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Role of 2,4-Dihydroxyquinoline (DHQ) in Pseudomonas Aeruginosa Pathogenicity

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Role of 2,4-Dihydroxyquinoline (DHQ) in *Pseudomonas aeruginosa* Pathogenicity

By

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A dissertation submitted to the faculty of the Medical University of South Carolina in

partial fulfillment of the requirements for the degree Doctor of Philosophy in the College

of Graduate Studies.

Department of Biochemistry and Molecular Biology

2015

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"Chance favors a prepared mind" – Louis Pasteur at Pouilly le Fort

In a public defense for the French Academy of Science, Pasteur inoculates several livestock using his inactivated anthrax vaccine. One change has been made to his protocol though; the inoculation dose of anthrax is now going to be significantly higher than what was thought. After inoculation, the press covering the event asks Pasteur if he is nervous for the results. He indicates that he is not because chance favors a prepared mind. After one week, all of the animals inoculated with the vaccine were alive, while all of the uninoculated were dead or dying.

Acknowledgements

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List of Abbreviations used:

Pqs – *Pseudomonas* quinolone system

PQS – 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal)

HHQ - 2-heptyl-4-quinolone

HQNO - 4-hydroxy-2-heptylquinolone N-oxide

DHQ – 2,4-Dihydroxyquinoline

ROS – Reactive oxygen species

HSL – Homoserine lactone

AI – Auto-inducer

QS – Quorum sensing

CF – Cystic fibrosis

EPS – Exopolymeric substance

eDNA – Extracellular DNA

Abstract

JORDON DALE GRUBER. Role of 2,4-Dihydroxyquinoline (DHQ) in *Pseudomonas aeruginosa* Pathogenicity. (Under the direction of Yong-Mei Zhang).

Bacterial group behaviors are advantageous during an infection to thwart immune cell attack and resist deleterious changes in the environment. Bacteria use a chemical messaging system in order to coordinate the phenotypes in the environment. In *Pseudomonas aeruginosa*, the Pseudomonas quinolone signal (Pqs) quorum-sensing system produces alkylquinolones that regulate virulence factor production and also perform extracellular roles. Two alkylquinolones, 2-heptyl-4-quinolone (HHQ) and 2-heptyl-3-hydroxy-4-quinolone (PQS) activate transcriptional regulator PqsR for subsequent production of quinolones and phenazines, iron chelation, and autolysis. The most abundant quinolone produced from the Pqs system is 2,4-Dihydroxyquinoline (DHQ); however, DHQ has no known function. We demonstrated mutants only able to produce DHQ maintained virulence towards a model of bacterial infection and *in vitro* virulence factor production. Furthermore, we identified a potential extracellular role for DHQ against both epithelial cells and macrophages that resulted in reduced replication, viability, and cytokine production. As a signaling molecule, DHQ activated PqsR to bind to the promoter region of *pqsA* for transcription. Finally, we determined the impact of DHQ on cystic fibrosis patient health and its correlation to lung function. Taken together, our findings suggest DHQ is capable of activating PqsR as a redundant QS molecule, but may play a significant role against host cells during infection.

Chapter 1: Introduction to Pseudomonas aeruginosa pathogenicity, quorum sensing, and the role of DHQ.

1.1 Significance of *P. aeruginosa* infections

Pseudomonas aeruginosa is a motile, Gram-negative bacterium found in different soil and aquatic environments. In order to survive on various carbon and nitrogen sources, *P. aeruginosa* possesses a versatile metabolic system that sustains the bacterium even in nutrient-poor conditions. Although *P. aeruginosa* uses oxygen as a terminal electron acceptor for oxidative phosphorylation, *P. aeruginosa* contains three separate systems for acquiring nitrogen and synthesizing nitrate as an alternative electron acceptor under anaerobic conditions. Among the different environments colonized by *P. aeruginosa*, the bacteria can live as a single cell or in a complex community.

P. aeruginosa is an increasingly prevalent environmental and nosocomial pathogen. Intact immune systems often prevent *P. aeruginosa* colonization, but furnishing a surface to grow on or a compromised immune system can predispose patients to infection [1, 2]. Implanted medical devices provide a surface for *P. aeruginosa* to colonize, which leads to biofilm formation and infection [3, 4]. Ventilated patients are also susceptible to *P. aeruginosa* infections because the ventilator system generates a warm, moist environment for bacterial growth and subsequent access to anesthetized or damaged tissue [5, 6]. Although not widely publicized in the media, contact-lenses can also be colonized by *P. aeruginosa*, which can lead to corneal infections severe enough to result in blindness [7].

Overall, patients with compromised immune systems are most at risk to acquire a *P. aeruginosa* infection. Diabetic patients have poor circulation as a result of damaged blood vessels and tissues not efficiently replenished with cycled blood. Subsequently,

pressure ulcers and wounds from these patients are no longer adequately protected from

the immune system and subject to *P. aeruginosa* chronic infections [8]. *P. aeruginosa*

colonizes burn wound patients because the charred dermal layers provide a surface not

protected by the immune system [9]. These patients face overwhelming bacterial

infections, especially those who have wounds covering over 40% of their bodies. Due to

the ability of *P. aeruginosa* to resist antibiotic treatments, patients infected with *P.

aeruginosa* require intense therapies to overcome infection. The cost of treating *P.

aeruginosa* is on the rise worldwide, and in America, the annual cost of treating *P.

aeruginosa* infections is in the billions [10, 11].

Cystic fibrosis (CF) is an autosomal recessive genetic disorder and the most

common heritable disorder among Caucasians. CF patients possess a mutation in the

cystic fibrosis transmembrane conductance regulator (CFTR), which results in a

dysfunctional chloride channel and disrupted surface-liquid homeostasis [12]. Over 2,000

different mutations exist that can cause disease, but the most common CF-causing

mutation is a deletion of phenylalanine 508 (ΔF508) [13]. Normal lungs contain a thin

layer of mucus over the epithelial cells, which bind to inhaled microorganisms. Over

time, expelling or swallowed mucus prevents most pathogens from establishing an

infection. CF patients accumulate thick mucus in their lungs, which contains

macromolecules and salt build-up above the epithelial cells [14]. As a result of increased

mucus viscosity and decreased ciliary beat, patients with CF cannot effectively clear their

lungs. In the thick mucus, pathogens establish infections, gather abundant nutrients, and

avoid attack by host immune cells. Altogether, the paradigm of CF disease revolves

around three deleterious effects: decreased lung clearance, increased infections with microbial pathogens, and increased inflammatory response [15].

In the US, more than 30,000 CF patients must receive intense, life-long treatments that include airway-clearance respiratory therapy, steroids, and daily antibiotics to prevent severe infection and lung damage [16]. Many CF patients are now living close to forty years (median survival) as a result of new antibiotics and better supportive therapies [17]. Within the CF population, *P. aeruginosa* colonizes up to 80% of patients, of which, *P. aeruginosa* colonization is associated with earlier morbidity and mortality [18, 19]. CF patients often acquire *P. aeruginosa* later on in life and may be previously infected with other pathogens. During their teenage years, CF patients initially isolate *P. aeruginosa* from their lungs and harbor the bacterium for the duration of the their disease [20]. Patient exacerbations, which may be related to increasing *P. aeruginosa* colonization, contribute to lung damage and lower patient expiratory lung volume over time. Eradication of *P. aeruginosa* is a priority in the care of CF patients, but traditional antibiotic therapies have had little success in eliminating the bacterium after an initial positive culture or following chronic colonization.

The success of pathogenic *P. aeruginosa* is based on its ability to adapt and control the environment within the lungs [21]. Specifically, *P. aeruginosa* utilizes a variety of nutrients and form communities that adjust quickly to changes in the environment. *P. aeruginosa* also 'senses' and attacks other microorganisms competing for the same niche [22]. This competition is highlighted in CF sputum samples that have been enumerated for bacterial growth and high densities of the bacterium, upwards of 10^8

to 10^9 CFU/ml, demonstrating the ability to thrive in the lungs while facing the host

inflammatory response and daily antibiotic therapy [23, 24].

1.2 Mechanisms of antibiotic resistance in *P. aeruginosa*

Eradicating *P. aeruginosa* is difficult due to both intrinsic and acquired antibiotic resistance mechanisms. Of the intrinsic resistance mechanisms, those contained in the membrane play major roles. Porins in the outermembrane allow for the exchange of small hydrophilic compounds [25]. Antibiotics take advantage of the porins to gain access to the inside of the cell [26]. *P. aeruginosa* porins can be smaller (allow molecules <200 da to pass through) than normally found in Gram-negative bacteria such as *E. coli* (allow for molecules <500 da to pass through), which impede antibiotics from moving across the membrane [27, 28]. Efflux pumps in the membrane also play a role in antibiotic resistance because they secrete a variety of toxic compounds and make entire classes of antibiotics useless [29, 30]. These mechanisms within the membrane protect individual *P. aeruginosa* cells, while other resistance mechanisms are community-dependent.

A biofilm is a community lifestyle of bacteria that provides cellular heterogeneity and protection from chemical assaults in the environment. *P. aeruginosa* forms biofilms on surfaces through a highly coordinated process of initial attachment, maturation, and dispersal. An exopolymeric substance (EPS - eDNA, proteins, lipids, carbohydrates) covers the cells and reduces the penetration of chemicals into the biofilm. By encasing the bacteria in an exopolymeric shield and reducing cellular activity, biofilms increase antibiotic resistance up to 1000x compared to planktonic cultures [31]. Another resistance feature is differentiated cells with more active cells closer to the surface and dormant cells closer to the core. Dormant cells naturally resist antibiotics because those drugs often target active processes in bacteria, which are reduced within the biofilm [32,

33]. Biofilms also contain small colony variants, which are highly antibiotic resistant when compared to planktonic cells [34]. Bacteria form biofilms or macrocolonies in response to the host environment; therefore, bacterial infections naturally contain resistance mechanisms that may be only controlled by a functioning immune system [35, 36].

Although not completely understood as an antibiotic resistance mechanism, *P. aeruginosa* contains a large genome, > 6Mb, which allows the bacteria to have specialization and redundancy within essential systems [37]. Apart from the large genome, >9% of the genes encode for regulatory systems that provide metabolic diversity and quick adaptation to an environment [38]. An example is the *P. aeruginosa* redundancy of key systems such as the Pel or Psl systems to produce polysaccharides for different components of the biofilm [39]. When one system is chemically inhibited, such as the Pel polysaccharide system, then another system, the Psl polysaccharide system, is able to perform the same function to help the bacterium survive [40]. To this end, true biocidal targets of *P. aeruginosa* may be difficult to identity because essential targets may not be easily found.

Acquired mechanisms of antibiotic resistance include those via transduction and transformation of DNA from the environment. Extracellular DNA from diverse bacterial sources can code for resistance mechanisms that are taken up and expressed. Those bacteria possessing the resistance mechanism will then pass on the gene to future progeny. *P. aeruginosa* strains that have acquired metallo-β-lactamases have been found to cause hospital-wide outbreaks and, in general, are difficult to control [41]. Another acquired mechanism, which doesn't require acquisition of extracellular DNA, is the

process of generating DNA-damaging reactive oxygen species (ROS) [42]. Damaged DNA can be repaired, but often at the expense of mutations that generate new phenotypes expressed within the bacterial population. *P. aeruginosa* metabolism, phenazines, and certain alkylquinolones generate ROS in the environment. ROS-damaged DNA within genes such as RecA may result in different bacterial phenotypes due to the loss of a global regulator [43]. The ability to actively promote DNA mutations under stress, known as the "Insurance Hypothesis", generates new phenotypes that are optimal in certain environments [43].

Traditional antibiotic treatments target essential cellular structures and functions such as the cell wall and protein synthesis. Therefore, antibiotics place a selective pressure on bacterial communities for those that can survive the treatment. Drug-resistant mutants pass on the mutation through horizontal and vertical gene transfer leading to widespread antibiotic resistance. In order to subvert the selection of resistance mutants, a new approach to treatment should target bacterial mechanisms such as virulence factor production or biofilm formation that are not essential for growth. This strategy would reduce the selective pressure of targeting those bacteria that are susceptible and place more emphasis on the immune system to naturally clear an infection. Bacterial communication, which is dispensable for growth, is one such target for anti-virulence because of its link to virulence factor regulation and community behaviors.

1.3 Bacterial quorum sensing

In order to colonize a new environment or cause an infection, *P. aeruginosa* communicates through chemical messaging systems to coordinate collective phenotypes [44-46]. Communication between bacteria, known as QS, provides a mechanism to determine the cell density and composition of the bacterial community. Although community behaviors have been widely notice by microbiologists, the first published study of QS was determined from *Vibrio fischeri* and its symbiotic behavior with the Hawaiian Bob-Tailed squid [47]. In the light-organ of the squid, bacteria produced light only in high densities following replication throughout the day. Further work found that a small molecule and a novel two-component system regulated the light system. Following the initial discovery of light generation, QS was determined to regulate community phenotypes such as biofilm formation, swarming motility, and the production of virulence factors. QS mutants are unable to establish effective infections and have been isolated from sites of bacterial infection (discussed in a later section).

Instead of the planktonic cultures that were traditionally envisioned, QS provides the ability to act as a higher multicellular organism. Interestingly, cells within the community share public and private goods [48]. Communication via QS arranges a mechanism to differentiate roles within a community, synchronize specific phenotypes, and regulate mutants that arise in the system [49]. Differentiated cells can display altered secondary metabolite synthesis, metabolism, virulence factor production, and DNA transformation [50]. Synchronization of phenotypes provides temporal regulation so that

bacteria can regulate the production of virulence factors or other secondary metabolites only when they are needed [51]. This 'timed-regulation' can save energy and resources.

Bacteria communicate by secreting small signaling molecules into the environment. To this end, the concentration of the signaling molecules increases with cell density. Consequently, increasing extracellular concentration drives QS molecules back into the cell by diffusion. Once the intracellular concentration of a QS molecule reaches a threshold, the QS molecule activates a cognate transcriptional regulator to enact changes in transcription [52]. In order to prevent early activation of the transcriptional regulators, orphan transcriptional regulators bind specific QS molecules and reduce the available intracellular concentration of QS molecules [53]. QS system regulation is also accomplished through limiting precursors incorporated into the QS molecules. In order to retain some QS molecules that have been secreted, biofilms utilize the constituents of the EPS to transiently interact with the QS molecules so that those molecules remain with the biofilm [54]. This polymer greatly reduces the loss of secreted metabolites and also provides more intimate signaling opportunities.

Apart from cellular density, environmental stimuli can also affect QS systems [21]. For example, low iron and phosphate activate the necessary QS systems, which induce extracellular systems responsible for gathering essential nutrients [55, 56]. Oxygen concentration has also been shown to have a relationship between QS and metabolism in *P. aeruginosa* [57]. Specifically, low oxygen concentrations affect the nitrate reductase system, which subsequently down-regulates the Pqs system. Stress response is another environmental factor that can alter QS [58, 59]. *P. aeruginosa* utilizes

the RelA-SpoT stress response system to produce the alarmone ppGp(p), which selectively activates the different QS systems [60].

Quorum-sensing molecules exhibit either broad or specific activity (Fig. 1.1). Bacteria universally produce Auto-inducer-2 (AI-2), but the individual receptors for the QS molecule have only been studied in a few species. Small cyclic peptides synthesized from Gram-positive bacteria target various two-component adaptive response proteins to regulate competence, sporulation, and virulence factor production [61, 62]. Acylated homoserine lactones (HSL) are conserved among Gram-negative bacteria and target LuxR-type transcriptional regulators [63]. HSL systems regulate certain community phenotypes, production of virulence factors, and acquisition of nutrients. Finally, some bacteria possess novel QS systems that are only shared among related species. *P. aeruginosa* produces alkylquinolones, which are conserved only to closely related *Burkholderia species*. Bacteria that contain these novel QS systems can use their own language to control certain processes that may not be intercepted by other bacteria. To date, *P. aeruginosa* produces AI-2, two separate acylated-homoserine lactones, and a diverse group of alkylquinolones. Current work has also identified other non-canonical QS systems that are environment-specific [64, 65].

QS molecules also play roles apart from signaling. In Gram-positive bacteria such as *Staphylococcus aureus*, the small signaling peptides have potent antibiotic properties. An example from Gram-negative bacteria, extracellular homoserine lactones and alkylquinolones from *P. aeruginosa* antagonize the host immune response during an infection [66]. Further explanation of alkylquinolones as multifunctional molecules will be discussed in another section (1.5).

Auto-inducer 2
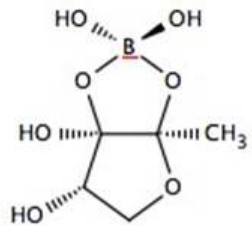
Produced throughout bacterial families.

Cyclic peptide thiolactone
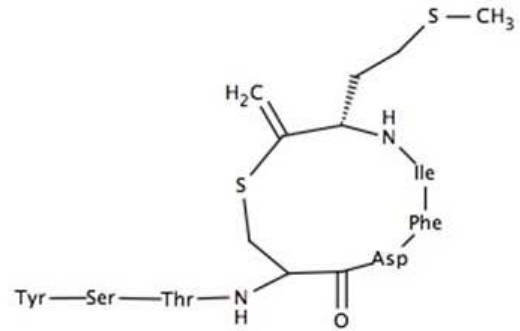
Conserved to Gram-positive bacteria.

N-acyl-Homoserine lactone
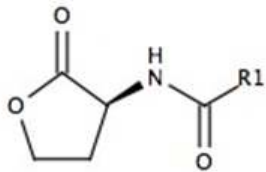
Conserved to Gram-negative bacteria

Modifications at R1 include:
- 3-oxoacly group
- 3-hydroyacyl group
- *p*-coumaroyl group

Alkylquinolone
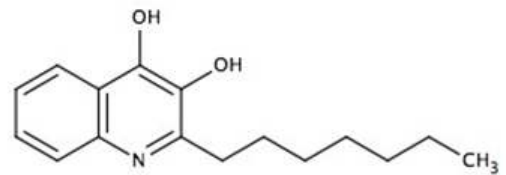
Conserved to *P. aeruginosa* and *B. thailandensis*

Figure 1.1 Diversity of QS molecules produced from bacteria.

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1.4 Major quorum-sensing systems in *P. aeruginosa*

In *P. aeruginosa*, there are three commonly studied QS systems; the Las, Rhl, and Pqs systems (Fig. 1.2). These QS systems control community phenotypes such as swarming, biofilm formation, and virulence factor production [67]. During replication to higher cellular densities, the QS systems function together in a hierarchal manner [68, 69]. The Las system, which is initiated earliest during growth, produces 3-oxo-C_{12}-Homoserine Lactone (C_{12}-HSL) and activates the LasR transcriptional regulator. An activated Las system synthesizes a protease and also induces the other QS systems [45, 70]. The Rhl system produces C_4-Homoserine Lactone (C_4-HSL) and activates the transcriptional regulator RhlR [58]. The Rhl system regulates the production of rhamnolipids, which aid in swarming and absorption of hydrophobic compounds [71, 72]. Secreted rhamnolipids also initiate apoptosis in neutrophils and reduce ROS in the environment [73]. Following a transposon mutant screen for genes regulated by the Las system, the Pqs system was discovered to be a novel system compared to traditional Gram-negative QS [74]. The Pqs system produces alkylquinolones that activate the transcriptional regulator PqsR (also known as MvfR). Signaling from the Pqs system controls production of virulence factors, iron chelators, and biofilm formation [75, 76]. Together, these three QS systems function together to control up to 12% of the genome and are essential for survival and community phenotype regulation [77, 78].

Interconnected QS systems in *P. aeruginosa*
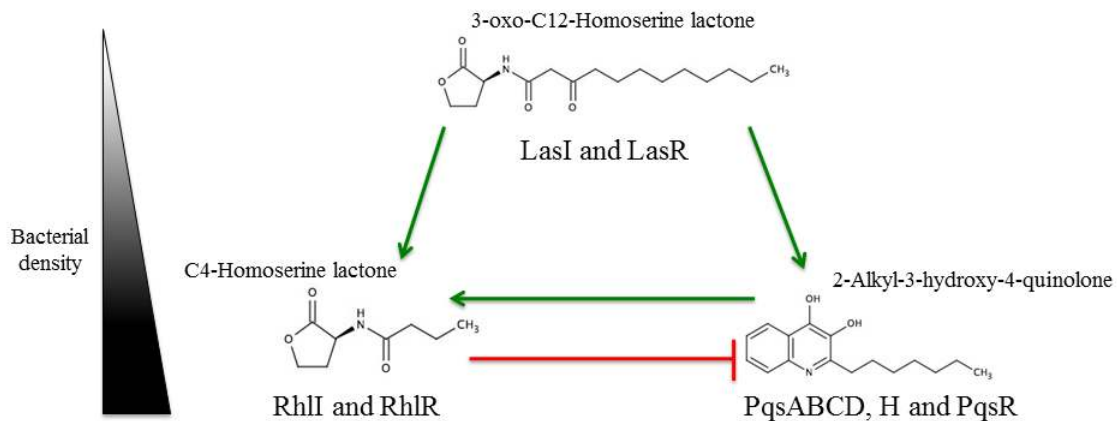
Figure 1.2 Interconnected QS systems in *P. aeruginosa*. Three core QS systems are both positively and negatively regulate each other. As bacterial density increases, there is a shift from Las-mediated transcriptional regulation to Rhl and Pqs transcriptional regulation.

Hierarchy of the QS systems in *P. aeruginosa* guides the production of virulence factors and community development in a new environment [21]. After initial growth, C_{12}-HSL signaling activates production of proteases that degrade peptides in the environment and affects host cell integrity [79]. The Las system also regulates biofilm formation following initial attachment to a surface. After Las system activation, the Rhl system participates in biofilm formation by forming water channels within the biofilm and also helps to secure fatty acids from the environment [80]. Rhamnolipids also block attack by the host immune system and participate in infiltration of airway epithelial cells [81]. The Pqs system contributes to biofilm formation by providing eDNA via autolysed cells and increases the production of virulence factors such as pyocyanin, hydrogen cyanide, and lectins [82, 83]. During an infection, the cascade of QS systems alters host immune cell reaction to bacteria, forms biofilms, and secures limited nutrients within the environment.

1.5. The Pseudomonas quinolone signal (Pqs) system

P. aeruginosa culture supernatants contain greater than fifty different quinolone molecules that vary in alkylation and modification on the quinolone ring [84]. Three alkylquinolones have garnered most of the attention for research; specifically, 2-heptyl-4-quinolone (HHQ), 4-hydroxy-2-heptylquinolone N-oxide (HQNO), and 2-heptyl-3,4-hydroxy-quinolone (PQS) [76]. Most of the other unmentioned quinolones exist in low concentrations and do not have specified function. Contrary to the HSLs, the quinolone ring is stable in different environments, but is affected by certain ring-hydrolyzing enzymes [85]. Those enzymes responsible for breaking down quinolones are not found in humans, which may allow quinolones to persist in the chronic wound environment for long periods of time.

The Pqs system consists of a transcriptional regulator, PqsR, and a five-gene operon, *pqsABCDE* (Fig. 1.3). Only *Burkholderia thailandensis* and *Burkholderia pseudomallei* possess homologous biosynthetic operons for quinolones, while no other *Pseudomonas* species use quinolones as a mechanism for QS [86]. PqsR belongs to the LysR-type transcriptional regulator family, which are structurally and functionally diverse DNA-binding proteins [87]. PqsR contains two domains; the C-terminus ligand-binding domain and the N-terminus DNA-binding domain. Crystal structures of PqsR bound to C9-PQS and a PQS-analogue identified two hydrophobic pockets that coordinate binding with the quinolones [88]. The peripheral hydrophobic pocket on the protein associates with the alkyl-moiety, while the interior pocket forms hydrogen bonds with the hydroxyl-groups and the hydrogen on the quinolone ring.

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Anthranilic acid, a precursor for tryptophan and quinolone synthesis, is formed from chorismate along with the enzymes PhnA and PhnB [89]. Three separate systems monitor anthranilic acid levels in the cell, which indicate the importance of this precursor to *P. aeruginosa* for maintaining a cellular pool to synthesize phenazines, catechol, tryptophan, and quinolones [90].

For quinolone synthesis, PqsA modifies anthranilic acid to form anthraniloyl-CoA, of which the product subsequently interacts with PqsD [91]. Following binding, CoA is released and a fatty acid is brought into the PqsD catalytic site for condensation with the anthranilate [92]. DHQ, the only terminate non-alkylated quinolone, is synthesized by PqsD from anthranilate and malonyl-CoA [93]. Alkylquinolone synthesis requires anthranilate condensation with longer-chain fatty acids such as β-keto fatty acid or octanoic acid and malonyl-CoA [94]. Condensation using PqsD, PqsB and PqsC form the initial alkylquinolone, HHQ. PqsD, PqsC, and PqsB share similar sequence homology and are described as FabH-type condensing enzymes [93]. However, PqsD is the only enzyme that contains the complete catalytic triad, Cys-His-Asn. Until recently, PqsE was not thought to participate in quinolone synthesis, but rather regulate the production of the virulence factor pyocyanin [95]. New data from Drees et al has now shown that PqsE may also be functioning as a thioesterase to increase precursors for alkylquinolone synthesis [96].
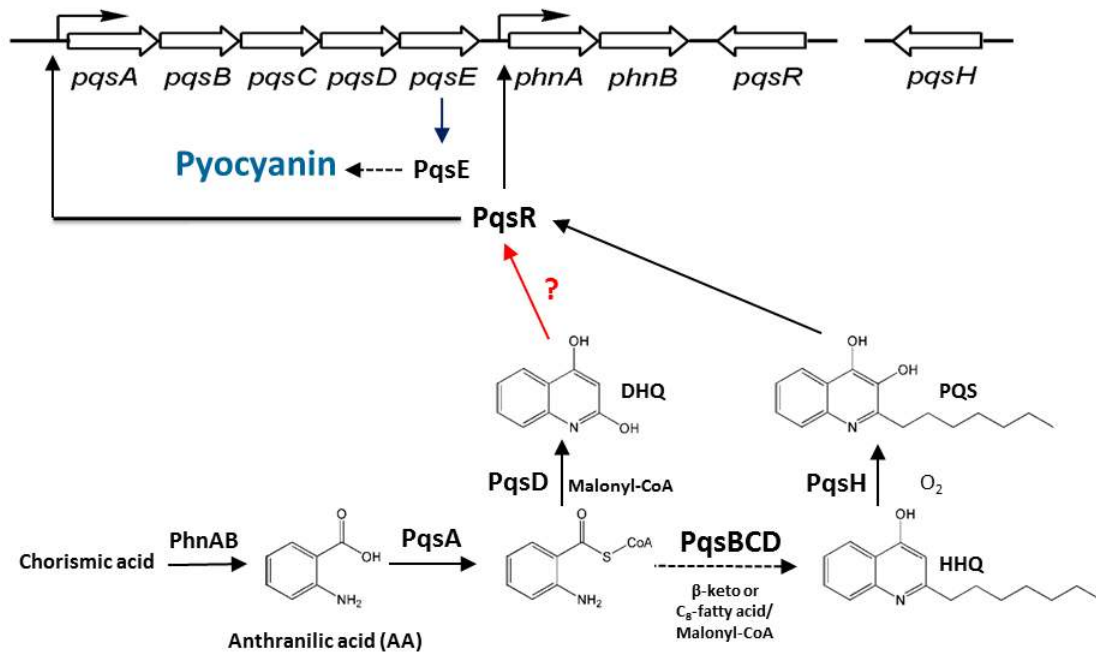
Figure 1.3 Synthesis and regulation of quinolones from the Pqs system. The red arrow indicates an undiscovered role for DHQ.

The *pqs* operon produces DHQ and HHQ, whereby HHQ is subsequently modified to generate other quinolones. PqsH synthesizes PQS, which adds a second hydroxyl-group to HHQ using oxygen [97]. Other *pqs* enzymes, such as PqsS and PqsL, are coded elsewhere on the genome and also modify the alkylquinolones [76].

Quinolone production can be expensive to the cell due to the requirements for precursors from fatty acid pools and anthranilic acid. However, *P. aeruginosa* requires an intact Pqs system to cause an infection [82, 98]. Quinolones play diverse roles for *P. aeruginosa*, but there are still several unanswered questions; specifically, the complete mechanism of quinolone synthesis and the function of a diverse set of quinolones.

1.6 Roles of the quinolones

Quinolones play both intracellular and extracellular roles for *P. aeruginosa* virulence, while only HHQ, HQNO, and PQS have established functions. HQNO is an N-oxide form of HHQ that is toxic towards Gram-positive bacteria [99]. HHQ performs similar functions compared to PQS, but is found at significantly lower concentration because it is a precursor for PQS that is actively turned over under aerobic conditions [84]. The major effector of the Pqs system is PQS (Fig. 1.4). As a signaling molecule, PQS regulates biofilm formation, swarming, and extracellular functions to control the environment [100, 101]. PQS signaling is also responsible for activating the Rhl operon, which synthesizes rhamnolipids that coat the surface and reduce the co-efficient of friction for swarming. Extracellular PQS can initiate cellular autolysis, which adds eDNA to the exopolymeric substance for biofilm formation and may have undiscovered roles in the PQS-laden blebs from the membrane [102, 103].

PQS-activated PqsR controls the production of virulence factors such as pyocyanin and hydrogen cyanide [98]. Pyocyanin, the characteristic blue-green pigment of *P. aeruginosa*, is a redox-active molecule that forms an electrocline surrounding a biofilm [104]. Due to its electrogenic potential, pyocyanin kills both eukaryotic and prokaryotic cells by creating reactive oxygen species [105]. Hydrogen cyanide has not been quantified from the environment during *P. aeruginosa* colonization, but was elucidated by infecting a model species with the bacterium [82]. Therefore, regulation of PqsR through PQS provides *P. aeruginosa* with an arsenal of virulence factors to unleash during infection.
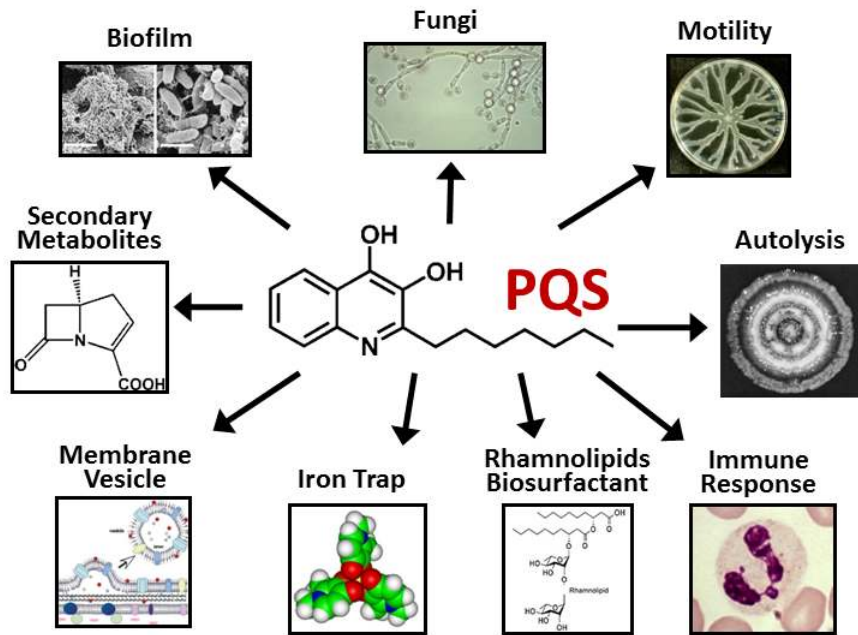
Figure 1.4 PQS is a multifunctional molecule and participates in several aspects of *P. aeruginosa* pathogenicity. PQS also functions to maintain *P. aeruginosa* within an environment and secure limiting nutrients.

Secreted PQS and HHQ antagonize immune cell signaling from host cells. Alkylquinolones inhibit the NF-κB pathway in macrophages causing a reduction in both IL-6 and TNF-α [106, 107]. PQS also blocks T-cell proliferation, which is important for the adaptive immune response by inhibiting dendritic cell antigen presentation [108]. Overall, control of the immune response is an important contribution to the ability of *P. aeruginosa* to cause chronic infections.

A unique trait to PQS is the sequestration of iron from the environment [109]. *P. aeruginosa* produces several iron siderophores, which trap iron from the environment [110, 111]. Three PQS molecules bind a single iron ion in the environment and reduce its availability to other organisms [109, 112]. It was proposed that PQS sequesters iron at the membrane of *P. aeruginosa* in order to increase the efficiency of siderophores [112]. Other quinolones possess similar structure, but do not appear to have the same ability to chelate. Although PQS has established chelation activity, further research is needed to understand the potential role of PQS-iron interactions during an infection.

Extracellular roles of the quinolones show how *P. aeruginosa* optimizes production of molecules for not only signaling, but also controlling the environment. However, most of the extracellular functions were elucidated using *in vitro* conditions and have not been demonstrated during an infection. Apart from pathogenicity, there are several links to quinolones and metabolic pathways. However, the metabolic flux from *P. aeruginosa* remains to be elucidated in conditions that represent an infection.

1.7 Mechanism of DHQ synthesis

Alkylquinolones HHQ and PQS have been extensively studied for their functions in *P. aeruginosa* pathogenicity. Critically, HHQ and PQS activate PqsR for transcriptional regulation of virulence factors and also provide extracellular functions. Of the quinolones produced, DHQ is the most abundant in *P. aeruginosa* planktonic culture [93, 113]. DHQ requires anthranilic acid, malonyl-CoA, and the enzymes PqsA and PqsD for its synthesis, which is significantly shortened compared to alkylquinolone synthesis. Given the truncated mechanism, increased DHQ synthesis is also perpetuated through more abundant precursors and no requirement for oxygen from the environment [114]. Because it has its own synthesis mechanism, DHQ is neither a precursor nor a degradation byproduct from the alkylquinolones, which highlight this molecule as unique and may play a novel role for the Pqs system.

Liquid chromatography and mass spectrometry can be used to quantify the different alkylquinolones from culture supernatant (Fig. 1.5). Supernatant from a *pqsA* KO-strain did not resolve any quinolone production. To identify mutants that altered DHQ synthesis, radiolabeled anthranilic acid was fed to wild-type and *pqs* mutants (Fig. 1.6). Following incubation of the *pqs* mutant strains, supernatants from the cultures were processed using TLC and monitored by autoradiography. Mutants of *pqsB* and *pqsC* maintained DHQ production, but did not produce the alkylquinolones. Mutants of *pqsA* and *pqsD* did not produce any quinolones, which indicated they were essential for all quinolone synthesis. The function of PqsA and PqsD for DHQ synthesis was confirmed in an *E. coli* strain, which maintained DHQ production using a plasmid containing both

pqsA and *pqsD*. DHQ production was also demonstrated *in vitro* with purified PqsA and PqsD. Both of the precursors, malonyl-CoA and anthranilic acid, sufficed for DHQ synthesis, while only activated forms of malonate were incorporated.

Virulence associated with DHQ production was initially visualized through inhibition of the yeast *Cryptococcus neoformans* (Cn) from a *pqs* mutant that was only capable of synthesizing DHQ [115] (Fig. 1.7). However, the DHQ-only mutant did not inhibit growth to the same degree compared to the wild-type. To determine toxicity towards host cells, DHQ was also incubated with mouse lung epithelial cells. DHQ treatment reduced epithelial cell number in a time-dependent and concentration- dependent manner [93]. These results demonstrated that the production of DHQ was associated with virulence factor production and induced extracellular effects.

Major quinolone species in culture supernatant

DHQ

HHQ

PQS

HQNO
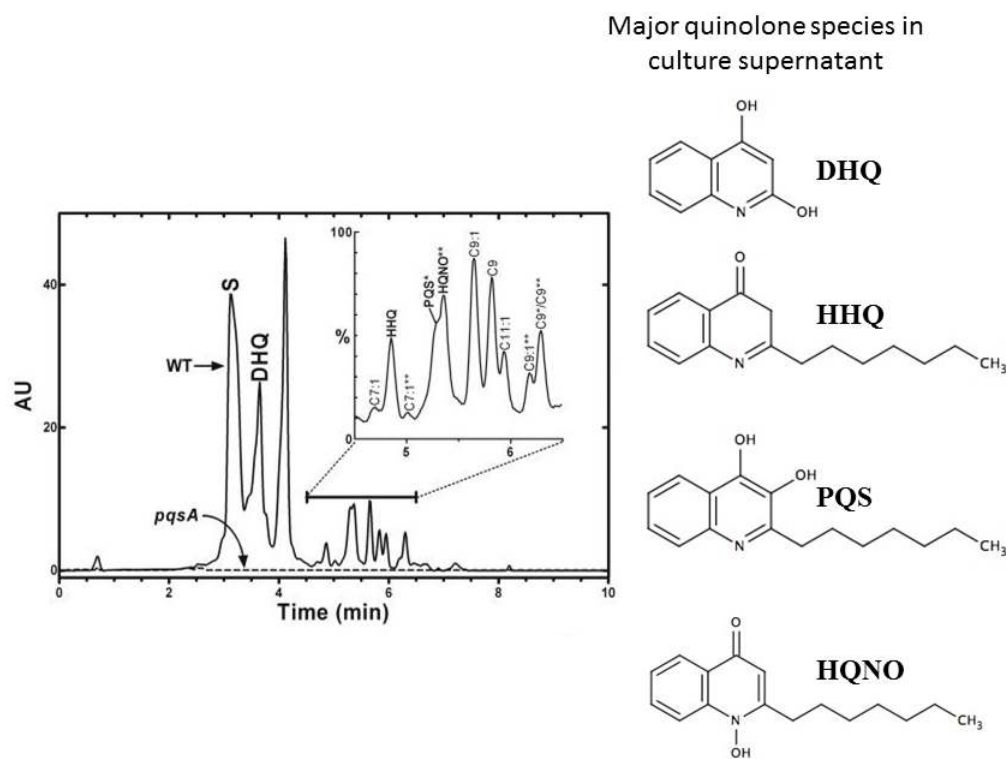
Zhang, JBC. 2008.

Figure 1.5 LC-MS trace of quinolones in *P. aeruginosa* supernatant. In wildtype supernatant, DHQ was the most abundant quinolone, followed by the alkylquinolones. From the *pqsA* mutant supernatant, no quinolones were quantified.

Anthranilic acid — HHQ/PQS — DHQ —

(Lanes: Medium, Wild type, *pqsA*, *pqsB*, *pqsC*, *pqsD*)
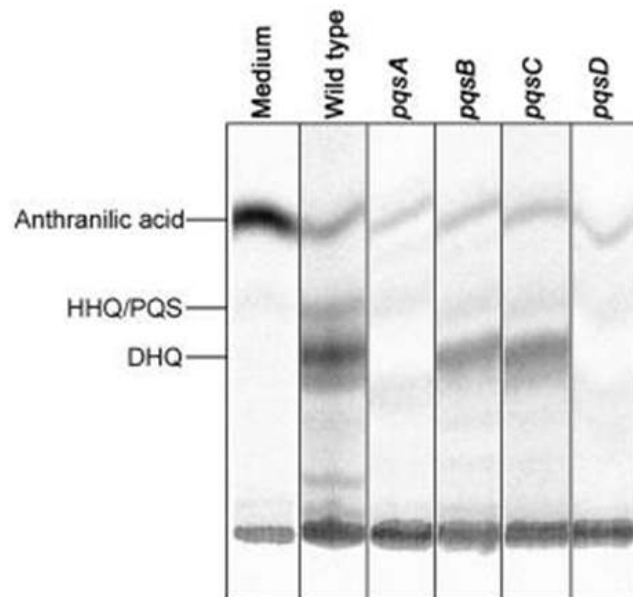
Zhang, JBC. 2008.

Figure 1.6 Mutants of the *pqs* operon displayed differences in synthesizing DHQ and the alkylquinolones. Only wild-type, *pqsB*, and, *pqsC* maintained production of DHQ, while *pqsA* and *pqsD* were devoid of quinolone production.

C. neoformans zones of inhibition
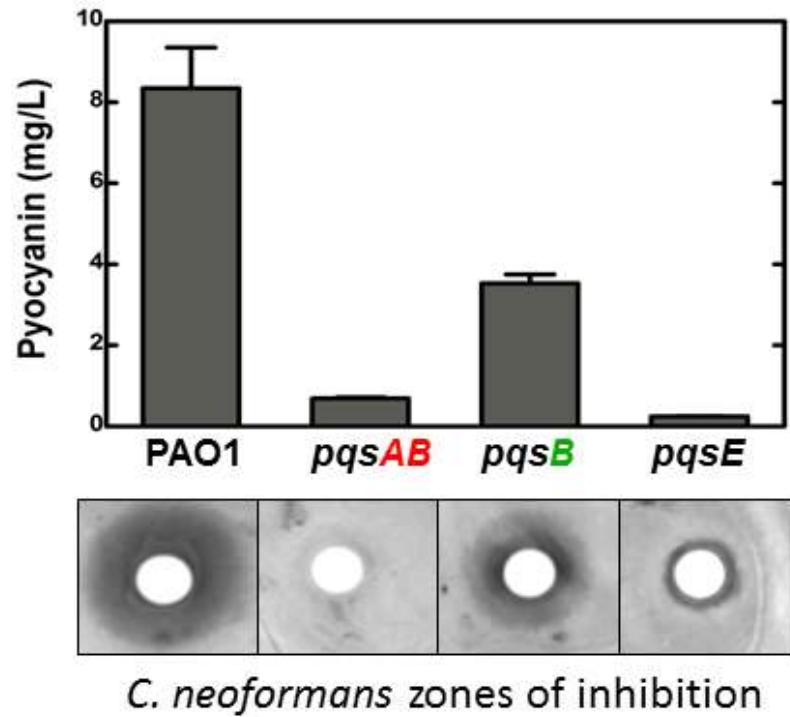
Figure 1.7 Pyocyanin negatively affects growth of yeast *Cryptococcus neoformans.* Lawns of *C. neoforms* were challenged against filter disks containing culture supernatant from *P. aeruginosa* PAO1 and *pqs* mutants. Pyocyanin from the *P. aeruginosa* strains were measured in vitro in normal laboratory conditions.

CF patients can harbor *P. aeruginosa* in their lungs for years. Therefore, CF patient sputum may contain secreted molecules that have accumulated over time. PQS has been quantified from sputum samples using various methods, while the composition of quinolones from the samples has not been assessed [116, 117]. Other studies of *P. aeruginosa* QS molecules from sputum have focused on the homoserine lactones. Struss et al. quantified the long chain HSL, 3-oxo-C_{12}-HSL, and identified an increasing trend in concentration prior to admission for an exacerbation and a decreasing trend following admission [118]. However, concentration of 3-oxo-C_{12}-HSL rebounded back to the levels found during stable disease after a week of IV antibiotic therapy. To date, no study to date has investigated all of the major QS molecules from *P. aeruginosa* and how the composition of the molecules may be related to disease severity. Because QS regulates virulence factor production and community behaviors, composition of QS molecules may predetermine certain negative events such as declining of lung function or an exacerbation. Also lacking are studies of quinolone composition on the basis of CF mutation, current antibiotic/lung-clearing therapy, and other cultured microorganisms.

1.8 Hypothesis and Specific Aims

The short-term goals of this study are to understand how DHQ production is involved in *P. aeruginosa* pathogenicity, the activity of DHQ with the transcriptional regulator PqsR, and dynamics of DHQ concentration in CF patient samples. Our long-term goal is to understand the fitness provided by DHQ production and its effect on chronic colonization of *P. aeruginosa*. No group has published a function for DHQ, only its high concentration in culture. Compared to PQS, DHQ does not require oxygen, which is especially limited within biofilms and the mucus of CF patients [119, 120]. Finally, DHQ possesses a similar structure compared to the alkylquinolones, which may allow for similar interactions with PqsR. The high concentration of DHQ warrants investigation into the extracellular effects against host cells. Overall, further understanding of the Pqs system makes it a good target for inhibition, but it is also important to understand the roles quinolones play during an infection to successfully block the communication system.

Hypothesis: DHQ plays a role in P. aeruginosa pathogenicity as a ligand for PqsR and controls host cell functions.

Specific Aim 1: Determine effect of DHQ production on *P. aeruginosa* pathogenicity. Alkylquinolones HHQ and PQS play a role in transcriptional regulation as ligands of the transcriptional regulator PqsR. Extracellular HHQ and PQS also exhibit anti-inflammatory effects. We infer DHQ plays a similar role in pathogenicity during an

infection, but may have specialized functions within certain environments. To investigate the mechanism of DHQ on virulence, we propose the following sub aims: **1)** determine virulence associated with DHQ production; **2)** determine effect of growth environment on synthesis and secretion of DHQ; and **3)** determine effect of DHQ on eukaryotic cells. We will use established methods for *Caenorhabditis elegans* infection to estimate total pathogenicity and quantify *in vitro* virulence factor production. We will also develop a model of *C. elegans* to investigate bacterial colonization in real-time. Liquid-chromatography with mass spectrometry (LC-MS) will be used to quantify DHQ and other QS molecules from samples. We will assess quinolone composition under the following conditions: in LB and cystic fibrosis mimic media (CFMM) using aerobic and anaerobic conditions. Finally, we will test the effect of DHQ on eukaryotic cells for viability and cytokine analysis by enzyme-linked immunosorbant assay (ELISA).

        Specific Aim 2: Determine effect of DHQ on PqsR for activating transcription of the *pqs* operon. HHQ and PQS activate PqsR to bind to the upstream regulatory site of the *pqsA* promoter. Transcription of the operon not only leads to virulence factor production, but also leads to further production of the quinolones by generating a positive feedback loop. To determine the role of DHQ on PqsR activation, we propose the following sub-aims: **1)** determine the activity of DHQ on transcription of the *pqs* operon through PqsR; and **2)** determine kinetic interaction of PqsR with DHQ. For these aims, we will quantify expression of *pqsA* in wild-type and *pqs* mutants using RT-PCR. We will also use a *pqsA'*-LacZ fusion reporter assay in *P. aeruginosa* strains and *E. coli* supplemented with exogenous quinolones. For the protein-ligand interaction

studies, we will use saturation transfer difference NMR (STD-NMR) that will identify the specific interactions of DHQ in the binding pocket of PqsR. Electrophoretic mobility shift (EMSA) *in vitro* assays will show the influence of DHQ on PqsR binding to the promoter region of *pqsA*.

Specific Aim 3: Determine DHQ concentration and composition of quinolones in CF sputum during stable and exacerbated disease states. Little is known about the dynamics of *P. aeruginosa* QS molecules during CF disease progression, especially since there are limited publications that report QS molecules in sputa. QS molecules provide a link to both bacterial density and virulence, which may be important in interpreting *in vivo* phenotypes. DHQ has not been quantified from CF sputum; however, PQS and homoserine lactones were previously identified in patient samples. Given that DHQ is secreted by *P. aeruginosa* in high concentrations *in vitro*, we predict that DHQ will be readily identified in both stable and exacerbated samples. The role of *P. aeruginosa* to initiate an exacerbation is not known, although declining lung function may be accompanied by increased colonization and density. To investigate the composition of quinolones during different disease states in CF patients, we propose the following sub-aims: **1)** determine QS molecule composition in stable CF patients and compare concentrations based on patient CFTR mutation, antibiotic treatment, lung volume, and organisms cultured from samples; and **2)** determine QS molecule composition during an exacerbation compared with stable levels. We will initiate a patient study of adult CF patients colonized with *P. aeruginosa* to obtain sputum samples

from clinic visits and hospitalized patients. QS molecules extracted from sputum will be

quantified using LC-MS.

Chapter 2: Role of DHQ in P. aeruginosa pathogenicity and extracellular effects against host cells.

2.1 Introduction

P. aeruginosa possesses a versatile metabolic system, which allows the bacterium to live in diverse soil and aquatic environments. People frequently come into contact with *P. aeruginosa*, but an intact immune system can block the bacterium from establishing an infection. However, immunocompetent and immunocompromised patients are still at risk for infection, particularly those who have diabetic or burn wounds, undergo surgery for an implantable device, or use contact lenses [121-124]. Patients with the genetic disorder cystic fibrosis (CF) are highly susceptible to *P. aeruginosa* infection, which is correlated with increased mortality [19, 125]. In CF lungs, *P. aeruginosa* replicates to high densities and forms antibiotic-resistant biofilms, a community lifestyle protected by exopolymeric substance [23, 126]. Treatment of chronic and acute *P. aeruginosa* infections is complicated due to numerous endogenous antimicrobial resistance mechanisms, including reduced outer-membrane porin size, increased expression of efflux pumps, and high mutation rate [127, 128].

Quorum sensing (QS) synchronizes group behaviors and is essential for *P. aeruginosa* to establish an infection [46]. The act of QS is comprised of releasing and uptake of signaling molecules that activate cognate transcriptional regulators at a critical intracellular concentration. Each QS system plays a role in community phenotypes and virulence factor production [74, 129-131]. The Las and Rhl systems, conserved to Gram-negative bacteria, produce acyl-homoserine lactones that activate cognate LuxR-type transcriptional regulators, LasR and RhlR [130, 132]. The Pqs system, conserved to *P. aeruginosa*, *Burkholderia thailandensis*, and *Burkholderia pseudomalia*, produces alkylquinolones that activate a LysR-type transcriptional regulator, specifically, PqsR in

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P. aeruginosa [86, 133]. Few quinolones have established functions for signaling, controlling the microenvironment, and facilitating biofilm formation; however, greater than 50 different quinolones are produced from the Pqs system [84, 133, 134].

Alkylquinolones are synthesized from the *pqs* operon and associated genes located elsewhere on the genome. The first step in quinolone synthesis requires PqsA to convert anthranilic acid, an intermediate of tryptophan biosynthesis, to anthraniloyl-CoA [91, 113]. The next step involves the transfer of the anthraniloyl-moiety to PqsD and the release of CoA [92]. Using simulations and surface Plasmon resonance, PqsD and the substrates display a ping-pong type mechanism, which is in agreement with other Claisen condensing enzymes [135]. PqsD condenses the anthraniloyl moiety with malonyl-CoA to form DHQ, the only terminate, non-alkylated quinolone species [93]. Formation of HHQ requires PqsD, PqsB, and PqsC to condense longer chain fatty acids with anthraniloyl-CoA [76]. PqsH converts HHQ to PQS in the presence of oxygen [97]. Unsaturations, variation in the length of the alkyl chain, and modification of the ring-substituted nitrogen generate the diversity of alkylquinolones [136, 137]. PQS and HHQ activate PqsR to initiate a positive-feedback mechanism that increases transcription of the *pqs* operon and further quinolone production [138, 139]. Among the genes regulated by PqsR for virulence, PqsE controls the production of pyocyanin, a potent redox-active virulence factor, through an unknown mechanism [75].

PQS plays numerous roles in *P. aeruginosa* pathogenicity [76, 137]. However, *P. aeruginosa* frequently colonizes hypoxic zones within CF lungs, which may reduce PQS production due to lack of oxygen [140, 141]. Deep sequencing has allowed for the

detection of anaerobic bacteria within the lungs of CF patients indicating the lack of oxygen found in certain parts of the lungs [142].

In comparison to other quinolones produced from the Pqs system, DHQ is the most abundant in *P. aeruginosa* planktonic cultures. DHQ also shares a similar structure with the alkylquinolones, which provides rationale in that DHQ functions similar to other established quinolones [93, 113]. Therefore, based on the high cellular expense of anthranilic acid to produce DHQ and numerous roles played by alkylquinolones to establish an infection, we hypothesize DHQ plays an important role in *P. aeruginosa* pathogenicity, potentially under hypoxic conditions. In this study, we investigated the role of DHQ on *P. aeruginosa* virulence, production of DHQ in different environments, and effect on epithelial cells and macrophages.

2.2 Results

Production of DHQ increased *P. aeruginosa* virulence in the *C. elegans* infection assay. In order to delineate function of DHQ from the other quinolones, we produced non-polar mutants of the *pqs* operon using the common lab strain PAO1 (originally isolated from a burn wound). Knockout of *pqsAB* resulted in abolished quinolone production, while knockout of *pqsB* or *pqsC* resulted in mutants that only produced DHQ. Previous results have indicated wild-type PAO1 required both virulence factor production and the ability to colonize the nematodes in order to kill *C. elegans* following several days of incubation (>200 h) [143-147]. We monitored the survival of *C. elegans* infected with PAO1 and *pqs* mutants to determine a role DHQ may play (Fig 2.1) [148]. None of the nematodes survived infections from PAO1, Δ*pqsB,* and Δ*pqsC* after 220 h of incubation, while 50% of the nematodes infected with Δ*pqsAB* remained viable. The reduction in virulence from the *ΔpqsAB* mutant was attributed to loss of quinolone production. In comparing survival trends, *C. elegans* survival incubated with PAO1 displayed the sharpest downward trend compared to the other strains. PqsB and PqsC are both essential for alkylquinolone synthesis but are not required to produce DHQ. Compared to the *ΔpqsAB* mutant, the trend of *C elegans* killing by the *ΔpqsB* and *ΔpqsC* decreased faster over time and also indicated that DHQ production maintained the same level of virulence in both mutants. Altogether, the survival trends demonstrated that production of DHQ was related to increased pathogenicity towards a model of bacterial infection.

Figure 2.1 Survival of *C. elegans* infected with PAO1 and *pqs* mutants. *P. aeruginosa* strains were incubated with *C. elegans* and worm survival was monitored daily for over 10 days. Each assay contained around 30 nematodes per strain and was performed in triplicate. Survival data of all of the assays were combined and plotted in a Kaplan-Meier survival curve and analyzed using the Log-Rank test *(significant * p = 0.05, ** p<0.05, ***p<0.0001)*.

None of the nematodes survived infections from PAO1, Δ*pqsB,* and Δ*pqsC* after 220 h of incubation, while 50% of the nematodes infected with Δ*pqsAB* remained viable. The reduction in virulence from the *ΔpqsAB* mutant was attributed to loss of quinolone production. In comparing survival trends, *C. elegans* survival incubated with PAO1 displayed the sharpest downward trend compared to the other strains. PqsB and PqsC are both essential for alkylquinolone synthesis but are not required to produce DHQ. Compared to the *ΔpqsAB* mutant, the trend of *C elegans* killing by the *ΔpqsB* and *ΔpqsC* decreased faster over time and also indicated that DHQ production maintained the same level of virulence in both mutants. Altogether, the survival trends demonstrated that production of DHQ was related to increased pathogenicity towards a model of bacterial infection.

Fluorescence-producing bacteria have been used to infect *C. elegans* in order to study the internalization of bacteria and their localization in different tissues [149]. To visualize colonization of *C. elegans* by the *P. aeruginosa* strains, we monitored nematodes infected with PAO1 and the *pqs* mutants carrying a plasmid that constitutively expressed GFP (Fig 2.2) [150]. After 4 h of co-incubation, GFP-expressing bacteria was present in the nematodes after 4 hours of incubation on the bacterial lawns. After 24 h, GFP-expressing bacteria were distributed throughout the intestinal tract of all the nematodes investigated. There was no overall qualitative difference between bacterial accumulation comparing the 4 and 24 hour time point. At 144 h, *C. elegans* infected with PAO1 and Δ*pqsB* showed higher GFP fluorescence than those infected with the Δ*pqsAB* mutant. Colonization was observed throughout 75% of the length of the nematodes that had been incubated with those strains.
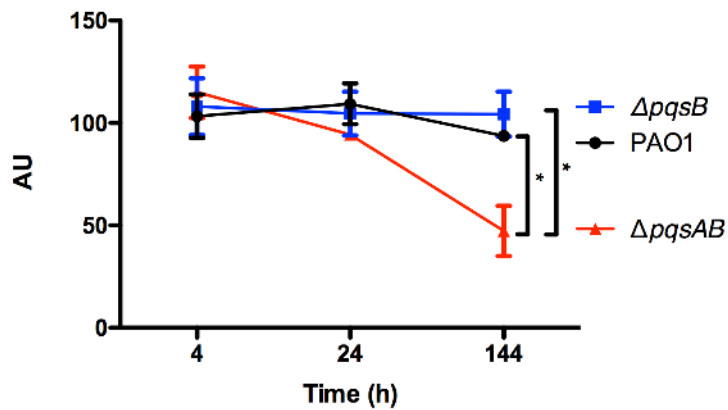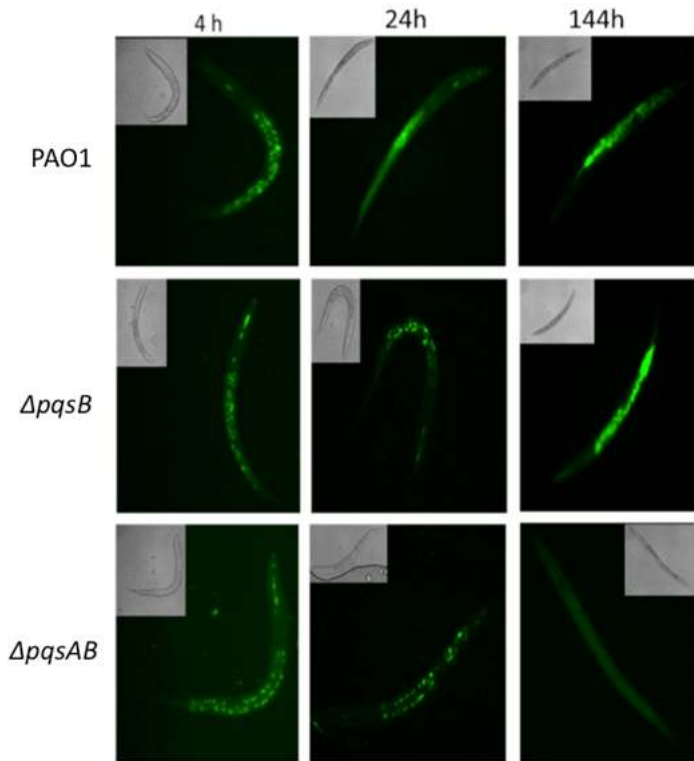
Figure 2.2 Real-time monitoring of *C. elegans* infected with *P. aeruginosa* strains expressing GFP. *C. elegans* were incubated on plates containing P. aeruginosa, which constitutively expressed GFP. At the indicated times, *C. elegans* was investigated for fluorescence at 435nm excitation and 485nm emission. Assay was completed in triplicate. Pictures shown were from the same assay. GFP fluorescence was quantified using ImageJ and graphed. Samples at 144 h were statistically compared using the student' t-test (* *p* < *0.05).*

Because of the disparity of fluorescence, we determined the difference in bacterial

colonization by comparing fluorescence of the strains following 1 second of excitation

versus 5 seconds of excitation. A 5 second excitation yielded oversaturated fluorescence

when analyzing nematodes infected with PAO1 and the $\Delta pqsB$ mutant, while minimal

fluorescence was detected from those infected with $\Delta pqsAB$. A 1 second excitation

resolved the oversaturation in PAO1 and $\Delta pqsB$ infected nematodes and demonstrated

only background fluorescence from nematodes infected with the $\Delta pqsAB$ mutant. The

144 h time point was important to show because it coincided with a sharp decrease in *C.*

elegans survival among the nematodes infected PAO1 and $\Delta pqsB$.

Over the course of an infection, *P. aeruginosa* displays several successive steps in

order to establish itself with the nematode. In our study, following several hours of

incubation, PAO1 and $\Delta pqsB$ both showed tissue invasion from the central gut-tract and

into the intestines and some gonadal tissue. The actions taken by the *P. aeruginosa*

strains appear to be initial attachment to the host tissue, protection from host anti-

infection mechanisms, and breakdown of tissues to move away from the central gut-tract.

Although fluorescence microscopy only visualized bacteria in different tissue sections of

the nematodes, comparison of the different strains indicated that those able to produce

DHQ maintained a similar infection progress compared to the wild-type.

Along with colonization of *C. elegans*, virulence factor production is also

important for nematode killing. Pyocyanin, a blue-green redox-active pigment, is a potent

virulence factor that is toxic towards other microorganisms and eukaryotic cells [115,

151-153]. Pqs enzymes PqsR and PqsE participate in the regulation of pyocyanin

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production. PqsR activates transcription of the *phz* operon, but the role of PqsE in pyocyanin production is not understood [154].

To demonstrate that changes in virulence were not due to defects in growth, we found all of the strains grew similarly regardless of their mutation (Fig 2.3). Next, we quantified pyocyanin from overnight cultures of PAO1 and *pqs* mutants grown in LB media. PAO1 produced the highest amount of pyocyanin followed by the Δ*pqsB* mutant. The Δ*pqsAB* mutant produced the least amount of pyocyanin. LB media is a rich media that contains an excess amount of nutrients to support high-density growth of bacteria. The media alone may help to potentiate pyocyanin production because of the low level found in Δ*pqsAB* mutant culture. However, this media does not represent of the environment colonized by bacteria during an infection. Nutrient conditions alter the bacterial phenotypes expressed; thereby, bacteria grown in LB compared to a less nutrient-dense media may demonstrate different phenotypes [155]. Therefore, we tested how growth in media more representative of an infection environment, cystic fibrosis mimic media (CFMM), would affect pyocyanin production. CFMM was chemically defined by LC-MS to quantify the abundance of carbon and nitrogen sources from cystic fibrosis sputa. Although the overall concentrations were lower, we found a similar trend in pyocyanin production among the strains compared to LB. Because of the loss of pyocyanin from the Δ*pqsAB* mutant, this demonstrates the requirement for quinolone production in the context of an infection.
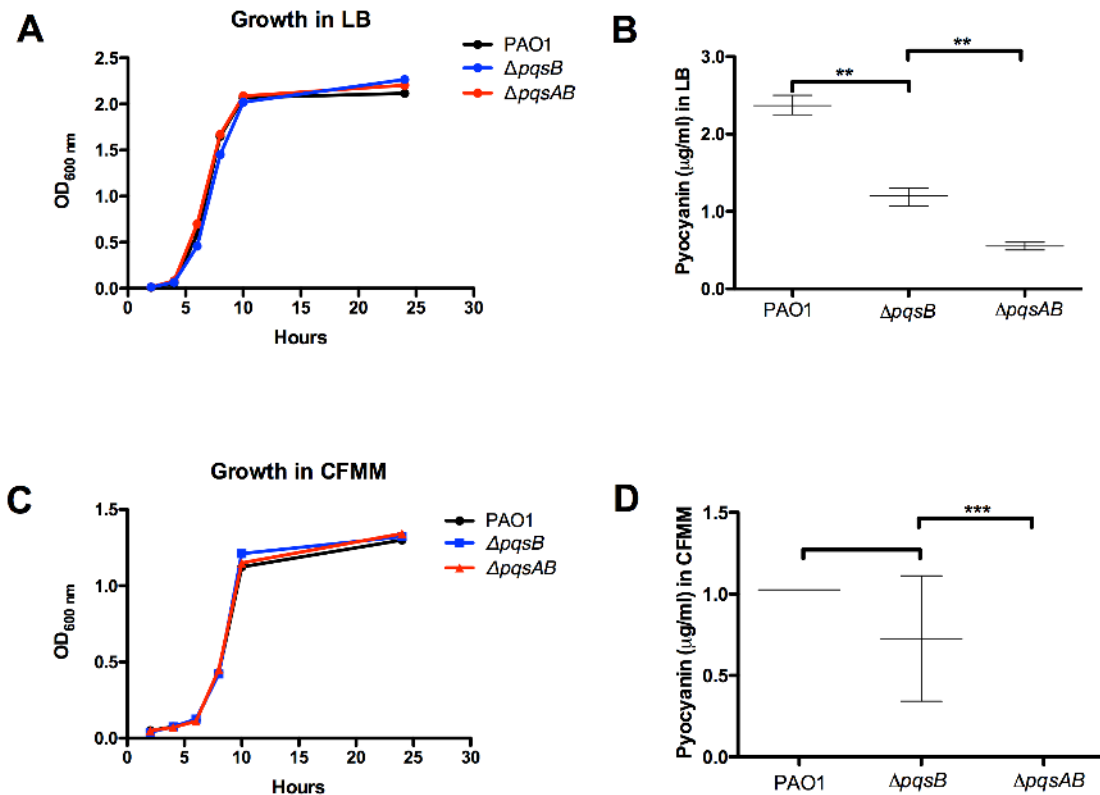
Figure 2.3 Growth and pyocyanin production by PAO1 and *pqs* mutants. (A) Growth curves of *P. aeruginosa* strains in LB over 24 h. (B) Pyocyanin was extracted from 18 h planktonic cultures grown in LB and quantified spectrophotometrically at 520 nm. (C) Growth curves of *P. aeruginosa* strains in SCFM over 24 h. (D) Pyocyanin was extracted from 24 h planktonic cultures grown in SCFM and measured the same as in LB. Experiments were performed in triplicate. C,D data is represented as box-and-whisker plots with the mean and range of the data. The data was statistically analyzed using a Student's T-test (** *p<0.005,* *** p < 0.0001).

Because pyocyanin production is regulated by Pqs signaling, we wanted to investigate if exogenous DHQ can rescue pyocyanin in the quinolone-null mutant. Culture supernatants from the different strains contain the quinolones in their natural form following secretion. Quinolones are not soluble in aqueous solutions; however, other secreted molecules may help solubilize the QS molecules. For the add-back experiments, we supplemented culture supernatants of the *P. aeruginosa* strains to *ΔpqsAB* cultures and incubated overnight. PAO1 and *ΔpqsB* supernatant supplied to the *ΔpqsAB* mutant rescued pyocyanin production, while the *ΔpqsAB* mutant with *ΔpqsAB* culture supernatant had little effect. Supplementation of pure DHQ back to the *ΔpqsAB* did not affect pyocyanin production, which indicated DHQ may have poor solubility in solution and/or require another factor to help cross the membrane. Overall, the importance of PqsR activation by the quinolones is underscored by the loss of pyocyanin.

The Pqs system activates both the Las and Rhl systems. Therefore, the effect of DHQ production apart from other quinolones on the concentration of virulence factors from the Las and Rhl systems are important to determine. Elastase and rhamnolipids are secreted from the cell and can be quantified from the culture supernatant. In the same growth conditions used for assessing pyocyanin production, production of DHQ did not significantly affect other virulence factor production (Fig 2.4). This result was surprising given the interconnected relationship of the QS systems found with PQS. The effect of the Pqs system on the other QS systems may need to be assessed during a time-course, which could resolve changes between the mutants.
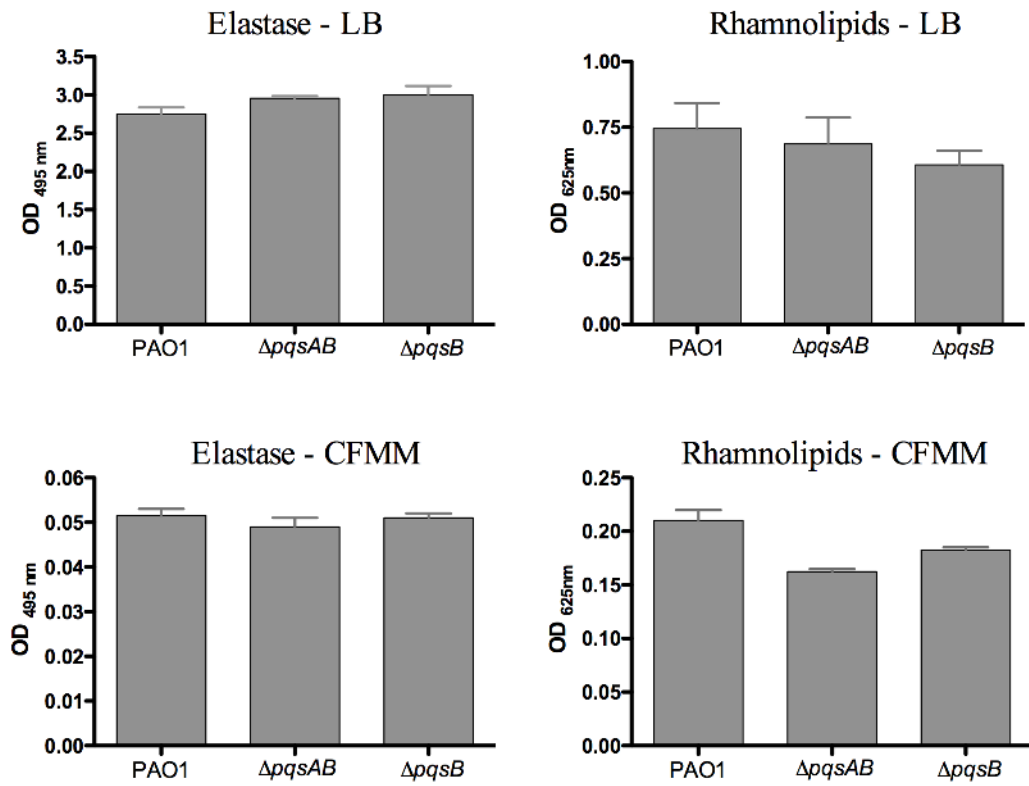
Figure 2.4 Elastase and rhamnolipids quantified from the *P. aeruginosa* strains grown in LB and CFMM. Samples were extracted from culture supernatants and measured at the indicated OD. Samples were measured in triplicate and the average and the error bars represent the standard deviation.

To date, long-term monitoring of the phenotypes from the QS systems has not been published. Biochemical modeling of the QS systems has indicated a circadian rhythm between activation of the Rhl system and the Pqs system, but has not been shown experimentally.

DHQ was the most abundant extracellular quinolone produced during aerobic and anaerobic planktonic growth. Our results have suggested that DHQ activates PqsR similarly to PQS and HHQ; however, conditions found during CF lung colonization may limit alkylquinolone production [120, 140]. Further complicating alkylquinolone syntheses are potentially altered fatty acid pools and lower cellular energy found in biofilms [156, 157]. Therefore, DHQ may play a role that is different from what has been traditionally considered for alkylquinolones during chronic colonization of the CF lung.

Strict anaerobic cultures of *P. aeruginosa* do not contain PQS and have fewer transcripts compared to aerobic cultures [158]. To determine how DHQ production might be affected during anaerobic growth, we quantified quinolones from PAO1 cultures grown aerobically and anaerobically (Fig 2.5). Increased C_4-HSL over 3-oxo-C_{12}-HSL concentrations in the anaerobic samples has been shown previously that QS molecule production was maintained without the presence of oxygen [119]. DHQ was the most abundant quinolone (65µM) produced under aerobic conditions in LB, but was less than then homoserine lactones (72 and 83µM).
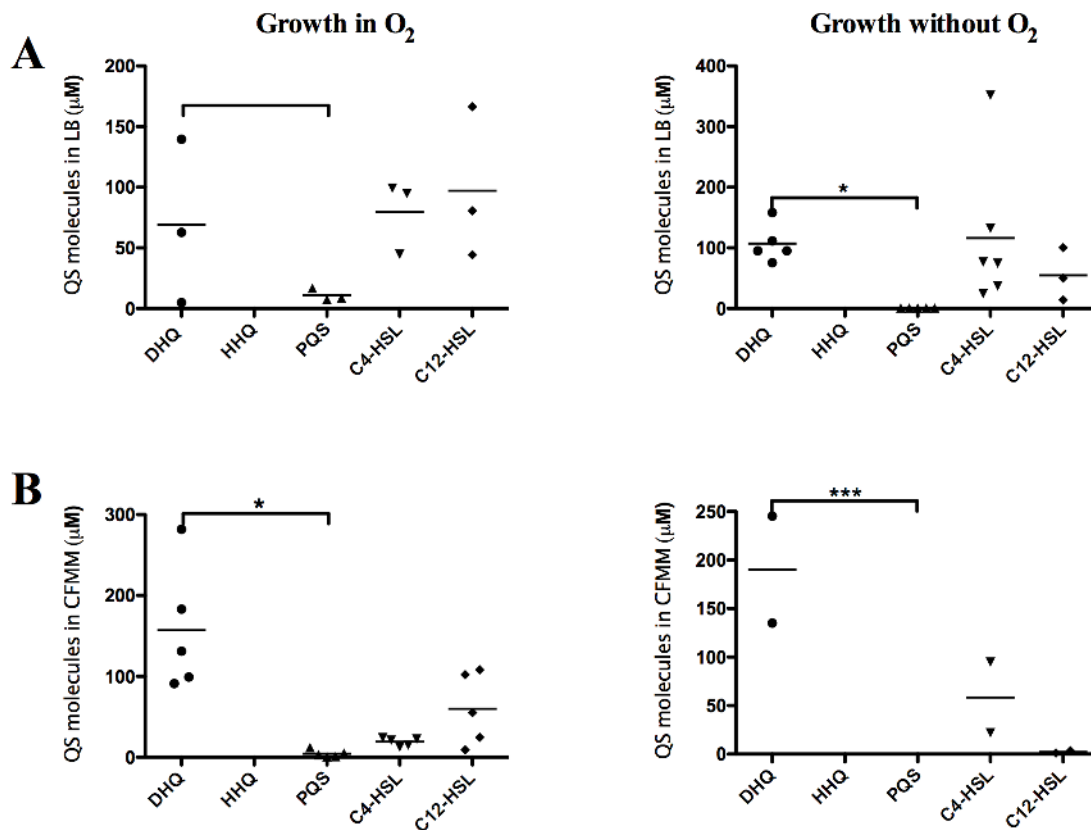
Figure 2.5 Quantification of extracellular levels of DHQ from aerobic and anaerobic PAO1 cultures. (A) PAO1 cultures were grown aerobically in LB and anaerobically in LB+400 µM sodium nitrate until the culture reached OD_{600} of 2.0. Culture supernatants were acidified with formic acid and QS molecules were analyzed using HPLC-MS. The concentrations were determined using calibration curves of QS standards. (B) PAO1 cultures in SCFM were treated in the same manner and grown aerobically and anaerobically to determine the concentrations of QS molecules. Data represented as scatter plots with the mean (central bar). The data was statistically analyzed using a Student's T-test (*$p = 0.05$, *** $p < 0.0001$).

When comparing the amount of DHQ between the different environments, anaerobic culture supernatants contained increased concentrations of DHQ compared to aerobic cultures. PQS was maintained at 10μM between both types of media; however, PQS was not detected under anaerobic conditions. To our surprise, HHQ was detected in aerobic and anaerobic cultures, but was below our limit of detection (<100 nM). Blinded controls verified that the extraction methods did not affect detection of the quinolones or homoserine lactones. Nutrient between the different types of media used appears to have an effect on QS molecule production because relative levels of the molecule shift between the different media regardless of oxygen availability. Comparison of the DHQ concentrations between LB and CFMM identified that DHQ production was slightly higher in the media most representing the CF lung environment, but was not statistically significant ($p>0.05$). The increase in DHQ and decrease in the alkylquinolones shows that precursors are possibly shunted over for DHQ production and less for HHQ. Importantly, DHQ was the most abundant QS molecule under anaerobic conditions in CFMM. This result indicates DHQ might have specific function within the Pqs system under anaerobic conditions; however, further research is needed to understand *P. aeruginosa* transcriptional regulation in hypoxic conditions and the dynamics of QS molecules within the mucous.

P. aeruginosa secretes PQS and HHQ through different mechanisms. PqsH, responsible for PQS synthesis, locates to the bacterial membrane and is thought to participate in membrane packaging of PQS into the blebs that are released into the environment. HHQ is not secreted through this mechanism, which was a surprise given

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the similarity in structure to PQS. An efflux pump, MexEF-OprN, was found to be responsible for HHQ secretion. The MexEF-OprN efflux pump is a RND-type efflux pump, which has a role in antibiotic resistance because those pumps are promiscuous with their target molecules. MexAB-OprM, another RND-type efflux pump, is responsible for ciprofloxacin resistance [159]. Although the fluoroquinolones have a similar structure to the quinolones, knocking out the Mex-EF-OprN pump resulted in increased pyocyanin production because the quinolones accumulate within the cells and activate the Pqs system (Fig 2.6). Loss of the negative regulator mexS slightly decreased pyocyanin, which is thought to be a result of increased efflux pump expression. MexT is a positive regulator of the MexEF-OprN pump; thereby, the loss of *mexT* also resulted in increased pyocyanin production. MexS negatively regulates the efflux pump and resulted in decreased pyocyanin production, presumably through the increased expression of the efflux pump. We hypothesized that the MexEF-OprN efflux pump would also be responsible for secretion of DHQ from the cell and found loss of *mexE* and *mexT* both resulted in decreased extracellular DHQ concentrations, while loss of *mexS* resulted in increased extracellular DHQ. Together, the data showed a relationship with DHQ secretion and a functional MexEF-OprN efflux pump.

It is important to note that the MexEF-OprN efflux pump is affected by the Rhl system, the Pqs system, and antibiotic selection (i.e. fluoroquinolones) [160]. There is currently no direct connection between the efflux pump and the Rhl system, but mutants of the pump dramatically affect rhamnolipid and swarming behavior. Antibiotic selection provides an interesting interaction because most wild-type *P. aeruginosa* strains possess a short insert within the positive regulator *mexT*.
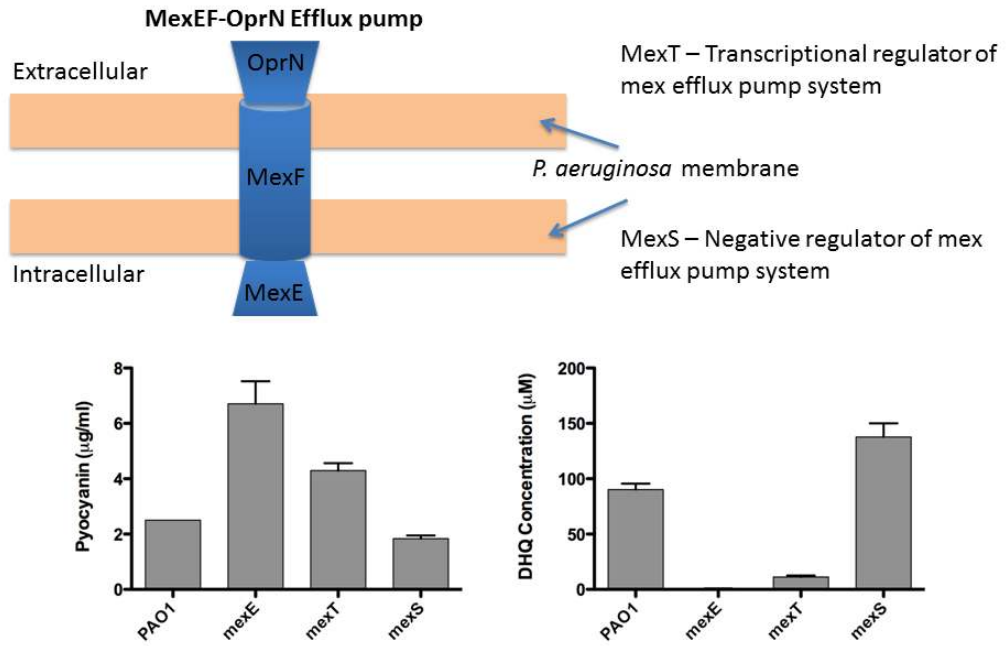
Figure 2.6 Effect of MexEF-OprN efflux pump on secretion of DHQ. Wild-type and transposon mutants were grown and assessed for pyocyanin production and extracellular DHQ. Samples were measured in duplicate. The average and standard deviation were plotted on the graph.

Following growth in the presence of antibiotics, mutants are selected that no longer contain the insertion and have increased expression of the efflux pump. As a consequence of the expression, the bacteria resist antibiotic treatment and quinolones secretion is increased. This new phenotype provides interesting context for *P. aeruginosa* during an infection and may have an impact on chronic colonization.

A hallmark of QS is the regulation of community behaviors, which involve the coordinated action of bacteria together. QS regulation proceeds through a stepwise process of signaling and transcriptional regulation. The outcome of the signaling cascade results in the bacteria acting like multicellular organisms. In *P. aeruginosa*, swarming and biofilm formation are well established for their QS regulation. Swarming bacteria move together across a surface. *P. aeruginosa* swarms coordinate both preparation of the surface by secreting rhamnolipids and flagellar movement in a uniform direction. Swarming on an agar surface shows *P. aeruginosa* move together as tendrils from a central inoculum. Signaling from *P. aeruginosa* prevents the tendrils from intersecting, even to the point of ceasing the tendril from moving in a direction that is too close to another tendril.

Swarming requires rhamnolipid section, which is regulated by the Rhl system. Rhamnolipids are composed of mono and di-rhamnose moieties coupled to C10 fatty acids. The Pqs system participates in regulation of the Rhl system by activating transcription of the *rhl* operon. In our study, we found that quinolone production was essential for swarming (Fig 2.7).
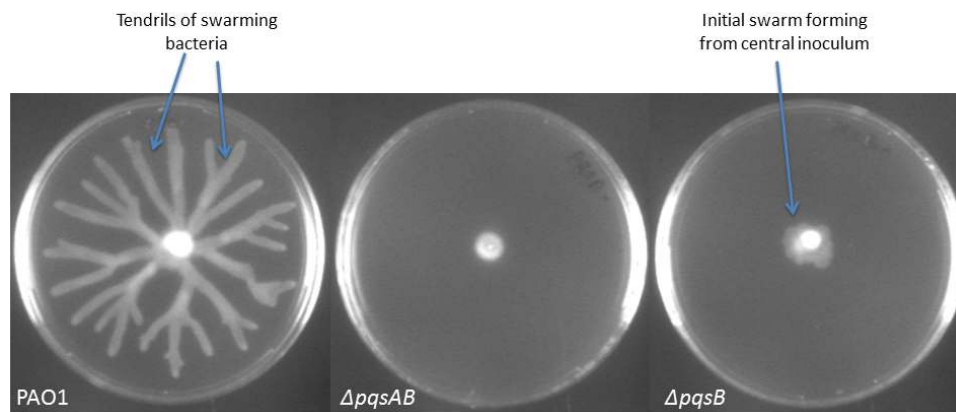
Figure 2.7 Swarming of wild-type and *pqs* mutants. Culture were inoculated at a central point on the agar and incubated overnight. Pictures of the agar plates were taken using a gel-dock with a positioned camera. Assays were performed in duplicate. Pictures were taken from the same assay.

Loss of quinolone production resulted in no movement from the central inoculum compared to growing tendrils of PAO1. Production of DHQ maintained some movement out from the central inoculum but no differentiated tendrils. These results demonstrated DHQ production was able to stimulate some activity from the Rhl system, but alkylquinolones may have a predominate role in cross-regulation for rhamnolipid production.

Biofilm formation also requires QS regulation to coordinate the sessile community phenotype. After initial attachment, a transcriptional signaling cascade initiates leading to maturation into a biofilm and dispersal of biofilm progenitor cells, which are all events that go on throughout the lifecycle of biofilm growth. Biofilms use an exopolymeric matrix that acts as 'glue' to hold the community together. The exopolymeric substance consists of both positively and negatively charged organic compounds such as polysaccharides, amino acids, DNA, and lipids. *P. aeruginosa* provides extracellular DNA for the matrix by lysing its own cells. This is known as autolysis and is essential for biofilm formation. PQS induces autolysis by acting as a oxidant and sensitizes the bacteria to ROS in the environment, which subsequently kills bacterial cells [136]. Using a long-term culture of the *P. aeruginosa* strains, we examined how the production of different quinolones affected autolysis (Fig 2.8). PAO1 cultures lost bacterial density overtime after initial log and stationary phase growth (after 24 h). This was previously established due to the effects of PQS production. Conversely, strains Δ*pqsAB* and Δ*pqsB* did not display as significant of losses in bacterial density and even displayed some rebounding over time. Because of the connection between autolysis and biofilm formation, we identified similar trends in biofilm formation among the strains.
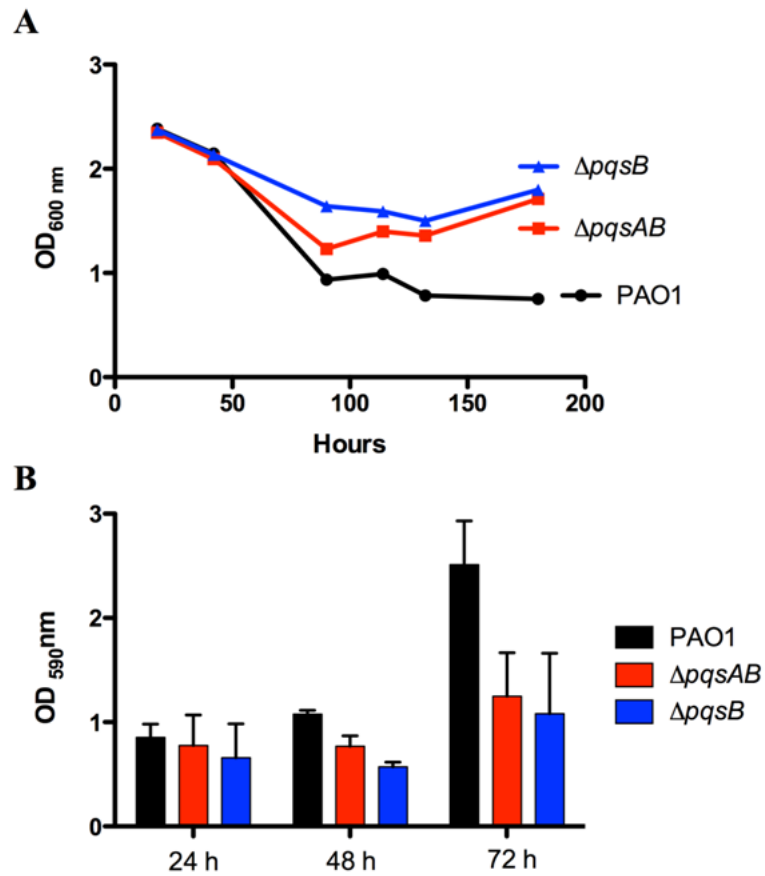
Figure 2.8 Long-term growth and biofilm formation of wild-type and *pqs* mutants. Cultures were measured following initial 24 h incubation at OD_{600} nm. The average from two assays was plotted per strain. Biofilm formation was assessed in 96-well plates over the indicated incubation times. Biofilm samples were measured in triplicate with the average and standard deviation listed.

PAO1 formed the largest biofilms after 72 h, followed by both $\Delta pqsAB$ and $\Delta pqsB$. Together, the trends in both autolysis and biofilm formation demonstrated DHQ was not involved in autolysis or the signaling involved to initiate those mechanisms.

Metabolic pathways oxidize and reduce diverse organic substrates in order to recycle or synthesize new precursors. As a consequence, electrons may accumulate and can be highly toxic if not neutralized. Pyocyanin is a redox molecule and is important for *P. aeruginosa* for virulence and electron shuttling [161]. To detoxify the cells and promote respiration under anaerobic conditions, pyocyanin accepts electrons and gives up them to other molecules that diffuse away from the cells [162]. The capacity to accept or give up electrons can be measured using cyclic voltammetry.

Data generated from CV experiments identify whether the molecule of interest is redox active, and if so, what are the reduction and oxidation peak potentials. To assess the oxidized and reduced states of the molecule, the potential of the working electrode cycles in reference to a second electrode. The oxidation and reduction peaks for pyocyanin were determined to be -84 mV and -294 mV, respectively. Redox properties of PQS and DHQ were also tested. PQS displayed a small oxidation peak compared to pyocyanin, but did not have a corresponding reduction peak. PQS was previously found to have a voltammetric response at 0.768V and 1.182 V using more sensitive apparatus [163]. DHQ did not show any changes in potential indicating that DHQ neither gained nor lost electrons under the conditions tested (Data not shown). We had hypothesized that the large extracellular concentration of DHQ may play a pivotal role protecting the cells from electrons during both aerobic and anaerobic conditions. In light of the long-term growth study, production of DHQ correlated with increased survival, but the protective

mechanism may not be through scavenging ROS. Rather, the mechanism may be from

not producing ROS. Therefore, the connection between DHQ and long-term survival

remains an important question.

DHQ reduced viability of host cells and antagonized cytokine production.

Zhang et al. showed mouse-derived lung epithelial cells treated with DHQ had reduced

viability in a dose-dependent manner [93]. In order to determine if DHQ had a similar

effect on other cell types, we also tested the effect of DHQ on A549 lung epithelial cells

and RAW264.7 alveolar macrophages for respiration and cell death (Fig 2.9). The MTS

assay examines cellular oxidoreductase activity, which can reflect metabolism of the cells

[164]. Sputum samples contained on average 150 μM DHQ from stable and exacerbated

cystic fibrosis patients and was included in the concentrations tested (Clinical data

covered in Chapter 4). RAW264.7 cells displayed increased sensitivity to DMSO;

therefore, we used lower concentration of DHQ for treatment. DHQ reduced the MTS

colorimetric conversion to formazan from both cell types in a dose-dependent manner,

but displayed a greater effect towards RAW264.7 cells. Total respiration was lowered by

25% from A549 cells and 50% for RAW264.7 cells at the highest DHQ concentration

tested. We predict that the lower total cell counts accounted for the lower A549

respiration in the MTS assay. This was supported by experiments with confluent cells

that did display reduced respiration (Data not shown). DHQ supplemented to RAW264.7

cells affected both cell replication and metabolic activity. The high concentrations of

DHQ secreted into the environment may be another mechanism used by *P. aeruginosa* to

protect itself from the host's immune system.
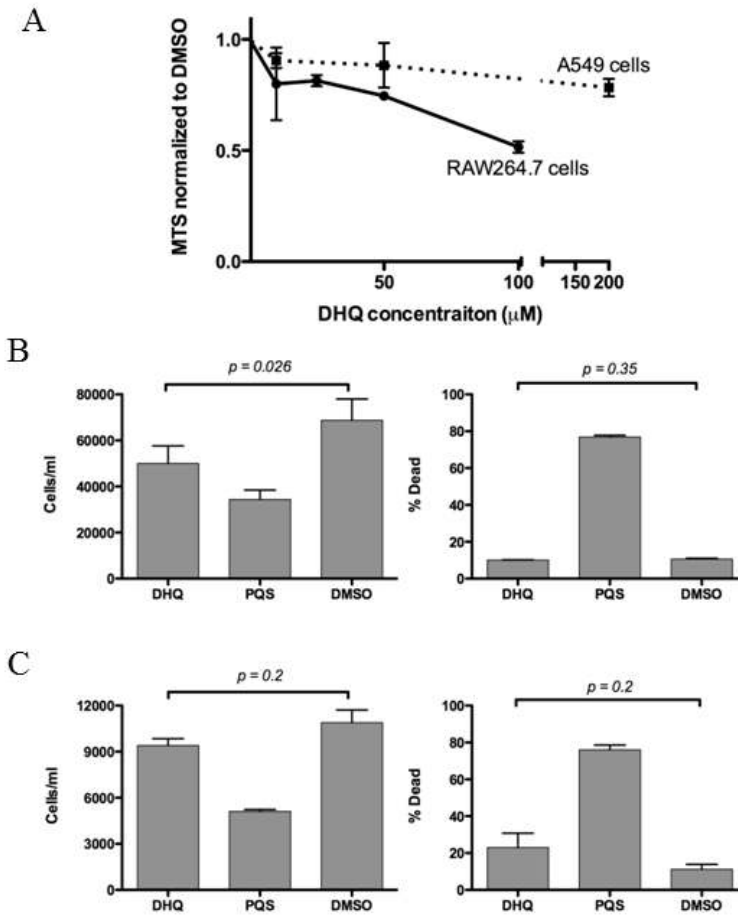
Figure 2.9 Viability of A549 lung epithelial cells and RAW264.7 macrophages treated with DHQ. (A) Cell viability was assessed using a commercial kit for respiration. (B) Total and dead cells were quantified from A549 lung epithelial cells follow treatment with 200 μM DHQ. (C) Total and dead cells were quantified from RAW264.7 cells treated with 100 μM DHQ. All Assays were completed in triplicate. The average and standard deviation are shown through column height and error bars. Student's T-test was used to compare samples.

Alkylquinolones HHQ and PQS were previously found to reduce IL-6 and TNF-α

production following treatment of macrophages with supernatant from wild-type and *pqs*

mutants. In the same study, the effect of quinolones on cytokine production identified

that HHQ and PQS reduced NF-κB binding function as a transcription factor [165].

It was also determined that PQS reduced IκB degradation, a repressor of NF-

κB[165]. Due to the similarity in structure, we predicted that DHQ may also lower

cytokine production through the same mechanisms as HHQ and PQS. In order to test the

effect of DHQ on cytokine production, DHQ was incubated with LPS-activated

macrophages (Fig 2.10). IL-6 production was depleted following increased

concentrations of DHQ, while the same treatment reduced TNF-α production more than

50%. Because of the antagonistic effect seen by DHQ on cytokine production, a

significant consequence of DHQ production may be the dramatic alteration of cytokine

signaling from host cells. The high extracellular concentration of DHQ supports this

hypothesis, but further clinical work remains in order to elucidate the role of DHQ during

disease.
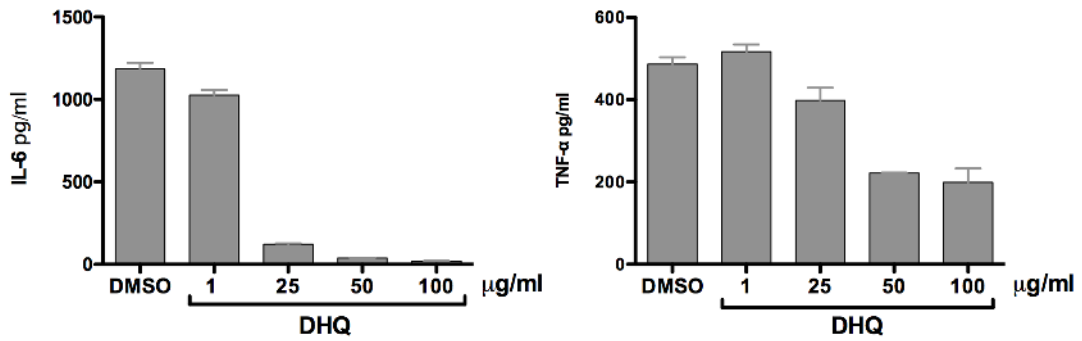
Figure 2.10 Cytokines measured from LPS-activated macrophages treated with DHQ. After treatment, extracellular aliquots were removed and tested using separate ELISA kits for both cytokines. Assays were conducted in duplicate with at least six replicates per sample. Concentration of cytokines was determined after generating a standard curve with purified TNF-α and IL-6. Bar graph shows mean and standard deviation of the samples.

2.3 Discussion

Our work provides the first evidence that DHQ is involved in *P. aeruginosa* pathogenicity and may have a specialized extracellular role. Those strains only able to produce DHQ maintained pathogenicity during infection of *C. elegans*. Specifically, the DHQ-only strain resulted in increased colonization in the nematodes and maintained some pyocyanin production compared to the quinolone-null mutant. DHQ was the most abundant quinolone regardless of oxygen availability and may be secreted via the MexEF-OprN efflux pump. We determined DHQ could participate in swarming, but may not have the redox properties to support autolysis and long-term biofilm formation. Finally, we found an extracellular effect of DHQ against host cells. DHQ reduced cell metabolism of both epithelial cells and macrophages, while having a strong effect on reducing cytokine production of macrophages stimulated with LPS.

P. aeruginosa and its host are involved in complicated interactions, which no infection model can completely resolve. *C. elegans* is easy to manipulate and has been previously studied for bacterial and host mechanisms that are important for infection [166]. However, *C. elegans* lacks tissue diversity and does not have an adaptive immune response. These properties limit *C. elegans* as a model for chronic infections, but *C. elegans* is useful as a model of acute bacterial infections. Results from studies using *P. aeruginosa* and *pqs* mutants have shown that a functional Pqs system is important for infection in *C. elegans* and other models [167, 168].

We compared mutants only able to produce DHQ to a quinolone-null mutant in order to determine the function of DHQ apart from the alkylquinolones. However, a

mutant that produces the alkylquinolones, but not DHQ would have been an important comparison to establish the functions DHQ play within the quinolone system. We were unable to identify residues in PqsD from the crystal structure that would block malonyl-CoA binding, yet maintain interactions with longer fatty acids. For our study, certain assays required the use of a quinolone-null mutant supplemented back with alkylquinolones.

Pyocyanin production was maintained in the *ΔpqsB* mutant compared to the quinolone-null mutant. Similarly, both PAO1 and Δ*pqsB* culture supernatant supplementation increased pyocyanin production when added to a growing *ΔpqsAB* culture. Exogenous supplementation of DHQ did not increase pyocyanin production in the *ΔpqsAB* mutant. We are currently studying the bioavailability of extracellular DHQ and whether rhamnolipids are important for solubilizing DHQ to pass through the membrane. In solution, multiple species of the quinolones may exist through tautomerization between the carbonyl-groups. DHQ is a planar molecule, but may be charged in solution, which could affect crossing of the bacterial membrane. Importantly, no quinolone has been studied for their ability to cross the membrane, but studies have identified the membrane of having a regulatory role with the QS systems [169]. Not only are the dynamics with the membrane not understood, but we are still attempting to understand the influence of the biofilm matrix and QS molecule retention. QS molecules possessing hydrophobic moieties transiently bind to amyloid fibers that can retain the QS molecules close to the biofilm [39]. DHQ does not contain a similar hydrophobic group and may be retained in a different manner within the matrix.

The MexEF-OprN efflux pump secretes HHQ; therefore, we hypothesized the pump would also secrete DHQ based on structural similarity. We found extracellular DHQ decreased and increased based on mutants of the Mex system that either blocked its function or increased its activity, respectively.

Anaerobic metabolism and regulation of the Pqs system is connected by the nitrate reduction system [158]. In our work, we showed that regardless of oxygen availability, DHQ was secreted in high concentration. During anaerobic growth, nitrogen assimilation systems provide nitrate as an alternative electron acceptor; however, regulators of nitrogen assimilation systems reduce activity of the Pqs system. We hypothesize that DHQ may maintain low activity of the *pqs* operon over time, or, DHQ may be stored in intracellular pools. Under anaerobic conditions, MexEF-OprN is up-regulated [101, 170]. In light of the secretion of DHQ through an efflux pump, anaerobiosis may be a trigger for a large release of DHQ into the environment. A large intracellular pool released under anaerobic conditions would explain why transcription of the Pqs system is reduced; yet we see an increase in DHQ concentration. Toxicity of DHQ towards host cells provides an extracellular role for DHQ to help *P. aeruginosa* maintain the environment under anaerobic conditions (24).

Community behaviors help to protect *P. aeruginosa* from environmental changes. We identified a strain that maintained DHQ production displayed some swarming compared to a quinolone-null mutant, but both strains appeared dramatically reduced compared to wild-type. This may be due to reduced cross-signaling of DHQ-PqsR with the Rhl system. Only PQS-PqsR has established function to activate the Rhl system and stimulate rhamnolipid production. After comparing the effects of DHQ on the community

behaviors, DHQ may not be primary initiator of multicellular phenotypes, but may maintain them under certain circumstances.

Redox chemistry plays an important role in metabolism and other exploited phenotypes from *P. aeruginosa*. DHQ did not display the same redox properties found with PQS as observed through autolysis, long-term biofilm formation, and cyclic voltammetry. Although redox of PQS is important for biofilm formation, autolysis requires close regulation so that the community is not disturbed by extensive cell breakdown. The anaerobic environment, which enriches for extracellular DHQ, may not require autolysis or that autolysis has already occurred for community development.

Our results help to show that different quinolone species may have specialized and redundant functions depending on the environment. Previous studies indicate that environmental and internal signals regulate QS and expand the functions of the molecules. The diversity of quinolones provides another level of complexity to understanding Pqs system regulation and activity, but may help to determine why *P. aeruginosa* is a successful human pathogen.

Chapter 3: DHQ activates PqsR for transcription of the pqs operon

3.1 Introduction

QS molecules target transcriptional regulators in order to synchronize community phenotypes and pathogenicity. In *P. aeruginosa*, homoserine lactones activate LuxR-type transcriptional regulators, while the alkylquinolones activate a LysR-type transcriptional regulator. The QS systems function together in a hierarchal manner, which may play a regulatory role between the QS systems [131]. The Las system is initiated earliest during growth and positively regulates both the Rhl and Pqs systems. Following the hierarchy, the Pqs system and Rhl system differentially regulate each other [87]. Although transcriptomic studies elucidated the changes in gene regulation following QS, synthesis of the QS molecules and interactions with target transcriptional regulators still remain an important area to investigate.

PqsR binds with alkylquinolones and is subsequently activated for DNA binding. LysR-type transcriptional regulators can function as both positive and negative regulators, but all share a similar size of about 300 amino acids and domain composition; a C-terminal ligand binding domain and a N-terminal DNA helix-turn-helix binding domain. LysR-type transcriptional regulators are often divergently transcribed from their target operon and require co-activators, which are part of a feedback loop [171]. PqsR is unique in that the regulator associates in the membrane until early stationary phase growth and is then released from the membrane into the cytoplasm [171]. Following release into the cytoplasm, a target ligand activates PqsR. Of the alkylquinolones, PQS is the most potent activator of PqsR; however, HHQ can also activate the transcriptional regulator [112]. Importantly, the preference of PqsR for PQS over HHQ includes activity with other target genes [112]. No other quinolone has been elucidated for their potential

to activate PqsR even though quinolones share a similar structure. Overall, both QS molecule production and a functional PqsR are essential for virulence in a model of infection [82, 172].

Illangova and Williams were the first group to publish a crystal structure of PqsR, 2.5 Å, of both a native and ligand-bound form [173]. This was a significant increase from the previous analysis at 3.25 Å [174]. From the structures, they elucidated two ligand-binding domains; Domain A and Domain B participate in coordinating alkylquinolone binding. While Domain A coordinates with the alkyl-moiety, Domain B encloses the bicyclic ring on either side and coordinate the molecule via two leucines and an isoleucine. Of the local amino acids, quinolones may form a hydrogen bond with Leu207.

Following activation, PqsR binds to a consensus region –45bp upstream of the *pqsA* transcription initiation site to coordinate the loading of RNA polymerase for transcription of the operon [175]. The -45 region contains the LysR-box, a symmetrical purine/pyrimidine dyad, which shares similar sequence homology to promoter regions upstream of *lasA* and *rhlA*. PqsR regulates transcription of over 200 genes, many of which are involved in pathogenicity [171]. Both PQS and HHQ activate PqsR similarly *in vitro*, but PQS was found to be more active in activating production of pyocyanin [138].

Our study seeks to understand the interaction of DHQ, the most abundant quinolone, with PqsR. We hypothesize DHQ activates PqsR similarly to HHQ and PQS for transcription of the *pqs* operon. Our results will determine whether DHQ plays a redundant role to PQS and if DHQ has a specialized function during conditions when alkylquinolone synthesis is reduced.

3.2 Results

Several studies and a crystal structure demonstrated PQS to be a ligand for PqsR; however, further work to determine the function of other quinolones remains an unanswered question. Quinolones share a similar structure, yet most quinolones remain undetermined because of the low concentration found in culture. DHQ is a novel quinolone with no previously determined function, while its concentration is highest among quinolones produced.

DHQ activated PqsR for transcription of the *pqs* operon. To determine how DHQ affects *pqs* operon transcription, we used quantitative real-time PCR (qRT-PCR) to monitor the expression of *pqsA* from PAO1 and *pqs* mutants grown to early stationary phase (Fig 3.1) ($OD_{600} = 1.8$). This phase of bacterial growth was selected because we had previously determined pyocyanin production at this bacterial density. Using the comparative threshold cycle numbers, levels of *pqsA* transcripts were 47% in the $\Delta pqsB$ mutant when normalized to PAO1. Comparatively, strains $\Delta pqsAB$, $\Delta pqsR$, and $\Delta pqsBR$ expressed *pqsA* at basal levels. We predicted the low transcriptional activity from the $\Delta pqsAB$ and $\Delta pqsR$ strains because it had been established that quinolone production and PqsR were required for transcription of the *pqs* operon [87, 138]. Transcription of *pqsA* from the $\Delta pqsBR$ strain, which produces DHQ but not PqsR, demonstrated that DHQ and PqsR are both essential for activity from the QS molecule.

Quinolone-activated transcription of the *pqs* operon further increases production of quinolones and pyocyanin. The later requires both PqsE and PqsR. Therefore, we examined if the mutants differentially expressed *pqsE*. Bacteria transcribe operons to create a poly-cistronic transcript.
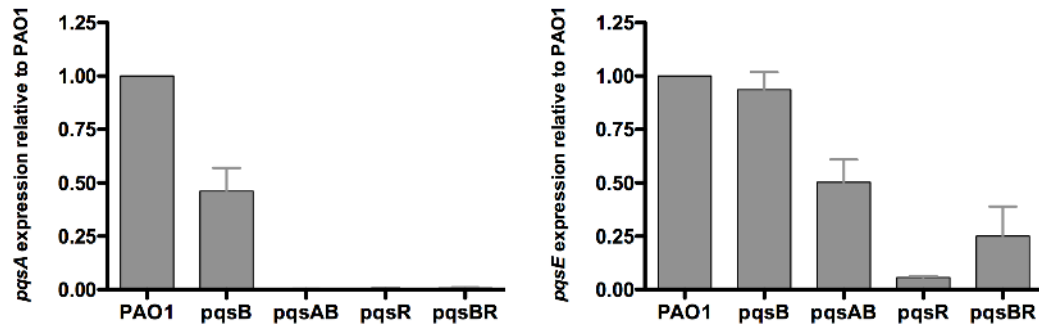
Figure 3.1 Expression of *pqsA* and *pqsE* in PAO1 and *pqs* mutants. Strains grown in LB until cell density reached OD_{600}nm of 1.8. House-keeping gene *rpoD* was used for normalization. Relative abundance of *pqsA* and *pqsE* transcript level determined using $\Delta\Delta C_t$ method and compared with PAO1, which was set at 1. Experiments were performed in triplicate with data shown as mean ± SE. Expression of *pqsA* and *pqsE* from the Δ*pqsB* mutant was significantly different ($p < 0.05$) compared to mutants of Δ*pqsAB*, Δ*pqsR,* and Δ*pqsBR* using the Student's T-test.

The genes are translated individually because the sequences contain their own ribosomal binding site, start site, and translational termination site. Although the general trend of expression mirrored what was found with *pqsA*, we were surprised to determine a difference in the expression of *pqsA* and *pqsE* from the strains. The Δ*pqsB* mutant maintained similar expression to wild-type, while the other mutants displayed reduced expression, although higher than previously expected. The increased expression of *pqsE* may indicate other systems are involved that regulates transcription at the end of the operon. However, due to the lack of pyocyanin from some of these strains, a threshold of expression may exist as another level of regulation. There may also be cofactors involved that are only available with quinolone production.

To further investigate the exogenous and endogenous activity of DHQ on PqsR, we performed promoter-fusion assays in *E. coli* and in the *P. aeruginosa* strains (Fig 3.2). The promoter-fusion construct contained the upstream regulatory element (-500 bp) of *pqsA* fused with the coding region of LacZ [176]. The construct also contained a copy of the *pqsR* gene, which was controlled by a *tac* promoter. DHQ supplemented to *E. coli* containing the reporter construct displayed a dose-dependent increase in LacZ activity. However, activity from 100 μM DHQ was significantly less compared to 1 μM PQS treatment. We also tested the effect of endogenous quinolone production on LacZ expression. DHQ from the Δ*pqsB* mutant was sufficient to activate LacZ activity similar to wild-type, while the Δ*pqsAB* strain displayed minimal activity.
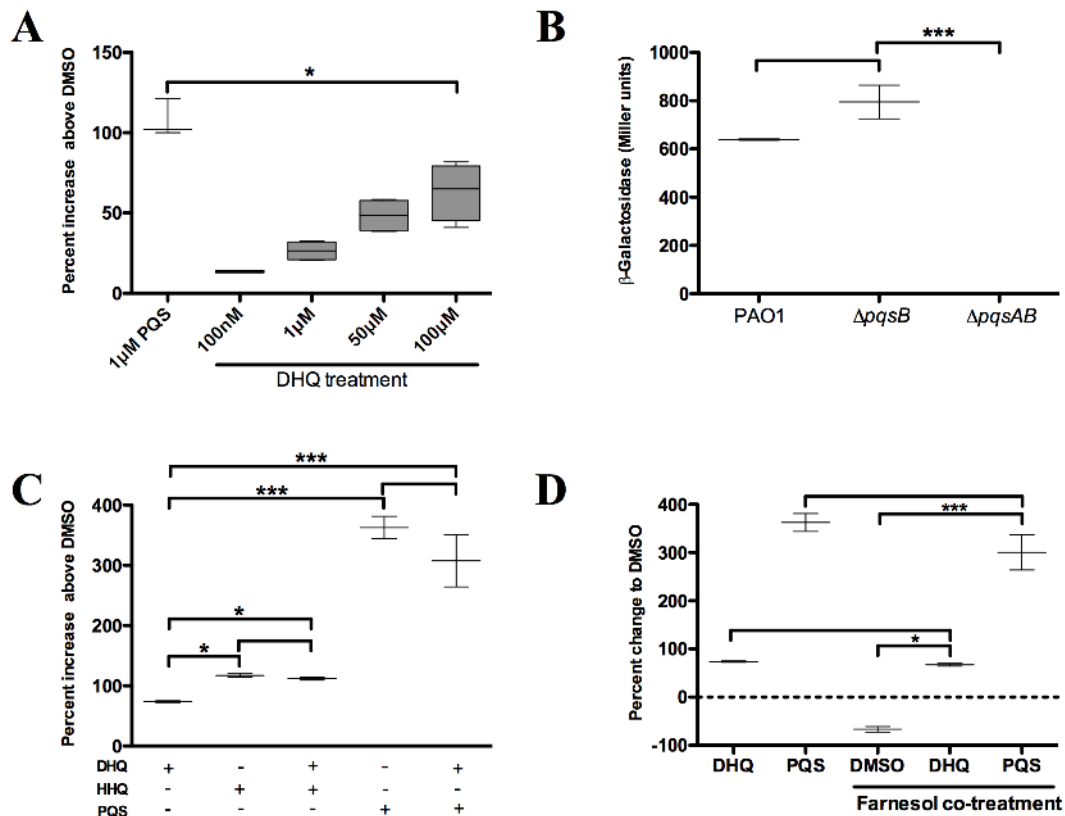
Figure 3.2 Reporter assay of PqsR activity in *E. coli* and *P. aeruginosa*. (A) DHQ supplemented to *E. coli* carrying the reporter construct pEAL08-2. LacZ activity in cells treated with DMSO was set as the baseline (1μM PQS – 100 μM DHQ, *p* = 0.013). (B) Overnight cultures of PAO1 and *pqs* mutants containing the reporter plasmid were used to determine the effect of endogenously generated DHQ on PqsR activation of *pqsA* promoter (no activity from *pqsAB* mutant). (C) Co-supplementation of 100 μM DHQ with 30 μM HHQ or 30 μM PQS in *E. coli* reporter (HHQ – HHQ/DHQ, no sig. difference; PQS – PQS/DHQ, no sig. difference). (D) Competition of 100 μM DHQ or 30 μM PQS with 250 μM farnesol in the *E. coli* reporter (DMSO/farnesol – DHQ/farnesol, *p* = 0.0023). Data gathered from at least duplicates of independent experiments and represented as mean ± SE. Data was statistically analyzed using the Student's T-test.

Due to potential differences in PqsR affinity for DHQ and PQS, we chose to look at the interactions of DHQ with HHQ and PQS co-supplemented to the *E. coli* reporter system. DHQ combined with either HHQ or PQS did not significantly alter LacZ expression compared to HHQ or PQS alone. No change in activity following co-supplementation may indicate that PqsR has significantly higher affinity for the alkylquinolones compared to DHQ.

Various fungal species produce farnesol as a quorum-sensing molecule [177]. Farnesol was discovered to be an antagonist of PqsR activity leading to decreased transcription and pyocyanin production in *P. aeruginosa* [176]. In the *E. coli* reporter system, farnesol reduced LacZ level below the background activity that was established by vehicle treatment. DHQ supplemented with farnesol rebounded activation of the reporter indicating DHQ out-competed the inhibitor of PqsR. The increase in activity was similar to what was found when PQS was supplemented with farnesol.

Taken together, endogenous transcription of *pqsA* from PAO1 and *pqs* mutants and activity from exogenously supplemented DHQ supplemented both show DHQ is able to activate PqsR for transcription of the operon.

In vitro assays demonstrate DHQ interacts directly with PqsR to bind with the promoter of *pqsA*. Few studies have investigated the direct interactions of PQS with PqsR until a crystal structure was solved [88, 139, 178]. Using this structure, we performed *in silico* docking of DHQ with PqsR to determine if DHQ interacted with similar amino acids compared to PQS (Data not shown). Modeling revealed DHQ potentially formed hydrogen bond to coordinating amino acids Ile236 and L208 similar to PQS, while also making novel hydrogen bonds with Ser196 and Glu194. Because DHQ

78

does not possess an alkylation, we did not observe any coordination to the second hydrophobic pocket identified with the alkylquinolones.

To look at direct binding of activated PqsR with the promoter region of *pqsA*, we used the electrophoretic mobility shift assay (EMSA) with biotinylated-*pqsA* (Bio-*pqsA*) supplemented to PAO1 and the *pqs* mutant strains (Fig 3.3). Similar experiments identified the interaction of PqsR with the *phn* operon sequences, so we also sought to confirm the interaction with DHQ as the co-activator [172]. Only PAO1 and Δ*pqsB* cell-lysates slowed *pqsA* migration through the gel, while the Δ*pqsAB* and Δ*pqsR* mutants displayed no interaction with the probe. This result confirmed that factors specific to a strain that produced PqsR and DHQ were required to interact with *pqsA*. Addition of unlabeled *pqsA* to PAO1 and Δ*pqsB* cell-lysates increased the free *pqsA* probe demonstrating the interaction was specific for *pqsA*.

Because other systems within *P. aeruginosa* may affect the activation of PqsR for DNA binding, we recombinantly expressed PqsR in *E. coli* Rosetta and monitored the interaction of the *pqsA* probe with the cell-lysate. *E. coli* does not possess a homologous quinolone system; therefore, the bacteria can be used to document the interaction with the non-native system. Incubation of PqsR-containing lysate with exogenous PQS or DHQ initiated binding to *pqsA*, but not with DMSO alone. Therefore, a quinolone was required to activate PqsR for binding to the target region of *pqsA*. The shifts of the probe were compared to a Rosetta strain containing a His-SUMO tag only, which would show any non-specific interactions. The activation of PqsR only in the presence of quinolones indicated a specific interaction was involved in order to initiate QS and transcriptional regulation.
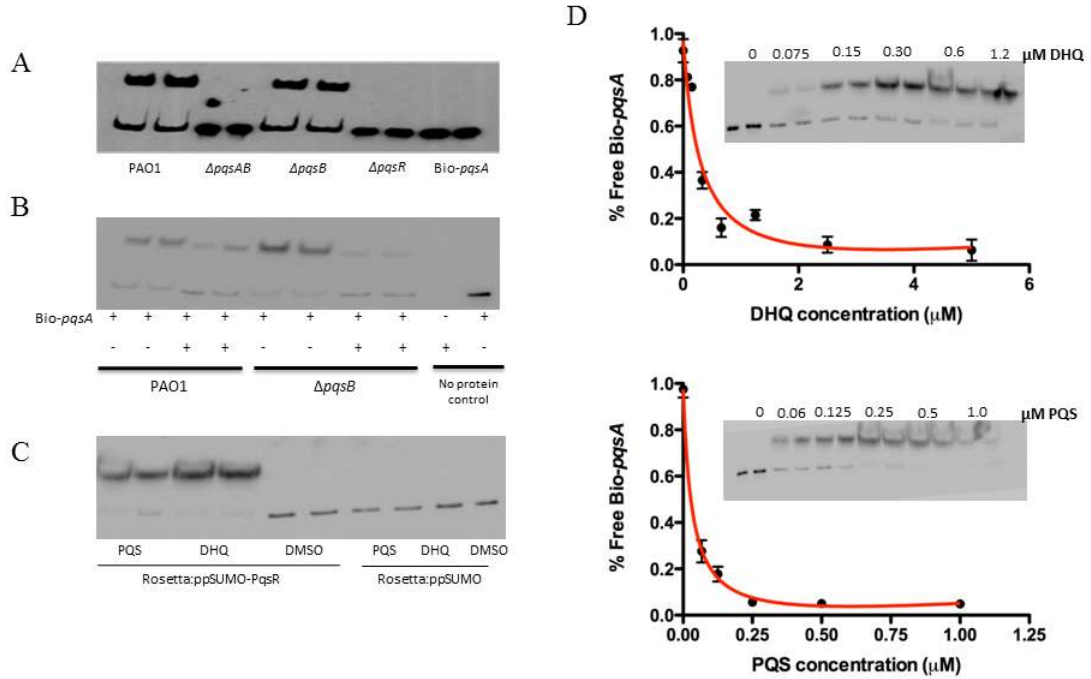
Figure 3.3 Electrophoretic mobility shift assay with *pqsA*. (A) EMSA assays with Biotinylated-*pqsA* (Bio-*pqsA*) probe incubated with cell-lysates of PAO1 and *pqs* mutants. (B) The interaction was specific for *pqsA* promoter as unlabeled-*pqsA* competed with Bio-*pqsA* probe. (C) Both DHQ (200μM) and PQS (30μM) promoted the interaction between recombinant PqsR with Bio-*pqsA*. (D) DHQ (0-5 μM) and PQS (0-1 μM) promoted recombinant PqsR binding to Bio-*pqsA* probe in a concentration dependent manner. Quantification of free Bio-*pqsA* was performed using ImageJ software and non-linear curve fitting was done using GraphPad Prism software to determine the apparent Kd of PqsR for DHQ and PQS.

EMSA was previously used to study acyl-homoserine lactones binding with QscR, an orphan regulator [179]. We were able to use a similar method to semi-quantitatively determine affinity of PqsR to DHQ and PQS by measuring free Bio-*pqsA* with titrations of the quinolones to the system. Increasing concentrations of DHQ and PQS resulted in decreased free probe and increased retarded probe. From the curve of concentrations with density of free probe, the affinity of PqsR for DHQ was determined to be 150 nM and 33 nM for PQS. The difference in affinity was consistent with trends found previously with DHQ and PQS supplemented to the *E. coli pqsA'*-LacZ reporter. Compared to other published work, they identified *in vitro* affinity of PqsR for PQS to be around 1 μM [180]. The Kd for the alkylquinolones from these experiments appears to be method-dependent. For our purposes, results from the EMSA demonstrate DHQ activates PqsR for subsequent transcription of the operon.

Saturation transfer difference NMR (STD-NMR) can be used to study protein-ligand biding in solution [181]. Saturation from the complex is assessed and the ligand signals are averaged following the overall effect in the free-state. A 1D ^{1}H NMR experiment is initially run at the thermal equilibrium for the off-resonance of the sample. Afterwards, the sample is run for on-resonance, during which some protons are selectively irradiated for a given saturation time. The off-resonance and on-resonance signals are subtracted, which leads to a positive difference between the molecules that are affected. After isolating those peaks that were derived from the ligand, changes in peak intensity throughout the titrations determine the Kd and identify residues on the ligand that directly interact with the protein.
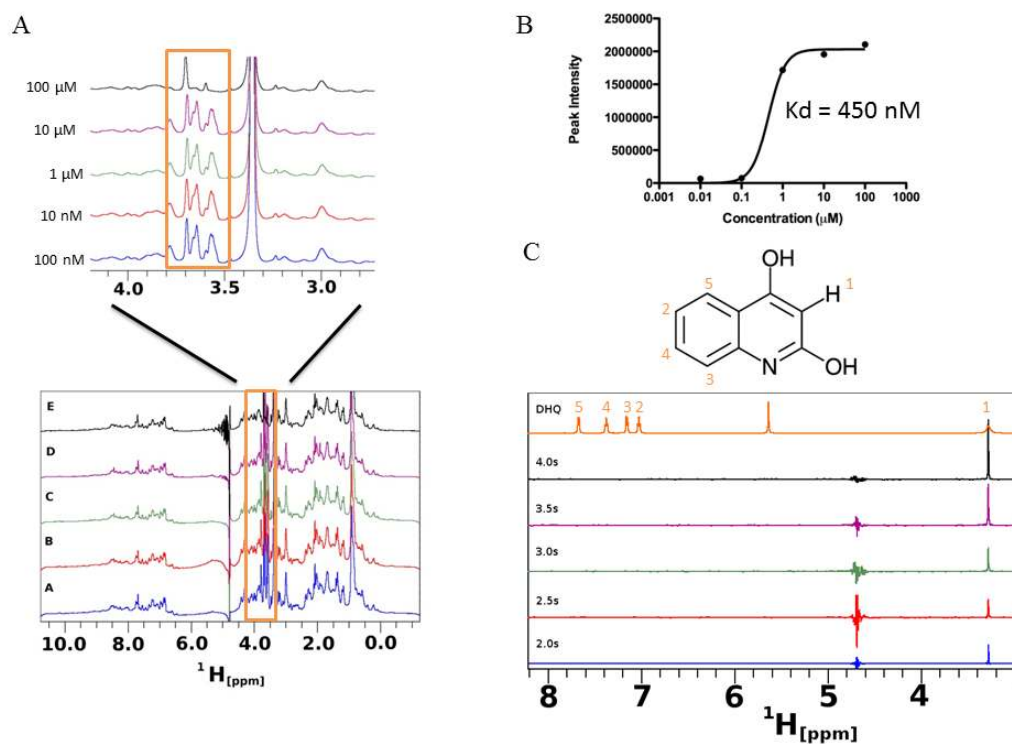
Figure 3.4 STD-NMR of DHQ titrated to PqsR-C87. (A) STD-NMR spectra of 1 μM SUMO-PqsR-C87 titrated with 10 nm to 100 μM DHQ. A close-up view of the changing spectra was used to highlight the differences in peak intensities following increased DHQ concentrations. (B) Peak intensity of the most prominent peak (close-up view) versus concentration of DHQ was used to determine Kd of binding. (C) STD-NMR of 100 μM DHQ added to 1 μM SUMO-PqsR-C87 and measured for saturation times in 0.5 sec intervals. Intervals selected to show saturation from initial incubation to 5 sec. D-COSY assignments of DHQ used to map interacting hydrogens (numbered in orange).

We found PqsR saturation transfer to DHQ after signals appeared in the STD experiments (Fig 3.4). Novel peaks in the PqsR spectra were quantified for intensity and modeled for single-site kinetic binding. The Kd was semi-quantitatively determined to be 450 nM. Interestingly, the saturation time showed a strong interaction after 3 seconds, while exhibiting fast-exchange interactions from 0.5 to 2.5 seconds. The fast-exchange may be explained by hydrophobic interactions within the hydrophobic pocket until DHQ is coordinated through hydrogen binding. Using the D-COSY assignments (Fig 3.5), we mapped the 1-D hydrogen spectra to show that the meta-hydrogen of DHQ participated in hydrogen-binding with PqsR. The ortho- and para-hydroxyl groups were not resolved because their hypothesized position was too close to the water peak to tell apart.

We also determined the interchange of the keto-enol tautomerization. In solution, both species exist, which allows for the coordination of the hydrogen through either site. PQS can also go through a similar keto-enol tautomerization, but it is not known how the keto and enol form would affect the overall function of the molecule. For DHQ, we predict the enol form participates in hydrogen binding; however, both in-solution chemistry and structure of PqsR bound to DHQ will be required to formally determine the precise interactions.
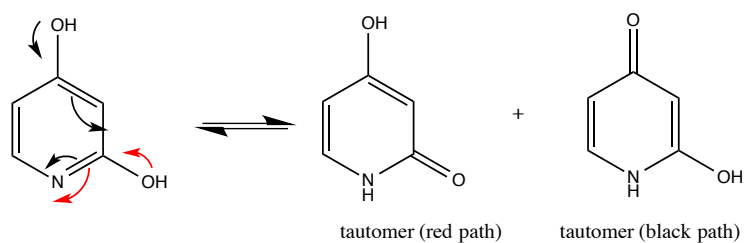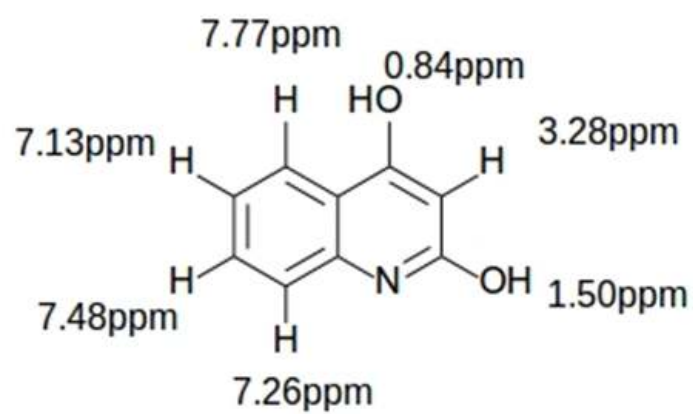
Figure 3.5 D-COSY assignments for DHQ and predicted tautomerization about the substituted pyridine ring.

3.3 Discussion

In order to elucidate the interaction of DHQ with PqsR, we performed assays to determine activity of DHQ on PqsR and the effect of DHQ on transcription of the *pqs* operon. Our previous work demonstrated that production of DHQ resulted in phenotypes associated with an active Pqs system. Based on the similar structure shard by DHQ and the alkylquinolones, we hypothesized DHQ would also activate PqsR for transcription of the operon. Both RT-PCR and a reporter construct for *pqsA* transcription showed that the strain only able to produce DHQ maintained transcription of the *pqs* operon similar to wild-type. We also examined the *in vitro* interaction of DHQ with PqsR using EMSA and STD-NMR. Both assays elucidated comparative Kd values for PqsR and DHQ. Importantly, the assays found DHQ could perform the similar function as PQS and bind within the same pocket on PqsR. Based on the newly identified transcriptional effect of DHQ for the Pqs system, we have determined that DHQ shares a role with PQS to activate transcription of the system and identified a structural scaffold to use for future inhibitor studies with PqsR.

QS controls target genes in order to regulate gene expression at certain bacterial densities. The mechanism of QS involves a transcriptional regulator, which is normally not functional until activated by a cofactor. We determined that production of DHQ resulted in similar transcriptional activity compared to a strain that possessed an intact Pqs system. However, we were surprised to find differences in the first and last gene transcribed in the operon. Previously published work identified a second possible transcription start site for the operon before the coding region of *pqsB* [182]. However,

this work was only examined using RNA-seq and RACE. No other group has published on this site as a second promoter of the system. Another group also found a short sequence in *pqsC* that can be mutated that results in a promoter with high activity [183]. Strains that possess that mutation do not require quinolone signaling for activation of transcription. Based on the requirement for both PqsR and a co-activator, we are confident our strains function through the traditional signaling system via activated PqsR. This is supported through our mutants not expressing Pqs-associated phenotypes unless activated by a quinolone at the previously established density.

To complement the results from RT-PCR of the *pqs* operon, we performed assays with a reporter plasmid that carried the upstream region of *pqsA* fused with the coding region of LacZ. DHQ supplied to *E. coli* harboring the plasmid resulted in a dose-dependent increase in LacZ activity, albeit not the level of a lower concentration of PQS. We also found endogenous production of DHQ activated the reporter system and that there were no other compensatory mechanisms that could activate the reporter without quinolones. Interestingly, we were able to recapture phenotypes associated with DHQ supplemented to *E. coli*, but not to *P. aeruginosa*. This may be due in part because of different membrane composition and the efflux pumps located in the membrane of *P. aeruginosa*. To our knowledge, no other group has elucidated the QS molecule dynamics of crossing the bacterial membrane. This is important because we are just beginning to understand cross-species and interkingdom signaling.

The Pqs system produces a number of different quinolones, but only HHQ and PQS have established signaling function. Because DHQ is also produced along with HHQ and PQS, it was important to determine whether DHQ has any competitive or

synergistic effects with the alkylquinolones on PqsR. DHQ supplemented with HHQ and PQS did not alter the level of LacZ activity compared to the alkylquinolones alone. Based on the lower activity of DHQ with PqsR, we predicted that higher concentrations of DHQ would affect the alkylquinolone binding. These results demonstrated the affinity of PqsR for the alkylquinolones were sufficiently higher enough to not be affected by the increased concentration of DHQ.

Yeast secrete farnesol, which inhibits PqsR activity. Yeast such as *Candida albicans* can colonize similar infection environments along with *P. aeruginosa*. Because of their close colonization, it is not surprising that both strains would try to inhibit each other [176]. PQS supplemented to a system containing farnesol showed PQS outcompetes the inhibitor in order to maintain operation of the Pqs system. We also tested whether DHQ could outcompete Farnesol and found DHQ supplementation increased LacZ activity despite the presence of the antagonist. The capacity for another organism to target PqsR in order to reduce its activity highlights the fact that PqsR is a good target. With this in mind, we are still struggling to understand the dynamics of *P. aeruginosa* within a polymicrobial environment. We predict that bacteria are often involved in complex synergy and chemical warfare over limited nutrients and space. Over time, systems among the microbes evolved so that they can compete with the other species. Successful combat would require blocking those systems that are most effective in controlling the environment, i.e. pyocyanin and the Pqs system. However, because PqsR function is important for establishing an infection and maintaining the local environment, *P. aeruginosa* has developed several redundant mechanisms to ensure its activity.

Molecular docking is a tool that can be used to generate hypotheses for how a ligand interacts with a protein. We used docking to determine whether DHQ binds with PqsR *in silico* and what could be the potential coordinating amino acids. We compared the structure of PqsR with an alkylquinolone and found DHQ could also bind within the same region. The core-quinolone moiety possessed by the molecules binds within an interior hydrophobic pocket of PqsR. This was not surprising because of the hydrophobicity of DHQ would most likely direct the molecule to the pocket. In order to more comprehensively study the interactions of DHQ and PqsR, *in vitro* methods EMSA and STD-NMR can be used to probe separate questions. The EMSA assay determined that DHQ was sufficient for activating PqsR to bind with the *pqsA* promoter. We also could measure the Kd by titrating DHQ into the assay and measuring the effect on the free-probe. Using software to fit the curve, we were able to elucidate the binding affinity of PqsR for both DHQ and PQS. Again, PqsR displayed higher affinity for PQS compared to DHQ. Other methods in publications determine affinity of PqsR for the alkylquinolones, again in a range from nanomolar to micromolar concentrations. Therefore, affinity found from the different experiments may be involved in an intricate relationship with the environment used to test the system and the data gathered from the assay.

STD-NMR can be used to determine the specific moieties involved in binding between the protein and ligand. DHQ was titrated into solution containing PqsR-C87, a truncation of PqsR missing the DNA-binding domain. The DNA-binding domain contains a helix-turn-helix binding motif, which can be insoluble and lead to poor protein purification. We found a dose-dependent shift in peaks from PqsR-C87, which indicated

DHQ interacts the protein. In order to determine the exact amino acids that are responsible for binding, double-labeled proteins can be pulsed using 2D and 3D NMR techniques. For our purpose, we solved the structure of DHQ and mapped the peaks of DHQ during the titration. Both keto-enol forms of the carbonyl fall too close to the central water peak to be identified individually. However, we were able to determine that the meta-hydrogen in between the two carbonyl-hydroxyl groups did display an increasing peak with higher concentrations of DHQ. The intensity of the peak was graphed based on concentration and used to determine the Kd of PqsR for DHQ. The Kd discovered from STD-NMR was similar to what we found using EMSA. We hypothesize that the increased Kd in STD-NMR is due to the environment of assay. STD-NMR requires the use of phosphate-buffered saline, while the EMSA used cell-lysate and glycerol. The cell-lysate along with glycerol may maintain the protein in solution better compared to the buffer, and therefore, yield higher affinity because of the increased stability.

Overall, we have found that PqsR binds to DHQ. Using both *in vivo* and *in vitro* techniques, we demonstrated that DHQ activated PqsR for transcription. It is important to note that no other group has discerned which species of quinolone may be required to participate in binding. DHQ should readily tautotomerize in solution and display two different charged species. Future work in this field should determine the natural state of DHQ in the environment and how the different tautomeric forms affect activity with PqsR. Similarly, the availability of the quinolones will be an important question to answer because charge and mass action kinetics will be involved in the system.

Chapter 4: DHQ is the most abundant quinolone in CF patient sputum and correlates

to patient health.

4.1 Introduction

Cystic fibrosis is a genetic disorder that results in increased sweat chloride and mucous production. Overtime, other pathologies predominate such as chronic lung infection and increased immune cell infiltration. *P. aeruginosa* is one of the most commonly isolated CF pathogens and becomes the dominant organism in the lungs [184]. During chronic colonization, *P. aeruginosa* infection leads to progressive lung damage and respiratory failure [185]

In vitro methods for studying host-pathogen interactions with *P. aeruginosa* in the lungs are limited. During chronic colonization, *P. aeruginosa* and the community together constantly change due to different selective pressures [186]. Due to the constant changes within the microbial community, it is not known how the resultant phenotypic changes affect patient health or the best method to assess virulence from the bacteria. Overall, the goal for treatment involves either eradication or maintenance of *P. aeruginosa*, but success with current therapies is limited.

Bacterial QS controls several community behaviors, which are essential for causing an infection. *P. aeruginosa* utilizes three QS systems to control virulence factor production and community lifestyles such as biofilm formation and swarming. Virulence factors have been extensively studied *in vitro*, but their direct involvement in lung pathology is not well understood. Biofilm and macrocolony formation, which are established antibiotic resistance mechanisms, have been identified from clinical samples of CF patients chronically infected with *P. aeruginosa [187]*. Swarming is less

understood, but has been hypothesized as a method for *P. aeruginosa* to further colonize areas in the lung.

Understanding the survival and fitness mechanisms from *P. aeruginosa* between different environments has not been well established. Within a planktonic culture, QS molecules function through a defined pathway, but these QS systems are altered during an infection. HSLs regulate community behaviors from the Las and Rhl systems, while quinolones alter behaviors from the Pqs system. Acidic conditions and certain enzymes break down HSLs [188]. Host cells may also hydrolyze the lactone ring of HSLs, which acts as a host defense mechanism [189]. Quinolones are more stable and have few known enzymes that can affect the ring structure, none of which are produced from a human host [190]. Based on the stability of the quinolone, those molecules may accumulate over time during chronic colonization. Of the QS molecules, C12-HSL and C4-HSL have been quantified from patient samples in order to show *P. aeruginosa* forms biofilms within the lungs [191]. Of the quinolones produced, only PQS has been isolated from patient samples [117]. Phenazines, which are regulated by the PQS systems, have also been assessed for their impact on *P. aeruginosa* infection and correlation to patient lung function [192]. Because the QS systems are inter-regulated, the consortia of molecules may yield a biomarker of *P. aeruginosa* community phenotype and virulence factor production.

For our investigation, we examined *P. aeruginosa* QS molecules from patients who maintained their lung function compared to those who experienced an exacerbation, which was diagnosed as a sudden decrease in lung function and increase in symptoms. Patients were enrolled in the study for up to three years and their clinical data was

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recorded with the QS molecules from their sputum. Clinical data included: patient sex, age at the time of sample, genotype, treatment therapies, and microbiology cultures. In total, 45 patients were recruited into our study and were monitored for both stable and exacerbation periods. During their clinic visits, patient FEV_1 was also recorded at the time of the sample. Concentrations of the QS molecules were used with statistical comparisons in order to determine the relationship of QS molecules with patient health status. We predominantly focused on the most abundant quinolone, DHQ, but also investigated QS molecules PQS, C4-HSL, and C12-HSL. During the study, we attempted to correlate QS molecules with maintenance of lung volume, initial exacerbation event, and treatment for an exacerbation.

4.2 Results

Inclusion of patients, sputum sample collection, and review of patient medical records were approved by the Medical University of South Carolina Institutional Review Board. Adult CF patients were sampled during stable periods of lung function and exacerbations over three years. Patients were selected using the following criteria: over 18 years old, had a positive culture-history of *P. aeruginosa*, non-smoker, did not culture *Burkholderia* species, readily produced sputum, and received consistent treatment at the Medical University of South Carolina Adult CF Center. Those patients included in the study were consented and were periodically consulted to discuss their view on their symptoms and pathology. Patients who maintained their lung function gave samples during clinic visits, and those who underwent an exacerbation gave samples during the hospitalization until they could no longer produce sputum. The patients enrolled in the study were assessed not only for QS molecules in their sputum, but also for their sex, genotype, age at sample, antibiotic therapy, and microbiology cultures (Table 4.1). This study included 45 patients, 23 female and 22 male, who gave over 80 samples during stable periods of disease and over 50 samples during exacerbations.

Patients ($n_1 = 45$)	Samples Received ($n_2 = 80$)
Sex	23 female, 22 male
CFTR genotype, n_2 (%)[a]	
ΔF508/ΔF508	34 (75.5)
ΔF508/other	8 (17.8)
Other/other	3 (6.7)
Age at time of sample, yr (+/- SD)[b]	30.88 (12.15)
Therapy at time of sample, n_2 (%)[c]	
single antibiotic	16 (20)
two antibiotics	28 (35)
three antibiotics	25 (31)
Tobramycin (Tobi)	46 (58)
Aztreonam (Cayston)	20 (25)
Microbiology (%)[d]	
multiple *Pa* strains	31 (39)
Co-culture *S. aureus* and *Pa*	29 (36)
Co-culture *Pa* and fungi	29 (36)

Table 4.1 Demographic and sample information for subjects enrolled in study. Patients were enrolled for three years and were sampled at both clinical visits and hospitalizations (or when available). [a]Percent of total patients (45) for comparison of CFTR mutation. [b]Age at time of sample is listed as mean and (standard deviation). [c]Per sample percent of antibiotic treatment at the time the sample was given. [d]Per sample percent of microorganisms found within the culture sputum given to the MUSC Microbiology Lab.

DHQ was the most abundant quinolone quantified from stable CF patient sputum colonized with *P. aeruginosa*. Stable patient samples contained several QS molecules from the three central QS systems in *P. aeruginosa* (Fig 4.1). Although there are several methods to detect QS molecules in a sample, mass spectrometry provides a definitive method for quantification [193]. Previously published work demonstrated that conditions in the CF lung foster the increased production of C4-HSL and decreased production of C12-HSL [191]. Our *in vitro* work similarly found that increased C4-HSL over C12-HSL was indicative of growth under anaerobic conditions. Results from the stable CF patient samples were consistent with this trend that *P. aeruginosa* colonized anaerobic areas within the lungs. PQS was quantified from the samples as well, but was significantly lower in concentration compared to DHQ. HHQ was detected in the samples, but was under the limit of detection, <100nM. Therefore, the rest of the data focused on C4-HSL, C12-HSL, PQS, and DHQ.

QS molecules between stable patient sputum displayed wide ranges in concentration (Table 4.2). DHQ was found to have a median concentration of 123 µM with an interquartile range of 25 to 464 µM. C4-HSL was another abundant QS molecule quantified in the sputum with a median concentration of 162 µM and an interquartile range of 41 to 452 µM. Both PQS and C12-HSL were significantly less compared to the other quinolone and HSL species, respectively.
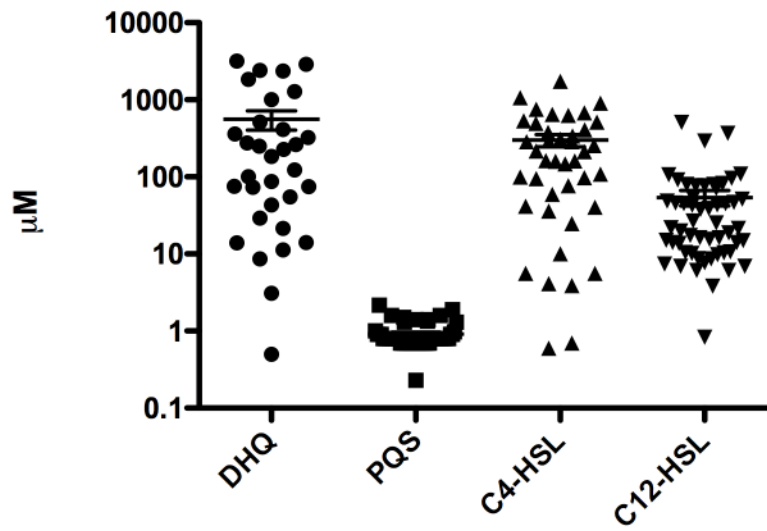
Figure 4.1 Distribution of QS molecules from stable CF patients. Samples quantified from clinic visits and graphed using a scatter plot. Samples (n=33) are listed with mean and standard deviation. HHQ is not shown because the QS molecule was below the limit of detection.

QS Molecules (µM)	DHQ	PQS	C4-HSL	C12-HSL
Minimum	0	0.229	0.6	0.829
25% Percentile	25.3	0.7	41	10.38
Median	123.2	0.8	162.6	21.65
75% Percentile	464.3	0.9	452.1	57.5
Maximum	3192	2.16	1732	507.1
Mean	560.8	0.917	299.4	54.02
Std. Deviation	910.5	0.3458	353.5	89.6
Std. Error	158.5	0.0475	55.2	12.19

Table 4.2 Descriptive statistics for QS molecules in stable patient sputum samples (n=33).

DHQ positively correlated with stable patient FEV$_1$. Patient FEV$_1$ is a measure of patient lung expiratory volume. Measured volumes estimate lung function and provides an ongoing test to monitor progression of the disease [194]. Although there is a correlation of increased mortality of CF patients colonized with *P. aeruginosa*, there is currently no published report that directly measures the effect of *P. aeruginosa* on lung tissue. Because *P. aeruginosa* caused dramatic changes in cultured cells and secreted a vast arsenal of virulence factors, we predicted *P. aeruginosa* destroyed lung tissue either through direct or indirect mechanisms.

P. aeruginosa produces several QS molecules that directly antagonize host cells. Both quinolones and HSLs alter host cell viability and cytokine response to LPS-activated macrophages [195, 196]. We determined DHQ positively correlated with FEV$_1$, indicating higher concentrations DHQ was associated with higher lung function (Fig 4.2). No other QS molecule statistically correlated with a change in FEV$_1$. However, C4-HSL was the next closest trend ($p = 0.12$), but demonstrated a negative correlation with FEV$_1$. Our previous *in vitro* data found that DHQ inhibited epithelial cell replication, while reducing the viability of macrophages. Another effect on the immune system was identified when DHQ dramatically reduced IL-6 and TNF-α production from LPS-stimulated macrophages. Alveolar macrophages are antigen-presenting cells in the lungs that react to foreign bacteria. The cytokines produced from the activated macrophages and other immune cells can induce a large influx of other host immune cells, which causes collateral damage to the lung tissue in the process. Inhibiting immune cell signaling would reduce the damage brought on by the immune system following *P. aeruginosa* infection [197].
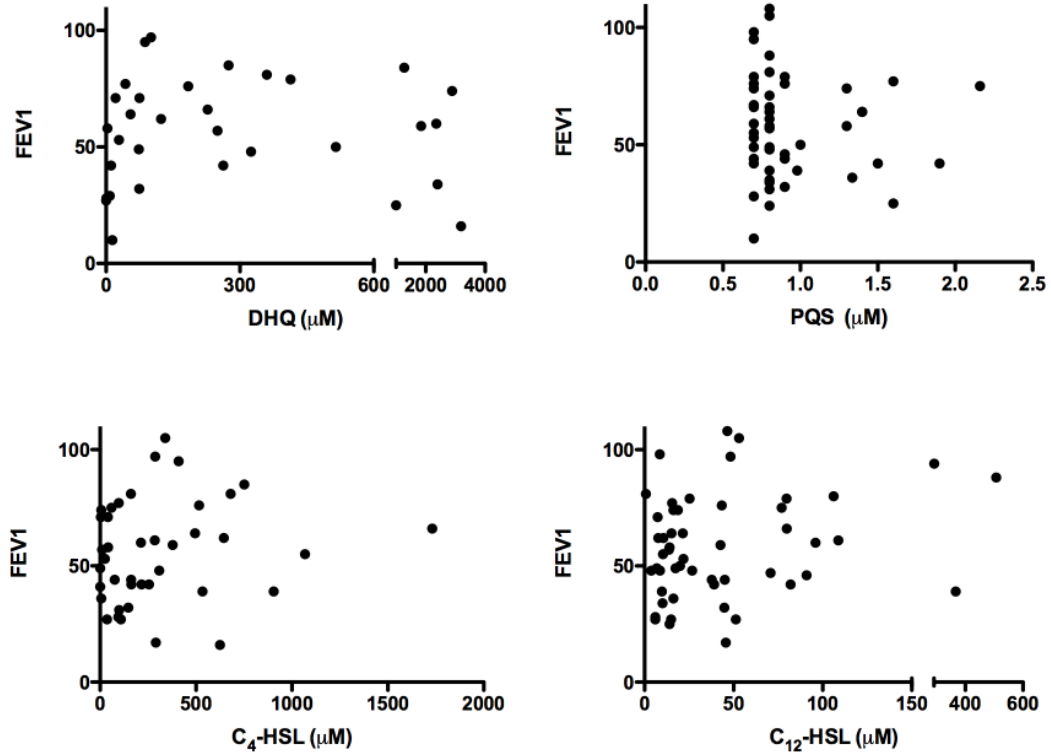
Figure 4.2 Correlation of QS molecules with patient FEV1. None of the QS molecules displayed a normal distribution; therefore, correlation of QS molecules with FEV1 analyzed using Pearsons's Correlation Coefficient (PCC). For all samples: n = 33. The PCC of DHQ = 0.36 ($p = 0.04$), PQS = 0.03 ($p = 0.5$), HHQ = -0.21 ($p = 0.12$), C12-HSL = -0.13 ($p = 0.17$).

Genotype and microbiology cultures of patients display changes in DHQ in stable patient sputum. Although the largest group of patients was homozygous for ΔF508, we found that patients with other mutations displayed differences in the abundance of DHQ in sputum (Fig 4.3). Other groups have investigated the trend of patients based on their genotype and overall mortality; however, we are the first to show trends involved with *P. aeruginosa* QS molecules [198]. We also investigated the concentration of DHQ from patients who cultured *P. aeruginosa* and other microorganisms. In co-culture, *P. aeruginosa* alters QS molecule production and subsequent phenotypes in response to other organisms [151]. We found patients who co-cultured *Staphylococcus aureus* or fungal species displayed increased DHQ in their sputum. This finding was consistent with other published work that demonstrated that the Pqs system was upregulated in response to other organisms [151].

We also examined the change in DHQ concentration based on the number of *P. aeruginosa* colony morphologies cultured from sputum. The diversity of colony morphologies is generated, in part, from the Pqs system [199]. We found that the increasing diversity of colonies was associated with larger concentrations of DHQ. In light of the correlation of different *P. aeruginosa* features and FEV_1, further work is necessary to understand the mechanism involved *in vivo* for the generation of diverse colonies either through the selection by antibiotics or the functions of the Pqs system.
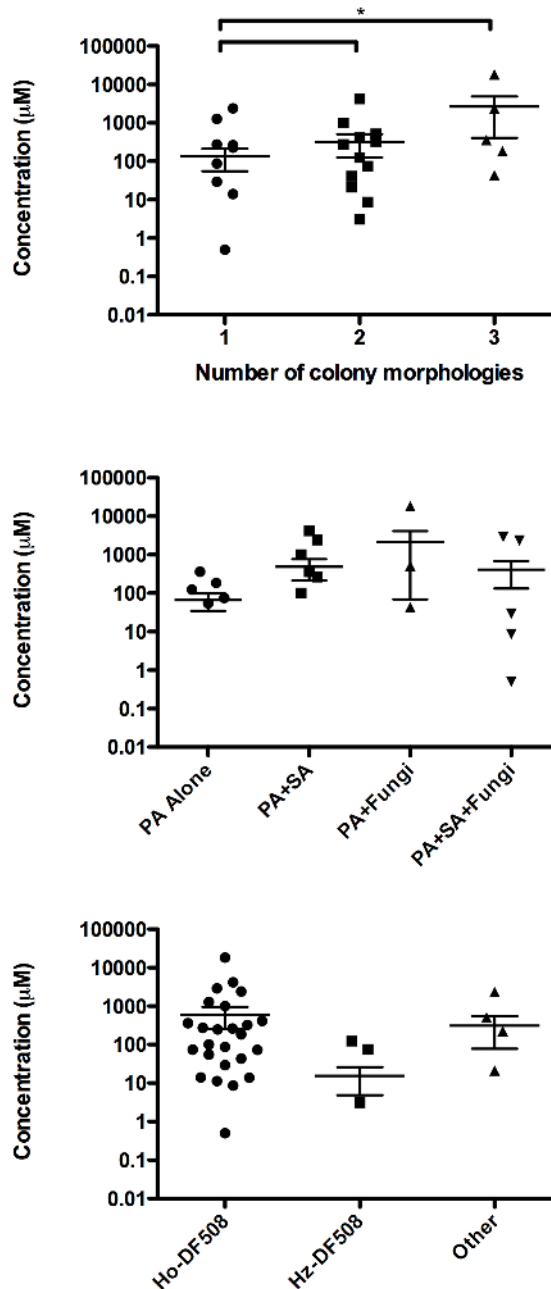
Figure 4.3 Correlation of DHQ with genotype, microbiology cultures, and colony variants. Data displayed as scatter plot. Correlation of DHQ with number of colony morphologies was measured using Student's T-test (* p = 0.024). Other data to be displayed as general trends among the samples.
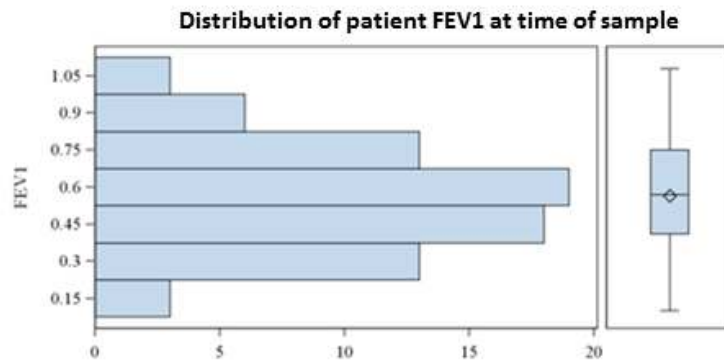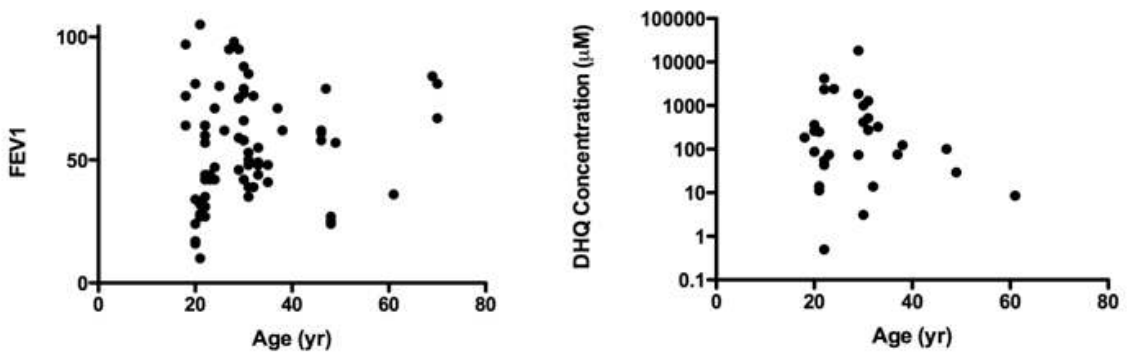
Figure 4.4 Correlation of Age, FEV1, and DHQ concentration. Patient FEV1 at the time of sampling displayed a normal distribution. For comparing FEV1 and age and also DHQ concentration and age, a Pearson's Correlation Coefficient (PCC) was used to determine the statistical relationship. No significant value was determined between either data set.

Quinolones are made up of two fused benzene rings with a single nitrogen-substitution. The nature of the ring structure provides a stable structure that could accumulate over time in an environment. Conversely, HSLs are broken down quickly over time, especially in acidic environments. We predicted that quinolones accumulate over time after patients are colonized with *P. aeruginosa*. First, we investigated if there was a significant trend in FEV_1 compared to age of stable patients (Fig 4.4). No significant trend existed between FEV_1 and age; therefore, we could interpret whether age and DHQ concentration could be dependent regardless of FEV_1. When we compared the age of patients with their respective DHQ concentration, again we found no significant trend. We initially hypothesized DHQ would increase overtime due to accumulation in sputum and in the lungs, but this did not take into account dynamics of QS molecules during acute and chronic periods of disease. The concentration of quinolones overall may change based on coughing out sputum and intense lung-clearing techniques. Although the concentration of QS molecule may fluctuate overtime, we still may be able to determine accumulation of QS molecules after following patients over time from their initial positive culture.

Concentration of DHQ does not predict an exacerbation event or change significantly during and exacerbation. Due to the use of QS systems to regulate bacterial pathogenicity, we predicted that increased QS molecule production would coincide with decreased lung function. During this time, we would also expect increased *P. aeruginosa* colonization and pathogenicity. Currently, there is no known connection between *P. aeruginosa* colonization and exacerbations.
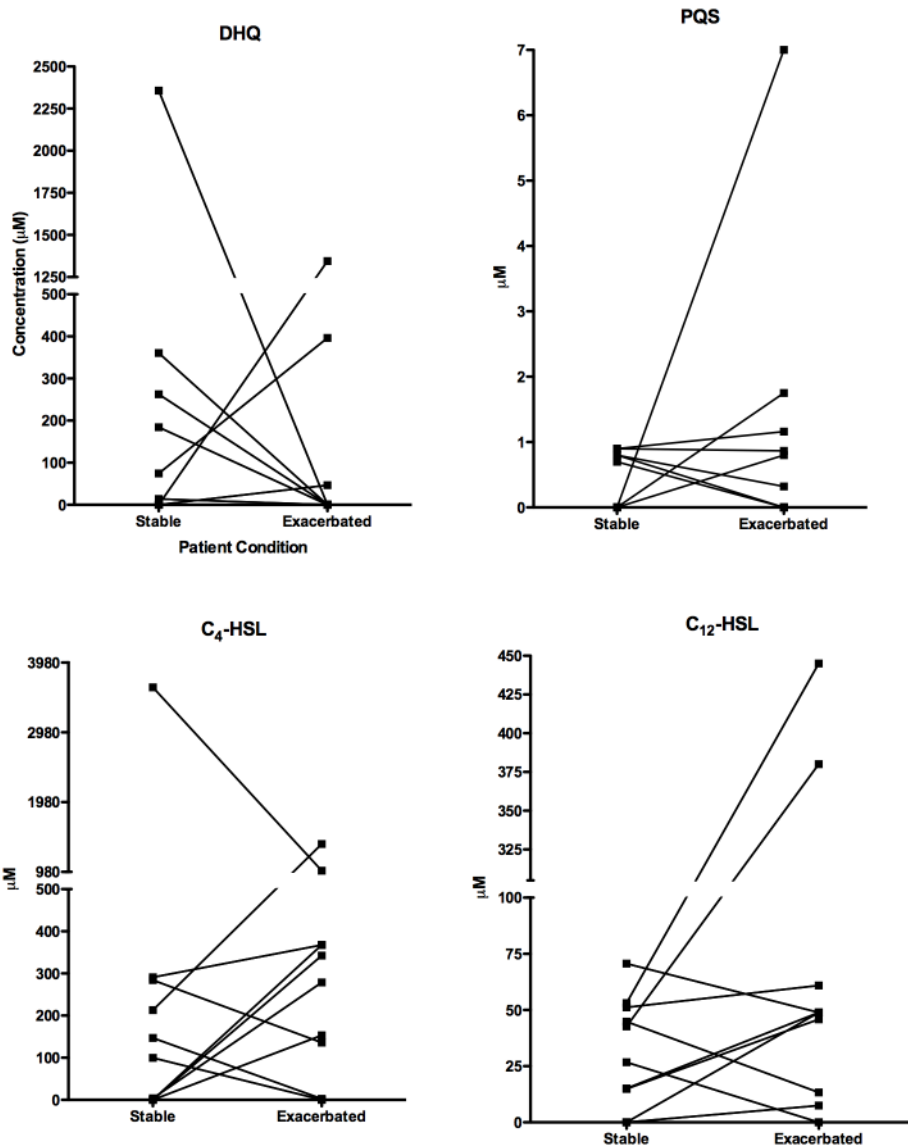
Figure 4.5 Before-and-after trends of QS molecules with exacerbations. 11 patients undergoing an exacerbation were included in this study because they had given sputum samples within 7 days of their exacerbation. Exacerbation samples were from samples given on day 1 of their hospitalization.

QS molecules and the relationship to pathogenicity may lead to potential biomarkers from *P. aeruginosa* that are related to disease severity.

Quinolones and HSLs were compared between stable periods of disease and the first day of a diagnosed exacerbation, which was identified as a sudden drop in FEV_1 and general health (Fig 4.5). None of the QS molecules demonstrated consistent trends between stable and exacerbation events. Both of the quinolones monitored showed intermixed increasing and decreasing concentrations. Of the HSLs, C12-HSL demonstrated a mix of trends between the patient health statuses, while C4-HSL displayed an increasing trend during exacerbations in > 70% of the patients monitored (n = 11). A much larger sample size is required to elucidate whether this trend is statistically significant. Importantly, this data provides initial evidence that the Rhl system is upregulated during an infection. Rhamnolipids are critical for both mobility of *P. aeruginosa* on a semi-solid surface and are important for reducing ROS caused by antibiotic treatment [119].
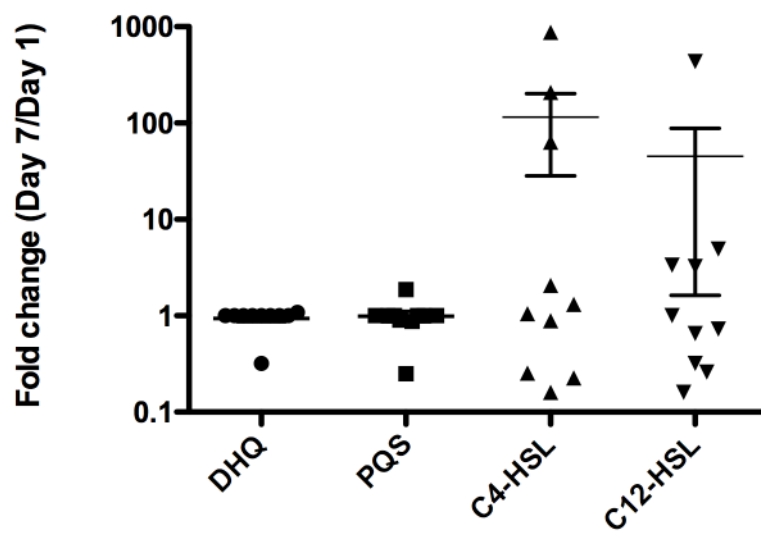
Figure 4.6 Change in QS molecule concentration from first day of inpatient treatment to day 7 of treatment (n=11). Samples displayed as a scatter plot with mean and SEM listed.

During an exacerbation, patients are aggressively treated with antibiotics and respiratory therapy. However, *P. aeruginosa* survival despite therapy or how antibiotic treatment affects *P. aeruginosa* phenotypes *in vivo* is not known. We monitored QS molecules from patients undergoing first seven days of an exacerbation (Fig 4.6). Interestingly, the concentration of DHQ and PQS did not significantly change overtime. We anticipated a decreasing trend in concentration because the antibiotic therapy should reduce *P. aeruginosa* density and/or increase lung clearance, which would affect the concentration of QS molecules in the lungs. We did determine changes to both of the HSLs throughout an exacerbation. The changes demonstrated a mix of increasing and decreasing concentrations. Overall, the trends identified between the QS molecules indicated *P. aeruginosa* density was not affected despite therapy, while the HSL signaling systems were altered during an exacerbation or in the presence of treatment.

4.3 Discussion

Studying QS from *P. aeruginosa* in CF patient samples will help to show the phenotypes of the bacterium during an infection. Because QS regulates community behavior and pathogenicity in a density-dependent manner, we wanted to determine the dynamics of QS molecule concentrations in order to determine the phenotype of *P. aeruginosa* during disease. We focused on adult patients because the majority of this patient population harbored *P. aeruginosa* for years. However, we were unable to know exactly how long the adult CF patients were colonized, which may have affect concentrations of the QS molecules in the samples or the relationship to the other pathologies. Therefore, our work concentrated on QS molecule and their relationship to current patient characteristics and exacerbation events.

We determined DHQ had a wide distribution of concentrations from patients who maintained their lung function. DHQ accumulated up to milimolar concentrations in sputum, which was significantly higher than found in planktonic cultures. It was also interesting to find the concentration of PQS was more consistent between samples, but at significantly lower concentrations. We predict the low concentration of PQS was related to lowered production from *P. aeruginosa* when grown under microaerophilic to anaerobic conditions. *P. aeruginosa* is often studied under aerobic conditions; however, this bacterium is able to utilize nitrate as a terminal electron acceptor, which is readily available in the lungs [200]. Growth within these different environments will alter bacterial phenotypes. In comparison among the different quinolones produced, DHQ was

secreted in high concentrations both in culture and within the lungs regardless of oxygen and may play an important extracellular role during an infection.

Our previous work tested the effect of DHQ exogenously supplied to LPS-activated macrophages and subsequent cytokine production. Other published reports indicated that alkylquinolones reduced cytokine production, but those bacterial small molecules were not readily available in CF patient sputum. We confirmed DHQ also reduced cytokine production from LPS-stimulated macrophages and were able to associate our findings with patient FEV_1. Although we did not directly test cytokines from patient samples, the high concentrations found in sputum support the extracellular function of DHQ to reduce host immune cell signaling [201]. Infiltrating immune cells cause collateral damage through normal immune processes that occur when the cells are fighting an infection [202]. Mice that displayed reduced immune activity did not contain as catastrophic lung damage compared to wild-type mice infected with *P. aeruginosa* *[203]*. Therefore, our findings are similar with *P. aeruginosa* colonization controlling the local immune system by reducing cytokine signaling and preserving the lung environment. Overall, patients whose *P. aeruginosa* strains secrete large amounts of DHQ may initially display higher lung function because the anti-inflammatory effect, but it is still not known how chronic exposure to DHQ may affect lung pathology.

Different CFTR mutations display different deleterious effects on patient lung function due to the activity, or lack thereof, from the CFTR [204]. The mutation most often found within our patients was ΔF508, but we also included patients that did not possess the F508 mutation. We found patients with different CF mutations displayed differences in DHQ concentration. However, we did not compare any other patient

conditions. Larger enrollment numbers may further elucidate a role for DHQ and the properties of *P. aeruginosa* infection in the different patients. We also identified DHQ concentrations change based on other organisms that were co-cultured with *P. aeruginosa*. In the presence of *Staphylococcus aureus*, *P. aeruginosa* increased expression of the Pqs system in order to produce a potent alkylquinolone-N-oxide [99]. We found sputum samples from patients that cultured both *P. aeruginosa* and *S. aureus* demonstrated increased concentration of DHQ compared to mono-cultures of *P. aeruginosa*. DHQ was also increased when *P. aeruginosa* was cultured with fungi. Both *P. aeruginosa* and yeast have been studied for their interactions; however, it is not clear if *P. aeruginosa* directly associates with the yeast or reacts to its metabolites in the environment [205]. This is an important question for the microbiology of CF patients and will help to understand another variable to the environment of *P. aeruginosa* during chronic colonization. In addition, discovery of two-component regulators and other sensor systems will be critical for also integrating how *P. aeruginosa* facilitates its interaction with the environment.

CF patients undergoing an exacerbation require extensive inpatient care. Based on the paradigm of CF pathologies, increasing colonization of bacteria in the lungs may play a primary role in initiating an exacerbation. Quorum sensing coordinates bacterial community development and virulence factor production; therefore, an increase in QS molecule concentration may be found before increased bacterial colonization or alteration of the lung environment. We studied QS molecules from the three established systems in order to identify how measuring QS molecules can be used as a biomarker for an exacerbation event. We found that the quinolones did not demonstrate a consistent trend

between stable and exacerbation periods, while the HSLs showed more uniforms trends. We initially hypothesized quinolones would show a sharp increase because of their relationship to growth under anaerobic conditions and virulence factor production. However, C4-HSL increased more often during the first day of an exacerbation. C4-HSL is responsible for the regulation of rhamnolipids, which play several roles for *P. aeruginosa*. Rhamnolipids are essential for swarming and may play a role in increasing *P. aeruginosa* colonization. Furthermore, we identified C4-HSL as a molecule that also fluctuated during an exacerbation. Limited studies have attempted to understand the interactions of the colonizing bacteria and the intense treatment received [206]. Previous work has already elucidated bacteria can react to small amounts of antibiotic in the environment by increasing biofilm formation [207]. It is reasonable to predict that IV antibiotic therapy and increased respiratory clearance will dramatically alter QS molecule synthesis from the bacteria, but these predictions remain to be concluded.

Bacteria within the lungs undergo genetic variation throughout chronic colonization. Antibiotic treatment, anaerobic metabolism, and host ROS are all drivers of genomic diversification [208]. The variation in the genome is a result from synonymous and non-synonymous mutations within genes creating diverse phenotypes within the environment that compete for dominance [209]. Mutations are generated through a variety of mechanisms, but most mutations may arise from loss of optimal DNA repair capability. Therefore, not only is it important to understand how the genome is altered throughout an infection, but also how global metabolism changes in response to the mutations and downstream effects on lung pathology. Ultimately, these changes are guided via selective pressures, which may be both disease and patient specific.

Based on the limited knowledge of bacterial reactions to antibiotic therapy and host factors, our data provides some of the initial evidences into understanding how the bacteria react to frequent changes in the environment. We provide a similar finding that increasing antibiotics caused alterations to the QS molecules secreted in the environment. Specifically, the HSLs displayed the most change, while the quinolones were relatively unaffected. HSL can signal for biofilm formation, but the link between HSL signaling and biofilm formation *in vivo* remains limited. The relatively small change in quinolones was interesting because patients are receiving aggressive therapy with the goal of reducing the bacterial concentration. If the bacterial community is reduced, we would expect a consequent reduction in QS molecules. This result would be the same for QS molecules that have accumulated in sputum. However, the QS molecules remained relatively unchanged; thereby, molecules that were removed by physical processes were either replaced or maintained in other pockets within the lungs despite treatment. Because the homoserine lactones are changing, we expect that those systems are upregulated in the presence of treatment. Further work is required to determine how long-term cultures are affected by treatment and how QS may be involved in the persistence of *P. aeruginosa* in the CF lungs.

Chapter 5: Conclusion and future outlook

DHQ and *P. aeruginosa* pathogenicity

P. aeruginosa produces several different quinolones, but few have designated function within the Pqs system. We determined DHQ maintains a redundant ability to activate PqsR, but also has specialized function for reducing inflammatory signaling. Our initial results to identify the link between DHQ and pathogenicity utilized the *C. elegans* colonization assay and found a strain that only produced DHQ displayed increased tissue penetration, which may represent a role during *P. aeruginosa* colonization. The strain only able to produce DHQ also maintained production of pyocyanin providing another link to its role in pathogenicity. Apart from the other quinolones, DHQ abundance was not affected by anaerobic conditions, which is a component of the environment *P. aeruginosa* frequently colonizes. Although our work did not identify any redox properties of DHQ, we did identify a potential role in community behaviors that are essential for initiating an infection. Importantly, our work also united the function of DHQ within CF patients who were chronically colonized because of reduced cytokine production and cell viability. Overall, initial evidence of DHQ and its role within the Pqs system was confirmed and demonstrated that the novel quinolone performs key roles during active colonization of a host.

P. aeruginosa stains only able to produce DHQ killed *C. elegans* significantly faster compared to a quinolone-null mutant. We were fortunate to have such polarizing results between the wild-type and the quinolone-null mutant in order to document the range of virulence. Although the DHQ-producing mutants did not completely display the total pathogenicity of the wild-type, the results clearly indicated DHQ was involved in

one or more virulence mechanisms that were perpetuated by the Pqs system, which may be required in order to activate optimal virulence. Because both DHQ-only mutants had similar trends in *C. elegans* death, we were confident that DHQ played the same role regardless of *pqs* gene deletion. The *C. elegans* infection only displays trends in total virulence, which is affected by both bacterial colonization and secreted virulence factors. In order to discern how DHQ production facilitated colonization in *C. elegans*, we monitored the real-time colonization of *P. aeruginosa* strains in the nematode.

C. elegans consumes bacteria as its food-source during its maintenance on agar plates. Therefore, we were able to incubate *C. elegans* with the GFP-expressing bacteria and assess nematodes overtime. Throughout the experiment, we found increasing bacterial density in the central gut-track. At 144 h, we saw a difference between the wild-type and Δ*pqsB* compared to the Δ*pqsAB* mutant. Those strains that produced DHQ displayed increased dissemination throughout the gut-track, while the quinolone-null mutant only had minimal fluorescence. This result indicated that the integrity of the surrounding tissue was compromised as a result of the quinolone or quinolone-mediated action. There are several mechanisms that can cause this tissue break-down; DHQ participated in this effect and can cause effects against host cells. This result may help to explain why CF patients have decreased lung function after reoccurring exacerbations.

Mammalian cells respond to foreign bacteria through complex signaling and direct cytopathic mechanisms. Numerous studies focusing on CF have attempted to find a model for chronic infections, but most have not succeeded due to key differences in biology [210]. Of the models tested, the *Porcine* model has had the most success and may hold promise for elucidating key interactions found only during chronic colonization

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[211]. *C. elegans* does not possess the same immune cell repertoire; thereby, nematodes respond to pathogenic bacteria via epithelial cell defensins and other basic regulatory signaling cascades (i.e. PAMPs and host damage) [212]. *P. aeruginosa* traditionally activates PAMP TLR4, but some *P. aeruginosa* strains have been found with altered O-linked oligoglycosides that may not activate the receptor as strongly [213]. *C. elegans* may primarily respond to *P. aeruginosa* infection following cell damage caused by the bacterium. This aspect of infection response is also found with *P. aeruginosa* infections in humans, which demonstrates another parallel for *C. elegans* as a model. Although *C. elegans* captures some key cellular attributes in responding to *P. aeruginosa* infection, the model cannot be used to study chronic infections.

Regardless of oxygen concentration, high concentrations of DHQ secreted into the environment demonstrated a unique feature among the quinolones. Anaerobic conditions limited all other quinolones, but DHQ was maintained around the same concentration compared to aerobic growth. These results provide the first evidence that Pqs molecules not only function during aerobic conditions, but also during anaerobic conditions. The ability to use quinolones under both conditions helps to show the Pqs system plays a role in fitness regardless of environment.

Due to alkylquinolones HHQ and PQS requiring an active mechanism to leave the cell, we also hypothesized DHQ required a secretion system. The initial candidate for DHQ secretion was the MexEF-OprN efflux pump because the pump was identified to secrete HHQ. The RND-type efflux pumps are known to be promiscuous and may also secrete DHQ because of the structural similarity [214]. Results from mutants of the Mex system showed that increased expression of the pump displayed higher extracellular

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concentrations of DHQ, while loss of pump machinery resulted in lower concentrations. There is no direct regulatory link between the Pqs system and the Mex system; however, environmental systems may affect both concurrently. During anaerobic growth, expression of the MexEF-OprN pump is increased, while antibiotic treatment also resulted in increased expression [215]. These two environmental changes may both be responsible for the increased efflux pump expression that has been isolated from strains in CF patient samples. Our preliminary work found that intracellular concentrations of DHQ are dramatically affected by anaerobic growth (data not shown). During aerobic growth, DHQ intracellular pools remain high, while anaerobic growth resulted in depleted intracellular pools. These results mirror those found with Mex efflux pump expression. Importantly, this provides an explanation for the anticipated reduced expression of the *pqs* operon during anaerobic growth, yet high extracellular concentration of DHQ. Future work may focus on quantifying transcription of both systems in order to confirm this conclusion.

The high extracellular concentrations indicated DHQ might have a specialized role for controlling the environment during colonization. QS molecules from *P. aeruginosa* displayed different effects toward host cells, but most demonstrated either altered cytokine signaling or reduced viability. We determined DHQ reduced the viability of alveolar macrophages and also limited the reproduction of epithelial cells. Both effects are advantageous during an infection. Macrophages both consume bacteria as professional antigen presenters and secrete signals to increase the localized inflammatory response. Limiting epithelial cell reproduction would reduce the loss of limiting nutrients to host cells; thereby, allowing for better access for the bacteria. In order to further study

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the effect of DHQ on damaged epithelial cells, scratch tests may be run in the presence of DHQ to determine how DHQ affects both cell migration and multiplication following injury. Inability to heal lung tissue following damage would perpetuate the disease and provide an explanation for the downward trend in lung function after consistent exacerbations. The effect of DHQ on the immune system demonstrated a direct role for DHQ with *P. aeruginosa* colonizing a host. Reducing the local inflammatory signaling cascade prevents other immune cells from moving in towards the bacteria in an attempt to kill the pathogens. Although a biofilm or other secreted products such as rhamnolipids can prevent phagocytosis, a secreted product that was able to reduce inflammation from a relative distance would be advantageous and help *P. aeruginosa* control the environment.

The dynamics of DHQ in the environment is still not clear. We hypothesize DHQ is stable and resists deleterious conditions found with homoserine lactones. Therefore, DHQ may accumulate overtime unless transported by physical forces. The dynamics of DHQ is easier to understand within a planktonic culture because the molecule would exist within the aqueous environment. However, *P. aeruginosa* can also live as a biofilm; thereby, the QS molecules interact directly with constituents of the biofilm following secretion. Biofilms are constructed using polymeric substances that possess different chemical moieties, which can be hydrophobic, hydrophilic, positively charged, and negatively charged. These interactions are further compounded in the lungs of a CF patient because biofilms form in mucus. In solution, DHQ tautomerizes leaving both the keto and enol form. Based on mass-action kinetics, sequestered DHQ within the biofilm may become predominantly charged as the keto form switches to the stabilized enol form. However, this arrangement may have altered properties from what is anticipated for a

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function. These properties include altered signaling, retention in a biofilm, or effect against host cells. Alkylquinolones possess a hydrophobic moiety that allows it to interact with the lipid components within the biofilm. DHQ does not have this moiety and may display different movement within the matrix. No work is available to determine how DHQ may or may not accumulate within the biofilm compared to the alkylquinolones. The biofilm matrix alone is a dynamic environment that is actively altered by *P. aeruginosa [216]*. Altering the polysaccharides secreted may be one such system elucidated in *P. aeruginosa* for modifying the biofilm matrix [217]. As the chemical dynamics of the biofilm changes, the molecules that are associated in the matrix will also change. The dynamics of DHQ in a biofilm and the subsequent changes in the biofilm demonstrate a niche adaptation mechanism that may be important to resist changes in the environment and the dissemination of the infection.

DHQ activates PqsR for transcription of the *pqs* operon

Quinolones not only enact extracellular functions, but also activate signaling processes via PqsR. We performed several experiments to test whether DHQ is capable of activating PqsR. However, PqsR activated by alkylquinolones displayed increased activity, which indicated DHQ may only perform the signaling role in a redundant manner. With this in mind, we still do not understand the effect or potential for *P. aeruginosa* to possess multiple quinolones that appear to initiate similar functions.

Initial assessment of *pqs* operon activity from the mutants identified that the production of DHQ increased transcription compared to a quinolone-null mutant. Late log/early stationary phase growth was significant because time-course experiments showed initial pyocyanin production at that period. Results from qRT-PCR of strains grown to late log/early stationary phase found DHQ and PqsR were both required for transcription. The difference in activity from *pqsA* and *pqsE* demonstrated that other mechanisms may be involved in maintaining transcription of the end of the operon. We hypothesize that production of pyocyanin may be more beneficial for the cell compared to quinolone production because of the bacteria's preference for maintaining *pqsE* transcription despite quinolone production.

Data from the real-time experiments were confirmed using a reporter containing a fusion of the *pqsA* promoter region and the *lacZ* gene. We determined that both exogenous supplementation and endogenous production of DHQ increased activity of the reporter compared to a vehicle control and quinolone-null control, respectively.

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Importantly, DHQ supplemented to an *E. coli* strain containing the reporter demonstrated a dose-dependent increase in activity. This result indicated that DHQ directly influenced activity. However, supplementation experiments to *P. aeruginosa* strains did not display a significant increase (data not shown). The ability to pass through the membrane may account for why DHQ did not affect co-supplementation with HHQ and PQS, which possess alkyl-moieties that may become associated with the membrane. We hypothesize that the charge of DHQ in solution prevents the molecules from crossing the *P. aeruginosa* membrane or those molecules that enter are quickly secreted.

In order to cross the membrane, DHQ may require association with another molecule to increase its hydrophobicity and decrease its charge. Rhamnolipids could increase the solubility of DHQ and help bring the molecule back into the cell. PqsR positively regulates the *rhl* operon for subsequent rhamnolipid production [154]. Together, rhamnolipids may play a role with quinolones to help facilitate their transport.

DHQ supplemented to PqsR *in vitro* activated the transcriptional regulator to bind to the promoter region of *pqsA*. PqsR alone did not bind to the DNA indicating ligand binding was essential for activity. Again, a dose-response in binding demonstrated DHQ was another ligand of PqsR and supported the *in vivo* results from the reporter and real-time experiments. The EMSAs were performed using cell-lysate, which supported the activity of recombinant PqsR. We performed EMSA in both *E. coli* producing recombinant PqsR and in the *P. aeruginosa pqs* mutants. Both sets of assays concluded DHQ was sufficient for activity. PqsR contains an insoluble DNA-binding domain and would precipitate from purification buffer quickly under ambient temperatures. Use of cell-lysate provided a vehicle to maintain PqsR in solution and may also facilitate the

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interaction of the transcriptional regulator with DNA because of the more physiologically-relevant environment. Future work may investigate the binding of DHQ-activated PqsR to other regions on the *pqs* operon. Previously published work has identified potential alternative binding sites for PqsR, but those sites remain to be substantiated using direct binding assays and defining what conditions those sites are used instead of the canonical *pqsA* promoter.

STD-NMR is a powerful structural technique to monitor the interaction between small molecules and proteins [181]. This *in vitro* assay confirmed the interaction of DHQ with PqsR and also began to determine the molecular interactions involved. We were able to use a truncated form of PqsR containing the ligand-binding domain to determine the Kd, residence time of the molecule, and moieties on DHQ responsible for binding. The technique relies on measuring the signal from the ligand before and during different titrations to a protein. Titrating DHQ to PqsR showed DHQ was in fast-exchange and that a certain hybridized C-H on DHQ is the donor group to form a hydrogen bond with PqsR. Both *in vitro* methods, STD-NMR and EMSA, determined the Kd for PqsR with DHQ to be in nanomolar concentrations. Further analysis of the interaction should focus on showing the structure of PqsR bound to DHQ. PqsR can be double-labeled, which allows for higher-order analysis of the structure via 2D and 3D techniques. Double-labeled protein can be used with two specific experiments in order to better determine the effects of titration on binding and the binding site. Structures of bound and unbound forms of PqsR would also show any potential structural changes that take place during binding and how that may preference activation for DNA binding.

In a single cycle of QS, QS systems are activated and produce molecules that leave the cell. At a certain intracellular concentration following diffusion, transcriptional regulators initiate and this process repeats in a positive manner. There is limited information as to what may continue to happen within a mature community after initial activation of QS systems. Often long term growth requires a balance with the environment and cellular stress response systems that can maintain DNA and cell integrity despite self-inflicted stress. It is not understood how QS systems are involved during long-term growth. We hypothesize the Pqs and Rhl systems continue to be active over time and initiate a circadian rhythm by which only one QS system is active and the other is repressed. The rationale is that products from these systems are both essential for gathering nutrients and also maintaining the local environment. Therefore, QS activation of the systems are important because these products may become depleted and need replenishing. An interesting question for future research may focus on how environmental signals alter QS in order to gather those nutrients to maintain the essential or pathogenic systems.

DHQ and QS molecule consortia in CF sputum samples

QS is involved in virulence factor production and biofilm formation in response to bacterial density in the environment. Most of the data gathered to support these interactions were performed *in vitro,* but only limited experiments have attempted to determine the properties of QS *in vivo*. Work on bacterial density has shown that bacteria do not need to grow to large numbers in order to communicate, rather a few cells in a small area can synchronize phenotypes [218]. Therefore, only a few bacterial cells are needed to enact QS regulation given the right environment together and capacity to exchange QS molecules. These small communities are significant because CF sputum contains small macrocolonies of bacteria that resemble small biofilm structures.

We understand QS in a test-tube, but the environment has a dramatic effect on QS regulation. Limited nutrients, oxygen, and loss of QS molecules to the environment are some examples of different environments that alter QS. The environment that *P. aeruginosa* colonizes in an infection has taken recent interest in the research community and will be an area that will help compare QS phenotypes between *in vitro* and *in vivo* conditions.

During chronic colonization of the CF lungs, *P. aeruginosa* undergoes several genotypic and phenotypic changes. These changes are both self-inflicted and induced by the environment. Following mutation, *P. aeruginosa* is under constant pressure for adaptation. The selective pressures in the lungs create a diverse community of *P. aeruginosa* cells that display different phenotypes and begin to resemble multicellular communities [219]. QS systems are frequently mutated throughout chronic colonization,

for example, the Las system is often mutated and losses function after colonization [220]. The Pqs system is another system that can also be mutated, but this is found much less often and requires the other QS systems to maintain function. When considering the interactions of QS and long-term growth, chronic colonization adds more complexity to understand how QS molecules can be interpreted as the active phenotype..

One limitation to studying QS molecules as a connection to disease severity and bacterial phenotypes is the poorly understood natural progression of molecules within the host environment. Bacteria synthesize QS molecules that accumulate in the environment, but the duration and integrity of the QS molecules is less understood. In the environment, molecules are subject to chemical conditions, which may cause them to breakdown. QS molecules may also be lost to physical processes and degradation by secreted enzymes. These changes to QS molecules will alter the concentration quantified from patient samples and may not represent the phenotypes found *in vitro*. The alterations in QS molecules will subsequently distort the conclusions reached based on QS molecules as biomarkers. Engineered *in vitro* systems may help to recapitulate those systems found within the lungs that alter the concentration of QS molecules and would help to assess phenotypes of bacteria.

In order to assess QS molecule dynamics within the sputum, imaging mass spectrometry may be used with sections of mucus to investigate the accumulation of molecules [221]. Another future focus for QS biomarker research is mucus production, which is affected by several different treatments, i.e. DNases, hypertonic saline, and respiratory therapy. Our patient study assessed sputum from different CF patients throughout their treatment. We could not control the quality of sputum, time of

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production, or the therapies the patients received, but we did ensure enough sputum was given in order to extract QS molecules. Working with the CF respiratory therapists, we were able to gather sputum both independently in the clinic and also following percussion therapy. Hospitalized patients gave samples during a morning routine with the respiratory therapists as well. Together, sputum sampling offered a convenient mechanism for sampling CF patient lungs, but will require further optimization for quality and reproducibility.

In order to reduce attack from the immune system, *P. aeruginosa* produces molecules that reduce signaling and viability of immune cells. For an opportunistic bacterium that resides in diverse natural environments, it is interesting to find a bacterial system that regulates host functions so efficiently. An important unanswered question to understand is what are the selective pressures that maintain production of DHQ. .We have found evidence that DHQ may be actively involved in dampening the host immune response. During an infection, reducing macrophage activity limits the inflammatory response that *P. aeruginosa* faces in the lungs. Other QS also affect the inflammatory response, which may have an effect on how *P. aeruginosa* protects its microenvironment during an infection. Future studies of the role of QS and inflammation should investigate the QS molecules together as a consortium in order to determine the complicated interactions that are involved.

Future Directions

DHQ plays both specialized and redundant roles for the Pqs system, but most importantly, DHQ is produced in high concentrations. Bacteria do not waste resources and quickly adapt their phenotypes. Those systems that are not essential for *P. aeruginosa* are selected against overtime. Our research focused on the importance of DHQ to *P. aeruginos*a during an infection. We found that production of DHQ maintained pathogenicity towards an infection model when synthesis of the other quinolones was stopped. However, DHQ did not perform as a signaling molecule to the same level compared to HHQ and PQS. What we did conclude was DHQ played a significant role as an extracellular molecule against host cells, regardless of oxygen present. An important unanswered question is what role DHQ plays for *P. aeruginosa* during its colonization of a soil or aquatic environment. These environments are the reservoir of *P. aeruginosa*. As an opportunistic infection, *P. aeruginosa* only causes disease when a barrier is broken down. Therefore, *P. aeruginosa* persists in the environment until it comes into contact with a host. For *P. aeruginosa* to maintain DHQ production host-to-host, DHQ must play a role that is not selected against. For most bacterial pathogens, this question is often unanswered for secondary metabolites.

From the Pqs system, a large group of quinolones is produced. However, apart from the alkylquinolones, DHQ is the only terminate non-alkylated product. With this in consideration, a future question may involve the evolution of DHQ synthesis away from the other quinolones. Two hypotheses provide possibilities as to why DHQ diverged within the Pqs system. First, DHQ formation preceded synthesis of alkylquinolones,

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which were developed for better signaling and transcriptional regulation. Also, the lowered ability for signaling may indicate that the extracellular role for DHQ was originally utilized by *P. aeruginosa* followed by some QS activity with the molecule. An alternative hypothesis is that DHQ was more recently developed from the Pqs system. As the Pqs system became more pivotal for survival, alkylquinolones were produced as an expensive signaling molecule. In order to produce quinolones optimally, DHQ was synthesized because its precursors are significantly more abundant in an environment. Development of DHQ production after establishment of alkylquinolone signaling would only come from a selective pressure that DHQ fulfills. The selective pressure for this adaptation would likely remain with *P. aeruginosa*. Research needs to determine what environmental factors potentiate the use of DHQ for increased *P. aeruginosa* survival.

The use of DHQ as an anti-inflammatory drug requires further investigation, but offers an exciting outlook for the molecule. The extracellular effects of DHQ were dramatic against host cells because the results indicated DHQ reduced cellular viability and key inflammatory signals. DHQ treated lung epithelial cells displayed reduced cellular replication, but no outward effect on cellular morphology or metabolism. Reduced MTS reading from the epithelial cells were a result of lower cell number. The effects of DHQ against alveolar macrophages were different in that DHQ was toxic towards the host cells based on reduced MTS reading and similar cell counts compared to vehicle treatment. LPS-activated macrophages also produced fewer cytokines as a result of DHQ treatment. Previous work demonstrated quinolones reduced NF-kB signaling, which is responsible for activating cytokine production. Future work may also determine if DHQ also has an effect on IL-10 production, which is an anti-inflammatory cytokine.

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Because of the low toxicity and low micromolar concentration required to reduce cytokines, DHQ is a good scaffold for further development of anti-inflammatory compounds.

Recently published work identified overactive inflammation as an agent of several diseases [222]. The reason for over-active inflammation was a result of the inability to reduce the signaling cascade after initiation. DHQ may be used in these situations to lower patient inflammation, but not critically limit the immune system to the point of predisposing those to opportunistic infections.

QS systems can be targets for inhibition that would reduce pathogenicity of bacteria. Because QS systems are essential for initiating an infection in models, inhibiting QS would allow the host's immune system to kill the bacteria. Several groups have identified compounds that block QS *in vitro* and also have some success *in vivo*. However, no drug has been brought to market as a result of the research. Researchers have encountered issues with access to targets, solubility of compounds, and identification of targets that have maximum effect. PqsR may be the most effective because of its regulation of the *pqs* and *rhl* operons [223]. Loss of PqsR would result in blocked quinolone production, reduced ability to form communities, and lowered pyocyanin production. These are all key factors that make *P. aeruginosa* a successful pathogen. In fact all transcriptional regulators are potential targets for inhibition because of their investment in QS, biofilm formation, and virulence factor production [51]. Future work can exploit the structural determination of the transcriptional regulators and new methods of drug delivery.

Methods

1. Bacterial strains, plasmids, and media

      P. aeruginosa wild-type strain PAO1, derived mutants, and *E. coli* strains were grown in Luria–Bertani (LB) medium at 37°C in a shaker incubator. Cystic fibrosis mimic media was created according to Palmer et al [23]. Hypoxic conditions were created by flushing media with N_2 for 10 min and incubating cultures within a screw-cap vial sealed with a Tephlon© insert. All strains and plasmids used are listed on Table 1. When necessary, 200 or 50 µg/ml carbenicillin (CBC), 30 µg/ml kanamycin (Km), and/or 34 µg/ml chloramphenicol was added to the culture medium. Culture density was assessed by measuring absorbance at 600 nm. DHQ (2,4-dihydroxyquinoline) was purchased from Sigma-Aldrich and PQS (2-heptyl-3-hydroxy-4-quinolone) and HHQ (2-heptly-4-quinolone) were purchased from Qingdao Vochem Co..

2. Generation of mutants

      P. aeruginosa mutant strains were generated by homologous recombination using a protocol described previously [224]. The mutant alleles were constructed by overlapping PCR to contain a gentamicin-resistance cassette flanked by 5' and 3' fragments of the gene to be deleted. The mutant fragments were inserted into pEX18ApGW, a suicide vector, to produce gene knockout plasmids. Each knockout plasmid was transformed into *E. coli* strain SM10 and conjugally transferred from SM10 to PAO1. The resultant integrants were selected on PIA medium containing 30 µg/ml gentamicin. To ascertain resolution of merodiploids, Gm^r colonies were streaked for single colonies on LB+Gm30 plates containing 5% sucrose. The unmarked deletion

mutants were generated by Flp-mediated marker excision utilizing pFLP2. Potential

mutants were screened by PCR using corresponding flanking primers, and were

confirmed by sequencing.

3. Generation of protein-expressing plasmids and protein purification

Gene fragments of PqsR and PqsR-C[87] were PCR amplified and digested with restriction

enzymes. Digested fragments were cloned into ppSUMO before transformation into

Promega JM109. Colonies isolated following transformation were sequenced to ensure

integrity and directionality of the insert. BL21(DE3) and Rosetta were transformed with

the expression plasmids for protein production. Briefly, *E. coli* containing the expression

plasmids were grown in LB to OD_{600} of 0.6 and heat-shocked at 45°C for 45 min,

followed by induction with 0.1mM isopropyl β-D-thiogalactopyranoside at 16°C for 16 h.

The cells were harvested by centrifugation and resuspened in Tris-HCL (pH 8.0), 500

mM NaCl and lysed using sonication (40% amplitude, 15 sec sonication with 45 sec

break for 5 min). His-tagged protein in the cell-free supernatant was purified using nickel

affinity chromatography. The fractions containing pure protein were pooled,

concentrated, and stored either at -80°C or with 50% v/v glycerol at -20°C. Protein

concentration was determined using the Bradford method [225].

4. Caenorhabditis elegans survival and imaging.

Bristol N2 strain of *C. elegans* was maintained and synchronized on nematode

growth media (NGM) containing *E. coli* OP50 according to the worm book [226].

Synchronized L4 nematodes (around 30 per plate) were transferred to 60 mm petri dishes

with bacterial lawns of the *P. aeruginosa* strains, which had been incubated overnight at 37°C. Assays were conducted at 25°C and nematodes were monitored daily for survival. Dead nematodes were determined after no movement following stimulation with a platinum wire. To assess *in vivo* bacterial colonization, *P. aeruginosa* strains carrying a GFP-expressing plasmid were used for infection. At the indicated time-points, nematodes were removed and allowed to move on fresh agar to remove bacteria attached to the outside of the nematodes. These nematodes were placed into a 10 µl drop of water on a glass slide and covered with a cover slip. Samples were viewed using a Nikon TE2000-S Epifluorescent microscope with CRI-Nuance imaging system. Images were color-enhanced and analyzed with ImageJ (NIH).

5. Pyocyanin quantification

Pyocyanin was measured using a modified protocol from previous work [227]. Briefly, 400 µl of overnight culture cell-free supernatant was mixed with 240 µl chloroform. After mixing vigorously, 200 µl of the organic phase was transferred to a new tube and mixed with 60 µl 0.2 N HCl. Pyocyanin was measured spectrophotometrically at 520 nm using a NanoDrop ND-1000 spectrophotometer.

6. Quantitative Real Time PCR (qRT-PCR)

P. aeruginosa strains were grown in LB until early stationary phase. Total RNA was harvested using 5-Prime PerfectPure Cultured Cell kit according to the manufacturer's instructions. RNA was treated with DNaseI and precipitated overnight at -20°C using the Ambion DNase treatment and removal kit. cDNA was generated using the

Bioline Tetro cDNA synthesis kit and manufacturer's instructions. Bioline SensiFAST

SYBR&Fluorescien kit with cDNA and primers for *pqsA* and *rpoD* were run on a Bio-

Rad MyIQ RT-PCR detection system. The expression levels of *pqsA* were measured as

previously described [228].

7. pqsA'-LacZ fusion reporter assay

LacZ reporter assay was performed in both *E. coli* and in *P. aeruginosa* strains as

previously described [176]. Briefly, overnight cultures of *E. coli*:pEAL08-2 was diluted

to OD 600 nm 0.05 and grown in the presence of quinolones for 2 h, followed by

centrifugation of the cells. *P. aeruginosa* strains with pEAL08-2 were grown overnight

after dilution to OD 600 nm 0.05. Cells were lysed and activity was measured using the

Promega β-galactosidase enzyme assay kit and measured at absorbance 420 nm on a

BioTek Synergy HT plate reader.

8. Electrophoretic Mobility Shift Assay (EMSA)

Protein-DNA gel retention assays were performed using cell lysates from PAO1,

pqs mutants, and Rosetta strains induced for SUMO or SUMO-PqsR production as

described [176]. For each sample, 10 μg of cell-lysate was incubated with 0.15 fmol of

biotinylated-*pqsA* that was generated from PCR amplification with biotinylated-primers

with the Pierce LightShift Chemiluminescent EMSA kit (10mM Tris-HCL, pH8.0, 1mM

EDTA, 50mM NaCl + 1mM Dithiothreitol + 1 μg/μl Poly(dIdC)) and 0.5x Tris Borate

EDTA (TBE) buffer for 20 min at room temperature (24°C). Unlabeled *pqsA* was

generated using the same primers as the Bio-*pqsA*, but without the biotinylation tag.

Samples were run at 100 V for 50 min on a 5% polyacrylamide gel and transferred onto a positively-charged nylon membrane at 40 V for 1 h. The nylon membrane was processed with the chemiluminescence kit and exposed to either X-ray film or GE ImageQuant RT-ECL.

9. Saturation Transfer Difference NMR

STD NMR experiments were prepared with 1 μM SUMO-PqsR-C87 with increasing concentrations of DHQ (10 nm to 100 μM). Data was collected at 298K on a Bruker Avance III 600 MHz NMR spectrometer equipped with a 5 mm cryogenically-cooled QC-Inverse and using a standard STD pulse sequence with 30 ms 8.4 kHz spin lock to minimize background protein resonances [229]. Solvent suppression was achieved using the excitation sculpting scheme. Saturation of the protein signals was performed using a train of 10, 20, or 59 selective 56 dB Gaussian pulses of 50 ms duration with total saturation times of 0.25 sec to 5.0 sec. The on-resonance frequency was set up at -0.5 ppm. STD spectra were acquired from 64 scans, 2050 receiver gain, and 14 ppm sweep width.

10. Quantification of quorum-sensing molecules

PAO1 aerobic and anaerobic cultures were grown overnight. Cultures were spun down and the supernatant was collected and acidified with 0.1% formic acid. HPLC purification was carried out using a WATERS HPLC system (HPLC 2767 Sample Manager), 1525 WATERS 2996 PDA and WATERS ZQ Single Quadrapole Mass Detector outfitted with MASS LYNX software (Waters Corporation, Milford, MA). The

HPLC/MS method was water/acetonitrile (ACN) gradient with 0.1% formic acid in both solvents. Samples were loaded onto an Ascentis Express C18 (Sulpeco Analytical) 5 μm particle size, 150 mm x 21 mm column. The flow scheme conditions were: 0.4 ml/min flow rate, 70:30 water/ACN (hold for 2 min). This was followed by a linear gradient over 30 min to 100% ACN. The 100% ACN was held for 5 min prior to re-establishment of original flow conditions. Column temperature was held at 30°C.

11. Cell viability and total cell counts

Human A549 lung carcinoma cell line and murine RAW264.7 alveolar macrophages were maintained in RPMI supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. Cells were maintained in flasks and transferred at confluency to 96-well plates ($2x10^4$ per well). After transferring, cells were treated with quinolones in DMSO. Viability of cells was assessed by MTS assay using the Promega CellTiter 96 aqueous non-radioactive cell proliferation kit according to manufacturer's instructions. Total cell counts were performed in 24-well plates ($2x10^4$ per well for A549 cells and $3x10^3$ per well for RAW264.7 cells) with a single quinolone concentration for treatment. A549 cells were removed for numeration using 0.05% trypsin and RAW264.7 cells were removed using a cell scraper. Cells were treated with Trypan blue and counted using a haemocytometer.

12. Molecular modeling

Docking studies were performed using GOLD software package 5.1. (Cambridge Crystallographic Data Centre, Cambridge, UK). The X-ray coordinates of PqsR (4JVD,

4JVC, and 4JVI) were downloaded from the Protein Data Bank. The active site was determined from both 4JVD and 4JVI. Docking of DHQ with PqsR was energy minimized and scored using ChemPLP. Of the possible sites generated by modeling, the highest fitness score provided the potential interactions to diagram. Molecular Operations Environment (MOE) was used to diagram DHQ within the PqsR hydrophobic binding pocket.

13. Extraction of QS molecules from sputum

Sputum was measured for volume and extracted with 2 volumes of ethyl acetate. The organic phase of the extraction was dried down under nitrogen and rehydrated in formic acid and ethanol. Samples were maintained at -20^0C until processed through LC-MS.

References:

1.   Flick MR, Cluff LE: **Pseudomonas bacteremia. Review of 108 cases**. *Am J Med* 1976, **60**(4):501-508.

2.   Morrison AJ, Jr., Wenzel RP: **Epidemiology of infections due to Pseudomonas aeruginosa**. *Rev Infect Dis* 1984, **6 Suppl 3**:S627-642.

3.   Cos P, Tote K, Horemans T, Maes L: **Biofilms: an extra hurdle for effective antimicrobial therapy**. *Curr Pharm Des* 2010, **16**(20):2279-2295.

4.   Costerton JW, Montanaro L, Arciola CR: **Biofilm in implant infections: its production and regulation**. *The International journal of artificial organs* 2005, **28**(11):1062-1068.

5.   Planquette B, Timsit JF, Misset BY, Schwebel C, Azoulay E, Adrie C, Vesin A, Jamali S, Zahar JR, Allaouchiche B *et al*: **Pseudomonas aeruginosa ventilator-associated pneumonia. predictive factors of treatment failure**. *American journal of respiratory and critical care medicine* 2013, **188**(1):69-76.

6.   Weems JJ, Jr.: **Nosocomial outbreak of Pseudomonas cepacia associated with contamination of reusable electronic ventilator temperature probes**. *Infect Control Hosp Epidemiol* 1993, **14**(10):583-586.

7.   Dart JK, Seal DV: **Pathogenesis and therapy of Pseudomonas aeruginosa keratitis**. *Eye (Lond)* 1988, **2 Suppl**:S46-55.

8. Lipsky BA, Peters EJ, Senneville E, Berendt AR, Embil JM, Lavery LA, Urbancic-Rovan V, Jeffcoate WJ: **Expert opinion on the management of infections in the diabetic foot**. *Diabetes Metab Res Rev* 2012, **28 Suppl 1**:163-178.

9. Church D, Elsayed S, Reid O, Winston B, Lindsay R: **Burn wound infections**. *Clin Microbiol Rev* 2006, **19**(2):403-434.

10. Bauer KA, West JE, O'Brien JM, Goff DA: **Extended-infusion cefepime reduces mortality in patients with Pseudomonas aeruginosa infections**. *Antimicrob Agents Chemother* 2013, **57**(7):2907-2912.

11. Peleg AY, Hooper DC: **Hospital-acquired infections due to gram-negative bacteria**. *N Engl J Med* 2010, **362**(19):1804-1813.

12. Bye MR, Ewig JM, Quittell LM: **Cystic fibrosis**. *Lung* 1994, **172**(5):251-270.

13. Rowe SM, Verkman AS: **Cystic fibrosis transmembrane regulator correctors and potentiators**. *Cold Spring Harb Perspect Med* 2013, **3**(7).

14. Wine JJ: **Cystic fibrosis: How do CFTR mutations cause cystic fibrosis?** *Curr Biol* 1995, **5**(12):1357-1359.

15. Davidson DJ, Dorin JR, McLachlan G, Ranaldi V, Lamb D, Doherty C, Govan J, Porteous DJ: **Lung disease in the cystic fibrosis mouse exposed to bacterial pathogens**. *Nature genetics* 1995, **9**(4):351-357.

16. Yankaskas JR, Marshall BC, Sufian B, Simon RH, Rodman D: **Cystic fibrosis adult care: consensus conference report**. *Chest* 2004, **125**(1 Suppl):1S-39S.

17. Stephenson AL, Tom M, Berthiaume Y, Singer LG, Aaron SD, Whitmore GA, Stanojevic S: **A contemporary survival analysis of individuals with cystic fibrosis: a cohort study**. *The European respiratory journal* 2014.

18. Emerson J, Rosenfeld M, McNamara S, Ramsey B, Gibson RL: **Pseudomonas aeruginosa and other predictors of mortality and morbidity in young children with cystic fibrosis**. *Pediatr Pulmonol* 2002, **34**(2):91-100.

19. Hoiby N: **Recent advances in the treatment of Pseudomonas aeruginosa infections in cystic fibrosis**. *BMC Med* 2011, **9**:32.

20. FitzSimmons SC: **The changing epidemiology of cystic fibrosis**. *J Pediatr* 1993, **122**(1):1-9.

21. Schafhauser J, Lepine F, McKay G, Ahlgren HG, Khakimova M, Nguyen D: **The stringent response modulates 4-hydroxy-2-alkylquinoline biosynthesis and quorum-sensing hierarchy in Pseudomonas aeruginosa**. *J Bacteriol* 2014, **196**(9):1641-1650.

22. Coggan KA, Wolfgang MC: **Global regulatory pathways and cross-talk control pseudomonas aeruginosa environmental lifestyle and virulence phenotype**. *Curr Issues Mol Biol*, **14**(2):47-70.

23. Palmer KL, Mashburn LM, Singh PK, Whiteley M: **Cystic fibrosis sputum supports growth and cues key aspects of Pseudomonas aeruginosa physiology**. *J Bacteriol* 2005, **187**(15):5267-5277.

24. Palmer KL, Aye LM, Whiteley M: **Nutritional cues control Pseudomonas aeruginosa multicellular behavior in cystic fibrosis sputum**. *J Bacteriol* 2007, **189**(22):8079-8087.

25.   Benz R, Bauer K: **Permeation of hydrophilic molecules through the outer membrane of gram-negative bacteria. Review on bacterial porins**. *Eur J Biochem* 1988, **176**(1):1-19.

26.   Delcour AH: **Outer membrane permeability and antibiotic resistance**. *Biochim Biophys Acta* 2009, **1794**(5):808-816.

27.   Shand GH, Anwar H, Brown MR: **Outer membrane proteins of polymyxin resistant Pseudomonas aeruginosa: effect of magnesium depletion**. *J Antimicrob Chemother* 1988, **22**(6):811-821.

28.   Pai H, Kim J, Lee JH, Choe KW, Gotoh N: **Carbapenem resistance mechanisms in Pseudomonas aeruginosa clinical isolates**. *Antimicrob Agents Chemother* 2001, **45**(2):480-484.

29.   Poole K, Krebes K, McNally C, Neshat S: **Multiple antibiotic resistance in Pseudomonas aeruginosa: evidence for involvement of an efflux operon**. *J Bacteriol* 1993, **175**(22):7363-7372.

30.   Giske CG, Buaro L, Sundsfjord A, Wretlind B: **Alterations of porin, pumps, and penicillin-binding proteins in carbapenem resistant clinical isolates of Pseudomonas aeruginosa**. *Microb Drug Resist* 2008, **14**(1):23-30.

31.   Mah TF, O'Toole GA: **Mechanisms of biofilm resistance to antimicrobial agents**. *Trends in microbiology* 2001, **9**(1):34-39.

32.   Anwar H, Strap JL, Costerton JW: **Establishment of aging biofilms: possible mechanism of bacterial resistance to antimicrobial therapy**. *Antimicrob Agents Chemother* 1992, **36**(7):1347-1351.

33.   Nichols WW, Dorrington SM, Slack MP, Walmsley HL: **Inhibition of tobramycin diffusion by binding to alginate**. *Antimicrob Agents Chemother* 1988, **32**(4):518-523.

34.   Evans TJ: **Small colony variants of Pseudomonas aeruginosa in chronic bacterial infection of the lung in cystic fibrosis**. *Future Microbiol*, **10**(2):231-239.

35.   Schaber JA, Triffo WJ, Suh SJ, Oliver JW, Hastert MC, Griswold JA, Auer M, Hamood AN, Rumbaugh KP: **Pseudomonas aeruginosa forms biofilms in acute infection independent of cell-to-cell signaling**. *Infection and immunity* 2007, **75**(8):3715-3721.

36.   Bjarnsholt T: **The role of bacterial biofilms in chronic infections**. *APMIS Suppl* 2013(136):1-51.

37.   Coggan KA, Wolfgang MC: **Global regulatory pathways and cross-talk control pseudomonas aeruginosa environmental lifestyle and virulence phenotype**. *Curr Issues Mol Biol* 2012, **14**(2):47-70.

38.   Venturi V: **Regulation of quorum sensing in Pseudomonas**. *FEMS Microbiol Rev* 2006, **30**(2):274-291.

39.   Irie Y, Borlee BR, O'Connor JR, Hill PJ, Harwood CS, Wozniak DJ, Parsek MR: **Self-produced exopolysaccharide is a signal that stimulates biofilm formation in Pseudomonas aeruginosa**. *Proc Natl Acad Sci U S A* 2012, **109**(50):20632-20636.

40.   Colvin KM, Irie Y, Tart CS, Urbano R, Whitney JC, Ryder C, Howell PL, Wozniak DJ, Parsek MR: **The Pel and Psl polysaccharides provide Pseudomonas**

aeruginosa structural redundancy within the biofilm matrix. *Environ Microbiol* 2012, **14**(8):1913-1928.

41.   Senda K, Arakawa Y, Nakashima K, Ito H, Ichiyama S, Shimokata K, Kato N, Ohta M: **Multifocal outbreaks of metallo-beta-lactamase-producing Pseudomonas aeruginosa resistant to broad-spectrum beta-lactams, including carbapenems**. *Antimicrob Agents Chemother* 1996, **40**(2):349-353.

42.   Webb JS, Thompson LS, James S, Charlton T, Tolker-Nielsen T, Koch B, Givskov M, Kjelleberg S: **Cell death in Pseudomonas aeruginosa biofilm development**. *J Bacteriol* 2003, **185**(15):4585-4592.

43.   Boles BR, Thoendel M, Singh PK: **Self-generated diversity produces "insurance effects" in biofilm communities**. *Proc Natl Acad Sci U S A* 2004, **101**(47):16630-16635.

44.   Pearson JP, Feldman M, Iglewski BH, Prince A: **Pseudomonas aeruginosa cell-to-cell signaling is required for virulence in a model of acute pulmonary infection**. *Infection and immunity* 2000, **68**(7):4331-4334.

45.   Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, Kumar N, Schembri MA, Song Z, Kristoffersen P *et al*: **Attenuation of Pseudomonas aeruginosa virulence by quorum sensing inhibitors**. *EMBO J* 2003, **22**(15):3803-3815.

46.   Rumbaugh KP, Griswold JA, Iglewski BH, Hamood AN: **Contribution of quorum sensing to the virulence of Pseudomonas aeruginosa in burn wound infections**. *Infection and immunity* 1999, **67**(11):5854-5862.

47. Fuqua WC, Winans SC, Greenberg EP: **Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators**. *J Bacteriol* 1994, **176**(2):269-275.

48. Dandekar AA, Chugani S, Greenberg EP: **Bacterial quorum sensing and metabolic incentives to cooperate**. *Science*, **338**(6104):264-266.

49. Wilder CN, Diggle SP, Schuster M: **Cooperation and cheating in Pseudomonas aeruginosa: the roles of the las, rhl and pqs quorum-sensing systems**. *ISME J*, **5**(8):1332-1343.

50. Whitehead NA, Barnard AM, Slater H, Simpson NJ, Salmond GP: **Quorum-sensing in Gram-negative bacteria**. *FEMS Microbiol Rev* 2001, **25**(4):365-404.

51. Rutherford ST, Bassler BL: **Bacterial quorum sensing: its role in virulence and possibilities for its control**. *Cold Spring Harb Perspect Med*, **2**(11).

52. Soulere L, Frezza M, Queneau Y, Doutheau A: **Exploring the active site of acyl homoserine lactones-dependent transcriptional regulators with bacterial quorum sensing modulators using molecular mechanics and docking studies**. *J Mol Graph Model* 2007, **26**(2):581-590.

53. Chugani S, Greenberg EP: **An evolving perspective on the Pseudomonas aeruginosa orphan quorum sensing regulator QscR**. *Front Cell Infect Microbiol* 2014, **4**:152.

54. Seviour T, Hansen SH, Yang L, Yau YH, Wang VB, Stenvang MR, Christiansen G, Marsili E, Givskov M, Chen Y *et al*: **Functional Amyloids Keep Quorum Sensing Molecules in Check**. *J Biol Chem* 2015.

55.   Oglesby AG, Farrow JM, 3rd, Lee JH, Tomaras AP, Greenberg EP, Pesci EC, Vasil ML: **The influence of iron on Pseudomonas aeruginosa physiology: a regulatory link between iron and quorum sensing**. *J Biol Chem* 2008, **283**(23):15558-15567.

56.   Jensen V, Lons D, Zaoui C, Bredenbruch F, Meissner A, Dieterich G, Munch R, Haussler S: **RhlR expression in Pseudomonas aeruginosa is modulated by the Pseudomonas quinolone signal via PhoB-dependent and -independent pathways**. *J Bacteriol* 2006, **188**(24):8601-8606.

57.   Hassett DJ, Cuppoletti J, Trapnell B, Lymar SV, Rowe JJ, Yoon SS, Hilliard GM, Parvatiyar K, Kamani MC, Wozniak DJ *et al*: **Anaerobic metabolism and quorum sensing by Pseudomonas aeruginosa biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets**. *Adv Drug Deliv Rev* 2002, **54**(11):1425-1443.

58.   Medina G, Juarez K, Diaz R, Soberon-Chavez G: **Transcriptional regulation of Pseudomonas aeruginosa rhlR, encoding a quorum-sensing regulatory protein**. *Microbiology* 2003, **149**(Pt 11):3073-3081.

59.   Nguyen D, Joshi-Datar A, Lepine F, Bauerle E, Olakanmi O, Beer K, McKay G, Siehnel R, Schafhauser J, Wang Y *et al*: **Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria**. *Science*, **334**(6058):982-986.

60.   Boes N, Schreiber K, Schobert M: **SpoT-triggered stringent response controls usp gene expression in Pseudomonas aeruginosa**. *J Bacteriol* 2008, **190**(21):7189-7199.

61. Kleerebezem M, Quadri LE, Kuipers OP, de Vos WM: **Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria**. *Mol Microbiol* 1997, **24**(5):895-904.

62. Miller MB, Bassler BL: **Quorum sensing in bacteria**. *Annu Rev Microbiol* 2001, **55**:165-199.

63. Parsek MR, Greenberg EP: **Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms**. *Proc Natl Acad Sci U S A* 2000, **97**(16):8789-8793.

64. Lee J, Wu J, Deng Y, Wang J, Wang C, Chang C, Dong Y, Williams P, Zhang LH: **A cell-cell communication signal integrates quorum sensing and stress response**. *Nat Chem Biol*, **9**(5):339-343.

65. Davies DG, Marques CN: **A fatty acid messenger is responsible for inducing dispersion in microbial biofilms**. *J Bacteriol* 2009, **191**(5):1393-1403.

66. Holban AM, Bleotu C, Chifiriuc MC, Bezirtzoglou E, Lazar V: **Role of Pseudomonas aeruginosa quorum sensing (QS) molecules on the viability and cytokine profile of human mesenchymal stem cells**. *Virulence*, **5**(2):303-310.

67. Wilder CN, Diggle SP, Schuster M: **Cooperation and cheating in Pseudomonas aeruginosa: the roles of the las, rhl and pqs quorum-sensing systems**. *ISME J* 2011, **5**(8):1332-1343.

68. Latifi A, Foglino M, Tanaka K, Williams P, Lazdunski A: **A hierarchical quorum-sensing cascade in Pseudomonas aeruginosa links the**

transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol Microbiol* 1996, **21**(6):1137-1146.

69.   Diggle SP, Winzer K, Chhabra SR, Worrall KE, Camara M, Williams P: **The Pseudomonas aeruginosa quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR**. *Mol Microbiol* 2003, **50**(1):29-43.

70.   Winson MK, Camara M, Latifi A, Foglino M, Chhabra SR, Daykin M, Bally M, Chapon V, Salmond GP, Bycroft BW *et al*: **Multiple N-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in Pseudomonas aeruginosa**. *Proc Natl Acad Sci U S A* 1995, **92**(20):9427-9431.

71.   Al-Tahhan RA, Sandrin TR, Bodour AA, Maier RM: **Rhamnolipid-induced removal of lipopolysaccharide from Pseudomonas aeruginosa: effect on cell surface properties and interaction with hydrophobic substrates**. *Appl Environ Microbiol* 2000, **66**(8):3262-3268.

72.   Wang S, Yu S, Zhang Z, Wei Q, Yan L, Ai G, Liu H, Ma LZ: **Coordination of swarming motility, biosurfactant synthesis, and biofilm matrix exopolysaccharide production in Pseudomonas aeruginosa**. *Appl Environ Microbiol* 2014, **80**(21):6724-6732.

73. Alhede M, Bjarnsholt T, Givskov M, Alhede M: **Pseudomonas aeruginosa biofilms: mechanisms of immune evasion**. *Adv Appl Microbiol* 2014, **86**:1-40.

74. Pesci EC, Milbank JB, Pearson JP, McKnight S, Kende AS, Greenberg EP, Iglewski BH: **Quinolone signaling in the cell-to-cell communication system of Pseudomonas aeruginosa**. *Proc Natl Acad Sci U S A* 1999, **96**(20):11229-11234.

75. Folch B, Deziel E, Doucet N: **Systematic mutational analysis of the putative hydrolase PqsE: toward a deeper molecular understanding of virulence acquisition in Pseudomonas aeruginosa**. *PLoS One* 2013, **8**(9):e73727.

76. Dubern JF, Diggle SP: **Quorum sensing by 2-alkyl-4-quinolones in Pseudomonas aeruginosa and other bacterial species**. *Mol Biosyst* 2008, **4**(9):882-888.

77. Schuster M, Lostroh CP, Ogi T, Greenberg EP: **Identification, timing, and signal specificity of Pseudomonas aeruginosa quorum-controlled genes: a transcriptome analysis**. *J Bacteriol* 2003, **185**(7):2066-2079.

78. Sifri CD: **Healthcare epidemiology: quorum sensing: bacteria talk sense**. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2008, **47**(8):1070-1076.

79. Conibear TC, Willcox MD, Flanagan JL, Zhu H: **Characterization of protease IV expression in Pseudomonas aeruginosa clinical isolates**. *Journal of medical microbiology* 2012, **61**(Pt 2):180-190.

80.   Espinosa-Urgel M: **Resident parking only: rhamnolipids maintain fluid channels in biofilms**. *J Bacteriol* 2003, **185**(3):699-700.

81.   Zulianello L, Canard C, Kohler T, Caille D, Lacroix JS, Meda P: **Rhamnolipids are virulence factors that promote early infiltration of primary human airway epithelia by Pseudomonas aeruginosa**. *Infection and immunity* 2006, **74**(6):3134-3147.

82.   Gallagher LA, Manoil C: **Pseudomonas aeruginosa PAO1 kills Caenorhabditis elegans by cyanide poisoning**. *J Bacteriol* 2001, **183**(21):6207-6214.

83.   Diggle SP, Stacey RE, Dodd C, Camara M, Williams P, Winzer K: **The galactophilic lectin, LecA, contributes to biofilm development in Pseudomonas aeruginosa**. *Environ Microbiol* 2006, **8**(6):1095-1104.

84.   Deziel E, Lepine F, Milot S, He J, Mindrinos MN, Tompkins RG, Rahme LG: **Analysis of Pseudomonas aeruginosa 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication**. *Proc Natl Acad Sci U S A* 2004, **101**(5):1339-1344.

85.   Hastings JW: **Bacterial quorum-sensing signals are inactivated by mammalian cells**. *Proc Natl Acad Sci U S A* 2004, **101**(12):3993-3994.

86.   Diggle SP, Lumjiaktase P, Dipilato F, Winzer K, Kunakorn M, Barrett DA, Chhabra SR, Camara M, Williams P: **Functional genetic analysis reveals a 2-Alkyl-4-quinolone signaling system in the human pathogen Burkholderia pseudomallei and related bacteria**. *Chem Biol* 2006, **13**(7):701-710.

87. Wade DS, Calfee MW, Rocha ER, Ling EA, Engstrom E, Coleman JP, Pesci EC: **Regulation of Pseudomonas quinolone signal synthesis in Pseudomonas aeruginosa**. *J Bacteriol* 2005, **187**(13):4372-4380.

88. Ilangovan A, Fletcher M, Rampioni G, Pustelny C, Rumbaugh K, Heeb S, Camara M, Truman A, Chhabra SR, Emsley J *et al*: **Structural basis for native agonist and synthetic inhibitor recognition by the Pseudomonas aeruginosa quorum sensing regulator PqsR (MvfR)**. *PLoS Pathog* 2013, **9**(7):e1003508.

89. Bredenbruch F, Nimtz M, Wray V, Morr M, Muller R, Haussler S: **Biosynthetic pathway of Pseudomonas aeruginosa 4-hydroxy-2-alkylquinolines**. *J Bacteriol* 2005, **187**(11):3630-3635.

90. Choi Y, Park HY, Park SJ, Park SJ, Kim SK, Ha C, Im SJ, Lee JH: **Growth phase-differential quorum sensing regulation of anthranilate metabolism in Pseudomonas aeruginosa**. *Mol Cells* 2011, **32**(1):57-65.

91. Coleman JP, Hudson LL, McKnight SL, Farrow JM, 3rd, Calfee MW, Lindsey CA, Pesci EC: **Pseudomonas aeruginosa PqsA is an anthranilate-coenzyme A ligase**. *J Bacteriol* 2008, **190**(4):1247-1255.

92. Bera AK, Atanasova V, Robinson H, Eisenstein E, Coleman JP, Pesci EC, Parsons JF: **Structure of PqsD, a Pseudomonas quinolone signal biosynthetic enzyme, in complex with anthranilate**. *Biochemistry* 2009, **48**(36):8644-8655.

93. Zhang YM, Frank MW, Zhu K, Mayasundari A, Rock CO: **PqsD is responsible for the synthesis of 2,4-dihydroxyquinoline, an extracellular metabolite**

produced by Pseudomonas aeruginosa. *J Biol Chem* 2008, **283**(43):28788-
28794.

94.   Dulcey CE, Dekimpe V, Fauvelle DA, Milot S, Groleau MC, Doucet N, Rahme LG,
Lepine F, Deziel E: **The end of an old hypothesis: the pseudomonas
signaling molecules 4-hydroxy-2-alkylquinolines derive from fatty
acids, not 3-ketofatty acids**. *Chem Biol* 2013, **20**(12):1481-1491.

95.   Farrow JM, 3rd, Sund ZM, Ellison ML, Wade DS, Coleman JP, Pesci EC: **PqsE
functions independently of PqsR-Pseudomonas quinolone signal and
enhances the rhl quorum-sensing system**. *J Bacteriol* 2008, **190**(21):7043-
7051.

96.   Drees SL, Fetzner S: **PqsE of Pseudomonas aeruginosa Acts as Pathway-
Specific Thioesterase in the Biosynthesis of Alkylquinolone Signaling
Molecules**. *Chem Biol* 2015, **22**(5):611-618.

97.   Schertzer JW, Brown SA, Whiteley M: **Oxygen levels rapidly modulate
Pseudomonas aeruginosa social behaviours via substrate limitation of
PqsH**. *Mol Microbiol* 2010, **77**(6):1527-1538.

98.   Gallagher LA, McKnight SL, Kuznetsova MS, Pesci EC, Manoil C: **Functions
required for extracellular quinolone signaling by Pseudomonas
aeruginosa**. *J Bacteriol* 2002, **184**(23):6472-6480.

99.   Hoffman LR, Deziel E, D'Argenio DA, Lepine F, Emerson J, McNamara S,
Gibson RL, Ramsey BW, Miller SI: **Selection for Staphylococcus aureus
small-colony variants due to growth in the presence of Pseudomonas
aeruginosa**. *Proc Natl Acad Sci U S A* 2006, **103**(52):19890-19895.

100.   Yang L, Nilsson M, Gjermansen M, Givskov M, Tolker-Nielsen T: **Pyoverdine and PQS mediated subpopulation interactions involved in Pseudomonas aeruginosa biofilm formation**. *Mol Microbiol* 2009, **74**(6):1380-1392.

101.   Lamarche MG, Deziel E: **MexEF-OprN efflux pump exports the Pseudomonas quinolone signal (PQS) precursor HHQ (4-hydroxy-2-heptylquinoline)**. *PLoS One* 2011, **6**(9):e24310.

102.   D'Argenio DA, Calfee MW, Rainey PB, Pesci EC: **Autolysis and autoaggregation in Pseudomonas aeruginosa colony morphology mutants**. *J Bacteriol* 2002, **184**(23):6481-6489.

103.   Schertzer JW, Whiteley M: **A bilayer-couple model of bacterial outer membrane vesicle biogenesis**. *MBio* 2012, **3**(2).

104.   Koley D, Ramsey MM, Bard AJ, Whiteley M: **Discovery of a biofilm electrocline using real-time 3D metabolite analysis**. *Proc Natl Acad Sci U S A* 2011, **108**(50):19996-20001.

105.   Gibson J, Sood A, Hogan DA: **Pseudomonas aeruginosa-Candida albicans interactions: localization and fungal toxicity of a phenazine derivative**. *Appl Environ Microbiol* 2009, **75**(2):504-513.

106.   Kim K, Kim YU, Koh BH, Hwang SS, Kim SH, Lepine F, Cho YH, Lee GR: **HHQ and PQS, two Pseudomonas aeruginosa quorum-sensing molecules, down-regulate the innate immune responses through the nuclear factor-kappaB pathway**. *Immunology* 2010, **129**(4):578-588.

107. Kim K, Kim SH, Lepine F, Cho YH, Lee GR: **Global gene expression analysis on the target genes of PQS and HHQ in J774A.1 monocyte/macrophage cells**. *Microb Pathog* 2010, **49**(4):174-180.

108. Skindersoe ME, Zeuthen LH, Brix S, Fink LN, Lazenby J, Whittall C, Williams P, Diggle SP, Froekiaer H, Cooley M *et al*: **Pseudomonas aeruginosa quorum-sensing signal molecules interfere with dendritic cell-induced T-cell proliferation**. *FEMS Immunol Med Microbiol* 2009, **55**(3):335-345.

109. Bredenbruch F, Geffers R, Nimtz M, Buer J, Haussler S: **The Pseudomonas aeruginosa quinolone signal (PQS) has an iron-chelating activity**. *Environ Microbiol* 2006, **8**(8):1318-1329.

110. Meyer JM, Neely A, Stintzi A, Georges C, Holder IA: **Pyoverdin is essential for virulence of Pseudomonas aeruginosa**. *Infection and immunity* 1996, **64**(2):518-523.

111. Buckling A, Harrison F, Vos M, Brockhurst MA, Gardner A, West SA, Griffin A: **Siderophore-mediated cooperation and virulence in Pseudomonas aeruginosa**. *FEMS Microbiol Ecol* 2007, **62**(2):135-141.

112. Diggle SP, Matthijs S, Wright VJ, Fletcher MP, Chhabra SR, Lamont IL, Kong X, Hider RC, Cornelis P, Camara M *et al*: **The Pseudomonas aeruginosa 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment**. *Chem Biol* 2007, **14**(1):87-96.

113. Lepine F, Dekimpe V, Lesic B, Milot S, Lesimple A, Mamer OA, Rahme LG, Deziel E: **PqsA is required for the biosynthesis of 2,4-dihydroxyquinoline (DHQ), a newly identified metabolite produced by**

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Pseudomonas aeruginosa and Burkholderia thailandensis. *Biol Chem* 2007, **388**(8):839-845.

114. Yuan Y, Leeds JA, Meredith TC: **Pseudomonas aeruginosa directly shunts beta-oxidation degradation intermediates into de novo fatty acid biosynthesis**. *J Bacteriol* 2012, **194**(19):5185-5196.

115. Rella A, Yang MW, Gruber J, Montagna MT, Luberto C, Zhang YM, Del Poeta M: **Pseudomonas aeruginosa inhibits the growth of Cryptococcus species**. *Mycopathologia* 2012, **173**(5-6):451-461.

116. Guina T, Purvine SO, Yi EC, Eng J, Goodlett DR, Aebersold R, Miller SI: **Quantitative proteomic analysis indicates increased synthesis of a quinolone by Pseudomonas aeruginosa isolates from cystic fibrosis airways**. *Proc Natl Acad Sci U S A* 2003, **100**(5):2771-2776.

117. Collier DN, Anderson L, McKnight SL, Noah TL, Knowles M, Boucher R, Schwab U, Gilligan P, Pesci EC: **A bacterial cell to cell signal in the lungs of cystic fibrosis patients**. *FEMS Microbiol Lett* 2002, **215**(1):41-46.

118. Struss AK, Nunes A, Waalen J, Lowery CA, Pullanikat P, Denery JR, Conrad DJ, Kaufmann GF, Janda KD: **Toward implementation of quorum sensing autoinducers as biomarkers for infectious disease states**. *Anal Chem* 2013, **85**(6):3355-3362.

119. Hassett DJ, Sutton MD, Schurr MJ, Herr AB, Caldwell CC, Matu JO: **Pseudomonas aeruginosa hypoxic or anaerobic biofilm infections within cystic fibrosis airways**. *Trends in microbiology* 2009, **17**(3):130-138.

120. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, Birrer P, Bellon G, Berger J, Weiss T *et al*: **Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients**. *J Clin Invest* 2002, **109**(3):317-325.

121. Ossipov MH, Morimura K, Porreca F: **Descending pain modulation and chronification of pain**. *Current opinion in supportive and palliative care* 2014, **8**(2):143-151.

122. Osmonov D, Ozcan KS, Erdinler I, Altay S, Yildirim E, Turkkan C, Ekmekci A, Gungor B, Gurkan K: **Cardiac device-related endocarditis: 31-Years' experience**. *Journal of cardiology* 2013, **61**(2):175-180.

123. Gordon SM, Schmitt SK, Jacobs M, Smedira NM, Goormastic M, Banbury MK, Yeager M, Serkey J, Hoercher K, McCarthy PM: **Nosocomial bloodstream infections in patients with implantable left ventricular assist devices**. *The Annals of thoracic surgery* 2001, **72**(3):725-730.

124. Chatterjee S, Maiti P, Dey R, Kundu A, Dey R: **Biofilms on indwelling urologic devices: microbes and antimicrobial management prospect**. *Annals of medical and health sciences research* 2014, **4**(1):100-104.

125. Konings AF, Martin LW, Sharples KJ, Roddam LF, Latham R, Reid DW, Lamont IL: **Pseudomonas aeruginosa uses multiple pathways to acquire iron during chronic infection in cystic fibrosis lungs**. *Infection and immunity* 2013, **81**(8):2697-2704.

126. Hoboth C, Hoffmann R, Eichner A, Henke C, Schmoldt S, Imhof A, Heesemann J, Hogardt M: **Dynamics of adaptive microevolution of hypermutable

Pseudomonas aeruginosa during chronic pulmonary infection in patients with cystic fibrosis. *The Journal of infectious diseases* 2009, **200**(1):118-130.

127.   Henrichfreise B, Wiegand I, Pfister W, Wiedemann B: **Resistance mechanisms of multiresistant Pseudomonas aeruginosa strains from Germany and correlation with hypermutation**. *Antimicrobial agents and chemotherapy* 2007, **51**(11):4062-4070.

128.   Xavier DE, Picao RC, Girardello R, Fehlberg LC, Gales AC: **Efflux pumps expression and its association with porin down-regulation and beta-lactamase production among Pseudomonas aeruginosa causing bloodstream infections in Brazil**. *BMC microbiology* 2010, **10**:217.

129.   Pesci EC, Pearson JP, Seed PC, Iglewski BH: **Regulation of las and rhl quorum sensing in Pseudomonas aeruginosa**. *Journal of bacteriology* 1997, **179**(10):3127-3132.

130.   Pearson JP, Pesci EC, Iglewski BH: **Roles of Pseudomonas aeruginosa las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes**. *Journal of bacteriology* 1997, **179**(18):5756-5767.

131.   McGrath S, Wade DS, Pesci EC: **Dueling quorum sensing systems in Pseudomonas aeruginosa control the production of the Pseudomonas quinolone signal (PQS)**. *FEMS Microbiol Lett* 2004, **230**(1):27-34.

132.   Fuqua C, Greenberg EP: **Listening in on bacteria: acyl-homoserine lactone signalling**. *Nature reviews Molecular cell biology* 2002, **3**(9):685-695.

133. Heeb S, Fletcher MP, Chhabra SR, Diggle SP, Williams P, Camara M: **Quinolones: from antibiotics to autoinducers**. *FEMS microbiology reviews* 2011, **35**(2):247-274.

134. Mashburn-Warren L, Howe J, Brandenburg K, Whiteley M: **Structural requirements of the Pseudomonas quinolone signal for membrane vesicle stimulation**. *Journal of bacteriology* 2009, **191**(10):3411-3414.

135. Steinbach A, Maurer CK, Weidel E, Henn C, Brengel C, Hartmann RW, Negri M: **Molecular basis of HHQ biosynthesis: molecular dynamics simulations, enzyme kinetic and surface plasmon resonance studies**. *BMC Biophys*, **6**(1):10.

136. Haussler S, Becker T: **The pseudomonas quinolone signal (PQS) balances life and death in Pseudomonas aeruginosa populations**. *PLoS Pathog* 2008, **4**(9):e1000166.

137. Diggle SP, Cornelis P, Williams P, Camara M: **4-quinolone signalling in Pseudomonas aeruginosa: old molecules, new perspectives**. *International journal of medical microbiology : IJMM* 2006, **296**(2-3):83-91.

138. Xiao G, Deziel E, He J, Lepine F, Lesic B, Castonguay MH, Milot S, Tampakaki AP, Stachel SE, Rahme LG: **MvfR, a key Pseudomonas aeruginosa pathogenicity LTTR-class regulatory protein, has dual ligands**. *Mol Microbiol* 2006, **62**(6):1689-1699.

139. Welch M, Hodgkinson JT, Gross J, Spring DR, Sams T: **Ligand binding kinetics of the quorum sensing regulator PqsR**. *Biochemistry* 2013, **52**(25):4433-4438.

140. Schobert M, Jahn D: **Anaerobic physiology of Pseudomonas aeruginosa in the cystic fibrosis lung**. *International journal of medical microbiology : IJMM* 2010, **300**(8):549-556.

141. Su S, Hassett DJ: **Anaerobic Pseudomonas aeruginosa and other obligately anaerobic bacterial biofilms growing in the thick airway mucus of chronically infected cystic fibrosis patients: an emerging paradigm or "Old Hat"?** *Expert opinion on therapeutic targets* 2012, **16**(9):859-873.

142. Tunney MM, Field TR, Moriarty TF, Patrick S, Doering G, Muhlebach MS, Wolfgang MC, Boucher R, Gilpin DF, McDowell A *et al*: **Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis**. *American journal of respiratory and critical care medicine* 2008, **177**(9):995-1001.

143. Harmer C, Alnassafi K, Hu H, Elkins M, Bye P, Rose B, Cordwell S, Triccas JA, Harbour C, Manos J: **Modulation of gene expression by Pseudomonas aeruginosa during chronic infection in the adult cystic fibrosis lung**. *Microbiology* 2013, **159**(Pt 11):2354-2363.

144. Mahajan-Miklos S, Tan MW, Rahme LG, Ausubel FM: **Molecular mechanisms of bacterial virulence elucidated using a Pseudomonas aeruginosa-Caenorhabditis elegans pathogenesis model**. *Cell* 1999, **96**(1):47-56.

145. Tan MW, Rahme LG, Sternberg JA, Tompkins RG, Ausubel FM: **Pseudomonas aeruginosa killing of Caenorhabditis elegans used to identify P.**

aeruginosa virulence factors*. *Proceedings of the National Academy of Sciences of the United States of America* 1999, **96**(5):2408-2413.

146. Tan MW, Mahajan-Miklos S, Ausubel FM: **Killing of Caenorhabditis elegans by Pseudomonas aeruginosa used to model mammalian bacterial pathogenesis**. *Proceedings of the National Academy of Sciences of the United States of America* 1999, **96**(2):715-720.

147. Hodgkin J, Partridge FA: **Caenorhabditis elegans meets microsporidia: the nematode killers from Paris**. *PLoS biology* 2008, **6**(12):2634-2637.

148. Papaioannou E, Utari PD, Quax WJ: **Choosing an appropriate infection model to study quorum sensing inhibition in Pseudomonas infections**. *International journal of molecular sciences* 2013, **14**(9):19309-19340.

149. Sifri CD, Begun J, Ausubel FM, Calderwood SB: **Caenorhabditis elegans as a model host for Staphylococcus aureus pathogenesis**. *Infection and immunity* 2003, **71**(4):2208-2217.

150. Bloemberg GV, O'Toole GA, Lugtenberg BJ, Kolter R: **Green fluorescent protein as a marker for Pseudomonas spp**. *Applied and environmental microbiology* 1997, **63**(11):4543-4551.

151. Korgaonkar A, Trivedi U, Rumbaugh KP, Whiteley M: **Community surveillance enhances Pseudomonas aeruginosa virulence during polymicrobial infection**. *Proc Natl Acad Sci U S A* 2013, **110**(3):1059-1064.

152. Morales DK, Grahl N, Okegbe C, Dietrich LE, Jacobs NJ, Hogan DA: **Control of Candida albicans metabolism and biofilm formation by Pseudomonas aeruginosa phenazines**. *mBio* 2013, **4**(1):e00526-00512.

153. Lau GW, Hassett DJ, Ran H, Kong F: **The role of pyocyanin in Pseudomonas aeruginosa infection**. *Trends in molecular medicine* 2004, **10**(12):599-606.

154. Williams P, Camara M: **Quorum sensing and environmental adaptation in Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules**. *Current opinion in microbiology* 2009, **12**(2):182-191.

155. Roszak DB, Colwell RR: **Survival strategies of bacteria in the natural environment**. *Microbiological reviews* 1987, **51**(3):365-379.

156. Subramanian C, Rock CO, Zhang YM: **DesT coordinates the expression of anaerobic and aerobic pathways for unsaturated fatty acid biosynthesis in Pseudomonas aeruginosa**. *Journal of bacteriology* 2010, **192**(1):280-285.

157. Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, Kamani MC, Allen HL, DeKievit TR, Gardner PR, Schwab U *et al*: **Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis**. *Developmental cell* 2002, **3**(4):593-603.

158. Lee KM, Yoon MY, Park Y, Lee JH, Yoon SS: **Anaerobiosis-induced loss of cytotoxicity is due to inactivation of quorum sensing in Pseudomonas aeruginosa**. *Infection and immunity* 2011, **79**(7):2792-2800.

159. Uwate M, Ichise YK, Shirai A, Omasa T, Nakae T, Maseda H: **Two routes of MexS-MexT-mediated regulation of MexEF-OprN and MexAB-OprM efflux pump expression in Pseudomonas aeruginosa**. *Microbiol Immunol*, **57**(4):263-272.

160. Kohler T, van Delden C, Curty LK, Hamzehpour MM, Pechere JC: **Overexpression of the MexEF-OprN multidrug efflux system affects cell-to-cell signaling in Pseudomonas aeruginosa**. *J Bacteriol* 2001, **183**(18):5213-5222.

161. Wang Y, Kern SE, Newman DK: **Endogenous phenazine antibiotics promote anaerobic survival of Pseudomonas aeruginosa via extracellular electron transfer**. *J Bacteriol* 2010, **192**(1):365-369.

162. Price-Whelan A, Dietrich LE, Newman DK: **Pyocyanin alters redox homeostasis and carbon flux through central metabolic pathways in Pseudomonas aeruginosa PA14**. *J Bacteriol* 2007, **189**(17):6372-6381.

163. Zhou L, Glennon JD, Luong JH, Reen FJ, O'Gara F, McSweeney C, McGlacken GP: **Detection of the Pseudomonas Quinolone Signal (PQS) by cyclic voltammetry and amperometry using a boron doped diamond electrode**. *Chemical communications* 2011, **47**(37):10347-10349.

164. Berridge MV, Herst PM, Tan AS: **Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction**. *Biotechnology annual review* 2005, **11**:127-152.

165. Kim K, Kim YU, Koh BH, Hwang SS, Kim SH, Lepine F, Cho YH, Lee GR: **HHQ and PQS, two Pseudomonas aeruginosa quorum-sensing molecules, down-regulate the innate immune responses through the nuclear factor-kappaB pathway**. *Immunology*, **129**(4):578-588.

166. Wetmore DR, Joseloff E, Pilewski J, Lee DP, Lawton KA, Mitchell MW, Milburn MV, Ryals JA, Guo L: **Metabolomic profiling reveals biochemical pathways**

and biomarkers associated with pathogenesis in cystic fibrosis cells. *The Journal of biological chemistry* 2010, **285**(40):30516-30522.

167.   Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ: **The multiple signaling systems regulating virulence in Pseudomonas aeruginosa**. *Microbiology and molecular biology reviews : MMBR* 2012, **76**(1):46-65.

168.   Erickson DL, Lines JL, Pesci EC, Venturi V, Storey DG: **Pseudomonas aeruginosa relA contributes to virulence in Drosophila melanogaster**. *Infection and immunity* 2004, **72**(10):5638-5645.

169.   Baysse C, Cullinane M, Denervaud V, Burrowes E, Dow JM, Morrissey JP, Tam L, Trevors JT, O'Gara F: **Modulation of quorum sensing in Pseudomonas aeruginosa through alteration of membrane properties**. *Microbiology* 2005, **151**(Pt 8):2529-2542.

170.   Fetar H, Gilmour C, Klinoski R, Daigle DM, Dean CR, Poole K: **mexEF-oprN multidrug efflux operon of Pseudomonas aeruginosa: regulation by the MexT activator in response to nitrosative stress and chloramphenicol**. *Antimicrobial agents and chemotherapy* 2011, **55**(2):508-514.

171.   Maddocks SE, Oyston PC: **Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins**. *Microbiology* 2008, **154**(Pt 12):3609-3623.

172.   Cao H, Krishnan G, Goumnerov B, Tsongalis J, Tompkins R, Rahme LG: **A quorum sensing-associated virulence gene of Pseudomonas aeruginosa encodes a LysR-like transcription regulator with a unique self-**

regulatory mechanism. *Proc Natl Acad Sci U S A* 2001, **98**(25):14613-14618.

173.   Ilangovan A, Fletcher M, Rampioni G, Pustelny C, Rumbaugh K, Heeb S, Camara M, Truman A, Chhabra SR, Emsley J *et al*: **Structural basis for native agonist and synthetic inhibitor recognition by the Pseudomonas aeruginosa quorum sensing regulator PqsR (MvfR)**. *PLoS Pathog*, **9**(7):e1003508.

174.   Xu N, Yu S, Moniot S, Weyand M, Blankenfeldt W: **Crystallization and preliminary crystal structure analysis of the ligand-binding domain of PqsR (MvfR), the Pseudomonas quinolone signal (PQS) responsive quorum-sensing transcription factor of Pseudomonas aeruginosa**. *Acta Crystallogr Sect F Struct Biol Cryst Commun*, **68**(Pt 9):1034-1039.

175.   Xiao G, He J, Rahme LG: **Mutation analysis of the Pseudomonas aeruginosa mvfR and pqsABCDE gene promoters demonstrates complex quorum-sensing circuitry**. *Microbiology* 2006, **152**(Pt 6):1679-1686.

176.   Cugini C, Calfee MW, Farrow JM, 3rd, Morales DK, Pesci EC, Hogan DA: **Farnesol, a common sesquiterpene, inhibits PQS production in Pseudomonas aeruginosa**. *Mol Microbiol* 2007, **65**(4):896-906.

177.   Leonhardt I, Spielberg S, Weber M, Albrecht-Eckardt D, Blass M, Claus R, Barz D, Scherlach K, Hertweck C, Loffler J *et al*: **The fungal quorum-sensing molecule farnesol activates innate immune cells but suppresses cellular adaptive immunity**. *MBio*, **6**(2):e00143.

178. Lu C, Kirsch B, Zimmer C, de Jong JC, Henn C, Maurer CK, Musken M, Haussler S, Steinbach A, Hartmann RW: **Discovery of antagonists of PqsR, a key player in 2-alkyl-4-quinolone-dependent quorum sensing in Pseudomonas aeruginosa**. *Chemistry & biology* 2012, **19**(3):381-390.

179. Oinuma K, Greenberg EP: **Acyl-homoserine lactone binding to and stability of the orphan Pseudomonas aeruginosa quorum-sensing signal receptor QscR**. *Journal of bacteriology* 2011, **193**(2):421-428.

180. Welch M, Hodgkinson JT, Gross J, Spring DR, Sams T: **Ligand binding kinetics of the quorum sensing regulator PqsR**. *Biochemistry*, **52**(25):4433-4438.

181. Angulo J, Nieto PM: **STD-NMR: application to transient interactions between biomolecules-a quantitative approach**. *Eur Biophys J*, **40**(12):1357-1369.

182. Dotsch A, Eckweiler D, Schniederjans M, Zimmermann A, Jensen V, Scharfe M, Geffers R, Haussler S: **The Pseudomonas aeruginosa transcriptome in planktonic cultures and static biofilms using RNA sequencing**. *PLoS One*, **7**(2):e31092.

183. Knoten CA, Wells G, Coleman JP, Pesci EC: **A conserved suppressor mutation in a tryptophan auxotroph results in dysregulation of Pseudomonas quinolone signal synthesis**. *J Bacteriol*, **196**(13):2413-2422.

184. Marvig RL, Sommer LM, Jelsbak L, Molin S, Johansen HK: **Evolutionary insight from whole-genome sequencing of Pseudomonas aeruginosa from cystic fibrosis patients**. *Future Microbiol* 2015, **10**:599-611.

185. Hoiby N: **Antibiotic therapy for chronic infection of pseudomonas in the lung**. *Annual review of medicine* 1993, **44**:1-10.

186. Marvig RL, Sommer LM, Molin S, Johansen HK: **Convergent evolution and adaptation of Pseudomonas aeruginosa within patients with cystic fibrosis**. *Nature genetics* 2015, **47**(1):57-64.

187. Staudinger BJ, Muller JF, Halldorsson S, Boles B, Angermeyer A, Nguyen D, Rosen H, Baldursson O, Gottfreethsson M, Guethmundsson GH *et al*: **Conditions associated with the cystic fibrosis defect promote chronic Pseudomonas aeruginosa infection**. *American journal of respiratory and critical care medicine* 2014, **189**(7):812-824.

188. Yates EA, Philipp B, Buckley C, Atkinson S, Chhabra SR, Sockett RE, Goldner M, Dessaux Y, Camara M, Smith H *et al*: **N-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of Yersinia pseudotuberculosis and Pseudomonas aeruginosa**. *Infection and immunity* 2002, **70**(10):5635-5646.

189. Chun CK, Ozer EA, Welsh MJ, Zabner J, Greenberg EP: **Inactivation of a Pseudomonas aeruginosa quorum-sensing signal by human airway epithelia**. *Proc Natl Acad Sci U S A* 2004, **101**(10):3587-3590.

190. Pustelny C, Albers A, Buldt-Karentzopoulos K, Parschat K, Chhabra SR, Camara M, Williams P, Fetzner S: **Dioxygenase-mediated quenching of quinolone-dependent quorum sensing in Pseudomonas aeruginosa**. *Chem Biol* 2009, **16**(12):1259-1267.

191. Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP: **Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms**. *Nature* 2000, **407**(6805):762-764.

192. Hunter RC, Klepac-Ceraj V, Lorenzi MM, Grotzinger H, Martin TR, Newman DK: **Phenazine content in the cystic fibrosis respiratory tract negatively correlates with lung function and microbial complexity**. *American journal of respiratory cell and molecular biology* 2012, **47**(6):738-745.

193. Fletcher MP, Diggle SP, Camara M, Williams P: **Biosensor-based assays for PQS, HHQ and related 2-alkyl-4-quinolone quorum sensing signal molecules**. *Nature protocols* 2007, **2**(5):1254-1262.

194. Taylor-Robinson D, Whitehead M, Diderichsen F, Olesen HV, Pressler T, Smyth RL, Diggle P: **Understanding the natural progression in %FEV1 decline in patients with cystic fibrosis: a longitudinal study**. *Thorax* 2012, **67**(10):860-866.

195. Kravchenko VV, Ulevitch RJ, Kaufmann GF: **Modulation of mammalian cell processes by bacterial quorum sensing molecules**. *Methods in molecular biology* 2011, **692**:133-145.

196. Pritchard DI: **Immune modulation by Pseudomonas aeruginosa quorum-sensing signal molecules**. *International journal of medical microbiology : IJMM* 2006, **296**(2-3):111-116.

197. Ulrich M, Worlitzsch D, Viglio S, Siegmann N, Iadarola P, Shute JK, Geiser M, Pier GB, Friedel G, Barr ML *et al*: **Alveolar inflammation in cystic fibrosis**.

Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society 2010, **9**(3):217-227.

198.   Amadori A, Antonelli A, Balteri I, Schreiber A, Bugiani M, De Rose V: **Recurrent exacerbations affect FEV(1) decline in adult patients with cystic fibrosis**. *Respiratory medicine* 2009, **103**(3):407-413.

199.   Starkey M, Hickman JH, Ma L, Zhang N, De Long S, Hinz A, Palacios S, Manoil C, Kirisits MJ, Starner TD *et al*: **Pseudomonas aeruginosa rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung**. *J Bacteriol* 2009, **191**(11):3492-3503.

200.   Line L, Alhede M, Kolpen M, Kuhl M, Ciofu O, Bjarnsholt T, Moser C, Toyofuku M, Nomura N, Hoiby N *et al*: **Physiological levels of nitrate support anoxic growth by denitrification of Pseudomonas aeruginosa at growth rates reported in cystic fibrosis lungs and sputum**. *Frontiers in microbiology* 2014, **5**:554.

201.   Osika E, Cavaillon JM, Chadelat K, Boule M, Fitting C, Tournier G, Clement A: **Distinct sputum cytokine profiles in cystic fibrosis and other chronic inflammatory airway disease**. *The European respiratory journal* 1999, **14**(2):339-346.

202.   Bhatia M, Moochhala S: **Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome**. *The Journal of pathology* 2004, **202**(2):145-156.

203.   Bruscia EM, Zhang PX, Ferreira E, Caputo C, Emerson JW, Tuck D, Krause DS, Egan ME: **Macrophages directly contribute to the exaggerated**

inflammatory response in cystic fibrosis transmembrane conductance regulator-/- mice. *American journal of respiratory cell and molecular biology* 2009, **40**(3):295-304.

204.   Rowntree RK, Harris A: **The phenotypic consequences of CFTR mutations**. *Annals of human genetics* 2003, **67**(Pt 5):471-485.

205.   McAlester G, O'Gara F, Morrissey JP: **Signal-mediated interactions between Pseudomonas aeruginosa and Candida albicans**. *Journal of medical microbiology* 2008, **57**(Pt 5):563-569.

206.   Hoiby N, Ciofu O, Bjarnsholt T: **Pseudomonas aeruginosa biofilms in cystic fibrosis**. *Future Microbiol* 2010, **5**(11):1663-1674.

207.   Kaplan JB: **Antibiotic-induced biofilm formation**. *The International journal of artificial organs* 2011, **34**(9):737-751.

208.   Dettman JR, Rodrigue N, Aaron SD, Kassen R: **Evolutionary genomics of epidemic and nonepidemic strains of Pseudomonas aeruginosa**. *Proc Natl Acad Sci U S A* 2013, **110**(52):21065-21070.

209.   Lieberman TD, Flett KB, Yelin I, Martin TR, McAdam AJ, Priebe GP, Kishony R: **Genetic variation of a bacterial pathogen within individuals with cystic fibrosis provides a record of selective pressures**. *Nature genetics* 2014, **46**(1):82-87.

210.   Scholte BJ, Davidson DJ, Wilke M, De Jonge HR: **Animal models of cystic fibrosis**. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society* 2004, **3 Suppl 2**:183-190.

211. Welsh MJ, Rogers CS, Stoltz DA, Meyerholz DK, Prather RS: **Development of a porcine model of cystic fibrosis**. *Transactions of the American Clinical and Climatological Association* 2009, **120**:149-162.

212. Irazoqui JE, Troemel ER, Feinbaum RL, Luhachack LG, Cezairliyan BO, Ausubel FM: **Distinct pathogenesis and host responses during infection of C. elegans by P. aeruginosa and S. aureus**. *PLoS Pathog* 2010, **6**:e1000982.

213. Dixon DR, Darveau RP: **Lipopolysaccharide heterogeneity: innate host responses to bacterial modification of lipid a structure**. *Journal of dental research* 2005, **84**(7):584-595.

214. Nikaido H, Pages JM: **Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria**. *FEMS Microbiol Rev*, **36**(2):340-363.

215. Schaible B, Taylor CT, Schaffer K: **Hypoxia increases antibiotic resistance in Pseudomonas aeruginosa through altering the composition of multidrug efflux pumps**. *Antimicrob Agents Chemother*, **56**(4):2114-2118.

216. Whitney JC, Whitfield GB, Marmont LS, Yip P, Neculai AM, Lobsanov YD, Robinson H, Ohman DE, Howell PL: **Dimeric c-di-GMP is Required for Post-translational Regulation of Alginate Production in Pseudomonas aeruginosa**. *J Biol Chem*.

217. Chew SC, Kundukad B, Seviour T, van der Maarel JR, Yang L, Rice SA, Doyle P, Kjelleberg S: **Dynamic remodeling of microbial biofilms by functionally distinct exopolysaccharides**. *MBio*, **5**(4):e01536-01514.

218. Connell JL, Wessel AK, Parsek MR, Ellington AD, Whiteley M, Shear JB:
      Probing prokaryotic social behaviors with bacterial "lobster traps".
      MBio, **1**(4).

219. Wilder CN, Allada G, Schuster M: **Instantaneous within-patient diversity of
      Pseudomonas aeruginosa quorum-sensing populations from cystic
      fibrosis lung infections**. *Infection and immunity* 2009, **77**(12):5631-5639.

220. Dekimpe V, Deziel E: **Revisiting the quorum-sensing hierarchy in
      Pseudomonas aeruginosa: the transcriptional regulator RhlR regulates
      LasR-specific factors**. *Microbiology* 2009, **155**(Pt 3):712-723.

221. Moree WJ, Phelan VV, Wu CH, Bandeira N, Cornett DS, Duggan BM, Dorrestein
      PC: **Interkingdom metabolic transformations captured by microbial
      imaging mass spectrometry**. *Proc Natl Acad Sci U S A*, **109**(34):13811-
      13816.

222. Wang WY, Lim JH, Li JD: **Synergistic and feedback signaling mechanisms
      in the regulation of inflammation in respiratory infections**. *Cell Mol
      Immunol*, **9**(2):131-135.

223. Lu C, Kirsch B, Zimmer C, de Jong JC, Henn C, Maurer CK, Musken M, Haussler
      S, Steinbach A, Hartmann RW: **Discovery of antagonists of PqsR, a key
      player in 2-alkyl-4-quinolone-dependent quorum sensing in
      Pseudomonas aeruginosa**. *Chem Biol*, **19**(3):381-390.

224. Darzins A, Chakrabarty AM: **Cloning of genes controlling alginate
      biosynthesis from a mucoid cystic fibrosis isolate of Pseudomonas
      aeruginosa**. *Journal of bacteriology* 1984, **159**(1):9-18.

225. Bradford MM: **A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding**. *Analytical biochemistry* 1976, **72**:248-254.

226. Girard LR, Fiedler TJ, Harris TW, Carvalho F, Antoshechkin I, Han M, Sternberg PW, Stein LD, Chalfie M: **WormBook: the online review of Caenorhabditis elegans biology**. *Nucleic acids research* 2007, **35**(Database issue):D472-475.

227. Das T, Manefield M: **Pyocyanin promotes extracellular DNA release in Pseudomonas aeruginosa**. *PloS one* 2012, **7**(10):e46718.

228. Zhu K, Choi KH, Schweizer HP, Rock CO, Zhang YM: **Two aerobic pathways for the formation of unsaturated fatty acids in Pseudomonas aeruginosa**. *Molecular microbiology* 2006, **60**(2):260-273.

229. Kemper S, Patel MK, Errey JC, Davis BG, Jones JA, Claridge TD: **Group epitope mapping considering relaxation of the ligand (GEM-CRL): including longitudinal relaxation rates in the analysis of saturation transfer difference (STD) experiments**. *Journal of magnetic resonance* 2010, **203**(1):1-10.