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Investigations into the Role of Aromatic Amino Acids in Quorum Sensing-mediated Virulence in *Pseudomonas aeruginosa*

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Investigations into the Role of Aromatic Amino Acids in Quorum Sensing-mediated Virulence in *Pseudomonas aeruginosa*

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Dedication

This dissertation is dedicated to my parents and sister for their unconditional love and support.

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I would like to thank Dr. Marvin Whiteley for supporting me and teaching me to be a better scientist. I would also like to thank members of the Whiteley Lab both past and present who have profoundly contributed to my development, especially Kelli Palmer and Peter Jorth. Lastly, I want to thank the teachers throughout my life who have contributed to my education including Dr. Ruthann Nichols, Michael Reed, Shirley Lemon, Lane Smith, Ben Quick, Charlotte Lothian, and most importantly my parents and sister.

Investigations into the Role of Aromatic Amino Acids in Quorum Sensing-mediated Virulence in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that is a primary constituent of chronic, polymicrobial infections in the lungs of individuals with cystic fibrosis (CF). A significant consequence of CF is production of thick mucus along epithelial surfaces. In the lungs, this mucus collects and serves as an excellent growth substrate for a range of bacteria including. CF lung fluids (sputum) also enhance the virulence of *P. aeruginosa*, as production of a signaling molecule critical for virulence, the *Pseudomonas* quinolone signal (PQS), is enhanced in the presence of phenylalanine and tyrosine in CF sputum. The goal of this dissertation is to better understand how phenylalanine and tyrosine affect PQS production and ultimately *P. aeruginosa* virulence. To address this, I use transcriptome profiling to determine that genes for phenylalanine and tyrosine catabolism, PQS biosynthesis, and a transcriptional regulator called PhhR are up-regulated in the presence of phenylalanine and tyrosine. I determine that PhhR regulates genes for aromatic amino acid catabolism but not genes for PQS biosynthesis. The PhhR regulon is further characterized by mapping of PhhR-regulated promoters with primer extension, and evidence for direct regulation is presented. To explain enhanced

production of PQS in CF sputum, I favor a model in which flux of a shared metabolic precursor, chorismate, toward PQS biosynthesis is enhanced when phenylalanine and tyrosine are present. I investigate this model by examining the first step in PQS biosynthesis, conversion of chorismate to anthranilate by an anthranilate synthase (AS). *P. aeruginosa* possesses two AS enzymes encoded by the *trpEG* and *phnAB* genes, with the former generating anthranilate specifically for tryptophan biosynthesis while the latter generates anthranilate for PQS biosynthesis. I investigate the evolutionary origins of these two enzymes and generate unmarked deletion mutants to dissect their roles in tryptophan and PQS biosynthesis. The ability of PhnAB to compensate for loss of TrpEG at high cell densities is documented, and a model explaining anthranilate sequestering is developed. Knowledge gained from these studies will be useful in developing novel therapeutic strategies.

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Chapter 1: Introduction

1.1 THE HOST AS A GROWTH MEDIUM AND *PSEUDOMONAS AERUGINOSA*

1.1.1 The host as a growth medium

In the 1870s Louis Pasteur developed a model of infection in which a host must provide the appropriate conditions for growth of an infecting microorganism, explaining that the host is the pathogen's culture vessel (54). While subsequent work has identified receptor-mediated interactions as the major explanation for tissue tropism, the importance of nutrients available to pathogens at the site of infection has continued to be an interesting, though occasionally overlooked area of study. In the 1950s Edward Garber demonstrated that strains of a plant pathogen carrying different amino acid auxotrophies exhibited varying abilities to infect plants, and experiments like these led him to describe the host as a growth medium in 1960 (45, 54). Since then the importance of nutrients such as D-serine, threonine, lactate, and aromatic amino acids have been implicated as nutritional cues involved in pathogenesis (13, 21, 101, 103, 112, 118, 122). As will become clear, a major phenotype of the genetic disease cystic fibrosis (CF) is a buildup of thick mucus along epithelial surfaces, and this represents a rich, complex growth substrate for a range of microbial pathogens. CF epithelial fluids will serve as a model infection environment for my studies of the role of nutrients in chronic infections throughout this work.

1.1.2 Cystic fibrosis and polymicrobial infections

CF is an autosomal recessive genetic disease caused by altered levels or activity of the cystic fibrosis transmembrane conductance regulator (CFTR) (117). CFTR is a chloride ion transporter present on epithelial surfaces that is also known to regulate transport of additional ions through interactions with other proteins like the sodium transporter ENaC (115, 129). The most common mutation (~70% of individuals with CF) causing CF results from the deletion of three nucleotides corresponding to phenylalanine 508 (ΔF508), and its product is a CFTR that is 100% degraded before exiting the endoplasmic reticulum (67, 72). Aberrant ion flux at the epithelial surface results in the most notable CF disease phenotype, the buildup of thick mucus that is particularly difficult to expectorate from the lungs (83). The complex mixture of mucus and factors derived from lysed bacterial and host cells is called sputum and serves as an excellent growth substrate for a range of bacteria including *Staphylococcus aureus*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa* (62, 83).

1.1.3 *Pseudomonas aeruginosa* as an opportunistic pathogen

P. aeruginosa is a Gram-negative, rod-shaped bacterium that is ubiquitously found in soil and water. It possesses an arsenal of virulence and competitiveness factors that include proteases, exotoxin, type III secretion, and hydrogen cyanide (14, 47, 50, 73, 125, 130, 150). Another significant virulence factor is a redox-active phenazine compound called pyocyanin. This secreted

small molecule gives *P. aeruginosa* its characteristic blue color, is destructive to host tissues, is produced in greater quantities in the presence of other bacteria, and may aid *P. aeruginosa* in iron acquisition (11, 78, 79, 113). *P. aeruginosa* has been associated with opportunistic infection of plants, insects, nematodes, and humans, and it is believed that the same virulence factors are correlated with infection across these diverse hosts (6, 29, 33, 66, 140, 141).

In humans, *P. aeruginosa* infections are common in immune compromised situations. The organism is known to cause infections in the eye (particularly in contact lens wearers) and burn wounds (6, 49, 116, 137, 138). It is also a frequent source of hospital-acquired infections (142, 157), however, the most studied infection of *P. aeruginosa* is in the lungs of individuals with CF. Most individuals with CF are colonized by *P. aeruginosa* in childhood, and these infections are usually maintained throughout the individual's life (62, 83). *P. aeruginosa* is extremely difficult to eradicate from the CF lung due to its inherent resistance to many antimicrobial therapies and propensity to form persistent biofilms or microcolonies (12, 76, 77, 83, 131, 134). Lung disease associated with *P. aeruginosa* is also the leading cause of morbidity and mortality among individuals with CF, emphasizing the need to better understand the physiology of these infections (34, 83).

1.1.4 Modeling *P. aeruginosa* growth in the CF lung

Animal models that adequately replicate the CF disease phenotype with regard to *P. aeruginosa* infection have been difficult to obtain. Several approaches have been used to generate mouse mutants with CF-like symptoms to varying degrees of success (17, 20, 30). Unfortunately, most of these models require extreme conditions such as repeat exposure or colonization in conjunction with agar beads for the mice to maintain *P. aeruginosa* infections, and one successful description of chronic infection reported only 33% of mice colonized for 24-53 weeks contained detectable *P. aeruginosa* in the lungs (18, 51, 155). The recent development of pig and ferret models that more accurately replicate human CF lung pathology is promising; however, *P. aeruginosa* was surprisingly not present among bacteria recovered from CF pigs (119, 135, 136).

In the absence of animal models that adequately replicate the chronic infection phenotype observed in CF lung infections, several groups developed an *in vitro* method for studying *P. aeruginosa* chronic infections (39, 46, 98, 101, 103, 134). A previous member of my research group, Kelli Palmer, used filtered, sterilized CF sputum as the sole source of carbon and energy in a buffered base as an *in vitro* growth medium (103). Because this medium is difficult to obtain and incredibly complex, she generated a synthetic version of this medium (SCFM) that mimicked the nutritional contents of genuine sputum (101). An important

feature of this medium compared to other attempts at synthetic sputum media is that it is defined. Thus, it can be used as a tool from which nutrients may be added or subtracted to determine their impact on *P. aeruginosa* physiology. Regardless of whether the contents are defined, media like these have identified nutritional cues implicated in several virulence/persistence phenotypes including density-dependent toxic factor production and microcolony formation (39, 101, 103, 134).

1.2 *P. AERUGINOSA* AND DENSITY-DEPENDENT GENE REGULATION

1.2.1 Overview of *P. aeruginosa* quorum sensing systems

Production of many *P. aeruginosa* virulence factors including proteases, pyocyanin, and hydrogen cyanide is regulated in a density-dependent manner referred to as quorum sensing (QS). The phenomenon of QS was discovered through the observation that light production in the bobtail squid symbiont *Vibrio fischeri* only occurs at high cell densities and requires a small molecule signal that can be recovered from spent media (32, 71, 94). This small molecule signal is called an autoinducer and is constitutively produced. The concentration of autoinducer increases with the number of cells producing it until reaching a threshold concentration where the signal interacts with a LuxR-type transcriptional regulator to alter gene expression (40, 41). QS is generally used to regulate behaviors that are more effective when performed as a group

including light production, biofilm formation, and toxic factor production (16, 22, 32, 44, 71, 75, 94, 108).

P. aeruginosa possesses three integrated QS regulatory systems that utilize four known autoinducers (Figure 1.1). The first system discovered was the LasI/LasR system in which LasI synthesizes 3-oxododecanoyl-homoserine lactone (3OC12-HSL), and LasR is the transcriptional regulator (44, 105, 106). The second system discovered was the RhlI/RhlR system in which, similar to the Las system, RhlI synthesizes butyryl-homoserine lactone (C4-HSL), and RhlR is the transcriptional regulator (96, 97, 107). Together these two systems have been shown to regulate more than 5% of the genome, and many of the genes they control are involved in production of toxic factors associated with virulence (126, 127, 144, 148). The third QS system is mediated by production of two quinolone signals: 2-heptyl-3-hydroxy-4-quinlone (*Pseudomonas* quinolone signal; PQS) and its direct biosynthetic precursor 2-heptyl-4-quinolone (HHQ) (109, 152). These two signals interact with the transcriptional regulator MvfR (PqsR), though PQS displays approximately 100-fold greater induction of MvfR-regulated promoters than does HHQ (16, 123, 152).

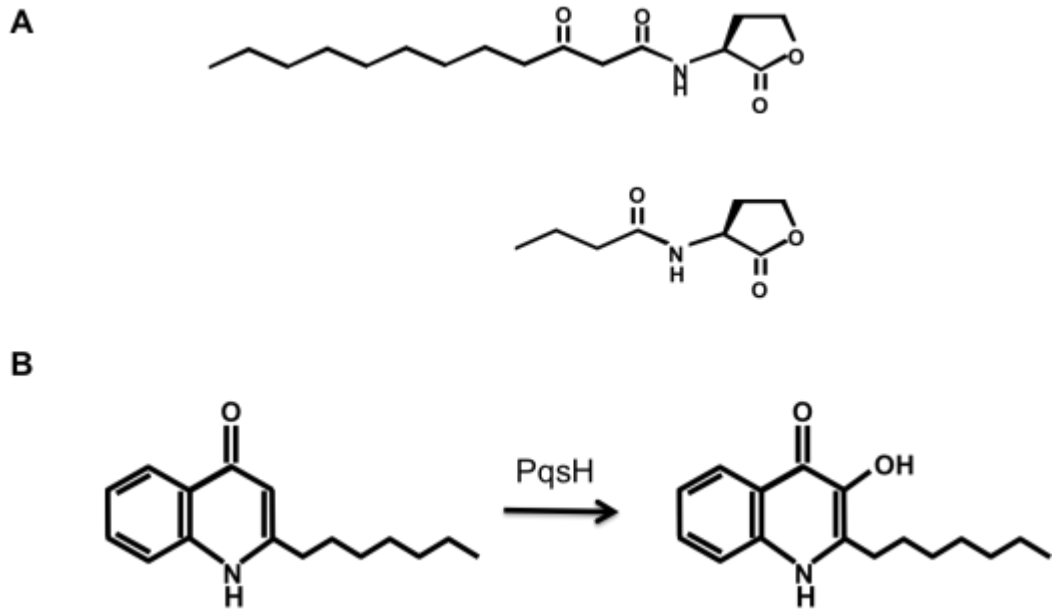

Figure 1.1 *P. aeruginosa* quorum-sensing signals.

P. aeruginosa produces four small molecules known to induce density-dependent gene regulation (QS). (A) *P. aeruginosa* produces two acyl-homoserine lactone quorum sensing signals: N-3-oxododecanoyl-homoserine lactone (top) and N-butyryl-homoserine lactone (bottom). (B) *P. aeruginosa* also produces two quinolone quorum sensing signals: 2-heptyl-4-quinolone (Left; HHQ) which is converted by PqsH to its more active form 2-heptyl-3-hydroxy-4-quinolone (Right; PQS).

1.2.2 The *Pseudomonas* quinolone signal: Biosynthesis

Unlike the acyl homoserine lactone (AHL) synthases, several enzymes are necessary to generate PQS (43). PQS biosynthesis is believed to begin with conversion of the central metabolite chorismate to anthranilate by the anthranilate synthase encoded in the *phnAB* operon, though *P. aeruginosa* possesses several mechanisms for generating anthranilate that are detailed in section 1.2.3 (15, 43). The products of genes in the *pqsABCD* operon synthesize HHQ through the head-to-head condensation of anthranilate with β-keto-decanioc acid derived from fatty acid metabolism (Figure 1.2B) (10, 43). HHQ is converted to PQS by the *pqsH* gene product in a reaction that is oxygen-dependent (123). Both the *phnAB* and *pqsABCD* operons are autoregulated by PQS via MvfR. Thus, the production of PQS results in a positive feedback loop generating greater amounts of PQS (16, 25).

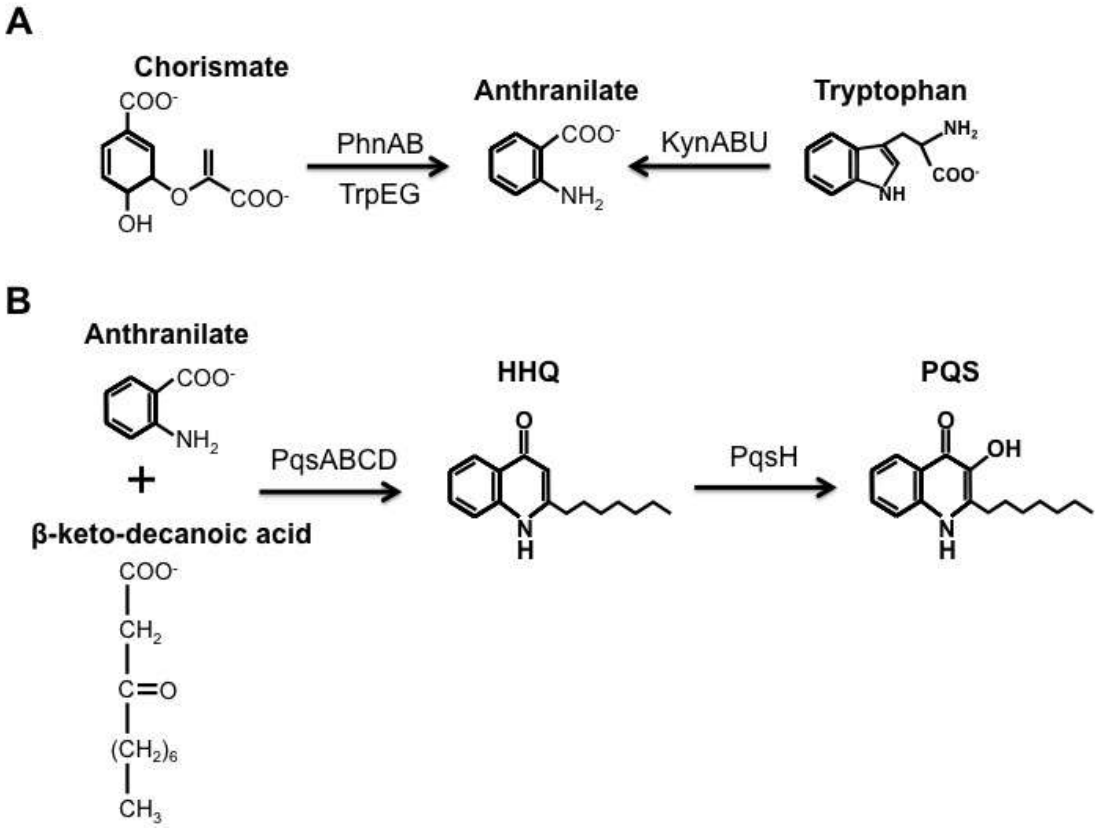

Figure 1.2 PQS biosynthesis pathway.

PQS biosynthesis requires several enzymes. (A) Anthranilate is generated by either degradation of tryptophan or conversion of chorismate by one of *P. aeruginosa's* two anthranilate synthases (TrpEG and PhnAB). (B) Anthranilate then participates in a head-to-head condensation with β-keto-decanoic-acid derived from fatty acid metabolism to generate HHQ via PqsABCD. HHQ is converted to PQS by PqsH.

1.2.3 *P. aeruginosa* anthranilate production

Anthranilate is required for production of PQS, and *P. aeruginosa* utilizes several mechanisms for generating anthranilate (Figure 1.2A). Degradation of tryptophan by products of the kynurenine pathway genes *kynABU* generates anthranilate that can be used for PQS production in the presence of tryptophan (37). Additionally, the central metabolite chorismate, which is a precursor for aromatic amino aids, quinolones, folate, ubiquinones, and siderophores, can be converted to anthranilate by an anthranilate synthase (AS) enzyme. *P. aeruginosa* possesses two AS enzymes with the large subunits encoded by *trpE* and *phnA* and the small subunits encoded by *trpG* and *phnB (15, 35, 36)*. Genetic evidence indicates that TrpEG generates anthranilate for tryptophan biosynthesis, as mutants in *trpEG* are tryptophan auxotrophs (35, 36). PhnAB is believed to generate anthranilate for PQS production, as *phnAB* mutants do not produce PQS under most conditions (43, 89). These results suggest that these two enzymes, while capable of performing the same reaction, are not functionally redundant, because *trpEG* mutants retain the ability to produce PQS and *phnAB* mutants retain the ability to grow in the absence of tryptophan (35-37).

A mechanism to explain the lack of metabolic crosstalk between tryptophan and PQS biosynthesis has not been identified. One possible model relies on the fact that *phnAB* expression is QS-controlled by PQS/MvfR (16). In this model, differential regulation or timing of expression could result in too little PhnAB present early in growth to compensate for the loss of TrpEG. Experiments to evaluate this model are presented in Chapter 3.

1.2.4 The *Pseudomonas* quinolone signal: Activities

The most notable biological activity of PQS is its regulation of toxic factor production. These factors include hydrogen cyanide, elastase, and pyocyanin (15, 42, 43, 152), and consistent with diminished toxic factor production, mutants unable to make PQS are attenuated in model infections (25, 42, 156). Since its discovery as a QS signal, however, several other PQS activities have been documented. Due to its hydrophobicity, extracellular trafficking of PQS occurs in vesicles formed from outer membrane blebs, and PQS promotes formation of these vesicles through its interactions with the outer membrane (86-88, 124). PQS may also function in iron acquisition as it has been reported to chelate the ion with high affinity (9, 28). Finally, though PQS does not possess antimicrobial activity, its autoinduction of the *pqsABCDE* quinolone biosynthetic operon results in PQS-dependent production of a range of other quinolones with antimicrobial activity (26, 80).

1.3 AROMATIC AMINO ACIDS AND *P. AERUGINOSA* VIRULENCE

1.3.1 Enhanced virulence in CF sputum

In order to better understand the physiology of *P. aeruginosa* infections in the CF lung, Kelli Palmer grew *P. aeruginosa* in a medium with CF sputum as the sole source of carbon and energy (103). Compared to a standard laboratory medium, growth to similar cell yields in CF sputum resulted in a five-fold increase in PQS, and production of PQS-regulated virulence factors was similarly induced (103). Because this medium is complex, she developed a synthetic sputum medium (SCFM) that mimics the nutritional contents of CF sputum, and she found that the phenotypes observed in CF sputum media were recapitulated in SCFM (101). The contents of SCFM are defined and based on concentrations measured from genuine CF sputum. Thus, the medium can be used as a tool from which nutrients can be added or subtracted to determine their effect on *P. aeruginosa* physiology (101).

CF sputum is rich in amino acids, and it was suspected that they may play a role in enhanced PQS production (103). This was confirmed as growth on tryptophan or a mixture of tryptophan, phenylalanine, and tyrosine resulted in increased PQS production (103). Degradation of tryptophan was later shown to be a source of anthranilate for PQS production. However, this is not expected to be relevant in CF sputum which contains very little (<10 μM) of the amino acid (37, 101). Tryptophan levels are consistent with its relatively infrequent incorporation into cellular proteins whose degradation are likely the source of free

amino acids in CF sputum. By contrast, approximately 500 μM phenylalanine and 800 μM tyrosine are present in CF sputum, and when these nutrients were removed from SCFM, enhanced PQS production was completely abolished (101).

1.3.2 Models for enhanced PQS production in CF sputum

Understanding the mechanism of enhanced PQS production in sputum is a major goal of this dissertation. I proposed two models to explain enhanced PQS production in the presence of phenylalanine and tyrosine (Figure 1.3). In the first model, transcriptional co-regulation of aromatic amino acid catabolic and PQS biosynthesis genes in the presence of phenylalanine results in increases of both activities during growth in CF sputum. This is supported by the fact that phenylalanine and tyrosine are important carbon sources in sputum beyond their role in cuing PQS production, and that an aromatic amino acid-responsive transcriptional regulator in a close relative, *P. putida*, is a global regulator of genes outside of aromatic amino acid utilization (58-60, 101). In the second model, flux of the central metabolite chorismate, a common precursor to aromatic amino acid and PQS biosynthesis, mediates enhanced PQS production; when aromatic amino acids are present their biosynthesis is feedback inhibited, resulting in more chorismate to be dedicated to PQS production (101, 103). Evaluation of the first model is the subject of Chapter 2, while the first attempts to evaluate the second model are the subject of Chapter 3.

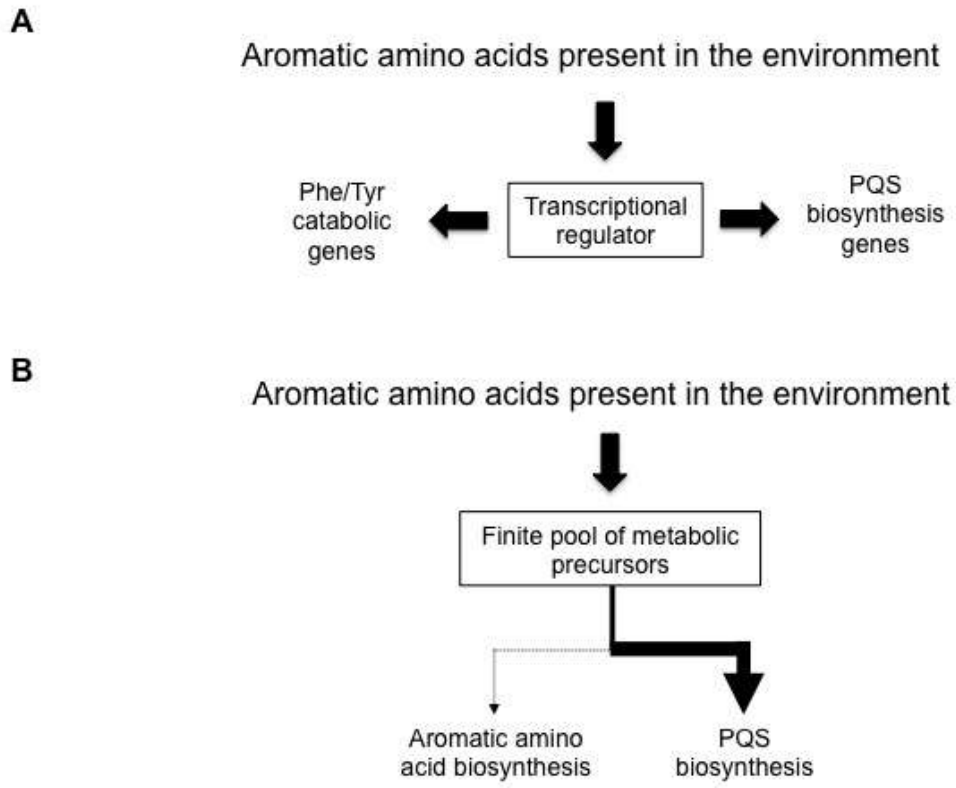

Figure 1.3 Models for enhanced PQS production in the presence of aromatic amino acids.

P. aeruginosa produces approximately five-fold more PQS in the presence of aromatic amino acids in CF sputum, and two models are proposed to explain this phenomenon. In the first model (A), PQS biosynthetic genes are co-regulated with genes for aromatic amino acid catabolism by an aromatic amino acid-responsive transcriptional regulator. In the second model (B), flux of a finite pool of shared metabolic precursors is altered in the presence of aromatic amino acids. When aromatic amino acids are present in the environment, their biosynthesis is feedback inhibited, allowing more of the shared precursor metabolites to be dedicated toward PQS production.

1.4 DISSERTATION OBJECTIVES

Many examples of nutritional cues promoting the pathogenesis of an organism have been identified, and the effect of aromatic amino acids on *P. aeruginosa* virulence is an excellent example of this. Now that this link has been established, it is important to better understand the physiology of the organism under these conditions, and knowledge gained about *P. aeruginosa* physiology in these infections could contribute to novel therapeutic strategies. The goal of my dissertation work has been to better understand the physiology of *P. aeruginosa* during lung infections by characterizing its response to nutrients critical to growth and pathogenesis in the CF lung.

This dissertation is comprised of 4 chapters. Chapter 1 is an overview of *P. aeruginosa* including a description of its virulence factors and their regulation in a density-dependent manner called quorum sensing. The role of aromatic amino acids in PQS production and *P. aeruginosa* virulence is also discussed, and the biosynthesis of PQS is described. Chapter 2 is a characterization of *P. aeruginosa's* response to aromatic amino acids via an aromatic amino acid responsive transcriptional regulator called PhhR. The PhhR regulon is delineated and direct regulation of PhhR-controled genes is demonstrated. Chapter 3 is an investigation of the two anthranilate synthase enzymes present in *P. aeruginosa.* Evidence that the second anthranilate synthase is likely the result of recent horizontal gene transfer is presented, and differential temporal expression of the two enzymes is presented as an explanation for their apparent lack of functional redundancy. Chapter 4 is a discussion of the conclusions from my studies of *P.*

aeruginosa metabolism and virulence, and I present several future directions that could be pursued based upon my work. A model for enhanced production of PQS in the presence of aromatic amino acids is described, and special attention is devoted to the use of degradative enzymes to modify the infection site as an antimicrobial strategy.

Chapter 2: Characterization of the *Pseudomonas aeruginosa* response to phenylalanine and tyrosine[1]

2.1 INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen commonly found in soil and water. It is notorious for causing persistent, chronic lung infections in individuals with the genetic disease cystic fibrosis (CF). A critical symptom of CF is a buildup of thick mucus (sputum) in the lungs, which inhibits the ability to clear invading pathogens (31). Additionally, sputum represents an excellent growth substrate for several bacteria including *P. aeruginosa* (55). Due to its natural resistance to most conventional antimicrobials, *P. aeruginosa* infections are particularly difficult to treat and are the leading cause of morbidity and mortality in individuals with CF (34).

To better understand the physiology of *P. aeruginosa* during growth in the CF lung, we previously developed a defined synthetic CF sputum medium (SCFM) that mimics the nutritional environment of CF sputum (101). Consistent with growth in authentic CF sputum, *P. aeruginosa* produces higher levels of the cell signaling molecule 2-heptyl-3-hydroxy-4-quinolone (Pseudomonas Quinolone Signal, PQS) in SCFM (101, 103). PQS is a quorum sensing signal (109) essential for production of a range of secreted virulence factors including phenazines and hydrogen cyanide (43). Interestingly, removal of aromatic amino

[1] This chapter was adapted from the following reference (99, Copyright © American Society for Microbiology, *Journal of Bacteriology*, 192**,** 2010, 2722-8, Doi: 10.1128/.

acids from SCFM decreased PQS production by approximately five-fold, implicating these amino acids as key mediators of enhanced PQS production in CF sputum (101). Further study revealed that the aromatic amino acids phenylalanine and tyrosine, but not tryptophan, were the primary inducers of PQS biosynthesis in SCFM (101, 103). While tryptophan has recently been reported to enhance production of PQS (37), this amino acid is likely not important in CF sputum as it is present at extremely low levels (~10 μM) (101).

In addition to enhancing PQS production, phenylalanine/tyrosine also serve as important carbon sources for *P. aeruginosa* in CF sputum (101). In *Pseudomonas putida*, the transcriptional regulator PhhR is required to induce genes encoding enzymes critical for catabolism of these amino acids (59, 60). Beyond regulation of genes involved in aromatic amino acids catabolism, PhhR regulates several other classes of genes in *P. putida* and has thus been described as a global transcriptional regulator (57). *P. aeruginosa* possesses a PhhR homolog (PA0873 in strain PAO1 and PA14_52980 in strain PA14) with 88% identity to *P. putida* PhhR that has been proposed to be critical for induction of phenylalanine catabolic genes (132).

The present study investigates the role of PhhR in mediating two important *P. aeruginosa* phenotypes observed during growth in CF sputum: catabolism of phenylalanine/tyrosine; and phenylalanine/tyrosine-mediated induction of PQS. Transcriptome analysis revealed that PhhR controls four putative transcriptional units involved in aromatic amino acid catabolism; however genes involved in PQS biosynthesis were unaffected. Promoters for

several identified genes were mapped, putative binding sites for PhhR were identified using *in silico* analysis, and the ability to specifically bind these promoter regions was determined using electrophoretic mobility shift assays (EMSAs).

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains and media

P. aeruginosa strain PA14 and the isogenic *phhR* mutant were obtained from the PA14 Non-Redundant Transposon Mutant Library (http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi; (81)). The transposon insertion site in *phhR* was confirmed by PCR. *P. aeruginosa* was routinely cultured on tryptic soy agar (69). *Escherichia coli* DH5α was used as the recipient for transformation and cultured on LB Miller broth/agar (Fisher Scientific). Cultures were grown at 37ºC with shaking at 250 rpm. Antibiotics were used at the following concentrations unless otherwise noted: ampicillin, 50 μg/ml for *E. coli*; carbenicillin, 300 μg/ml for plasmid selection and 25 μg/ml for plasmid maintenance in *P. aeruginosa*.

P. aeruginosa was also grown in a defined synthetic cystic fibrosis sputum medium (SCFM) (101). SCFM normally contains 0.8 mM tyrosine, 0.5 mM phenylalanine, and 10 µM tryptophan; however when SCFM without aromatic amino acids was used, equimolar serine replaced these amino acids. Serine was

chosen because it has been shown previously not to affect PQS production (101, 103). To evaluate growth of *P. aeruginosa* with tyrosine or phenylalanine as a carbon and energy source, a MOPS (morpholinepropanesulfonic acid)-buffered salts base (50 mM MOPS, pH 7.2, 93 mM NH_4Cl, 43 mM NaCl, 3.7 mM KH_2PO_4, 1 mM $MgSO_4$, 3.5 µM $FeSO_4 \cdot 7H_2O$, 2 mM proline) was supplemented with 10 mM tyrosine or 10 mM phenylalanine. Proline was added to reduce the considerable lag in growth commonly observed when phenylalanine/tyrosine is used as a sole carbon and energy source.

2.2.2 DNA manipulations

Standard methods were used to manipulate plasmids and DNA fragments (2). Restriction endonucleases and DNA modification enzymes were purchased from New England Biolabs. Chromosomal DNA from *P. aeruginosa* was isolated using DNeasy Tissue kits (Qiagen), and plasmid isolations were performed using QIAprep spin miniprep kits (Qiagen). DNA fragments were purified using QIAquick mini-elute PCR purification kits (Qiagen), and PCR was performed using the Expand Long Template PCR System (Roche).

2.2.3 PQS extractions

Overnight cultures of *P. aeruginosa* PA14 or the *phhR* mutant grown in SCFM were subcultured into fresh SCFM to an OD_{600nm} of 0.05. Cells were grown to exponential phase (OD_{600nm} of 0.45 to 0.55), pelleted by centrifugation

at 9,000 x g for 5 min, washed twice with carbon-free SCFM, and starved for 2 hours in carbon-free SCFM. Starved cultures were used to inoculate SCFM or SCFM without aromatic amino acids to an OD_{600nm} of 0.01. Cultures were allowed to reach near maximum growth yields (OD_{600nm} ~3) and extracted twice with an equal volume of acidified ethyl acetate (150 μL acetic acid per L ethyl acetate; Fisher). Extracts were dried under a constant stream of N_2 gas, resuspended in methanol (Fisher), and analyzed by thin layer chromatography (TLC) as described previously (86, 101, 109). PQS was identified by co-migration with 500 ng synthetic PQS standard. PQS on TLC plates was visualized by fluorescence after excitation with long wave UV light using a G:Box gel imager (Syngene).

2.2.4 Global expression profiling

For Affymetrix GeneChip analysis, *P. aeruginosa* PA14 was grown in SCFM or SCFM without aromatic amino acids and the *phhR* mutant was grown in SCFM. To prepare cells for RNA extraction, exponentially growing cells were diluted to an OD_{600nm} of 0.001 and allowed to grow to an OD_{600nm} of 0.35-0.45. Cultures were mixed 1:1 with RNALater (Ambion), and total RNA was isolated. DNA contamination within RNA samples was removed by DNase treatment (Promega) and monitored by PCR of the *rplU* gene as previously described (85, 101-103, 127). RNA integrity was monitored by agarose gel electrophoresis. cDNA synthesis from total RNA and cDNA fragmentation and labeling were performed as previously described (85, 101-103, 127). Processing of Affymetrix

P. aeruginosa GeneChips was performed at the University of Iowa DNA Facility. All experiments were performed in duplicate, and data were analyzed using GeneChip operating software version 1.4. Differentially regulated genes were identified by pair-wise comparisons (4 or 6 total) of all GeneChips ($P \leq 0.05$).

2.2.5 Complementation of the *P. aeruginosa phhR* mutant

The *phhR* gene was PCR amplified from *P. aeruginosa* PA14 chromosomal DNA using the primers *phhR*-comp-for (5'-TCCCCCGGGGAACGACAACACNNNNNCACGCC-3') and *phhR*-comp-rev (5'-TCCCCCGGGCCGCCGTTTCTTTCCCAGCCTG-3'). The *phhR*-comp-for primer was designed such that the native Shine-Dalgarno sequence of *phhR* was replaced with N_5. The resulting 1628 bp fragments were cloned into the pGEM-T Easy vector (Promega) per the manufacturer's instructions. Plasmids were isolated from 10 pooled white colonies and digested with SmaI to excise *phhR*. The *phhR* DNA fragments were gel-purified and then ligated into SmaI-digested pUCP18 (147). Resulting pUCP18 plasmids containing *phhR* were purified and transformed into the *P. aeruginosa phhR* mutant by $MgCl_2$ transformation (149). Transformants were screened for the ability to grow with tyrosine as a carbon source. One plasmid was identified that restored growth with tyrosine, pKP-phhR. The sequence of the *phhR* gene in pKP-phhR was confirmed by DNA sequencing at the University of Texas Institute for Cell and Molecular Biology DNA Facility. In pKP-phhR, *phhR* expression is controlled by a constitutive *lac* promoter.

2.2.6 Primer extension analysis

To obtain RNA for primer extension analyses, *P. aeruginosa* was grown to late exponential phase in SCFM (OD_{600nm} of 1.3) and mixed 1:1 with RNALater (Ambion). Total RNA was isolated using the RNeasy Mini kit (Qiagen), and DNA contamination and RNA integrity were monitored as described above for Affymetrix GeneChip analysis. Primer extension was performed using fluorescently (6-carboxyfluorescein) labeled primers as previously described (7, 82). Briefly, 1 μL of a 0.4 μM 5'-FAM (carboxyfluorescein) labeled primer was added to 20-30 μg total RNA in a final volume of 20 μL and incubated at 70ºC for 10 min. Reactions were quickly chilled in an ice-water bath then incubated at 65ºC for 20 min. The temperature was shifted to 42ºC, and reagents for cDNA synthesis were added (SuperScript II system (Invitrogen): 8 μL 5X buffer, 4 μL 0.1 M DTT, 4 μL 10 mM deoxyribonucleotides, 4 μL SuperScript II enzyme). Reactions were incubated at 42ºC for 2 h, ethanol precipitated, and submitted for DNA sizing analysis at the University of Oklahoma Health Sciences Center Laboratory for Genomics and Bioinformatics. Some reactions were treated with 1 μL RNase H (Invitrogen) at 37ºC for 20 min prior to precipitation. Primers used for primer extension are shown in Figure 2.2. When more than one fluorescence peak was present, the highest peak, which corresponds to the major primer extension product, was reported. Primer extension for each gene was performed at least twice.

2.2.7 PhhR purification

The *phhR* gene was PCR amplified from *P. aeruginosa* PA14 chromosomal DNA using the primers *phhR*-for-NdeI (5'-GGAATTCCATATGCGTATCAAAGTGCACTGCCAG-3') and *phhR*-rev-XhoI (5'-CCGCTCGAGTCAGCCCTCGCCTTGCCCCAC-3'). The resulting 1560-bp fragment was digested with NdeI/XhoI and ligated into pET15b (Novagen) to generate pKP501. This construct adds a 6-histidine tag (his_6) to the N-terminus of PhhR. For *E. coli* transformations, 1% glucose was added to growth media to suppress transcription of *phhR*. Sequence of the pKP501 *phhR* gene was confirmed at the University of Texas Institute for Cell and Molecular Biology DNA Facility. For over-expression of his_6-PhhR, the *E. coli* host strain BL21(DE3) (Novagen) was used. BL21(DE3) carrying pKP501 was subcultured from LB Miller broth supplemented with 100 mM glucose and ampicillin into fresh LB Miller broth with ampicillin. During exponential growth (OD_{600nm} of 0.65-0.85), 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce expression of his_6-*phhR*. Cultures were harvested for protein purification after 1 h incubation with IPTG.

To purify his_6-PhhR, cells were pelleted by centrifugation at 10,000 x *g* for 5 min and resuspended in 3 ml cold buffer A (25 mM potassium phosphate buffer, pH 7.4; 0.5 M NaCl; 5 mM dithiothreitol [DTT]; 20 mM imidazole). Cells were lysed by two passes through a French press at ~20,000 pounds per square inch at 4ºC, and the resulting lysate was centrifuged at 15,600 x g for 20 min to

pellet insoluble material. The supernatant was then passed over a 1 mL His-Trap HP column (GE Healthcare) equilibrated with cold buffer A. The column was washed twice with 3 ml cold buffer A, and protein bound to the column was eluted with 3 ml cold buffer B (25 mM potassium phosphate buffer, pH 7.4; 0.5 M NaCl; 5 mM DTT; 500 mM imidazole). All fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. Western blotting on nitrocellulose membranes with the Monoclonal Anti-Polyhistidine-Alkaline Phosphatase Conjugate Clone His-1 antibody (Sigma) and the Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega) was used to confirm the presence of his_6-PhhR in eluted fractions. Purified his_6-PhhR was desalted with an Amicon Ultra-4 Centrifugal Filter Device (10 kDa cut-off) by successive concentration and dilution in storage buffer (50 mM Tris•HCl, pH 7.4; 100 mM NaCl; 5 mM DTT; 10% glycerol) until the imidazole concentration was $\leq$ 10 mM. Protein concentrations were quantified with the Bio-Rad Protein Assay (Bio-Rad). Purified his_6-PhhR was stored in storage buffer at 4ºC and -80ºC.

2.2.8 Electrophoretic mobility shift assays (EMSAs)

Primers used to generate probes for EMSA are shown in Table 2.1. Probes were generated by PCR and gel purified. Probes (5-10 pmol each) were labeled with γ-^{32}P-ATP (Sigma-Aldrich) using the KinaseMax kit (Ambion) per the manufacturer's instructions. Unincorporated radiolabeled nucleotides were removed using NucAway Spin Columns (Ambion). Unlabelled probes targeting

intragenic regions of relevant genes were used as cold competitors in EMSAs (see below).

Table 2.1 Primers used for EMSA analysis.

Probe[a]	Primers	Probe size (bp)
phhA[b]	**GGGGTACCGACCAGCAGGTTGAGGATGTC**	**321**
	AACTGCAGGGGTCTTTGTTGTTGTCGTTGC	
phhA	CAGCTCGACGAGATCAACAGGG	510
	CAGGCGCTTGAGGTCGGGCAG	
hpd	**CCGTCGCGGAGTAAAGACGCAG**	**266**
	GCCGGCGACATGGGAGATGCC	
hpd	GGAAGGCATCGGCGGTTCGC	500
	CGAGCGGCTCGCCATGCCCG	
dhcA	**GCCACGGCGAGGAAGGCGC**	**130**
	GCAGTCTGACTGCCGGGTCG	
dhcA	GATTCCCGAGAACCTGATCGCG	513
	GTCGGGTCCAGTTCGCCGGG	
hmgA	**CTCGCGCCCCAGCGAGTAATG**	**352**
	GGACCTGGCACGCTGGCTGC	
hmgA	CAGTACCTGGCCAACCGCTCG	498
	GGTCAGCACGGTGAAGATCGAC	

[a] Primers used to amplify promoter probes for EMSA analysis are shown in bold text; primers used for non-specific competitor probes from intragenic regions are shown in plain text.

[b] Primers used to amplify the *phhA* promoter contained recognition sites for restriction endonucleases (underlined).

For EMSA, probes (10^4 counts per minute (cpm)) were incubated with varying concentrations of his_6-PhhR (0, 100, 250, and 500 nM) in 1X DNA binding buffer (20 mM Tris•HCl, pH 7.5; 50 mM KCl; 1 mM EDTA; 1 mM dithiothreitol; 2% glycerol; 100 μg/mL bovine serum albumin; 10 μg/mL poly(dI•dC); modified from ref. (128)). For each cold competition reaction, a 20- (*hpd* and *dhcA*) or 50- (*hmgA* and *phhA*) fold molar excess of unlabeled probe was added to the binding reaction. Competitions were performed with 100 (*hpd)*, 250 (*dhcA*), or 500 (*phhA* and *hmgA*) μM his_6-PhhR. The *hmgA* promoter binding reaction included 100 μM each phenylalanine and tyrosine; all other reactions did not contain free amino acids. EMSA reactions were incubated at 30ºC for 30 min prior to separation on 5% native polyacrylamide gels. Gels were pre-run at 80V for 1 h prior to loading EMSA reactions. Gels were dried and exposed to phosphorimager screens overnight, and ^{32}P-labeled bands were visualized with the Personal Molecular Imager (BioRad) or Storm 860 imaging system (GE Healthcare Life Sciences).

2.2.9 Microarray accession number

The microarray data have been deposited in the EMBL-EBI data bank (www.ebi.ac.uk/miamexpress) under experiment accession number E-MEXP-2593.

2.3 Results and Discussion

2.3.1 The *P. aeruginosa* transcriptional response to phenylalanine and tyrosine

Previous work from our laboratory demonstrated that aromatic amino acids within CF sputum serve not only as carbon and energy sources but also enhance synthesis of the cell signaling molecule PQS (101, 103). Although these studies provided new insight into the *P. aeruginosa* response to nutritional cues, it was not clear if phenylalanine and tyrosine provoke other phenotypic responses. To begin to address this question, the global transcriptional response of *P. aeruginosa* to aromatic amino acids was assessed using Affymetrix GeneChips. For these experiments, *P. aeruginosa* was grown in a synthetic CF sputum medium (SCFM) designed to mimic the nutritional conditions of sputum from the CF lung. Twenty-two genes were differentially expressed greater than four-fold when grown in SCFM as compared to SCFM in which the aromatic amino acids had been removed (Table 2.2). As expected, genes involved in biosynthesis and response to PQS as well as genes important for catabolism of phenylalanine/tyrosine were highly induced in the presence of aromatic amino acids.

Table 2.2 Gene expression with aromatic amino acids and in the *phhR* mutant

ORF[a]	Gene[a]	Function or Class[a]	Aromatic aa[b]	*phhR* mutant[c]
PQS biosynthesis and response				
PA0996	*pqsA*	Coenzyme A ligase	5	NC
PA0997	*pqsB*	Predicted beta-keto-acyl-acyl-carrier synthase	10	NC
PA0998	*pqsC*	Predicted beta-keto-acyl-acyl-carrier synthase	13	NC
PA0999	*pqsD*	3-oxoacyl-[acyl-carrier-protein] synthase III	11	NC
PA1000	*pqsE*	Quinolone signal response protein	10	NC
PA1001	*phnA*	Anthranilate synthase component I	22	NC
PA1002	*phnB*	Anthranilate synthase component II	10	NC
Aromatic amino acid catabolism				
PA0865	*hpd*	4-hydroxyphenylpyruvate dioxygenase	31	-47
PA0866	*aroP2*	Aromatic amino acid transport protein	10	-12
PA0870	*phhC*	Aromatic amino acid aminotransferase	11	-4
PA0871	*phhB*	Pterin-4-alpha-carbinolamine dehydratase	4	-2
PA0872	*phhA*	Phenylalanine-4-monooxygenase	16	-4
PA0873	*phhR*	Transcriptional regulator	4	NT
PA1999	*dhcA*	Dehydrocarnitine CoA transferase, subunit A	4	-30
PA2000	*dhcB*	Dehydrocarnitine CoA transferase, subunit B	4	-31
PA2001	*atoB*	Acetyl-CoA acetyltransferase	8	-18
PA2002	*atoE*	Conserved hypothetical protein	2	-7
PA2006		Probable MFS transporter	5	-8
PA2007	*maiA*	Maleylacetoacetate isomerase	8	-9
PA2008	*fahA*	Fumarylacetoacetase	12	-12
PA2009	*hmgA*	Homogentisate 1,2-dioxygenase	12	-12
Other genes				
PA2393		Probable dipeptidase precursor	-7	NC
PA4033		Hypothetical protein	-5	NC

[a] From the *P. aeruginosa* genome website; www.pseudomonas.com

[b] Fold regulation of genes differentially expressed during *P. aeruginosa* growth in SCFM containing aromatic amino acids medium as compared to SCFM in which the aromatic amino acids have been replaced with serine; a positive number indicates an up-regulation of the gene during growth with aromatic amino acids. The generation times of wt *P. aeruginosa* with and without aromatic amino acids were similar (data not shown).

[c] Fold regulation of genes differentially expressed in wt *P. aeruginosa* as compared to the isogenic *phhR* mutant. Bacteria were grown in SCFM and displayed similar growth rates. A negative number indicates a down-regulation of the gene in the *phhR* mutant. NC indicates no change in mRNA levels as determined by GeneChip Operating Software version 1.4. NT indicates that the gene could not be tested due to insertion of the MAR2xT7 mariner transposon in *phhR*. The generation times of wt *P. aeruginosa* and the *phhR* mutant in SCFM were similar (data not shown).

2.3.2 Determining the PhhR regulon

In addition to genes for PQS biosynthesis and phenylalanine and tyrosine catabolism, *phhR* was also induced in the presence of aromatic amino acids (Table 2.2). PhhR has been implicated as a phenylalanine/tyrosine-responsive transcriptional regulator critical for induction of phenylalanine/tyrosine catabolic genes (132). Based on these data, we hypothesized that PhhR was the transcriptional regulator mediating differential expression of the aromatic amino acid responsive genes observed in Table 2.2. To test this hypothesis, global expression profiling of wild-type (wt) *P. aeruginosa* and the isogenic *phhR* mutant was performed in SCFM. Twelve genes were down-regulated greater than four-fold in the *phhR* mutant as compared to the wild-type strain (Table 2.2). All of the genes down-regulated in the *phhR* mutant are putatively involved in phenylalanine/tyrosine degradation to fumarate and acetyl-CoA (Figure 2.1A) or in amino acid transport. Of note, we propose that DhcA and DhcB constitute the acetoacetyl-CoA transferase (Figure 2.1A), as these proteins share high similarity with the AtoD (64% similarity, E value $<10^{-43}$ using BLASTp) and AtoA (62% similarity, E value $<10^{-48}$ using BLASTp) components of this enzyme from the *E. coli* acetoacetate degradation pathway respectively. Recent observations that *dhcA* and *dhcB* likely comprise a CoA transferase required for growth with carnitine further support this designation (146). The carnitine catabolic intermediate 3-dehydrocarnitine and acetoacetate are structurally similar, both being 3-ketoacids, differing only by the presence of a tri-methylated amine on carbon four of 3-dehydrocarnitine. Finally, isogenic *dhcA* and *dhcB* mutants reached only 28% and 30%, respectively, of the final growth yield of wild-type *P.*

aeruginosa when grown with phenylalanine as the major carbon and energy source (data not shown), which strongly suggests these genes are involved in the complete degradation of phenylalanine/tyrosine to fumarate and acetyl-CoA (Figure 2.1A).

2.3.3 PhhR regulates phenylalanine/tyrosine catabolism but not PQS biosynthesis

Interestingly, genes involved in PQS biosynthesis were not differentially regulated in the *phhR* mutant (Table 2.2) supporting the hypothesis that while PhhR is likely important for catabolism of phenylalanine/tyrosine, it is not responsible for aromatic amino acid-mediated increases in PQS production. To test this hypothesis, the ability of aromatic amino acids to support growth and stimulate PQS production was assessed for wt *P. aeruginosa* and the *phhR* mutant. As expected, the *P. aeruginosa phhR* mutant demonstrated poor growth with phenylalanine and tyrosine as the major carbon and energy source (Figure 2.1B); however, growth with tryptophan was unaffected (data not shown). Similar to wt *P. aeruginosa*, the *phhR* mutant demonstrated increased PQS production during growth with aromatic amino acids (Figure 2.1C). These results indicate that PhhR is an aromatic amino acid responsive transcriptional regulator that controls – either directly or indirectly -- genes involved in phenylalanine/tyrosine catabolism but not PQS production.

Initial attempts to genetically complement the *phhR* mutant using a variety of methods, including heterologous inducible promoters, were unsuccessful. We

reasoned this may be due to improper expression of PhhR. To overcome this problem, the *phhR* Shine-Dalgarno sequence (ribosome binding site) was randomized during PCR amplification by incorporating N_5 into the 5' amplification primer in place of the native *phhR* Shine-Dalgarno sequence, CGGGC. Amplicons were ligated into the multi-copy plasmid pUCP18 and transformed into *E. coli*. Plasmids were then pooled from the resulting *E. coli* transformants and transformed into the *P. aeruginosa phhR* mutant. Plasmids from *P. aeruginosa phhR*$^-$ transformants that grew with tyrosine as a carbon source were selected for DNA sequencing. This approach yielded a plasmid (pKP-*phhR*) containing *phhR* with a Shine-Dalgarno sequence of GTGCT. The new sequence is likely less favorable to ribosome binding, resulting in tolerable levels of PhhR in the cells. Introduction of pKP-phhR into the *P. aeruginosa phhR* mutant restored growth with phenylalanine and tyrosine as the primary carbon source (Figure 2.1B).

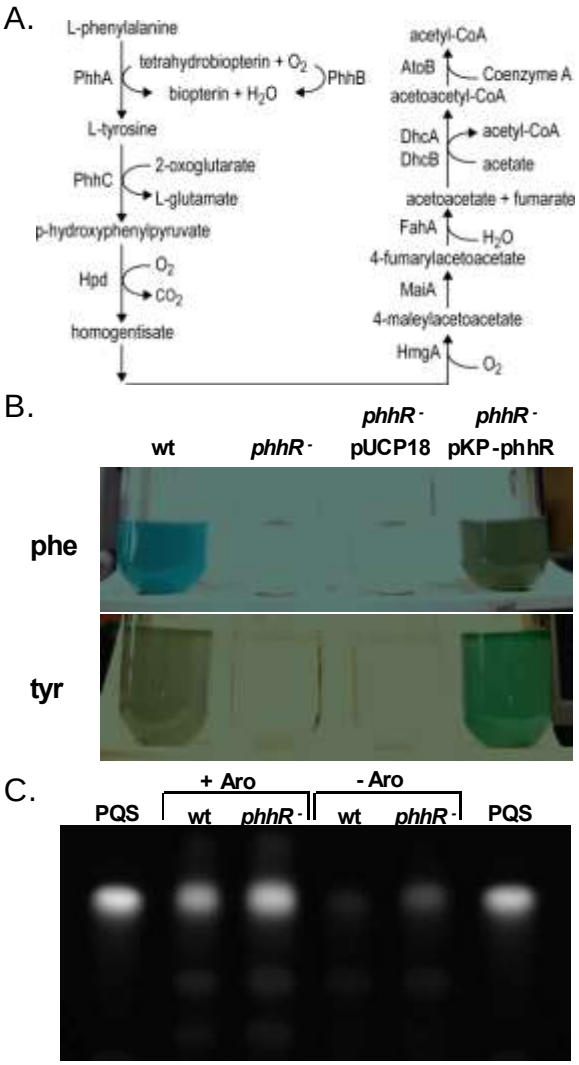

Figure 2.1 PhhR is critical for tyrosine/phenylalanine catabolism

(A) Predicted pathway of phenylalanine and tyrosine catabolism in *P. aeruginosa* (from the Pseudomonas Genome Database, www.pseudomonas.com). The acetoacetate catabolic pathway is from the Encyclopedia of *E. coli* K-12 Genes and Metabolism (www.ecocyc.org). *PhhA*, phenylalanine-4-hydroxylase; PhhB, pterin-4-α-carbinolamine dehydratase; PhhC, aromatic amino acid aminotransferase; *Hpd*, 4-hydroxyphenylpyruvate dioxygenase; *HmgA*, homogentisate-1,2-dioxygenase; MaiA, maleylacetoacetate isomerase; FahA, fumarylacetoacetase; AtoD and AtoA, acetoacetyl-CoA transferase; AtoB, acetyl-CoA transferase. (B) PhhR is required for *P. aeruginosa* growth with phenylalanine (phe) and tyrosine (tyr) as a carbon and energy source. Shown from left to right are: wt *P. aeruginosa*; *phhR*$^-$; *phhR*$^-$ carrying the plasmid pUCP18; *phhR*$^-$ carrying the complementation plasmid pKP-*phhR*. (C) PhhR is not required for PQS stimulation by aromatic amino acids. Thin layer chromatography of ethyl acetate extractions from wt *P. aeruginosa* and the *phhR* mutant grown in the presence (+ Aro) or absence (- Aro) of aromatic amino acids and visualized by fluorescence. Increased PQS levels in the *phhR* mutant were not consistently observed. Chemically synthesized PQS is provided as a standard (99).

2.3.4 Mapping promoters of PhhR-regulated genes

In silico analysis revealed that the PhhR-regulated phenylalanine/tyrosine catabolic genes are arranged into four operons. To identify important transcription regulatory DNA elements, promoter regions from these four transcriptional units were mapped using primer extension (Figure 2.2 and Table 2.3). To map the transcriptional start sites, a non-radioactive primer extension assay previously used to map *Helicobacter pylori* and *P. aeruginosa* transcriptional start sites was employed (7, 82). Briefly, a fluorescently labeled primer homologous to the gene of interest was used to generate cDNA from total cellular RNA. The size and quantity of the cDNA product was determined using a standard DNA sequencer and used to map the transcriptional start site of the gene of interest (Table 2.3). This analysis revealed that: *phhA* possesses a transcriptional start site located 48 bp upstream of the translational start codon (Figure 2.2A and Table 2.3); *hpd* possesses a transcriptional start site located 64 bp upstream of the translational start codon (Figure 2.2B and Table 2.3); *dhcA* possesses a transcriptional start site located 56 bp upstream of the translational start codon (Figure 2.2C and Table 2.3); and *hmgA* possesses a transcriptional start site located 88 bp upstream of the translational start codon (Figure 2.2D and Table 2.3).

Table 2.3 Summary of primer extension data.

Gene	cDNA fragment size (bp)	Peak height[a]	Distance from translational start site
phhA	108	310	-48
hpd	125	178	-64
dhcA	95	119	-56
hmgA	152	96	-88

[a] Fluorescent peaks were identified using Peak Scanner software (Applied Biosystems) and peak heights are reported in fluorescence units.

Promoter regions of *phhA*, *hpd*, *hmgA*, and *dhcA* were subjected to *in silico* analyses to identify putative regulatory DNA sequences. A consensus DNA binding site has been proposed for *P. putida* PhhR (TGTAAAGATAGTTTTACA) (57) and the *E. coli* PhhR homolog TyrR (TGTAAA-N_6-TTTACA) (110). In addition, two potential PhhR binding sites have previously been mapped to the *phhA*-*phhR* intergenic region of a non-PA14 *P. aeruginosa* strain (132). Examination of the *phhA* promoter region in *P. aeruginosa* PA14 revealed that these two sites are conserved in this strain, centered at -86 and -159 bp respective to the *phhA* transcriptional start site (Figure 2.2A). Putative PhhR binding sites were also identified upstream of the *hpd* (-138 bp) (104) and *dhcA* (-40 bp) transcriptional start sites (Figure 2.2B and 2.2C); however unlike *P. putida* (57), a DNA sequence similar to the consensus PhhR and TyrR binding sites was not identified in the *hmgA* promoter (Figure 2.2D).

PhhR has been proposed to modulate transcription from σ^{54} and σ^{70} dependent promoters (59, 60, 132). Both σ^{54} and σ^{70} DNA binding sites have been identified on the *P. aeruginosa* chromosome between *phhR* and *phhA* (132), and PhhA production is σ^{54}-dependent in *P. aeruginosa* (132). σ^{54} binding sites in promoters of interest can be difficult to identify *in silico*. σ^{54}-dependent genes generally possess a conserved GG-N_{10}-GC sequence centered from -24 to -12 bp relative to their transcriptional start sites, although this may vary between promoters (5). For some σ^{54}-dependent promoters, the conserved GG-N_{10}-GC sequence is not centered at -24 to -12 but is instead shifted several bases upstream or downstream (5). Of particular note is the *algD* promoter of *P. aeruginosa*, which has a GG-N_{10}-GC sequence centered at -34 to -22 (151). In

addition, while the GG and GC sequences are the most highly conserved sites in the consensus σ^{54}-binding sequence (5), these sites can be variable. Examination of the promoter sequences revealed putative σ^{54}-binding sites upstream of *phhA*, *hpd*, and *dchA* but not *hmgA* (Figure 2.2A-D).

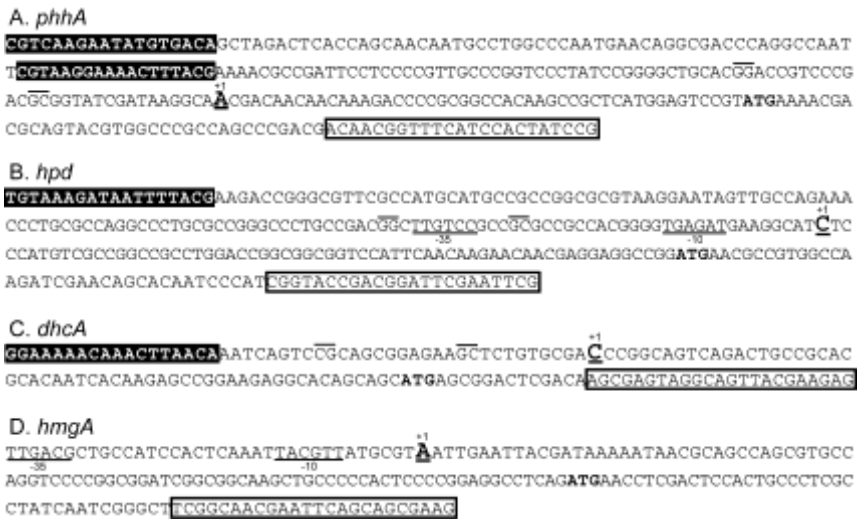

Figure 2.2 Promoter architecture of PhhR-controlled genes.

Primer extension analysis was used to map transcriptional start sites (Table 2.2). Shown are the promoter sequences for four PhhR-dependent transcriptional units: (A) *phhA*, (B) *hpd*, (C) *dhcA*, and (D) *hmgA*. The target DNA sequence of the primer extension oligonucleotide primer is boxed. Transcriptional start sites are indicated in large, bold, underlined letters (+1). Putative σ^{70} promoters containing homology to the canonical -10 and -35 sequences are indicated by single underlines. Putative σ^{54} promoters are indicated by overlines. Proposed PhhR binding sequences are included within black boxes (99).

2.3.5 PhhR directly regulates genes involved in phenylalanine/tyrosine catabolism

To examine if PhhR binds directly to the promoter regions of PhhR-regulated genes, *P. aeruginosa phhR* was cloned into an expression vector (pET15b) to create N-terminal his_6-tagged PhhR. Affinity purification using a nickel column resulted in nearly pure his_6-PhhR with a prominent band at approximately 58-kDa on Coomassie stained SDS-PAGE (Figure 2.3). Binding of his_6-PhhR to the *phhA*, *hpd*, *hmgA*, and *dhcA* promoters was characterized by electrophoretic mobility shift assays (EMSAs). DNA fragments containing promoter regions were generated by PCR (Figure 2.4A-D) and 5'-labeled with ^{32}P for use as EMSA probes. Each probe was incubated with increasing concentrations of PhhR, and was submitted to competition with non-radioactive (cold) excess specific and non-specific competitor DNA to confirm specificity of PhhR binding.

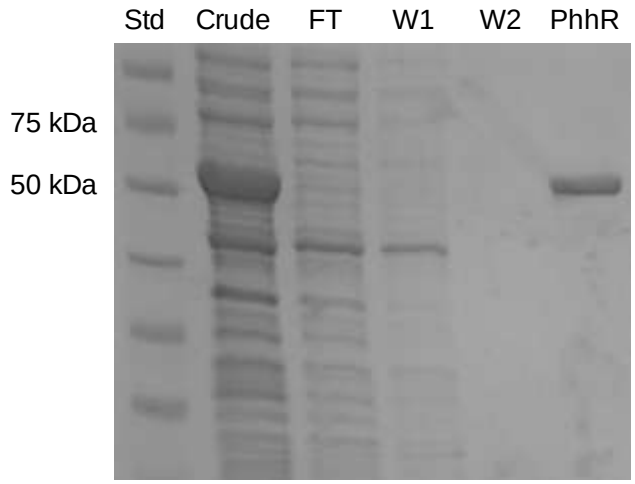

Figure 2.3 Purification of his_6-PhhR.

His_6-PhhR was purified using a HisTrap nickel column, separated on a 10% SDS-PAGE gel, and visualized after staining with Coomassie blue. The lanes contained: molecular weight markers (Std); soluble crude extract (Crude); column flow-through after soluble cell extract addition (FT); first wash with Buffer A containing 20 mM imidazole (W1); second wash with Buffer A containing 20 mM imidazole (W2); elution of his_6-PhhR with Buffer B containing 500 mM imidazole (PhhR). Protein eluted from the Buffer B wash (PhhR) was desalted and used for EMSA (99).

Purified his_6-PhhR bound and retarded mobility of the *phhA*, *hpd*, and *dhcA* promoters in the absence of aromatic amino acids, and addition of specific, but not non-specific cold competitor DNA eliminated this binding (Figure 2.4A-C). These results suggest that PhhR is a direct regulator of the *phhA*, *hpd*, and *dhcA* operons. The observation that purified his_6-PhhR bound to the *phhA*, *hpd*, and *dhcA* promoters in the absence of aromatic amino acid ligands is not unexpected as *P. putida* PhhR binds target promoters *in vitro* in the absence of amino acid inducers (57). Of note, regulation of the *dhcA* operon may be more complex as it was recently reported to also be under control of the divergently transcribed transcriptional regulator DhcR, although direct binding of this regulator to the *dhcA* promoter was not examined (146).

We could not detect binding of his_6-PhhR to the *hmgA* promoter (Figure 2.4D) even upon addition of phenylalanine and tyrosine to the binding reaction. The *P. aeruginosa hmgA* promoter does not possess a DNA sequence with high homology to a PhhR binding sequence (Figure 2.2). This is in contrast to the *hmgA* promoter in *P. putida*, which contains a functional PhhR binding sequence (57). How could *P. aeruginosa* PhhR regulate *hmgA* transcription without binding the promoter? Clues were provided by a recent study demonstrating that the transcriptional regulator HmgR also controls *hmgA* transcription in *P. putida* (1). HmgR binds and represses transcription of the *hmgA* promoter unless it is bound to its ligand, homogentisate. Homogentisate is produced during catabolism of phenylalanine/tyrosine by the enzyme Hpd (Figure 2.1A). The *P. aeruginosa phhR* mutant produces significantly less *hpd* mRNA (Table 2.2) suggesting that this mutant likely produces very little intracellular homogentisate even in the

presence of phenylalanine/tyrosine. The low levels of homogentisate would not allow de-repression of HmgR, thereby resulting in lower levels of *hmgA* mRNA in the *phhR* mutant. In support of this hypothesis, *P. aeruginosa* possesses an HmgR homolog (PA2010 in strain PAO1 and PA14_38500 in strain PA14) immediately upstream of *hmgA*, and the *hmgA* promoter possesses a putative HmgR binding site (1).

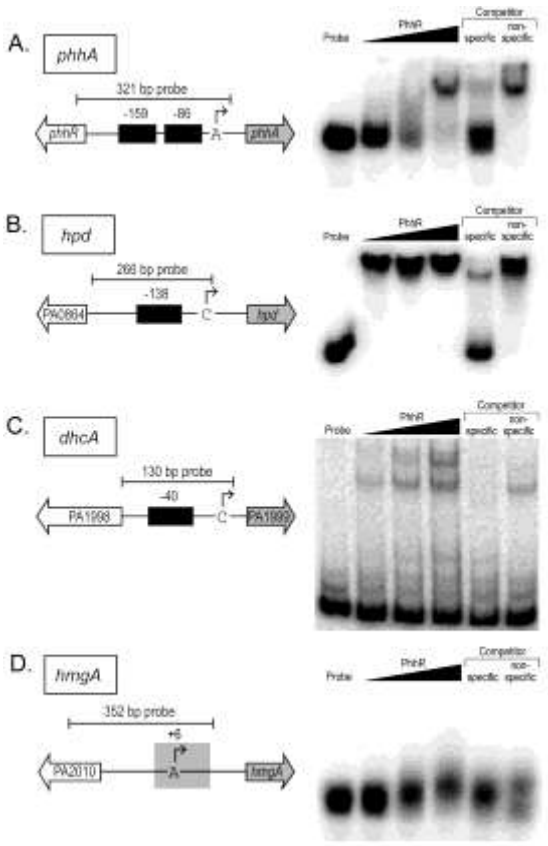

Figure 2.4 His_6-PhhR binds to the promoter regions of phenylanine/tyrosine catabolic genes.

(Left) Diagrammatic representation of the (A) *phhA*, (B) *hpd*, (C) *dhcA*, and (D) *hmgA* promoters. Line arrows indicate transcriptional start sites (Table 2.2) and block arrows represent ORFs. Putative PhhR binding sites are shown as black boxes, and the gray box in (D) represents a putative HmgR binding site. Numbers above putative binding sites represent the location of the center of the binding site relative to the transcriptional start site. The locations of promoter probes used for EMSA are also shown. Figure is not drawn to scale. (28) EMSA analysis of his_6-PhhR binding to (A) *phhA*, (B) *hpd*, (C) *dhcA*, and (D) *hmgA*. The lanes contained: ^{32}P-labeled promoter probe alone (probe); ^{32}P-labeled probe with 100 nM (lane 2), 250 nM (lane 3), or 500 nM his_6-PhhR (lane 4); ^{32}P-labeled probe with 100-500 nM his_6-PhhR and 20-50 fold molar excess unlabeled probe (specific); ^{32}P-labeled probe with 100-500 nM his_6-PhhR and 20-50 fold molar excess unlabeled non-specific DNA competitor (non-specific) (99).

2.3.6 Discussion and conclusion

The goal of this study was to expand on previous work from our laboratory demonstrating that aromatic amino acids within CF lung secretions (sputum) serve not only as carbon and energy sources but also to enhance PQS synthesis. This study provides evidence that PhhR controls genes important for catabolism of phenylalanine and tyrosine. The role of PhhR in regulation of phenylalanine catabolic genes is reminiscent of *P. putida* PhhR (57); however, it is intriguing that in contrast to *P. putida*, *P. aeruginosa* PhhR does not control expression of aromatic amino acid biosynthesis genes (57). This study also provides evidence that PhhR is not critical for induction of PQS biosynthetic genes. Instead, the data support a previous model of enhanced PQS production in which the presence of phenylalanine/tyrosine allows increased flux of the common biosynthetic precursor chorismate away from aromatic amino acid production and into PQS biosynthesis (101).

Chapter 3: Density-dependent expression of a *P. aeruginosa* anthranilate synthase promotes metabolite sequestration

3.1 INTRODUCTION

The Gram-negative, opportunistic pathogen *Pseudomonas aeruginosa* is a primary constituent of chronic, polymicrobial infections in the lungs of individuals with the genetic disease cystic fibrosis (CF) (24, 55). Because it is intrinsically resistant to many conventional antimicrobial therapies, *P. aeruginosa* is generally the most challenging pathogen to eradicate from these infections, and is the leading cause of morbidity and mortality for individuals with CF (34, 63). *P. aeruginosa* utilizes numerous virulence factors to colonize and persist in the CF lung, and many of those virulence factors are regulated in a density-dependent manner through a process called quorum sensing (QS) (25, 120, 126, 148). In a canonical Gram-negative QS system, a small molecule called an autoinducer is synthesized constitutively, and the concentration of autoinducer increases as cell density increases. Upon reaching a threshold concentration, autoinducers interact with LuxR-type transcriptional regulators to alter gene expression. *P. aeruginosa* has three known QS systems: two require the production of acyl homoserine lactone autoinducers, and one utilizes quinolone signals (109, 126). The most potent quinolone signal is the Pseudomonas Quinolone Signal (2-heptyl-3-hydroxoy-4-quinlone; PQS) which regulates several

genes critical for virulence (25, 42). PQS signaling is likely relevant in the CF lung, as *P. aeruginosa* produces five times more PQS when grown in CF lung fluids (sputum) compared to a standard laboratory medium (103). Using a defined synthetic CF sputum medium that mimics the nutritional contents of sputum, our laboratory determined that phenylalanine and tyrosine are responsible for enhanced PQS production in CF sputum (101). Subsequent work demonstrated aromatic amino acid induction of PQS production is not due to co-regulation of phenylalanine/tyrosine catabolism and PQS biosynthesis (99).

Our favored model for enhanced PQS production in the presence of phenylalanine and tyrosine involves flux of the central metabolite chorismate, a shared metabolic precursor for aromatic amino acid and PQS biosynthesis (99, 101). Chorismate is converted to anthranilate by anthranilate synthase in the first step of PQS biosynthesis. However, degradation of tryptophan via the products of the *kynABU* operon has also been reported to be a significant source of anthranilate (37). Our measurements of CF sputum have indicated extremely low tryptophan levels (<10 μM) in the CF lung. Thus, chorismate-mediated synthesis of anthranilate for PQS production is likely the relevant biosynthetic scheme in the CF lung (101).

It was discovered in 1990 that *P.* aeruginosa possess two functional anthranilate synthases, each comprised of a large and small subunit encoded by

the products of the *trpE* and *trpG* or *phnA* and *phnB* genes, respectively (35, 36). Interestingly, early evidence indicated that these enzymes are not functionally redundant, as growth experiments using a marked *trpE* mutant demonstrated tryptophan auxotrophy while a similar *phnA* mutant did not (35, 36). The authors also observed that the *phnA* mutant was deficient in production of the virulence factor pyocyanin while the *trpE* mutant was not (35, 36). At the time it was believed that PhnAB generated anthranilate for pyocyanin production, but later work determined that anthranilate is instead a precursor for PQS production, which is required to induce genes important for pyocyanin biosynthesis (15, 43, 89). These observations led to the hypothesis that TrpEG generates anthranilate for tryptophan biosynthesis while PhnAB generates anthranilate for PQS biosynthesis. However, a mechanism explaining the lack of crosstalk between these pathways has not yet been determined.

In the present work, we investigate the two *P. aeruginosa* anthranilate synthases, TrpEG and PhnAB, in order to better characterize their roles in physiology and pathogenesis and determine why anthranilate does not appear to be shared between the tryptophan and PQS biosynthetic pathways. The phylogenetic relationship of the two anthranilate synthases is analyzed and previously reported tryptophan auxotrophy and PQS production phenotypes are confirmed in unmarked *trpE* and *phnAB* deletion mutants, respectively. We demonstrate that over-expression of the two enzymes forces crosstalk between

tryptophan and PQS biosynthesis pathways, and present direct evidence for differential expression of *trpEG* and *phnAB* as an explanation for their lack of redundancy.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and media

Strains and plasmids used in this work are listed in Table 3.1. Luria-Bertani (LB) broth (Fisher) was used for growth of *E. coli* and *P. aeruginosa* for molecular cloning and genetic manipulations. For growth and physiology experiments, *P. aeruginosa* cultures were grown in a MOPS (morpholinepropanesulfonic acid) minimal medium, a MOPS-buffered salts base (50 mM MOPS, pH 7.2, 93 mM NH_4Cl, 43 mM NaCl, 3.7 mM KH_2PO_4, 1 mM $MgSO_4$, 3.5 μM FeSO4 heptahydrate) supplemented with 20 mM succinate as a sole source of carbon and energy. When necessary for growth of tryptophan auxotrophic strains (see Table 3.1), MOPS minimal medium was additionally supplemented with 200 μM tryptophan unless otherwise stated. MOPS buffer was used to wash and starve cells and is comprised of MOPS minimal medium without a carbon source. Tetracycline concentrations for *E. coli* and *P. aeruginosa* were 10 and 50 μg/mL for selection and 5 and 25 μg/mL for maintenance, respectively. Gentamicin concentrations for *P. aeruginosa*

containing pJN105-derived plasmids were 50 μg/mL for selection and 25 μg/mL for maintenance. Growth conditions were 37°C shaking at 250 rpm.

Table 3.1 Strains and plasmids

Strain or plasmid	Description[a]
Strains	
E. coli	
DH5α	Wild-type strain for molecular cloning (121)
SM10	Conjugation strain for deletion mutant generation (23)
P. aeruginosa	
PA14	Wild-type *Pseudomonas aeruginosa* strain (81)
Δ*trpE*	*trpE* clean deletion mutant in PA14 background
Δ*kynA*	*kynA* clean deletion mutant in PA14 background
Δ*phnAB*	*phnAB* clean deletion in PA14 background
Δ*trpE* Δ*kynA*	*trpE* and *kynA* double clean deletions in PA14 background
Δ*trpE* Δ*phnAB*	*trpE* and *phnAB* double clean deletions in PA14 background
Δ*trpE* Δ*pqsA*	*trpE* and *pqsA* double clean deletions in PA14 background
Plasmids	
pEX18Tc	gene replacement vector, Tc^R (61)
pJN105	*araC-pBAD* expression vector, Gm^R (95)
pGPKO-*trpE*	pEX18-derived *trpE* deletion vector, Tc^R
pGPKO-*kynA*	pEX18-derived *kynA* deletion vector, Tc^R
pGPKO-*phnAB*	pEX18-derived *phnAB* deletion vector, Tc^R
pGPKO-*pqsA*	pEX18-derived *pqsA* deletion vector, Tc^R
pGP-*trpEG*	pJN105-derived *trpEG* over-expression vector, Gm^R
pKP-*phnAB*	pJN105-derived phnAB over-expression vector, Gm^R

[a] All mutant strains and plasmids were generated in this study unless otherwise referenced.

3.2.2 DNA manipulations

Standard methods were utilized for handling DNA fragments and plasmids (3). Restriction endonucleases and other DNA-modifying enzymes were purchased from New England Biolabs. Genomic DNA preparations were performed using DNeasy Tissue kits (Qiagen). Plasmid DNA preparations were performed using a GeneJet Plasmid Miniprep kit (Fermentas), and purification of DNA fragments was done using a GeneJet PCR Purification kit (Fermentas). PCR was performed using the Expand Long Template PCR System (Roche). DNA sequencing to confirm newly generated constructs was performed at the University of Texas at Austin Institute for Cell and Molecular Biology DNA core facility.

3.2.3 Anthranilate synthase phylogenetic analysis

The amino acid sequences for *P. aeruginosa* PA14 PhnA and TrpE were obtained from http://www.pseudomonas.com and used to probe the database of non-redundant protein sequences at http://blast.ncbi.nlm.nih.gov with BLAST. Sequences for the top 30 hits from unique species were aligned using the MEGA 5 muscle alignment feature and phylogenetic trees were generated using the MEGA 5 maximum likelihood tree generator with 100 bootstrap replicates (139).

3.2.4 Generation of deletion mutants

Unmarked deletion mutants were constructed by allelic exchange as described previously with some modifications (61). To generate deletion constructs, ~1000bp regions upstream and downstream of the desired locus to be deleted were PCR amplified using *P. aeruginosa* PA14 DNA as template and primers listed in Table 3.2. Amplicons were concatenated by overlap extension PCR, digested with EcoRI and XbaI or KpnI and XbaI (see underlined restriction sites in Table 3.2), and ligated into pEX18Tc to generate the deletion vectors listed in Table 3.1. Deletion constructs were conjugated into PA14, or a previously generated mutant strain for double mutants, using the conjugation-competent *E. coli* strain SM10. Mutants were selected by first growth on LB plates containing 50 μg/mL tetracycline and 25 μg/mL nalidixic acid for merodiploid transconjugants. Subsequent growth on LB plates containing 10% sucrose was used to select for excision of the integrated plasmid with the desired deletion locus. Deletions were confirmed by PCR using confirmation primers outside the amplified flanking regions (Table 3.2) as well as phenotypic characterization for tryptophan auxotrophy (Δ*trpE,* Δ*trpE* Δ*phnAB*, Δ*trpE* Δ*pqsA*), PQS production (Δ*phnAB*, Δ*trpE* Δ*phnAB*, Δ*trpE* Δ*pqsA*), and ability to grow on tryptophan (Δ*kynA*, Δ*trpE* Δ*kynA*).

Table 3.2 Primers for deletion/expression vectors, mutant confirmation, and RT-PCR

Description	Sequence
Deletion	
trpE 1 for	CTGAATTCGTCAACGTCAAGAACATCCGTGAG
trpE 1 rev	GTCCAGCCGAGCACAAAAAAGGCATCGGAGGAGAGAACGGTCAAC
trpE 2 for	GTTGACCGTTCTCTCCTCCGATGCCTTTTTTGTGCTCGGCTGGAC
trpE 2 rev	CTTCTAGAGCTGTACAAGGAAGTGGAGATGTC
kynA 1 for	CTGGTACCCGACTTTTTCCGTTCCCTGC
kynA 1 rev	GTACTACAGGTTGGAACGGAGCGTGAATCTCCTGAATTCGGC
kynA 2 for	GCCGAATTCAGGAGATTCACGCTCCGTTCCAACCTGTAGTAC
kynA 2 rev	CTTCTAGAGACATGACCGACGACATCGAC
phnAB 1 for	CTGAATTCGGTCAGCAACCTGGAAATCG
phnAB 1 rev	CGATGATGAACATGCCGTTGCCATCCCGAGTCGATTCTCAC
phnAB 2 for	GTGAGAATCGACTCGGGATGGCAACGGCATGTTCATCATCG
phnAB 2 rev	CTTCTAGACGTAAACCTGAGGAGGTGAAC
pqsA 1 for	CTGAATTCCCAGAATTGCCACCAAGACTC
pqsA 1 rev	GTCCACATTGGCCAACCTGACCCCTTTATCACGACAACCTTC
pqsA 2 for	GAAGGTTGTCGTGATAAAGGGGTCAGGTTGGCCAATGTGGAC
pqsA 2 rev	CTTCTAGACTATGGCAAGGTGCAACAATGG
Confirmation	
trpE for	GGTACCCTCGACAAGTTGC
trpE rev	CATTGGTGCTGGAACCGCTG
kynA for	CGTACTGCGTTGGTGATGG
kynA rev	CCTCCATCGCATTACTCAGG
phnAB for	CGTGAACATGTTCCTCCAGG
phnAB rev	GGATCGTCTGGGCAACATG
pqsA for	CCAGGCTGAACTCGTTCTCG
pqsA rev	GGTTTCCAAACGCAGCAACC
Expression	
phnAB for	GACTAGTGCGCGCTAGCGTCGCGCAGG
phnAB rev	GCTCTAGACCTGGCAACCGAGCATCGTCG
trpE for	GCCTGCAGCGTTTGCACCCTGTTGACC
trpE rev	GCAGAGCGTCGAGTAAGACGGAAATCAAGAGGTTACAGCC
trpG for	GGCTGTAACCTCTTGATTTCCGTCTTACTCGACGCTCTGC
trpG rev	GCTCTAGAGGTTGACGATGCGATTGAGG
RT-PCR	
phnA for	CGTTGAACGCCAATGGACG
phnA rev	CGGTACGATCTGGAACACG
rplU for	CGCAGTGATTGTTACCGGTG
rplU rev	AGGCCTGAATGCCGGTGATC

3.2.5 Growth curves

To determine growth characteristics of PA14, Δ*phnAB,* and Δ*trpE*, cultures of each were grown overnight in MOPS minimal medium supplemented with 200 μM tryptophan. Overnight cultures were subcultured into fresh MOPS minimal medium supplemented with 200 μM tryptophan and grown to mid-exponential phase (OD_{600} = 0.05) at which point cells were washed twice, concentrated, and starved for 2 hours in MOPS buffer. Washed, starved, exponential phase cells were used to inoculate 25 mL MOPS minimal medium with no tryptophan to OD_{600} = 0.01, and growth was measured by OD_{600} readings every half hour upon the initiation of exponential growth. To demonstrate tryptophan-dependent growth of Δ*trpE*, tryptophan was added to a final concentration of 200 μM three hours after inoculation. Growth was monitored for 8 hours after inoculation, and the generation times for each culture were calculated. The growth curve was repeated 3 times.

3.2.6 PQS extractions

Overnight cultures of indicated strains grown in MOPS minimal medium supplemented with 200 μM tryptophan when necessary were subcultured into fresh MOPS minimal medium to OD_{600} = 0.05 and grown to mid-exponential phase (OD_{600} = 0.5). Mid-exponential phase cells were washed twice, concentrated, and starved for 2 hours in MOPS buffer. Washed, starved, exponential phase cells were used to inoculate

12.5 mL of MOPS minimal medium containing 20 mM succinate supplemented with 5 mM of the stated aromatic amino acid. Cultures were grown for 24 hours (final OD_{600} values were consistently within a range of 1.5-2.0) and 10 mL were removed and extracted twice with an equal volume of ethyl acetate (Fisher) acidified with 150 μL per liter glacial acetic acid (Fisher). Ethyl acetate extracts were dried completely under a constant stream of N_2 gas and resuspended in methanol (Fisher). PQS in extracts was resolved by separation on thin layer chromatography plates (EMD) as described previously (99-101). PQS spots were imaged with excitation by long-wave UV light, and spots were quantified by densitometry using GeneTools software compared to a standard curve of synthetic PQS standards spotted on the same plate.

3.2.7 Over-expression of TrpEG and PhnAB

Over-expression constructs were generated by PCR amplification of the *trpE* and *trpG* genes and the *phnAB* operon using PA14 genomic DNA as template and primers listed in Table 3.2. Because the *trpE* and *trpG* genes are not adjacent on the *P. aeruginosa* chromosome (36), overlap extension PCR was used to generate a fused construct. This was not necessary for *phnAB,* which are present in an operon (16, 35). The *trpEG* and *phnAB* PCR products were digested with PstI and XbaI or SpeI and XbaI (see restriction sites in Table 3.2), respectively, and ligated into the arabinose-inducible vector pJN105 to generate pGP-*trpEG* and pKP-*phnAB* (Table 3.1). Expression constructs and empty

pJN105 were introduced into relevant *P. aeruginosa* strains by $MgCl_2$ transformation as described previously (149).

To determine whether over-expression of PhnAB could restore growth to PA14 Δ*trpE*, washed, starved, exponential phase Δ*trpE* cells containing pKP-phnAB or empty pJN105 were prepared as described above and used to inoculate 3 mL MOPS minimal medium with 0.5% arabinose. After overnight growth (~16 hours), a photograph was taken to demonstrate final growth yield. To determine whether over-expression of TrpEG restores PQS production in a minimal medium, washed, starved, exponential phase PA14 and Δ*phnAB* cells containing either pGP-*trpEG* or empty pJN105 cells were prepared as described above and used to inoculate 12.5 mL MOPS minimal medium with 0.5% arabinose. PQS was extracted and quantified as described above.

3.2.8 Tryptophan molar growth yield and semi-quantitative RT-PCR

To determine the tryptophan molar growth yield for Δ*trpE*, washed, starved, exponential phase Δ*trpE* or Δ*trpE* Δ*phnAB* cells prepared as described above and used to inoculate 100 μL MOPS minimal medium supplemented with increasing concentrations of tryptophan from 0 to 200 μM in the wells of a Nunc 96 well plate. Each well contained a 3 mm borosilicate glass bead (Fisher) to improve aeration. Plates were incubated overnight (~16 hours) after which glass

beads were removed, and the volume in each well was increased to 200 μL by addition of MOPS buffer. Final growth yield for each well was determined by measuring OD_{600} using a BioTek SynergyMx 96 well plate reader with Gen5 software. Growth yields were averaged from five wells for each tryptophan concentration, and each plate set containing the entire range of tryptophan concentrations was repeated 3 times with unique biological replicates each time. For experiments with exogenous PQS, the same protocol and media were used with the addition of 20 μM PQS throughout growth and starvation. Reported OD_{600} readings are corrected for dilution in MOPS buffer and spectrophotometric path length.

To determine whether *phnAB* expression correlates with abolishment of Δ*trpE* tryptophan auxotrophy, washed, starved, exponential phase Δ*trpE* cells were inoculated into 96 well plates as described above except that all wells contained MOPS minimal medium supplemented with 200 μM tryptophan. The RNA-stabilizing agent RNALater (Ambion) was added 1:1 volume:volume to wells containing cultures grown to OD_{600} = 0.07, 0.2, and 1.0. Total RNA was extracted using RNA bee (Tel-test) per the manufacturer's instructions. DNA contaminants were removed by DNase treatment (Promega) and confirmed by PCR amplification of the *rplU* gene using primers listed in Table 3.2 as previously described (99, 101, 127). RNA integrity was confirmed by gel electrophoresis, and cDNA was synthesized using a SuperScript II First-Strand cDNA synthesis

kit (Invitrogen) with random primers as described previously (99, 101, 127). cDNA was purified and used as the template for semi-quantitative RT-PCR using *phnA* primers listed in Table 3.2 to amplify *phnA* from 25 ng cDNA, and *rplU* primers listed in Table 3.2 to amplify *rplU* from 5 ng cDNA as a constitutively expressed loading control. RNA alone and genomic DNA served as negative and positive controls, respectively.

3.3 RESULTS AND DISCUSSION

3.3.1 Evidence that PhnAB was acquired by horizontal gene transfer

P. aeruginosa appears to be rare in that it possesses two functional anthranilate synthases, and it is likely not a coincidence that this bacterium also produces over 50 unique quinolone compounds that require anthranilate for biosynthesis (35, 36, 80). A complete quinolone biosynthesis operon has been identified in *Burkholderia pseudomallei* and *B. thailandensis,* and *P. putida* contains quinolone biosynthesis genes throughout its genome (27). These organisms have been reported to produce the immediate precursor to PQS, 2-heptyl-4-quinoline (HHQ) (27), however, BLAST analysis using the *P. aeruginosa* PA14 TrpEG and PhnAB sequences indicated that these organisms only possess homologs of TrpEG. As a first step toward determining how *P. aeruginosa* came to possess two anthranilate synthases, we used BLAST

analysis of the TrpE, and PhnA protein sequences to identify the most closely related anthranilate synthase homologs. Identified sequences were aligned and used to generate phylogenetic trees. Our results demonstrate that TrpE shares significant homology with the tryptophan biosynthesis enzymes of closely related species including many pseudomonads (Figure 3.1A). As a reference, the 30 closest *P. aeruginosa* relatives are presented in a phylogenetic tree based on 16S ribosomal RNA sequence (Figure 3.1B). By contrast, the protein sequence of PhnA was most closely related to multiple divergent species (Figure 3.1A). These results are consistent with previous observations that *P. aeruginosa* acquired the *phnAB* operon from the tryptophan biosynthetic machinery of another bacterium via horizontal gene transfer, and that this event likely occurred after the diversification of fluorescent pseudomonads, as no close relatives of *P. aeruginosa* possess a *phnAB*-like operon (90, 154).

A

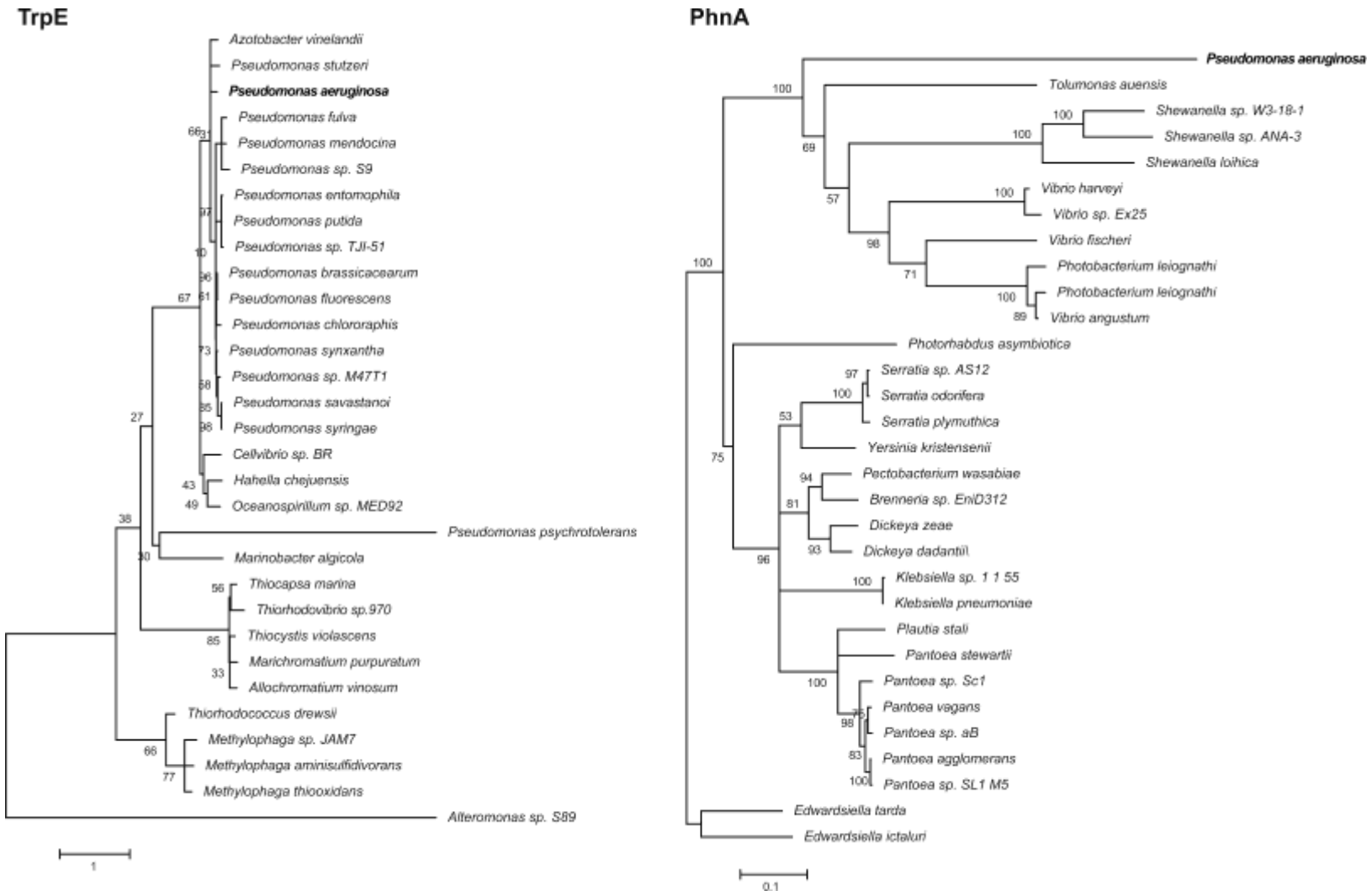
TrpE
Azotobacter vinelandii
Pseudomonas stutzeri
Pseudomonas aeruginosa
Pseudomonas fulva
Pseudomonas mendocina
Pseudomonas sp. S9
Pseudomonas entomophila
Pseudomonas putida
Pseudomonas sp. TJI-51
Pseudomonas brassicacearum
Pseudomonas fluorescens
Pseudomonas chlororaphis
Pseudomonas synxantha
Pseudomonas sp. M47T1
Pseudomonas savastanoi
Pseudomonas syringae
Cellvibrio sp. BR
Hahella chejuensis
Oceanospirillum sp. MED92
Pseudomonas psychrotolerans
Marinobacter algicola
Thiocapsa marina
Thiorhodovibrio sp.970
Thiocystis violascens
Marichromatium purpuratum
Allochromatium vinosum
Thiorhodococcus drewsii
Methylophaga sp. JAM7
Methylophaga aminisulfidivorans
Methylophaga thiooxidans
Alteromonas sp. S89
1
PhnA
Pseudomonas aeruginosa
Tolumonas auensis
Shewanella sp. W3-18-1
Shewanella sp. ANA-3
Shewanella loihica
Vibrio harveyi
Vibrio sp. Ex25
Vibrio fischeri
Photobacterium leiognathi
Photobacterium leiognathi
Vibrio angustum
Photorhabdus asymbiotica
Serratia sp. AS12
Serratia odorifera
Serratia plymuthica
Yersinia kristensenii
Pectobacterium wasabiae
Brenneria sp. EniD312
Dickeya zeae
Dickeya dadantii
Klebsiella sp. 1 1 55
Klebsiella pneumoniae
Plautia stali
Pantoea stewartii
Pantoea sp. Sc1
Pantoea vagans
Pantoea sp. aB
Pantoea agglomerans
Pantoea sp. SL1 M5
Edwardsiella tarda
Edwardsiella ictaluri
0.1

Figure 3.1 continued from previous page.

B

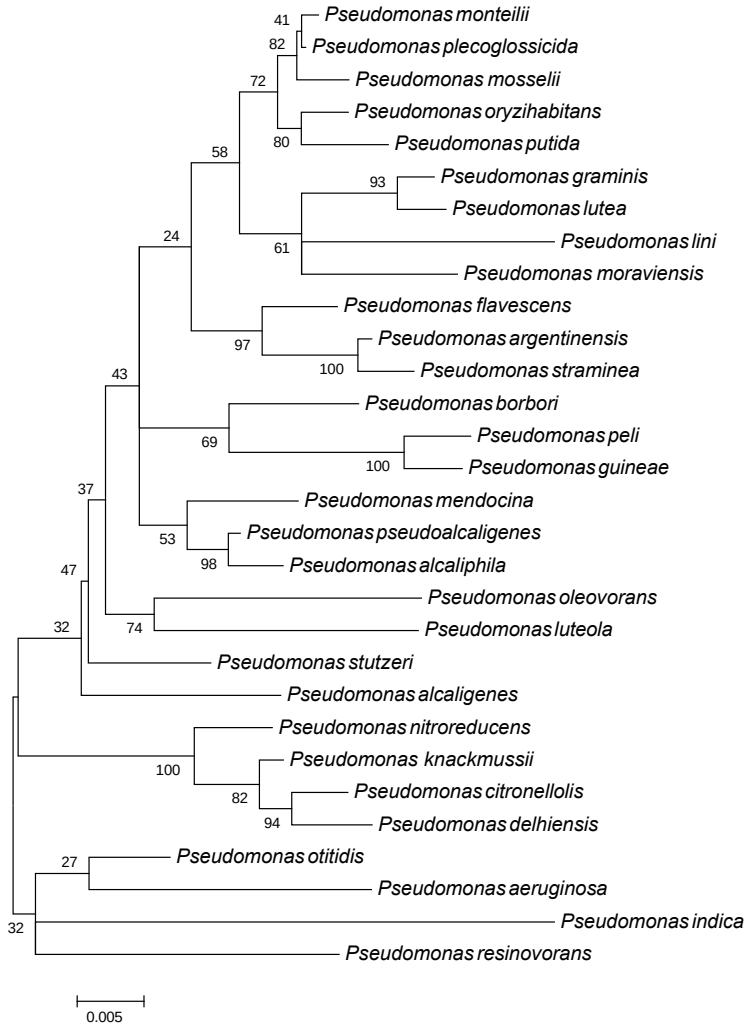

Figure 3.1 Anthranilate synthase phylogenetic trees.

Anthranilate synthase phylogenetic trees. (A) The top 30 homologs identified by BLAST analysis using the *P. aeruginosa* TrpE and PhnA sequences reveal the evolutionary relationships of each anthranilate synthase enzyme to those of other species. TrpEG are most closely related to anthranilate synthases from other members of the fluorescent pseudomonad family, while PhnAB is most closely related to anthranilate synthases from more distantly related organisms. The absence of a *phnAB*-like operon in other pseudomonads is evidence that PhnAB acquisition occurred after the family's diversification. (B) A reference tree of the top 30 most related organisms to *P. aeruginosa* by 16S ribosomal RNA gene sequence. Bootstrap values at tree nodes indicate the likelihood that the node represents a genuine phylogenetic relationship. Alignments and trees were constructed using Mega5 (139).

3.3.2 TrpE is required for growth without tryptophan

It was previously reported that a mutation in *trpE* results in tryptophan auxotrophy in *P. aeruginosa*, while *phnA* and *phnB* mutants retain the ability to grow in the absence of tryptophan (35, 36). To confirm this phenotype, Δ*trpE* and Δ*phnAB* mutants were generated. *P. aeruginosa* PA14, Δ*trpE*, and Δ*phnAB* were grown to exponential phase, washed, starved, and inoculated into MOPS minimal medium with no tryptophan, and growth was monitored over 8 hours. *P. aeruginosa* Δ*phnAB* grew at the same rate without tryptophan. However, upon addition of tryptophan to a final concentration of 200 μM after 3 hours of non-growth, Δ*trpE* grew at the same rate as wt *P. aeruginosa* (Figure 3.2). These results confirmed the fact that Δ*trpE* is a tryptophan auxotroph and endogenous *phnAB* is unable to complement tryptophan auxotrophy in Δ*trpE*.

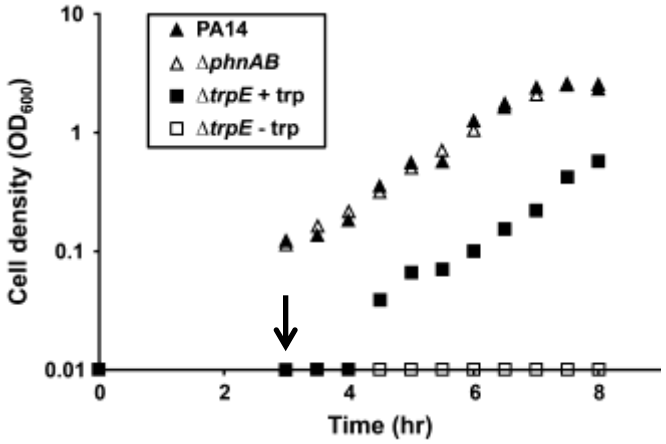

Figure 3.2 Confirmation of Δ*trpE* tryptophan auxotrophy.

Washed, starved, exponential phase PA14, Δ*phnAB,* and Δ*trpE* were grown in MOPS minimal medium with no tryptophan. Wild-type growth was observed for Δ*phnAB*. However, Δ*trpE* did not grow until the addition of tryptophan to a final concentration of 200 μM after three hours of incubation (arrow). Δ*trpE* did not grow in the absence of tryptophan.

3.3.3 PhnAB supplies anthranilate for PQS production

P. aeruginosa enhances production of PQS in the presence of aromatic amino acids (37, 101, 103). For tryptophan, catabolism via the products of the *kynABU* genes, generates anthranilate for PQS production (37); however the role of PhnAB during growth with tryptophan was not known. Examination of PQS production in the presence of tryptophan revealed that wt *P. aeruginosa* and Δ*phnAB* produced ~6 and ~3.5 μM PQS, respectively (Figure 3.3). The decreased levels of PQS in Δ*phnAB* suggest that PhnAB generates anthranilate for PQS biosynthesis even in the presence of high levels of tryptophan, indicating that degradation of tryptophan via KynABU is not the only source of anthranilate when the amino acid is in excess (37). This was confirmed as Δ*kynA*, which is unable to degrade tryptophan and Δ*trpE* Δ*kynA* produced low levels (~1 μM) of PQS in the presence of 5mM tryptophan (Figure 3.3). Because PhnAB is the only known source of anthranilate in Δ*trpE* Δ*kynA*, it is likely that PhnAB is a source of anthranilate for quinolone biosynthesis in the presence of tryptophan.

Our group has previously reported that in addition to tryptophan, phenylalanine and tyrosine also enhance PQS production (101, 103). However unlike tryptophan, this induction is not due to catabolism of these amino acids to anthranilate, but instead increased flux of shared precursors of anthranilate biosynthesis to PQS biosynthesis. Thus, we hypothesized that deletion of *phnAB* would completely eliminate PQS production in the presence of phenylalanine and tyrosine unless *trpEG* could complement this deletion. To test this, PQS was extracted and quantified from cultures where starved, exponential phase cells

were inoculated into MOPS minimal medium supplemented with 5 mM phenylalanine or tyrosine. *P. aeruginosa* PA14, Δ*trpE*, and Δ*trpE* Δ*kynA* produced ~2 μM PQS in the presence of 5 mM phenylalanine and ~5-6 μM PQS in the presence of 5 mM tyrosine (Figure 3.3). No detectable PQS was produced by Δ*phnAB*, indicating PhnAB is the anthranilate synthase responsible for PQS production in the presence of phenylalanine and tyrosine (Figure 3.3). Taken together, these experiments indicate that TrpEG-generated anthranilate is used for tryptophan biosynthesis while PhnAB-generated anthranilate is used for quinolone biosynthesis.

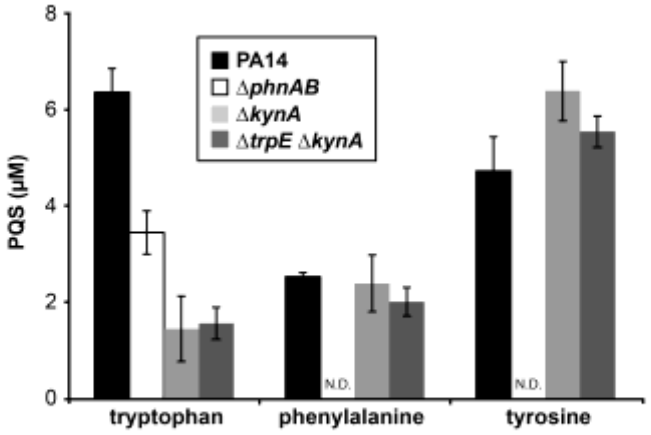

Figure 3.3 PQS production in the presence of aromatic amino acids.

PQS was extracted and quantified from whole cultures of PA14, Δ*phnAB,* Δ*kynA,* and Δ*trpE* Δ*kynA* grown for 24 hours in MOPS minimal medium supplemented with 5 mM of the indicated aromatic amino acid. N.D. indicates no detectable levels of PQS, and error bars represent the standard error of the mean.

3.3.4 Over-expression of either AS enzyme complements the loss of the other

The inability of these seemingly redundant anthranilate synthases to compensate for each other in genetic experiments is an interesting conundrum. To determine whether it is possible for anthranilate to cross pathways, over-expression constructs were generated for TrpEG and PhnAB, and their ability to cross complement each of the respective anthranilate synthase mutant phenotypes was tested (Figure 3.4). Over-expression of PhnAB restored growth of Δ*trpE* in the absence of tryptophan, while no growth was observed for Δ*trpE* cells containing empty vector (Figure 3.4A). To determine whether PQS production could be restored in Δ*phnAB*, PQS was extracted and quantified from Δ*phnAB* cells over-expressing TrpEG or containing empty vector. Expression of TrpEG in wild-type *P. aeruginosa* and Δ*phnAB* resulted in high levels of PQS production (Figure 3.4B). This effect was dependent upon TrpEG, as Δ*phnAB* cells containing empty vector did not produce detectable levels of PQS (Figure 3.4B). These experiments indicate that it is possible for metabolic crosstalk to occur between the tryptophan and quinolone biosynthesis pathways. However, why this does not occur with the endogenous enzymes remains unclear.

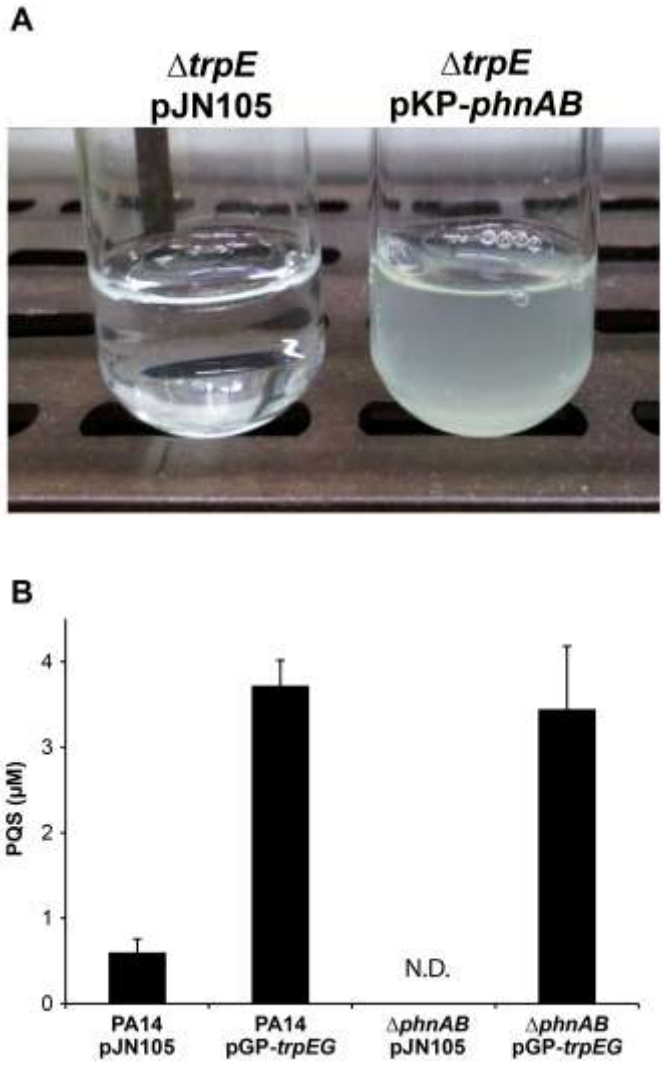

Figure 3.4 Over-expression of AS genes results in pathway crosstalk.

(A) Over-expression of *phnAB* in Δ*trpE* restored growth in MOPS minimal medium without tryptophan, while empty vector did not. (B) Over-expression of *trpEG* restored the ability of Δ*phnAB* to generate PQS in a minimal medium, while empty vector did not. N.D. indicates no detectable levels of PQS, and error bars represent the standard error of the mean.

3.3.5 Δ*trpE* tryptophan auxotrophy is dependent upon phnAB expression

One possible explanation for the lack of crosstalk of TrpEG- and PhnAB-generated anthranilate is that PhnAB is expressed at levels too low to compensate for loss of TrpEG. Expression of *phnAB* is cell density dependent, showing increased expression at mid- to late-exponential phase (16). Thus, it is possible that sufficient PhnAB levels are not present at low cell densities to complement Δ*trpE*-mediated tryptophan auxotrophy. However at high cell densities, we hypothesize that tryptophan auxotrophy would be eliminated in Δ*trpE* due to increased *phnAB* expression. To test this hypothesis, starved, exponential phase Δ*trpE* cells were grown overnight in MOPS minimal medium supplemented with tryptophan ranging from 0 to 200 μM, and final growth yields were determined. Results revealed that Δ*trpE* final growth yields were dependent upon tryptophan concentrations until mid-exponential phase, at which point Δ*trpE* reached maximal growth yields regardless of the concentration of tryptophan in the medium (Figure 3.5A). When *phnAB* was deleted in Δ*trpE*, growth yields remained dependent on the tryptophan concentration in the medium (Figure 3.5A).

The previous results suggest that PhnAB is a source of anthranilate for tryptophan biosynthesis at high cell densities. If this is the case, we hypothesized that addition of PQS to low density cultures would eliminate the Δ*trpE* auxotrophy since *phnAB* is induced by PQS. Upon induction of *phnAB* expression with a physiologically relevant concentration of PQS (20 μM), Δ*trpE* tryptophan auxotrophy was completely rescued, as Δ*trpE* reached maximal growth yields

regardless of the concentration of tryptophan in the medium (Figure 3.5B). To further confirm the ability of PQS-dependent PhnAB expression to mitigate Δ*trpE* tryptophan auxotrophy, Δ*trpE* Δ*pqsA* was generated. Because the *pqsA* gene product is required for PQS biosynthesis, its deletion renders this strain unable to generate PQS endogenously (10, 19, 86). In the absence of endogenous PQS, Δ*trpE* Δ*pqsA* growth yields were similar to those of Δ*trpE* Δ*phnAB*, and thus, were entirely dependent on the concentration of tryptophan in the medium (Figure 3.5B). As a final line of evidence for the correlation of Δ*trpE* tryptophan auxotrophy and low levels of *phnAB* expression, RT-PCR was used to examine *phnA* expression levels during growth in the presence of tryptophan, compared to the constitutively expressed *rplU* gene. Consistent with other PQS-regulated genes (16, 153), *phnA* displayed low levels of expression in lag (OD_{600} = 0.07) and early exponential (OD_{600} = 0.20) phases of growth. However, expression increased substantially as density increased (OD_{600} = 1.0) (Figure 3.5C). Taken together these results are consistent with cell density-dependent PhnAB expression rescuing Δ*trpE* tryptophan auxotrophy.

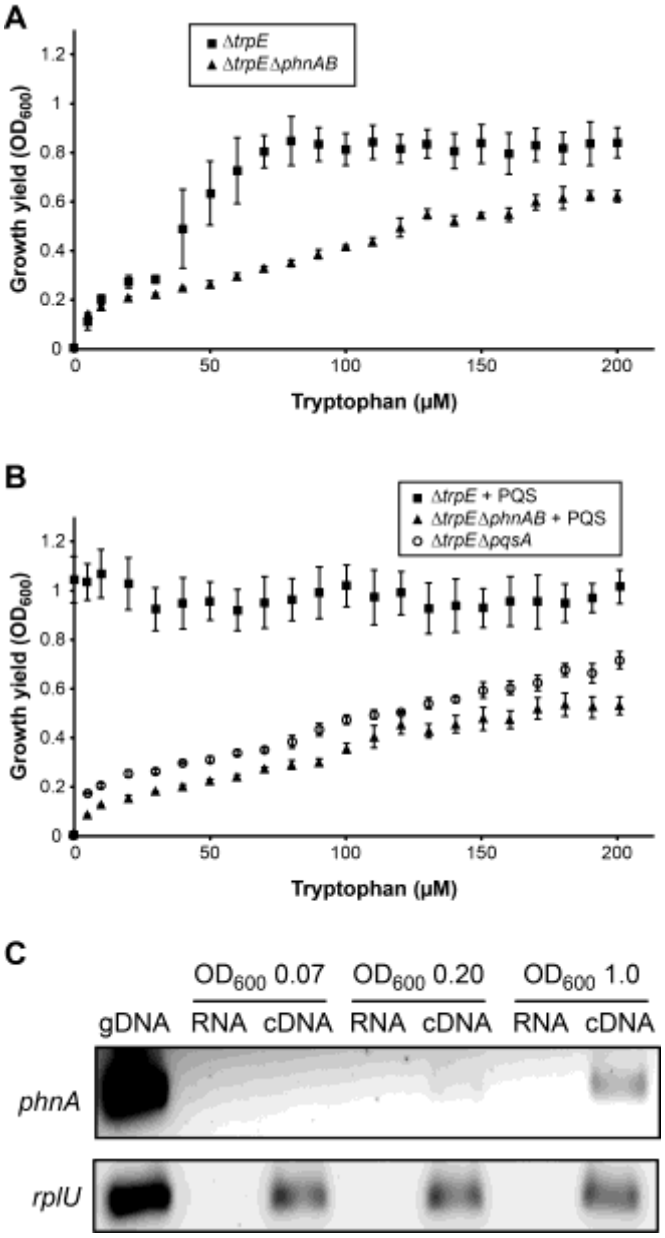

Figure 3.5 Δ*trpE* tryptophan auxotrophy is dependent upon *phnAB* expression.

(A) The final growth yields of Δ*trpE* and Δ*trpE* Δ*phnAB* on increasing levels of tryptophan were determined by optical density at 600 nm. Δ*trpE* growth became tryptophan independent upon reaching OD_{600} ~ 0.5. Growth of the Δ*trpE* Δ*phnAB* was tryptophan-dependent. Error bars represent the standard error of the mean. (B) Exogenous addition of 20 μM PQS rescued Δ*trpE* tryptophan auxotrophy, while Δ*trpE* Δ*phnAB* remained auxotrophic for tryptophan in the presence of PQS. Growth yields were also tryptophan-dependent in Δ*trpE* Δ*pqsA* which is unable to generate PQS. Error bars represent the standard error of the mean. (C) RT-PCR confirms *phnA* expression levels correspond to the loss of Δ*trpE* tryptophan auxotrophy. Expression of *phnA* was analyzed at three stages of growth (OD_{600} 0.07, 0.2, and 1.0) by PCR amplification from 25 ng cDNA, and levels are compared to constitutive expression of *rplU*, amplified from 5 ng cDNA. Genomic DNA (gDNA) and RNA serve as positive and negative controls, respectively. Gel images are inverted for clarity.

3.3.6 Discussion and conclusion

P. aeruginosa has been known to possess two anthranilate synthases for over 20 years, and the unique phenotypes resulting from mutations in the anthranilate synthase genes suggested that TrpEG generates anthranilate exclusively for tryptophan biosynthesis while PhnAB generates anthranilate exclusively for quinolone biosynthesis (35, 36). Here we present evidence consistent with the proposal that a second anthranilate synthase system, *phnAB*, was acquired through horizontal gene transfer at some point after the diversification of fluorescent pseudomonads. Based on its organization in an operon and quinolone-dependent regulation, PhnAB has likely evolved to generate anthranilate for quinolone biosynthesis (35, 36, 90). Over-expressed TrpEG and PhnAB forced crosstalk of anthranilate between the tryptophan and quinolone biosynthesis pathways, suggesting that regulation of biosynthetic enzymes explains the observed anthranilate synthase mutant phenotypes. While our data do not rule out the physical sequestration of tryptophan and quinolone biosynthesis precursors, the importance of temporal expression of *phnAB* was confirmed, as *phnAB* expression levels are the primary determinant of Δ*trpE* tryptophan auxotrophy (Figure 3.5). These results lead to a model for *P. aeruginosa* anthranilate production (Figure 3.6) in which temporal expression of PhnAB explains the apparent lack of redundancy between TrpEG and PhnAB. At low cell densities PhnAB expression is low, and TrpEG is the primary anthranilate synthase enzyme present. As PQS-mediated *phnAB* expression is activated at higher cell densities, PhnAB becomes the primary anthranilate synthase enzyme present. The results presented here underscore the

importance of studying basic microbial physiology and metabolism to understand bacterial signaling and pathogenesis.

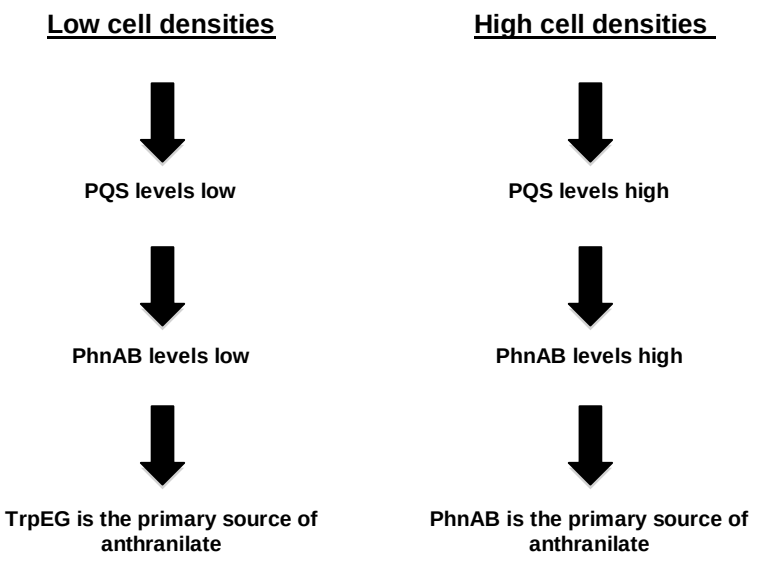

Figure 3.6 Model for *P. aeruginosa* anthranilate production

At low cell densities, insufficient levels of PhnAB are produced and TrpEG is the primary source of anthranilate in the cell. As growth yields increase and quorum sensing regulation via PQS activates *phnAB* expression, PhnAB becomes the primary source of anthranilate in the cell.

Chapter 4: Conclusion and future directions

4.1 OVERVIEW OF RESULTS

4.1.1 PhhR and the transcriptional response to aromatic amino acids

Our research group has previously reported that phenylalanine and tyrosine enhance PQS production in CF sputum, though a mechanism to explain this phenomenon had not been elucidated (101, 103). In Chapter 2, I began to investigate how aromatic amino acids affect PQS production by performing transcriptome analysis using Affymetrix microarrays comparing *P. aeruginosa* grown in the presence and absence of phenylalanine and tyrosine. I found that genes for PQS biosynthesis and aromatic amino acid catabolism were up-regulated in the presence of phenylalanine and tyrosine (Table 2.2) (99). Among the aromatic amino acid catabolic genes was a gene encoding the aromatic amino acid-responsive transcriptional regulator PhhR (Table 2.2) (99). To determine whether PhhR regulates both aromatic amino acid catabolism and PQS biosynthesis, I identified the PhhR regulon using a second set of Affymetrix microarrays comparing a *phhR* mutant to wild-type *P. aeruginosa*, and found that only aromatic amino acid catabolic genes were altered in the *phhR* mutant (Table 2.2) (99). I confirmed that PhhR does not regulate PQS production with the observation that there was no difference in PQS extracted from PA14 and *phhR* mutant cells both in the presence and absence of aromatic amino acids (Figure 2.1C) (99).

The rest of Chapter 2 is dedicated to evaluation of the PhhR regulon. From the genes differentially expressed in the *phhR* mutant, I was able to propose a complete pathway for degradation of phenylalanine to TCA cycle intermediates (Figure 2.1A), and I showed that PhhR is required for growth on phenylalanine and tyrosine as a sole source of carbon and energy (Figure 2.1B) (99). The genes for aromatic amino acid catabolism comprised four transcriptional units. I mapped the transcriptional units' promoters using primer extension to identify transcriptional start sites and *in silico* analysis to identify potential PhhR operator sequences (Figure 2.2) (99). I purified a his-tagged PhhR variant (Figure 2.3) and used it to demonstrate direct regulation of promoters containing putative PhhR binding sites with electrophoretic mobility shift assays (EMSAs) (Figure 2.4) (99). These results indicate that co-regulation of aromatic amino acid catabolism and PQS biosynthesis does not explain enhanced PQS production in the presence of phenylalanine and tyrosine.

4.1.2 Differential regulation of anthranilate synthase activity

The absence of co-regulation of aromatic amino acid catabolism and PQS biosynthesis by PhhR led me to favor a model in which flux of the central metabolite chorismate, which is a shared precursor for aromatic amino acid and PQS biosynthesis, explains enhanced PQS production in CF sputum (Figure 1.3B). To evaluate this model, I began by investigating the enzyme responsible for the first step in PQS biosynthesis, conversion of chorismate to anthranilate by

an anthranilate synthase (AS). *P. aeruginosa* possesses two AS enzymes, TrpEG and PhnAB (35, 36). Phylogenetic analysis demonstrated that *P. aeruginosa* PhnAB is more closely related to distant species, while TrpEG is more closely related to the tryptophan biosynthetic enzymes of other pseudomonads (Figure 3.1). *P. aeruginosa* is rare among bacteria to have a second AS enzyme, and the previous results combined with the fact that other closely related pseudomonads do not possess a second anthranilate synthase suggests that PhnAB was acquired by horizontal gene transfer after the diversification of fluorescent pseudomonads.

Previous genetic evidence suggested that these two enzymes are not functionally redundant, as mutants in *trpEG* result in tryptophan auxotrophy, while mutants in *phnAB* result in the loss of PQS production in the absence of tryptophan (15, 35-37, 43, 89). I confirmed these observations as an unmarked deletion mutant in *trpE* does not grow without tryptophan, and an unmarked deletion mutant in *phnAB* does not produce PQS without tryptophan (Figures 3.2 and 3.3). Additionally, I demonstrated that PhnAB is responsible for enhanced PQS production in the presence of phenylalanine and tyrosine, as Δ*phnAB* did not produce PQS in the presence of those amino acids (Figure 3.3). The previous observation that tryptophan degradation can be a source of anthranilate for PQS production was also confirmed, though diminished PQS production by Δ*phnAB* suggests that PhnAB is also capable of generating anthranilate in the presence of tryptophan (Figure 3.3). These results confirm the lack of crosstalk between TrpEG-generated anthranilate and PQS biosynthesis as well as between PhnAB-generated anthranilate and tryptophan biosynthesis. To

determine whether anthranilate crosstalk between these two pathways was possible, I demonstrated that over-expressed *phnAB* complements Δ*trpE* tryptophan auxotrophy (Figure 3.4A), and over-expressed *trpEG* complements the loss of PQS production by Δ*phnAB* in the absence of tryptophan (Figure 3.4B).

Once I established that it is possible to force crosstalk of anthranilate between tryptophan and PQS biosynthesis, I wanted to determine why this does not occur in the cell. To answer this question, I examined growth yields of Δ*trpE* and Δ*trpE* Δ*phnAB* on increasing levels of tryptophan. If no alternative source of anthranilate is present in the cell, growth yields would be completely dependent on the amount of tryptophan in the medium. This was not the case as Δ*trpE* grown in enough tryptophan to reach mid-exponential phase consistently reached the maximum growth yield regardless of whether any additional tryptophan was present in the medium (Figure 3.5A). When PhnAB is removed in Δ*trpE* Δ*phnAB* growth yields are completely dependent on the amount of tryptophan in the medium (Figure 3.5A). PQS-mediated induction of *phnAB* upon growth to mid-exponential phase could explain this phenomenon. To test this, I induced early *phnAB* expression with exogenously added PQS and observed that Δ*trpE* tryptophan auxotrophy was abolished at any tryptophan concentration (Figure 3.5B). I confirmed that *phnAB* expression levels correlate to the loss of tryptophan auxotrophy using semi-quantitative RT-PCR of the *phnA* gene, and *phnA* expression increased substantially when PhnAB would be predicted to be active (Figure 3.5C). These results led me to propose a model for Δ*trpE* tryptophan auxotrophy in which expression of *phnAB* dictates whether Δ*trpE*

exhibits tryptophan auxotrophy (Figure 3.6). At low cell densities, PhnAB levels are low, and Δ*trpE* requires tryptophan to grow; however, as PQS-mediated induction of *phnAB* begins around mid-exponential phase, PhnAB levels are sufficient to compensate for the loss of TrpEG.

4.2 FUTURE DIRECTIONS

4.2.1 Validating the metabolite flux model for enhanced PQS production in sputum

While my investigations into *P. aeruginosa's* two AS enzymes have provided insight into how regulation of aromatic amino acid and PQS biosynthetic enzymes can dictate metabolite flux of common precursors, further studies should be undertaken to confirm the metabolite flux model for enhanced PQS production in the presence of aromatic amino acids. One approach toward this characterization could be to grow *P. aeruginosa* with uniformly ^{14}C-labeled chorismate in the presence and absence of aromatic amino acids. The metabolite flux model predicts that more ^{14}C-labeled PQS would be generated when aromatic amino acids are present. Additionally, previous investigators have used whole metabolome profiling to identify differences between *P. aeruginosa* strains and planktonic versus biofilm modes of growth (38, 48). These same approaches could be applied to evaluate the metabolite flux model and identify new pathways and metabolic nodes affected by aromatic amino acids or other nutrients in CF sputum. Knowledge gained from studies like these could lead to

better treatment strategies or novel antimicrobials to address *P. aeruginosa* infections in the CF lung.

4.2.2 Degradation of phenylalanine and tyrosine as a novel therapeutic strategy

The discovery that phenylalanine and tyrosine enhance production of PQS in CF sputum suggests that removal of these nutrients from *P. aeruginosa* infection sites could be a novel therapeutic strategy. This approach could be advantageous over conventional antimicrobials that kill or slow the growth of bacteria, because there is no immediate incentive to evolve resistance. Enzymatic degradation of DNA by inhalation of recombinant human DNase I is currently used in the CF lung to decrease sputum viscosity and aid in expectoration (64, 68). It is possible that phenylalanine/tyrosine-degrading enzymes could also be used in the CF lung. I began to investigate this approach by purifying a his_6-tagged version of phenylalanine ammonia lyase (PAL), an enzyme capable of deaminating phenylalanine and tyrosine to generate cinnamic and coumaric acid, respectively (84). The PAL homolog from the cyanobacterium *Anabaena variabilis* was particularly useful in these studies because a crystal structure is available, and importantly, the enzyme has already been modified for use as an injected therapeutic to treat the genetic disease phenylketonuria (92, 145). Attempts to degrade phenylalanine and tyrosine in SCFM with his_6-PAL were unsuccessful due to low enzyme activity in the medium; however, it is likely that directed evolution to generate a PAL with more robust activity could produce a functional therapeutic. Alternatively, the use of PQS precursor metabolite

analogs to inhibit PQS biosynthesis enzymes may also represent a workable therapeutic strategy (15).

4.2.3 Enhancing *in vitro* CF sputum models

CF sputum is incredibly complex, and there are several nutrients present in that environment that are not included in the current iteration of SCFM (83, 101). In the interest of more accurately replicating the nutrient environment of the CF lung, I have developed a new version of SCFM that includes several nutrients not present in the published version. N-acetylglucosamine (GlcNAc) is a common sugar in many environments that enhances pyocyanin production and *P. aeruginosa* competitiveness against other bacteria (79). I have added 300 μM GlcNAc to SCFM based on Aishwarya Korgaonkar's measurements of CF sputum (79). CF sputum also contains high concentrations of DNA that alters the viscosity of sputum and may represent an alterative carbon source for *P. aeruginosa* (83, 93). Reported measurements of DNA in CF sputum vary over a range of micrograms to milligrams per mL; thus I have added 600 μg/mL salmon sperm DNA to SCFM, which is near the median of that range (8, 52, 56, 74, 114, 143). Lipids have also been reported to be an important carbon source in CF sputum (4, 70). The most prevalent lipid in CF sputum is phosphatidylcholine, and I have added 100 μg/mL of dioleoylphosphatidylcholine to SCFM, which is also consistent with the range of phosphatidylcholine concentrations reported from CF sputum measurements (53, 65, 91, 111). Finally, mucin is a glycosylated protein secreted along epithelial surfaces that may induce biofilm/microcolony

formation in the CF lung, and I have added 5 mg/mL bovine submaxillary mucin to SCFM, consistent with other synthetic CF sputum media (39, 46, 133).

To determine the effect of these additional nutrients on *P. aeruginosa,* I performed one round of transcriptome profiling using Affymetrix microarrays comparing cells grown to early stationary phase (OD_{600nm} ~ 1.5) in the published SCFM and this medium. Tables listing genes that were differentially expressed in the enhanced medium are present in the appendix, and genes for GlcNAc utilization were the only obvious catabolic genes induced in enhanced SCFM. As it is clear that nutrients available in CF sputum affect the physiology and pathogenicity of *P. aeruginosa*, improvements in SCFM that result in more accurately replicating the nutritional environment of CF sputum will improve the medium as a tool to reveal nutrition-influenced phenotypes.

4.3 MODELS FOR THE INFLUENCE OF AROMATIC AMINO ACIDS ON PQS PRODUCTION

Once it was demonstrated that aromatic amino acids affect PQS production, the next question was why does this happen? In the case of tryptophan it is clear that degradation of tryptophan generates anthranilate that can be then converted to HHQ, PQS, and other quinolones (37, 80). The question remained, though, how do phenylalanine and tyrosine affect PQS production? In Chapter 2, I demonstrated that co-regulation of phenylalanine and tyrosine catabolism with PQS biosynthesis does not occur, leading me to favor an alternative model involving flux of shared precursor metabolites. In this model,

(Figure 4.1A), the presence of aromatic amino acids results in regulation of aromatic amino acid biosynthetic enzymes (TrpEG and PheA) by feedback inhibition. Diminished activity of these enzymes allows more chorismate to be dedicated toward PQS biosynthesis.

In the interest of testing this model, I began to investigate *P. aeruginosa's* two AS enzymes capable of converting chorismate to anthranilate. While these enzymes do not appear to be functionally redundant by genetic analysis, I demonstrated in Chapter 3 that differential regulation of *trpEG* and *phnAB* expression explains the lack of anthranilate crosstalk between tryptophan and PQS biosynthesis (Figure 4.1B). When *phnAB* expression is induced by PQS through either growth to mid-exponential phase or exogenous addition of PQS, PhnAB levels increase to a sufficient level to compensate for the loss of TrpEG. My results indicate that *P. aeruginosa* has recently acquired a second enzyme, PhnAB, and placed it under QS control to generate anthranilate for quinolone production late in growth. The presence of this quinolone biosynthesis machine (*phnAB* with *pqsABCDE*) combined with *P. aeruginosa's* unique ability among *Pseudomonads* to grow at 37°C may explain this organism's enhanced ability to colonize human hosts.

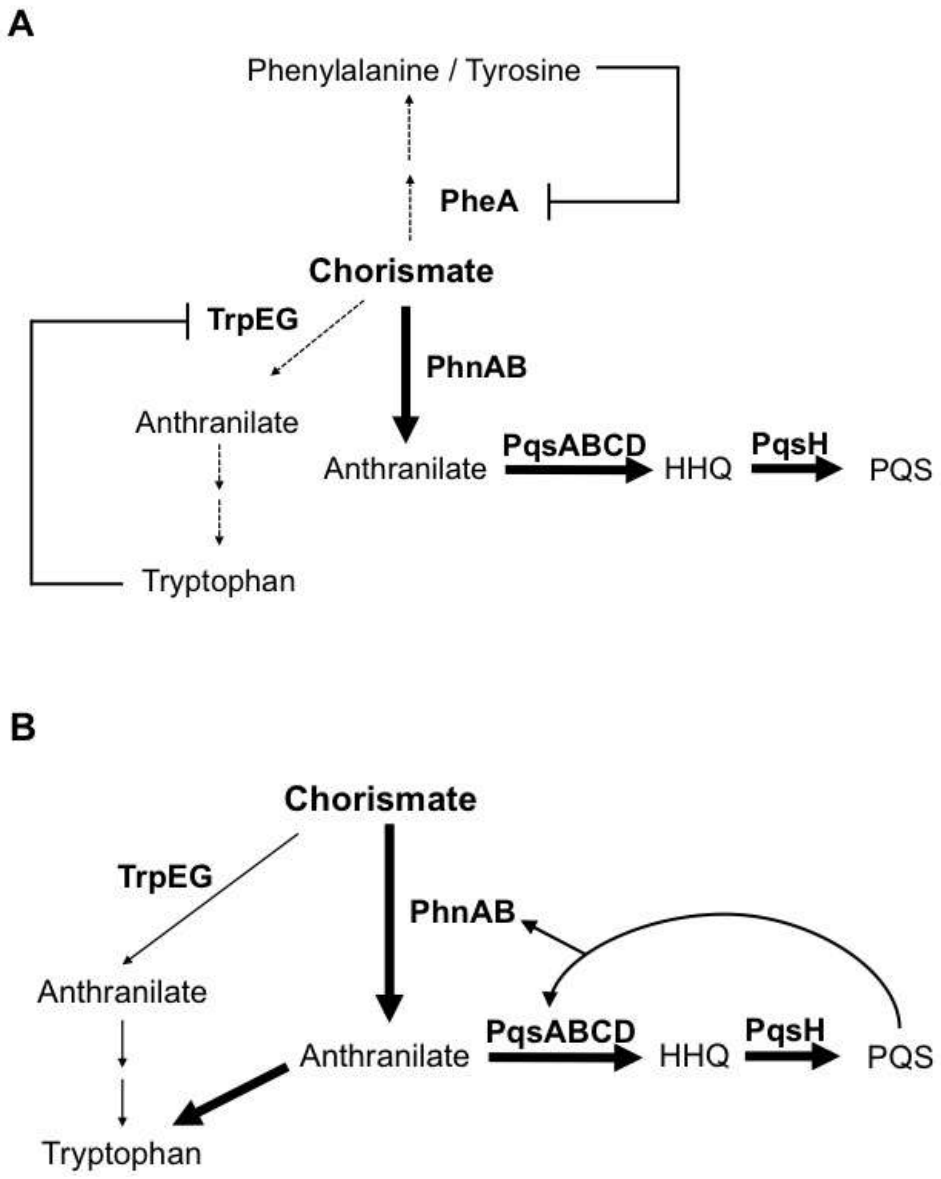

Figure 4.1 Models for PQS and anthranilate production.

Models for (A) enhanced PQS production in the presence of aromatic amino acids and (B) the effect of PQS-mediated PhnAB expression on anthranilate production.

4.4 FINAL DISCUSSION

Since Pasteur's work in the late 1800s, the importance of nutrients at the site of infection have been known to microbiologists, yet the *in vivo* carbon sources for many pathogenic organisms have not been identified. Chronic infections in the lungs of individuals with the genetic disease cystic fibrosis have served as a model environment for my studies of *P. aeruginosa* physiology and pathogenicity. I determined that enhanced production of PQS in the presence of aromatic amino acids is not due to co-regulation of aromatic amino acid catabolism and PQS biosynthesis, which led me to favor a metabolite flux model of enhanced PQS production in CF sputum. I began to test that model by investigating *P. aeruginosa's* two AS enzymes, and determined that the reason they do not appear to be redundant is their differential temporal expression. These studies have contributed to knowledge of a clinically significant organism and could provide the basis for novel strategies to eradicate *P. aeruginosa* infections.

Appendix: Genes up- and down-regulated in enhanced SCFM

Genes up-regulated in enhanced SCFM:

Locus_gene	Fold change	Description
PA0141	7	conserved hypothetical protein
PA0177	4	probable purine-binding chemotaxis protein
PA0506	9	probable acyl-CoA dehydrogenase
PA0509_nirN	4	nirN /DEF=probable c-type cytochrome
PA0510	12	probable uroporphyrin-III c-methyltransferase
PA0511_nirJ	6	nirJ /DEF=heme d1 biosynthesis protein NirJ
PA0512	13	conserved hypothetical protein
PA0513	9	probable transcriptional regulator
PA0514_nirL	11	heme d1 biosynthesis protein NirL
PA0515	8	probable transcriptional regulator
PA0516_nirF	9	heme d1 biosynthesis protein NirF
PA0517_nirC	8	probable c-type cytochrome precursor
PA0518_nirM	10	cytochrome c-551 precursor
PA0519_nirS	11	nitrite reductase precursor
PA0523_norC	7	nitric-oxide reductase subunit C
PA0524_norB	6	nitric-oxide reductase subunit B
PA0526	8	hypothetical protein
PA0527_dnr	5	dnr
PA0713	6	hypothetical protein
PA0918	5	cytochrome b561
PA1049_pdxH	4	pyridoxine 5 -phosphate oxidase
PA1076	5	hypothetical protein
PA1123	17	hypothetical protein
PA1337_ansB	6	glutaminase-asparaginase
PA1421_speB2	4	agmatinase
PA1429	4	probable cation-transporting P-type ATPase
PA1546_hemN	5	hemN /DEF=oxygen-independent coproporphyrinogen III oxidase
PA1550	4	hypothetical protein
PA1555	5	probable cytochrome c
PA1556	6	probable cytochrome c oxidase subunit
PA1557	6	probable cytochrome oxidase subunit (cbb3-type)
PA1673	8	hypothetical protein
PA1746	14	hypothetical protein
PA1789	8	hypothetical protein
PA2127	4	conserved hypothetical protein
PA2662	5	conserved hypothetical protein
PA2663	10	hypothetical protein
PA2664_fhp	7	flavohemoprotein
PA2753	6	hypothetical protein
PA2953	6	electron transfer flavoprotein-ubiquinone oxidoreductase
PA3278	6	hypothetical protein
PA3309	9	conserved hypothetical protein

PA3336	4	probable MFS transporter
PA3337_rfaD	9	ADP-L-glycero-D-mannoheptose 6-epimerase
PA3391_nosR	12	regulatory protein NosR
PA3458	5	probable transcriptional regulator
PA3465	6	conserved hypothetical protein
PA3520	5	hypothetical protein
PA3572	11	hypothetical protein
PA3614	4	hypothetical protein
PA3758	9	probable N-acetylglucosamine-6-phosphate deacetylase
PA3759	12	probable aminotransferase
PA3761	11	probable phosphotransferase system protein
PA3839	4	probable sodium:sulfate symporter
PA3870_moaA1	5	molybdopterin biosynthetic protein A1
PA3871	10	probable peptidyl-prolyl cis-trans isomerase, PpiC-type
PA3872_narI	6	respiratory nitrate reductase gamma chain
PA3873_narJ	6	respiratory nitrate reductase delta chain
PA3876_narK2	23	nitrite extrusion protein 2
PA3877_narK1	42	nitrite extrusion protein 1
PA3880	10	conserved hypothetical protein
PA3911	6	conserved hypothetical protein
PA3912	6	conserved hypothetical protein
PA3913	11	probable protease
PA3915_moaB1	39	molybdopterin biosynthetic protein B1
PA4129	4	hypothetical protein
PA4131	5	probable iron-sulfur protein
PA4132	5	conserved hypothetical protein
PA4133	8	cytochrome c oxidase subunit (cbb3-type)
PA4134	6	hypothetical protein
PA4236_katA	9	catalase
PA4348	6	conserved hypothetical protein
PA4352	8	conserved hypothetical protein
PA4357	5	conserved hypothetical protein
PA4358	6	probable ferrous iron transport protein
PA4514	5	probable outer membrane receptor for iron transport
PA4523	5	hypothetical protein
PA4542_clpB	5	ClpB protein
PA4571	7	probable cytochrome c
PA4577	15	hypothetical protein
PA4587_ccpR	28	cytochrome c551 peroxidase precursor
PA4610	6	hypothetical protein
PA4611	5	hypothetical protein
PA4916	5	hypothetical protein
PA4990	10	SMR multidrug efflux transporter
PA5027	13	hypothetical protein
PA5053_hslV	5	heat shock protein HslV
PA5054_hslU	5	heat shock protein HslU
PA5170_arcD	18	arginine/ornithine antiporter
PA5171_arcA	4	arginine deiminase
PA5427_adhA	17	alcohol dehydrogenase
PA5440	4	probable peptidase

PA5475	10	hypothetical protein

Genes down-regulated in enhanced SCFM:

Locus_gene	Fold change	Description
PA0730	-5	probable transferase
PA0781	-30	hypothetical protein
PA0848	-5	probable alkyl hydroperoxide reductase
PA1318_cyoB	-10	cytochrome o ubiquinol oxidase subunit I
PA1863_modA	-5	molybdate-binding periplasmic protein precursor ModA
PA1864	-10	probable transcriptional regulator
PA2204	-4	probable binding protein component of ABC transporter
PA2392	-4	hypothetical protein
PA2393	-4	probable dipeptidase precursor
PA2397_pvdE	-5	pyoverdine biosynthesis protein PvdE
PA2401	-4	probable non-ribosomal peptide synthetase
PA2425	-5	probable thioesterase
PA2450	-4	hypothetical protein
PA2912	-6	probable ATP-binding component of ABC transporter
PA3452_mqoA	-6	malate:quinone oxidoreductase
PA3598	-9	conserved hypothetical protein
PA3600	-42	conserved hypothetical protein
PA3601	-34	conserved hypothetical protein
PA4063	-16	hypothetical protein
PA4064	-14	probable ATP-binding component of ABC transporter
PA4065	-9	hypothetical protein
PA4066	-5	hypothetical protein
PA4170	-15	hypothetical protein
PA4171	-28	probable protease
PA4218	-5	probable transporter
PA4219	-7	hypothetical protein
PA4220	-5	hypothetical protein
PA4221_fptA	-7	Fe(III)-pyochelin receptor precursor
PA4222	-5	probable ATP-binding component of ABC transporter
PA4223	-6	probable ATP-binding component of ABC transporter
PA4224	-11	hypothetical protein
PA4225_pchF	-5	pyochelin synthetase
PA4226_pchE	-7	dihydroaeruginoic acid synthetase
PA4228_pchD	-11	pyochelin biosynthesis protein PchD
PA4229_pchC	-9	pyochelin biosynthetic protein PchC
PA4230_pchB	-9	salicylate biosynthesis protein PchB
PA4231_pchA	-5	salicylate biosynthesis isochorismate synthase
PA4467	-4	hypothetical protein
PA4470_fumC1	-7	fumarate hydratase
PA4836	-60	hypothetical protein
PA4838	-6	hypothetical protein

PA5530	-7	probable MFS dicarboxylate transporter
PA5540	-26	hypothetical protein

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