

A $\sigma$ factor and anti-$\sigma$ factor that control swarming motility

and biofilm formation in \textit{P. aeruginosa}
Attributions

Isabelle Vallet-Gely performed the PA2896-TAP, RpoS-TAP, and AceF-TAP purifications discussed in this chapter and created the necessary plasmids and strains. She also performed the two-hybrid experiments to test the interaction between PA2895-NTR and PA2896 and cloned the necessary plasmids to do so. Kirsty McFarland assisted in analyzing DNA microarray data. Heather McManus created pP30ΔFRT-PA2896-VSV-G. Keith Turner created pEXG2-ΔwspF. I performed all other work described in this chapter and wrote the text of this chapter, with editorial assistance from Simon Dove.
Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic human pathogen notorious for being the principal cause of morbidity and mortality in cystic fibrosis (CF) patients [1]. In patients with CF, chronic pulmonary colonization by *P. aeruginosa* leads to chronic inflammation, progressive loss of lung function, and eventually respiratory failure and death [1]. *P. aeruginosa* is also the fifth leading cause of nosocomial infections overall in the US and is the second most common cause of ventilator-associated pneumonia (VAP) and catheter-associated urinary tract infections (CAUTI) [2], [3]. In patients with VAP or CAUTI, *P. aeruginosa* grows as a biofilm on endotracheal tubes and catheters, respectively [4]-[6]. In addition, *P. aeruginosa* is thought to grow as a biofilm in the CF lung [7]. *P. aeruginosa* biofilms are associated with chronic infection and exhibit increased antibiotic resistance and resistance to clearance by the immune system [8]. Thus, the ability to form biofilms contributes significantly to the clinical burden of *P. aeruginosa* infection.

In *P. aeruginosa*, growth as a biofilm is inversely regulated with a cooperative form of multicellular surface motility called swarming [9]-[12]. Swarming motility is flagella-dependent and requires the secretion of surfactants regulated by quorum sensing (reviewed in [13])[14]-[16]. In addition, swarming motility correlates with increased expression of virulence factors and is associated with acute infection [9], [17]. Several systems are known to mediate the transition from motile, swarming cells, to cells growing as sessile biofilms, including c-di-GMP signaling and the GacS/GacA two-component system [9], [11], [12].

PA2895 was identified in a signature-tagged mutagenesis (STM) screen as being required for persistence in a rat-lung model of chronic *P. aeruginosa* respiratory infection [18]. PA2895 encodes a protein that has no homology to any previously characterized
protein and is predicted to contain a transmembrane α-helix from residues 65-87 that separates a predicted cytoplasmic N-terminus and a periplasmic C-terminus. PA2895 is encoded in a putative bicistronic operon downstream of an uncharacterized putative extracytoplasmic function (ECF) σ factor, PA2896. The gene encoding the putative ECF σ factor PA2896 has been reported to be upregulated in response to osmotic shock and in response to treatment of cells with the cell wall inhibitory antibiotic D-cycloserine [19], [20]. As a group, ECF σ factors are frequently cotranscribed with their own negative regulator, a transmembrane anti-σ factor (reviewed in [21]). Upon stimulation by the appropriate extracytoplasmic signal, the ECF σ factor is released from the anti-σ factor, allowing it to associate with RNAP and activate expression of its regulon. ECF σ factors are frequently autoregulated and control the expression of relatively small regulons [21].

Here we present evidence that PA2896 and PA2895 are an ECF σ and anti-σ factor pair. We identify the PA2896 regulon and show that PA2895 and PA2896 influence biofilm formation and swarming motility by controlling the expression of PA1494. In particular, we show that cells lacking PA2895 are unable to engage in swarming motility and exhibit increased biofilm formation due to the PA2896-dependent increase in PA1494 expression observed in these cells. Thus, PA2895 and PA2896 represent a novel set of regulators of swarming motility and biofilm formation that mediate their effects through PA1494, a protein that had not previously been implicated in biofilm formation or swarming motility.
Results

PA2896 interacts with RNA polymerase

PA2895 was previously identified through a signature tagged transposon mutagenesis screen as a gene that is essential for the persistence of P. aeruginosa in a chronic respiratory infection model [18]. PA2895 is predicted to be a component of a bicistronic operon together with PA2896 (Fig. 2.1A). A four base pair overlap in the coding sequences of PA2895 and PA2896 suggests strong transcriptional coupling of these genes. While PA2895 has no homology to any previously characterized proteins, PA2896 is annotated as a probable ECF σ factor on the Pseudomonas Genome Database and shares significant sequence homology with other ECF σ factors (www.pseudomonas.com). To determine if PA2896 might function as a σ factor, we purified PA2896 from cells of P. aeruginosa and asked whether subunits of RNAP co-purify with it.

To facilitate the purification of PA2896 from P. aeruginosa we constructed a strain of PAO1 that synthesized PA2896 with a tandem affinity purification (TAP) tag fused to its C-terminus (PA2896-TAP) from its native chromosomal location. As a positive control for our ability to detect an association between a σ factor and RNAP, we also constructed a second strain that synthesized the stationary phase-specific σ factor RpoS with a C-terminal TAP-tag (RpoS-TAP). As a negative control we used a previously constructed strain that synthesizes AceF (a subunit of pyruvate dehydrogenase that is not expected to interact with RNAP), with a TAP-tag fused to its C-terminus (AceF-TAP) [22]. We then purified PA2896, RpoS, and AceF by TAP and analyzed those proteins that co-purified by SDS-PAGE followed by staining with Coomassie blue. As Figure 2.1B shows, proteins with the expected molecular weights for the β, β’, and α subunits of RNAP co-purify with both RpoS-TAP and PA2896-TAP,
but not the negative control AceF-TAP. This suggests that PA2896 associates with RNAP in vivo.

Figure 2.1. The PA2896-PA2895 operon and the results of TAP immunoprecipitation to detect association of PA2895 and RNAP. (A) The putative PA2896-PA2895 operon. (B) The β, β', and α subunits of RNAP copurify with RpoS-TAP and PA2896-TAP, but not with AceF-TAP. Purified proteins were separated by SDS-PAGE and stained with Coomassie blue. AceF-CBP, RpoS-CBP, and PA2896-CBP indicate the purified proteins with the calmodulin binding protein (CBP) moiety that remains after cleaving the protein A moiety of the TAP tag during purification. (C) Anti-VSVG Western blot of purified protein from PAO1 β'-TAP (lane 1) and PAO1 β'-TAP PA2896-V (lane 2). The protein A moiety of β'-TAP cross-reacts with the anti-VSVG antibody at the top of the gel.

As a further test of whether PA2896 interacts with RNAP we purified RNAP directly from cells of P. aeruginosa and asked whether PA2896 co-purified. To do this we constructed a strain of PAO1 that synthesized both the β' subunit of RNAP with a TAP-tag fused to its C-terminus (β'-TAP), and synthesized PA2896 with a vesicular stomatitis
virus-glycoprotein (VSV-G) epitope tag fused to its C-terminus (PA2896-V), at their respective native loci. PAO1 β′-TAP, which did not contain any epitope-tagged PA2896, was used as a control strain. Following TAP, Western blotting revealed that PA2896 co-purified with β′-TAP (Fig. 2.1C). Taken together our findings suggest that PA2896 associates with RNAP in vivo, consistent with its predicted function as a σ factor.

Interestingly, PA2896 appears as a doublet in Figure 2.1 B and C, suggesting it can exist as a high molecular weight and low molecular weight species. It is unclear if this doublet represents PA2896 processing, the use of an alternative translational start site, or if there is any functional difference between these two forms. However, both forms co-purify with β′-TAP (Fig. 2.1C), suggesting that both forms are capable of associating with RNAP.

**PA2895 negatively influences PA2896 protein abundance**

The genomic arrangement of PA2896 and PA2895 suggests that they form an ECF σ factor and anti-σ factor pair. In some cases, ECF σ factors exhibit reduced activity in the absence of their cognate anti-σ factor, which are thought to stabilize and protect the σ factor from degradation [23], [24]. To determine if PA2895 influences the abundance of PA2896 we performed anti-VSV-G Western blots using PAO1 PA2896-V and PAO1 ΔPA2895 PA2896-V cells. In ΔPA2895 mutant cells, PA2896-V levels were increased relative to wild type (WT) (Fig. 2.2A), indicating PA2896 is more abundant in the absence of PA2895. This suggests that PA2895 negatively influence the abundance of PA2896 and that PA2895 might inhibit expression of PA2896.
**Figure 2.2.** PA2895 negatively regulates PA2896 expression. (A) Anti-VSV-G Western blot of WT PAO1 (lane 1), PAO1 PA2896-V (lane 2), and PAO1 ΔPA2895 PA2896-V (lane 3). (B) β-galactosidase activity of PAO1 $P_{PA2896}^{lacZ}$ and the indicated mutant derivatives containing the indicated plasmids. EV indicates pPSV38 empty vector. 2895 indicates pPA2895. NTR indicates pPA2895-NTR. 2896 indicates pPA2896. (C) Schematic representation of PA2895 and the location of its predicted transmembrane (TM) domain.
Expression from the PA2896 promoter is negatively regulated by PA2895

After observing increased PA2896 protein levels in the ΔPA2895 mutant strain, we were interested in determining whether PA2895 represses transcription from the PA2896 promoter. To test this, we integrated a construct with the putative PA2896 promoter region upstream of a lacZ reporter at the φCTX phage attachment site on the PAO1 chromosome to generate the reporter strain PAO1 attB::P_{PA2896}-lacZ. We then created an in-frame ΔPA2895 deletion in this strain to test the effects of PA2895 on expression from the PA2896 promoter. In cells of the ΔPA2895 mutant reporter strain, β-galactosidase activity increased 45-fold relative to that observed in cells of the WT reporter strain (Fig. 2.2B), suggesting that PA2895 inhibits expression from the PA2896 promoter. This increase was restored to WT levels by complementation with PA2895 from a plasmid (Fig. 2.2B). Cells of the ΔPA2895 ΔPA2896 double mutant strain exhibited basal levels of β-galactosidase activity similar to that observed in cells of the WT reporter strain (Fig. 2.2B), suggesting that expression from the PA2896 promoter is PA2896-dependent. Ectopic expression of PA2896 in cells of the ΔPA2895 ΔPA2896 mutant strain resulted in a modest increase in β-galactosidase activity (Fig. 2.2B), confirming the positive regulatory effect of PA2896 on its own promoter. Together, these results suggest that PA2895 inhibits PA2896-dependent transcription and that PA2896 is positively autoregulated, consistent with a model in which PA2896 is an ECF σ factor that controls its own expression and PA2895 is the cognate anti-σ factor.

The N-terminal region of PA2895 inhibits the activity of PA2896

It has been shown that the N-terminal cytoplasmic region of ECF anti-σ factors can be sufficient for anti-σ factor activity [24]-[27]. PA2895 is predicted to contain a single transmembrane α-helix from residue 65 to 87, with a cytoplasmic N-terminal...
domain and a periplasmic C-terminal domain, consistent with the membrane topology of other ECF anti-σ factors (Fig. 2.2C). To determine if the N-terminal region of PA2895 was capable of inhibiting PA2896-dependent expression, we truncated PA2895 at the start of the predicted transmembrane domain to produce PA2895-NTR (residues 1-64) (Fig. 2.2C). Levels of β-galactosidase activity in the ΔPA2895 reporter strain expressing PA2895-NTR are indistinguishable from those expressing full length PA2895 (Fig. 2.2B), which suggests PA2895-NTR contains the region of PA2895 necessary for inhibiting PA2896 activity. It further suggests that PA2895-NTR may contain a domain that is capable of interacting with PA2896.

*The N-terminal region of PA2895 interacts with PA2896*

ECF anti-σ factors have been shown to inhibit their cognate σ factors by binding to them directly and preventing their association with RNAP [25], [27]-[29]. We have shown that both PA2895 and PA2895-NTR inhibit PA2896-dependent gene expression (Fig. 2.2B), and we next sought to determine whether PA2895 directly interacts with PA2896 using a bacterial two-hybrid system.

In this two-hybrid system, the detection of a protein-protein interaction relies on the observation that an interaction between a DNA-bound protein and a subunit of RNAP can result in transcription activation of a test promoter [30], [31]. In the version of the assay used here, contact between a protein fused to the α subunit of *E. coli* RNAP and another protein (or protein domain) fused to the λcI DNA-binding protein activates the transcription of a *lacZ* reporter gene situated downstream of an appropriate test promoter containing a λcI binding site (Fig. 2.3A).
Figure 2.3. PA2895-NTR and PA2896 interact by bacterial two-hybrid. (A) Schematic representation of the bacterial two-hybrid system. Interaction between the N-terminal region of PA2895 (PA2895-NTR) fused to λcI and PA2896 fused to the α subunit of RNAP activates transcription from the test promoter driving expression of lacZ. The diagram depicts test promoter PlacO2-62, which bears the λ operator O2 centered 62 bp upstream from the transcription start site of the lac core promoter. This test promoter is linked to lacZ and located on the chromosome. (B) Effect of λcI-PA2895NTR on transcription from PlacO2-62 in the presence of the α-PA2896 chimera. Cells harboring compatible plasmids directing the synthesis of the indicated proteins were grown in the presence of the indicated concentrations of IPTG and assayed for β-galactosidase activity.

To test whether PA2895 could interact directly with PA2896 we created two compatible plasmids, one expressing PA2895-NTR (residues 2-64) fused to the C-terminus of λcI and the other expressing an α fusion protein where the C-terminal domain (CTD) of α has been replaced with full-length PA2896 (residues 2-194). We then determined whether the resulting λcI-PA2895-NTR fusion protein could activate transcription from the test promoter in cells that also synthesized the α-PA2896 fusion
protein. Plasmids directing the synthesis of the $\lambda CI$-PA2895-NTR and the $\alpha$-PA2896 fusion proteins were used to transform *E. coli* strain KS1, which harbors the *Plac-O_{n}2-62* test promoter (depicted in Fig. 2.3A) linked to *lacZ* and integrated in single copy in the *E. coli* chromosome [31]. We found the $\lambda CI$-PA2895-NTR fusion protein strongly activated the transcription of the *lacZ* reporter in cells that also synthesize the $\alpha$-PA2896 fusion protein, but not in cells that contained WT $\alpha$ (Fig. 2.3B). Additional controls revealed that WT $\lambda cl$ fails to activate expression of the *lacZ* reporter in the presence of the $\alpha$-PA2896 fusion protein or in the presence of WT $\alpha$ (Fig. 2.3B). These results suggest that PA2895 and PA2896 directly interact, consistent with the hypothesis that PA2895 is the cognate anti-\(\sigma\) factor of PA2896.

**Defining the PA2896 regulon using DNA microarray analysis**

Next, we wanted to identify the genes controlled by PA2895 and PA2896. Based on our above results that show PA2895 inhibits PA2896 expression and that PA2896 is positively autoregulated, we reasoned the PA2896 regulon would be constitutively expressed in $\Delta$PA2895 mutant cells. Using DNA microarrays, we compared changes in gene expression in $\Delta$PA2895 mutant cells and $\Delta$PA2895 $\Delta$PA2896 mutant cells relative to WT to identify the genes that might be regulated by PA2895 and PA2896. To test whether any changes in gene expression we observed in cells of the PAO1 $\Delta$PA2895 mutant relative to WT PAO1 were due to a polar effect of the PA2895 deletion, we transformed cells of the PAO1 $\Delta$PA2895 mutant strain with a plasmid that expresses PA2895 (pPA2895). We also transformed WT PAO1 cells, PAO1 $\Delta$PA2895 mutant cells, and PAO1 $\Delta$PA2895 $\Delta$PA2896 mutant cells with the empty vector control plasmid pPSV38 (pEV) to allow for the direct comparison of gene expression between all of the strains.
Based on our hypothesis that PA2896 would be constitutively active in ΔPA2895 mutant cells, we expected to see an increase in the expression of genes that are positively regulated by PA2896 in ΔPA2895 cells (PAO1 ΔPA2895 pEV) relative to WT cells (PAO1 pEV). In addition, we expected the expression of those genes that were upregulated in ΔPA2895 mutant cells (PAO1 ΔPA2895 pEV) to return to WT expression levels in the complemented strain (PAO1 ΔPA2895 pPA2895). Lastly, by comparing the relative expression of genes in ΔPA2895 mutant cells (PAO1 ΔPA2895 pEV) to that in cells of the ΔPA2895 ΔPA2896 double mutant (PAO1 ΔPA2895 ΔPA2896 pEV), we could determine which changes in gene expression in ΔPA2895 mutant cells were dependent upon PA2896.

Compared to WT cells, the expression of 21 genes changed >2-fold in cells of the ΔPA2895 mutant (Table 2.1). The expression of three genes in particular was strongly influenced by the deletion of PA2895. Specifically, the expression of PA1494 was 103-fold higher in cells of the ΔPA2895 mutant than in WT cells, while expression of PA4495 and PA2896 was 22- and 18-fold higher, respectively, in cells of the ΔPA2895 mutant when compared to WT (Table 2.1). The effects of the ΔPA2895 deletion on PA1494, PA4495 and PA2896 expression levels could be complemented by providing PA2895 in trans from a plasmid (Table 2.1). Furthermore, upregulation of PA1494, PA4495, and PA2896 did not occur in cells of the ΔPA2895 ΔPA2896 double mutant, suggesting that the upregulation of these genes observed in cells of the ΔPA2895 single mutant is dependent upon PA2896 (Table 2.1). In addition, expression of PA1494 was reduced 2-fold in cells of the ΔPA2895 ΔPA2896 mutant relative to WT cells, suggesting that PA2896 positively influences the expression of PA1494 in WT cells even in the presence of PA2895. Taken together, these findings suggest that expression of PA1494, PA4495, and PA2896 is positively controlled by PA2896 and negatively controlled by PA2895.
**Table 2.1.** DNA Microarray results comparing fold changes in gene expression relative to WT PAO1 pEV. “−” indicates no change in expression.

<table>
<thead>
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<th>Gene number</th>
<th>∆PA2895 pEV</th>
<th>∆PA2895 pPA2895</th>
<th>∆PA2895 pEV</th>
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<td>−</td>
<td>−</td>
<td>−</td>
</tr>
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<td>−</td>
</tr>
<tr>
<td>PA0971</td>
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<td>−</td>
<td>−</td>
<td>−</td>
</tr>
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Several genes exhibited differential expression in ΔPA2895 cells relative to WT cells that did not respond to complementation with PA2895, suggesting the expression of these genes may not be controlled by PA2895. Indeed, previous work in our lab may explain the differential regulation of three genes (PA1202, PA1203, and PA2432) that fit this profile. We have previously shown that expression of PA1202, PA1203, and PA2432 (bexR) is bistable and subject to control by a bistable switch mediated by the transcription regulator BexR [32]. Specifically, expression of these three genes, including bexR, switches on when there are high levels of BexR and switches off when levels of BexR fall below a critical threshold. BexR controls its own expression in a positive feedback loop that is responsible for producing and maintaining the bistable switch.

Another gene with differential expression in ΔPA2895 mutant cells relative to WT that was unaffected by PA2895 complementation was PA3876 (narK2) (Table 2.1). Expression of this gene was also elevated in ΔPA2895 ΔPA2896 double mutant cells relative to WT (Table 2.1), suggesting that changes in narK2 expression may be a polar effect of the ΔPA2895 deletion.

Several genes were found to be downregulated in ΔPA2895 cells (PAO1 ΔPA2895 pEV) relative to WT (PAO1 pEV) (Table 2.1). In particular, the mmsAB operon was found to be down-regulated roughly 9-fold in ΔPA2895 mutant cells relative to WT. MmsA and MmsB are enzymes involved in valine metabolism [33]. The mmsAB operon is positively regulated by the divergently transcribed AraC-like transcription regulator MmsR [33], however no changes in mmsR expression were observed by DNA microarray. These findings suggest that PA2895 might also exert positive effects on the expression of some genes.
PA2895 inhibits PA2896-dependent expression from the putative PA1494 and PA4495 promoters

Our genome-wide expression analyses revealed that the PA1494, PA4495, and PA2896 genes were strongly regulated by PA2895 in a manner that was dependent upon PA2896 (Table 2.1), suggesting their respective promoters are controlled by PA2896. To test whether expression from the putative promoter regions of PA1494 and PA4495 are subject to regulation by PA2895 and PA2896, we used the same method used to generate the PAO1 attB::P_{PA2896-lacZ} reporter strain to create the PAO1 attB::P_{PA1494-lacZ} and PAO1 attB::P_{PA4495-lacZ} reporter strains. We then generated ΔPA2895 single mutant and ΔPA2895 ΔPA2896 double mutant derivatives of these reporter strains to test the regulatory effects of PA2895 and PA2896 on expression from the putative promoters.

In the ΔPA2895 mutants derived from these two reporter strains (PAO1 attB::P_{PA1494-lacZ} ΔPA2895 and PAO1 attB::P_{PA4495-lacZ} ΔPA2895), expression of lacZ from P_{PA1494} and P_{PA4495} increased 17- and 57-fold, respectively (Fig. 2.4A and B). Ectopic expression of PA2895 or PA2895-NTR from a plasmid restored WT levels of β-galactosidase activity in cells of both ΔPA2895 mutant reporter strains (Fig. 2.4A and B), consistent with the results from the P_{PA2896-lacZ} reporter strain (Fig. 2.2B). Together, these results indicate that PA2895 inhibits expression from the PA1494, PA4495, and PA2896 promoters, consistent with our data showing PA2895 has anti-σ factor activity.
Figure 2.4. The PA1494 and PA4495 promoters are negatively controlled by PA2895 and are PA2896-dependent. β-galactosidase activity of (A) PAO1 \( \text{attB}:P_{\text{PA1494}}\text{lacZ} \) and (B) PAO1 \( \text{attB}:P_{\text{PA4495}}\text{lacZ} \), and their derivative mutant strains complemented with plasmids as indicated. WT: wild type. EV: empty vector. 2895: pPA2895. NTR: pPA2895-NTR. 2896: pPA2896.
Next, we tested if the increase in expression from the promoters in the ∆PA2895 mutant reporter strains was PA2896-dependent. In cells of the ∆PA2895 ∆PA2896 double mutant strains, expression of the $P_{PA1494}$ and $P_{PA4495}$ promoter-lacZ reporters returned to WT levels (Fig. 2.4A and B), suggesting that expression from these promoters is PA2896-dependent. Ectopic expression of PA2896 in cells of the ∆PA2895 ∆PA2896 double mutant reporter strains resulted in an increase in β-galactosidase activity, albeit to a lesser extent than that observed in cells of the ∆PA2895 single mutant reporter strains (Fig. 2.4A and B), indicating expression from these promoters is positively controlled by the ECF σ factor PA2896. Taken together these results suggest that transcription from the $P_{PA1494}$, $P_{PA4495}$, and $P_{PA2896}$ promoters is positively regulated by the ECF σ factor PA2896 and inhibited by PA2896’s cognate anti-σ factor PA2895.

### ∆PA2895 mutants have a severe swarming motility defect

*P. aeruginosa* is capable of several types of motility, including twitching, swimming, and swarming. Twitching motility is a form of surface motility and is mediated by type-IV pili. Swimming motility occurs in low viscosity liquid environments and is a unicellular behavior dependent upon flagella and the chemotaxis system. Swarming is a form of cooperative multicellular motility that occurs on hydrated surfaces and in viscous liquid environments. Swarming motility is dependent upon flagella and secreted surfactants, the production of which is controlled by quorum sensing [14], [15]. On agar plates, WT *P. aeruginosa* colonies grown overnight expand their borders via surface motility [34]. When we compared our WT and ∆PA2895 mutant strains, we noticed colonies of ∆PA2895 mutant cells were slightly smaller (data not shown), suggesting these cells might have a motility defect. To determine if PA2895 influences motility, we compared twitching, swimming, and swarming motility in wild-type and ∆PA2895
mutants of *P. aeruginosa* strains PAO1 and PA14.

Twitching motility can be observed in the laboratory by stab inoculating cells to the bottom of a petri dish containing media solidified with 1% agar measuring the diameter of the zone of motile cells that forms at the agar-plastic interface. Compared to WT, PAO1 $\Delta$PA2895 and PA14 $\Delta$PA2895 mutants exhibit reductions in twitching motility of 14% and 20%, respectively (Fig. 2.5A). These findings suggest that although $\Delta$PA2895 mutants have reduced twitching motility, cells of these mutants continue to produce functional type-IV pili and engage in twitching motility.

![Figure 2.5](image.png)

**Figure 2.5.** Twitching, swimming, and swarming motility of WT and $\Delta$PA2895 PAO1 and PA14 strains. (A) The relative diameter of twitching motility zones for the indicated strains, normalized to WT for each strain. (B) The relative diameter of swimming motility zones of the indicated strains, normalized to WT for each strain. (C) The relative area of swarming motility zones of the indicated strains. (D) Representative image of swarming motility exhibited by WT PA14 and PA14 $\Delta$PA2895 strains.
Next we tested the swimming motility of our strains. Swimming motility can be assayed by observing a zone of motility that forms when cells are grown on low percentage agar plates (0.3% agar). In this assay bacteria migrate out from the initial point of inoculation through the agar via swimming motility, forming a circular zone of motile cells. Measuring the diameter of the resulting zone of motile cells allows for the relative motility of different strains to be determined. PAO1 and PA14 \( \Delta \text{flgK} \) mutants that do not produce flagella were unable to swim from the point of inoculation (data not shown). Compared to WT, PAO1 and PA14 \( \Delta \text{PA2895} \) mutants exhibited reductions in swimming motility of 12% and 19%, respectively (Fig. 2.5B). Thus, swimming motility is only slightly reduced in \( \Delta \text{PA2895} \) mutant cells, suggesting that these mutant cells continue to engage in chemotaxis and continue to produce functional flagella.

To test whether cells of our \( \Delta \text{PA2895} \) mutant strains exhibited a swarming defect, we inoculated cells from overnight cultures onto plates containing a minimal media solidified with 0.5% agar. WT PA14 was a robust swarmer, forming colonies with dendrites that extended radially from the point of inoculation in an irregular starburst (Fig. 2.5D). In contrast to our WT PA14 strain, cells of our WT PAO1 strain did not swarm appreciably under these conditions, precluding an analysis of swarming motility in this strain background (data not shown). When we examined our PA14 \( \Delta \text{PA2895} \) mutant strain we discovered it does not spread from the point of inoculation and is completely defective for swarming motility (Fig. 2.5C and D). This suggests that PA2895 promotes swarming motility or that PA2895 represses an inhibitor(s) of swarming motility.

Swarming motility in PA14 is dependent upon flagella and the secreted surfactants HAA and rhamnolipid [14]-[16]. As demonstrated by our swimming assays, PA14 \( \Delta \text{PA2895} \) mutants are capable of chemotaxis and produce functional flagella (Fig. 2.5). On the 0.5% agar plates used to observe swarming, surfactant production can be
observed as an area of wetness surrounding surfactant-producing colonies [15], [16]. We observed this region surrounding non-swarming PA14 ΔPA2895 mutant colonies, suggesting this strain continues to produce surfactants (data not shown). These findings suggest the swarming motility defect of PA14 ΔPA2895 is not necessarily caused by a defect in rhamnolipid production.

*The effect of PA2895 on swarming motility is dependent upon PA2896 and PA1494*

Given PA2895’s role as an anti-σ factor, we reasoned that constitutive activation of PA2896 might be responsible for the motility defect in ΔPA2895 mutant cells. To determine if the swarming defect in PA14 ΔPA2895 mutants was dependent upon PA2896, we constructed the double mutant PA14 ΔPA2895 ΔPA2896. Swarming motility of cells of the PA14 ΔPA2895 ΔPA2896 strain was restored to WT levels (Fig. 2.6A), demonstrating that PA2896 is necessary for swarming inhibition in PA14 ΔPA2895.
Figure 2.6. PA1494 inhibits swarming motility. (A) Inhibition of swarming motility in PA14 \( \Delta \text{PA2895} \) is PA2896- and PA1494-dependent. Area is normalized to WT. (B) The effect of ectopic PA1494 expression on swarming motility. Strains were transformed with the indicated plasmids. Expression of PA1494 from pPA1494 was induced by the addition of 0.1% arabinose. Normalized to WT. EV = pEV. 1494 = pPA1494.
Next, we reasoned that PA2896-dependent inhibition of swarming motility in PA14 ΔPA2895 mutants was likely due to the constitutive expression of a gene(s) in the PA2896 regulon. The three most highly upregulated genes in the cells of the ΔPA2895 mutant in our microarrays were PA1494, PA4495, and PA2896 itself (Table 2.1). PA1494 and PA4495 have no significant homology to any previously characterized proteins, are predicted to encode N-terminal secretion signals, and have been found experimentally in the periplasm [35]. We focused on the most highly upregulated gene by microarray, PA1494, and created an in-frame deletion of PA1494 in PA14 ΔPA2895. Unlike the cells of a ΔPA2895 single mutant, cells of a ΔPA2895 ΔPA1494 double mutant did not exhibit a swarming motility defect and instead resembled WT PA14 with respect to their ability to swarm (Fig. 2.6A). This finding demonstrates that PA1494 is necessary for swarming inhibition in ΔPA2895 mutant cells. In addition, the PA14 ΔPA1494 mutant strain exhibited a 17% increase in swarming motility that was not statistically significant (Fig. 2.6A). The PA14 ΔPA2895 ΔPA1494 mutant strain could be complemented by ectopic expression of PA1494, which restored swarming inhibition (Fig. 2.6B). These results suggest that the inhibition of swarming motility in ΔPA2895 mutant cells is the result of increased PA2896-dependent expression of PA1494.

Expression of PA1494 is sufficient for the inhibition of swarming motility

In cells of the ΔPA2895 mutant, the expression of PA1494, PA4495, and PA2896 becomes activated (Table 2.1), and swarming motility is inhibited (Fig. 2.5C and D). Moreover, PA1494 is necessary for the inhibition of swarming motility in the ΔPA2895 mutant strain (Fig. 2.6A). Therefore we wondered whether ectopic expression of PA1494 would suffice to inhibit swarming motility, or if PA1494-mediated inhibition of swarming motility in the ΔPA2895 mutant strain required activation of the entire PA2896 regulon.
To test this, we transformed WT PA14 cells with the same PA1494 expressing plasmid used to complement the PA14 ΔPA2895 ΔPA1494 double mutant (pPA1494) and an empty vector control (pEV). Swarming motility was inhibited to levels comparable to that of the ΔPA2895 mutant strain in WT PA14 cells transformed with pPA1494 (PA14 pPA1494), but not in cells transformed with the empty vector control (PA14 pEV), (Fig. 2.6B). Thus, ectopic expression of PA1494 is sufficient for the inhibition of swarming motility and may not require the increased expression of other genes in the PA2896 regulon.

**ΔPA2895 mutant cells exhibit enhanced biofilm formation**

The surface-associated behaviors of swarming motility and biofilm formation have been shown to be inversely co-regulated in *P. aeruginosa* PA14 [9]-[12]. That is, strains that exhibit increased swarming motility produce less biofilm, while strains that produce increased levels of biofilm exhibit reduced or inhibited swarming motility. Given this relationship, we next asked whether our PA14 ΔPA2895 mutant were altered with respect to their ability to form biofilms. Following 8 hours of static growth, the non-swarming PA14 ΔPA2895 mutant strain formed ~2-fold more biofilm than the WT strain (Fig. 2.7A). This finding suggests that PA2895 reduces biofilm formation or inhibits factors that facilitate biofilm formation.
Figure 2.7. PA1494 enhances biofilm formation. (A) PA14 ΔPA2895 forms increased amounts of biofilm. This effect is PA2896- and PA1494-dependent. (B) The effect of ectopic PA1494 expression on biofilm formation. Strains were transformed with the indicated plasmids and grown in the presence of 200 μg/ml carbenicillin. Expression of PA1494 from pPA1494 was induced by the addition of 1% arabinose. EV = pEV. 1494 = pPA1494.
The matrix exopolysaccharide Pel is a major component of *P. aeruginosa* PA14 biofilms [36] and increased Pel production correlates with enhanced PA14 biofilm formation [11], [12], [37]. Increased Pel production can be observed qualitatively by growing cells on solid media containing Congo red (CR); CR binds to Pel, which stains Pel-producing colonies red. For example, \(\Delta wspF\) mutant cells produce large amounts of Pel [37] and stain red, while the \(\Delta pelA\) mutant does not (Fig. 2.8). When \(\Delta PA2895\) mutant cells are grown on CR containing plates they do not stain red, suggesting that enhanced biofilm formation in the \(\Delta PA2895\) mutant strain may not be the result of increased Pel production.

**Figure 2.8.** Congo Red binding as a qualitative measure of Pel production. Overnight cultures were spotted on CR plates to qualitatively assess levels of Pel production. PA14 \(\Delta wspF\) cells produce high levels of Pel and stain red. PA14 \(\Delta pelA\) mutant cells are unable to produce Pel and do not stain red. PA1494 \(\Delta PA2895\) does not stain deep red.
The effect of PA2895 on biofilm formation is dependent on PA2896 and PA1494

Next, we tested the PA14 ∆PA2895 ∆PA2896 double mutant strain to determine if the increase in biofilm formation in the ∆PA2895 mutant strain was PA2896-dependent. PA14 ∆PA2895 ∆PA2896 formed biofilms at levels similar to WT (Fig. 2.7A), suggesting that in addition to inhibiting swarming motility, increased expression of the PA2896 regulon in the ∆PA2895 mutant strain also promotes biofilm formation.

Our previous finding that the swarming motility defect of the ∆PA2895 mutant strain was PA1494-dependent led us to ask whether PA1494 was also required for enhanced biofilm formation in the ∆PA2895 strain. Cells of a PA14 ∆PA2895 ∆PA1494 mutant strain formed biofilms at levels similar to WT (Fig. 2.7A), indicating PA1494 is necessary for enhanced biofilm formation in the ∆PA2895 mutant strain. In addition, biofilm formation in PA14 ∆PA2895 ∆PA1494 mutants could be restored to ∆PA2895 mutant levels by expressing PA1494 from a plasmid (Fig. 2.7B). These findings suggest that enhanced biofilm formation in the ∆PA2895 mutant strain may be the result of increased PA2896-dependent expression of PA1494. Lastly, while PA1494 is required for increased biofilm formation in the ∆PA2895 mutant strain, the PA14 ∆PA1494 mutant strain formed biofilms at WT levels (Fig. 2.7A). This suggests PA1494 does not significantly influence biofilm formation in WT cells of P. aeruginosa PA14, at least under the conditions of our experiments.

Expression of PA1494 is sufficient to promote biofilm formation

Ectopic expression of PA1494 in WT PA14 results in a swarming motility defect equivalent to that observed in cells of the ∆PA2895 mutant strain (Fig. 2.6B), suggesting it is sufficient for the inhibition of swarming motility. In light of this observation and the inverse relationship between swarming motility and biofilm formation, we next asked
whether ectopic PA1494 expression was also sufficient to enhance biofilm formation. In WT PA14 cells transformed with a PA1494 expression construct (PA14 pPA1494), but not those transformed with an empty vector (PA14 pEV), there is an increase in biofilm formation that is comparable to that seen in ∆PA2895 mutants (Fig. 2.7B), demonstrating that ectopic expression of PA1494 is sufficient to promote biofilm formation. Taken together, our findings suggest that in ∆PA2895 mutant cells, constitutive activation of PA2896 results in high levels of PA1494 expression, which enhances biofilm formation via an unknown mechanism.
Discussion

We have presented evidence that PA2896 and PA2895 form a cognate ECF σ factor and anti-σ factor pair. We show that PA2896 associates with RNAP (Fig. 2.1B and C) and that PA2895 inhibits PA2896-dependent gene expression. We also show that the first 64 residues of PA2895 (the predicted cytoplasmic portion of the protein) directly interact with PA2896 (Fig. 2.3B) and inhibit PA2896-dependent gene expression (Fig. 2.2B, 2.4A and B). Our results therefore suggest PA2895 may be oriented in the cytoplasmic membrane such that the N-terminal cytoplasmic domain is able to bind PA2896 and prevent it from associating with RNAP. We show that PA2896 is positively autoregulated and our DNA microarray results suggest PA2896 likely controls the expression of a small number of genes (Table 2.1). Using promoter-\textit{lacZ} fusion reporter strains we demonstrated PA2896-dependent expression from the promoters of PA2896, PA1494, and PA4495 is inhibited by the anti-σ factor PA2895 (Fig. 2.2B, 2.4A and B). In \textit{P. aeruginosa} PA14, we show that ΔPA2895 mutant cells are defective for swarming motility and produce increased amounts of biofilm, and that these phenotypes are PA2896- and PA1494-dependent (Fig. 2.5C, 2.6A and 2.7A). PA14 ΔPA2895 mutant cells continue to produce surfactants and do not bind increased amounts of CR (Fig. 2.8), suggesting these phenotypes may not be readily explained by defects in rhamnolipid production or increases in exopolysaccharide production. Taken together, these results suggest that in ΔPA2895 mutant cells, PA2896-dependent expression of PA1494 results in the inhibition of swarming motility and enhanced biofilm formation.

\textit{The PA2896 regulon}

Our transcription profiling experiments identified a relatively small number of genes that appear to be controlled by PA2895 and PA2896. In particular, the expression
of the putative PA2896-PA2895 operon, PA1494, and PA4495 exhibited the largest changes in expression in cells of the ΔPA2895 anti-σ factor mutant relative to WT (Table 2.1). Using promoter-lacZ fusion reporter strains we determined that the promoters of these genes are PA2896-dependent (Fig. 2.2B, 2.4A and B). Alignment of the transcription start sites of these genes derived from RNA-seq studies reveals they likely share identical promoter -10 and -35 elements, with the putative promoter sequence TAACCG-\textit{N}^{16}\textit{C}G\textit{C}T\textit{C}T\textit{C}A-N^{6}\textit{A} (+1) [38]-[40]. Using the Find Individual Motif Occurrences (FIMO) program to search for this putative promoter sequence in the PAO1 and PA14 genomes reveals statistically significant matches to this consensus only occur in the promoters of PA2896, PA1494, and PA4495 (False Discovery Rate q-value ≤0.01) (data available in Appendix XXX). The additional hits are statistically likely to be false positives (FDR q-values ≥0.1), are almost never found in potential promoter regions, and their potential -10 and -35 elements are poorly conserved. ECF σ factors have been reported to exhibit increased stringency in promoter sequence recognition [41], suggesting these additional hits may indeed represent false positive results and may not represent promoters that are recognized by PA2896. We therefore believe the PA2896 regulon likely consists of PA2896-PA2895, PA1494, and PA4495. This regulon is not unusually small for ECF σ factors, which frequently control the expression small regulons [21].

Our findings also indicate that PA2896 is positively autoregulated – a feature shared by many ECF σ factors [21]. Interestingly, complementation of cells of our PAO1 $P_{PA2896}\text{-}\text{lacZ}$ ΔPA2895 ΔPA2896 reporter strain with PA2896 \textit{in trans} resulted in considerably lower lacZ expression than in cells of the otherwise isogenic ΔPA2895 mutant reporter strain harboring pPA2896 (Fig. 2.2B). This suggests expression of PA2896 from its native promoter creates a positive feedback loop that drives constitutive
expression of PA2896 in the absence PA2895, while expression of PA2896 from the heterologous \( P_{\text{lac}} \) promoter (when supplied \textit{in trans} from a plasmid) does not result in the same effect. Indeed, PA2896 is more abundant in \( \Delta \text{PA2895} \) mutant cells (Fig. 2.2A). Such positive autoregulation among ECF \( \sigma \) factors is thought to serve as a mechanism to amplify the signal produced by the release of the \( \sigma \) factor from its anti-\( \sigma \) factor [21]. Thus the expression of an autoregulated ECF \( \sigma \) factor regulon can be rapidly increased in response to the appropriate activating signal.

\textit{PA1494 controls swarming motility and biofilm formation in \textit{P. aeruginosa} PA14}

PA1494 was the most highly upregulated member of the PA2896 regulon in \( \Delta \text{PA2895} \) mutant cells relative to WT (103-fold) and expression from the \( P_{\text{PA1494}} \) promoter was found to be PA2896-dependent (Table 2.1, Fig. 2.4A). We have shown that increased PA2896-dependent expression of PA1494 in \( \Delta \text{PA2895} \) mutant cells inhibits swarming motility and enhances biofilm formation, and that ectopic expression of PA1494 in WT cells has the same effect. Although we do not know the mechanism by which PA1494 influences swarming motility and biofilm formation, several systems have been shown to exert reciprocal control over swarming motility and biofilm formation in \textit{P. aeruginosa}, including c-di-GMP and the GacS/GacA two-component system [9], [11], [12]. Future work will be aimed at identifying the mechanism by which PA1494 represses swarming motility and enhances biofilm formation in \textit{P. aeruginosa} PA14.

\textit{A model for the PA2896 regulon}

Taken together, our results suggest a model in which the anti-\( \sigma \) factor PA2895 binds to PA2896 and sequesters it at the membrane, preventing it from associating with RNAP (Fig. 2.9). In response to an unknown cell envelope stress signal, PA2896 is
released from PA2895, associates with RNAP, and begins expression of the PA2896 regulon (Fig. 2.9). The PA2896 regulon likely consists of the putative PA2896-PA2895 operon, PA1494, and PA4495. PA2896-dependent expression of PA2896 results in a positive feedback loop, amplifying the expression of the PA2896 regulon. PA1494 and PA4495 are expressed at high levels and exported to the periplasm, leading to PA1494-dependent inhibition of swarming motility and enhanced biofilm formation. Thus we propose a model in which cell envelope stress is sensed by PA2895 and PA2896 to trigger a transition from motile swarming cells to growth as a sessile biofilm (Fig. 2.9). This work has elucidated a regulatory pathway controlling the expression PA1494, which we found to be a novel regulator of swarming motility and biofilm formation, and demonstrated anti-σ activity for the previously uncharacterized gene PA2895.

Figure 2.9. Model of PA2896, PA2895, PA1494, and PA4495.
Materials and Methods

Bacterial strains

*E. coli* DH5αF′IQ (Invitrogen) was used as the recipient strain for all plasmid constructions. *E. coli* SM10 λpir was used to mate plasmids into *P. aeruginosa*. *P. aeruginosa* strains used included PAO1 (provided by A. Rietsch) and PA14 (provided by L. Rahme). Bacterial cultures were routinely grown at 37°C in lysogeny broth (LB), or on plates containing LB solidified with 1.5% agar unless otherwise noted. When appropriate, gentamicin (30 μg/ml) and carbenicillin (200 μg/ml) were used for selection in *P. aeruginosa* cultures.

Plasmids and strains for tandem affinity purification (TAP)-tag experiments

Plasmid pP30Δ-PA2896-TAP was made by cloning an ~300 bp fragment of DNA corresponding to a 3’ portion of the PA2896 gene into pP30Δ-YTAP cut with HindIII and NotI; the portion of the PA2896 gene was cloned such that it was in-frame with the DNA specifying the TAP-tag. PAO1 PA2896-TAP was constructed by mating PAO1 with SM10 containing pP30Δ-PA2896-TAP and selected on Pseudomonas isolation agar (Difco) containing gentamicin (60 μg/ml) as previously described [22]. Strain PAO1 RpoS-TAP was constructed in a similar way using vector pP30Δ-RpoS-TAP, which contains a portion of the *P. aeruginosa rpoS* gene fused in-frame to DNA specifying the TAP-tag. The PAO1 AceF-TAP strain, which expresses AceF-TAP and serves as a control, has been described previously [22]. Plasmid pP30ΔFRT-PA2896-VSV-G was made by subcloning the HindIII/NotI PA2896 fragment from pP30Δ-PA2896-TAP into pP30ΔFRT-mvaT-VSV-G to replace mvaT, such that the 3’ end of PA2896 is in-frame with the VSV-G tag [42]. PAO1 β′-TAP PA2896-V was constructed in a similar manner
by integrating pP30ΔFRT-PA2896-VSV-G into the previously described strain PAO1 β'-TAP [43].

**Reporter strain and plasmids for bacterial two-hybrid assays**

Bacterial two-hybrid assays were performed with the *E. coli* reporter strain KS1 [30]; KS1 harbors on its chromosome the *placO*2-62 test promoter driving expression of a linked *lacZ* reporter gene. Plasmids pACλ.cI32 and pBRαLN have been described previously [31], and were used to create fusions to the C-terminus of λ.cI and the C-terminus of the α-linker, respectively. Plasmid pACλ.cI-PA2895-NTR encodes λ.cI (residues 1-236) fused to residues 2-64 of PA2895 from *P. aeruginosa* via a small linker composed of three alanine residues. Plasmid pACλ.cI-PA2895-NTR was made by cloning the appropriate NotI-BamHI-digested PCR product into pACλ.cI32 that had been digested with NotI and BstYI, thus placing expression of the λ.cI-PA2895-NTR fusion protein under the control of the IPTG-inducible lacUV5 promoter. Plasmid pBRα-PA2896 encodes residues 1-248 of the α subunit of *E. coli* RNA polymerase fused to residues 2-194 of PA2896 from *P. aeruginosa* via a small linker composed of three alanine residues. Plasmid pBRα-PA2896 was made by cloning the appropriate NotI-BamHI-digested PCR product into pBRαLN digested with NotI and BamHI, thus placing the α-fusion under the control of tandem *lpp* and IPTG-inducible lacUV5 promoters. Plasmid pBRα encodes wild type α under the control of tandem *lpp* and IPTG-inducible lacUV5 promoters and has been described previously [30].
Promoter-lacZ fusion reporter strains

The PAO1 promoter-lacZ fusion reporter strains PAO1 attB::P<sub>PA2896-lacZ</sub>, PAO1 attB::P<sub>PA1494-lacZ</sub>, and PAO1 attB::P<sub>PA4495-lacZ</sub> contain the putative promoter regions of PA2896, PA1494, and PA4495, respectively, fused to the lacZ gene and integrated in single copy into the ΦCTX locus in the PAO1 chromosome. The putative PA2896 promoter region consisted of the 327 bp intergenic region upstream of the PA2896 start codon (see www.pseudomonas.com). This region was amplified by the PCR and cloned into mini-CTX-lacZ as a BamHI/PstI fragment to generate mini-CTX-P<sub>PA2896-lacZ</sub>. The upstream intergenic regions of PA1494 (122 bp) and PA4495 (275 bp) were also PCR amplified and cloned into mini-CTX-lacZ on HindIII/BamHI fragments to generate mini-CTX-P<sub>PA1494-lacZ</sub> and mini-CTX-P<sub>PA4495-lacZ</sub>, respectively. The resulting plasmids were integrated in single copy into the ΦCTX site to create reporter strains PAO1 attB::PPA2896-lacZ, PAO1 attB::PPA1494-lacZ, and PAO1 attB::P<sub>PA4495-lacZ</sub> as previously described [44].

Construction of deletion mutant strains

The deletion construct for the PA2895 gene was generated by amplifying regions ~700 bp in length that flank PA2895 in the PAO1 genome by PCR and then splicing the flanking regions together by overlap extension PCR. Due to a 4 bp overlap between the 3’ end of PA2896 and the 5’ end of PA2895, the deletion was designed such that PA2896 would not be disrupted by the PA2895 deletion construct. The deletion was in-frame and contained a 9-bp NotI-linker sequence 5’-GCGGCCGCC-3’ separating the two flanking regions. The resulting PCR product was cloned on a HindIII/XbaI fragment into plasmid pEXG2 [45], yielding plasmid pEXG2-ΔPA2895. E. coli SM10 transformed with this plasmid was mated with PAO1 and PA14 and allelic replacement was
performed as previously described to create the PAO1 \(\Delta\)PA2895 and PA14 \(\Delta\)PA2895 deletion strains [46]. This plasmid was also used to generate the \(\Delta\)PA2895 mutant reporter strains in a similar manner. The \(\Delta\)PA2895 \(\Delta\)PA2896 deletion construct was generated by amplifying the ~700 bp 5’ flanking region of PA2896 in the PAO1 genome by PCR. This PCR product was digested with Xbal and NotI and cloned into pEXG2-\(\Delta\)PA2895 digested with Xbal and NotI, such that the 5’-flanking-PA2896 Xbal/NotI fragment replaced the 5’ flanking region used for deleting PA2895, yielding plasmid pEXG2-\(\Delta\)PA2895-\(\Delta\)PA2896. This plasmid was used to create the \(\Delta\)PA2895 \(\Delta\)PA2896 deletion strains as previously described [46]. The PA1494 deletion construct was made in a similar fashion to the PA2895 deletion construct. Flanking regions ~700 bp in length on either side of PA1494 in the PAO1 genome were amplified by the PCR and spliced together by overlap extension PCR. The deletion was in-frame and included the NotI-linker as above. This PCR product was cloned into pEXG2 to generate pEXG2-\(\Delta\)PA1494. The resulting plasmid was used to delete PA1494 as described above. Deletions were confirmed by PCR.

\textit{Tandem affinity purification}

Cells were grown at 37°C with aeration in 200 ml of LB in 1L flasks to an \(\text{OD}_{600}\) of ~1, then harvested by centrifugation at 4°C. TAP was then performed as described [45]. Purified proteins were concentrated using Amicon Ultra-4 centrifugal filtration units with a 10 kDa molecular weight cut-off (Millipore), separated on 4-12% Bis-Tris NuPAGE gel (Invitrogen) and stained with Coomassie blue.
**Western blots**

Purified proteins and cell lysates were separated on 4-12% Bis-Tris NuPAGE (Invitrogen) and Western blotting was performed as described previously [22]. The VSV-G-tag was detected using polyclonal rabbit anti-VSV-G (Sigma-Aldrich) and peroxidase-conjugated goat anti-rabbit IgG antibodies (Sigma-Aldrich).

**Bacterial two-hybrid assays**

Cells were grown with aeration at 37°C in LB supplemented with kanamycin (50 µg/ml), carbenicillin (100 µg/ml), chloramphenicol (25 µg/ml), and IPTG at the concentration indicated. β-galactosidase assays were performed as described [31]. Assays were performed three times in duplicate on separate occasions. A representative data set is shown. Values are averages based on one experiment; duplicate measurements differed by <10%.

**Construction of PA2895, PA2896, and PA1494 expression plasmids**

To make expression plasmid pPA2895, a DNA fragment containing the *P. aeruginosa* PAO1 PA2895 coding sequence flanked by HindIII and BamHI sites was amplified by the PCR and cloned into pPSV38 [47]. pPA2895 directs IPTG-inducible synthesis of PA2895 and confers resistance to gentamicin. The same process was used to generate expression plasmid pPA2896. To construct pPA2895-NTR, a DNA fragment encoding the first 64 residues of PA2895 flanked by HindIII and BamHI sites was amplified by the PCR and cloned into pPSV38. To make pPA1494, a DNA fragment containing the *P. aeruginosa* PA14 PA1494 coding sequence flanked by XbaI and PstI
was amplified by the PCR and cloned into pHERD20T [48]. pPA1494 directs arabinose-inducible synthesis of PA1494 and confers resistance to ampicillin.

**Microarray experiments.**

Cells of PAO1 pPSV38, PAO1 ΔPA2895 pPSV38, PAO1 ΔPA2895 pPA2895, and PAO1 ΔPA2895 ΔPA2896 pPSV38 were grown with aeration at 37°C in 200 ml LB supplemented with gentamicin (30 μg/ml). Triplicate cultures of each strain were inoculated at a starting OD$_{600}$ of 0.01 and grown to an OD$_{600}$ of ∼0.5 (corresponding to the mid-logarithmic phase of growth). RNA isolation, cDNA synthesis, cDNA fragmentation, and labeling were performed as described previously [49]. Labeled cDNA was hybridized to Affymetrix GeneChip *P. aeruginosa* genome arrays (Affymetrix) and GeneSpring GX was used to analyze data for statistically significant changes in gene expression. The genes with changes in expression ≥2-fold ($P$ value of ≤0.01) are listed in Table 2.1.

**Reporter strain β-galactosidase assays**

Cells were grown at 37°C with aeration in LB supplemented with gentamicin (30 μg/ml). Cells were permeabilized with sodium dodecyl sulfate and CHCl$_3$ and assayed for β-galactosidase activity as described previously [31]. Assays were performed at least twice in biological triplicate. Representative data sets are shown. The values are averages based on one experiment.
Motility assays

Swimming motility and swarming motility assays were performed as previously described [50], [51]. Agar plates for assessing swimming motility and swarming motility consisted of M8 medium supplemented with glucose, MgSO₄, CAA, and 0.3% agar for swim plates or 0.5% agar for swarm plates. 0.1% arabinose was added where indicted. Swim plates were stab inoculated from colonies grown overnight on LB agar plates. Swarm plates were inoculated with 3μl of liquid culture grown overnight in LB. Swim plates and swarm plates were incubated ~20 hours at 37°C. Quantification of swim and swarm zones was performed using ImageJ software. Experiments for testing swimming motility and swarming motility were performed in biological triplicate or quadruplicate on three separate days. The data shown is an aggregate of 3 separate experiments normalized to WT. Subsurface twitching motility was assayed as described previously [52]. Bacteria were stab inoculated through a layer of LB agar (1% agar) to the bottom of the petri dish. After incubation for ~24 hours at 37°C, the twitching motility was examined by removing the agar and staining the attached cells with Coomassie blue (Sigma-Aldrich). Quantification of twitching motility was performed by measuring the maximum diameter in millimeters of the circular zones formed by attached cells. Twitching motility experiments were performed in quadruplicate on two separate occasions. Data shown represent the results of those experiments shown in aggregate and normalized to WT.

Congo red plates

Congo red (CR) plates were used to assess exopolysaccharides production as previously described with slight modifications [36]. Tryptone broth (10 g/l) with salt (5 g/l) solidified with 1% agar was supplemented with Congo red (40 mg/ml) and Coomassie brilliant blue (20 mg/ml). Plates were inoculated from overnight cultures grown in LB
broth and incubated at 37°C for approximately 24 hours. CR plates were further incubated for 1 or 2 days at room temperature to allow for improved color as previously described [11].

**Biofilm formation assay**

Biofilm formation in 96-well microtiter plates was assayed as previously described with slight modifications [53]. Overnight cultures grown in LB were normalized and used to inoculate fresh media to a starting OD$_{600}$ of 0.1. Media was supplemented with carbenicillin (200ug/ml) and 1% arabinose as indicated. 100 μl of each bacterial suspension was dispensed in quadruplicate into the wells of a Costar 96-well polyvinylchloride microtiter plate and incubated for 8 hours at 37°C. Following incubation, plates were washed twice with water and adherent biofilms were stained with 150 μl of 0.1% Crystal violet for 15 minutes. Following staining, plates were washed twice with water and allowed to dry overnight. Stained biofilms were solubilized with 200 μl of 33% acetic acid and absorbance at 595 nm was read with a Tecan Infinite 200 plate reader. Experiments were performed on at least two separate occasions. Representative results are shown.
### Table 2.2: Strains used in this study

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<th><strong>Description</strong></th>
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References


Chapter 3

Evidence for a flagellar brake in *P. aeruginosa*
Attributions

pEXG2-ΔpslD was provided by Joseph Mougous. Keith Turner provided pEXG2-ΔwspF. I performed all experimental work described in this chapter and wrote the text of this chapter, with editorial assistance from Simon Dove.
Introduction

In many bacteria, the transition from motile free-swimming bacteria to a sessile biofilm mode of growth is coordinated by the second messenger c-di-GMP. Flagellar-dependent swimming and swarming motility are often downregulated in response to c-di-GMP, either by inhibiting flagellar gene expression, or by post-translationally controlling flagellar rotation (reviewed in [1])[2]-[5]. c-di-GMP is synthesized by GGDEF domain-containing proteins called diguanylate cyclases (DGCs) and degraded by EAL and HD-GYP domain-containing phosphodiesterases (PDEs) (reviewed in [6]). This second messenger can be sensed by a variety of effectors, notably c-di-GMP-binding PilZ domain-containing proteins. In the Gram-negative bacteria *E. coli* and *Salmonella*, the PilZ domain-containing protein YcgR has been shown to bind c-di-GMP and reduce swimming motility, functioning as a “flagellar brake” by interacting with components of the flagellar basal body to slow flagellar rotation and decrease flagellar reversals, inducing a counter-clockwise rotational bias [3]-[5], [7].

*P. aeruginosa* encodes a YcgR homolog, PA3353, which has been shown to bind c-di-GMP [8], but its role in regulating flagellar motility in response to c-di-GMP is unclear. In a recent study investigating the effect of c-di-GMP on flagellar motility in *P. aeruginosa*, Kulasekara *et al.* concluded PA3353 does not control flagellar rotation after observing ΔPA3353 mutant cells and wild type (WT) cells exhibited similar swimming speeds and rates of flagellar reversal [9]. In *E. coli* and *Salmonella*, the effect of YcgR on flagellar motility can only be observed in strains producing elevated levels of c-di-GMP [7]. In these bacteria, this is achieved by deletion of *yjH*, which encodes a PDE. The Kulasekara study did not test the effect of mutating PA3353 in a background known to produce elevated levels of c-di-GMP, so these results may not be surprising. While PA3353 has not been shown to control flagellar motility in *P. aeruginosa*, there are hints
of YcgR-like activity in *P. aeruginosa* strains that produce elevated levels of c-di-GMP. Specifically, in strains that produce elevated levels of c-di-GMP, swimming motility is reduced or inhibited, and rates of flagella reversals are reduced [10]-[12]. We therefore hypothesized that the *P. aeruginosa* YcgR homolog PA3353 would have a negative effect on flagellar motility in a strain background that produces elevated levels of c-di-GMP.

Here, we present evidence that the *P. aeruginosa* YcgR homolog PA3353 reduces flagellar motility in a *P. aeruginosa* mutant background that has been shown to exhibit increased levels of c-di-GMP. We further show that in this mutant background, the effects of PA3353 on swimming motility are masked by the exopolysaccharide Psl.
Results

PA3353 has no effect on swimming motility in WT or ΔwspF mutant cells

We first tested whether deleting PA3353 in *P. aeruginosa* PAO1 had an effect on swimming motility. As shown in Figure 3.1, WT cells and ΔPA3353 mutant cells were able to swim equally well. This finding is consistent with those of Kulasekara *et al.*, who reported that PA3353 had no effect on swimming motility in strain PAO1 [9]. Given that the effects of YcgR can only be observed in *E. coli* and *Salmonella* Δ*yhjH* mutants that produce high levels of c-di-GMP [7], we reasoned PA3353 would likely influence swimming motility only in cells of *P. aeruginosa* in which the intracellular concentration was elevated. Inactivation of the *wspF* gene has been shown to substantially increase the intracellular concentration of c-di-GMP in *P. aeruginosa*, resulting in the inhibition of swimming and swarming motility [10], [11]. Therefore, to test whether PA3353 influences swimming motility under conditions of high intracellular c-di-GMP, we created a Δ*wspF* single mutant and a Δ*wspF* ΔPA3353 double mutant in the PAO1 strain background. Consistent with previous findings, cells of the Δ*wspF* mutant exhibited a ~70% reduction in swimming motility relative to WT cells (Fig. 3.1). Surprisingly, there was no statistically significant difference in swimming motility between Δ*wspF* cells and Δ*wspF* ΔPA3353 cells (Fig. 3.1), suggesting that PA3353 might not influence flagellar motility.
Figure 3.1. Exopolysaccharides mask the effect of PA3353 on swimming motility in ΔwspF mutant cells of PAO1. Bars represent the relative motility of the indicated strains relative to WT. The relative difference in swimming motility between ΔwspF and ΔwspF ΔPA3353 was not statistically significant. ΔEPS: ΔpslD ΔpelA Δalg8.

**PA3353 controls swimming motility in PAO1 ΔwspF ΔEPS mutant cells**

It was recently discovered that in the cells of a *Salmonella* ΔyhjH mutant, swimming motility is negatively affected by c-di-GMP-stimulated production of the *Salmonella* EPS cellulose, which can partially mask the effects of a *ycgR* deletion in that strain [13]. In *P. aeruginosa*, elevated levels of c-di-GMP in the PAO1 ΔwspF mutant strain have been shown to result in increased EPS production [11], [14]. We therefore hypothesized that the effects of PA3353 on swimming motility in the ΔwspF mutant background might be masked by the dominant negative effects of increased EPS production. To test this hypothesis, we created a ΔwspF mutant strain that was incapable of synthesizing the three EPSs produced by *P. aeruginosa* (Psl, Pel, and alginate). In particular, to create a strain that was incapable of producing Psl, Pel, and alginate (referred to here as ΔEPS), we created a strain that contained deletions of *pslD*,
pelA, and alg8 [8], [11], [15], [16]. Compared to cells of the PAO1 ΔwspF mutant strain, cells of the PAO1 ΔwspF ΔEPS mutant exhibited a ~30% increase (p = 0.018) in swimming motility, suggesting that the production of EPS has a modest negative effect on swimming motility in the ΔwspF mutant strain (Fig. 3.1). When the effect of a ΔPA3353 deletion was tested in the ΔwspF ΔEPS strain background, motility reproducibly increased approximately 1.5-fold (p = 0.002) (Fig. 3.1). This finding suggests that PA3353 reduces swimming motility in cells of the ΔwspF mutant, but this effect is masked by the dominant negative effect of EPS production. Ectopic expression of PA3353 was able to complement the ΔwspF ΔEPS ΔPA3353 mutant strain and restored swimming motility to the same degree as that observed in cells of the ΔwspF ΔEPS mutant (Fig. 3.2). Together, these results demonstrate that PA3353 can negatively regulate flagellar motility in P. aeruginosa under conditions of elevated c-di-GMP.

**Figure 3.2.** Complementation of ΔwspF ΔEPS ΔPA3353 by ectopic expression of PA3353. Bars represent the relative swimming motility of the indicated strains relative to PAO1 ΔwspF ΔEPS transformed with the empty vector pHERD20T (pEV). PAO1 ΔwspF ΔEPS ΔPA3353 pEV cells exhibit increased swimming motility, which can be complemented by supplying PA3353 in trans from the vector pPA3353. Expression of PA3353 from pPA3353 was induced by the addition of 0.1% arabinose.
PsI masks the effect of PA3353 on swimming motility in PAO1 $\Delta wspF$

Next, we were interested in determining which of the three different EPSs was responsible for masking the effect of a PA3353 mutation on swimming motility. Alginate, while clinically important to *P. aeruginosa* biofilms, is not produced in significant amounts or required for biofilm formation in the non-mucoid PAO1 strain [17], [18]. Therefore we reasoned increased production of either Pel, PsI, or both were likely responsible for this masking effect in the $\Delta wspF$ mutant background. To test the contribution of each EPS, we created $\Delta wspF \Delta pelA$ and $\Delta wspF \Delta pslD$ mutant strains and tested the effect of deleting PA3353 in these two mutant backgrounds. Swimming motility in cells of a $\Delta wspF \Delta pelA$ mutant was unaffected by the deletion of PA3353 (Fig. 3.3), suggesting that Pel is not responsible for the dominant negative effect of EPS on swimming motility in the $\Delta wspF \Delta PA3353$ mutant. However, cells of the $\Delta wspF \Delta pslD \Delta PA3353$ mutant exhibited a 2-fold increase in swimming motility relative to $\Delta wspF \Delta pslD$ mutant cells (Fig. 3.3), suggesting that in the $\Delta wspF$ mutant background, the effects on swimming motility of deleting PA3353 are masked by PsI.

![Figure 3.3](image)

**Figure 3.3.** PsI masks the effects of PA3353 on swimming motility in PAO1. Bars represent the relative motility of the $\Delta PA3353$ mutant strains relative to their isogenic parental strains.
Discussion

Here, we have presented evidence that the *P. aeruginosa* YcgR homolog PA3353 can act as a negative regulator of flagellar motility in response to elevated c-di-GMP. Furthermore, we show that PA3353 only appears to function as a negative regulator of swimming motility in cells lacking Psl. Thus, Psl appears to mask the inhibitory effect of PA3353 on swimming motility that can occur when the intracellular concentration of c-di-GMP is elevated.

In agreement with previous studies, we found that cells of a PAO1 ΔPA3353 mutant strain were unaltered with respect to swimming motility when compared to WT PAO1 cells (Fig. 3.1) [9]. We were also unable to observe any effects on swimming motility by deleting PA3353 in a ΔwspF mutant background (Fig. 3.1). However, by inactivating EPS production we were able to observe PA3353-mediated reductions in swimming motility (Fig. 3.1). Finally, we found that the EPS Psl, but not Pel, was responsible for masking the effects of PA3353 in the cells of the ΔwspF mutant (Fig. 3.3). Psl is known to be the primary EPS produced by PAO1 [19] and its inhibitory effect may be the result of cells becoming physically trapped in secreted Psl polysaccharide. However, Psl has also been shown to serve as a secreted signal that stimulates c-di-GMP production by the DGCs SadC and SiaD [20]. Although our findings do not reveal whether Psl masks the inhibitory effect of PA3353 by physically interfering with swimming motility or via its signaling effects, they demonstrate that PA3353 can influence swimming motility in *P. aeruginosa*. Based on the findings that YcgR homologs in other bacteria can function as a c-di-GMP-responsive flagellar brake [3]-[5], we suggest that PA3353 may interfere with swimming motility by functioning as a flagellar brake in *P. aeruginosa*. 
The observation of the masking effects of EPS production on motility may have implications for the interpretation of results obtained by a recently published study that examined the function of the PA3353 ortholog FlgZ in *Pseudomonas fluorescens*. In *P. fluorescens*, ectopic expression of FlgZ resulted in reduced swimming motility, however FlgZ’s effect on swimming motility was not found to be dependent upon levels of intracellular c-di-GMP [21]. Specifically, the authors of this study reported that deletion of *flgZ* had no effect on swimming motility in a WT background, nor did it influence motility when intracellular concentrations of c-di-GMP were increased by the deletion of *bifA*, a PDE [21]. This led the authors to conclude that c-di-GMP inhibits swimming motility independently of FlgZ in *P. fluorescens* [21]. The results presented in this Chapter suggest the effects of increased EPS production in response to elevated c-di-GMP in *Pseudomonas* species are likely to mask the effects of YcgR homologs on swimming motility and may explain why FlgZ did not appear to control flagellar motility in response to c-di-GMP. Therefore, the inhibitory effects of exopolysaccharides on motility should be considered in future studies of c-di-GMP-mediated control of bacterial motility.
Materials and methods

Bacterial strains

_E. coli_ DH5α F′IQ (Invitrogen) was used as the recipient strain for all plasmid constructions. _E. coli_ SM10 λpir was used to mate plasmids into _P. aeruginosa_. _P. aeruginosa_ strains PAO1 was provided by A. Rietsch. Bacterial cultures were routinely grown at 37°C in lysogeny broth (LB), or on plates containing LB solidified with 1.5% agar unless otherwise noted. When appropriate, gentamicin (30 μg/ml) and carbenicillin (200 μg/ml) were used for selection in _P. aeruginosa_ cultures.

Construction of deletion mutant strains

The deletion construct for the PA3353 gene was generated by amplifying regions ~ 700 bp in length that flank PA3353 in the PAO1 genome by PCR and then splicing the flanking regions together by overlap extension PCR. The deletion was in-frame and contained a 9-bp NotI-linker sequence 5′- GCGGCCGCC-3′ separating the two flanking regions. The resulting PCR product was cloned on a BamHI/HindIII fragment into plasmid pEXG2 [22], yielding plasmid pEXG2-ΔPA3353. The pEXG2-ΔpelA and pEXG2-Δalg8 plasmids were created in identical fashion. pEXG2-ΔpslD was shared by J. Mougous and pEXG2-ΔwspF was shared by K. Turner. _E. coli_ SM10 were transformed with the pEXG2-based deletion constructs and mated with PAO1 recipient strains. Allelic replacement was performed as previously described to create unmarked deletion mutant strains [23]. Deletions were confirmed by PCR.
**Construction of the PA3353 expression plasmid**

To make pPA3353, a DNA fragment containing the *P. aeruginosa* PAO1 PA3353 coding sequence flanked by EcoR and HindIII was amplified by the PCR and cloned into pHERD20T [24]. pPA3353 directs arabinose-inducible synthesis of PA3353 and confers resistance to ampicillin.

**Motility assays**

Swimming motility assays were performed as previously described [25]. Agar plates for assessing swimming motility consisted of M8 medium supplemented with glucose, MgSO₄, CAA, and 0.3% agar. 0.1% arabinose was added where indicted. Swim plates were stab inoculated from colonies grown overnight on LB agar plates. Swim plates were incubated ~20 hours at 37°C and the diameter of the swim zone was measured and normalized as described in figure legends. Each experiment was performed in biological triplicate on at 2 separate occasions. Representative data is shown.
Table 3.1: Strains used in this study

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References


Chapter 4

Discussion
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Overview

*P. aeruginosa* is the primary cause of morbidity and mortality in cystic fibrosis (CF) patients and is a leading cause of nosocomial infections. *P. aeruginosa* is thought to grow as a biofilm in the CF lung and the growth of *P. aeruginosa* biofilms on indwelling medical devices is a significant factor in nosocomial infections. In most bacteria, including *P. aeruginosa*, biofilm formation and swarming motility are reciprocally regulated. In Chapter 2, I described the results of a study that identified PA2896 and PA2895 as a σ and anti-σ factor pair with previously undescribed regulatory effects on swarming motility and biofilm formation in *P. aeruginosa*. PA2896 and PA2895 were found to control the expression of PA1494, which we identified as a novel regulator of swarming motility and biofilm formation. In addition, the results presented in Chapter 3 provide evidence for a c-di-GMP-responsive regulator of flagellar motility in *P. aeruginosa*.

A σ factor and anti-σ factor that control swarming motility and biofilm formation in *P. aeruginosa*

**Summary of results**

In Chapter 2, I described the characterization of the extracytoplasmic function (ECF) σ factor PA2896 and its cognate anti-σ factor PA2895. We presented evidence that PA2896 interacts with RNAP and that PA2895 directly interacts with PA2896 and inhibits PA2896-dependent gene expression. We reported the results of DNA microarrays that suggested the PA2896 regulon likely comprises the putative PA2896-PA2895 operon, PA1494, and PA4495, and found expression from the promoters of these genes is positively controlled by the ECF σ factor PA2896 and negatively controlled by the anti-σ factor PA2895. We presented evidence that increased
expression of the PA2896 regulon in ΔPA2895 mutants cells leads to the inhibition of swarming motility and enhanced biofilm formation in \textit{P. aeruginosa} PA14, and showed that these effects are the result of increased expression of PA1494. Taken together, these results describe a previously uncharacterized ECF σ factor/anti-σ factor system that controls the expression of PA1494, a novel regulator of swarming motility and biofilm formation in \textit{P. aeruginosa}. I will address some of the unanswered questions about the system below.

\textit{How does PA1494 control swarming motility and biofilm formation?}

The mechanism by which PA1494 inhibits swarming motility and enhances biofilm formation remains undetermined. There are several previously described \textit{P. aeruginosa} signaling pathways that are capable of inhibiting swarming motility and enhancing biofilm formation, raising the possibility that PA1494 might exert its effects on these behaviors by influencing one of these pathways, either directly or indirectly. These pathways include the GacS/GacA two-component system, c-di-GMP signaling, and the PQS signaling system.

In the GacS/GacA two-component system, the GacS sensor kinase located in the cytoplasmic membrane phosphorylates the GacA response regulator upon stimulation, eventually leading to inhibition of swarming motility and enhanced biofilm formation \cite{1}, \cite{2}. PA1494 also inhibits swarming motility and enhances biofilm, suggesting PA1494 might control these behaviors by activating the GacS/GacA two-component system. PA1494 is predicted to be secreted to the periplasm and has been identified in periplasmic fractions \cite{3}. It is therefore possible to imagine periplasmic PA1494 stimulating GacS, which has the downstream effect of inhibiting swarming motility and enhancing biofilm formation. Activation of the Gac system results in
increased expression of the small RNAs RsmY and RsmZ, which modulate the activity of the RNA binding protein RsmA [1], [4]. Although RsmA is thought to function primarily through its effects on the translation of target mRNAs, translation can govern mRNA stability and the Gac system (through RsmA) has been shown to influence the abundance of many target transcripts using DNA microarrays [1], [5]. Interestingly, expression of PA1494 and PA4495 increases in a mutant background where the Gac system is activated [1]. However, we did not observe changes in the expression of any additional Gac-controlled genes in the ΔPA2895 mutant strain, which highly expresses PA1494. This suggests the GacS/GacA system might not be involved in PA1494-mediated control over swarming motility and biofilm formation. However, it would be possible to specifically test whether PA1494 exerts its regulatory effects via the Gac system by monitoring the expression of RsmY and RsmZ in response to ectopic expression of PA1494. If PA1494 activates the Gac system, ectopic expression of PA1494 would be expected to result in increased RsmY and RsmZ expression. Alternatively, ectopic expression of PA1494 in ΔgacA mutants, which exhibit hyperswarming and reduced biofilm formation [4], [6], could be used to test for the involvement of the Gac system in mediating PA1494’s effect on swarming motility and biofilm formation. If the Gac system were involved, we would expect that ectopic expression of PA1494 would no longer result in the inhibition of swarming motility and biofilm formation in the ΔgacA mutant background.

The second messenger c-di-GMP has also been shown to control swarming motility and biofilm formation in *P. aeruginosa*. In cells with elevated intracellular concentrations of c-di-GMP, swarming is inhibited and biofilm formation is enhanced [7]-[10]. Expression of PA1494 has the same inhibitory effect on swarming motility and enhanced biofilm formation as elevated levels of c-di-GMP, suggesting PA1494 might
exert its regulatory effects by increasing intracellular c-di-GMP. Regulation of these behaviors in *P. aeruginosa* by c-di-GMP can occur without significant changes in the expression of motility and biofilm related genes [9]-[11], consistent with our microarray results. It is therefore possible that PA1494 exerts its effects on swarming motility and biofilm formation via the second messenger c-di-GMP. PA1494 is not predicted to contain a GGDEF domain and is therefore unlikely to synthesize c-di-GMP itself. However, it may control other proteins that are capable of increasing intracellular concentrations of c-di-GMP. For example, periplasmic PA1494 might stimulate either the *Wsp* chemosensory system or the diguanylate cyclase SadC, both of which are found at the cytoplasmic membrane and positively influence c-di-GMP levels [8], [12]. The most direct way to determine if expression of PA1494 results in elevated c-di-GMP levels is to directly measure c-di-GMP levels in strains expressing PA1494. This can be done using thin layer chromatography (TLC) or liquid chromatography-mass spectrometry (LC-MS). One way to test whether PA1494’s effects on swarming motility and biofilm formation requires c-di-GMP is to ectopically express a c-di-GMP-degrading phosphodiesterase (PDE) in ∆PA2895 mutant cells. In cells that ectopically express PDEs, swarming motility is enhanced and biofilm formation is reduced [7], [9]. If PA1494 inhibits swarming motility and enhances biofilm formation by indirectly increasing intracellular c-di-GMP, we would predict ectopic expression of a PDE to reduce or eliminate the effects of PA1494 on swarming motility and biofilm formation.

The *P. aeruginosa* quorum signaling molecule 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS) and its precursor 2-heptyl-4-quinolone (HHQ) have also been shown to inhibit swarming motility and promote biofilm formation in *P. aeruginosa* [13]-[15]. Specifically, the addition of exogenous PQS to *P. aeruginosa* cultures has been shown to inhibit swarming motility and promote biofilm formation [13], [15], and HHQ has been
shown to mediate the suppression of swarming motility when cells are grown on arginine as a nitrogen source [14]. Expression of PA1494 also results in inhibition of swarming motility and enhanced biofilm formation, suggesting PA1494 may exert its effects via the PQS signaling pathway. The mechanism by which HHQ and PQS control swarming motility is not clear, however they are thought to promote biofilm formation by stimulating autolysis, which generates extracellular DNA (eDNA) that contributes to the biofilm matrix, and by increasing the expression of the lectin LecA, which is thought to promote biofilm development by cross-linking matrix polysaccharides (reviewed in [16])[13]. In addition, PQS signaling positively controls the expression of the phenazine biosynthetic genes, which results in increased production of the blue pigment pyocyanin [13]. We did not observe increased pyocyanin production in cultures of ΔPA2895 mutant strains or in strains ectopically expressing PA1494 (data not shown), which suggests that PQS signaling is not increased in these cells. We also did not detect changes in the expression of PQS-controlled genes in the ΔPA2895 mutant by microarray. If PA1494 stimulated PQS signaling, we might expect to see increased expression of genes such as lecA and the phenazine biosynthesis genes. However, we performed our microarrays using cells in the exponential phase of growth, while PQS signaling does not become active until early stationary phase [13]. Therefore it is possible we would have missed any changes in gene expression that result from PQS signaling in our DNA microarrays. Nevertheless, whether PA1494 stimulates or enhances PQS signaling could be more carefully tested by measuring the effect of PA1494 expression on several of the known endpoints of PQS mediated signaling, such as pyocyanin production, eDNA release, or the expression of lecA and the phenazine biosynthetic genes, at multiple points in the growth curve.
Alternatively, we could use an unbiased genetic screen rather than taking a candidate approach to determine the mechanism by which PA1494 controls swarming motility and biofilm formation. For example, a suppressor screen or transposon screen could identify mutants that regain the ability to swarm in cells ectopically expressing PA1494. Or a transposon screen could be used to identify mutants that no longer exhibit increased biofilm formation when PA1494 is ectopically expressed.

It would also be interesting to determine if PA1494 and PA4495 function together in any capacity to control swarming motility or biofilm formation. PA1494 and PA4495 are coregulated by PA2896, are predicted to be exported to the periplasm, and have both been identified experimentally in periplasmic fractions [3]. The role of PA4495 in this system has not been explored, in part because PA1494 was found to be sufficient to explain the phenotypes we observed in ΔPA2895 mutant cells. However, the creation of a ΔPA2895 ΔPA4495 mutant strain would allow for PA4495’s contribution to swarming inhibition and biofilm enhancement to be tested.

**Bioinformatic analysis of PA1494 and PA4495**

Bioinformatic analyses do not offer any obvious clues as to the function of PA1494. PA1494 encodes a hypothetical 556 amino acid protein with no homology to previously characterized proteins. The protein is predicted to contain an N-terminal signal peptide and to localize to the outer membrane. PA1494 has been provisionally named *muiA*, for *mu*co*dy i*nh*ibitor A*, after the discovery that ectopic expression of PA1494 inhibited alginate production in certain mucoid *P. aeruginosa* strains via an unknown mechanism [17]. While PA1494 is highly conserved amongst *P. aeruginosa* strains, it appears to be absent from almost all other sequenced *Pseudomonas* species. Homologs of PA1494 are present in several bacterial phyla, including proteobacteria,
cyanobacteria, and actinobacteria, as well as in nematodes. In particular, a PA1494 homolog appears to be well conserved in *Bordetella holmesii*, an opportunistic pathogen that can cause septicemia, endocarditis, and respiratory infections in immunocompromised patients [18].

PA4495 is predicted to encode a 236 amino acid protein with no homology to characterized proteins. It is predicted to secreted to the periplasm and has been experimentally identified in periplasmic fractions [3]. Pfam domain analysis (http://pfam.xfam.org/) identifies PA4495 as a possible member of the SIMPL (signaling molecule that associates with the mouse pelle-like kinase) family of proteins. SIMPL domain proteins have been identified in bacteria, mice, rats, and humans. In mammals, SIMPL regulates the activity of NF-κB, a key regulator of the immune system [19]. Structural analysis performed with the Phyre2 web server predicts the structure of PA4495 is similar to the crystal structure of the *Brucella* SIMPL family protein BP26 with 100% confidence [20]. While the function of BP26 is unknown, the protein is a major antigen produced by *Brucella* during infection and is widely used as a diagnostic marker and for vaccination against Brucellosis in animals [21].

**A possible role in virulence**

Our initial interest in studying the PA2896/PA2895 σ factor/anti-σ factor system stemmed from the results of a transposon screen that identified PA2895 as being required for persistence in a rat lung model of chronic respiratory infection [22]. This study attributed the defect in persistence of the PA2895 mutant to a protease secretion defect. However, our PAO1 ΔPA2895 and PA14 ΔPA2895 mutant strains did not exhibit a defect in protease secretion (Appendix I, Figure A.2). The work presented here suggests an alternative explanation for the requirement of PA2895 during respiratory
infection. Specifically, inhibition of swarming motility in ΔPA2895 mutant cells prevents them from persisting in this chronic model of infection. Interestingly, 20/36 (55.6%) of the transposon mutants harboring insertions in novel virulence genes identified by the screen that identified PA2895 exhibited swarming motility defects [22]. This suggests that in this infection model, swarming motility may be a crucial virulence determinant. Our findings raise the possibility that the defect in persistence exhibited by the PA2895 transposon mutant during respiratory infection in the original study may be attributable to a defect in swarming motility mediated by PA1494.

While the results of the study described above suggest inactivation of PA2895 and resulting activation of PA2896 and its regulon may be detrimental to virulence, there is evidence to suggest that the system may also contribute to virulence. In addition to respiratory infections, *P. aeruginosa* is capable of mucosal colonization and systemic dissemination in patients with neutropenia [23]. A recent study utilizing a mouse infection model that mimics the course of human infection in patients with cancer or patients who have undergone bone marrow transplantation identified PA2896 as being required for colonization of the murine gastrointestinal (GI) tract [23]. This study also found biofilm related genes from the pel, psl, cupA, and cupB gene clusters were required for colonization, raising the possibility that the ability of *P. aeruginosa* to form a biofilm is important for its ability to colonize the GI tract [23]. Our data suggest that the colonization defect exhibited by PA2896 transposon mutants in this study may be related to PA2896-dependent effects on biofilm formation.

Additional evidence suggests PA1494 and PA4495 may be expressed in the CF lung. Strikingly, sera from CF patients contain PA1494-reactive antibodies, indicating PA1494 is expressed and is immunogenic in CF patients [24]. Recently, the *P. aeruginosa* Australian epidemic strain 1-R (AES-1R) was found to secrete elevated
levels of PA4495 when grown in artificial sputum medium, a model of CF sputum [21], [25]. The finding that PA4495 is secreted is particularly interesting given its similarity to the potent *Brucella* antigen BP26 and that it contains a domain that is known to effect the immune system by modulating NF-κB signaling (SIMPL domain). In light of these observations, it would be interesting to test whether PA1494 or PA4495 have an effect on *P. aeruginosa* virulence. Moreover, the prediction that PA1494 localizes to the outer membrane and evidence of secreted PA4495 in AES-1R raises the exciting possibility that PA1494 may even secrete PA4495. Future experimental work could explore this possibility more fully by testing whether PA4495 is secreted, and if so, whether secretion is PA1494-dependent. Thus the genes of the PA2896 regulon represent an interesting subject for future studies to investigate.

*What is the PA2896 activation signal?*

Identifying the physical stimuli or genetic changes that induce expression of a σ factor regulon can be used to infer the possible functions of genes within that regulon [26]. With regard to PA2896, there is evidence that the system can be induced by an undefined cell envelope stress signal. A DNA microarray study investigating the effects of osmotic shock on the *P. aeruginosa* transcriptome found PA2896 was upregulated 7.5-fold within 15 minutes of exposure to osmotic shock [27]. Another study reported that treatment of cells with the cell wall-inhibitory antibiotic D-cycloserine resulted in upregulation of PA2895 (2-fold), PA2896 (5-fold), PA1494 (16-fold), and PA4495 (6-fold) [28]. Yet another possible connection to cell wall stress was suggested by a study that found PA1494 was upregulated 19-fold in response to depletion of GlmU, an essential enzyme required for the biosynthesis of N-acetylglucosamine, a major component of peptidoglycan [29]. Finally, a recent study by Seo and Darwin reported that
overexpression of the periplasmic protease CtpA induces expression of PA2896, PA1494, and PA4495 [30]. Interestingly, ΔctpA mutants exhibited separation of their outer membrane and inner membranes [30], suggesting CtpA may be involved in maintaining the integrity of the cell envelope. Seo and Darwin also reported that increased expression of PA2896, PA1494, and PA4495 in response to D-cycloserine treatment was not CtpA-dependent, suggesting CtpA and D-cycloserine activate the system via independent mechanisms, that CtpA may activate the system by generating a cell envelope stress signal similar to that produced by D-cycloserine treatment, or that CtpA directly activates PA2896, possibly by degrading PA2895 directly [30].

It is possible that PA2896 is activated in response to a specific cell wall stress signal, or it may be part of a general stress response. To test if PA2896 is activated in response to a specific perturbation of the cell wall, compounds known to effect different stages of peptidoglycan biogenesis could be tested for their ability to activate PA2896. By also testing antimicrobials that do not specifically target the cell wall for their ability to activate PA2896, we could discern whether PA2896 is activated by a cell wall stress signal, or whether the system responds generally to stress. Alternatively, a transposon screen could be used to identify genes that control activation of PA2896. We would expect to recover insertions in PA2895 from such a screen, however any additional hits could provide insight into the signal that this system responds to and may suggest possible functions for PA1494 and PA4495.
Evidence for a flagellar brake in *P. aeruginosa*

Summary of results

In Chapter 3, I presented evidence that the YcgR homolog PA3353 reduces swimming motility in a strain background in which the intracellular concentration of c-di-GMP is known to be elevated compared to wild type.

Future directions

The molecular mechanism by which YcgR controls flagellar function is unclear and two possible models have been suggested to explain how it functions. One model has been proposed in which YcgR interacts with the rotor proteins FliG and FliM to control flagellar rotation [31], [32], while an alternative model involves an interaction between YcgR and the MotA stator protein [33]. The FliG/FliM-YcgR model is supported by observation that YcgR copurifies with FliG and FliM [31], [32]. In addition, evidence of an interaction between YcgR and FliG was observed using a bacterial two-hybrid system [32]. The YcgR-MotA interaction model is based on the observation that suppressor mutations in MotA allow cells to escape YcgR-mediated inhibition of swimming motility [33]. The suppressor mutations in MotA were found in residues that participate in electrostatic interactions with FliG and appear to generally increase flagellar motility overall [33], suggesting these MotA suppressor mutants produce stronger motors that are able to overcome the effects of YcgR.

*P. aeruginosa* may provide the opportunity to test the hypothesis that YcgR functions by interacting with the stator proteins. *P. aeruginosa* encodes two flagellar stator complexes, MotAB and MotCD [34], [35]. Interestingly, it was recently reported that c-di-GMP-mediated inhibition of swarming motility in *P. aeruginosa* is dependent upon the MotAB stator complex, which is dispensable for swarming motility [36].
Sequence alignment of the homologous stator proteins MotA and MotC reveals a 17 amino acid region present in MotA, but absent in MotC. Intriguingly, this 17 amino acid “MotA-domain” is predicted to be on a cytoplasmic loop, where it could interact with YcgR/PA3353. Moreover, this MotA-domain is present in MotA homologs from species that also encode YcgR homologs, but largely absent from those that do not. I propose that this MotA-domain may constitute a potential YcgR/PA3353 interaction domain on the MotA stator. If PA3353 is also found to inhibit swarming motility in response to c-di-GMP in *P. aeruginosa*, the presence of the MotA-domain in MotA and its absence in MotC may explain why MotA is required for c-di-GMP-mediated inhibition of swarming motility, but MotC is not. This hypothesis could be tested by deletion of the MotA-domain from MotA, which should relieve c-di-GMP-mediated inhibition of swarming motility if this hypothesis is correct. Additionally, inserting the MotA-domain in-frame into the corresponding location in MotC should restore c-di-GMP-mediated swarming inhibition in ΔmotAB mutant cells, which would otherwise not be subject to swarming inhibition by c-di-GMP. The interaction between the YcgR homolog PA3353 and the MotA-domain could be further explored using a bacterial two-hybrid approach. Given the conservation of the MotA domain in cells that also encode YcgR homologs, the results of these experiments could have general implications for the mechanism by which YcgR functions to control flagellar rotation.
Concluding remarks

The work described in this thesis has implications for the study of \textit{P. aeruginosa} motility and biofilm formation. It will be interesting to determine how PA1494 controls swarming motility and enhances biofilm formation, what the signal is that activates PA2896, and to identify the physiological functions of PA1494 and PA4495 in the cell. Additionally, several lines of evidence suggest the PA2896/PA2895 $\sigma$/anti-$\sigma$ factor system may also be important during infection, and it will be interesting to learn whether they effect virulence. It will also be interesting to test the specific model described above for how PA3353 influences flagellar motility, as the results of those experiments might have implications for the function of YcgR homologs in general.
References


Appendix I – Supplementary figures and tables

Figure A.1. Weblogo of the putative PA2896 promoter consensus sequence created by aligning the sequence upstream of the putative PA2896, PA1494, and PA4495 transcription start sites.
Table I.1. Top 10 results from FIMO search for occurrences of the putative PA2896 promoter motif in the PAO1 genome. The putative -35 element, -10 element, and transcription start site have been bolded in the putative promoter sequences of PA4495, PA2896, and PA1494. An “X” in the “Upstream?” column indicates the matched sequence was located upstream of a gene on the chromosome. An “X” in the “In coding?” column indicates the matched sequence was located in the coding sequence of a gene.

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<th>Upstream?</th>
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<th>Score</th>
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Figure A.2. Milk agar secreted protease assay. 5 μl of overnight liquid culture was inoculated on T-broth agar plates containing 1% milk. Digestion of the milk protein casein results in clearing of the agar, indicating the production of secreted proteases. Top row: WT PAO1 and PAO1 \(\Delta \text{PA2895}\). Bottom row: WT PA14 and PA14 \(\Delta \text{PA2895}\). Scale bars represent 1 cm.