THE REGULATION OF ANTHRANILATE SYNTHESIS FOR THE PRODUCTION OF PQS IN *PSEUDOMONAS AERUGINOSA*

by

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(Under the direction of Everett C. Pesci, Ph.D)

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The ubiquitous bacterium *P. aeruginosa* is an important human pathogen that causes devastating infections in immunocompromised patients and chronic infections in cystic fibrosis patients. The ability of *P. aeruginosa* to cause such debilitating infections is in part due to its three known cell-to-cell signals. The *Pseudomonas* quinolone signal (PQS, 2-heptyl-3-hydroxy-4-quinolone) is one of these intercellular signals and is required for virulence in multiple models of infection. The pathway needed for biosynthesis of PQS is also responsible for the production of at least 56 other 4-quinolone molecules. The synthesis of PQS, as well as the other 4-quinolone molecules, requires the condensation of anthranilate and a β-keto fatty acid. The precursor anthranilate is an important branch point molecule as it can be converted into PQS (or other metabolites), used for tryptophan synthesis, or catabolized as a carbon and nitrogen source. In *P. aeruginosa,* anthranilate is synthesized by either the

breakdown of tryptophan through the kynurenine pathway or the conversion of chorismate via one of the two anthranilate synthases, TrpEG and PhnAB. The kynurenine pathway and the secondary anthranilate synthase PhnAB have been shown to provide anthranilate for the production of PQS. Our laboratory previously showed that the kynurenine pathway is the main source of anthranilate for PQS production in the presence of tryptophan, while PhnAB provides anthranilate under nutrient limiting conditions. In this work we aimed to gain a better understanding of the regulation of each of these metabolic routes to anthranilate. We described the transcriptional regulator KynR that is responsible for the upregulation of the kynurenine pathway genes in the presence of kynurenine. We also discovered that a single nucleotide change at nucleotide 1041 in *pqsC* resulted in a new transcript and the upregulation of *pqsD, pqsE,* and *phnAB* transcripts. This work provides insight into the regulation of anthranilate synthesis and the production of PQS.

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Doctor of Philosophy in Microbiology and Immunology

by

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CHAPTER ONE: INTRODUCTION

This work has developed around the discovery of the biosynthetic route for the *P. aeruginosa* cell-to-cell signal PQS (*Pseudomonas* Quinolone Signal). PQS is synthesized via a multistep reaction involving the condensation of the two precursors, anthranilate and a β-keto fatty acid. The precursor anthranilate is of interest because though the pathways that produce anthranilate are known, the regulation of the pathways is not well understood. Three pathways in *P. aeruginosa* produce anthranilate, yet only two of the pathways provide anthranilate used for the biosynthesis of PQS (37). The first pathway is the kynurenine pathway and the second is mediated by the anthranilate synthase PhnAB. These two pathways alternatively produce anthranilate when grown under different nutrient conditions and each is uniquely regulated. The goal of the current research was to understand the regulation of each pathway with regard to its role in the production of PQS. To fully recognize the significance of this research it is important to first have a basic understanding of *P. aeruginosa* as a human pathogen, its ability to communicate using cell-to-cell signaling, and the biosynthetic pathways for anthranilate.

1.1 *Pseudomonas aeruginosa* is an important human pathogen

The opportunistic pathogen *P. aeruginosa* is a ubiquitous, Gram-negative, facultative anaerobe that is able to flourish under environmental stress and nutrient limiting conditions. Due to the adaptive nature of *P. aeruginosa*, it is able to cause a wide range of infections from bacterial keratitis (113) to infections in the immunocompromised (72). *P. aeruginosa* is a common nosocomial pathogen and is known to cause 16.3% of ventilator-associated pneumonia, 10.0% of catheter-

associated urinary tract infections, and 5.6% of surgical site infections (50). Burn patients are also commonly infected with *P. aeruginosa,* and these infections can lead to bacterial sepsis, pneumonia, and an increased mortality rate (26). The chronic infection of *P. aeruginosa* in the lungs of cystic fibrosis (CF) patients is especially devastating. The majority of CF patients are infected by the age of 3 and over time, *P. aeruginosa* develops into a mucoid, multi-drug resistant phenotype that makes eradication nearly impossible (10). Chronic *P. aeruginosa* infections lead to decreased respiratory function, eventually resulting in increased morbidity and mortality in CF patients (73, 112). The ability for *P. aeruginosa* to cause such a wide-range of infections makes it an important bacterium to study and understand.

Treatment for *P. aeruginosa* infections is through multi-antibiotic drug treatment, yet with the ever-increasing antibacterial resistance of *P. aeruginosa* strains this approach may not be effective in the near future. Treatment of CF patients consists of a multi-antibiotic approach that includes inhaled tobramycin or colistin along with oral ciprofloxacin (112). Current studies suggest elimination of the bacterial infection from children is possible through a multi-drug treatment regimen, but long term eradication of *P. aeruginosa* from these patients is not expected because of continued environmental exposure that leads to reinfection (112). The antibiotic treatment in older CF patients is mildly effective until the multi-drug resistant phenotype appears in the lung (10). The treatment for ventilator-associated pneumonia is also a multi-drug approach and has decreased the ventilator-associated pneumonia mortality rate from 35% to 29% in some reported cases (49, 83). Though multi-drug therapies have benefited patients, *P. aeruginosa* is rapidly evolving, and multi-drug resistance has greatly increased over the

years. Current studies suggest that 31% of *P. aeruginosa* isolates in the United States are fluoroquinolone resistant and increasing resistance has been noted worldwide for multiple classes of antibiotics (49, 50, 104). Hospital isolates from 2006-2007 revealed that 10% of *P. aeruginosa* strains were resistant to three classes of antibiotics (56), suggesting that nosocomial infections will be increasingly difficult to treat in the future. New therapies to treat *P. aeruginosa* infections are in high demand. The development of novel treatment and prevention strategies is focused on targeting biosynthetic pathways through drug therapies and using extracellular structures to make vaccines. Current therapies in development include vaccines with *P. aeruginosa* outer-membrane proteins OprI/OprF or purified flagellin protein, immunoglobulin research, and new antibiotic delivery systems (1, 9, 136).

1.2 Quorum Sensing in *Pseudomonas aeruginosa*

Bacterial communication via quorum sensing has been well described in many species and occurs through multiple mechanisms. Three cell-to-cell signaling systems in *P. aeruginosa* are utilized to precisely activate expression of virulence factors and quickly adapt to the changing environmental conditions. *P. aeruginosa* utilizes two acyl homoserine lactone (AHL) signals and one quinolone based signal. The three signal systems employed by *P. aeruginosa* include the *las, rhl,* and PQS systems. (Figure 1.1) (19). The *las* and *rhl* regulons are considered true quorum sensing systems and synthesize *N*-acylhomoserine lactone signals (96). The AHL systems function by producing the signal at a basal rate and as the population increases the signal concentration also increases (96). Once the signal reaches an intracellular threshold concentration, it will bind to the transcriptional regulator (“R” protein) and then regulate

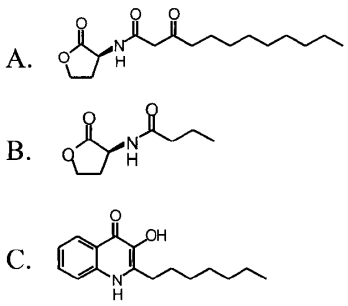

Figure 1.1: The three known cell-to-cell signals in *P. aeruginosa*. A) 3-oxo-C_{12}-HSL B) C_4-HSL C) 2-heptyl-3-hydroxy-4-quinolone (PQS) (95).

gene expression accordingly (96). The signal for the *las* system is *N*-3-oxo-dodecanoyl homoserine lactone (3-oxo-C_{12}-HSL), which is produced by the signal synthase LasI, and binds to the transcriptional regulator LasR (42, 92). The *rhl* system is similar to the *las* system, where the signal *N*-butyryl homoserine lactone (C_4-HSL) is produced by RhlI, and binds to the transcriptional regulator RhlR (84, 85, 93). The two systems are arranged in a hierarchy where LasR-3-oxo-C12-HSL positively regulates the *rhl* system, as well as the quinolone-based system (63, 96). Together, the *las* and *rhl* systems regulate up to 10% of the *P. aeruginosa* genome, suggesting that the quorum sensing systems coordinate a highly influential signal cascade (107, 122). This influence is further shown by the attenuation of *P. aeruginosa las* and *rhl* signal system mutants in multiple infection models (27).

1.3 The *Pseudomonas* Quinolone Signal

1.3.1 The biosynthesis and regulation of PQS

The third cell-to-cell signal was discovered in 1999 by Pesci *et al*. and was termed the *Pseudomonas* Quinolone Signal (PQS) (95). PQS was detected when spent culture media from wild-type *P. aeruginosa* activated a transcriptional fusion of *lasB (*encoding for the virulence factor elastase) in a *lasR* mutant (95). This suggested that a novel signal could activate *lasB* in the absence of the known regulator LasR (95). The signal was purified and identified to be 2-heptyl-3-hydroxy-4-quinolone (95). The structure was confirmed when synthetic PQS activated *lasB-lacZ* in a similar manner to natural PQS (95). The bioassay results also suggested that PQS required the *las* system for production of the signal and the *rhl* system for bioactivity (95). The role of PQS in the quorum sensing hierarchy was further defined by testing *lasR, lasI, rhlR,* and *rhlI*

transcriptional gene fusions in the presence and absence of PQS. The results suggested that PQS positively influences the signal synthase RhlI and, to a lesser degree, LasR and RhlR (81). This complicates the idea of a quorum sensing hierarchy and leads us to believe that each of the signals have influence on the other systems performing checks and balances to ensure the precise regulation of quorum controlled genes. McKnight *et al.* also showed that maximal production of PQS occurs during stationary phase, unlike true quorum sensing molecules that reach maximal concentration during late exponential phase (81). The positive influence of PQS on the activity of AHL quorum sensing genes and the late stationary phase production suggests that PQS does not sense cell density, but is a link between the *las* and *rhl* quorum sensing systems (81).

Since the initial identification of PQS, the genes required for the biosynthesis of this molecule have been identified. The PQS biosynthetic locus (Figure 1.2) is composed of a five gene operon, *pqsABCDE,* that contains the genes responsible for the synthesis of 4-quinolone compounds (*pqsABCD)* (80). The last gene in the operon, *pqsE,* encodes a putative metallo-β-lactamase/hydrolase that is not required for PQS biosynthesis, but rather for the production of PQS-controlled virulence factors (38). Directly downstream from *pqsE* is the *phnAB* operon that encodes an anthranilate synthase. Lastly in the biosynthetic gene cluster is *pqsR,* which is convergently transcribed from *phnAB* and encodes the transcriptional regulator that induces the expression of the PQS biosynthetic operon. The final gene required for PQS biosynthesis is *pqsH* and it is located separately on the chromosome. The PQS biosynthetic genes are responsible not only for the production of PQS, but also for at

least 56 other 4-hydroxy-2-alkylquinolines (HAQ) produced by *P. aeruginosa* (67). HAQs have multiple roles in *P. aeruginosa* virulence and understanding the synthesis of these molecules could lead to potential drug therapy targets (67).

The structural similarity of HAQ and previously described pyo-compounds suggested that the synthesis of these molecules required the condensation of anthranilate and a fatty acid precursor (21, 66). The biosynthesis of PQS is diagramed in Figure 1.3. Anthranilate is provided for the production of PQS by two separate pathways, the anthranilate synthase *phnAB* or the kynurenine pathway (37). Calfee *et al*. first showed that anthranilate was a precursor to PQS and Bredenbruch *et al.* confirmed that PQS was produced via a "head to head" condensation reaction between anthranilate and a β-keto fatty acid (7, 11). The first four genes in the PQS biosynthetic operon, *pqsABCD,* are responsible for the production of the PQS precursor HHQ (4-hydroxy-2-heptylquinoline), as well as the production of 56 other quinolones (67). The synthesis of HHQ occurs via PqsA, an anthranilate CoA ligase that activates anthranilate, and PqsD, which condenses the fatty acid with the activated thioester of anthranilate to make the precursor HHQ (5, 18). PqsB and PqsC are necessary for PQS biosynthesis, though it is unclear exactly what role they play in the production of PQS (47). The final step in PQS biosynthesis occurs via PqsH, a FAD-dependent monoxygenase that hydroxylates HHQ to make PQS (106). Together, these proteins produce may quinolone molecules that influence virulence and aid in the survival of *P. aeruginosa.*

As explained above, the production of PQS is highly coordinated and requires the expression of multiple genes. The role of traditional AHL quorum sensing regulators

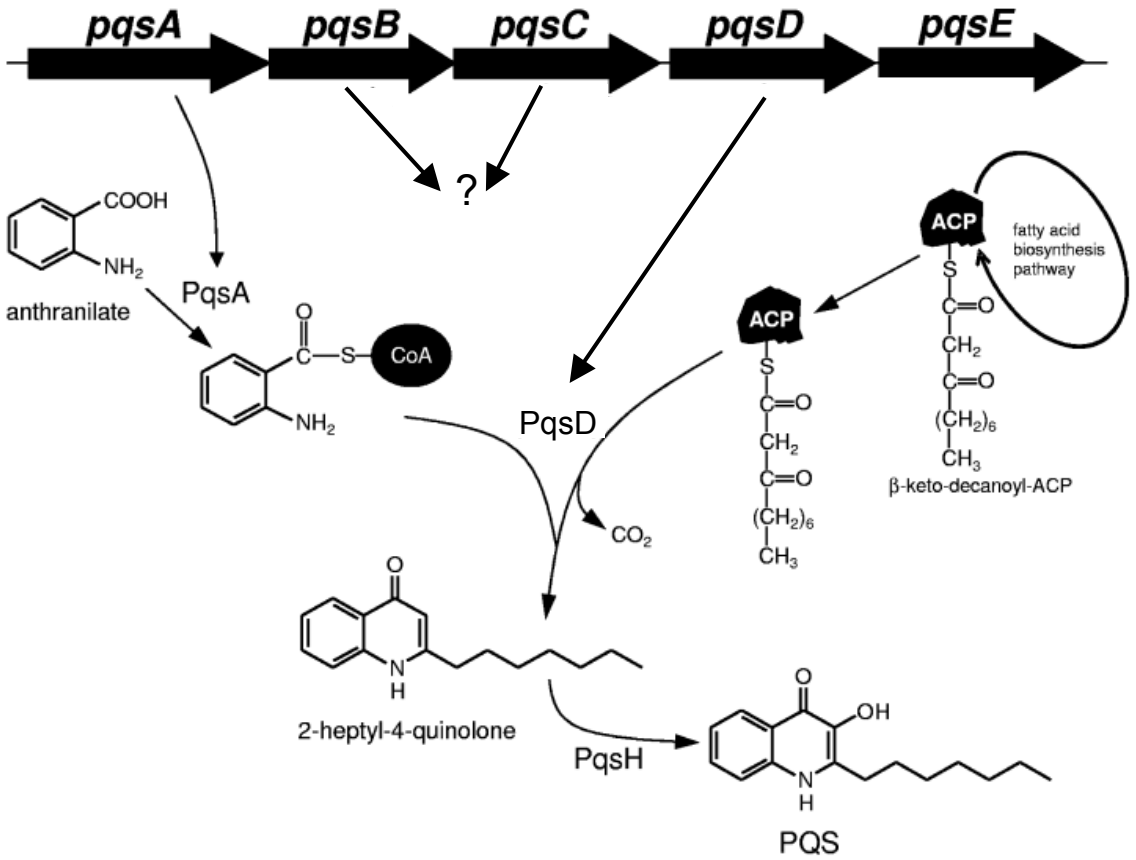

Figure 1.2. Proposed pathway for PQS biosynthesis. Anthranilate is activated by PqsA to form anthraniloyl-CoA, and this is condensed with 2-oxo-decanoyl-ACP which is recruited from the fatty acid biosynthetic pathway. The recruitment and condensation are postulated to be catalyzed by PqsB, PqsC, and PqsD. The resulting 2-heptyl-4-quinolone is then hydroxylated at the third carbon by the PqsH monooxygenase to produce PQS. Adapted from the figure published by Coleman *et al.* (18)

and the PQS induced transcriptional regulator PqsR are well described. The AHL quorum sensing regulators LasR and RhlR regulate quinolone production in a counterintuitive loop, with LasR and RhlR respectively upregulating and downregulating PQS biosynthesis (80, 81, 95, 121). The regulator LasR directly induces the expression of *pqsR, pqsA,* and *pqsH* and LasR binding boxes were identified in the promoters of these genes (43, 80, 121, 127). LasR is important for the induction of PQS biosynthetic genes, yet a mutation in *lasR* only delays the expression of PQS genes, indicating that the PQS biosynthetic genes are transcribed despite the absence of LasR (31). While LasR has a positive effect on PQS gene regulation, RhlR has a negative impact on the transcription of PQS related genes. This was shown both by a 50% increase in *pqsR* transcription in an *rhlI* mutant, as well as increased transcription of *pqsA* (80). Two predicted *las/rhl* binding boxes were identified in the *pqsA* promoter, and studies suggested that both LasR and RhlR could bind to each box resulting in either activation or repression of the transcription of *pqsA* (43, 132). The transcriptional regulator PqsR is activated by PQS to positively regulate gene expression (29, 95, 131). PqsR is necessary for the production of 4-quinolone based molecules (29, 41, 80). Both microarray and transcriptional fusion experiments have shown the importance of PqsR on both *pqsABCDE* and *phnAB* operons expression (28, 29, 80). Direct binding of PqsR to the *pqsA* promoter has been shown by both Wade *et al*. (121) and Xiao *et al.* (132), indicating the direct importance of PqsR to the production of 4-quinolone molecules. In addition to quorum sensing regulation, other factors have been found to influence PQS regulation. These include PmpR, a negative regulator of *pqsR*, and environmental factors such as iron and phosphate levels (55, 71, 86). Overall, the

regulators of PQS genes work in concert to ensure the correct timing of PQS production and PQS-mediated gene regulation.

1.3.2 The bioactivity and virulence of PQS

PQS and the other 56 HAQs produced by *P. aeruginosa* are extremely important to the survival of the bacteria through the bioactivity of these molecules. PQS induces the production of membrane vesicles and is incorporated into the bacterial outer membrane and membrane vesicles at high concentrations (77, 78). The PQS-containing membrane vesicles are also bioactive as they are able to induce PQS-mediated virulence factors in a PQS null strain (77). The membrane vesicles are believed to protect PQS from degradation when grown in the environment, thus ensuring that PQS cell-to-cell signaling would still be active (127). The induction of rhamnolipids, a virulence factor controlled by PQS and the quorum sensing regulator RhlR, is important for PQS bioactivity because the presence of rhamnolipids increases the solubility and activity of the molecule (121). PQS is also able to chelate ferric iron and the addition of PQS to cultures induces siderophore production and induction of siderophore-mediated iron transport systems (7, 30). The production of PQS is also controlled by the iron-inducible regulator Fur and small RNAs PrrF1 and PrrF2 (86). Lastly the production of over 56 HAQ molecules is advantageous to *P. aeruginosa* as some of the molecules exhibit antibiotic properties (47). The HAQs produced by *P. aeruginosa* are able to inhibit the growth of *Staphylococcus aureus, Staphylococcus epidermidids,* and *Candida albicans,* which would important during infections, as it would eradicate the other microorganisms (74, 77). Overall, it is clear that the bioactivity of PQS and the 56 other HAQs produced by the PQS pathway are important

for *P. aeruginosa* survival.

PQS affects *P. aeruginosa* virulence in two ways. First it activates virulence factor production. PQS is essential for virulence in *Caenorhabditis elegans, Drosophila melanogaster,* lettuce leaf, and multiple rodent infection models (28, 41, 64, 101, 130). The influence of PQS on the virulence of *P. aeruginosa* is also possible during infection of CF patients, as PQS was directly extracted from CF patient sputum and the mucopurulent airway fluid of a freshly resected CF lung (19). It also was produced by 9 of 10 *P. aeruginosa* isolates from CF patients (19). In addition, Guina *et al*. showed an overall deregulation of PQS production by *P. aeruginosa* isolates from infant CF patients, with strains exhibiting a 7-15 fold increase in PQS production (44). The importance of PQS in infections can be attributed to the positive regulation of many virulence factors such as pyocyanin, elastase, rhamnolipids, PA-IL lectin, RpoS, C_4-HSL, and biofilm production (31, 41, 64, 81, 91, 95). Both elastase and pyocyanin are directly attributed to aiding in the immune system modulation by *P. aeruginosa* (28, 31, 81, 95). Elastase cleaves collagen, immunoglobulins, complement, and fibronectin (31). Pyocyanin causes severe damage to the epithelial cells of the lung and is directly responsible for a 10% increase of neutrophils, macrophages, and lymphocytes during a *P. aeruginosa* infection (31, 60, 128). PQS also causes a directly increased apoptosis and decreased cell viability in two mouse cell lines at physiological concentrations (5 to 10 uM) (12). Finally, PQS directly affects the immune system by downregulating the innate immune response through both nuclear factor kappa B (NF-KB) and hypoxia-inducible factor 1 (HIF-1) (58, 65). As an adaptive human pathogen *P. aeruginosa* employs PQS to aid in evading the immune system through virulence factor regulation

and modulation of host factors, thus making its biosynthesis a very important pathway to understand.

1.4 The Pathways to Anthranilate

The production and degradation of anthranilate is an important branch point in *P. aeruginosa,* as it balances the production of virulence factors via PQS and growth of the bacterium by being broken down into tricarboxylic acid cycle intermediates (17). Since the identification of anthranilate as the precursor of PQS the importance of understanding both the pathways that synthesize and degrade anthranilate has become clear. Anthranilate is synthesized by three main pathways in *P. aeruginosa*, the kynurenine pathway and the two anthranilate synthases TrpEG and PhnAB (Figure 1.3) (37, 45). Other enzymes, PhzE1, PhzE2, PabB, and PchA, are anthranilate synthase homologs, but are not responsible for the cellular pool of anthranilate (25, 34, 35). The existence of multiple pathways to synthesize anthranilate further supports the importance of this molecule to *P. aeruginosa* survival, and these three pathways will be discussed in depth in the following paragraphs. The degradation of anthranilate into pyruvate is via AntABC and CatBCA, and the expression of these genes is tied into quorum sensing and iron availability (17, 86). The balance between production and degradation is maintained via similar regulators including quorum sensing and iron regulated small-RNA's, autoinduced regulators, and yet to be identified regulators (17, 29, 35, 86). The complex nature of the anthranilate branch point makes understanding the production and regulation of this key molecule, as well as its role in PQS synthesis, our priority.

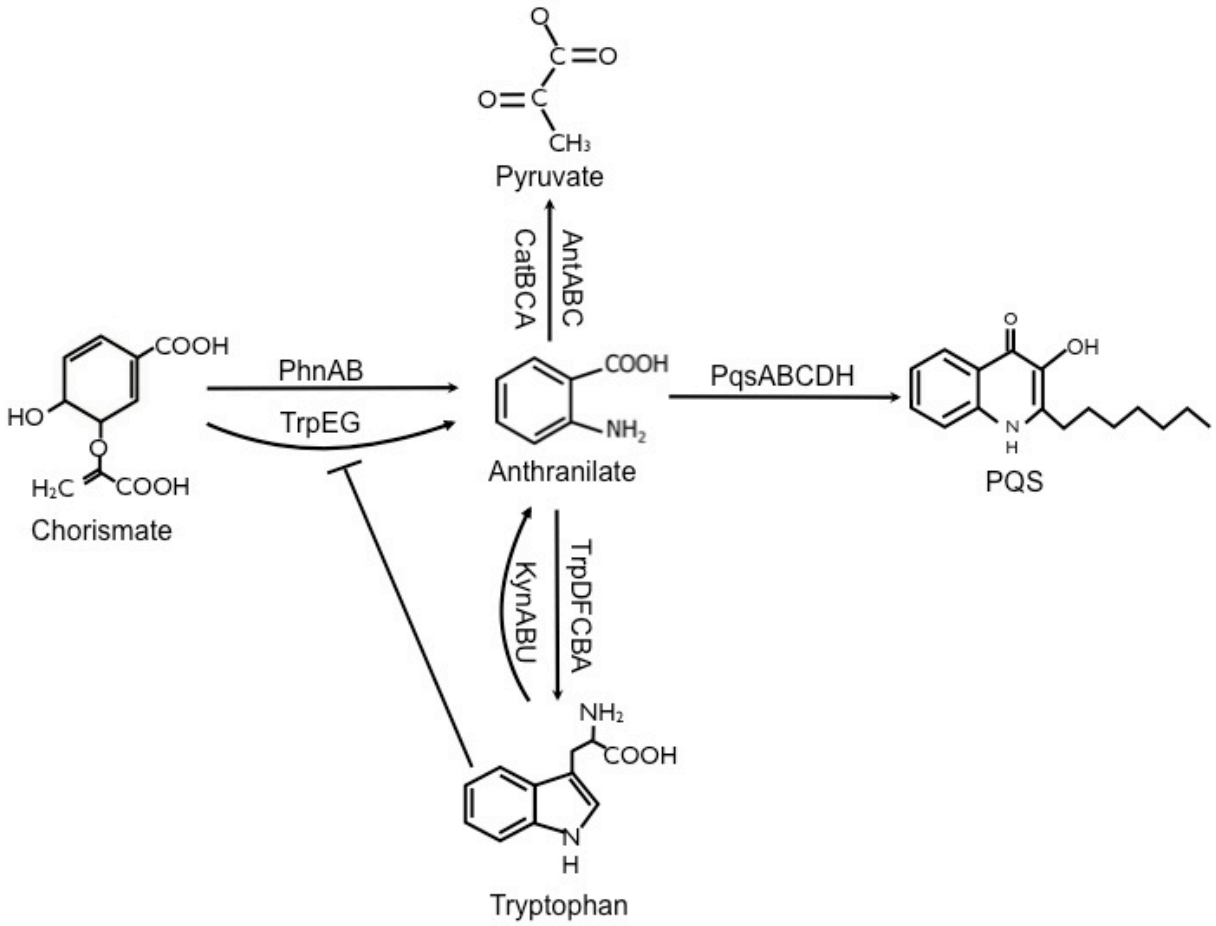

Figure 1.3 : The three biochemical pathways to anthranilate in *P. aeruginosa*

1.4.1 The Kynurenine Pathway

The kynurenine pathway is responsible for the oxidative catabolism of tryptophan to anthranilate and has been well described in eukaryotes (62). The ability of a bacterium to utilize this amino acid as a carbon and nitrogen source is advantageous in the environment, as it would preserve energy for the bacteria due to the energy expense to synthesize tryptophan and further decrease the concentration of free tryptophan for use by other organisms. The existence of the kynurenine pathway was originally predicted in prokaryotes in 1943 and verified in *Pseudomonad* species during the early 1950's (82, 110). In 1962, a strain of *P. aeruginosa* was shown to catabolize L-tryptophan, D- tryptophan, and kynurenine into anthranilate via the kynurenine pathway and biochemically confirmed the pathway in this species of *Pseudomonas* (4). The anthranilate branch of the kynurenine pathway is pictured in Figure 1.3 and consists of three enzymes: tryptophan 2,3-dioxygenase (*kynA*), kynurenine formamidase (*kynB*), and kynureninase (*kynU*) (62). In 2003, the eukaryotic genes for *kynA* and *kynU* were used to search for genetic homologs in bacteria, and this comparative genomics approach provided putative genetic homologs in multiple bacteria (62). At the time of the analysis the eukaryotic *kynB* was not sequenced and putative prokaryotic *kynB* genes were identified by searching predicted protein functions near the other kynurenine pathway genes (62). A conserved gene was identified in many of the bacteria near the kynurenine pathway genes and was suggested to encode KynB (62). The predicted KynB protein from *Ralstonia metallidurans* was purified and enzymatically converted formyl-kynurenine to kynurenine *in vitro,* confirming that the predicted gene encoded a kynurenine formamidase *(62).* The entire pathway in *R.*

metallidurans was confirmed to convert tryptophan to anthranilate and proved the predicted genes encoded a functional kynurenine pathway (62). The genetic identification of the kynurenine pathway in *P. aeruginosa* confirmed the earlier biochemical findings in the 1960's. The genetic organization of the kynurenine pathway is pictured in Figure 1.4**.** The *kynA* gene is located separately on the chromosome, while *kynB* and *kynU* are encoded in a putative two or three gene operon that may also contain an amino acid permease (PA2079) (www.pseudomonas.com). We have shown that the kynurenine pathway mutants cannot grow on defined media with tryptophan as the sole carbon source indicating that this is the only pathway that can catabolize tryptophan in *P. aeruginosa* (Figure 2.2) (59). The identification of this pathway provides for a unique alternative for the synthesis of the branch point molecule anthranilate.

The importance of the kynurenine pathway in *P. aeruginosa* virulence was indicated when it was identified as the major pathway of anthranilate for PQS production. Chugani and Greenberg also have suggested the kynurenine pathway is a main source of anthranilate for nutritional acquisition for *P. aeruginosa* (17). The role of the kynurenine pathway in PQS production was suspected due to the increase in PQS when *P. aeruginosa* was grown in tryptophan-supplemented media (62, 91). To investigate if the kynurenine pathway provided anthranilate for PQS production, radiolabeled tryptophan was added to growing cultures and PQS extractions were performed (37). The results indicated that [^{3}H]-tryptophan was incorporated into PQS in wild-type PAO1 **(**Figure 1.5A) (37). The lack of PQS production in the kynurenine pathway mutants was surprising because *P. aeruginosa* contains two other pathways

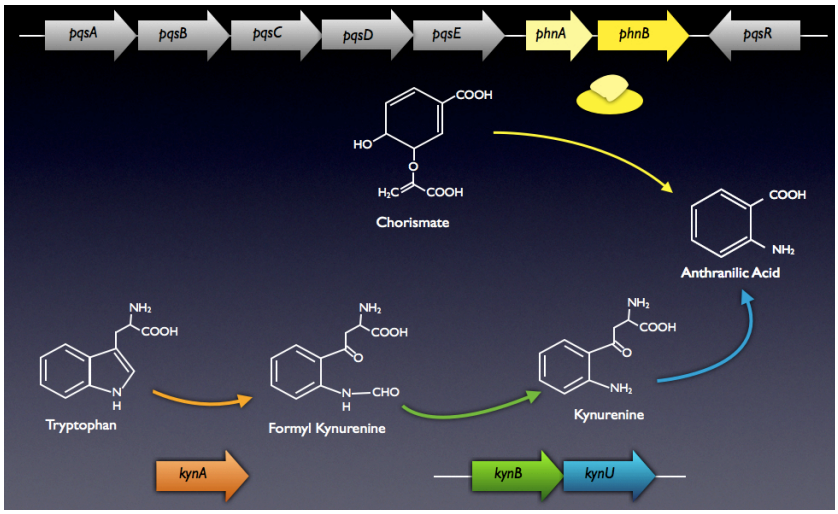

Figure 1.4: The genetic organization and enzymatic reactions of PhnAB and the kynurenine pathway. The color of the gene corresponds to the enzymatic reaction depicted by the colored arrow.

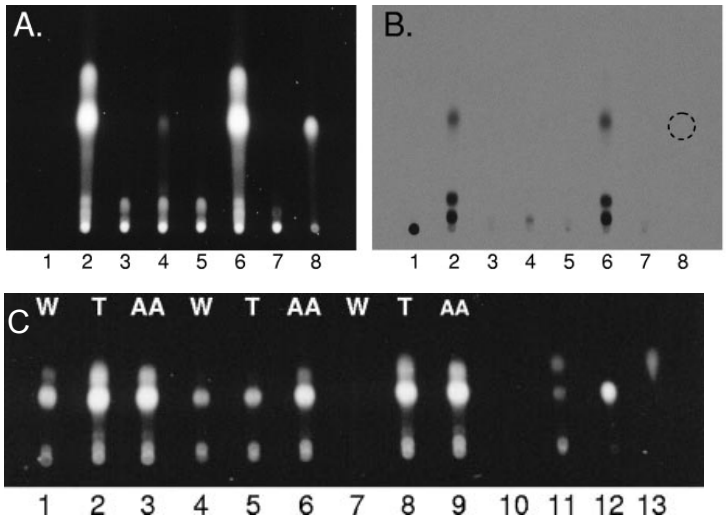

Figure 1.5: PQS extractions in kynurenine pathway and *phnAB* mutants. A) and B) PQS extractions of multiple *P. aeruginosa* strains grown in the presence of [^{3}H]-Tryptophan Photograph (A) and autoradiograph (B) of TLC. Lanes 1 contain 0.001 µCi L-[5-3H]tryptophan, and lanes 8 contain 25 ng synthetic PQS. Lanes 2 to 6 contain culture extracts from strains PAO1, PJF-KA1 (Δ*kynA)*, PJF-KB1 (Δ*kynB)*, PJF-KU1 (Δ*kynU)*, and PJF-PHNA1 (Δ*phnA)*, respectively. Lanes 7 contain an extract of uninoculated culture medium. C) PQS extracts from growth in minimal media supplemented with water (W), 1 mM L-tryptophan (T), or 0.5 mM anthranilic acid (AA) were analyzed by TLC. Lanes 1 to 3 contain extracts from strain PAO1 cultures, lanes 4 to 6 contain extracts from strain PJF-KA1 cultures, and lanes 7 to 9 contain extracts from strain PJF-PHNA1 cultures. Lane 10 contains an extract from a culture of PJF-PHNA1 carrying vector plasmid pUCP22, and lane 11 contains an extract from a culture of PJF-PHNA1 carrying *phnAB* expression plasmid pJF100. Lane 12 contains 25 ng of synthetic PQS, and lane 13 contains 34 ng of anthranilic acid. These data were originally published by Farrow and Pesci in 2008 (37).

that can produce anthranilate (35, 37), yet the results showed the kynurenine pathway is the main source of anthranilate for PQS production when grown in complex media. The importance of these genes in the production of PQS and other HAQs was also demonstrated when the kynurenine mutants exhibited a decreased ability to kill *S. aureus* (37). Finally it is suspected that the kynurenine pathway would be important for nutritional acquisition and virulence factor production in the CF lung due to the increased concentration of amino acids, as well as the production of proteases to liberate tryptophan from proteins within sputum (2, 48, 90, 115).

As the kynurenine pathway was found to be a major source of anthranilate for the synthesis of PQS, elucidating the regulation of this pathway would be beneficial for further understanding PQS production. Previous enzymatic studies in *Pseudomonas aureofaciens* and *Bacillus megaterium* suggested that the kynurenine pathway in *P. aeruginosa* would be positively influenced by the presence of tryptophan or kynurenine (6, 105). Transcriptional *lacZ* fusions of the *kynA* (pJFO3) and *kynB* (pJFO1) promoters were analyzed in wild-type *P. aeruginosa* (PAO1) and PJF-KA1 (a *kynA* mutant) to investigate the role of tryptophan, kynurenine, and anthranilate in the expression of the kynurenine pathway (Figure 1.6) (6, 37). The results indicated that *kynA* and *kynB* have similar regulation, and are induced in the presence of tryptophan and kynurenine in strain PAO1 (37). To determine if tryptophan or kynurenine induced the expression of *kynA* and *kynB* transcriptional fusions, the two fusions were tested in the *kynA* mutant strain, which is unable to break down tryptophan into kynurenine pathway intermediates (Figure 1.6)(37). In this strain, the expression of both fusions was only induced in the presence of kynurenine, and not tryptophan (37). Also, these experiments showed that

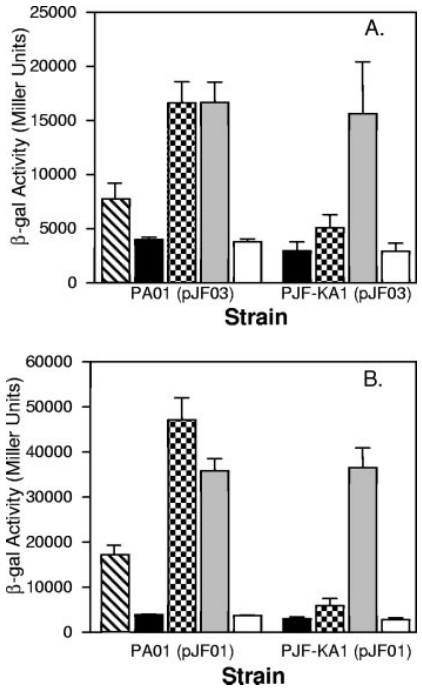

Figure 1.6: Kynurenine increased the transcription of *kynA* and *kynB.* β-Galactosidase activity of *kynA-lacZ* (pJF03) (A) or a *kynB-lacZ* (pJF01) (B) in strains PAO1 and PJF-KA1 was assayed in cultures grown in LPSM supplemented with either water (black bars), 1 mM L-tryptophan (checkered bars), 1 mM L-kynurenine (light gray bars), or 1 mM anthranilic acid (white bars) for 24 h. For reference, the striped bars represent data derived from unsupplemented cultures grown in PTSB medium. β-Gal activity is presented in Miller units as the mean±σ^{n-1} of results from duplicate assays from at least three separate experiments. As a reference, parental vector plasmid pLP170 in strain PAO1 cultured in minimal media supplemented with water produced 814 ± 292 Miller units of β-Gal activity. These data were originally published by Farrow and Pesci in 2008 (37).

neither gene is responsive to anthranilate (Figure 1.6). The influence of kynurenine on the transcriptional expression of the kynurenine pathway genes indicated the possibility of a direct transcriptional regulator that was influenced in the presence of this metabolite. A ChIP-chip assay performed with the quorum sensing regulator LasR indicated that it directly binds to the *kynB* promoter region and this was confirmed by an electrophoretic mobility shift assay by our lab (unpublished data) (43). Yet our lab ruled out LasR as the major transcriptional regulator of the kynurenine pathway due to the small change in β-galactosidase activity of a *kynB* lacZ fusion in a *lasR* mutant (unpublished data). In the search for the kynurenine responsive transcriptional regulator our lab identified gene PA2082, which encodes a putative transcriptional regulator that is divergently transcribed from *kynB*. This putative regulator, which we have designated KynR, is also located near the kynurenine pathway genes in multiple other bacteria (Figure 2.1) (59). KynR is homologous to Lrp or AsnC-type regulators of which many function as feast/famine regulators (FFRP) (135). FFRPs often induce amino acid metabolism genes, and though Lrp has a global regulatory role in *E. coli,* many Lrp-like proteins have more specific roles in regulation (8). FFRPs typically function as dimers and have two functional domains, an N-terminal DNA binding domain and a C-terminal assembly domain (135). These characteristics and the location of *kynR* suggested that *kynR* could encode the kynurenine responsive transcriptional regulator of the kynurenine pathway genes. The experiments that investigated the transcriptional regulator KynR are detailed in Chapter 2.

1.4.2 The Anthranilate Synthases TrpEG and PhnAB

Anthranilate is typically synthesized within bacteria by an anthranilate synthase. Anthranilate synthases are composed of a α subunit and a β subunit. The α subunit is the larger of the two and is responsible for the conversion of chorismate to anthranilate (23, 133). The β subunit provides ammonia from glutamine for the α subunit enzymatic reaction *in vivo* (133). Typically anthranilate synthases have two levels of tryptophan inhibition, at the post-transcriptional and post-translational levels (89, 100, 116). To initially characterize the *P. aeruginosa* anthranilate synthase, Crawford *et al.* used a plasmid containing *P. aeruginosa* genes to complement an *E. coli* tryptophan auxotroph (22, 23). The *P. aeruginosa* genes provided a five-fold regulatory response to tryptophan deprivation and suggested that the plasmid encoded an anthranilate synthase (22, 23). The suspected anthranilate synthase, however, did not exhibit any type of tryptophan inhibition (22, 23). Sequence alignments of the suspected *P. aeruginosa* anthranilate synthase with previously sequenced *trpE* and *trpG* from *Pseudomonas putida* suggested that the plasmid encoded an anthranilate synthase that differed significantly in sequence and localization on the chromosome (22, 23). In 1990, a series of papers by Essar *et al.* demonstrated that the anthranilate synthase on the plasmid was not the *P. aeruginosa* TrpEG*,* but rather contained genes encoding for a secondary anthranilate synthase (35, 36). This was an unexpected find, as the majority of bacteria contain only one anthranilate synthase. The newly identified anthranilate synthase was linked to secondary metabolites and the genes were named *phnAB* (35). Together TrpEG and PhnAB are the only two true anthranilate synthases in *P. aeruginosa* and results in a genetic redundancy that provides for uniquely functioning

anthranilate synthases, with TrpEG providing the first step in tryptophan synthesis and PhnAB supplying anthranilate for PQS production.

Tryptophan is the most energetically expensive amino acid to produce and to compensate this the production of tryptophan is highly regulated (137). Tryptophan synthesis is the result of five separate enzymatic reactions and the first step involves the anthranilate synthase TrpEG (45). The regulation of tryptophan synthesis involves multi-level regulation of the anthranilate synthase both at the post-transcriptional and post-translational levels. The *trpE* and *trpGDC* operons are regulated by post-transcriptional tryptophan attenuation in *Pseudomonas putida,* and are suspected to be regulated similarly in *P. aeruginosa* (89). Tryptophan also post-translationally inhibits the *P. aeruginosa* TrpE via competitive feedback inhibition by binding in a hydrophobic pocket and inhibiting the catalytic residues from interacting with chorismate (100, 116). It is unlikely that TrpEG is providing anthranilate for PQS biosynthesis. This was shown when the additional two pathways that produce anthranilate were not functional, TrpEG was unable to supplement PQS production (37). Furthermore, the tryptophan operons are down regulated when *P. aeruginosa* is grown in CF sputum, suggesting that TrpEG would not be responsible for supplying anthranilate for PQS production *in vivo* (91). With all of the presented data, it is unlikely that TrpEG is the source of anthranilate for the synthesis of PQS.

During the early investigation for the source of anthranilate used for the production of PQS, *phnAB* became the main focus due to the two gene operon clustering within the PQS synthetic locus (35, 41). Also, previous studies have shown that *phnA* is positively regulated by the transcriptional regulator PqsR, which positively regulates the PQS

operon *pqsABCDE,* and further suggested that it would have a major role in the production of anthranilate for PQS biosynthesis (13). However, results shown by Farrow and Pesci suggested otherwise (37). The PQS extractions performed on the kynurenine pathway mutants in complex media revealed that PhnAB could not supply anthranilate for PQS production in kynurenine pathway mutants (Figure 1.5A) (37). To further understand these results Farrow *et al.* investigated the source of anthranilate for PQS production during growth in a defined medium in the presence or absence of tryptophan (37). In a defined minimal media with no tryptophan, the *kynA* mutant produced PQS and suggested that PhnA provided anthranilate for PQS production under these growth conditions (Figure 1.5C, lanes 4-6) (37). This was confirmed when the *phnA* mutant did not produce PQS in the defined media and a *phnAB* complementing plasmid restored PQS production in the absence of tryptophan (Figure 1.5C, lane 11) (37). These results showed that PhnA is necessary for PQS production under nutrient limiting conditions and is negatively controlled during growth in rich media.

The regulation of *phnAB* is quite complex and it is expected to be regulated at both the transcriptional and translational level. Analyses by RNA slotblot, microarray, and transcriptional fusions show that *phnA* expression peaks during stationary phase (13, 16, 35). At the transcriptional level, *phnAB* has been shown to be positively influenced by LasR, and negatively influenced by both RhlR and the orphan quorum sensing regulator QscR, though this influence could be through PqsR as it is regulated similarly (16, 88). As stated previously PqsR has been shown to regulate *phnAB* (30, 31). A purified recombinant PqsR protein was demonstrated to have a direct interaction with

phnAB, however the direct interaction was not shown in DNA binding assays with purified native, full-length PqsR performed by our lab (unpublished data) (13). As for post-translational regulation, enzyme assays by Essar *et al.* suggested that PhnA is not inhibited in the presence of tryptophan (35). Sequence alignment with the known tryptophan binding residues of anthranilate synthases revealed that PhnA only has one of the three residues known to interact with tryptophan and may explain the lack of post translational inhibition by this molecule (109). To further complicate the regulation of *phnAB,* a *P. aeruginosa trpE* mutant (tryptophan auxotroph) was able to grow on tryptophan deplete media with a mutation frequency of 10^{-5} to 10^{-6} (35). The source of this mutation was not determined, but transcript levels of *phnAB* in the revertants are at high levels during all growth phases, which contrasts significantly with the stationary phase expression seen in the wild-type strain (16, 35). This suggests that PhnAB can supply the anthranilate used for tryptophan synthesis, and that the mutation caused dysregulation of *phnAB* to allow for *P. aeruginosa* survival. This mutation also resulted in a twenty percent increase in pyocyanin, which is a PQS controlled virulence factor (35). Experiments that investigate the dysregulation of *phnAB* transcription by this unknown mutation are further explained in chapter three. Overall, the regulation of the secondary anthranilate synthase *phnAB* appears to be complex, and elucidating this regulation should help us to better understand the source of anthranilate for PQS production.

CHAPTER TWO: KYNR, A LRP/ASN-C-TYPE TRANSCRIPTIONAL REGULATOR, DIRECTLY CONTROLS THE KYNURENINE PATHWAY IN *PSEUDOMONAS AERUGINOSA*

2.1 Summary

The opportunistic pathogen *Pseudomonas aeruginosa* can utilize a variety of carbon sources and produces many secondary metabolites to help survive harsh environments. *P. aeruginosa* is part of a small group of bacteria that use the kynurenine pathway to catabolize tryptophan. Through the kynurenine pathway, tryptophan is broken down into anthranilate, which is further degraded into tricarboxylic acid cycle intermediates or utilized to make numerous aromatic compounds, including the *Pseudomonas* quinolone signal (PQS). We have previously shown that the kynurenine pathway is a critical source of anthranilate for PQS synthesis and that the kynurenine pathway genes (*kynA* and *kynBU)* were upregulated in the presence of kynurenine. A putative Lrp/AsnC-type transcriptional regulator (gene PA2082, hereafter called *kynR*), is divergently transcribed from the *kynBU* operon and is highly conserved in Gram-negative bacteria that harbor the kynurenine pathway. We show that a mutation in *kynR* renders *P. aeruginosa* unable to utilize L-tryptophan as a sole carbon source and decreases PQS production. In addition, we found that the increase of *kynA* and *kynB* transcriptional activity in response to kynurenine was completely abolished in a *kynR* mutant, further indicating that KynR mediates the kynurenine-dependent expression of the kynurenine pathway genes. Finally, we found that purified KynR specifically bound the *kynA* promoter in the presence of kynurenine, and the *kynB*

promoter in the absence or presence of kynurenine. Taken together, our data show that KynR directly regulates the kynurenine pathway genes

2.2 Introduction

Pseudomonas aeruginosa is a Gram-negative bacillus that is ubiquitous throughout nature and infects a variety of hosts. It is a common nosocomial pathogen known to cause serious opportunistic infections in immunocompromised individuals (72). *P. aeruginosa* also causes a chronic infection in cystic fibrosis (CF) patients that is difficult, if not impossible, to eradicate and ultimately leads to increased morbidity and mortality in this population (10, 112). In order to survive during such infections and in many other harsh environments, *P. aeruginosa* can utilize numerous different carbon sources and produces a wide-range of secondary metabolites (57, 67, 79, 102, 114). One potential nutrient, tryptophan, can be used by some bacteria to provide building blocks for many secondary metabolites, some of which function as siderophores, signaling molecules, and protective compounds (2, 37, 79, 123). Similar to eukaryotes, *P. aeruginosa* catabolizes tryptophan through the kynurenine pathway (37, 61). Kurnasov *et al*. utilized comparative genetics to identify several bacteria with putative kynurenine pathways, including *Bacillus anthracis, P. aeruginosa,* and *Bordetella pertussis* (61). The kynurenine pathway contrasts significantly from the major tryptophan catabolic pathway of *Escherichia coli* (and many other species of bacteria), which catabolizes L-tryptophan anaerobically into indole, pyruvate, and ammonia via a pyridoxal phosphate-dependent tryptophanase (119, 134). Nevertheless, with either catabolic pathway the ability to utilize both tryptophan and tryptophan-breakdown products as a carbon and nitrogen source, and as precursors for many secondary

metabolites, provides a unique advantage for survival within nutrient-limited and harsh environments.

The conversion of L-tryptophan into quinolinate via the kynurenine pathway has been demonstrated in multiple bacterial species (61). However, *P. aeruginosa* only encodes the genes for the anthranilate branch of the kynurenine pathway (62). This branch catabolizes L-tryptophan into anthranilate via a three-step enzymatic pathway (61). The three enzymes are encoded by *kynA* (which encodes a tryptophan-2,3-dioxygenase), *kynB* (kynurenine formamidase), and *kynU* (kynureninase) (see Fig. 2.7 for pathway) (37, 62). The *kynA* gene is located separately on the *P. aeruginosa* chromosome, while *kynB* and *kynU* are encoded in a putative operon. The kynurenine pathway was linked to *P. aeruginosa* virulence when it was shown that radiolabeled tryptophan was incorporated into the *Pseudomonas* quinolone signal (PQS) (37), which is important for virulence in multiple models of infection (29, 40, 76, 101, 130). These data also showed that despite the presence of two alternative pathways for anthranilate production, the kynurenine pathway is the main source of anthranilate for PQS production when *P. aeruginosa* is grown in the presence of tryptophan or tryptophan breakdown metabolites (37, 91).

Due to both the importance of the kynurenine pathway in the production of PQS and because tryptophan is a costly amino acid to synthesize (37, 137), it would be advantageous for *P. aeruginosa* to strictly regulate the catabolism of tryptophan. Multiple transcriptome arrays have shown that quorum sensing may regulate the kynurenine pathway (107, 122), and the quorum-sensing regulator LasR was predicted through ChIP-chip analysis to bind to the promoter region of *kynB* (43). Our laboratory

has also shown that the transcription of both *kynA* and *kynB* was significantly increased in the presence of kynurenine (37). With these data in mind, we began to search for a transcriptional regulator that could specifically regulate *kynA* and *kynB* in the presence of kynurenine. We identified a putative transcriptional regulator encoded by gene PA2082, which is divergently transcribed from *kynB* and is homologous to the Lrp/AsnC family of transcriptional regulators. Lrp/AsnC-type regulators are known to control amino acid metabolism, and though Lrp has a global regulatory role in *E. coli,* many other regulators within the family have more specific regulons (8, 135). We demonstrate here that the protein encoded by gene PA2082 directly binds to and regulates the kynurenine pathway genes, and therefore propose that this protein be named the kynurenine pathway regulator (KynR).

2.3 Material and Methods

2.3.1 Bacterial strains, plasmids, and culture conditions

E. coli and *P. aeruginosa* strains were respectively maintained in 30% glycerol and 10% skim milk (Difco) at -80°C, and were freshly plated to begin each experiment. All strains and plasmids used are listed in Table 2.1. Bacteria were cultured at 37°C in Luria Bertani medium (LB), Vogel Bonner minimal media supplemented with 0.5% glycerol (VBG) (120), or Sole Carbon Source Media (SCM) containing 73.4 mM K_2HPO_4, 16.76 mM $NaNH_5PO_4{\cdot}4H_2O$, 0.8 mM $MgSO_4$; and with either 10 mM L-tryptophan (TSCM) or 10 mM L-alanine (ASCM) (pH 7.0). Cultures were supplemented with the kynurenine pathway metabolites as indicated below. Growth was monitored spectrophotometrically by optical density at 600 nm (OD_{600}) for *E. coli* or OD_{660} for *P. aeruginosa.* To maintain plasmids, 30 µg/ml chloramphenicol, 100 µg/ml ampicillin, 200

Table 2.1. List of strains and plasmids used in this study.

Strain or Plasmid	Relevant genotype or phenotype	Reference
E. coli DH5α	F'/*endA1, hsdR17, supE44 thi-1, recA1, gyrA, relA1,* (*lacZYA-argF) U169, deoR* [Φ80d*lac*Δ(*lacZ*)*M15recA1]*	(129)
P. aeruginosa strains		
PAO1	Wild-type	(53)
PAO-R1	*lasR*::Tc deletion mutant derived from strain PAO1	(42)
PJF-KA1	*kynA* deletion mutant derived from strain PAO1	(37)
PJF-KB1	*kynB* deletion mutant derived from strain PAO1	(37)
PJF-KU1	*kynU* deletion mutant derived from strain PAO1	(37)
PAΔKynR	*kynR* deletion mutant derived from strain PAO1	This study
PAO-R1DKynR	*kynR, lasR* deletion mutant derived from strain PAO-R1	This study
Plasmid		
pHERD30T	P_{BAD}- based shuttle vector, Gm^r	(99)
pKynRex	KynR expression vector, Gm^r	This study
pACYC184	General purpose cloning vector, Tet^r and Chl^r	(14)
pKynRsubex	KynR expression vector derived from pKynRex, Chl^r	This study
pEX18Ap	Suicide vector, Amp^r	(51)
p*kynR*del	*kynR* deletion suicide vector, Amp^r	This study
pLP170	*lacZ* transcriptional fusion vector, Amp^r	(98)
pJF01	*kynB'-lacZ* transcriptional fusion, Amp^r	(37)
pJF03	*kynA'-lacZ* transcriptional fusion, Amp^r	(37)

Abbreviations: Gm^r, gentamicin resistance; Tet^r, tetracycline; Chl^r, chloramphenicol; and Amp^r, ampicillin

μg/ml carbenicillin, or 15 μg/ml (*E. coli*) or 30 μg/ml (*P. aeruginosa)* gentamicin was added when appropriate.

In order to generate an expression plasmid for KynR, a 568 bp DNA fragment, which began at the *kynR* start codon (ATG) and ended 46 bp downstream from the stop codon was amplified by PCR using strain PAO1 chromosomal DNA as the template. The oligonucleotide primers used for this amplification were engineered to contain a single HindIII site downstream from the stop codon (Table 2.2). Vector plasmid pHERD30T, which contains an *araBAD* (P_{BAD}) promoter to control gene expression, was digested sequentially with SmaI and HindIII. The digested plasmid DNA was ligated with the *kynR* PCR fragment, which had also been digested with HindIII, to create the plasmid pKynRex. This plasmid was used to amplify *kynR* and regulatory elements for use in a two-plasmid system in *E. coli.* PCR was used to amplify both the *kynR* and divergently transcribed *araC* to ensure all elements necessary for controlled expression of KynR were included in the subcloned plasmid. Primers were located 52 bp downstream from *araC* and 46 bp downstream from *kynR*. The resulting PCR fragment, as well as pACYC184, were sequentially digested with SalI and HindIII and ligated together, generating plasmid pKynRsubex.

2.3.2 Generation of *kynR* mutants

A splicing by overlap extension protocol was used to generate mutant alleles (126). Alleles were constructed to contain in-frame deletions in the coding DNA sequences corresponding to amino acids 25 to 140 for *kynR* (73% of protein sequence). Primers were designed to contain approximately 1 kb of DNA both upstream and downstream from the splice junction and each primer added an EcoRI restriction site to

Table 2.2. List of primers used in this study.

Primer Name	Sequence (5' to 3')[a]
Primers for Mutagenesis	
2082-F1-remake	AAAAAA**GAATTC**GAGGTAGATCACGCCGTC
PA2082Rev1	CCGGCCTTGATCTCCTGCAGTCCTGGTTGCTCATCCGCCC
PA2082Fwd2	GGGCGGATGAGCAACCAGGACTGCAGGAGATCAAGGCCGG
PA2082Rev2	AAAAAA**GAATTC**GAACTTGGCGGCGATGG
Primers for KynR expression	
2082atgfwd	ATGTCCCTGGACGCCATCGA
2082 Rev Herd	AAAA**AAGCTT**CGTCCCTCTCATTGCACT
Herd2082exsubRev	AAAA**GTCGAC**CGAAGCAGGGTTATGCAGC
EMSA	
kynApromoterfwd	GAGTGAGGGCAAGGACACAT
kynApromoterrev	CGCGAGTGATCCGAAATTCG
kynBpromoterfwd	GACTGATGTCCCAGTAGCGG
kynBpromoterrev	GACGGAGAATGCGCAGATCG
FwdKynU-EMSA	GGTACTTGTAGGTGCAGCCG
RevKynU-EMSA	CAAGACCGGCTACCTGCACG

[a]Restriction sites indicated by bold type.

both ends of the PCR product. Both the *kynR*del-PCR product and pEX18Ap (suicide vector) were digested with EcoRI and ligated together. The resulting plasmid, p*kynR*del, was transformed into strains PAO1 and PAO-R1 by electroporation (15). Mutants were selected for by plating transformants on medium containing carbenicillin and then on medium containing 6% sucrose to remove the vector sequence (51). PCR was used to screen colonies and DNA sequencing of PCR products was used to confirm mutants.

2.3.3 PQS production

Washed cells from overnight cultures were used to inoculate 10 ml cultures of LB or VBG supplemented either with water, 1 mM L-tryptophan, 1 mM L-kynurenine, or 1 mM anthranilate (final concentrations) to an OD_{660} of 0.05. After 6 and 24 h of growth, 300 µl samples of each culture were extracted with 900 µl of acidified ethyl acetate as previously described (20). One-half of the resulting organic phase was evaporated to dryness at 37°C and 50 µl of 1:1 acidified ethyl acetate:acetonitrile was used to reconstitute the extract. Samples were analyzed by thin-layer chromatography, visualized by long-wave UV light, and photographed (20).

2.3.4 β-galactosidase (β-gal) assays in *P. aeruginosa*

Cells from overnight cultures of *P. aeruginosa* grown in VBG were washed and resuspended in fresh medium to an OD_{660} of 0.05 and incubated at 37°C with shaking at ≥180 rpm for 6 h. At that time either water or 1 mM L-kynurenine was added and cultures continued to grow at 37°C with shaking at ≥180 rpm for an additional 18 h. The cells were collected by centrifugation at 12,000 x g for 2 min, resuspended in fresh

VBG, and assayed for β-gal activity in duplicate. Data are presented in Miller units as the mean ± the standard deviation (σ^{n-1}) of at least 3 separate experiments.

2.3.5 β-gal assays in *E. coli.*

Overnight cultures of *E. coli* carrying the appropriate plasmids were grown in LB and used to inoculate 10 ml cultures of fresh media to an OD_{600} of 0.08. The subcultures were incubated at 37°C with shaking at ≥180 rpm until an OD_{600} of 0.3 was reached. Then 2 ml aliquots were transferred to tubes that contained 0.1% L-arabinose to induce *kynR* expression and either water, 1 mM L-kynurenine, 1 mM L-tryptophan, or 1 mM anthranilic acid (final concentrations) was added. The cells were incubated for 2 h at 37°C with shaking at ≥180 rpm. After incubation, the β-gal activity produced by each culture was assayed and data are reported as the mean ± the standard deviation (σ^{n-1}) of at least 3 independent experiments.

2.3.6 Purification of KynR

The method used for purification of KynR is a modification of those described by Madhusudhan *et al.* for the purification of BkdR from *Pseudomonas putida* (75). A 10 ml overnight culture of *E. coli* strain DH5α (pKynRex) was used to inoculate 250 ml of LB and was incubated at 37°C with shaking at ≥ 180 rpm until an OD_{600} of 0.7 was reached. At this point, 1% L-arabinose (Sigma-Aldrich) was added to induce *kynR* expression and the culture was grown for an additional 3 h at 37°C with shaking at ≥ 180 rpm. Cells were harvested by centrifugation at 6,000 x g for 10 minutes and then resuspended in 20 mM Tris-HCL pH 7.4 and 1 mM dithiothreitol (DTT) (buffer A). The cells were lysed by sonication and the lysate was cleared by centrifugation at 37,000 x g for 1 h at 4°C (Beckman Coulter Optima L-100 XP Ultracentrifuge). KynR purification

was performed at room temperature and all samples were kept on ice or at 4°C until purification was complete. The soluble fraction (90 mg total protein) was applied to a HiTrap DEAE Sepharose Fast Flow column (1 ml, GE Healthcare) in buffer A at a flow rate of 0.5 ml/min. The column was washed with buffer A until the A_{280} returned to 0 (~50 ml). Bound proteins were eluted with a 30 ml linear gradient of 0 to 0.3 M NaCl at a flow rate of 1 ml/min, and 3 ml fractions were collected. Samples from the collected fractions were precipitated using deoxycholic acid and tricholoracetate acid (DCA/TCA) and then separated by 15% SDS-PAGE to identify fractions containing KynR. These fractions were collected and dialyzed against a buffer containing 20 mM Tris-HCL pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT (buffer B) overnight at 4°C. The dialyzed fractions were then applied to a HiTrap Heparin (5 ml, GE Healthcare) column equilibrated with buffer B. The column was washed with buffer B until the A_{280} returned to 0 (~24 ml). Bound proteins were eluted with a 50 ml linear gradient of 0.1 to 0.4 M NaCl in buffer B at a flow rate of 1 ml/min and 3 ml fractions were collected. Again, proteins from the collected fractions were precipitated with DCA/TCA and were separated on a 15% SDS-polyacrylamide gel to identify the fractions containing KynR. All fractions containing KynR were pooled and dialyzed against 20 mM Tris-HCL pH 7.8 and 1 mM DTT (buffer C) overnight at 4°C. The dialyzed fraction was then added to a HiTrap DEAE Sepharose Fast Flow column (1 ml, GE Healthcare) equilibrated with buffer C. The column was washed with buffer C until the A_{280} returned to 0 (~12 ml) and bound proteins were eluted with a 30 ml linear gradient of 0 to 0.3 M NaCl at a flow rate of 1 ml/min and 3 ml fractions were collected. Samples from collected fractions were precipitated with DCA/TCA and separated on a 15% SDS-PAGE to identify the fractions

that contained KynR. The fractions containing KynR resulted in 95% purity of KynR as judged by SDS-PAGE (data not shown). Glycerol was added to the pooled KynR fractions to a final concentration of 15% and the protein was stored at -80°C.

Column fractions during purification were monitored by absorbance measurements at 280 and 260 nm with a NanoDrop ND-1000 spectrophotometer. After purification, the final protein concentration was 4.34 mg/ml (244 mol) of protein, as determined by a Bradford assay using BioRad reagents. In addition, protein concentrations for electrophoretic mobility shift assays (EMSA) were determined by the Bradford assay using Bio-Rad reagents.

2.3.7 EMSA

PCR was used to generate DNA fragments containing the *kynA* (228 bp, +208 bp to -20 bp relative to ATG) and *kynB* (200 bp, +169 bp to -31 bp relative to the ATG) promoter regions. An internal fragment of *kynU* (157 bp, +527 bp to +685 bp relative to the ATG) was also generated by PCR as a negative control. DNA fragments were labeled with [γ-P^{32}] ATP (Perkin-Elmer, Wellesley, MA) by using T4 polynucleotide kinase (Invitrogen, Carlsbad, CA). The binding assays were carried out in buffer containing 20 mM Tris-HCl (pH 7.4), 50 mM $NaCl_2$, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 10% glycerol. Each reaction mixture contained 0.3 µg of salmon sperm DNA, approximately 1.5 x 10^4 cpm radiolabeled DNA, and 0 to 488 pmol of protein. Binding reactions were performed in both the presence and absence of 0.1 mM L-kynurenine or 0.1 mM L-tryptophan. Reactions mixtures were incubated at room temperature for 20 min and separated by electrophoresis at 4°C on a native 10% polyacrylamide gel [29:1 acrylamide to bis-acrylamide, 1X Tris-borate buffer (89 mM Tris and 89 mM Borate, pH

8.0), and 2.5% glycerol] for 5 minutes at 200 V and then 4 hours at 100 V. After electrophoresis, the gels were dried and visualized by autoradiography.

2.4 Results

2.4.1 The identification of a putative regulator of the kynurenine pathway

To begin our search for potential regulators of the kynurenine pathway, we first analyzed the predicted function of annotated genes located near the kynurenine pathway operons in *P. aeruginosa* (www.pseudomonas.com). The kynurenine pathway genes are located separately on the *P. aeruginosa* chromosome, with *kynB* and *kynU* located in a putative polycistronic operon and *kynA* located on a distant putative monocistronic operon (Figure 2.1A). A potential transcriptional regulator encoded by gene PA2082, was divergently transcribed from *kynB* in *P. aeruginosa* and appeared to be a member of the Lrp/AsnC family of transcriptional regulators (Figure. 2.1B). These regulators are known to be important for controlling amino acid metabolism and are often encoded adjacent to pathway genes that they regulate (8). We used a comparative genomics approach and began to examine other bacteria that harbor the kynurenine pathway genes to determine if they also possessed similar regulator proteins. Both the *Burkholderiacea* and *Pseudomonadaceae* families encoded a PA2082 homolog divergently oriented from either *kynB* or *kynU* in the *Burkholderia, Ralstonia, Cupavarius, Bordetella,* and *Pseudomonas* genera (Fig. 2.1B). *P. aeruginosa* only contains the anthranilate branch of the kynurenine pathway, while some of the *Burkholderia* species and *P. fluorescens* apparently contain the pathway to catabolize tryptophan into quinolinate or the siderophore quinolobactin (61, 79). Also,

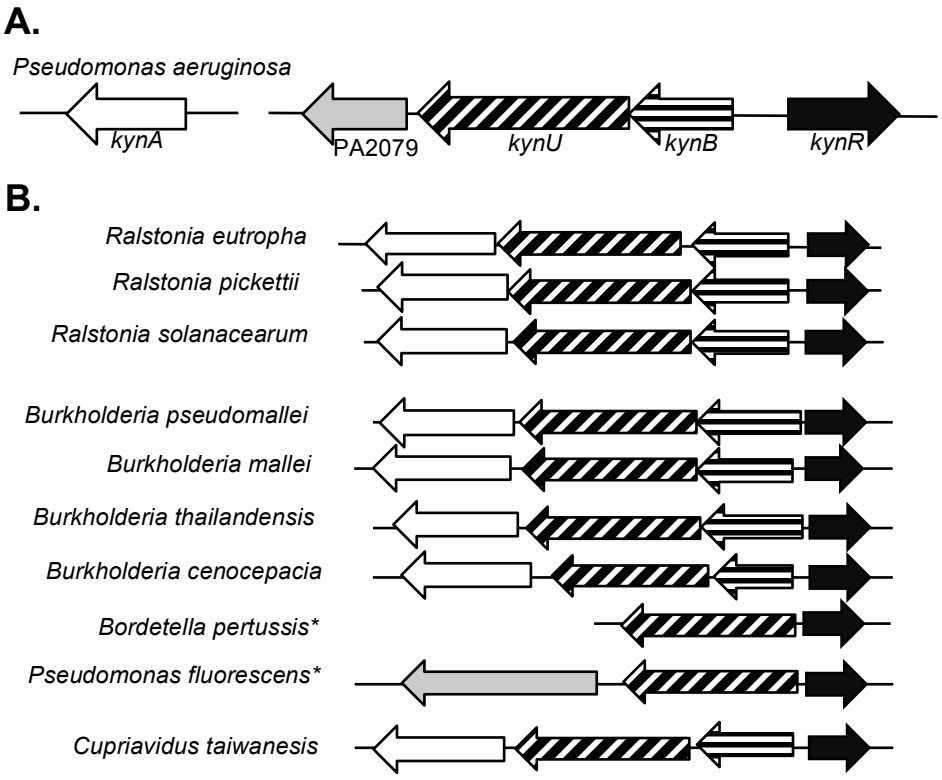

Figure 2.1. The *kynR* gene is conserved in other Gram-negative bacteria that have the kynurenine pathway genes. A. The two loci of the kynurenine pathway genes in *P. aeruginosa.* B. A schematic to show the conservation of *kynR.* The shading on each gene corresponds to the homologous genes in part A. * Indicates that *kynB* and *kynA* are located separately on the chromosome in a putative operon.

Bacillus cereus and *Bacillus anthracis* contain the kynurenine pathway genes (62), but these species have a TetR-like regulator divergently oriented from *kynU* that may be responsible for an alternative regulatory scheme. Overall, these findings suggested that the putative transcriptional regulator encoded by PA2082, which we hereby designate as KynR (kynurenine pathway regulator), might play a role in regulating tryptophan catabolism in *P. aeruginosa.*

In order to examine the role of KynR in tryptophan catabolism we assessed the impact of KynR on kynurenine pathway-dependent phenotypes. The kynurenine pathway is the only enzymatic pathway identified in *P. aeruginosa* capable of degrading tryptophan (61). To confirm this, we grew mutants containing deletions of the kynurenine pathway genes *kynA* (PJF-KA1)*, kynB* (PJF-KB1)*,* and *kynU* (PJF-KU1) in a minimal salts media with tryptophan as the only carbon source (TSCM) (Figure 2.2A). The wild-type strain PAO1 utilized tryptophan as a sole carbon source and grew to an OD_{660} of over 0.5 (Figure 2.2A). However, the kynurenine pathway mutants were unable to grow with only tryptophan as a carbon source (Figure 2.2A). As a control, the strains were also grown on alanine as a sole carbon source (ASCM) and the kynurenine pathways mutants were able to grow to wild-type levels (data not shown). These results confirmed that the kynurenine pathway is required for tryptophan catabolism in *P. aeruginosa.* Next, we tested whether the *kynR* mutant strain PAΔKynR was able to grow on tryptophan as a sole carbon source (Figure 2.2B). The results indicated that, like the kynurenine pathway mutants, PAΔKynR was unable to grow on tryptophan alone. In order to determine if the inhibition of growth was due to the loss of *kynR,*

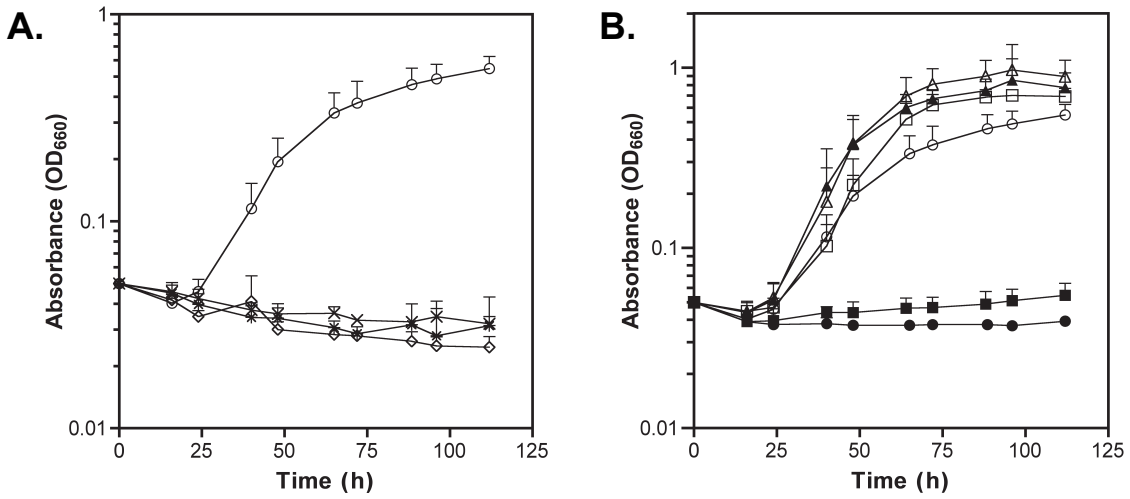

Figure 2.2. Utilization of L-tryptophan as a sole carbon source. A. Bacterial strains PAO1 (circles), PJF-KA1 (X), PJF-KB1 (diamonds), PJF-KU1 (asterisks) were grown in TSCM and OD_{660} was measured. B. Bacterial strains PAO1 (open circles), PAO1 + 0.1% arabinose (open squares), PAO1(pKynRex) + 0.1% arabinose (open triangles), PAΔKynR (closed circles), PAΔKynR + 0.1% arabinose (closed squares), and PAΔKynR(pKynRex) + 0.1% arabinose (closed triangles) were grown in TSCM and OD_{660} was measured. Values depict the mean ± σ^{n-1} from at least three separate experiments.

strains PAΔKynR and PAO1 were transformed with a plasmid harboring wild-type *kynR* under an arabinose inducible promoter (pKynRex). Expression of KynR in strain PAΔKynR caused growth on tryptophan to be similar to that of the parent strain PAO1 (Figure 2.2B). Since *P. aeruginosa* can utilize arabinose as a carbon source, we also included control cultures to ensure that the arabinose that was added for KynR expression was not affecting the growth phenotypes and observed that the effect was negligible (Figure 2.B). Overall, these data indicated that a *kynR* mutant was unable to grow on tryptophan as a sole carbon source, and further supported the notion that KynR has a role in the regulation of the kynurenine pathway.

2.4.2 PQS production is affected in a *kynR* mutant.

Previous studies showed that the kynurenine pathway mutants produced little to no detectable PQS and suggested that the kynurenine pathway is the main source of anthranilate for PQS production when tryptophan or its breakdown metabolites are present (37). Therefore, we were interested in testing whether a mutation in *kynR* would also have an effect on PQS production. Strains PAO1 and PAΔKynR were grown in LB and VBG supplemented with tryptophan, kynurenine, anthranilic acid, or water (as a control). The cultures were grown for either 6 or 24 h and then were extracted with acidified ethyl acetate. The extracts were analyzed by TLC to assay both PQS and anthranilate production. After 6 and 24 h of growth in LB or LB supplemented with tryptophan, strain PAΔKynR produced less PQS (Figure 2.3A and 3B, lanes 6 and 7) than the wild-type strain PAO1 (Figure 2.3A and B, lanes 2 and 3). This suggested that *kynR* affects the supply of anthranilate for PQS production by positively controlling an element of the kynurenine pathway.

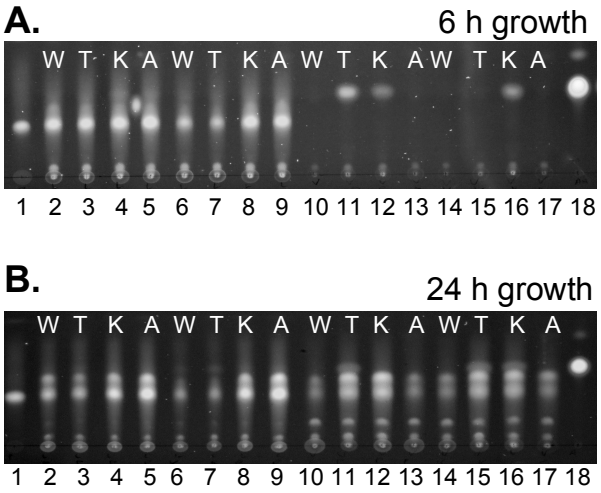

Figure 2.3: The production of anthranilate and PQS is decreased in the *kynR* mutant. Ethyl acetate extracts from cultures grown for 6 (A) and 24 h (B) were analyzed by TLC. Equal volumes of extracts were resolved in each lane, and the addition of water (W), 1 mM L-tryptophan (T), 1 mM L-kynurenine (K), or 1 mM anthranilic acid (A) to the respective culture is indicated at the top of each lane. Lane 1 contains 75 ng of PQS and lane 18 contains 1.25 ng of anthranilic acid as controls. Lanes 2 to 5 contain extracts from strain PAO1 grown in LB; lanes 6-9 contain extracts from strain PAΔKynR grown in LB; lanes 10-13 contain extracts from strain PAO1 grown in VBG; and lanes 14-17 contain extracts from strain PAΔKynR grown in VBG.

We then saw something unexpected when the addition of kynurenine to LB resulted in wild-type amounts of PQS being produced by strain PAΔKynR after both 6 and 24 h (Figure 2.3A and B, lane 8). This suggested that KynR was not the only factor that controls *kynBU,* which must be induced for the conversion of kynurenine to anthranilate to be used for PQS synthesis (see Figure 2.7 for pathway). PQS was also extracted from both strains grown in a minimal media to assess the effects of supplementing with individual kynurenine pathway metabolites. The production of PQS by strains PAO1 and PAΔKynR grown in VBG differed somewhat from those in LB, but followed a similar trend. PQS was not detectable from either strain after 6 h of growth in VBG, but the addition of tryptophan or kynurenine to the media induced the production of anthranilate by the wild-type strain PAO1 (Figure 2.3A, lanes 11 and 12). Unlike the wild-type strain PAO1, strain PAΔKynR only produced anthranilate with the addition of kynurenine and not when tryptophan was added (Figure 2.3A, lanes 15 and 16). After 24 h of growth in VBG, PQS was detectable from both strains under all conditions and anthranilate was no longer detectable after presumably being used by the cells (Figure 2.3B, lanes 10-17). Taken together, the data suggested that KynR indirectly regulates PQS production, presumably through the kynurenine pathway, but that it is not absolutely required for PQS to be produced.

2.4.3 KynR is required for kynurenine dependent induction of kynurenine pathway genes.

Thus far the phenotypes of the *kynR* mutant have implied that KynR plays a role in the kynurenine pathway. In addition, our previous work had shown that the transcription of both *kynA* and *kynB* were induced in the presence of kynurenine (37).

Therefore, we decided to determine if KynR affected the expression of *kynA* and *kynB* in a defined medium (VBG) where we could control the concentration of kynurenine. To study this, we performed β-gal assays in strains PAO1 and PAΔKynR harboring the respective *kynA'-lacZ* and *kynB'-lacZ* transcriptional fusion plasmids pJF03 and pJF01. Our data showed that after 24 h of growth in VBG media in the absence of kynurenine, the expression of both *kynA* and *kynB* were similar in both the parental and *kynR* mutant strains (Figure 2.4A and B). However, the addition of 1 mM kynurenine resulted in a large induction of both *kynA* and *kynB* in the wild-type strain PAO1, but this induction did not occur in the *kynR* mutant (Figure 2.4A and B). These data led us to conclude that in the presence of kynurenine, KynR is positively regulating *kynA* and *kynB* transcription.

It has been suggested from microarray experiments that *kynB* is directly regulated by the quorum sensing regulator LasR (43). Since our data in Figure 2.3 also suggested that a factor in addition to KynR was regulating *kynBU*, it seemed logical that we should examine whether LasR affected *kynBU* transcription. The data of Figure 2.4B showed that *kynB* transcription was greatly decreased in a *lasR* mutant when kynurenine is not present but that the addition of kynurenine will override the *lasR* mutation. In addition, a *lasR*, *kynR* double mutant exhibited no transcription of *kynB* in the presence or absence of kynurenine (Figure 2.4B). Taken together, the data of Figure 2.4 lead us to conclude that *kynBU* is controlled by LasR when kynurenine is absent and by KynR when kynurenine is available.

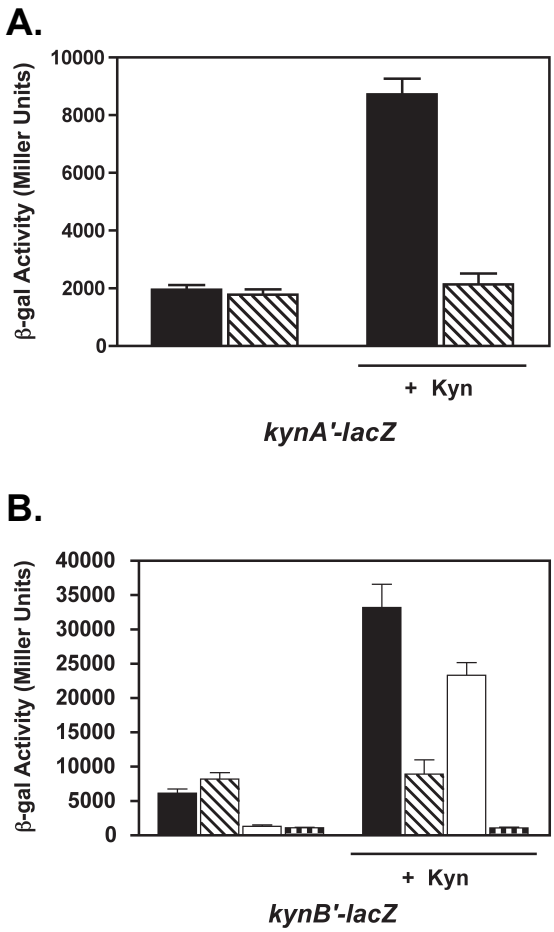

Figure 2.4: The kynurenine pathway genes are not induced by kynurenine in a *kynR* mutant. β-gal activity of (A) *kynA'-lacZ* fusion (pJF03) and (B) *kynB'-lacZ* fusion (pJF01) in strains PAO1 (solid bars), PAΔKynR (striped bars), PAO-R1 (open bars), and PAO-R1ΔKynR (stippled bars) were assayed in cultures grown in VBG or 6 h and then supplemented with either water (as a control) or 1 mM L-kynurenine for 18 h. β-gal activity is presented in Miller units as the mean ± σ^{n-1} of results from duplicate assays from at least three separate experiments.

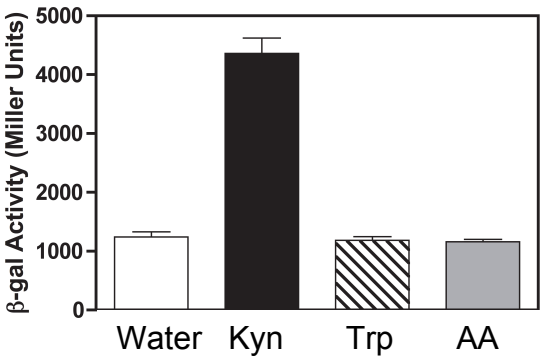

Figure 2.5: KynR can induce expression of *kynB'-lacZ* in the presence of L-kynurenine in *E. coli*. *E. coli* strain DH5α (pJF01)(pKynRex) was grown in LB media in the presence of water as a control water (white bar), 1 mM L-kynurenine (Kyn, black bar), 1 mM L-tryptophan (Trp, striped bar), or 1 mM anthranilate (AA, grey bar). After 3 h of growth the cultures were induced with 0.1% arabinose and grown for an additional 3 h. The cultures were then assayed for β-gal activity, which is presented in Miller units as the mean ± σ^{n-1} of results from at least three experiments.

2.4.4 KynR directly regulates the kynurenine pathway.

Our data have shown so far that KynR is required for the kynurenine-dependent increase of both *kynA* and *kynB* transcription (Figure 2.4A and B), but whether this regulation was direct or indirect was unknown. To eliminate any possibility of endogenous *P. aeruginosa* factors altering the results of our *kynA* and *kynB* transcriptional fusion assays, we utilized an *E. coli* two-plasmid system to try to establish a more direct link between KynR and the induction of *kynA* and *kynB.* [Note: It is important to point out here that *E. coli* does not contain the genes for the kynurenine pathway.] *E. coli* strain DH5α harboring either a *kynA'-lacZ* (pJF03) or a *kynB'-lacZ* (pJF01) transcriptional fusion and the KynR overexpression plasmid (pKynRsubex) was grown in LB in the presence of tryptophan, kynurenine, or anthranilate, and the expression of KynR was induced with the addition of 0.1% arabinose. The results presented in Figure 2.5 showed that the expression of *kynB'-lacZ* was only induced in the presence of KynR and kynurenine, and not when tryptophan or anthranilate was present. Unfortunately, the similar assays performed with the *kynA'-lacZ* fusion showed that it was constitutively active in *E. coli* and direct regulation by KynR could not be demonstrated in this experiment (data not shown). Nevertheless, the results of Figure 2.5 suggested that KynR can directly activate the expression of *kynB*, and that kynurenine acts as a coinducer for KynR.

To determine if KynR directly binds to the *kynA* and *kynB* promoter regions in response to kynurenine, we purified KynR and performed electrophoretic mobility shift assays (EMSA). KynR was purified from *E. coli* strain DH5α (pKynRex) and incubated with either the *kynA* or *kynB* promoter region in the presence or absence of kynurenine.

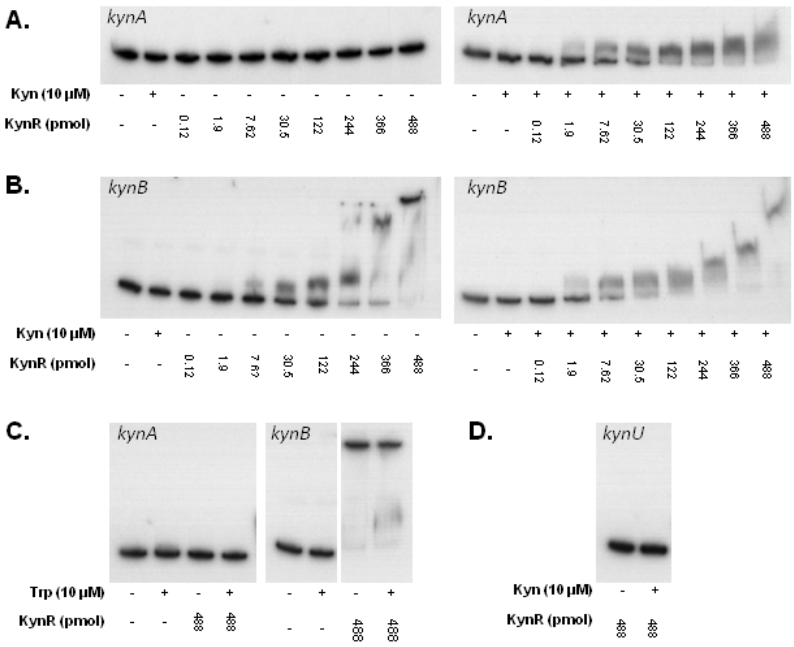

Figure 2.6: KynR binds to the *kynA* and *kynB* promoter regions. A. KynR was added to [γ-P^{32}]*kynA* in both the presence and absence of 10 µM kynurenine (kyn). B. KynR was added to [γ-P^{32}]*kynB* in both the presence and absence of 10 µM kynurenine. C. KynR was added to [γ-P^{32}]*kynA* and [γ-P^{32}]*kynB* in both the presence and absence of 10 µM tryptophan (trp). D. KynR was added to [γ-P^{32}]*kynU* DNA fragment in both the presence and absence of 10 µM kynurenine. The total amounts of KynR are indicated below each lane. Total binding reaction mixtures were electrophoresed on nondenaturing 6% polyacrylamide gels. Gels were dried, and overlaid X-ray film was exposed for 2 days before being developed.

We also added tryptophan in separate binding reactions to determine if it had an effect on the ability of KynR to bind DNA. As a non-specific binding control, an internal *kynU* fragment was radiolabeled and utilized in binding reactions with KynR and kynurenine. The results of all EMSAs are shown in Figure 2.6. These data indicated that KynR only bound to the *kynA* promoter in the presence of kynurenine (Figure 2.6A, right panel), and KynR is unable to bind *kynA* in the absence of a coinducer (Figure 2.6A, left panel). Additionally, tryptophan had no effect on the ability of KynR to bind to the *kynA* promoter (Figure 2.6C, left panel). In contrast to *kynA,* the *kynB* promoter was bound by KynR in both the presence and absence of kynurenine (Figure 2.6B). This binding was not affected by tryptophan (Figure 2.6C, right panel) and indicated that the *kynA* and *kynB* promoters are recognized by KynR in different manners. It should also be noted that in the presence of kynurenine, an observable shift with *kynB* occurred with 1.9 pmol of KynR (Figure 2.6B, right panel) while 7.62 pmol KynR was needed for a *kynB* shift to occur in the absence of kynurenine (Figure 2.6B, left panel). This showed that KynR has a greater affinity for the *kynB* promoter in the presence of kynurenine. Furthermore, when compared to the interaction with *kynA,* the binding of KynR to *kynB* exhibited a more complex pattern of DNA migration retardation as the protein concentration increased. These results suggest that the binding of KynR-kynurenine to the *kynA* promoter represented the binding of a single complex to the DNA, and that multiple KynR complexes, with or without a kynurenine coinducer, most likely interact with the *kynB* promoter. Taken together, these data indicated that KynR bound to the *kynA* promoter in a kynurenine-dependent manner, while the interaction of KynR with

the *kynB* promoter occurred both in the presence and absence of kynurenine in a less constricted manner.

2.5 DISCUSSION

We have previously shown that the transcription of both *kynA* and *kynB* was induced in the presence of kynurenine (37). This led us to believe that a transcriptional regulator was responsible for the induction of the kynurenine pathway genes in the presence of kynurenine. With this in mind, we identified KynR as the transcriptional regulator for the kynurenine pathway. The *kynR* gene is divergently transcribed from *kynB,* and is found in the same genomic context in other Gram-negative bacteria with kynurenine pathway genes (Figure 2.1). To begin to characterize the role of KynR on the expression of the kynurenine pathway genes, we first determined that *P. aeruginosa* required *kynA, kynB,* and *kynU* to grow on tryptophan as a sole carbon source (Figure 2.2). This phenotype provided a testable method to determine if KynR was important for expression of the kynurenine pathway. When the *kynR* mutant was grown in TSCM, it was unable to utilize tryptophan as a sole carbon source, thereby indicating that KynR was involved in tryptophan degradation and leading us to explore its role as the regulator of the kynurenine pathway genes.

Due to the similar phenotypes of the kynurenine pathway mutants and *kynR* mutants when grown on tryptophan as a sole carbon source, we felt that this regulator would probably affect PQS production. We have previously shown that the kynurenine pathway is the main source of anthranilate for PQS production in rich media and that *kynA* and *kynU* mutants produce no detectable PQS, while *kynB* mutants produce trace amounts of PQS (37). To our surprise, the data indicated that a mutation in *kynR*

caused only a partial decrease in PQS production (Figure 2.3). This was probably due to the fact that we have mutated a pathway regulator as opposed to a structural gene (i.e. *kynA, B,* or *U*) that directly acts to breakdown tryptophan. We also found that the addition of kynurenine to the *kynR* mutant resulted in a wild-type level of PQS production, which means that *kynBU* was at least partly induced in the absence of KynR. This was explained by showing that *kynB* is positively regulated by LasR (Figure 2.4), indicating that multiple regulators control this gene.

To determine how KynR was effecting the kynurenine pathway genes, we analyzed the transcriptional activity of *kynA* and *kynB* in our *kynR* mutant. Our data showed that both *kynA* and *kynB* were induced only in the presence of both KynR and kynurenine (Fig. 4), thereby implying that kynurenine was acting as a coinducer for KynR. These studies were then taken a step further when we showed that KynR and kynurenine were required and sufficient for the activation of *kynB* in *E. coli* (Figure 2.5). This suggested that the regulation of *kynB* by KynR was direct, and made it clear that the regulation of the kynurenine pathway in *P. aeruginosa* differs from tryptophan degradation pathways in other bacteria where the expression of the pyridoxal phosphate-dependent tryptophanase is post-transcriptionally regulated through attenuation (134). Taken together, the data we accumulated suggested that the kynurenine pathway is directly regulated in a positive way by KynR in a kynurenine-dependent manner.

To explore this potential direct regulation, we utilized purified KynR in DNA binding assays to study its interaction with the *kynA* and *kynB* promoters (Figure 2.6). These studies showed that KynR bound to the *kynA* promoter only in the presence of

kynurenine, and that it would bind to the *kynB* promoter in the presence or absence of kynurenine. The addition of kynurenine did cause KynR to have a higher affinity for the *kynB* promoter, but the ability to bind *kynB* in the absence of kynurenine was interesting and not entirely unexpected. Ligand independent binding by Lrp/AsnC-type regulators is typically observed when the regulator and target gene are divergently transcribed and adjacent to each other, as is the case with *kynB* and *kynR* (8). Such genetic organization often results in decreased transcription of either the regulator or both the regulator and target gene (8), but we have not demonstrated this for *kynB* or *kynR*.

Another interesting observation from the EMSA experiments was the differences that were observed between the binding complexes that KynR formed with the *kynA* and *kynB* promoter regions. The *kynA*-KynR-kynurenine complexes migrated the same regardless of the KynR concentration, while the *kynB*-KynR complexes had different migration rates with increasing concentrations of KynR (with and without kynurenine present). This type of migration pattern could be the result of KynR binding to multiple sites within the promoter region. Multiple binding sites, as well as DNA bending, have been reported in several Lrp/AsnC-type regulators in *E. coli* and other bacteria (24, 39, 54, 124, 125). In addition, Lrp/AsnC type transcriptional regulators can bind as multimers both in the presence and absence of a ligand and are capable of acting as both activators and repressors (8, 135), so this seems to be the most likely explanation for the results seen with the *kynB* promoter binding assays.

Overall, our findings show that tryptophan degradation in *P. aeruginosa* is upregulated by a positive feedback mechanism and suggests that the pathway would be activated in tryptophan-rich environments. To help understand this, we have included a

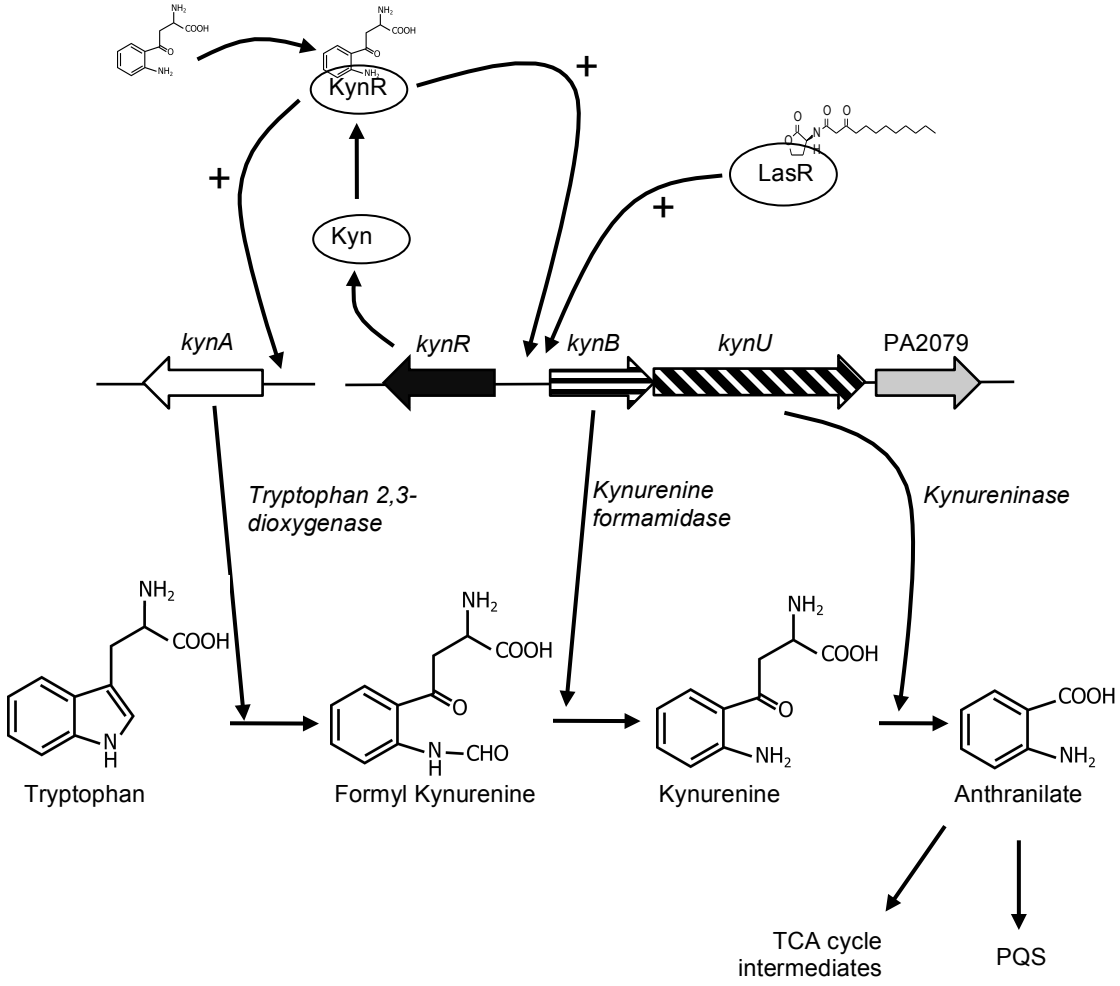

Figure 2.7: Proposed model for KynR regulation and the kynurenine biosynthetic pathway. In this model tryptophan is degraded via three separate enzymes and made into anthranilate. Anthranilate can either be used for the production of 4-quinolones such as PQS or is broken down into TCA cycle intermediates. KynR interacts with kynurenine to become a transcriptional activator for both *kynA* and *kynBU*.

proposed model of tryptophan degradation by *P. aeruginosa* (Figure 2.7). This model incorporates our previous results which showed that the kynurenine pathway is a major source of anthranilate for PQS production in the presence of tryptophan and tryptophan-breakdown products (37). Similarly, Chugani and Greenberg (17) demonstrated that the kynurenine pathway was necessary for the expression of *catB*, a gene encoding an enzyme involved in anthranilate catabolism. These findings suggest that the kynurenine pathway could be active in amino-acid rich environments, such as in the CF lung (2, 90, 115). While one study found only 10 μM free tryptophan in CF sputum (90), it is well known that CF sputum also contains increased amounts of protein (3, 52, 87, 117). *P. aeruginosa* is well-known to utilize proteases during infections within the CF lung (48, 115) and these enzymes could readily liberate tryptophan from host or bacterial proteins found in sputum. The ability of *P. aeruginosa* to catabolize tryptophan via the kynurenine pathway would provide a good source of both carbon for growth and anthranilate for the production of 4-quinolones (and many other secondary metabolites) during human infections (37). Whether the kynurenine pathway is providing anthranilate for the production of PQS or for nutrient acquisition through anthranilate catabolism, this pathway provides a unique tool for *P. aeruginosa* to regulate virulence through PQS or survival through carbon and nitrogen source acquisition.

CHAPTER THREE: A CONSERVED SILENT MUTATION IN A TRYPTOPHAN AUXTOTROPH RESULTS IN DYSREGULATION OF *PSEUDOMONAS* QUINOLONE SIGNAL SYNTHESIS

3.1 Summary

Pseudomonas aeruginosa is a common nosocomial pathogen that relies on three cell-to-cell signals to regulate multiple virulence factors. The *Pseudomonas* quinolone signal (PQS, 2-heptyl-3-hydroxy-4-quinolone) is one of the three intercellular signals and it has been shown to be important for pathogenesis of *P. aeruginosa.* PQS is synthesized by the condensation of anthranilate and a β-keto fatty acid. In *P. aeruginosa* anthranilate is produced via the kynurenine pathway and the two separate anthranilate synthases, TrpEG and PhnAB. The anthranilate synthase PhnAB is important for the synthesis of PQS and is regulated similarly to the PQS biosynthetic operon, where PQS acts as a co-inducer for the transcriptional regulator PqsR. In a series of papers by Essar *et al.* describing the anthranilate synthases in *P. aeruginosa,* they discovered that a tryptophan auxotroph was able to grow on tryptophan deplete media with a frequency of 10^{-5} to 10^{-6} and revealed that the tryptophan auxotrophy revertant produced increased pyocyanin and exhibited constitutive levels of *phnA* transcript (35, 36). Our lab made several tryptophan auxotrophy revertants and found that the source of the reversion mutation was a silent G to A nucleotide conversion in the 3' end of *pqsC.* The single nucleotide change resulted not only in increased pyocyanin and *phnA* transcript, but also a three-fold increase in *pqsD* and *pqsE* transcript. Through Northern blots, we identified a unique transcript in the tryptophan auxotroph revertant that corresponds to the size of *pqsD* and *pqsE.* To determine if the

single nucleotide change resulted in the transcription of *pqsD* and *pqsE* from a separate promoter, we constructed reporter fusions with the *pqsD* upstream region containing wild-type sequence or the reversion we identified. The single nucleotide change resulted in a 40-fold increase in activity from the reporter fusion when compared to the wild-type fusion. These results suggest a new or dysregulated transcript occurs in the PQS biosynthetic operon that allows for the constitutive transcription of *pqsD, pqsE,* and *phnAB,* which would provide for PQS-independent regulation of virulence factors and production of anthranilate for basic cellular functions.

3.2 Introduction

The Gram-negative bacillus *Pseudomonas aeruginosa* is a common nosocomial pathogen that causes devastating opportunistic infections in immunocompromised individuals and chronic infections in cystic fibrosis (CF) patients (10, 72, 112). *P. aeruginosa* is notoriously resistant to multiple classes of antibiotics making many infections difficult to treat. The ability of *P. aeruginosa* to evade the immune system and cause these serious infections can be attributed to the numerous virulence factors employed by the bacteria. Many of these virulence factors are controlled by the three known cell-to-cell signals. The three known signals compose a complex communication network that ensures for precise expression of many genes in *P. aeruginosa*. Two of the cell-to-cell signaling systems are acyl-homoserine lactone signals that are regulated by their cognate transcriptional regulators LasR and RhlR (42, 92). Together these two signaling systems regulate 6 to 11% of the *P. aeruginosa* genome (107, 122). The third signal is the *Pseudomonas* quinolone signal (PQS, 2-heptyl-3-hydroxy-4-quinolone)

(95). PQS acts as a coinducer for the transcriptional regulator PqsR, which drives the transcription of the PQS biosynthetic gene locus and positively regulates multiple virulence factors (33, 41, 64, 75, 85, 88). PQS has also been shown to be important to *P. aeruginosa* pathogenesis in *Caenorhabditis elegans, Drosophila melanogaster,* lettuce leaf, and mouse infection models (28, 41, 64, 101, 130). Lastly, PQS has been extracted from the lungs and sputum of infected CF patients, further suggesting its importance in *P. aeruginosa* infections (19).

With the overwhelming evidence for the role PQS has in virulence and pathogenesis, our lab has set out to understand both the regulation of the genes required for PQS production and the biosynthesis of PQS. The biosynthetic genes required for PQS synthesis, *pqsABCD* and *pqsH,* are also responsible for the production of at least 56 other 4-hydroxy-2-alkylquinolines (HAQs) (67, 80). The biosynthesis of all of the HAQ's depends on the condensation of a β-keto fatty acid and anthranilate (5, 18). The precursor anthranilate is an important branch-point metabolite in *P. aeruginosa* that can either be synthesized into PQS or the amino acid tryptophan, as well as catabolized into tricarboxylic acid cycle intermediates (86). The cellular pool of anthranilate in *P. aeruginosa* is maintained via three separate pathways (37). The first pathway is the kynurenine pathway, which catabolizes tryptophan into anthranilate, and is the main source of anthranilate for PQS production (37). This pathway is regulated by KynR, which upregulates the pathway genes in the presence of kynurenine (59), as well as through direct regulation by LasR (43, 59). Anthranilate is also supplied by the two anthranilate synthases TrpEG and PhnAB, which convert chorismate into anthranilate. TrpEG catalyzes the first step in tryptophan synthesis and is regulated by

tryptophan through attenuation and post-translational competitive inhibition of TrpE (100, 116). Though this enzyme could provide anthranilate to the cellular pool, previous data from our lab suggests that TrpEG is unable to provide anthranilate for PQS production (37). PhnAB is the secondary anthranilate synthase in *P. aeruginosa* and was first described by Essar *et al.* (35). PhnAB is encoded directly downstream from the PQS biosynthetic operon and is important for the production of anthranilate for PQS biosynthesis (37, 80). The transcriptional regulation of *phnAB* is similar to the regulation of the PQS biosynthetic locus (13, 121). The positive influence of PqsR on *phnAB* expression has been noted in both microarray and transcriptional fusion results (13, 29). Also, a truncated, recombinant PqsR was shown to bind to the *phnA* promoter, but a PqsR binding site has yet to be identified (13). The influence of LasR, QscR, and RhlR on *phnA* is similar to the regulatory effects that these three transcription factors have on the PQS biosynthetic operon. LasR positively influences *phnAB* expression, but RhlR and QscR negatively effect the expression of *phnAB* (16, 86). Whether the influences of these transcriptional regulators are due to direct regulation or through regulation of PqsR is not known. The transcription of *phnAB* follows that of PQS production, where *phnAB* expression increases during late exponential growth and peaks in stationary phase (13). Due to both the location and regulation of *phnAB,* it was assumed that PhnAB provided the majority of anthranilate for PQS synthesis, yet it was shown to only be a main source of anthranilate for PQS under nutrient limiting conditions (37). With three separate and uniquely regulated pathways to anthranilate, it is clear that production of anthranilate is important to *P. aeruginosa* survival both for basic cellular functions and the production of PQS.

In a series of papers describing both TrpEG and PhnAB, an interesting finding about *phnAB* regulation was revealed (35, 36). When a tryptophan auxotroph was plated on tryptophan deplete media, auxotrophy-negating mutations occurred at a frequency of 10^{-5} to 10^{-6} (35). When one of the revertants was investigated, it was discovered that it had constitutive levels of *phnAB* transcript expression, as opposed to the peak stationary levels exhibited by wild-type *P. aeruginosa* (35). Also, the revertant had increased levels of pyocyanin, a virulence factor influenced by the PQS signaling system. Our lab found the results of the tryptophan auxotroph revertant intriguing and set out to identify the source of the mutation. We made tryptophan auxotroph revertants and performed whole genome sequencing to identify the mutation. In this paper, we describe the mutation and how it uniquely regulates not only *phnAB,* but also other PQS biosynthetic genes.

3.3 Materials and Methods

3.3.1 Bacterial strains, plasmids, and culture conditions

E. coli and *P. aeruginosa* strains were freshly plated to begin each experiment and were maintained in 30% glycerol and 10% skim milk (Difco) at -80°C, respectively. All strains and plasmids used are listed in Table 3.1. Bacteria were cultured at 37°C in Luria Bertani medium (LB) or Vogel Bonner minimal media supplemented with 0.5% glycerol (VBG) (120). Growth of bacterial cultures was monitored spectrophotometrically based on the optical density at 600 nm (OD_{600}) for *E. coli* and OD_{660} for *P. aeruginosa*. To maintain plasmids, 100 µg/ml of ampicillin or 200 µg/ml of

Table 3.1. List of strains and plasmids used in this study.

Strain or Plasmid	Relevant genotype or phenotype	Reference
Strains		
E. coli DH5α	F'/*endA1, hsdR17, supE44 thi-1, recA1, gyrA, relA1,* (*lacZYA-argF) U169, deoR* [Φ80d*lac*Δ(*lacZ*)*M15recA1*	(129)
P. aeruginosa		
PAO1	Wild-type	(53)
PJF-QR1	*pqsR* deletion mutant derived from strain PAO1	(38)
PAΔTrpE	*trpE* deletion mutant derived from strain PAO1	This study
PME-QD1	*pqsD* deletion mutant derived from strain PAO1	(38)
PJF-QE1	*pqsE* deletion mutant derived from strain PAO1	(37)
PJF-PhnA	*phnA* deletion mutant derived from strain PAO1	This study
PAQC-G1041A	G to A mutation in *pqsC* at base pair 1041 derived from strain PAO1	This study
TrpErev1	Selective pressure mutation(s) derived from strain PAΔTrpE*	This study
TrpErev2	Selective pressure mutation(s) derived from strain PAΔTrpE *	This study
TrpErev3	Selective pressure mutation(s) derived from strain PAΔTrpE *	This study
TrpErev4	Selective pressure mutation(s) derived from strain PAΔTrpE *	This study
TrpErev5	Selective pressure mutation(s) derived from strain PAΔTrpE *	This study
Plasmids		
pLP170	*LacZ* transcriptional fusion vector, Amp^r	(98)
pPqsDwtTcLacZ	*pqsD'-lacZ* transcriptional fusion, Amp^r	This study
pPqsDG1041ATcLacZ	*pqsDpqsC*G1041A1041*'-lacZ* transcriptional fusion, Amp^r	This Study
pEX18AP	Suicide vector, Amp^r	(51)
pPAQC-G1041Adel	G to A reversion in *pqsC* suicide vector, Amp^r	This study
p*trpE*del	*trpE* deletion suicide vector, Amp^r	This study
pΔ*pqsD*-suc	*pqsD* deletion suicide vector, Amp^r	(121)
pDSW8	*tacp-pqsR* on pEX1.8, Amp^r	This study
pBluescript II SK+	Cloning vector, Amp^r	Aligent Technologies
pSLM100	*phnA* expression vector, Amp^r	This study

Abbreviations: Amp^r, ampicillin
*See Table 3.3 for the location of mutation(s)

Table 3.2. List of primers used in this study.

Primer use and name	Sequence (5' to 3')[a]
Primers for Mutagenesis	
TrpEF1-HindIII	AAAA**AAGCTT**CCTGATCGATGCCTCCGGTC
TrpEdelF2	TCACCGTTCTTGATCACCGCAGTCGGCAAGGGTCTCGA
TrpEdelR1	TCGAGACCCTTGCCGACTCGCGGTGATCAAGAACGGTGA
TrpER2-HindIII	AAAA**AAGCTT**ACCGGCAACAGCATGGAAC
PqsCUp1	AAAA**AAGCTT**AGATTCCCTCGCACATGCTG
PqsCDown2	AAAA**AAGCTT**ACCTCCTCAGGTTTGCGGTA
PqsD UP-O	AAA**AAGCTT**TGGCAATTCTGGGGCGTC
PqsD UP-I	GTCCGGCCAGAACATCGCGTTTCGGCAGGCTGAA
PqsD DOWN-O	TTCAGCCTGCCGAAACGCGCGATGTTCTGGCCGGAC
PqsD DOWN-I	AAAA**AAGCTT**TCGCCGCAATGGATGTCC
Expression plasmids	
pqsClacZfwd	AAAAA**GAATTC**CCGACCGGCGAGGCCAA
pqsClacZrev	AAAA**AAGCTT**TGCTGACCTGGCGTTTCGGC
phnAXba1up	TTT**TCTAGA**TGATGACCTGTGCCTGTT
phnAHindIIIdown	TTT**AAGCTT**GTGCAATGCCTCCCTCGA
EMSA	
phnA5fwd	TGCCGACCCTGCTGCAACTG
phnA5rev	CGGGCAGGAAGTCGACGCTC
phnA8fwd	CTGAGCGAGGAGCTGCACCG
phnA8rev	TCGCCGCAATGGATGTCCCG
phnA9fwd	AGGCGCTGCCTCTGGACTGA
phnA9rev	CAACCCCGCTCACGAGCCAC
phnA10fwd	CAGCGGTCGCAATTGGGGGA
phnA10rev	GGCCATGCGCCAGATCGACA
pqsAfwd	TACGAAGCCCGTGGTTCT
pqsArev	TGGGCGAAGCGCGATATT
EMSApqsCDfwd	GGCGATCGTATCGTCATGGC
Real-Time PCR	
RPLU RT1	GGTGGCAAGCAGCACAAAGTCACCG
RPLU RT2	GCGGACCTTGTCGTGACGGCCGTGG
*pqsA*RTF1	GCCCTTTGCTCGACGATTTCTCG
*pqsA*RTR2	AACCCGAGGTGTATTGCAGGAAACA
*pqsC*RTF1	CGAGTCCTGGTGGCAATTCT
*pqsC*RTR1	TCAGCATGTCCACGCTATCC
*pqsD*RTF1	GTGGATCGTCTGGGCAACAT
*pqsD*RTR1	CTCCTCAGGTTTGCGGTACA
*pqsE*RTF1	TGATGACCTGTGCCTGTTGG

*pqsE*RTR1	GTCGTAGTGCTTGTGGGTGA
*phnA*RTF1	ACGTGGACAGCAAGGCTGCG
*phnA*RTR1	GGGCCGGACAGTCCTCGCTC
Northern Blot	
NorthphnAFwd2	AGGAAGAGTCGCATGCGGAC
NorthphnARev2	GACCTGGTAGTTGCCGGCCCGTACG
NorthphnARev4	GATGTCCAGCAGCAACTCCG
NorthpqsDFwd1	CGCTATCACGTCGAGCCGGAAC
NorthpqsDRev1	GCGAACCTGTCGTGGAGCG
NorthpqsDFwd2	GCGAGGTGCTGTCCAAGCG
NorthpqsDRev2	CGCAGGACATTCTCGTCGAGG
NorthpqsEFwd1	AGGTGCCGGTGTTCCTGCTG
NorthpqsERev1	GGACCTGTACGTTCGGCAGC
NorthpqsEFwd2	GTGGTCGAGCGCTTGAACCG
NorthpqsERev2	TCGAACTCGCCCAGGGCATC
Primer Extension	
PE*pqsD*1	AGGATCGGATTACCCATGTGCAG
PE*pqsD*3	ATGTGCAGCCCTCCTCGGACA
PE*phnA*1	AACCCCGCTCACGAGCCACC
PE*phnA*2	AGCCACCTCCTCGCGCCCAT
PE*phnA*3	AGTCGAACAGCATCCGGTTGGC
PE*phnA*4	CGATCGCCGCAATGGATGTCCC
PE*phnA*5	GCGCAACGGCATGTTCATCATCGG
PE*phnA*6	AGAGAATCTCCAGCATCCGGCGC
PE*phnA*7	AGGAAGTCGACGCTCTGCCC
PE*phnA*8	AGAGCAACCGCCGGCACA
Reverse-Transcriptase PCR	
E1	CCACGGCCACAGCGACGATCA
E2	CCAGACTTTCTTCCAGTCGATAGC
F1	(80)
F2	(80)

[a]Restriction sites indicated by bold type.

carbenicillin was added when appropriate. Also, 10 mM L-tryptophan was added to VBG cultures when indicated.

Generation of a transcriptional fusion for *pqsD,* a 499 bp DNA fragment, extending from begins 441 bp upstream of the *pqsD* start codon (ATG) to 58 bp downstream of the ATG, was amplified by PCR using chromosomal DNA as a template from either strain PAO1 or TrpErev1. The oligonucleotide primers used for the amplification contained either an EcoRI (pqsClacZfwd) or a HindIII (pqsClacZrev) restriction site (Table 3.1). The amplified fragment was digested with EcoRI and HindIII, purified from an agarose gel, and ligated into pLP170 digested with the same enzymes. This resulted in the reporter plasmids pPqsDwtTcZ and pPqsDG1041ATcZ. Both fusions were sequenced to ensure that mutations did not occur during cloning. Plasmids were transformed into *P. aeruginosa* strains by electroporation (33).

The plasmid pSLM100 was used as a sequencing template for *phnA* primer extensions. The DNA fragment, which begins 993 bp upstream from the ATG and 1000 bp downstream from the stop codon, was created by PCR using strain PAO1 chromosomal DNA as a template. The primers used are noted in Table 3.2 and added an XbaI to the 5' end and a HindIII to the 3' end for the PCR product. The DNA fragment and pBluescriptIIsk+ were digested with the two restriction enzymes and ligated together.

3.3.2 Generation of mutants

A splicing by overlap extension protocol was used to generate mutant alleles for both the *trpE* and *pqsD* deletion strains (126). The mutations were constructed to contain in-frame deletions in the coding DNA sequences corresponding to amino acids

26 to 445 for *trpE* (85% of protein sequence) and 16 to 298 for *pqsD* (83% of protein sequence). Primers were designed to contain approximately 1 kb of DNA both upstream and downstream from the splice junction and each primer added a HindIII restriction site to both ends of the PCR product. Both PCR fragments and pEX18Ap (suicide vector) were digested with HindIII and ligated together to make the suicide vectors p*trpE*del and pΔ*pqsD*-suc. To generate the PAQC-G1041A mutant, a PCR fragment was created using TrpErev1 and the primers listed in Table 3.2. The PCR fragment was digested with HindIII and then ligated into similarly digested pEX18AP. The resulting plasmids, p*trpE*del, pΔ*pqsD*-suc and pPAQC-G1041A, were transformed into strain PAO1 by electroporation (15). Mutants were selected for by plating transformants on a medium containing carbenicillin and then on a medium containing 6% sucrose to remove the vector sequence (51). PCR was used to screen colonies and DNA sequencing of PCR products was used to confirm mutants.

Tryptophan auxotroph revertants were made through selective pressure on media deplete of tryptophan. The tryptophan auxotroph strain PAΔTrpE was grown overnight in VBG supplemented with 10 mM L-tryptophan at 37°C with ≥180 rpm shaking. Overnight cultures were washed with fresh, unsupplemented VBG and 100 µL of washed culture was added to 10 ml of unsupplemented VBG. The culture was grown for 3 h at 37°C with ≥180rpm. After 3 h of growth, cells were harvested at 6,000 x g for 10 min. The cell pellet was resuspended in 1 ml of VBG and then plated onto VBG plates. The random mutations were then identified through Illumina sequencing and bioinformatics performed by the Genomic Sciences Laboratory at North Carolina State University.

3.3.3 PQS production

Washed cells from overnight cultures were used to inoculate 10 ml cultures of LB or VBG to an OD_{660} of 0.05. After 24 h of growth, 300 µl samples of each culture were extracted with 900 µl of acidified ethyl acetate as previously described (20). One-half of the resulting organic phase was evaporated to dryness at 37°C and 50 µl of 1:1 acidified ethyl acetate:acetonitrile was used to reconstitute the extract (19). Samples were analyzed by thin-layer chromatography, visualized by long-wave UV light, and photographed (20).

3.3.4 RNA isolation

For all RNA extractions the cultures were grown at 37°C in either LB or VBG media until an OD_{660} of 0.2 or 0.6 was reached. At that point total RNA was isolated using multiple methods. RNA for RT-PCR and real-time PCR was purified using the RNeasy mini spin columns (Qiagen). RNA for the northern and primer extension experiments was either isolated using a RNeasy midi column (Qiagen) or through Trizol isolation (Invitrogen). For Trizol isolation, RNAprotect (Qiagen), two times the volume of the culture, was added and allowed to incubate at room temperature (RT) for 5 minutes. The cells were harvested by centrifugation at 6,000 x g for 5 minutes. The cells were then suspended in lysis buffer (30 mM Tris pH 8.0, 1 mM EDTA, 10 mg/ml Lysozyme, and 2 mg/ml Proteinase K) and allowed to react for 5 min at RT. Then 2.5 times the culture volume of Trizol was added to the lysed cells and incubated for 5 minutes at RT. After incubation, 0.2 volumes of chloroform was added, vigorously shaken, and then incubated at RT for 2 to 3 minutes. The mixture was centrifuged at 12,000 x g for 15 minutes at 4°Cj and the aqueous phase was removed. Next, 0.5 vol of isopropanol was

added to the aqueous phase and this was centrifuged at 12,000 x g for 20 minutes at 4°C to pellet RNA. The pellet was washed with 75% EtOH-DEPC and centrifuged for 15 minutes at 12,000 x g at 4°C. RNA pellets were dried at 37°C and resuspended in DEPC-treated water. All RNA samples, regardless of the purification method, were treated with RQ1 DNase (Promega) for 2 hours at 37°C. To remove RQ1 DNase, phenol/chloroform extraction was performed, followed by an ethanol precipitation. RNA was stored in DEPC-treated water at -80°C.

3.3.5 Real-Time RT-PCR (qRT-PCR)

cDNA synthesis was performed on a Mastercycler (Eppendorf) using 2.5 µg of DNase-free RNA. The cDNA was made using Superscript III (Invitrogen) following manufactures instructions. Primers to generate cDNA were Random Primers (Invitrogen), as well as Random Hexamers 72% GC (Gene Link). qRT-PCR was performed on a CFX96 (BioRad) using the PerfeCTa SYBR Green FastMix for iQ (Quanta Biosystems). The qRT-PCR reactions contained 5 µl of cDNA diluted (1:500) in a 15 µl volume. Relative transcript levels were determined using the Pfaffle method by comparing experimental gene expression (*phnA, pqsA, pqsC, pqsD,* or *pqsE)* to the control gene *rplU* (97). Standard curve and efficiency values were determined by using gene-specific primers with 10 ng to 0.1 pg of RNA-free genomic DNA from strain PAO1. A melting curve step was included at the end of each PCR to ensure that nonspecific variation between samples, as well as the absence of primer dimers did not occur. Each qRT-PCR was performed on at least three biological replicates of each RNA sample with technical replicates performed in duplicate.

3.3.6 RT-PCR

For reverse transcriptase-PCR, the AccessQuick Kit from Promega was used. 100 ng of PAO1 chromosomal DNA was used as a positive control and 300 ng of RNA was used in all experimental samples. Two biological RNA samples per strain were used to verify results. Primers utilized in the assay are noted in Table 3.2.

3.3.7 Northern Blot

RNA was purified from strain PAO1, PJF-PhnA, PME-QD1, PJF-QE1, and TrpERev1 from cultures grown in VBG at 0.6 OD_{660} as stated previously. RNA (30 µg of sample RNA and 5 µg Ambion millennium marker) was electrophoresed on a 6% formaldehyde-1X DEPC-MOPS -1% agarose gel at 100V for 10 minutes followed by 35V overnight. Samples were photographed under UV light. Downward alkaline transfer onto a BrightStar-Plus membrane (Ambion) was used to transfer the RNA from the gel to the membrane according to manufactures instructions. RNA was crosslinked by exposing the membrane to UV light. DNA fragments for Northern probes were synthesized by PCR using strain PAO1 chromosome as a template and then were labeled with [α-P^{32}] CTP (Pierce) using the Prime-a-Gene system (Promega). Hybridization of the labeled probes to the membrane was done according to the manufactures protocol (BrightStar-Plus membrane, Ambion). The membranes were then exposed to film.

3.3.8 Primer Extension

RNA was purified as stated previously. Primers used for primer extension are listed in Table 3.2. Primers were radiolabeled using [γ-^{32}P]ATP (Pierce) and T4 polynucleotide kinase (Invitrogen). A sequenase Cycle Sequencing Kit (USB Thermo)

was used according to the manufacturer's instructions to sequence DNA using pSLM100 (for *phnA)* and p*pqsC*G1041Adel (for *pqsD)* as template. The only deviation from the protocol is ddNTPs from USB Affymetrix were used instead of those included in the kit. For the extensions, between 30-80 µg of total RNA was used, primers were annealed, and Superscript III Reverse Transcriptase (Invitrogen) was used to extend each primer. The sequencing and extension reactions were electrophoresed as stated previously (94).

3.3.9 β-galactosidase (β-gal) assays in *P. aeruginosa*

Cells from overnight cultures of *P. aeruginosa* grown in VBG were washed and resuspended in fresh medium to an OD_{660} of 0.05 and incubated at 37°C with shaking at ≥180 rpm for 6 h. At that time either water or 1 mM L-kynurenine was added and cultures continued to grow at 37°C with shaking at ≥180 rpm for an additional 18 h. The cells were collected by centrifugation at 12,000 x g for 2 min, resuspended in fresh VBG, and assayed for β-gal activity in duplicate. Data are presented in Miller units as the mean ± the standard deviation (σ^{n-1}) of at least three separate experiments.

3.3.10 Overproduction and Purification of PqsR.

Plasmid pDSW8 in *E. coli* strain DH5α (Table 3.1) was used for over-expression of PqsR. Culture medium (500 ml LB with 100 µg/ml ampicillin) was inoculated with 10 ml overnight culture and grown on a rotary shaker at 250 rpm at 37°C to 0.6 $OD_{600\ nm}$. Cultures were cooled to room temperature and IPTG was added to a final concentration of 0.5 mM. Growth was continued at 25°C on a rotary shaker for 2 h. Cells were harvested by centrifugation at 4°C, washed once with phosphate-buffered saline, and stored at -20°C.

For protein purification, cell pellets (~2.5 g) were resuspended in 20 ml TES buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 mM DTT) containing 0.02% Tween 20 and 10 µl/ml HALT protease inhibitor (Thermo Scientific) and passed twice through a French pressure cell at 12,000 lb/in^2. Following incubation on ice for 30 min, cell debris was removed by centrifugation at 10,000 x g for 30 min. The supernatant was removed and the protein concentration estimated by measuring absorbance at 280 nm. The sample was adjusted to a protein concentration of 2 mg/ml by addition of TES + 0.02% Tween 20. Solid ammonium sulfate was then added to 35% saturation. After incubation at 4°C for 30 min, the suspension was centrifuged at 10,000 x g for 30 min. The pellet was resuspended in 25 ml Buffer A (20 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 MM DTT, 10% glycerol) and dialyzed 3 h or overnight against the same buffer. The dialysate was centrifuged for 10 min to remove any precipitate and applied to a Buffer A-equilibrated heparin agarose column (4.6 mm x 5.5 cm) at a flow rate of 0.5 ml/min. The run-through was reapplied to the column once and the column was then washed with Buffer A until the $A_{280\ nm}$ reading reached a stable baseline. Proteins were then eluted with a 150 ml linear gradient of 0 – 1 M NaCl in Buffer A. Fractions of ~3.5 ml volume were collected and the presence of PqsR was determined by SDS-PAGE and mobility shift assays. PqsR eluted at approximately 0.5 M NaCl. Samples were stored at -20°C.

3.3.11 Electrophoretic Mobility Shift Assays (EMSA).

Labeled probes were prepared by ϒ-^{32}P-ATP / T4 polynucleotide kinase labeling of PCR amplified promoter fragments (Table 3.2 , note that PEpqsD1 was used with EMSApqsCDFWD to make the *pqsCD* probe). For all promoter fragments strains PAO1

chromosome was used as template, with the exception of the G1041A mutated *pqsD* promoter in which chromosomal DNA from strain TrpERev1 was used. EMSA was performed on 6% polyacrylamide gels (5% crosslinker) containing 2.5% glycerol and running buffer containing 25 mM Tris, 0.19 M glycine, and 1 mM EDTA. Gels were pre-run at 4°C for 30 min before loading. Binding reactions (15 µl) contained 1 nM ^{32}P-labeled probe, 100-200 ng purified PqsR, 20 mM Tris·HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 100 µg/ml acetylated BSA, 10 µg/ml poly(dIdC) and 5% glycerol. Samples were incubated for 20 min at 25°C and then loaded. Electrophoresis was conducted at 4°C at 10 V/cm and then gels were soaked in 10% methanol – 5% acetic acid for 15 minutes, blotted dry, covered with plastic wrap; and exposed to X-ray film.

3.4 Results

3.4.1 A conserved mutation rescues a tryptophan auxotroph.

P. aeruginosa possesses two anthranilate synthases, TrpEG and PhnAB. The existence of two anthranilate synthases, as well as the kynurenine pathway, ensures that the cellular pool of anthranilate can be maintained for tryptophan synthesis, PQS production, and the use of anthranilate as a carbon and nitrogen source. In the series of papers describing both TrpEG and PhnAB, Essar *et al.* discovered that a tryptophan auxotroph reverted to tryptophan prototrophy at a high frequency (10^{-5} to 10^{-6}) when grown on tryptophan deplete media (35, 36). The tryptophan auxotroph revertant exhibited increased pyocyanin and constitutive levels of *phnAB* transcript, which was dysregulated to provide anthranilate for the synthesis of tryptophan and ensure survival

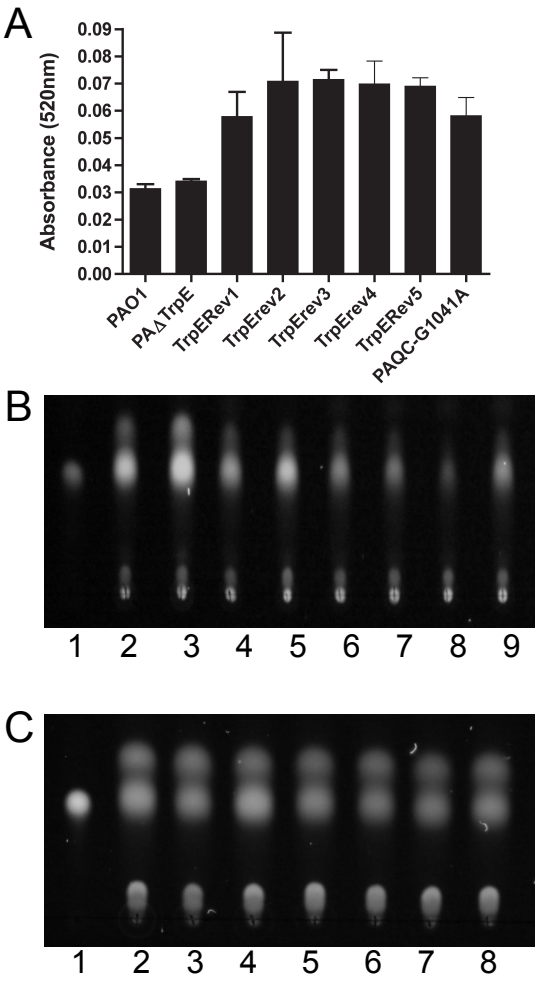

Figure 3.1 Under tryptophan starvation, a mutation in a tryptophan auxotroph results in increased pyocyanin and decreased PQS. (A) Pyocyanin production after 18 h growth in LB. Results shown are the mean ± σ^{n-1} of results from duplicate assays from at least three separate experiments. (B and C) Ethyl acetate extracts from cultures grown in LB (B) and VBG (C) after 24 h were analyzed by TLC. Equal volumes of extracts were resolved in each lane. For B: Lane (1) 50 ng of synthetic PQS, (2) strain PAO1, (3) strain PAΔTrpE, (4) strain PAQC-G1041A, and (5-9) strains TrpErev 1,2,3,4, and 5. For C: Lane (1) 50 ng of synthetic PQS, (2) PAO1, (3) PAQC-G1041A, and (4-8) TrpErev 1, 2,3 4, and 5.

(35). The exact revertant strain is no longer available, so our lab selected revertants in the same manner and set out to identify the mutation(s) responsible for the loss of tryptophan auxotrophy. In order to do this we constructed a tryptophan auxotroph (PAΔTrpE) and grew it on minimal media deplete of tryptophan. This was repeated three times and we selected five revertants to analyze. All five tryptophan revertants showed increased pyocyanin as seen by Essar *et al.* (35). up to double that of the wild-type strain PAO1 (Figure 3.1). Pyocyanin is linked to PQS production through the expression of *pqsE,* which is transcriptionally connected to *pqsABCD* and necessary for pyocyanin to be produced (31, 41). It is because of this link that we investigated the production of PQS in each of the tryptophan revertants. All of the revertants made PQS, albeit at a decreased level in both complex (Figure 3.1B) and tryptophan deplete media (Figure 3.1C). The decreased PQS in the tryptophan revertants was more pronounced when extracted from complex media than in the tryptophan deplete media. These results were unexpected because the PQS operon must be expressed to allow for pyocyanin, yet we observed decreased PQS production, which implies that the mutation in the tryptophan auxotroph revertants may also affect the PQS biosynthetic operon.

When analyzing their revertant, Essar *et al.* performed RNA slotblots to show that *phnAB* transcription had increased during exponential phase (35). To determine if our revertants had a similar change in *phnAB* transcript levels, we extracted RNA from the revertants grown in either complex or tryptophan deplete media to an OD_{660} of 0.2 or 0.6 and performed quantitative real-time PCR. These time points were based on the

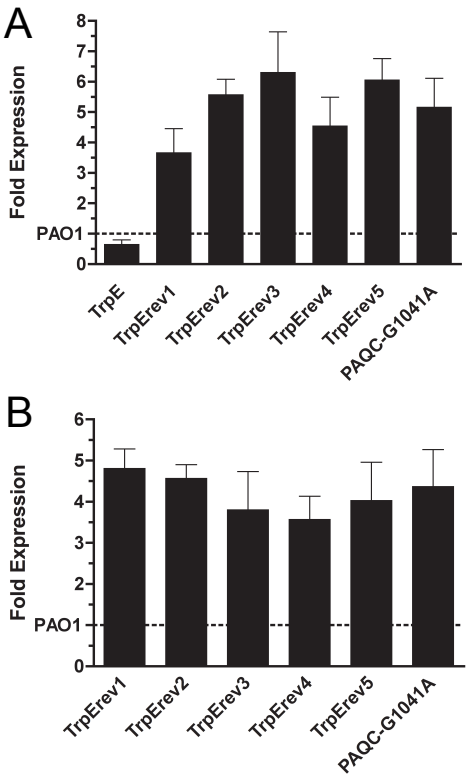

Figure 3.2: The mutation in the tryptophan auxotroph revertants results in increased *phnA* transcript levels. Real-time PCR was used to measure the expression of *phnA* mRNA from the indicated strains grown at 37°C in LB (A) and VBG (B). Wild-type strain PAO1 transcript levels are set at 1, which is indicated by the dashed line. Error bars indicate the standard deviation of three independent experiments performed in duplicate.

slot blot results presented by Essar *et al.,* which showed a respective eight or four fold increase of *phnA* transcript in the revertants compared to wild-type the during early exponential and late exponential phases (35). The real-time data for RNA extracted at OD_{660} 0.2 are shown in Figure 3.2 A and B. The results indicate that in either media the *phnA* transcript is greater in our revertants. As a control, *phnA* expression in the tryptophan auxotroph strain PAΔTrpE grown in LB is shown in Figure 3.2A. This control was not performed in VBG because this media is deplete of tryptophan and the strain would not have grown without tryptophan supplementation. The results indicated that *phnAB* levels are lower in the *trpE* mutant strain when compared to wild-type strain PAO1 grown in the same media. As stated above, the assay was also performed on RNA isolated from cultures at an OD_{660} of 0.6 in both media, and those results mirrored the data of Figure 3.2 (data not shown). Our real-time data confirmed that each of our tryptophan auxotroph revertants had increased expression of *phnA* during early growth phases and agrees with the data reported by Essar *et al.* (35). Taken together, our data lead us to believe that we are studying the phenomenon first reported by Essar *et al.* and that an unknown regulatory event occurs when cells are starved for tryptophan (35).

To understand what has happened in the revertant stains, the full genome sequence of the first three tryptophan revertants was determined. An Illumina GAIIX at the North Carolina State University Gene Sequencing Laboratory was used to sequence the wild-type strain PAO1 and three revertants (TrpErev1, 2, and 3). The sequencing provided between 5.8 and 7.3 million reads for a high quality map. To determine the location of the mutation(s), the published wild-type strain PAO1 genome from

Revertant Strain	*pqsC* SNP 1041	*ntrC* SNP 1227
TrpErev1	G ➔A	Wild-type
TrpErev2	G ➔A	G ➔A
TrpErev3	G ➔A	G ➔A
TrpErev4	G ➔A	Wild-type
TrpErev5	G ➔A	Wild-type

Table 3.3 Location of the mutation(s) in tryptophan auxotroph revertants.

www.pseudomonas.com was compared to the sequenced lab wild-type PAO1 strain and tryptophan revertant strains. The Burrows-Wheeler Aligner (BWA) was used to align the raw reads and provided the high quality map (68). The *trpE* deletion in each of the revertants was confirmed by using the Integrative Genomics Viewer (IGV) (103, 118). Both Joint Genotyper for Inbred Lines (JGIL) and SAMtools/BCFtools were used to identify single nucleotide polymorphisms (SNP) and the SAMtools/BCFtools were used specifically to identify any insertions and/or deletions (69, 111). The JGIL is designed to look for SNPs in inbred lines, but is appropriate to use for bacteria due to all strains being derived from the same parent strain. The two programs each identified two SNPs shown in Table 3.3 and no insertions or deletions were noted. The SNP at *pqsC* nucleotide 1041 had over 100 depth of coverage and SNP at *ntrC* nucleotide 1227 had over 55 for each of the revertants and wild-type strain PAO1. Once the location of the SNPs were identified the TrpErev4 and TrpErev5 strains were sequenced separately using PCR fragments. The results are shown in Table 3.3. The only SNP that occurred in all five strains was the G to A conversion at basepair 1041 of *pqsC*. Due to the G1041A mutation in *pqsC* occurring in all five tryptophan auxotroph revertant strains and the fact that it is relatively close to *phnA,* we felt it warranted further investigation. The G1041A mutation is two amino acids from the PqsC stop codon and is in the third base of a valine codon, resulting in a silent mutation. There is a 43 base pair intergenic region between the termination of the *pqsC* coding sequence and the start codon of *pqsD,* so this change does not affect the PqsD protein. To ensure that the G1041A mutation in *pqsC* resulted in the effects observed in the tryptophan auxotroph revertants, we created a mutant that contained only the G1041A mutation.

Most interestingly, the G1041A mutation resulted in increased pyocyanin (Figure 3.1A), decreased PQS (Figure 3.1 B lane 4 and C lane 3), and increased *phnA* transcript (Figure 3.2 A and B). This confirmed that a single base change at nucleotide 1041 of *pqsC* caused the observed results and is a mechanism by which *P. aeruginosa* can survive when starved for tryptophan, and probably anthranilate. Our next goal was to try to understand how this single base change allows this to occur.

3.4.2 A silent mutation in *pqsC* results in differential expression within the PQS biosynthetic operon.

The G1041A mutation in *pqsC* affected the production of pyocyanin and PQS so we decided to investigate the transcription levels of the PQS biosynthetic operon genes. We utilized quantitative real-time PCR to look at the transcript levels of *pqsA*, *pqsC, pqsD,* and *pqsE.* The level of *pqsB* transcript was not investigated because we wanted to specifically look at the first gene in the operon (*pqsA)* and the genes that may have been affected by the mutation (*pqsC, D, and E).* As above, RNA was taken at an OD_{660} of 0.2 in both LB and VBG media. The results from the quantitative real-time PCR are shown Figure 3.3 A and B. These data show that both the G1041A mutant (PAQC-G1041A) and the tryptophan auxotroph revertant (TrpErev1) have a greatly decreased level of *pqsA* transcript when compared to the wild-type strain PAO1. The decrease in *pqsA* transcript is likely due to lower PQS levels resulting in less co-inducer for the transcriptional regulator PqsR to drive transcription of *pqsA* (121). The most striking results were that both *pqsD* and *pqsE* transcript levels were increased three fold in both strain TrpErev1 (Figure 3.3A) and strain PAQC-G1041A (Figure 3.3B) when compared to the wild-type strain PAO1. This result is perplexing because previous data suggested

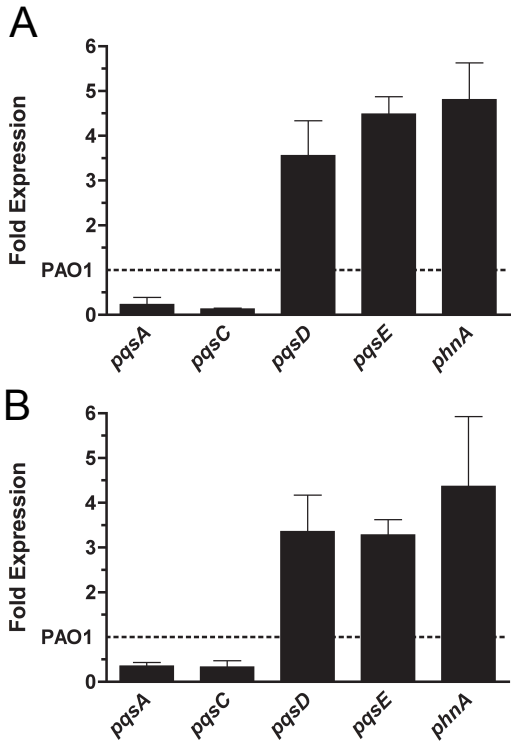

Figure 3.3 The expression of *pqsD* and *pqsE* is increased by the single nucleotide mutation in *pqsC.* Real-time PCR was used to measure the expression of *pqsA, pqsC, pqsD,* and *pqsE* mRNA from strains TrpErev1 (A) and PAQC-G1041A (B) grown at 37°C in VBG to an OD_{660} of 0.2. Wild-type strain PAO1 transcript levels are set at 1, which is indicated by the dashed line. Error bars indicate the standard deviation of three independent experiments performed in duplicate.

that the PQS biosynthetic operon transcript is derived from the *pqsA* promoter and that the transcript included all five genes (80). A recent transcriptome analysis of transcriptional start sites in *P. aeruginosa* strain PA-14 suggested that an alternative start site for the PQS biosynthetic operon was located upstream from *pqsB* (32). This alternative start site could not account for the differential levels in transcript between *pqsA* and *pqsD/E* because the levels of *pqsC* transcript are also significantly decreased in both mutants. All of the real-time results in LB mimicked those of VBG and are not shown. Our results suggest that the G1041A mutation results in (1) the induction of a transcript upstream from *pqsDE* or (2) stabilization of the *pqsDE* transcript.

Due to the increased levels of *pqsDE* transcript, along with *phnA,* it is possible that the mutation has allowed for a new transcript including *pqsDE* and *phnAB.* To first investigate this possibility, we employed reverse transcriptase PCR (RT-PCR)(Figure 3.4 B). The oligionucleotides used for RT-PCR are illustrated in Figure 3.4 A and included a set from the paper by McGrath *et al.,* who had previously used the oligionucleotides to determine that the PQS biosynthetic operon only included *pqsABCDE* and not *phnAB.* The results from the RT-PCR were surprising and contradicted what had been published by McGrath *et al.* The RT-PCR, using four separate oligionucleotides in three different combinations, suggests that there is a transcript between our outermost oligonucleotides, not only in the G1041A mutation strain PAQC-G1041A, but also in wild-type strain PAO1. These results were also confirmed in an independent RNA extraction from each strain. The outermost oligionucleotides (Figure 3.4A, E1 and F2) are 556 nucleotides upstream from the *phnA* start codon (438 nucleotides of the *pqsE* genes) and 468 nucleotides downstream from

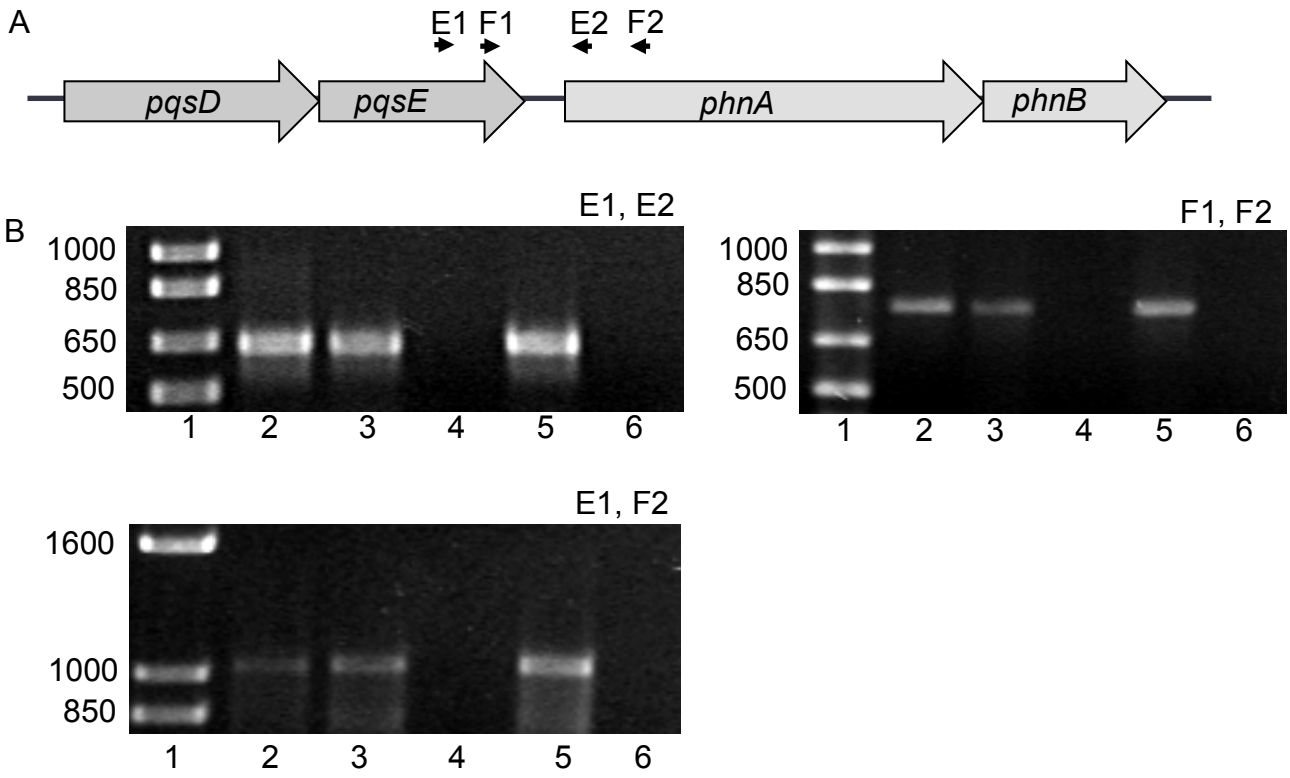

Figure 3.4: Analysis of the *pqsE* and *phnA* transcript by RT-PCR. A.) Diagram indicating the location of the primers (primer sequences are shown in Table 3.2) B.) A 1.0% agarose gel was loaded with products form RT-PCR experiments performed with the indicated oligonucleotide primer pairs shown in A. Lanes 3 and 4 contain total RNA from wild-type strain PAO1 and lanes 5 and 6 contain total RNA from the strain PAQC-G1041A. RNA was isolated from VBG media at an OD_{660} of 0.2. Total RNA was used as a template for experimental reactions (lanes 3 and 5 in each panel). Chromosomal DNA was used as a template for positive control reactions (lane 2 in each panel). For negative controls, reverse transcriptase was omitted from reactions (lanes 4 and 6 in each panel). Lane 1 contains molecular mass standards (1 kb DNA plus ladder, Invitrogen) and relevant standards are marked on the left side of each panel.

the *phnA* ATG. Three possibilities are likely for the RT-PCR results: (1) either *phnA* has a large leader sequence on the transcript, (2) *pqsE* has over a 500 basepair tail on the transcript, or (3) that *pqsE* and *phnAB* are transcribed together. The results are perplexing as a previously reported *phnAB-lacZ* fusion was active and a transcriptional start site would have to be upstream from *phnA* in order to have an active fusion for the bioassay (13). Our lab has also utilized a plasmid that only included the *phnAB* coding region and was able to complement a *phnA* mutant, which would also suggest that there is a separate transcriptional start site for *phnAB* (37). Our lab has attempted to identify a transcriptional start site by utilizing primer extension, as well as other methods, but we have not been successful (data not shown). The only thing that we can conclude from the RT-PCR results is that there is a transcript between the two outermost oligonucleotides, but what is contained on that transcript is unknown.

To help understand the RT-PCR results, we performed Northern blots probing for *phnA, pqsD,* and *pqsE* (Figure 3.5 B). In our Northern blots, we included a negative control using RNA isolated from an isogenic deletion mutant strain for each probe, which ruled out non-specific binding for our probes. For the *phnA* blot (Figure 3.5B, left panel), our results indicate that the *phnA* probe is binding specifically to a transcript that is centered at approximately 2 kilobases (kb). This size would account for the coding regions of *phnAB* together, which is 2174 nucleotides long, but does not account for any mRNA leader or tail. Our blot also suggests that the transcript(s) that contains *phnA* is unstable as the probe bound to transcript(s) extending from above 3.5 kb to below 1 kb (Figure 3.5B, left panel), which indicated that the transcript(s) is at least 3.5 kb. Yet, we can conclude that the G1041A mutation did not result in a notable change in the *phnA*

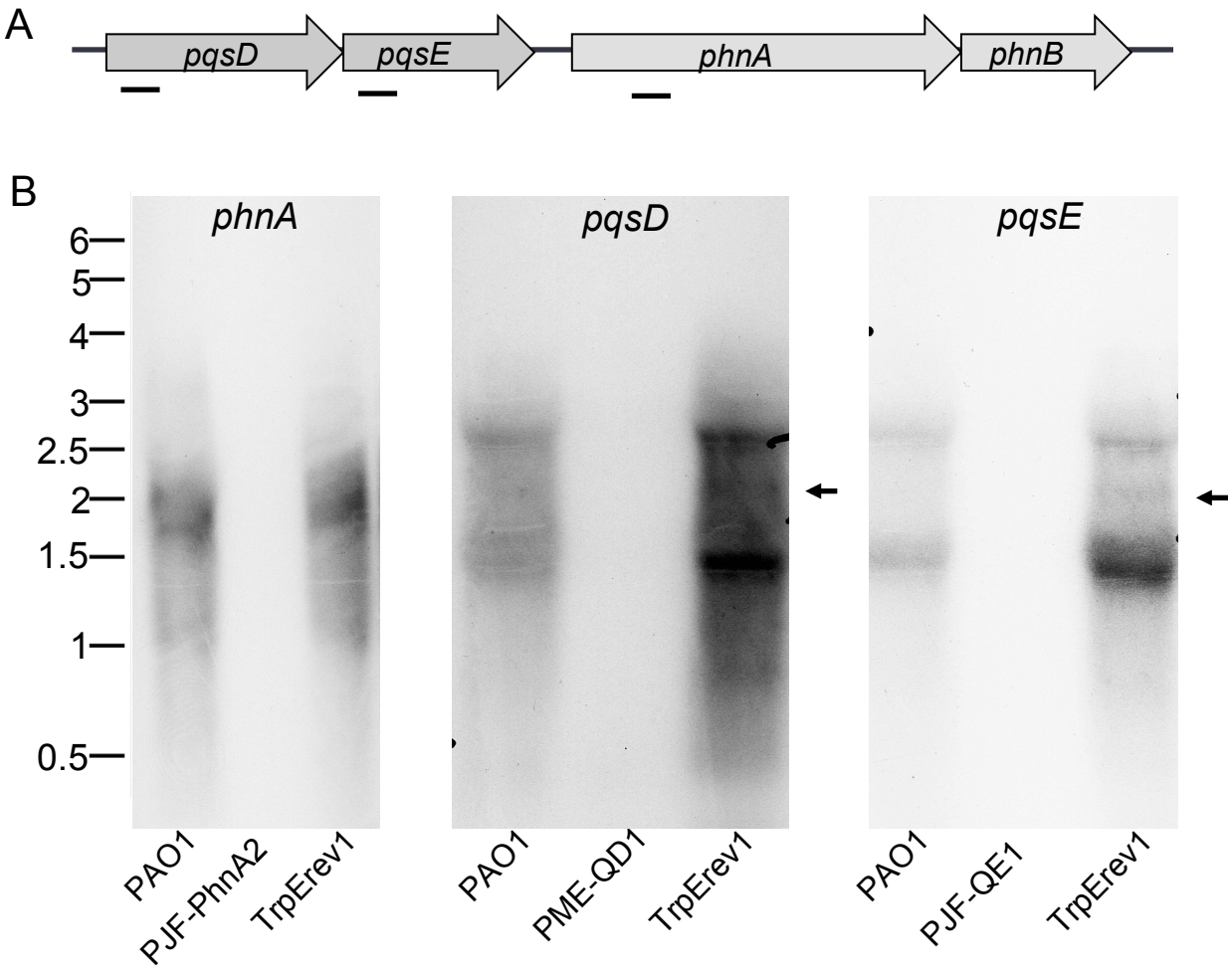

Figure 3.5 Northern blot results reveal a novel transcript that contains *pqsD* and *pqsE*. (A) A map of the probe locations for the *pqsD, pqsE,* and *phnA* pictured Northern blots. A second set of probes for each blot were also used (not pictured) and all primers for the blots are indicated in Table 3.2 (B) Northern blot results for *phnA, pqsD,* and *pqsE* of RNA extracted from cutlures grown in VBG to an OD_{660} of 0.2 extracted from the indicated *P. aeruginosa* strains. The arrows indicate the light band the novel *pqsDE* transcript.

transcript(s) or stability as both the wild-type strain PAO1 and strain TrpErev1 blots are nearly identical (Figure 3.5B, left panel). We also performed Northern blots with *pqsD* and *pqsE* probes to further investigate the RT-PCR results and determine the size of the transcript(s) containing these genes (Figure 3.4B, center and right panels). The blots did not aid in clearing up the transcript(s) size as they both contain a smear from above 3 kb and below 1 kb and again suggest that the transcript(s) are unstable. One surprise is that no transcript(s) are indicated above 4 kb in the *pqsD* and *pqsE* blots, which would be the size of the *pqsABCDE* transcript. Lastly, a band is visible in the tryptophan revertant strain TrpErev1, but not the wild-type strain PAO1, in both the *pqsD* and *pqsE* blots. This band (indicated by the arrow) is approximately 2 kb in size, which would account for the coding regions of *pqsD* and *pqsE* (1908 nucleotides), as well as for a leader and/or tail on the mRNA. This fragment could encode for a transcript that includes only *pqsD* and *pqsE,* and leads us to believe that the mutation may have resulted in the creation of a new or dysregulated transcriptional start site upstream of *pqsD.*

3.4.3 The single nucleotide mutation in *pqsC* induces transcription of *pqsD* from a new transcriptional start site in the PQS biosynthetic operon.

To determine if the 2kb band identified in the *pqsD* and *pqsE* Northern blots represents a novel transcript containing only *pqsD* and *pqsE,* we made a transcriptional *lacZ* fusion containing 500 nucleotides upstream from the *pqsD* start codon from both the wild-type strain PAO1 and the tryptophan auxotroph revertant strain TrpErev1. The resulting transcriptional fusions were electroporated into the wild-type strain PAO1, and the β-gal activity of the fusions was assayed at an OD_{660} of 0.2 in VBG media. The

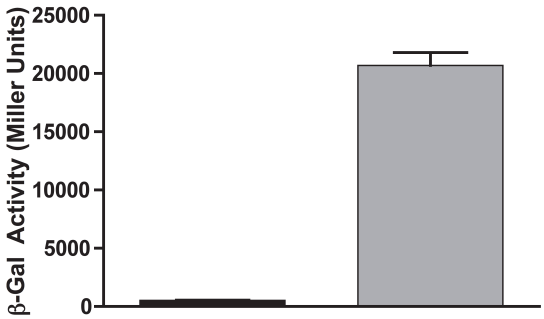

Figure 3.6 The G1041A mutation results in the transcriptional activation of a new transcript upstream from *pqsD*. The *P. aeruginosa* wild-type strain PAO1 with either p*pqsD*wtTcZ (black bar) or p*pqsD*G1041ATcZ (grey bar) was grown in VBG media until an OD_{660} of 0.2 was reached. The cultures were then assayed for β-gal activity, which is presented in Miller units as the mean ± σ^{n-1} of results from at least three separate experiments.

results show a 40-fold increase in the activity of the G1041A mutant *pqsD-lacZ* fusions when compared to the wild-type *pqsD-lacZ* fusion (Figure 3.6). The assay was also performed at an OD660 of 0.6 and after 24 hours of growth in both LB and VBG media with identical results (data not shown). The drastic difference in activity of the two fusions suggests that the G1041A mutation has provided an increase in activity of p*pqsD*G1041ATcZ and the G1041A mutation resulted in the transcriptional upregulation of *pqsD* and *pqsE* from a new transcriptional start site. These results taken together with the increase in pyocyanin (Figure 3.1), decreased PQS (Figure 3.1 A and B), three fold increase in *pqsD* and *pqsE* transcripts (Figure 3.3), and the band identified in the *pqsD* and *pqsE* northern blots (Figure 3.5B, center and right panels) would suggest that the G1041A mutation in *pqsC* results in a new transcript that starts upstream from *pqsD* start codon. We have performed electrophoretic mobility shift assays (EMSA) with purified PqsR to see if it regulated the *pqsD* promoter and no binding was noted (data not shown). We are currently pursuing experiments to identify the transcriptional start site of the new promoter and begin searching for possible regulators of this promoter.

3.5 Discussion

The intriguing results reported for the Essar *et al.* (35) tryptophan revertant left our lab wondering what natural event would lead to changes in pyocyanin and *phnA* transcription. Our isolation of five tryptophan auxotroph revertants that mirrored the results shown by Essar *et al.* suggested that we had recreated the mutation that allowed for survival on tryptophan deplete media (Figure 3.1 and Figure 3.2). Full genome sequencing of the revertants led to the discovery of a point mutation in *pqsC* at nucleotide 1041 that occurred in all five examined strains (Table 3.3). The *pqsC*

G1041A mutation was surprising, but since its location was near *phnAB* and within the PQS biosynthetic operon we believed that it was worthy of investigation. The G1041A mutant strain PAQC-G1041A that we constructed confirmed that the single nucleotide change in *pqsC* was responsible for the increase in pyocyanin production and *phnA* transcription exhibited by our revertants (Figures 3.1 and 3.2). The second point mutation was found in *ntrC* in only two out of the five strains examined. This gene encodes for the response regulator in a two-component system that regulates nitrogen assimilation and is part of an intricate network that balances nitrogen and carbon source utilization in *P. aeruginosa* (70). This mutation was likely an unrelated secondary mutation and could be due to growing under nutrient limiting conditions. It is also possible this was a secondary mutation that resulted from over expression of *phnA(B)* causing increased anthranilate production that shifts the nitrogen and/or carbon source demand. Regardless of what resulted in the *ntrC* mutation, it is clear that a single mutation of a G to A in nucleotide 1041 of *pqsC* is sufficient to produce the same results as the tryptophan auxtroph revertants.

Further investigation of the G1041A mutation in *pqsC* lead us to discover that not only was the single nucleotide change responsible for elevated levels of *phnA* transcript and pyocyanin production (Figures 3.1 and 3.2), but it also resulted in dysregulation of the PQS biosynthetic operon (Figure 3.3). The G1014A mutation resulted in decreased levels of *pqsA* and *pqsC* transcript. This most likely resulted in decreased co-inducer (PQS) for PqsR, the transcriptional regulator of the PQS biosynthetic operon (121). The most striking results were the elevated levels of *pqsD* and *pqsE* transcripts (Figure 3.3). The elevated level of *pqsE* transcript explains the increased production of pyocyanin, as

pqsE is necessary for the increased production of pyocyanin (38, 41). The increased transcription of *pqsE,* and likely overproduction of the PqsE protein, may also account for the decrease in PQS production in the revertants (Figure 3.1), as it has been shown that overproduction of PqsE results in decreased levels of PQS and HAQ's (46).

The elevation of *pqsD, pqsE,* and *phnA* transcripts in the G1041A mutant was intriguing and led us to believe that the mutation resulted in either a new transcript or increased stability of transcript(s) that contained each of the genes. To elucidate if the mutation resulted in a transcript that contained *pqsE* and *phnA(B),* which had been previously reported to be separate, we utilized RT-PCR (80). This experiment showed that a transcript containing *pqsE* and *phnA* was possible. The RT-PCR also revealed that if *pqsE* and *phnA* are on the same transcript it was not a result of the G1041A mutation in *pqsC* (Figure 3.4B), as the results were the same in both the wild-type strain PAO1 and the G1041A mutant strain PAQC-G1041A. Our results suggested that *pqsE* and *phnA* are on the same transcript, yet we believe that a separate promoter for just the transcription of *phnAB* exists. This has been shown through the activity of *phnA* transcriptional fusions, as well as expression plasmids from previous results by both our lab and others (13, 37). The ability of *phnAB* to be transcribed both from its own promoter and read-through from *pqsE* transcription would further ensure the production of anthranilate for PQS production and basic cellular functions.

In order to understand the RT-PCR results, we performed Northern blots on *pqsD, pqsE,* and *phnA* (Figure 3.5B). In each of the Northern blots, it was clear that the transcripts that contained *pqsD, pqsE,* and *phnA* were degraded and did not result in the conclusion of how many transcripts or an exact size of the transcripts. We can

speculate that the transcript(s) size containing *pqsD, pqsE,* and *phnA* are all at least 3.5 kb (Figure 3.5B). Previous Northern blot attempts on the PQS biosynthetic locus genes by our lab using multiple different probes and RNA extraction methods have consistently shown binding that extended from above 4 kb to below 1kb (data not shown). These results further suggested that the transcripts for the biosynthetic locus are highly unstable (data not shown). The Northern blots for *pqsD* and *pqsE* from this study did show a novel band at approximately 2kb in the tryptophan auxotroph revertant strain TrpErev1 that was not in the wild-type strain PAO1 (Figure 3.5, center and right panels). The size of this band suggested that the transcript would contain both *pqsD* and *pqsE* and led us to the possibility that the G1041A mutation in *pqsC* resulted in the creation of this transcript. One problem with the size of this fragment is that it most likely does not contain both *pqsDE* and *phnAB.* If the transcript only contains *pqsDE*, it would call into question our RT-PCR results and exactly how the transcript is upregulated. Future efforts are underway to understand exactly what is causing the RT-PCR results, the stability of the PQS biosynthetic locus genes, and the size of these transcripts

The next step to understanding the quantitative real-time PCR and Northern blot results for *pqsD* and *pqsE* was to determine if the G1041A mutation resulted in the creation of a new promoter. We made two *lacZ* fusions, one with wild-type sequence and the other with G1041A mutant sequence extending 500 nucleotides upstream from the *pqsD* start codon, and β-gal activity of the G1041A fusion was significantly increased compared to the wild-type fusion (Figure 3.6). The result from the β-gal assay taken together with both our real-time PCR and Northern results indicated that

the G1041A at nucleotide 1041 of *pqsC* resulted in the expression of a new transcript. This result explains the upregulation of pyocyanin (Figure 3.1) and the potential upregulation of *phnA(B)* (3.2), if *pqsE* and *phnA(B)* are indeed transcribed together. The upregulation of just the final two genes of the PQS biosynthetic operon and *phnAB* would be important for *P. aeruginosa* to circumvent the need for PQS and PqsR to drive the transcription of both *pqsABCDE* and *phnAB*. This would allow for the anthranilate produced from the transcription of just *pqsDE* and *phnAB* to provide for tryptophan and other cellular functions, rather than for the production of PQS. Furthermore, PqsE is known to regulate virulence factors independent of PQS production and to affect the expression of 90% of genes regulated by PqsR (38, 46). The expression of just *pqsDE* and *phnAB* would allow the bacteria to regulate virulence factors independently of PQS production and to produce anthranilate for basic cellular functions. We are currently undertaking experiments to identify the location of the start site in the newly identified transcript, as well as looking into the regulation of this transcript.

CHAPTER FOUR: GENERAL SUMMARY

The goal of these studies was to understand the regulation of the pathways that produce anthranilate for PQS production in *P. aeruginosa*. These investigations were motivated by previous data which indicated that both the kynurenine pathway and the secondary anthranilate synthase PhnAB were important for the production of PQS (37). This work revealed that the systems are utilized during different growth conditions, with the kynurenine pathway being the main source of anthranilate for PQS production during growth in complex media and PhnAB being the main source of anthranilate for PQS production during growth in minimal media without tryptophan (Figure 1.5)(37). Most bacteria do not possess the kynurenine pathway or a secondary anthranilate synthase, yet *P. aeruginosa* encodes each set of unique genes and utilizes each of the pathways. The maintenance of redundant pathways for the production of anthranilate implies the importance of this branch point molecule to *P. aeruginosa* survival.

The kynurenine pathway functions as the main source of anthranilate when tryptophan, or its breakdown products are available. The kynurenine pathway has only been identified in a small number of sequenced bacteria (Gram negative bacteria are listed in Figure 2.1), and *P. aeruginosa* contains only the anthranilate branch of this pathway, which catabolizes tryptophan into anthranilate. This pathway provides *P. aeruginosa* with a growth advantage as *P. aeruginosa* could deplete this essential amino acid from the intra- and extracellular environments. The kynurenine pathway genes are upregulated by the transcriptional regulator KynR in the presence of the co-inducer kynurenine (Figures 2.3, 2.4, and 2.5). Use of the pathway intermediate kynurenine as the co-inducer for KynR ensures that tryptophan is not catabolized unless

kynurenine is present and provides a check-point to ensure tryptophan concentrations for protein synthesis are maintained. It has also been shown that the kynurenine pathway genes *kynBU* are upregulated by LasR. LasR activates *kynBU* transcriptional activity (Figure 2.3) and directly binds to the intergenic region between *kynB* and *kynR* (43). The induction of *kynBU* by LasR would allow for this pathway to be active once *P. aeruginosa* cell density has reached a quorum. This would ensure that the kynurenine pathway would be primed for upregulation when *P. aeruginosa* encounters the intermediate molecule kynurenine. Further studies need to be conducted to understand the availability of kynurenine during infection to determine if this pathway is critical for PQS production during *P. aeruginosa* infections, as this pathway is highly dependent on its presence to upregulate the transcription of the kynurenine pathway genes.

The understanding of *kynR* regulation is in its infancy, as we know very little about how this gene is regulated. We have constructed a transcriptional *lacZ* fusion of *kynR* and performed β-gal assays in multiple mutants (Figure 4.1). Our data suggest that *kynR* is not autoregulated, unlike many feast/famine regulatory proteins, as the transcription of *kynR* is only slightly increased in a *kynR* mutant (Figure 4.1) (135). We also looked at the quorum sensing regulators LasR and RhlR because as stated above, LasR was shown to bind in the intergenic region between *kynBU* and *kynR* (43). The transcriptional activity of *kynR* is slightly increased in both *lasR* and *rhlR* mutants suggesting a slight negative effect on *kynR* transcription (Figure 4.1). The regulation of *kynR* has not been noted in any published microarray data. Even the catabolite

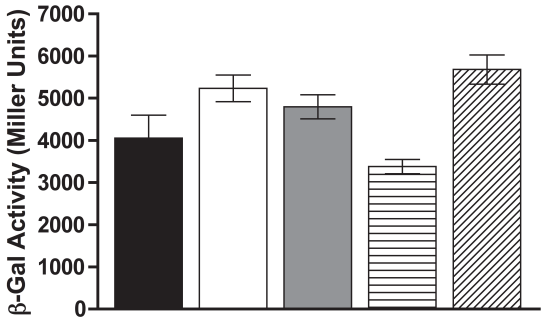

Figure 4.1: The transcription of *kynR* is minimally affected by itself or the AHL quorum sensing regulators. The *kynR* transcriptional *lacZ* fusion p*kynR*TcZ activity was monitored in the *P. aeruginosa* strains PAO1 (black bar), *kynR* mutant strain PAΔKynR (white bar), *lasR* mutant strain PAOR-1 (grey bar), *kynR, lasR* double mutant PAOR1ΔKynR (horizontal striped), and *rhlR* mutant PJF-RLR1 (diagonal striped). The cultures were assayed for β-gal activity after 24 hours growth in LB at 37°C, which is presented in Miller units as the mean ± σ^{n-1} of results from at least two experiments.

control system CbrAB/Crc that plays a role in the regulation of other amino acid catabolism pathways did not influence *kynR* transcription (108). To find any direct regulators of *kynR* we plan to incubate the *kynR* promoter with cell lysate and then elute off any protein that may bind to the promoter. We can then utilize mass spectrophotometry to identify the bound proteins. This technique will identify any proteins that directly bind to the intergenic region between *kynR* and *kynBU* and we can begin to investigate if and how they regulate *kynR*.

Originally, the secondary anthranilate synthase PhnAB was believed to be the main source of anthranilate for PQS production due to its location and regulation (37). However, PhnAB is unable to provide anthranilate for the production of PQS when grown in complex media in the absence of the kynurenine pathway (37). This was perplexing as *phnAB* is transcribed during growth in complex media (13). We believe the inability of PhnAB to synthesize anthranilate for PQS production under complex growth conditions despite the seemingly high stationary phase transcription is due to potential post-translational regulation of the anthranilate synthase α-subunit, as the activity of anthranilate synthase α-subunits typically exhibit inhibition by tryptophan (100). Essar *et al.* showed that tryptophan does not inhibit PhnAB activity and suggested that another molecule is causing this inhibition. Furthermore, overexpression of a His-tagged PhnA in *kynA* and *kynU* mutants allowed for the production of PQS, which has been abolished in these strains (Figure 4.2). We do not currently know what is causing the post-translational inhibition of PhnA and to ascertain this, our lab plans to first purify PhnA and then perform enzymatic assays with organic extract fractions of *P.*

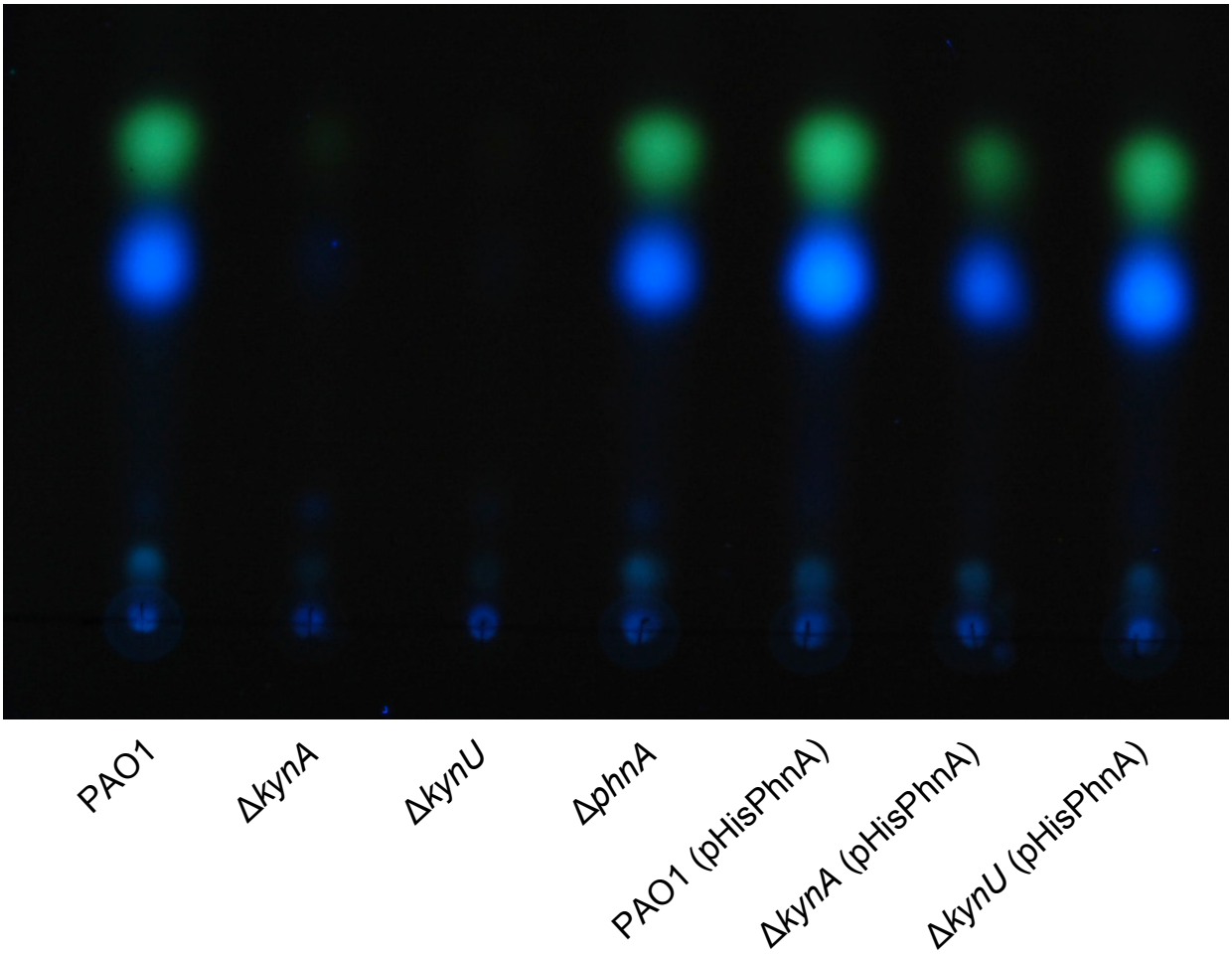

Figure 4.2: The overexpression of His-tagged PhnA provides for anthranilate for PQS production in kynurenine pathway mutants. Ethyl acetate extracts from cultures grown in LB after 24h were analyzed by TLC. Equal volumes of extracts were resolved in each lane. The strains are listed below the extractions.

aeruginosa culture grown in complex media. Though this process will be time consuming, it will identify the post-translational inhibitory molecule of PhnA.

Our recent research identifying a single nucleotide mutation in *pqsC* that induced the expression of *pqsD, pqsE,* and *phnA* suggested that there is a new transcript that starts upstream from the *pqsD* start codon (Figure 3.2, 3.3, and 3.5). The single nucleotide change was confirmed to increase the activity of the *pqsD-lacZ,* that was shown to have a 40-fold increase in activity compared to the wild-type strain. The next step in understanding the new transcript is to identify the promoter and how it is regulated. The transcriptional regulator PqsR does not regulate this promoter, but we do not know if other quorum sensing regulators could possibly be involved in the regulation. It is possible that the single nucleotide change resulted in either the relief of a repressor or the binding of an activator that has promoted the activation of the new transcript. The benefit of this transcript for *P. aeruginosa* is the PQS-independent regulation of virulence factors, as PqsE is thought to be the main player in the quinolone signaling regulation cascade and has been titled the PQS response gene (31, 38, 41, 46). Furthermore, the creation of a new transcript that is PQS signaling independent would allow for dysregulation of virulence factors in *P. aeruginosa*, which could be advantageous to bacterial growth in the environment or within a host.

Taken together, our data have provided a better understanding of how *P. aeruginosa* regulates the pathways that provide anthranilate for PQS production, as well as basic cellular function. Though a redundancy exists with multiple metabolic routes for anthranilate production, the unique regulation of each pathway ensures that anthranilate can be produced under all conditions, whether it is in feast or in famine.

Within the environment or during an infection, the ability of *P. aeruginosa* to have three pathways that ensure anthranilate production would be advantageous. Anthranilate concentrations being high within the bacteria or environment would allow for production of PQS for virulence factor regulation, tryptophan production for protein synthesis, or as a carbon or nitrogen source. No matter the reason, *P. aeruginosa* possesses the ability to ensure that anthranilate is produced to allow for its survival in many different environments.

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