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Biofilm-Mediated Regulation of Siderophore Production in *Pseudomonas aeruginosa*

by

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ABSTRACT

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Pseudomonas aeruginosa is a gram-negative, multidrug-resistant, nosocomial pathogen that threatens the lives of immunocompromised patients. A key virulence factor in this pathogen is the siderophore pyoverdine. Due to its extremely high affinity for ferric iron, pyoverdine gives the pathogen a significant advantage over the host in their competition for iron. In addition, pyoverdine can regulate the production of multiple bacterial virulence factors and perturb host mitochondrial homeostasis. To elucidate the regulation of pyoverdine production, we developed a high-throughput genetic screen to identify genes necessary for its biosynthesis. Through this screen, we demonstrated that biofilm formation is necessary for pyoverdine production. Consistent with this result, upstream regulators of biofilm, notably intracellular c-di-GMP, regulated pyoverdine production in a biofilm-dependent manner. Furthermore, inhibiting biofilm formation using 2-amino-5,6-dimethylbenzimidazole was sufficient to attenuate pyoverdine production, suggesting that biofilm inhibitors may have multiple therapeutic benefits in addition to disrupting the pathogen's main mechanism of antimicrobial resistance.

Acknowledgements

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Chapter 1

Introduction

This chapter was adapted from (Kang and Kirienko, 2018) and (Kang et al., 2017).

Antibiotic resistance is a catastrophic, re-emerging threat to health care. Gram-negative, multidrug-resistant bacteria, including *Pseudomonas aeruginosa*, are one of the leading causes of nosocomial infections in intensive care units (Peleg and Hooper, 2010). Antimicrobial resistant infections are associated with pronounced increases in morbidity and mortality, along with drastically increasing healthcare costs (Brusselaers et al., 2011; Peleg and Hooper, 2010). Beyond expressing a wide-variety of resistance genes acquired via horizontal gene transfer, *P. aeruginosa* utilizes two methods to defend against antimicrobial agents. First, during chronic infections, bacteria form dense biofilm structures on the surface of host tissue and at interfaces with medical implants. Bacteria embedded in these structures are virtually impervious to antibiotics and often evade recognition by the host's innate immune system (Anderson and O'Toole, 2008; Mah and O'Toole, 2001). Second, the bacterium expresses multi-drug efflux pumps that efficiently reduce intracellular concentration of harmful toxic molecules (Lomovskaya et al., 2001).

These innate defense mechanisms complicate treating chronic *P. aeruginosa* infections. For example, over half of patients with cystic fibrosis suffer from chronic colonization, and infection is estimated to shorten their life expectancy by nearly ten years (C.F. Foundation, 2007; Li et al., 2005). In addition, the prevalence of *P. aeruginosa* in nosocomial infections is rising, making it imperative that we search for new therapeutic approaches to support the dwindling identification of new antimicrobials.

One promising alternative approach for mitigating *P. aeruginosa* infections is to identify mechanisms to limit its virulence, rather than its growth. This target is complicated however, as mechanisms underlying pathogenesis vary widely, depending on infection conditions and environmental factors. A short, and incomplete, list of virulence factors encoded by *P. aeruginosa* includes cyanide- and phenazine-based toxins (Cezairliyan et al., 2013; Mahajan-Miklos et al., 1999), type three secretion effectors (Diaz and Hauser, 2010; Hauser, 2009), phospholipases (Jiang et al., 2014), and other exotoxins (McEwan et al., 2012). Interestingly, Visca and colleagues recently reported that the pathogen's iron uptake systems also play a crucial role during infection (Minandri et al., 2016). For instance, *P. aeruginosa* mutants impaired in various iron uptake functions (i.e: siderophores pyoverdine and pyochelin biosynthesis, heme uptake, ferrous iron transport) exhibited attenuated virulence in a murine pneumonia model (Minandri et al., 2016), demonstrating the utility of targeting these systems during infection.

1.1. Nutritional Immunity: Host Inhibition of Pathogen Iron Acquisition

Disrupting pathogen iron uptake attenuates virulence because iron is a scarce nutrient within the host. During infection, hosts and pathogens compete for several bioavailable transition metals (including iron, copper, zinc, manganese, and molybdenum) (Hood and Skaar, 2012). These metals are required for fundamental cellular processes such as gene transcription and various redox-reactions. Hosts have evolved mechanisms to sequester these metals, restricting them from bacteria. This phenomenon is colloquially referred to as "nutritional immunity" (Hood and Skaar, 2012). Host cells withhold intracellular iron using iron-storage proteins such as ferritin or iron-containing complexes like heme, and restrict extracellular iron availability by secreting iron-sequestering proteins such as transferrin and lactoferrin (Kelson et al., 2013; Skaar, 2010) (**Figure 1.1**). Transferrin and lactoferrin have been shown to exhibit bacteriostatic or even bactericidal activity against a number of gram-negative and gram-positive bacteria, including *Staphylococcus aureus* and *Escherichia coli* (Aguila et al., 2001; Arnold et al., 1982; Rainard, 1986; Spik et al., 1978).

Interestingly, transferrin and lactoferrin also inhibit bacterial biofilm formation. In a seminal study, Singh and colleagues demonstrated that *P. aeruginosa* iron deprivation severely disrupts the bacteria's ability to form biofilms (Singh et al., 2002). Based on these observations, a number of studies tested the impact of synthetic iron chelators (such as deferasirox, ethylenediaminetetraacetic acid, ethylenediamine-*N*,*N*-bis(2-hydroxyphenylacetic acid), and others) on *P. aeruginosa* biofilm formation (Banin et al.,

2006; Kang and Kirienko, 2017; Moreau-Marquis et al., 2009; O'May et al., 2009). In each case, these compounds disrupted the formation of biofilms, indicating that iron acquisition is necessary for biofilm formation. Transferrin and lactoferrin function similarly to chemical iron chelators; by restricting environmental iron, bacterial biofilm formation is compromised (**Figure 1.1**). For example, apo-lactoferrin significantly attenuates biofilm formation in *P. aeruginosa* (Banin et al., 2005; Kamiya et al., 2012; Singh et al., 2002). The removal of apo-lactoferrin (or replacing it with iron-saturated lactoferrin) permits re-establishment of biofilm formation and bacterial adhesion in these systems.

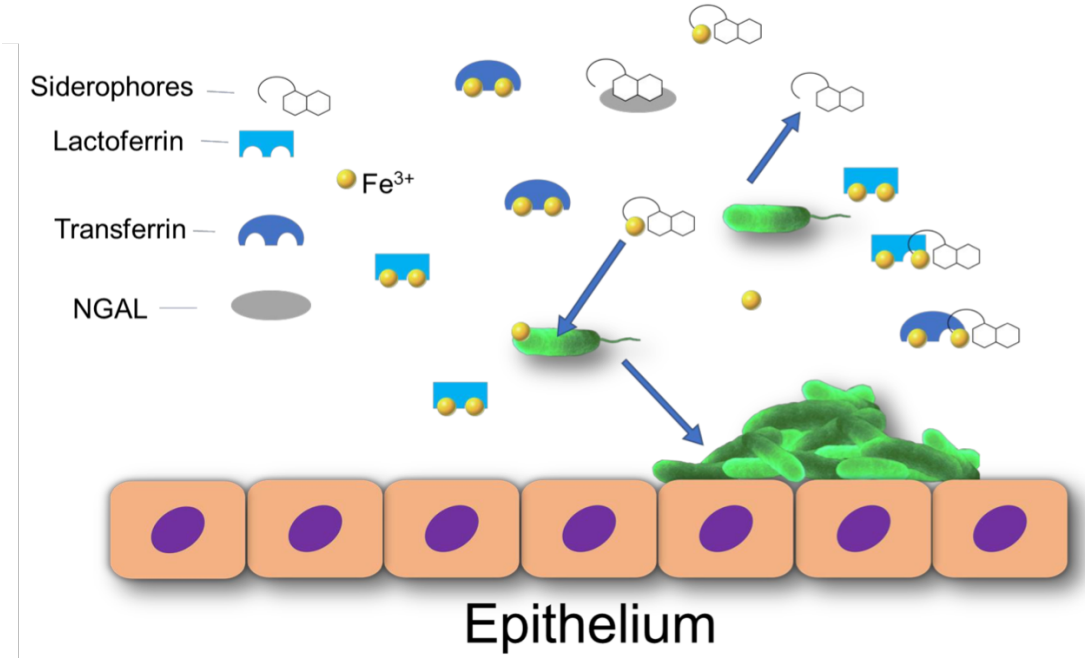

Figure 1.1. Hosts and pathogens compete to sequester iron from the environment. Host cells secrete iron-sequestering proteins such as transferrin and lactoferrin to minimize free extracellular iron. Some bacterial pathogens secrete siderophores to compete against these proteins and scavenge trace amounts of free iron. Certain siderophores, such as enterobactin (from *E. coli* and *S. typhimurium*) and pyoverdine (from *P. aeruginosa*), can directly remove ferric iron from iron-bound transferrin and lactoferrin, increasing pathogen iron uptake and promoting biofilm formation. To interfere with siderophore activity, certain host cells secrete lipocalin-2 (also known as NGAL) to recognize and bind to siderophores, preventing their function. Production of siderophores that can evade lipocalin-2 has also been linked to pathogenicity. Figure adapted was from (Kang and Kirienko, 2018).

1.2. Siderophores: Pathogen Evasion of Host Iron Restriction

In response to host iron-sequestering mechanisms, pathogens attempt to overcome iron limitation in at least three ways. First, some pathogens express receptors for lactoferrin or transferrin, in a bid to acquire the proteins and their associated iron (Beddek and Schryvers, 2010; Pogoutse and Moraes, 2017). Second, many human pathogens have heme acquisition pathways comprised of heme-binding receptors and/or even heme-binding molecules called hemophores (Cescau et al., 2007; Huang and Wilks, 2017). Finally, and most commonly, most pathogenic and many non-pathogenic species of bacteria (and fungi) produce small molecule iron chelators called siderophores. These molecules evolved to improve the aqueous solubility of iron (III). To facilitate their biological role, these molecules have exceptionally high affinities to ferric iron. This also helps them overcome host iron restriction mechanisms by directly chelating ferric iron from host iron-sequestering proteins (Skaar, 2010) (**Figure 1.1**). For instance, both enterobactin (a high-affinity siderophore produced by a variety of Enterobacteriaceae, including *E. coli* and *S. typhimurium*) and pyoverdine (produced by *P. aeruginosa*) can acquire iron from human iron storage proteins such as transferrin or ferritin (Carrano and Raymond, 1979; Guterman et al., 1978; Harris et al., 1979; Kvach et al., 1977; Meyer et al., 1996; Tidmarsh et al., 1983; Wolz et al., 1994; Xiao and Kisaalita, 1997).

To inhibit siderophore activity, mammalian hosts secrete the siderophore binding protein lipocalin-2 (also known as neutrophil gelatinase-associated lipocalin, or NGAL, to differentiate it from lipocalin-1, which is derived from tears) to recognize and bind to siderophores such as enterobactin (**Figure 1.1**) (Flo et al., 2004; Goetz et al., 2002). Lipocalin-2 is critical for innate immunity, as lipocalin-2-deficient mice exhibit increased

bacteremia and sepsis during infection with *E. coli* (Berger et al., 2006; Flo et al., 2004). *In vitro*, when bacteria are grown in iron-limited media, lipocalin-2 treatment has a growth-inhibitory and antivirulent effect, which is mitigated by the supplementation of enterobactin or ferrichrome (as a source of iron), suggesting that lipocalin-2 rescues hosts by depriving the pathogen of iron (Flo et al., 2004). However, while lipocalin-2 production has been shown to be an effective host immune response against some pathogens, others (including *Klebsiella pneumoniae*, *Salmonella enterica*, and *P. aeruginosa*) have evolved mechanisms to circumvent this defense. For example, lipocalin-2 does not efficiently bind pyoverdine (Peek et al., 2012), while *K. pneumoniae*, *E. coli*, *and S. enterica* can evade lipocalin-2 by secreting a glycosylated version of enterobactin known as salmochelin (Fischbach et al., 2006).

1.3. Siderophore Pyoverdine as a Virulence Factor

In addition to facilitating pathogen iron uptake within host (and thus promoting biofilm formation), pyoverdine has additional functions in *P. aeruginosa* virulence. First, iron-bound pyoverdine (known as ferripyoverdine) functions as a signaling molecule that triggers the release of the alternate σ factor PvdS from sequestration by the intermembrane FpvA/FpvR complex (Beare et al., 2003). Once released, PvdS promotes the expression of at least two secreted toxins (the translational inhibitor ToxA and the protease PrpL) and also its own biosynthetic machinery (Lamont et al., 2002; Ochsner et al., 1996; Wilderman et al., 2001). Second, we recently demonstrated pyoverdine can directly translocate into *Caenorhabditis elegans* host tissue, disrupting host iron and mitochondrial homeostasis in the absence of live pathogen (Kang et al., 2018; Kirienko et al., 2015) (**Figure 1.2**). Exposing *C. elegans* to *P. aeruginosa* thus induces a distinct host

response that resembles that of acute iron removal (by synthetic chelator 1,10-phenanthroline) and ultimately causes a lethal hypoxic response and host death (Kang et al., 2018; Kirienko et al., 2013; Tjahjono and Kirienko, 2017). Due to a combination of these functions, pyoverdine is essential for *P. aeruginosa* pathogenesis in various mammalian and invertebrate host models (Imperi et al., 2013; Kirienko et al., 2013; Lopez-Medina et al., 2015; Meyer et al., 1996; Minandri et al., 2016; Takase et al., 2000). Furthermore, pyoverdine biosynthetic inhibitors (i.e: fluorocytosine, fluorouridine) (Imperi et al., 2013; Kirienko et al., 2016) or novel functional inhibitors (Kirienko et al., 2019) have been shown to effectively limit *P. aeruginosa* pathogenesis, validating pyoverdine as a promising therapeutic target.

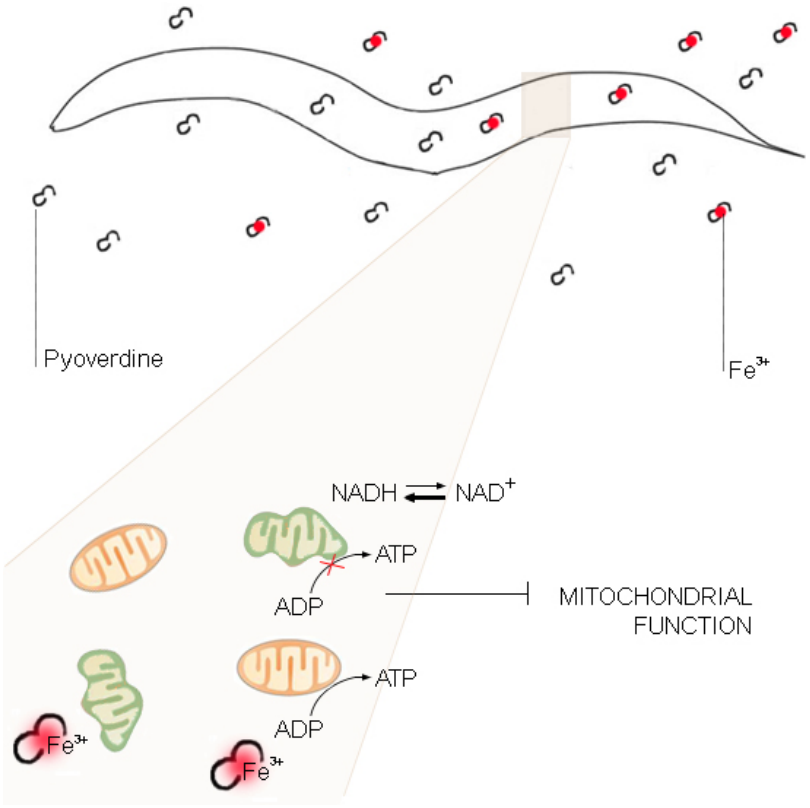

Figure 1.2. Pyoverdine translocates into *C. elegans*, removes iron, and disrupts host mitochondrial function.
Pyoverdine secreted from *P. aeruginosa* translocates into *C. elegans* tissue. Within hosts, pyoverdine binds intracellular iron, disrupting host iron homeostasis. Since iron is necessary for mitochondrial redox reactions, pyoverdine exposure diminishes *C. elegans* mitochondrial function. For instance, pyoverdine exposure results in the accumulation of cytosolic NADH and activation of mitochondrial autophagy (Kang et al., 2018).

Chapter 2

Results

***This chapter was adapted from (Kang and Kirienko, 2017) and (Kang et al., 2017).**

2.1. High-Throughput Genetic Screen Reveals that Biofilm Formation is Necessary for Pyoverdine Production

To elucidate the regulation of pyoverdine production in *P. aeruginosa*, we performed a high-throughput genetic screen of 5,810 mutants using a non-redundant *P. aeruginosa* PA14 transposon mutant library (Liberati et al., 2006). The high-throughput screen was performed under static growth conditions in 96-well plates, which resulted in robust biofilm formation in most wells, supporting the potential for a relationship between the generation of biofilm and production of pyoverdine. Fluorescence spectrophotometry (Ex 405 nm, Em 460 nm) was used to monitor pyoverdine biosynthesis in 96-well plates over a 24 h span. Of the mutants screened, 485 showed severe impairments in pyoverdine fluorescence (as defined as production below 30% of wild-type levels). Eliminating targets with growth defects (i.e., mutants where reduced pyoverdine biosynthesis might be caused by poor growth) left 338 hits. These were

narrowed further to include only 296 hits that had predicted gene function (**Figures 2.1A, B**). 55 of these (18.6%) were associated with various stages of biofilm formation (**Figure 2.1C**), including flagellin biosynthesis, chemotaxis, type IV pili assembly, Cup fimbriae biogenesis, and exopolysaccharide synthesis (**Table 2.1**).

Table 2.1. Full list of screen hits associated with biofilm formation. Hits that reoccurred are not listed.

Gene Name	PAO1 Homologue	Gene Function
Initial Surface Contact		
motA	PA4954	chemotaxis protein
motB	PA4953	chemotaxis protein
pctA	PA4309	chemotactic transducer
flgA	PA3350	putative flagella basal body P-ring formation protein
flgB	PA1077	flagellar basal-body rod protein
flgC	PA1078	flagellar basal-body rod protein
flgE	PA1080	flagellar hook protein
flgF	PA1081	flagellar basal-body rod protein
flgI	PA1084	flagellar P-ring protein precursor
flgJ	PA1085	flagellar protein
flgK	PA1086	flagellar hook-associated protein 1
flgL	PA1087	flagellar hook-associated protein type 3
flgM	PA3351	putative negative regulator of flagellin synthesis
flhA	PA1452	flagellar biosynthesis protein
fliA	PA1455	motility sigma factor
fliC	PA1092	flagellin type B
fliD	PA1094	flagellar capping protein
fliF	PA1101	Flagella M-ring outer membrane protein precursor
fliG	PA1102	flagellar motor switch protein
fliI	PA1104	flagellum-specific ATP synthase
fliJ	PA1105	flagellar protein
fliK	PA1441	putative flagellar hook-length control protein
FliL	PA1442	putative flagellar protein

fliM	PA1443	flagellar motor switch protein
fliN	PA1444	flagellar motor switch protein
fliP	PA1446	flagellar biosynthetic protein
fliQ	PA1447	flagellar biosynthetic protein
	PA1103	probable flagellar assembly protein
Attachment and Adhesion Factors Production		
pilB	PA4526	type 4 fimbrial biogenesis protein
pilC	PA4527	type 4 fimbrial biogenesis protein
pilF	PA3805	type 4 fimbrial biogenesis protein
pilR	PA4547	two-component response regulator
pilX	PA4553	type IV pilus biogenesis protein
pilZ	PA2960	type 4 fimbrial biogenesis protein
fimU	PA4550	type 4 fimbrial biogenesis protein
cupA1	PA2128	fimbrial subunit CupA1
Biofilm Maturation		
pelG	PA3058	Pel exopolysaccharide transport protein
Transcriptional Regulators that Affect Multiple Steps of Biofilm Formation		
fleQ	PA1097	Regulates flagellum motility and exopolysaccharide
fleS	PA1098	two-component sensor: regulates flagellum and adhesion factors
fleR	PA1099	two-component response regulator: regulates flagellum and adhesion factors

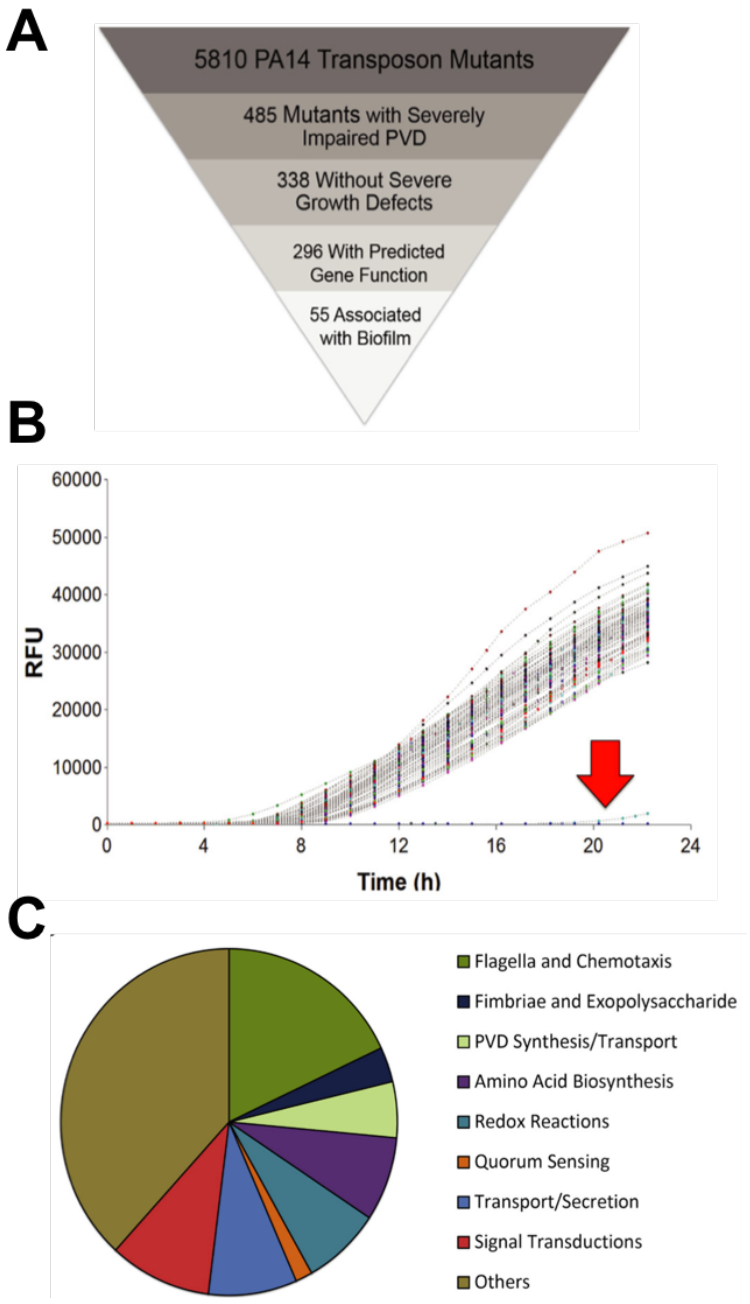

Figure 2.1. PA14 transposon mutant library screen for genes essential for pyoverdine production. (A) Schematic diagram of library screening process with criteria for identifying screen hits. **(B)** Kinetic data from a sample plate showing two mutants with severely impaired pyoverdine production (< 30% of normal, below the red arrow). **(C)** Families of gene functions identified and their relative enrichment among hits identified in the screen. Figure was adapted from (Kang and Kirienko, 2017).

To verify our findings, we selected genes representative for each stage of biofilm formation (initial surface attachment: *flgK,* a flagellum biosynthesis gene, and *motAB,* required for chemotaxis; adhesion factor production: *pilY1,* a type IV pili mutant; and bioflm maturation: *pelA*, an exopolysacharide mutant) (Vogeleer et al., 2014). As expected, these strains exhibited impairments in both biofilm formation (**Figure 2.2A**) and pyoverdine production (**Figure 2.2B**). We also observed a strong linear correlation between the amount of biofilm and pyoverdine produced by these mutants (**Figure 2.2C**), indicating a role for biofilm formation in pyoverdine production.

Quantitative real-time PCR (qRT-PCR) using RNA harvested from WT *P. aeruginosa* PA14, Δ*pilY1,* and Δ*flgK* planktonic cells after 8 h growth corroborated this finding. We used qRT- PCR to analyze expression of the alternative sigma factor *pvdS* and *pvdS*-dependent pyoverdine biosynthesis genes [including *pvdA* and *pvdF* (which are responsible for generating the non-standard amino acid *N*-formyl-*N*-hydroxyornithine), *pvdE* (predicted to transport nascent pyoverdine into the periplasm), *pvdP* and *pvdQ* (involved in periplasmic maturation)] (Drake and Gulick, 2011; Visca et al., 2007). All genes were significantly downregulated in Δ*flgK* mutant (**Figure 2.2D**). As may be hypothesized by the weaker pyoverdine biosynthesis phenotype (**Figure 2.2B**), the Δ*pilY1* mutant showed significantly less impact, although several genes were still disrupted, including *pvdF, pvdP*, and *pvdQ*. The PvdRT-OpmQ system is thought to be responsible for recycling pyoverdine. Once ferripyoverdine is imported into the bacterium, ferric iron is reduced to ferrous iron, which exhibits lower affinity for pyoverdine. Iron is then liberated from pyoverdine, and apo-pyoverdine is secreted back out via the PvdRT-OpmQ system (Imperi et al., 2009). Unsurprisingly (*pvdT* is also

transcriptionally regulated by *pvdS*), *pvdT* expression was diminished in the Δ*flgK* mutant, although regulation of *opmQ*, which is in the same operon as *pvdR* and *pvdT*) was unaffected. Our qRT-PCR data also suggest a possible role for ferripyoverdine uptake in biofilm-mediated regulation of pyoverdine production. *fpvA*, the ferripyoverdine receptor (Shen et al., 2002), and *tonB1*, which provides the energy for ferripyoverdine translocation into the cell (Shirley and Lamont, 2009), were significantly downregulated in the Δ*flgK* mutant (**Figure 2.2D**).

To determine whether the regulatory relationship between pyoverdine and biofilm is bidirectional (i.e., whether pyoverdine production was necessary for biofilm formation), we assayed biofilm development in two mutants (PA14Δ*pvdA* and PA14*pvdE*). Pyoverdine production was abolished in PA14Δ*pvdA* (**Figure 2.2B**), while PA14*pvdE* produced a small amount of pyoverdine (**Figure 2.2B**). Both mutants showed nearly normal biofilm formation (**Figure 2.2A**). This is consistent with previous results from Banin and colleagues, who showed that active iron acquisition was necessary for biofilm formation, but that pyoverdine itself is not (Banin et al., 2005).

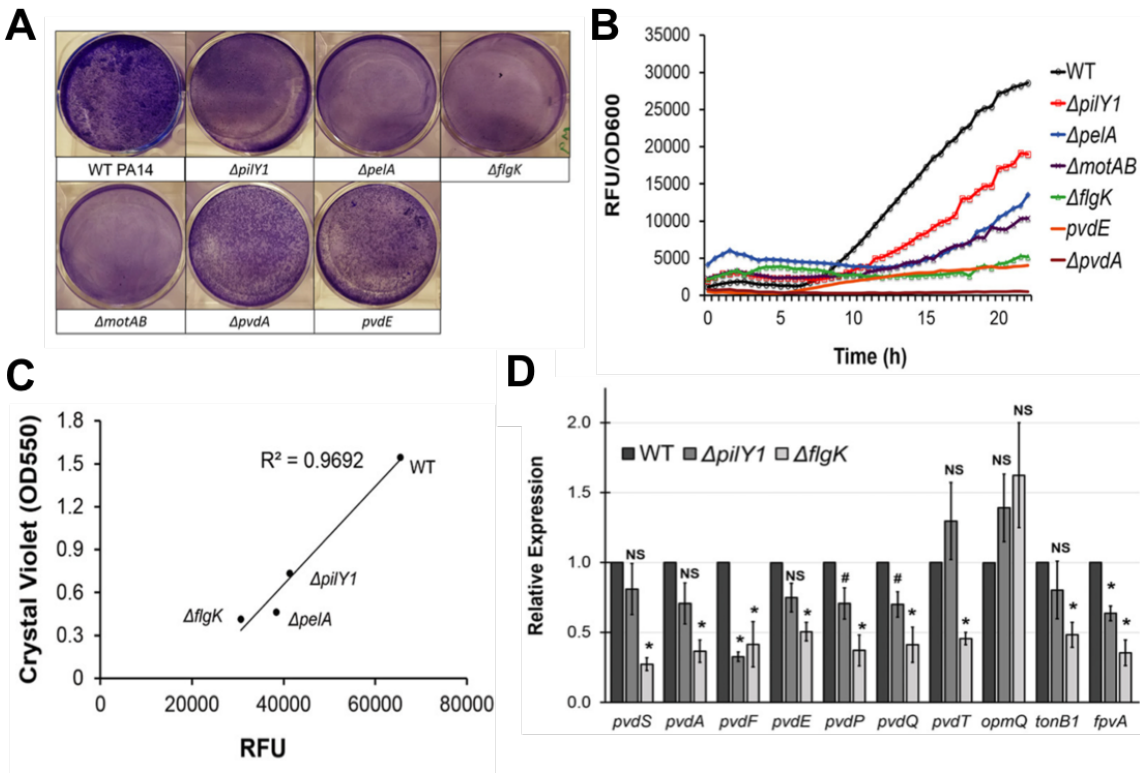

Figure 2.2. Biofilm formation is necessary for pyoverdine production. (A) Biofilm matrix of PA14 or 6 PA14 mutants in 6-well plates stained with 0.1% crystal violet solution. **(B)** Pyoverdine fluorescence normalized to bacterial growth, measured kinetically over 24 h in biofilm mutants. **(C)** Scatterplot of pyoverdine and biofilm produced in PA14 biofilm mutants grown in 6-well plate cultures. Biofilm was quantified by resuspending crystal violet stain in 30% acetic acid solution and measuring absorbance at 550 nm. **(D)** qRT-PCR of genes involved in pyoverdine biosynthesis, secretion, and signaling in WT *P. aeruginosa* PA14, Δ*pilY1* mutant, and Δ*flgK* mutant. Gene expressions in biofilm mutants are normalized to those in wild-type. Error bars in **(D)** represent SEM between three biological replicates. NS corresponds to $p > 0.05$, # corresponds to $p < 0.05$, and * corresponds to $p < 0.01$ (based on Student's *t*-test). Figure was adapted from (Kang and Kirienko, 2017).

2.2. Cell Aggregation Promotes Pyoverdine Production

Based on these observations, we hypothesized that sessile and planktonic cells would exhibit differences in pyoverdine production. These differences were observed in earlier stages of bacterial growth when pyoverdine biosynthesis is initiated. Using fluorescence microscopy, we visualized intracellular pyoverdine levels in sessile cells aggregated in the biofilm matrix and planktonic cells collected from the growth media after 8 h (when pyoverdine production is initiated), and 16 h of growth (**Figure 2.3A**). At 8 h, pyoverdine fluorescence is detected only in cells in the biofilm matrix, but at 16 h, pyoverdine fluorescence is present in both biofilm matrix and concentrated planktonic cells (**Figure 2.3A**). In a Δ*pvdA* mutant, neither biofilm matrix cells nor planktonic cells exhibit fluorescence, verifying that the fluorescence detected is from pyoverdine (**Figure 2.3B**). These results suggest one possible model for biofilm-dependent regulation of pyoverdine. First, pyoverdine production is initiated in the microcolonies that will nucleate biofilm formation. Secreted pyoverdine binds to ferric iron, returning to the pathogen via the ferripyoverdine receptor protein FpvA (Shen et al., 2002). This increases the activity of PvdS, upregulating pyoverdine biosynthesis genes in both sessile and planktonic cells (Lamont et al., 2002; Llamas et al., 2014).

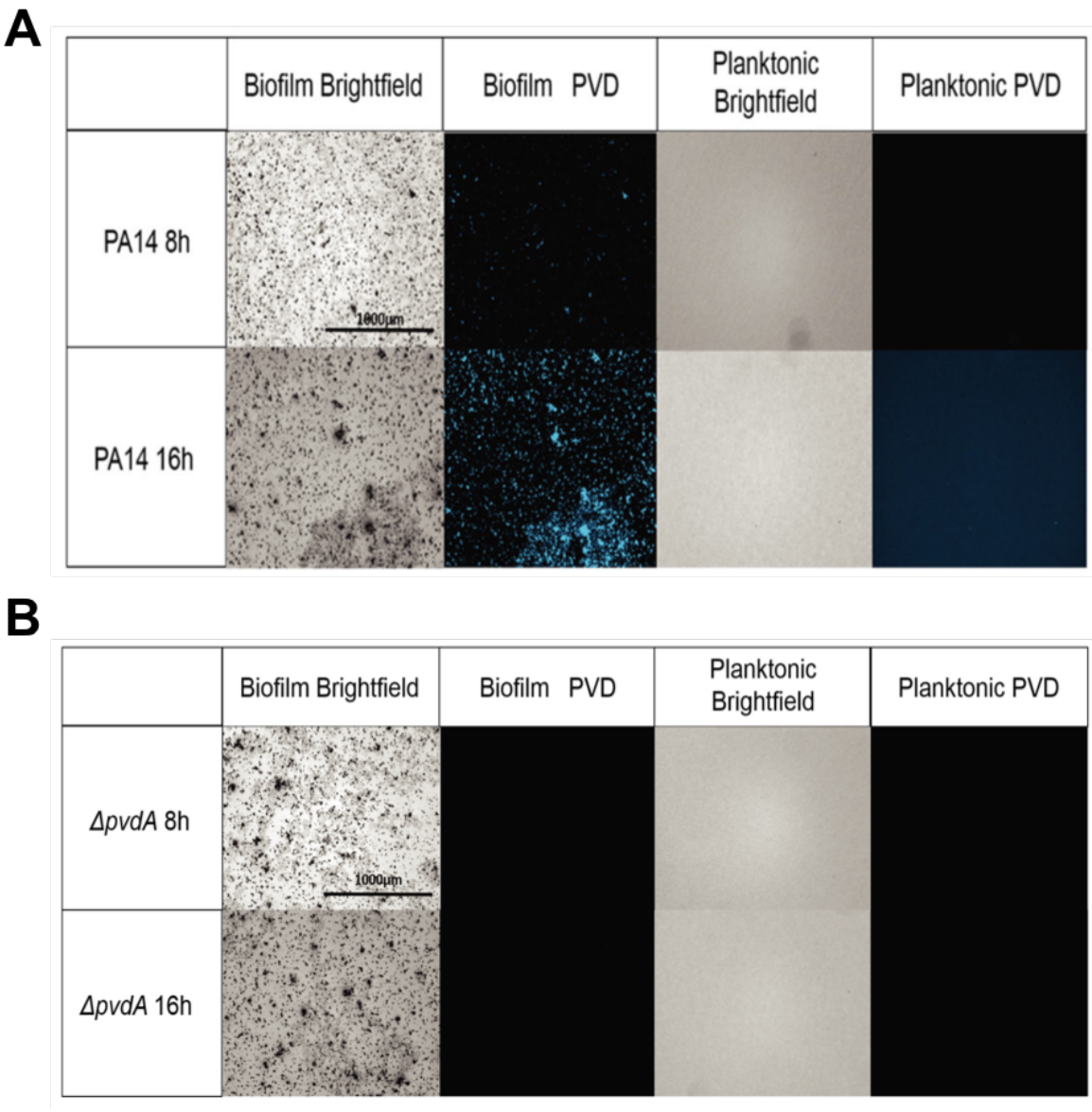

Figure 2.3. Cell aggregates in the biofilm matrix exhibit high pyoverdine production. Pyoverdine production in wild-type PA14 **(A)** or PA14Δ*pvdA* **(B)** biofilm matrix and planktonic cells imaged using pyoverdine-specific fluorescence filter. Figure was adapted from (Kang and Kirienko, 2017).

Consistent with these results, the aggregation of planktonic cells via quorum sensing molecule, 2-heptyl-3,4-dihydroxyquinoline, also known as Pseudomonas quinolone signal or PQS, was sufficient to enhance pyoverdine production. Within 4 h of PQS treatment, we observed aggregation and sedimentation of planktonic cells (**Figure 2.4A**), which resulted in significantly earlier activation of pyoverdine production (**Figure 2.4B**). We used fluorescence microscopy to visualize pyoverdine expression and observed that aggregated planktonic cells, which congregated after PQS treatment, exhibited high levels of pyoverdine production, as shown by increased pyoverdine-specific fluorescence (**Figure 2.4C**). When a pyoverdine-deficient mutant, *P. aeruginosa* PA14Δ*pvdA*, was treated with PQS, cell aggregation still occurred, but fluorescence was abolished, verifying that the fluorescence observed was pyoverdine-sourced. We also measured the expression of pyoverdine biosynthesis genes in PQS-induced cell aggregates by qRT-PCR. As expected, *pvdS*, *pvdA*, and *pvdE* were expressed more highly in PQS-treated *P. aeruginosa* cells (**Figure 2.4D**). This was not due to changes in expression of the ferric uptake regulator (*fur*).

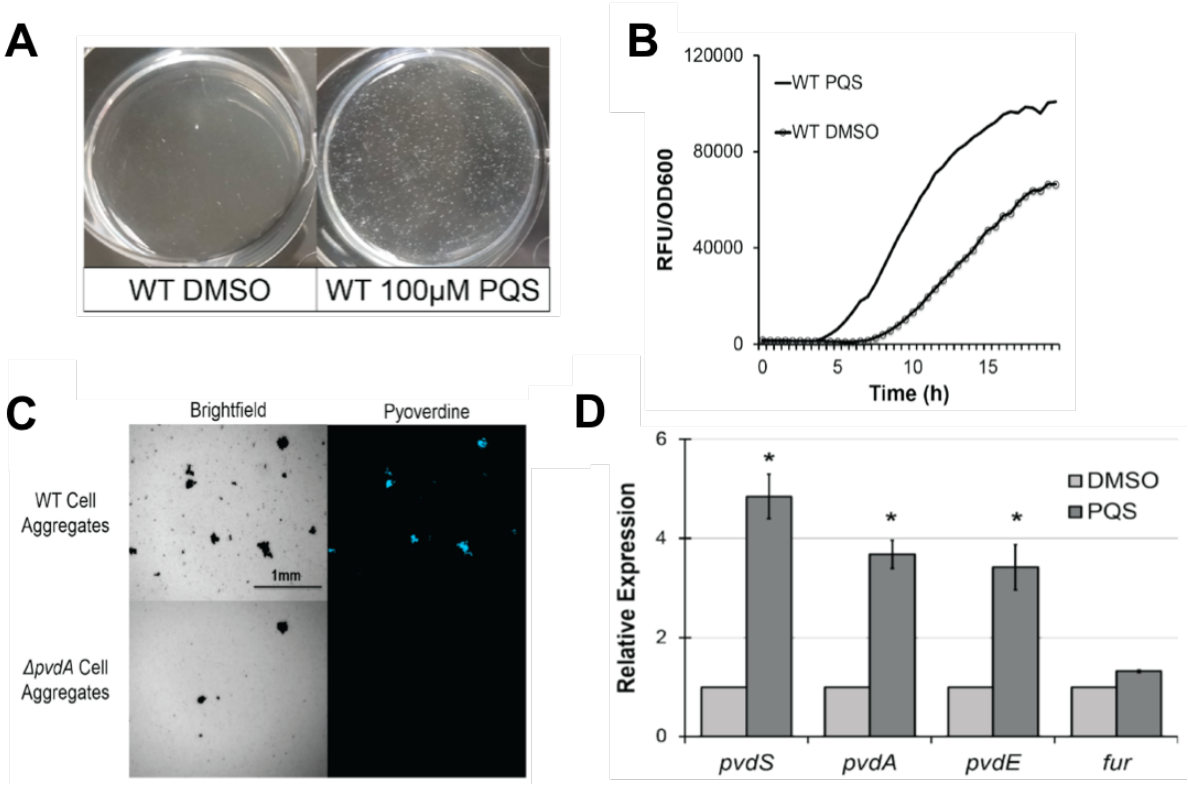

Figure 2.4. Exogenous Pseudomonas quinolone signal (PQS) induces cell aggregation and promotes pyoverdine production. (A) Cell aggregate formation in *P. aeruginosa* PA14 treated with either dimethyl sulfoxide (DMSO) (left) or 100 μM PQS (right) after a 4 h growth period. (B) Pyoverdine fluorescence normalized to bacterial growth, measured over 24 h in *P. aeruginosa* treated with DMSO or 100 μM PQS. (C) Brightfield (left) or fluorescence (right) micrographs of pyoverdine expression in either wild-type PA14 (top) or PA14Δ*pvdA*, a pyoverdine biosynthesis mutant (bottom). Cell aggregates were visualized with a pyoverdine-specific fluorescence filter. (D) Expression of pyoverdine biosynthesis genes in bacteria treated with 100 μM PQS or DMSO after 6 h growth, as measured by quantitative, real-time PCR (qRT-PCR). Gene expression in PQS-treated bacteria was normalized to that of the solvent control. Error bars represent standard error of the mean (SEM) between three biological replicates. * corresponds to $p < 0.01$ based on Student's *t*-test. Figure was adapted from (Kang et al., 2017).

Furthermore, we postulated that PQS-induced cell aggregation may be sufficient to induce pyoverdine production in biofilm mutants (PA14Δ*pelA*, PA14Δ*flgK*) even in the absence of the normally required biofilm signal. As expected, pyoverdine production in the Δ*pelA* mutant was restored by exogenous PQS (**Figures 2.5A, B**). In the Δ*flgK* mutant, the lag before pyoverdine production was significantly shortened after PQS supplementation (**Figures 2.5A, B**). These results are consistent with previous findings by Visaggio and colleagues, who observed that artificial cell aggregation restored pyoverdine production in exopolysaccharide-deficient *P. aeruginosa* mutants (Visaggio et al., 2015). Overall, these findings support the model where cell aggregation, rather than biofilm itself, promotes pyoverdine production.

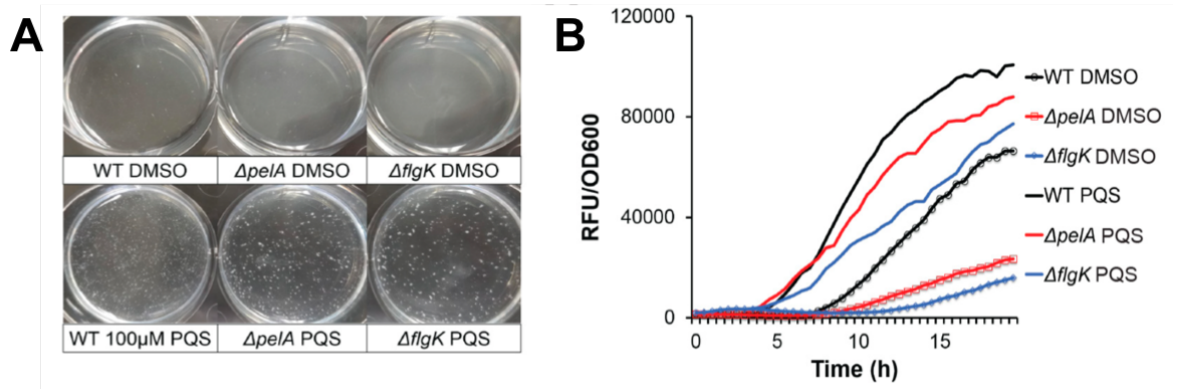

Figure 2.5. Exogenous PQS restores pyoverdine kinetics in biofilm-defective mutants. (A) Cell aggregate formation in wild-type PA14 or biofilm mutants treated with either DMSO (top) or 100 μM PQS (bottom) after 4 h growth. (B) Pyoverdine fluorescence normalized to bacterial growth measured over 24 h in biofilm mutants treated with DMSO or 100 μM PQS. Figure was adapted from (Kang et al., 2017).

2.3. c-di-GMP Regulates Pyoverdine Production in Biofilm-Dependent Manner

Next, we were interested if upstream regulators of biofilm formation would affect pyoverdine production. Cyclic diguanylate monophosphate (c-di-GMP) is a crucial secondary messenger in *P. aeruginosa* that transcriptionally regulates a wide variety of virulence factors, including type III and type VI secretion (Moscoso et al., 2011). Previous research has also linked intracellular c-di-GMP concentration to biofilm formation. For example, diguanylate cyclases (i.e., SadC) are involved in c-di-GMP synthesis and support increased formation of biofilms, while phosphodiesterases (i.e., BifA) hydrolyze c-di-GMP and limit biofilm formation (Kuchma et al., 2007; Merritt et al., 2007). Under our screening conditions, PA14Δ*sadC* (low c-di-GMP) and PA14Δ*bifA* (high c-di-GMP) mutants exhibited less and more biofilm respectively (**Figure 2.6A, B**), which is consistent with previously published data (Kuchma et al., 2007; Merritt et al., 2007). As predicted, pyoverdine production was attenuated in Δ*sadC* and enhanced in Δ*bifA* mutants (**Figure 2.6C**).

To test the regulatory relationship between c-di-GMP concentration and biofilm formation in pyoverdine production, we repeated the kinetics experiment with a PA14Δ*bifA*Δ*pelA* double mutant that exhibits high intracellular c-di-GMP concentrations, but poor biofilm formation (**Figures 2.6A, B**) (Kuchma et al., 2007). Since pyoverdine production was not completely abolished in the PA14Δ*pelA* mutant background, the ramifications of *bifA* deletion on pyoverdine production (whether augmented or decreased) can be observed. Pyoverdine production in the PA14Δ*bifA*Δ*pelA* double

mutant was similar, if not lower, to Δ*pelA* single mutant (**Figure 2.6C**), indicating that regulation of pyoverdine via intracellular c-di-GMP concentrations is hypostatic to biofilm formation. This confirms that c-di-GMP's effect on pyoverdine production is mediated indirectly, via biofilm formation.

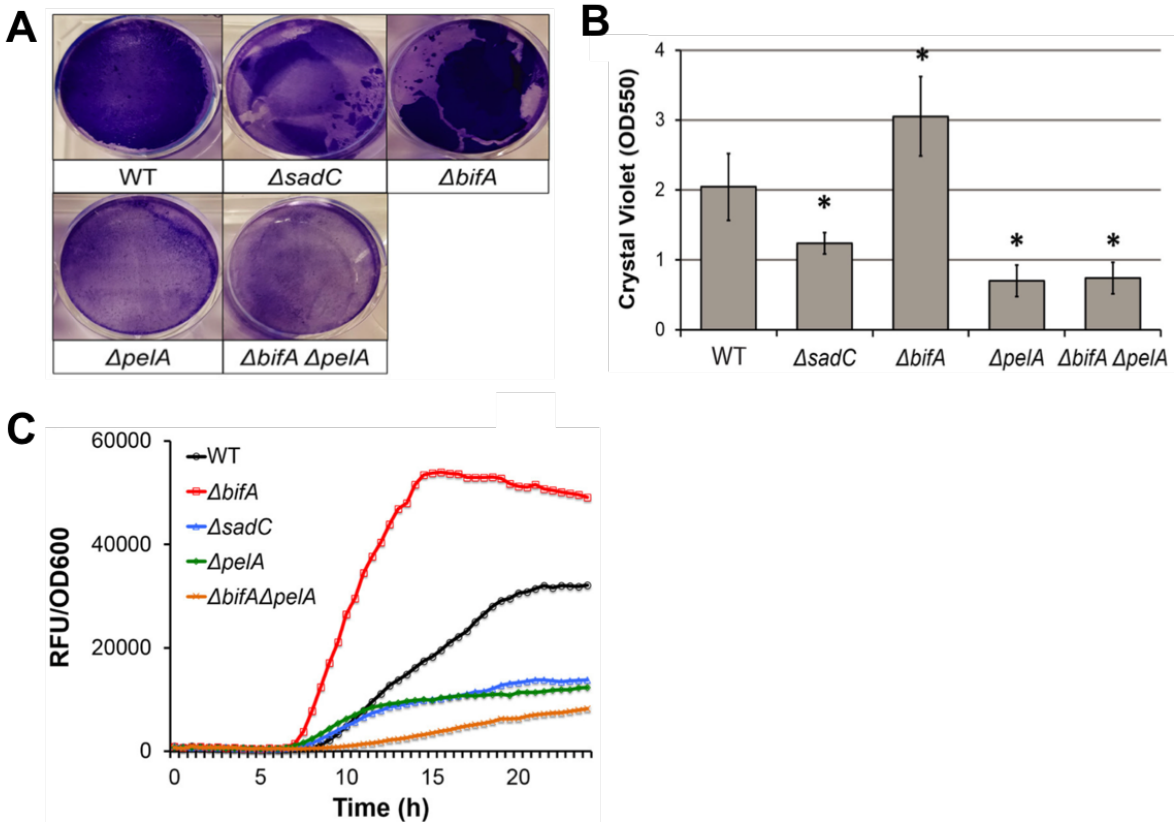

Figure 2.6. Intracellular c-di-GMP concentrations modulate pyoverdine production in a biofilm-dependent manner. (A) Biofilm matrix of c-di-GMP biosynthesis mutants stained with 0.1% crystal violet solution. **(B)** Crystal violet concentration measured by absorbance at 550 nm after biofilm matrix stain was solubilized in 30% acetic acid. **(C)** Pyoverdine fluorescence normalized to bacterial growth was measured kinetically over 24 h in diguanylate cyclase and phosphodiesterase mutants and the phosphodiesterase mutant with biofilm defect. Data presented in **(A, C)** are representative results from three biological replicates. Error bars in **(B)** represent SEM between three biological replicates. Asterisks indicate significant difference between conditions (p-value < 0.01, based on Student's t-test). Figure was adapted from (Kang and Kirienko, 2017).

2.4. Chemical and Physical Modifiers of Biofilm Formation Affect Pyoverdine Production

We hypothesized that chemical inhibition of biofilm formation, like genetic disruption, would reduce pyoverdine production. Several compounds are known to compromise *P. aeruginosa* biofilm formation, including the aromatic heterocycle 2-amino-5,6-dimethylbenzimidazole (2-ABI) (Frei et al., 2012). Addition of 25 μM 2-ABI to *P. aeruginosa* culture reduced biofilm formation by 40% compared to the solvent control (**Figure 2.7A**), with a concomitant decrease (60%) in pyoverdine production (**Figure 2.7B**). The decrease in these exoproducts was not a consequence of bacterial growth inhibition; bacterial titer was unaffected by the presence of the compound (**Figure 2.7C**

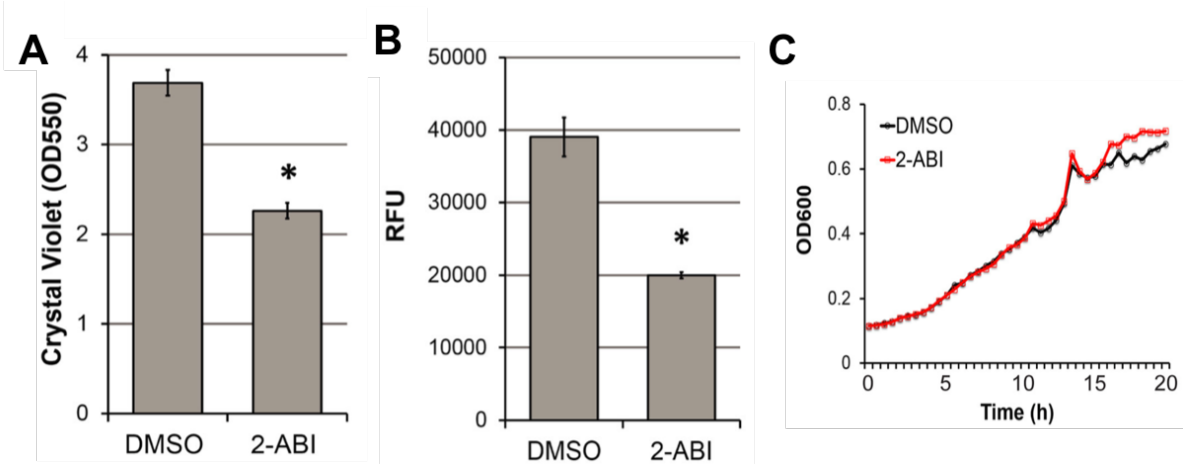

Figure 2.7. Biofilm inhibitor significantly impairs pyoverdine production and attenuate *P. aeruginosa* virulence. (A) Quantification of crystal violet-stained biofilm matrix solubilized in acetic acid for PA14 grown in M9 media with 25 μM 2-ABI or DMSO solvent control. **(B)** Pyoverdine fluorescence of bacterial supernatant after 24 h of static growth for PA14 grown in conditions as above. **(C)** Bacterial growth measured kinetically over 24 h by absorbance at 600 nm for PA14 grown in conditions as above. Error bars in **(A, B)** represent SEM between three biological replicates. Asterisks indicate significant difference between conditions (p-value < 0.01, based on Student's t-test). Figure was adapted from (Kang and Kirienko, 2017).

Another way to significantly alter biofilm formation is by changing the surface characteristics of the vessel for *P. aeruginosa* static cultures. In this case many of the off-target effects that may be induced by chemical inhibitors or genetic manipulations will be eliminated. On a plastic surface, *P. aeruginosa* PA14 formed approximately one-half of the biofilm as a glass surface (**Figure 2.8A**). This led to ~25% decrease in pyoverdine production (**Figure 2.8B**).

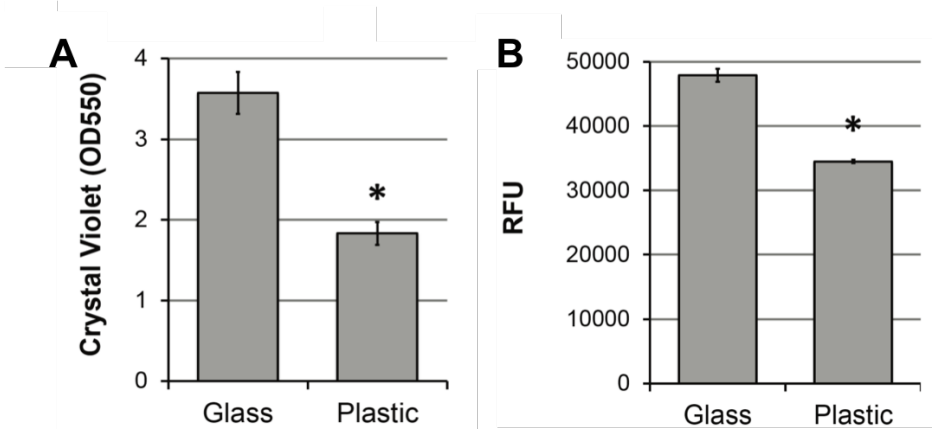

Figure 2.8. Modification of attachment surface affects biofilm formation and subsequently pyoverdine production. (A) Quantification of crystal violet-stained biofilm matrix solubilized in acetic acid for *P. aeruginosa* PA14 grown on plastic or glass surfaces. **(B)** Pyoverdine fluorescence in bacterial supernatant after 16 h of static growth for PA14 grown on plastic or glass surfaces. All error bars in represent SEM between three technical replicates. Asterisks indicate significant difference between conditions (p-value < 0.01, based on Student's *t*-test). Figure was adapted from (Kang and Kirienko, 2017)

Furthermore, nutrient availability can also contribute to pyoverdine production by affecting bacterial biofilm formation. In a previous report, biofilm mutants showed no attenuation of virulence in *C. elegans* Liquid Killing (where pyoverdine production is indispensable for pathogenesis) (Kirienko et al., 2013). The media used for those studies is comprised of low concentrations of sodium chloride and peptone, diluted into buffered inorganic salts to match the osmolarity of the host (SK media). This contrasts with the M9 media used in this study, which is better defined and more nutritionally rich. Kinetic measurements of biofilm mutants grown in SK media showed no significant decrease in pyoverdine fluorescence (**Figure 2.9A**). In contrast to the M9 media used in this study, SK media does not support dense bacterial growth nor formation of dense biofilms (**Figure 2.9B, C**). This alleviates pyoverdine biosynthesis from biofilm regulation. Likewise, pyoverdine production in c-di-GMP biosynthesis mutants (PA14Δ*sadC,* PA14Δ*bifA*) in SK media also did not significantly differ from that of wild-type bacteria. This further supports our model where c-di-GMP affects pyoverdine production indirectly via biofilm formation.

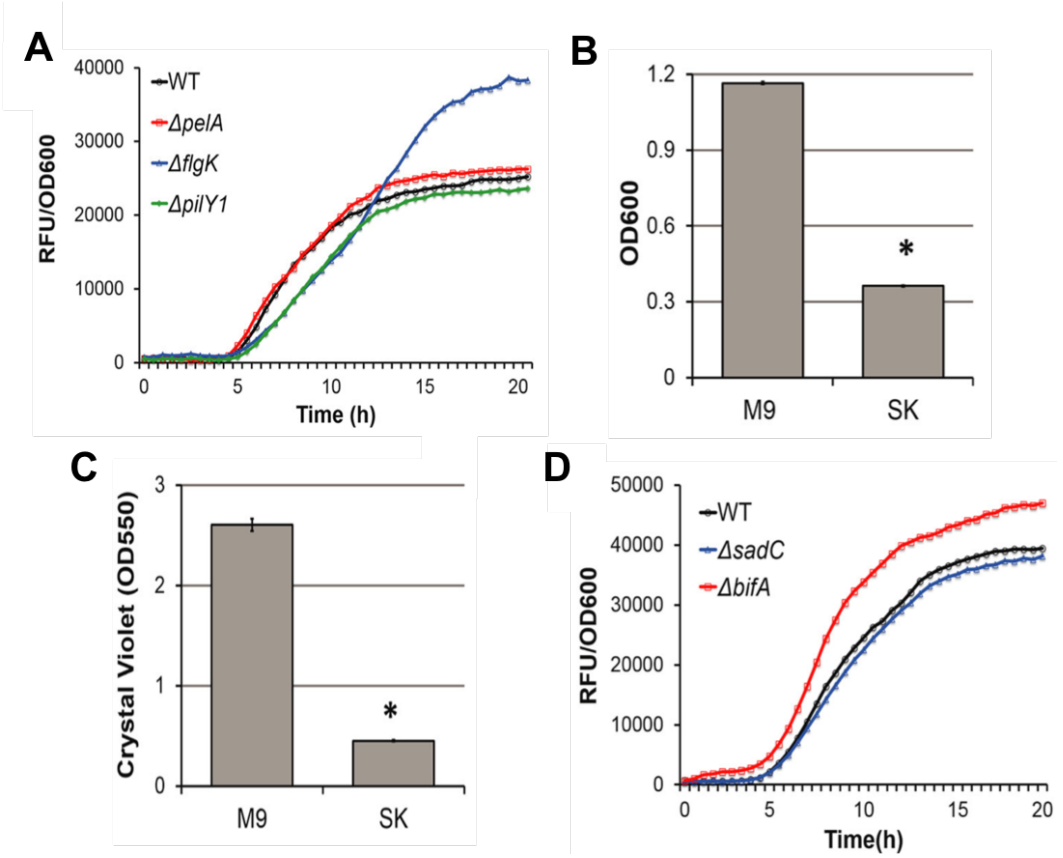

Figure 2.9. Biofilm formation doesn't affect pyoverdine production in SK media. (A) Pyoverdine fluorescence normalized to bacterial growth measured kinetically over 24 h in biofilm mutants grown in SK media. **(B)** Difference in bacterial growth for bacteria grown in M9 or SK media measured by absorbance at 600 nm. **(C)** Difference in biofilm formation for bacteria grown in M9 or SK media measured by crystal violet biofilm matrix stain solubilized in acetic acid. **(D)** Pyoverdine fluorescence normalized to bacterial growth measured kinetically over 24 h in c-di-GMP biosynthesis mutants grown in SK media. All error bars represent SEM between four technical replicates. Asterisks indicate significant difference between conditions (p-value < 0.01, based on Student's t-test). Figure was adapted from (Kang and Kirienko, 2017).

Chapter 3

Summary of Results and Discussion

This chapter was adapted from (Kang and Kirienko, 2017), (Kang et al., 2017), and (Kang and Kirienko, 2018).

3.1. Complexity of Pyoverdine Regulation

The increasing prevalence of multidrug resistant pathogens demands a new therapeutic approach to treating nosocomial infections. One possibility is to supplement antibiotics with novel drugs that compromise pathogen virulence. However, in order to develop these treatments, we need to first identify the relevant determinants and clearly understand their regulatory relationships. Toward this end, we screened a *P. aeruginosa* transposon mutant library to identify genes necessary for the production of pyoverdine. The innate fluorescence of pyoverdine enabled us to take a high-throughput, kinetic approach, monitoring pyoverdine production over 24 h. This revealed a relationship between pyoverdine, c-di-GMP, and biofilm in *P. aeruginosa* (summarized in **Figure 3.1**). Due to their physiological significance, these three virulence factors have been

extensively studied. For example, multiple studies have demonstrated the importance of pyoverdine in various mammalian and non-mammalian models, most notably in GI tract colonization and lung infection models in mice (Imperi et al., 2013; Kirienko et al., 2013; Lopez-Medina et al., 2015; Meyer et al., 1996; Minandri et al., 2016; Takase et al., 2000). The secondary messenger c-di-GMP functions as a master switch between motility and biofilm formation. And biofilms form a critical *in vivo* reservoir of infection that is particularly resistant to the immune system and antimicrobials (Costerton et al., 1999). Many previous studies have demonstrated that siderophores, including pyoverdine, play an important role in biofilm formation through their iron-scavenging activity (Banin et al., 2005; Chhibber et al., 2013; Ojha and Hatfull, 2007). Recent studies (including the data presented here) have suggested that biofilms may also regulate pyoverdine (Kang and Kirienko, 2017; Visaggio et al., 2015) (**Figure 3.1**). For example, Chen *et al*., suggested that exopolysaccharides may regulate pyoverdine production through the Gac/Rsm pathway and the diguanylate cyclase SadC, via an unknown mechanism (Chen et al., 2015). It is difficult to know how relevant their conditions were, however, since they overexpressed the diguanylate cyclase YedQ, leading to levels of c-di-GMP that may not reflect biologically relevant conditions. Visaggio, *et al*., also saw a link between the Pel and Psl exopolysaccharides in pyoverdine production. However, their data suggest that the relevant function of these sugars is to drive cell aggregation in the PAO1 strain. This aggregation appears to be the driving force for pyoverdine production in their conditions (Visaggio et al., 2015). We observed a similar phenomenon when cell aggregation was artificially induced using the quorum-sensing molecule PQS (Kang et al., 2017) (**Figure 3.1**).

Although our high-throughput genetic screen provided valuable insight into the possibility of biofilm formation regulating pyoverdine, further work demonstrated the complexity of this regulation that was not interpretable from the screen alone. For instance, qRT-PCR of genes associated with pyoverdine biosynthesis and fluorescence microscopy of biofilm matrices suggested that ferripyoverdine uptake may be involved in biofilm-dependent regulation of pyoverdine production. These findings suggest that biofilm formation, a process which is not directly related to iron metabolism in the bacterium, utilizes iron-sensitive mechanisms to regulate pyoverdine production.

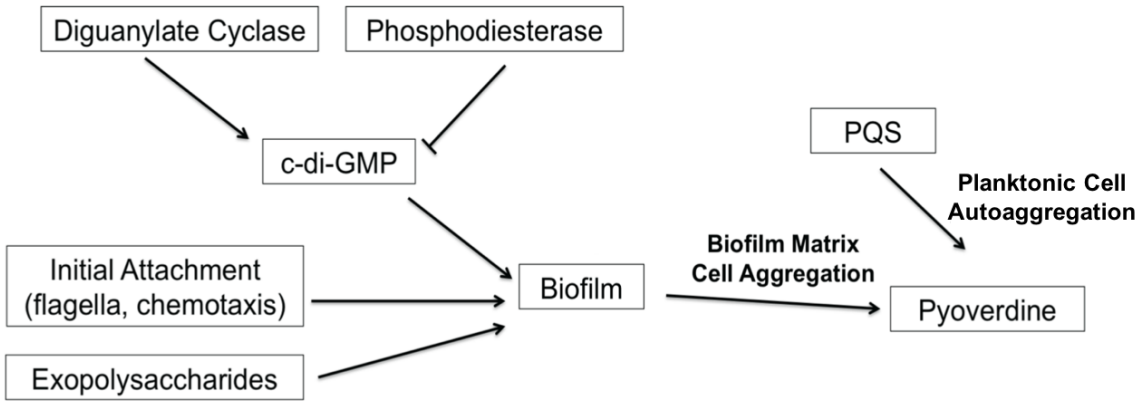

Figure 3.1. Model for biofilm-mediated regulation of pyoverdine production. This model summarizes factors involved in biofilm formation that have been shown to affect pyoverdine production. Figure was adapted from (Kang et al., 2017).

Furthermore, we were able to break the connection between biofilm synthesis and pyoverdine production in PA14 by using low concentrations of the macromolecules that provide carbon and nitrogen. For instance, in nutrient-poor SK media, bacterial density is artificially restrained, which is likely to diminish cell aggregation and decrease production of quorum sensing molecules. M9 media, which is richer in nutrients, permits

more robust growth. This difference may explain the variations in pyoverdine production and regulation that we observed. Our data indicate that the regulation of virulence factors like pyoverdine are highly complex, multifactorial, and are likely to take into account the conditions both within and outside of the bacterium.

3.2. Utility of High-Throughput Screens

It is important to note however, that the innate fluorescence of pyoverdine dramatically simplifies the process of identifying regulators of pyoverdine biosynthesis using high-throughput screening approaches; fluorescence is a nearly ideal readout for this type of screen. It should be admitted that regulators of virulence factors lacking ready detection techniques would be much harder to identify in this fashion. Arguably, the most effective method in these cases is to leverage model organisms to screen libraries (small molecule, transposon, RNAi, etc.) for pathogenesis; *C. elegans* and zebrafish are commonly used for this purpose (Begun et al., 2005; Feinbaum et al., 2012; Garvis et al., 2009; Kirienko et al., 2019; Kirienko et al., 2016; Kizy and Neely, 2009; Miller and Neely, 2005) because they are small, have rapid generation times, and exhibit strong evolutionary similarity to humans. This approach has been particularly successful in identifying drugs that might be repurposed to treat infectious diseases (Carvalho et al., 2011; Kim et al., 2017; Kirienko et al., 2016). However, as noted above, this approach strictly requires that the virulence mechanisms, and their regulation, can be recapitulated in these models. If this demand can be met, this approach seems an invaluable resource for making discoveries ranging from the basic (e.g., the interconnectivity of regulatory networks that are involved in a variety of metabolic processes) to the clinical (i.e., the identification of therapeutics that can transform health care and postpone the rapidly

approaching antimicrobial crisis).

3.3. Interdependence between Biofilm Formation and Iron Acquisition

Finally, the results presented in Chapter 2 contributes to our current model where biofilm formation and iron acquisition are interrelated. As mentioned in Chapter 1, iron acquisition is necessary for proper biofilm formation in a number of microorganisms. However, our results suggest that biofilm formation can also have the reciprocal effect, influencing siderophore production.

Another intriguing phenomenon that further demonstrates this interdependent relationship between biofilm formation and iron acquisition is the discovery that biofilms can store iron. Although iron is essential for most living organisms, it is also quite toxic at high concentrations as it can catalyze the Fenton reaction, which produces reactive oxygen species (ROS). Therefore, bacteria must maintain a delicate balance, acquiring sufficient iron for growth but not enough to allow the wide-spread production of ROS. It now appears that components of the *P. aeruginosa* biofilm matrix help the bacteria maintain this balance. A recent study by Ma and colleagues demonstrated that all three exopolysaccharides produced by *P. aeruginosa* (alginate, Pel, Psl) can sequester free environmental iron (**Figure 3.2**). In brief, alginate binds ferric iron, Pel binds ferrous iron, and Psl binds to both (Yu et al., 2016). Importantly, *P. aeruginosa* is capable of utilizing iron bound to Psl to support its growth during in iron-limiting environments (Yu et al., 2016). This ability to sequester iron is not unique to polysaccharides from *P. aeruginosa*. Exopolysaccharides from *Xanthomonas campestris*, *Paracoccus*

zeaxanthinifaciens, and *Klebsiella oxytoca* have also been shown to bind iron (Baldi et al., 2009; Javvadi et al., 2018; Moppert et al., 2009). Like Psl, cyclic β-(1,2) glucans from *X. campestris* can store iron that is utilized by the bacteria to support growth under iron-restricted conditions (Javvadi et al., 2018).

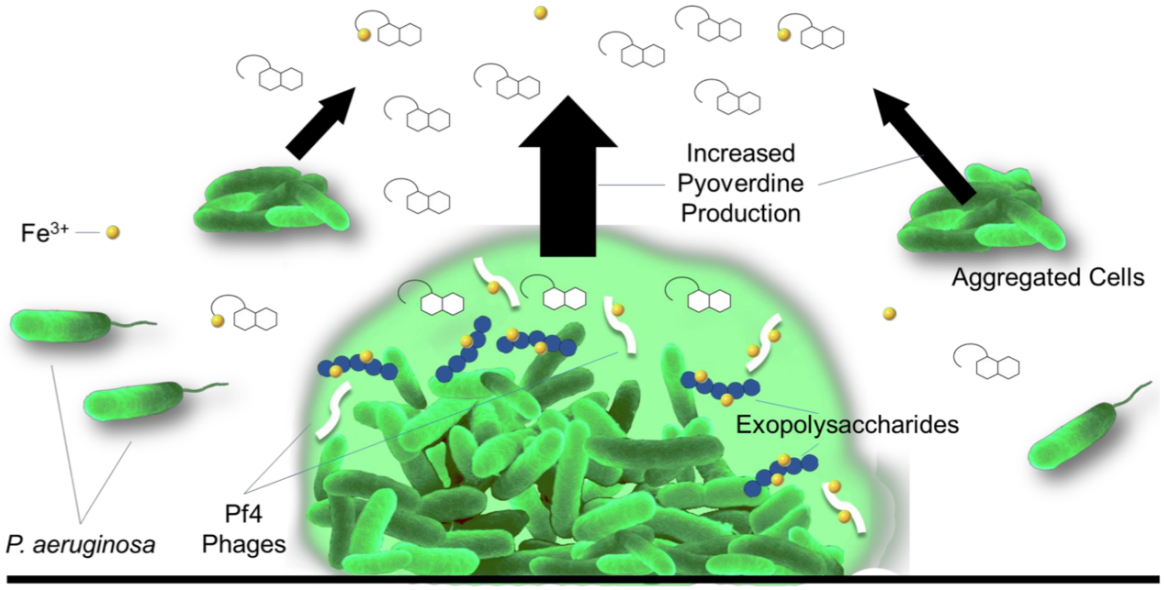

Figure 3.2. *P. aeruginosa* biofilm can store excess iron. In *P. aeruginosa* biofilms, secreted exopolysaccharides and Pf4 bacteriophages can bind iron, sequestering it in the extracellular matrix. Alginate and Pf4 can sequester ferric iron, Pel exopolysaccharide can sequester ferrous iron, and Psl exopolysaccharide can sequester both. In the case of Psl, *P. aeruginosa* can utilize iron-bound Psl for growth and biofilm formation under conditions of iron restriction. Furthermore, aggregated cells (in both planktonic and sessile states) exhibit increased pyoverdine production, suggesting another regulatory mechanism for iron uptake. Figure was adapted from (Kang and Kirienko, 2018).

Another component of the *P. aeruginosa* biofilm matrix that is capable of sequestering iron is the filamentous bacteriophage Pf4 (**Figure 3.2**). The Pf4 prophage within the *P. aeruginosa* genome is highly expressed in biofilm cells, resulting in orders of magnitude greater phage production in biofilms than planktonic cell cultures (Webb et al., 2004; Whiteley et al., 2001). Pf4 activity is necessary for normal biofilm development and maturation, as well as pathogen virulence (Rice et al., 2009). Phage activity also triggers death of *P. aeruginosa* cells in CF infection isolates (Kirov et al., 2007; Webb et al., 2004), and has been posited to drive *P. aeruginosa* to a mucoid state (Hoiby et al., 2001; Miller and Rubero, 1984). Pf4 bacteriophage in *P. aeruginosa* biofilms can also directly bind to ferric iron, as demonstrated by Raman-binding analysis and the induction of phage cross-linking in the presence of ferric iron (Penner et al., 2016). This iron-chelating activity gives *P. aeruginosa* an advantage during polymicrobial interactions. For instance, *P. aeruginosa* inhibits *Aspergillus fumigatus* biofilm formation via Pf4-mediated iron sequestration (Ferreira et al., 2015; Penner et al., 2016). Pf4 bacteriophage can inhibit *A. fumigatus* biofilms even in the absence of live *P. aeruginosa*, but this inhibition is deterred by supplementation of ferric iron (Penner et al., 2016). It is currently unknown whether *P. aeruginosa* can utilize iron-bound Pf4 as a source of iron, either directly or indirectly.

Overall, the ability of *P. aeruginosa* biofilms to regulate siderophore production and sequester extracellular iron provides exciting insight into a possible interdependent relationship between biofilm formation and iron acquisition.

Chapter 4

Materials and Methods

This chapter was adapted from (Kang and Kirienko, 2017).

4.1. Bacterial Strains and Growth Conditions

Strains used are listed in **Table 4.1**. For all pyoverdine production and biofilm formation assays, bacteria were seeded in M9 media (M9 salts (1% w/v) and casamino acids (1.3% w/v), supplemented with 1 mM $MgSO_4$ and 1 mM $CaCl_2$) in static 6-well plates (Greiner, North Carolina) at 30°C. SK media was composed of 0.35% (w/v) Bacto-Peptone and 0.3% (w/v) NaCl, supplemented with 1 mM $MgSO_4$ and 1 mM $CaCl_2$ (Conery et al., 2014).

Table 4.1. Bacterial strains used in this study.

Strains	Relevant Genotype	Source or Reference
PA14	WT	(Rahme et al., 1995)
ΔpelA	PA14*ΔpelA*	(Kuchma et al., 2007)
ΔflgK	PA14*ΔflgK*	(Kuchma et al., 2010; Shanks et al., 2006)
ΔpilY1	PA14*ΔpilY1*	(Kuchma et al., 2010)
ΔmotAB	PA14*ΔmotAΔmotB*	(Kuchma et al., 2015)
ΔpvdA	PA14*ΔpvdA*	(Kuchma et al., 2010; Shanks et al., 2006)
pvdE	PA14*pvdE* Gent^R	(Liberati et al., 2006)
ΔsadC	PA14*ΔsadC*	(Merritt et al., 2007)
ΔbifA	PA14*ΔbifA*	(Kuchma et al., 2007)
ΔbifAΔpelA	PA14*ΔbifAΔpelA*	(Kuchma et al., 2007)

Gent^R: gentamicin resistant

4.2. Transposon Mutant Library Screen

PA14 transposon mutants were inoculated into 96-well plates with LB media containing 15 μg/mL gentamicin. Inoculated plates were incubated overnight at 35°C. 10 μL of LB culture from each well were transferred into 96-well, clear, flat-bottom plates (Greiner, North Carolina) containing 90 μL of M9 media per well. Bacterial plates were grown at room temperature for 24h. Pyoverdine production (Ex 405 nm, Em 460 nm) and bacterial growth ($O.D._{600}$) were measured every hour in these plates using a Cytation5 (BioTek, Vermont) multimode plate reader

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4.3. Pyoverdine Production Kinetics Assay

Bacterial strains were grown in LB media with appropriate antibiotics overnight with shaking. Two milliliters M9 media were dispensed into each well in six-well plates (Greiner, North Carolina) and inoculated with 100 μL from overnight LB cultures. The plate was incubated at 30°C inside a plate reader for 24 h with pyoverdine fluorescence measurements and bacterial growth absorbance measurements made every 30 min. Each experiment consisted of at least three biological replicates.

4.4. Biofilm Formation Assay

This procedure was adapted from (Merritt et al., 2005). In brief, bacterial strains were grown under conditions identical to those described above. After incubation at 30°C for 24 h, bacterial cultures were aspirated and the biofilm matrix on the bottom of the plate was stained with 2 mL of 0.1% (w/v) crystal violet in 20% (v/v) ethanol/water for 30 min. The stain was removed and excess stain was washed with two consecutive rinses of PBS (Gibco, Maryland). Plates were dried and then photographs were taken. For biofilm quantification, the remaining crystal violet was solubilized in 30% acetic acid and absorbance was measured at 550 nm. Each experiment consisted of at least three biological replicates. Statistical significance was determined using Student's *t*-test.

4.5. RNA Purification and qRT-PCR

After 8 h growth in six-well plates, planktonic cells were collected from 1.5 mL of supernatant. RNA was extracted and purified using Trizol reagent (Invitrogen, California) according to manufacturer's protocols with minor adjustments. To ensure cell lysis, cells

resuspended in Trizol reagent were heated at 95°C for 15 min prior to phase separation. Purified RNA was treated with DNase I (Thermo Scientific, Massachusetts). Reverse transcription was performed using random decamers and Retroscript kit (Ambion). qRT-PCR was conducted using SYBR green PerfeCTa SYBR Green Fastmix (Quantabio, Massachusetts) in a CFX-96 real-time thermocycler (Bio-Rad, California). Fold-changes were calculated using a ΔΔCt method, and compared to expression from wild-type *P. aeruginosa*.

4.6. Biofilm and Planktonic Cells Pyoverdine Imaging

Two milliliters of M9 media were dispensed into each of three wells in a six-well plate (Greiner, North Carolina) and inoculated with 100 μL of *P. aeruginosa* grown overnight in LB. After 8 or 16 h incubation at 30°C, supernatant was carefully collected from all wells. Planktonic cells were collected from 2 mL of media. Planktonic cells and biofilm-associated cells attached to the bottom of the plate were washed twice with PBS. Planktonic cells were resuspended in 0.5 mL PBS buffer and dispensed into a six-well plate. Biofilms and concentrated planktonic cells were imaged using a custom filter (445/45 excitation, 510/42 emission, 482 nm dichroic) using a Cytation5 multimode reader (Biotek, Vermont). To image planktonic cell aggregates (after PQS treatment), after 6 h of bacterial growth, media with planktonic cell aggregates were collected from the plate. Cells were gently washed and resuspended in 1mL PBS buffer (Gibco, Waltham, MA, USA). Resuspended cells were transferred to a new 6-well plate for imaging. All images were taken under identical conditions, and each experiment consisted of at least three biological replicates.

4.7. Quantification of Biofilm and Pyoverdine in Different Surface Growth Conditions

25 μL of *P. aeruginiosa* PA14 grown overnight in LB was inoculated into 475 μL of M9 media in 17 mm diameter glass or plastic culture tubes. Tubes were grown in 30°C under static growth conditions for 16 h. Bacterial supernatant was collected from the tubes, and pyoverdine fluorescence was determined. Biofilms in tubes were stained and crystal violet staining was measured as described above. Each experiment consisted of at least three biological replicates. Statistical significance was determined using Student's *t*-test.

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