LAYERED REGULATION OF A PATHWAY CAPABLE OF ALTERING QUINOLONE PRODUCTION IN *PSEUDOMONAS AERUGINOSA*

by

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

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Pseudomonas aeruginosa is a gram negative opportunistic pathogen which commonly infects hospitalized patients and those afflicted with cystic fibrosis. *P. aeruginosa* communicates intercellularly via a system of small diffusible molecules in the process of cell-to-cell signaling. The *Pseudomonas* quinolone signal (PQS) is one of these molecules and its synthesis is required for production of virulence factors such as pyocyanin. PQS is synthesized by the gene products of *pqsABCD* and *pqsH*, and it is a cofactor for the transcriptional regulator PqsR which then positively regulates the *pqsABCDE* operon. Many gene deletions have been identified that eliminate or suppress PQS production including biosynthetic and regulatory protein encoding genes. We have identified a gene which encodes an RpiR-type transcriptional regulator (*PA5506*; now designated *qapR* for quinolone alteration pathway regulator) that modulates PQS production through an unknown mechanism. A *qapR* deletion mutant was constructed in strain PAO1 and the phenotype of this mutant was characterized. The Δ*qapR* mutant produced less PQS and pyocyanin than the wild type strain PAO1.

We found that transcription from the *pqsA* promoter is reduced in the Δ*qapR* mutant compared to wild type strain PAO1 and this reduction is due to decreased PQS concentration. Expression of PqsABCD in the Δ*qapR* mutant was able to complement PQS and pyocyanin production which indicates that PQS precursor pools are unaffected by *qapR* mutation. Using gel-shift assays we showed that *qapR* encodes a transcriptional regulator capable of binding to the *qapR* operon (containing genes *qapR*, *PA5507*, *PA5508*, and *PA5509*) promoter. Quantitative real-time PCR showed a large increase in transcription of genes *PA5507*, *PA5508*, and *PA5509* when comparing the *qapR* mutant to the wild type. All three of these genes are required to reduce PQS concentration when transcriptional repression of the operon is alleviated. Further investigation of the *qapR* operon lead us to construct a point mutant that introduced a premature stop codon in the *qapR* open reading frame. This mutation led to derepression of the *qapR* operon but did not affect PQS concentration. We found expression of gene *PA5507* restored the low PQS phenotype to the *qapR*-C90G point mutant. Reporter fusions translationally linked to *PA5507* allowed us to show that translation of this gene is coupled to that of the upstream regulator *qapR*. This second level of regulation allows *P. aeruginosa* to have intricate control over the *qapR* operon which can reduce PQS concentration.

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CHAPTER ONE: INTRODUCTION

The impetus for conducting this research came from a study which identified genes required to produce 2-alkyl-4-hydroxyquinolones (HAQs) in *Pseudomonas aeruginosa* [14]. It has been shown that signaling via HAQs is important for infection of several model organisms including plants [87], nematodes [27], and mammals (mice) [9] and these molecules have been isolated from the lungs of cystic fibrosis patients colonized with *P. aeruginosa* [13]. In this study, an autolytic colony phenotype was produced by introducing a lesion into the *pqsL* gene. This phenotype reverted back to that of the wild type after a second mutation reduced quinolone production. This screen was utilized to identify genes required for quinolone biosynthesis, for quinolone precursor production, and for a transcriptional regulator encoded by gene *PA5506* [105]. This gene is homologous to RpiR-type transcriptional regulators, but this did not indicate a clear role pertaining to quinolone production in *P. aeruginosa* [2].

1.1 *Pseudomonas*

Pseudomonads are members of the phylum Proteobacteria and class γ-Proteobacteria which contains other bacteria of medical importance, including the Enterics and Vibrios. The genus *Pseudomonas* was named in the 1890s by Walter Migula [78]. This genus now contains over 100 species which are mainly environmental organisms found in soil and water, and some of these act as pathogens of plants and animals [78]. The pseudomonads are able to grow on a wide range of nutrient sources that include various carbohydrates, fatty acids, amino acids, and long-chain hydrocarbons [95]. This metabolic diversity allows these organisms to reside in almost

every niche imaginable. In fact, *Pseudomonas* sp. have been isolated from refrigerated meat [67], sewage [51], and even ash byproducts of coal-fired power plants [82]. Some Pseudomonads, such as *P. putida*, are also utilized for biotechnology applications [73] and in bio-remediation of contaminated soils [49].

1.1.1 *P. aeruginosa.*

P. aeruginosa is the most well-known *Pseudomonas* species due to its prevalence as an opportunistic pathogen often encountered in the nosocomial environment [63]. This organism is a gram-negative bacterium that has long been acknowledged as a ubiquitous environmental organism, and is noted for its sophisticated ability to metabolize a vast array of compounds [95]. The genome of *P. aeruginosa* is comprised of approximately 6.3 million base pairs containing greater than 5500 open reading frames (ORFs) [96]. These ORFs allow *P. aeruginosa* to exhibit wide nutritional diversity and complex regulatory patterns to adapt to changing niches [3]. Capable of growing anaerobically with a terminal electron acceptor such as nitrate or aerobically in the presence of atmospheric oxygen, *P. aeruginosa* is considered a facultative anaerobe [110]. These bacteria are motile via swimming with a polar flagellum or twitching with type 4 pilus [70]. *P. aeruginosa* is capable of sessile growth in the form of robust, multicellular communities called biofilms which form on surfaces of all types in nature and the nosocomial environment [103]. These biofilms form and differentiate into highly ordered structures, directed by the organisms within that sense and respond to various nutritional and environmental cues [44]. In a pathogenic setting, biofilms provide *P. aeruginosa* a means to resist physical processes such as

desiccation, provide a physical barrier to penetration by molecules (i.e., antibiotics) and immune cells (when in the context of an infection), and allow the organisms to exist in a low metabolic state which can also aid in antibiotic resistance [101].

P. aeruginosa is known to be intrinsically resistant to many antimicrobials due to its hydrophobic lipopolysaccharide anchored to the outer membrane [60], chromosomally encoded enzymes such as *ampC* (which encodes a cephalosporinase) [61], and several efflux pumps [56]. Mutations in antibiotic targets can also lead to increased resistance as is the case for the quinolone class of antibiotics which target the DNA gyrase subunit A protein encoded by *gyrA* [108]. Antibiotic resistance can also be acquired laterally and lead to pan-resistance which has been noted for some clinical isolates [62].

1.1.2 *P. aeruginosa* pathogenesis.

Recently, *P. aeruginosa* has been recognized as a pathogen of many different organisms including plants, nematodes, animals, and as an opportunist in humans [9, 21, 86, 87, 97]. *P. aeruginosa* was reported as the most frequently isolated organism from burn wounds by 46 of 104 U.S. burn centers [4]. Another study found that *P. aeruginosa* was the most commonly isolated causative agent of bacterial keratitis, causing approximately 40-70% of these infections [104]. The nosocomial environment is also rife with *P. aeruginosa* as evidenced by the large number of patients that it infects in the hospital setting [21]. Aside from immunocompromised and hospitalized patients, *P. aeruginosa* frequently colonizes and causes pathology in the lungs of those afflicted with cystic fibrosis (CF) [31]. Up to 25% of children with CF are colonized by

age 5 and 80% of adults with CF (age 25-34 years) are infected by *P. aeruginosa,* which causes significant morbidity and mortality [59]. This is especially troubling due to the increasing rate of antibiotic and multidrug resistant strains mentioned previously. One study conducted at the University of Colorado Hospital from 1998-2002 found multi-drug resistant isolates (resistant to imipenem, ceftazidime, ciprofloxacin and tobramycin) to have increased from 17% to 32% in the five year sampling period [41]. *P. aeruginosa* is known to be a prevalent causative agent of ventilator-acquired pneumonia (VAP) and significantly increases mortality in patients with VAP [33]. Since *P. aeruginosa* can be found in diverse environments, adaptability is a hallmark of this organism when free-living in nature, in a pathogenic setting, or in the transition between environmental and pathogenic lifestyle. This remarkable ability to adapt to environmental cues is borne from an intricate regulatory network and the utilization of small, diffusible chemicals produced for inter-cellular signaling. *P. aeruginosa* constantly keeps one ‘ear’ toward the environment and one toward other members of a local population by sensing and responding to these signals in the process of quorum sensing [26].

1.2 Cell-to-cell signaling.

P. aeruginosa encodes three cell-to-cell signaling systems that are arranged in a hierarchy, but significant cross-talk exists between the systems [18, 52, 74]. Two systems have a function simliar to the well-studied *N*-acylhomoserine lactone (HSL) based LuxRI system of *Vibrio fischeri* [42]. These HSL signaling molecules are produced by a signal synthase, and as the concentration of these molecules increases

in the local environment it reaches a threshold where the signal can bind to its cognate receptor protein, which then alters gene expression at the transcriptional level [23].

1.2.1 *las* signaling system.

One of the HSL-based signaling systems of *P. aeruginosa* is the LasRI quorum sensing system which is placed at the top of a signaling hierarchy (see Fig. 1.1) due to the positive influence this system exerts on the other HSL system (*rhl*) and the quinolone signaling system [52, 92]. In the *las* system, the *N*-(3-oxododecanoyl) homoserine lactone (3-oxo-C_{12}-HSL) signal (Fig. 1.2) is produced by the LasI enzyme, and as this signal concentration increases in the environment, LasR (the transcriptional regulator) binds 3-oxo-C_{12}-HSL and the complex then binds DNA to regulate gene expression [29, 77]. Many genes are activated by LasR-3-oxo-C_{12}-HSL such as *lasB* (encoding the virulence factor elastase) and *lasI* (encoding the 3-oxo-C_{12}-HSL signal synthase), but many more genes are regulated indirectly via influence of the other cell-to-cell signaling systems [29, 74]. In acute infections the *las* system is important for virulence [28], but in a chronic infection setting (such as the CF lung), LasR is dispensable for virulence and is often mutated [35].

1.2.2 *rhl* signaling system.

It has been shown that LasR-3-oxo-C_{12}-HSL is required for activating transcription of the *rhlR* gene that encodes a transcriptional regulator which responds to the *N*-butyryl homoserine lactone (C_4-HSL) signal (Fig. 1.2) produced by the signal

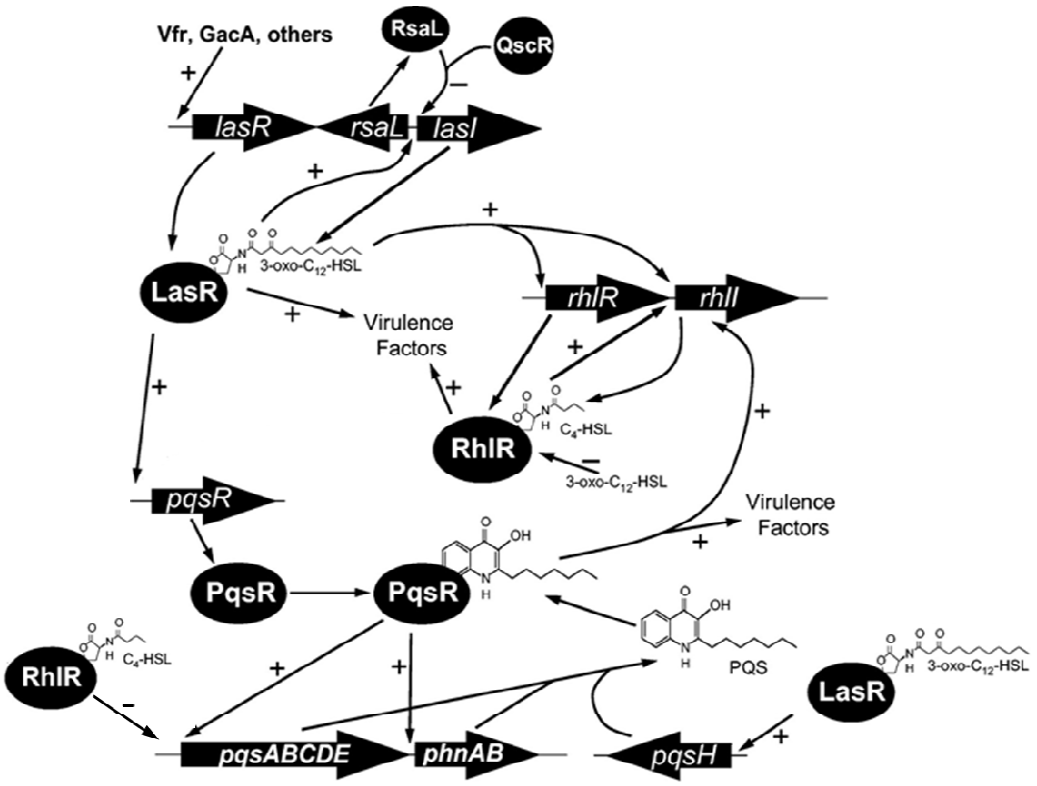

Figure 1.1. Schematic of cell-to-cell signaling hierarchy of *P. aeruginosa*. Three signaling systems are interconnected and all three influence virulence factor production. Adapted from Wade *et al.* [99].

synthase, RhlI [75, 80]. RhlR-C_4-HSL can then activate transcription of numerous genes including *rhlA,* which encodes an enzyme required for synthesis of the biosurfactant rhamnolipid [71]. It must also be noted that a large portion (up to 6%) of the *P. aeruginosa* genome is influenced by the *las* and *rhl* signaling systems [92]. While the *las* system activates quinolone signaling, the *rhl* system antagonizes quinolone signaling to further fine tune virulence determinant expression [6, 65].

1.2.3 Quinolone signaling system.

The *Pseudomonas* quinolone signal (2-heptyl-3-hydroxy-4-quinolone) or PQS (Fig. 1.3) is one molecule of a series of 56 related HAQ compounds produced by *P. aeruginosa* [55]. This signaling molecule is important for virulence in multiple models of infection including the mouse lung and the nematode *Caenorhabditis elegans* [27, 53]. Furthermore, PQS has been detected in sputum from the lungs of CF patients infected with *P. aeruginosa* pointing to a role for PQS in human disease [13]. The LasRI system activates transcription of the genes *pqsH* and *pqsR* which are required for activation of the quinolone signaling system of *P. aeruginosa* [79, 99]. It has been shown that PQS can still be produced in a *las* signaling mutant, but production is delayed and the concentration is lower than wild type [20]. While the RhlR regulator can decrease PQS biosynthesis by binding to the *pqs* operon promoter [6], it is also required to activate virulence factors with the function of PqsE [25]. This illustrates that the cross-talk between the signaling systems allows for genetic regulation in response to a very broad continuum of stimuli which help *P. aeruginosa* prevail as a pathogen (Fig. 1.1). To regulate quinolone synthesis, the transcriptional regulator PqsR binds PQS so that

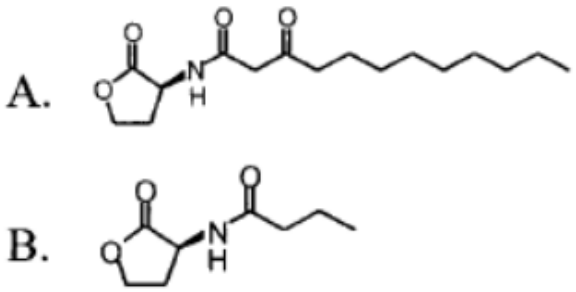

Fig. 1.2. Chemical structures of N-acylhomoserine lactone signaling compounds produced by *P. aeruginosa*. (A) *N*-3-oxo-C_{12}-HSL. (B) C_4-HSL. [79].

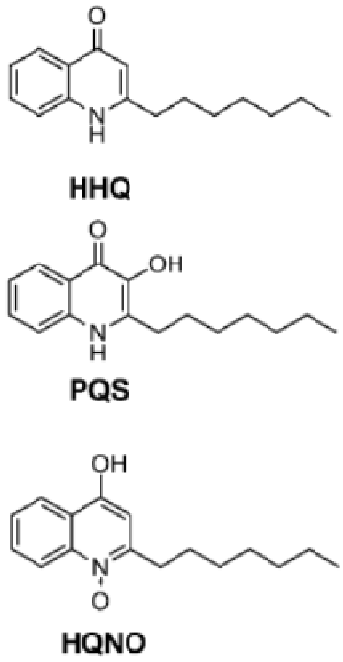

Fig. 1.3. Chemical structures of major alkylquinolones produced by *P. aeruginosa*. [84].

PqsR-PQS can then activate transcription of the *pqsABCDE* biosynthetic operon [14, 27]. *P. aeruginosa* also integrates environmental signals into the regulation scheme for quinolone signaling by increasing PQS production in response to low phosphate or low iron growth conditions [40].

The *pqs* operon is responsible for production of all quinolones including the antibiotic HQNO (Fig. 1.3) and HHQ (Fig. 1.3) which is the direct precursor to PQS [19, 54]. The accessory gene *pqsH* encodes a monooxygenase that is required for the conversion of HHQ into PQS to allow full bioactivity of the quinolone signaling system [90]. It has been demonstrated that the precursors anthraniloyl-CoA, malonyl-CoA, and octanoyl-CoA are combined through the actions of PqsABCD to form HHQ [12, 22]. The study by Dulcey *et al.* [22] proved that quinolones are not produced from 3-keto-fatty acids as originally hypothesized [5]. Just as the other signaling systems have regulons of indirectly regulated genes, the PQS signaling system regulon is no different, being comprised of an estimated 237 genes [88]. PqsR-PQS has been shown to bind to the *pqsA* promoter, and it has been postulated that binding to the *phnAB* promoter also occurs [9], but this only accounts for 5-7 genes directly regulated by PqsR-PQS [9, 99]. The larger global regulatory role of PQS is attributed to the fifth gene of the *pqs* operon, *pqsE*, which encodes a protein of unknown function that is not required for PQS biosynthesis but is indispensable for responding to PQS and affecting transcription of PQS regulon genes [88]. However, it has also been shown that overexpression of *pqsE* can compensate for a loss of quinolone production by synergizing with the *rhl* system to affect production of many virulence factors including rhamnolipid and pyocyanin [25]. Recently, it was shown that an alternate transcript originating in the coding region of

pqsC could drive expression of *pqsDE-phnAB* in a PQS-independent manner in response to tryptophan-deplete growth conditions [45], illustrating that *P. aeruginosa* has evolved several ways to produce PqsE even without PQS signaling.

Since quinolone biosynthesis provides another layer of regulation for fine-tuning *P. aeruginosa* virulence, it makes an attractive target for the development of anti-infective compounds that could diminish virulence without necessarily being bactericidal. D'Argenio *et al.* identified many of the genes in *P. aeruginosa* that abolish or drastically reduce PQS concentration by use of an elegant screen that identified suppressor mutations in the autolytic *pqsL* mutant background [14]. The autolytic phenotype identified by D'Argenio *et al.* was shown to correlate with PQS overproduction but the direct mechanism contributing to autolysis is unknown [14]. Fig. 1.4 shows the genes identified by the suppressor mutagenesis screen of D'Argenio *et al.* This study identified not only the biosynthetic operon for quinolone production but also accessory genes for PQS and HQNO production such as *pqsH* and *pqsL* (respectively), and genes that provide required precursors such as kynurenine [24]. One gene (*PA5506*) shown to modulate PQS concentration in the D'Argenio study encodes a homolog of an RpiR-type transcriptional regulator. Organic extracts from a PA5506 mutant are shown in Fig. 1.5 visualized by thin layer chromatography. This figure shows increased PQS produced by the *pqsL* mutant compared to the wild type strain and the reduced PQS in the *PA5506*, *pqsL* double mutant. Gene *PA5506* affected the concentration of the PQS signal in *P. aeruginosa* through an unknown mechanism, and its role in PQS production is the basis for this dissertation.

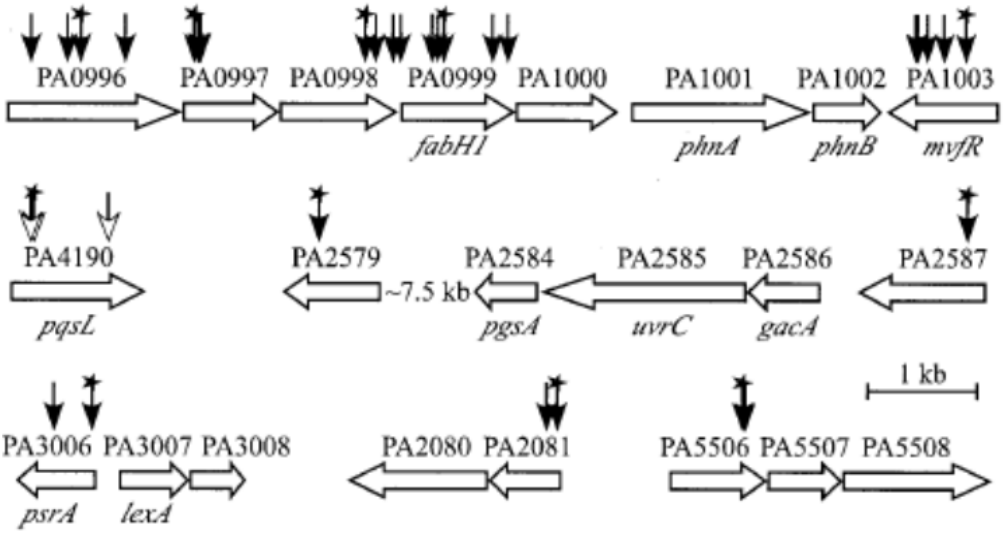

Figure 1.4. Genes identified in autolysis reversion screen in *P. aeruginosa*. Open white arrowheads denote insertions which yielded the autolytic phenotype. Filled arrowheads denote insertions in genes which reverted autolytic colonies to wild type morphology. Stars indicate insertions that were further characterized by D'Argenio *et al.* [14].

1.3 RpiR-type regulators.

This family of transcription factors is so named for the prototype from *Escherichia coli* that represses expression of *rpiB*, which encodes ribose-phosphate isomerase B (a part of the oxidative pentose phosphate pathway) [94]. Most members of this family regulate genes of central metabolism in response to a pathway intermediate or precursor. These transcription factors contain an N-terminal helix-turn-helix (HTH) DNA binding domain and C-terminal sugar isomerase [SIS] domain shown to bind a cofactor [15, 94]. GlvR, an RpiR homolog from *Bacillus subtilis*, has been shown to activate transcription of genes required for maltose metabolism in response to maltose-6-phosphate [107]. However, most characterized RpiR homologs function as repressors of carbohydrate catabolic enzymes. This is the case for ribose and *N*-acetylmuramic acid catabolism in *E. coli* and hexose catabolism in *P. putida* which are all repressed by RpiR homologs [15, 39, 94]. In the case of *P. putida*, the RpiR homolog HexR is named for its role in the repression of genes of the glucose phosphorylative pathway and Entner-Doudoroff (ED) pathway [15]. It was also shown that a metabolic intermediate of the ED pathway, 2-keto-3-deoxy-6-phosphogluconate, served as the co-factor for HexR, which upon binding allowed the protein to dissociate from DNA *in vitro* and derepress transcription of target genes [15]. RpiR homologs can regulate not only enzymes of the pentose phosphate pathway but also virulence factor expression through RNAIII transcription in the opportunistic pathogen *Staphylococcus aureus* [109]. Interestingly, in *S. aureus*, RNAIII transcription is linked to the *agr* small peptide signaling system [69]. While small peptide signaling is not homologous to cell-to-cell signaling in *P. aeruginosa,* it is thought-provoking that another opportunistic pathogen would

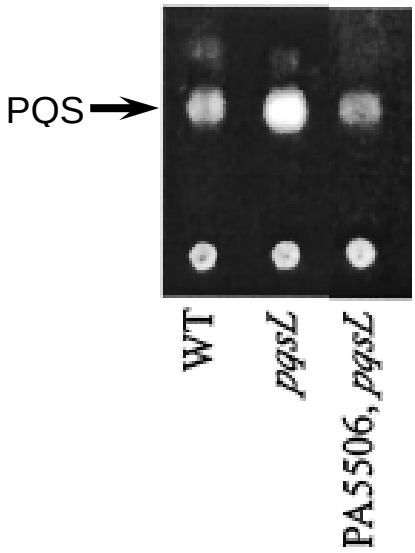

Fig. 1.5. Thin layer chromatography of PQS extracts. Wild type strain PAO1 (WT), *pqsL* (pqsL64 transposon mutant), PA5506, *pqsL* (PA5506 transposon mutant in Δ*pqsL* background) [14].

encode transcription factors that allow concerted regulation of virulence via intercellular signaling in response to metabolic cues.

The RpiR-type regulator investigated here was found to repress transcription of an operon that contains itself and three downstream genes (*PA5507*, *PA5508*, and *PA5509*). *PA5507* encodes a protein with sequence homology to isochorismatases, *PA5508* encodes a protein with sequence homology to glutamine synthetases and has been shown to exhibit γ-glutamyl aromatic monoamine ligase activity *in vitro* [50], and *PA5509* encodes a protein with sequence homology to amidohydrolases [2]. The putative functions of these enzymes does not suggest a reaction directly involving HAQs, but we have found that overexpression PA5507, PA5508, and PA5509 has a negative effect on PQS concentration.

1.4 Translational coupling.

Bacterial genes are sometimes co-regulated by a single promoter when genes are arranged into operons that are transcribed as polycistronic mRNAs. Translation of each ORF in a polycistronic mRNA is most commonly initiated at a ribosome binding site (RBS) upstream from the ORF. Each ORF typically has an RBS dedicated to initiating translation of a single protein but some ORFs have been found with no identifiable RBS. ORFs that overlap one another or that lack a functional RBS can potentially be regulated through post-transcriptional mechanisms. Translational coupling is a post-transcriptional regulatory mechanism which allows for control of protein stoichiometry produced from operons in bacteria and bacteriophage [38, 72]. In most cases of translational coupling, translation of a downstream ORF is positively affected

by translation of an ORF directly upstream. Translation of the upstream ORF can affect the downstream ORF by either alleviating mRNA secondary structure to allow internal ribosomal initiation of the downstream ORF [7], or by re-initiation of ribosomes that have translated the upstream ORF and terminated near the translational start codon of the downstream ORF [48]. Maintenance of protein stoichiometry through translational coupling is important for structures composed of multiple protein subunits such as the DNA injection apparatus from *Agrobacterium tumefaciens* [68]. Careful control of the translation of regulatory proteins such as those composing two component systems that sense environmental cues can be post-transcriptionally regulated by translational coupling [58]. Commonly, translationally coupled ORFs are overlapping one another or have their respective stop and start codons in close proximity. However, genes can be transcribed in operons with overlapping ORFs that are not regulated by translational coupling [43]. In Chapter 3 of this dissertation, we present data which show that translational coupling of an ORF to the PA5506 transcriptional regulator plays a role in the modulation of PQS concentration. This regulatory mechanism may have evolved to allow for tight control of expression of PA5507 which acts with PA5508 and PA5509 to reduce PQS concentration through an unknown mechanism [98].

CHAPTER TWO: QAPR (PA5506) REPRESSES AN OPERON THAT NEGATIVELY AFFECTS THE *PSEUDOMONAS* QUINOLONE SIGNAL IN *PSEUDOMONAS AERUGINOSA*

2.1 Summary

Pseudomonas aeruginosa is a gram negative, opportunistic pathogen that can cause disease in varied sites within the human body and is a significant source of morbidity and mortality in those afflicted with cystic fibrosis. *P. aeruginosa* is able to coordinate group behaviors, such as virulence factor production, through the process of cell-to-cell signaling. There are three intercellular signaling systems employed by *P. aeruginosa* and one of these systems utilizes the small molecule 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal; PQS). PQS is required for virulence in multiple infection models and has been found in the lungs of cystic fibrosis patients colonized by *P. aeruginosa*. In this report, we have identified an RpiR-family transcriptional regulator, QapR, which is an autoregulatory repressor. We found that mutation of *qapR* caused overexpression of the *qapR* operon. We characterized the *qapR* operon to show that it contains genes *qapR*, PA5507, PA5508, and PA5509, and that QapR directly controls the transcription of these genes in a negative manner. We also show that derepression of this operon greatly reduces PQS concentration in *P. aeruginosa*. Our results suggest that *qapR* affects PQS concentration by repressing an enzymatic pathway that acts on PQS or a PQS precursor to lower PQS concentration. We believe that this operon comprises a novel mechanism to regulate PQS concentration in *P. aeruginosa*.

2.2 Introduction

Pseudomonas aeruginosa is a ubiquitous, gram negative bacterium that can infect a broad range of hosts including insects, plants, and animals [16, 87, 97]. This prevalent opportunistic pathogen is frequently acquired in the nosocomial setting and causes intractable infections in the lungs of cystic fibrosis patients [13, 21, 31]. The ability of this organism to colonize and cause disease is tied to its vast array of virulence factors such as pyocyanin, alkaline protease, hydrogen cyanide, elastase, and rhamnolipid which are produced in response to intercellular signals [18, 74, 92, 100].

There are three cell-to-cell signaling systems that *P. aeruginosa* utilizes to coordinate expression of numerous genes for metabolic processes and virulence factor production [92, 100]. The *lasRI* signaling system is at the top of the cell-to-cell signaling hierarchy and positively regulates the *rhlRI* and quinolone signaling systems [80]. The *las* and *rhl* systems produce and respond to the acyl-homoserine lactone signals, *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butyryl-L-homoserine lactone (C_4-HSL), respectively [75, 77]. The other cell-to-cell signaling system functions through the quinolone compound 2-heptyl-3-hydroxy-4-quinolone (the *Pseudomonas* quinolone signal (PQS)) [79]. Production of PQS is mediated by the products of the *pqsABCD* operon and the *pqsH* gene, and occurs through the condensation of the precursors anthranilate and β-ketodecanoic acid [5]. PQS then interacts with and activates PqsR, which positively controls expression of the PQS biosynthetic operon (*pqsABCDE*) in an autoregulatory loop [99]. Transcription of the *pqsR* gene is regulated in a complex manner by the *las* and *rhl* signaling systems [30, 99]. The quinolone

biosynthetic machinery also produces 55 other 4-quinolone compounds, many of which function as antimicrobials [55].

A prior study of factors that control the autolytic phenotype of *P. aeruginosa* identified 10 mutated genes that altered PQS production [14]. Subsequent investigations of these genes have yielded a wealth of knowledge about the precursors and biosynthesis of PQS in *P. aeruginosa* [12, 24, 46, 90]. One of the mutants was found to have a disruption in gene *PA5506* [hereafter referred to as *qapR* for quinolone alteration pathway regulator] which encodes an RpiR-family transcriptional regulator homolog. RpiR homologs are usually transcriptional repressors of carbohydrate metabolic genes [15, 94]. In this report, we present data demonstrating that QapR is a negative autoregulatory transcription factor that controls a four gene operon. We show that the *qapR-PA5509* operon has a negative effect on PQS concentration to provide another layer of regulation for quinolone signaling.

2.3 Materials and Methods

2.3.1 Bacterial strains, plasmids, and growth conditions.

Bacterial strains used in this study are listed in Table 1. Strains of *P. aeruginosa* and *Escherichia coli* were maintained at -70^{o}C in 10% skim milk (Becton-Dickinson) or 15% glycerol, respectively. Bacteria were freshly plated from frozen stocks to begin each experiment and bacteria were grown in Luria-Bertani [LB] medium as noted below [89]. When required to maintain plasmids, cultures were supplemented with 200 µg/ml carbenicillin for *P. aeruginosa* and 100 µg/ml ampicillin for *E. coli*. Arabinose (Sigma-Aldrich) was added to cultures to induce expression of genes under the control of the P_{BAD} promoter where indicated.

Plasmids used in this study are listed in Table 2.1. To generate an expression plasmid for *qapR*, a 1078 bp DNA fragment, which began at the *qapR* start codon (ATG) and ended 220 bp downstream from the stop codon, was amplified by PCR using chromosomal DNA from strain PAO1 as a template. The oligonucleotide primers used for PCR were engineered to include an XbaI site upstream from the start codon and a HindIII site downstream from the stop codon. The fragment was purified from an agarose gel with a gel extraction kit (MPBio). Vector plasmid pHERD20T [85], which contains a P_{BAD} promoter to control gene expression, was digested with XbaI and HindIII restriction enzymes (New England Biolabs). PCR-amplified DNA fragments were also digested with XbaI and HindII and prepared fragments were ligated to produce the P_{BAD}-*qapR* expression plasmid pqapROE.

To generate a PA5507-5509 expression plasmid, a 2,818 bp DNA fragment that began at the PA5507 start codon (ATG) and ended 100 bp downstream from the

PA5509 stop codon was amplified using PAO1 genomic DNA as a template. Primers were engineered to include an NcoI restriction site upstream from the PA5507 start codon and a HindIII site downstream from the PA5509 stop codon. Vector pHERD20T and PA5507-5509 DNA were both digested with NcoI and HindIII and the resulting fragments were gel purified. Fragments were then ligated to produce the P_{BAD}-PA5507-5509 expression plasmid p5507-09OE.

A PQS biosynthetic operon expression plasmid was generated by amplifying a 4,577 bp fragment that began at the *pqsA* start codon (ATG) and ended 83 bp downstream from the *pqsD* stop codon. Primers were engineered to include an NcoI restriction site upstream from *pqsA* and an XbaI restriction site downstream from *pqsD*. Vector pHERD20T and the *pqsABCD*-containing fragment were digested with NcoI and XbaI and both fragments were gel purified. Fragments were then ligated to produce the P_{BAD}-*pqsABCD* expression plasmid pPQSsynOE.

In order to generate a *qapR'-lacZ* reporter plasmid, a 300 bp DNA fragment corresponding to bp -230 to +70 relative to the *qapR* translational start site was amplified via PCR with strain PAO1 genomic DNA as a template. PCR primers were engineered to include a HindIII site and an XbaI site upstream and downstream from the fragment, respectively. The DNA fragment and pLP170 were digested with HindIII and XbaI and were ligated together. The resulting plasmid, pLP5506, harbors the *qapR* promoter transcriptionally fused to the *lacZ* gene.

To generate the *pqsR'-lacZ* chromosomal reporter fusion, an 850 bp DNA fragment corresponding to bp -776 to +94 relative to the *pqsR* translational start site was amplified via PCR with strain PAO1 genomic DNA as a template. PCR primers

were engineered to include a PstI site and a HindIII site upstream and downstream from the fragment, respectively. The DNA fragment and pUC18-mini-Tn7T-Gm-*lacZ* were digested with PstI and HindIII and were ligated together. The resulting plasmid, pJF-Tn7T-pqsR'Tc, harbors the *pqsR* promoter transcriptional fusion that can be integrated into strains as a chromosomal fusion as in Choi & Schweizer [11].

All plasmids were confirmed by DNA sequencing of insert DNA. All primers for these studies are listed in Table 2.2.

2.3.2 Generation of mutant strains.

Mutant *P. aeruginosa* strains were generated as described previously [34]. Mutant alleles were generated using a splicing by overlap extension (SOE) PCR protocol [37]. The mutant alleles contain in-frame deletions in the coding DNA sequence corresponding to amino acids 30 to 275 for *qapR* (86% of protein sequence), 19 to 204 for PA5507 (84% of protein), 28 to 421 for PA5508 (89% of protein), and 32 to 198 for PA5509 (75% of protein). Oligonucleotide primers used to generate mutant alleles also contained the following restriction sites at each end: BamHI for *qapR* and PA5509, XbaI for PA5507 and PA5508. The fragments were digested with the appropriate enzyme and ligated into pEX18Ap that had been previously digested. This ligation produced the suicide vectors pΔqapR, pΔ5507, pΔ5508, pΔ5509. To transfer the mutant alleles onto the *P. aeruginosa* PAO1 chromosome, each plasmid was electroporated into cells and integrants were selected as described by Choi & Schweizer [11]. Potential mutants were screened by PCR using appropriate primers and mutants were further confirmed by sequencing of generated PCR products.

2.3.3 Assays for PQS and pyocyanin.

To assay for PQS production, bacteria from frozen skim milk stocks were plated on LB medium with antibiotics as necessary. Isolated colonies were used to inoculate 10 ml LB broth cultures which were incubated at 37^{o}C with vigorous shaking for 18 h. PQS was extracted as described by Calfee *et al.* [8] and PQS extracts were visualized as described by D'Argenio *et al*. [14]. Images were then analyzed using Quantity One software (Invitrogen) to quantify PQS by densitometry.

For pyocyanin extraction, cultures were grown from freshly plated bacteria as above. Isolated colonies were used to inoculate 10 ml LB broth cultures which were incubated at 37^{o}C for 18 h with vigorous shaking. Samples of cultures were centrifuged at 16,000 x *g* to remove cells and 500 µl of supernatant was transferred to a clean tube. The supernatant was then extracted and analyzed for pyocyanin production as previously described by Farrow *et al.* [24].

2.3.4 β-Gal assays in *P. aeruginosa*.

Cells from overnight cultures were washed in LB medium and used to inoculate 10 ml LB cultures to an OD_{660} of 0.05. Cultures were incubated at 37^{o}C with vigorous shaking. At 6 and 24 h, aliquots were collected and β-Gal activity was assayed in duplicate. Activity is reported in Miller units as the mean ± standard deviation of at least three separate replicates .

For experiments including the addition of PQS, cells from overnight culture were washed in LB medium and used to inoculate 2 ml LB cultures with or without the

addition of 30 µM PQS in acidified ethyl acetate. PQS or ethyl acetate (as a control) was evaporated under N_2 gas in 13 ml capped tubes prior to the addition of culture. Cultures were then incubated at 37^{o}C with vigorous shaking. β-Gal activity was assayed at 24 h in duplicate as above. Activity is reported in Miller units as the mean ± standard deviation of at least three independent experiments [66].

2.3.5 RNA isolation.

Overnight cultures of strains PAO1 and PKT-QapR1 were washed in LB medium and used to inoculate 10 ml LB broth cultures to an OD_{660} 0.05. Cultures were incubated at 37^{o}C for 3 h or to an OD_{660} of approximately 1.5 prior to centrifugation at 4^{o}C to harvest bacterial cells. Total cellular RNA was isolated from *P. aeruginosa* cultures using the RNeasy Midiprep kit following the manufacturer's protocol (Qiagen). Contaminating DNA was removed by treatment of RNA samples with RQ1 DNase following the manufacturer's protocol (Promega). RNA was then extracted with 1:1 phenol:chloroform and collected by ethanol precipitation. Purified RNA was resuspended in nuclease-free water. RNA concentration was quantified with a Nanodrop ND-1000 spectrophotometer.

2.3.6 Transcript analysis by reverse transcriptase PCR (RT-PCR).

Purified RNA (50 ng) from strain PAO1 was used as a template for RT-PCR performed with the Promega Access RT-PCR system following the manufacturer's protocol. Primer pairs were designed to span intergenic regions of all genes of interest. All cDNA synthesis and PCR amplification steps were performed in an Eppendorf

Mastercycler with the following parameters: cDNA synthesis at 45^{o}C for 45 min; 95^{o}C for 2 min; 30 cycles of 95^{o}C for 30 sec, 55-62^{o}C for 30 sec (different annealing temperatures were required to optimize different primer sets), 72^{o}C for 45 sec. The final cycle was followed by heating the samples at 72^{o}C for 5 min. Positive controls were performed using genomic DNA and negative controls were performed without the addition of reverse transcriptase enzyme. Reaction products were analyzed by agarose gel electrophoresis.

2.3.7 cDNA synthesis for quantitative real time-PCR.

Total RNA was isolated from strains PAO1 and PKT-QapR1 and purified as described above. cDNA was synthesized in a 21 µl reaction volume from 5 µg of total RNA using a 1:1 mixture of GC-rich hexamers (Gene Link) and random hexamers (Invitrogen) for priming with 40 µM dNTPs (USB). Reactions were then heated at 65^{o}C for 5 minutes followed by cooling to 4^{o}C for 1 minute. Following this step 200 units of SuperScript III reverse transcriptase in First Strand buffer (Invitrogen) with dithiothreitol and RNase Out RNase Inhibitor (Invitrogen) were added to each reaction yielding a final volume of 30 µl. Reactions were then heated to 25^{o}C for 5 minutes, followed by 50^{o}C for 60 minutes, then 75^{o}C for 15 minutes.

2.3.8 Quantitative Real Time-PCR.

cDNA and total RNA to be used as template and negative control, respectively, were diluted 1:200 in nuclease-free water. Oligonucleotide primer pairs for qRT-PCR were generated by the Primer-Blast program available at

www.ncbi.nlm.nih.gov/tools/primer-blast/. Primers were designed to amplify a 200 bp fragment of *rplU* (control), a 213 bp fragment of *PA5507,* a 140 bp fragment of *PA5508,* and a 199 bp fragment of *PA5509* as target genes. qRT-PCR was performed using FastStart SYBR Green Master mix (Roche Diagnostics) with the BioRad CFX96. The following cycle was utilized to amplify and quantify fragments: 95^{o}C for 10 minutes; 95^{o}C for 15 seconds, 55^{o}C for 15 seconds, and 72^{o}C for 20 seconds - repeated 40 times. Melt curve data was collected to ensure amplification of one fragment by heating samples from 65^{o}C to 95^{o}C in 0.5^{o}C increments. Data were generated from three separate RNA/cDNA preparations and at least two technical replicates for each primer set. Relative expression of each gene was determined by comparing target genes with the control gene (*rplU*) using the Pfaffl method [81].

2.3.9 Preparation of *E. coli* lysate containing QapR.

Overnight cultures of *E. coli* strain DH5α harboring the empty vector pHERD20T or the expression vector pqapROE were subcultured to an OD_{600} of 0.08. Cultures were incubated at 37^{o}C with vigorous shaking for 2.5 h and then l-arabinose was added to a final concentration of 1% to induce expression of QapR. The cultures were allowed to grow for another 2.5 h following addition of arabinose. After 5 h of total growth, cells were harvested from cultures by centrifugation at 6,000 x *g* for 10 min at 4^{o}C. Bacterial cell pellets were resuspended in 1 ml of STE buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl) and this suspension was passed through a French pressure cell at 16,000 psi to yield whole cell lysate. Protein concentration for DNA mobility shift assay was assessed using a Bradford assay (Bio-Rad).

2.3.10 DNA mobility shift assay.

PCR was used to generate DNA fragments containing the *qapR* (198 bp) or *pqsA* (300 bp) promoter region. DNA probes were labeled with ^{32}P using [γ-^{32}P]ATP (Perkin-Elmer) and T4 polynucleotide kinase (Invitrogen). Binding reactions were carried out in buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol [15]. Each reaction contained 0.3 µg salmon sperm DNA, 10^6 cpm of radiolabeled probe and 0 to 1 µg of lysate protein. Reaction mixtures were incubated at room temperature for 20 min and separated by electrophoresis on a native 6% polyacrylamide gel in 0.5X tris-borate-EDTA buffer at 4^{o}C. Gels were then exposed to X-ray film to visualize radiolabeled bands.

2.4 Results

2.4.1 *qapR* controls PQS concentration.

Previously, it was shown that disruption of *qapR* (gene *PA5506*) caused decreased PQS production and suppression of autolysis in a *pqsL* mutant that overproduced PQS [14]. In order to investigate this phenotype, we constructed an isogenic *qapR* mutant and analyzed PQS levels. This mutant exhibited a reduction in PQS concentration in 18 h cultures compared to strain PAO1 (Fig. 2.1A). PQS concentration could be restored to wild type levels in the *qapR* mutant when *qapR* was provided on a plasmid (Fig. 2.1A). In addition, mutation of *qapR* caused a decrease in pyocyanin production in a manner similar to the effect seen for PQS (Fig. 2.1B). This was expected since pyocyanin biosynthesis is positively controlled by PQS. Taken together, the data of Fig. 2.1 confirm that PQS concentration is controlled by *qapR*.

2.4.2 Transcription of the PQS biosynthetic operon, but not *pqsR*, is altered in the *qapR* mutant.

In order to dissect the mechanism by which *qapR* modulates PQS concentration, we first assessed its effects on the transcription of the PQS biosynthetic operon. We found that *pqsA* transcription decreased in the *qapR* mutant as compared to the wild type strain PAO1 (Fig. 2.2A). Since the *pqsA* promoter is activated by PqsR-PQS [99], deficiency of either PqsR or PQS can cause reduced activation of the biosynthetic operon. To test whether PqsR was active in the *qapR* mutant, we added synthetic PQS to cultures and assessed *pqsA* activation. As can be seen in Fig. 2.2A, the addition of PQS to cultures restored *pqsA'-lacZ* transcription in the *qapR* mutant strain to the level

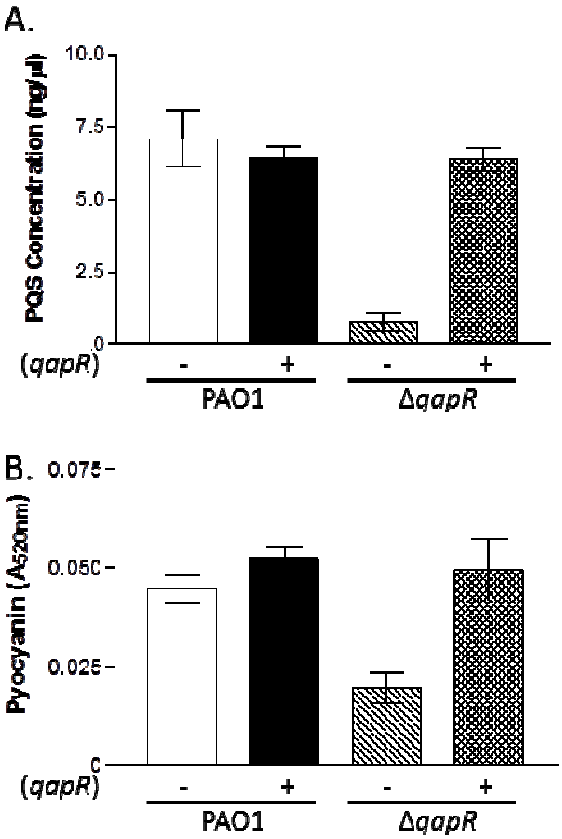

Figure 2.1. Mutation of qapR decreased PQS concentration in *P. aeruginosa*. Strains are indicated below each bar and the presence of *qapR* expression plasmid (pqapROE) or control vector (pHERD20T) is indicated with a plus (+) or minus (-), respectively. PAO1 is the wild type strain and Δ*qapR* is strain PKT-QapR1. (A.) Cultures were grown for 18 h in LB medium supplemented with 1% $_{L}$-arabinose and PQS was extracted and quantified as described in Materials and Methods. Data are presented as the averages +/- standard deviation (SD) of three independent experiments. (B.) Pyocyanin was extracted and quantified from cultures grown for 18 h in LB medium supplemented with 1% $_{L}$-arabinose. Data are presented as averages +/- SD of three independent experiments.

produced by strain PAO1. This suggested that PqsR expression is unaffected in the *qapR* mutant and we confirmed this by assessing transcriptional activity from the *pqsR* promoter (Fig. 2.2B). These data implied that PQS concentration is the cause of the decreased *pqsA* transcription in the *qapR* mutant. To confirm this, we expressed the PQS biosynthetic operon (*pqsABCD*) from an inducible promoter in the *qapR* mutant strain and assayed for PQS and pyocyanin. We found that expression of the PQS biosynthetic operon restored PQS concentration and pyocyanin production in the *qapR* mutant background (Fig. 2.2C and 2.2D). These results showed that the *qapR* mediated modulation of PQS concentration can be overcome by PQS-independent expression of the *pqsABCD* operon. This implies that the precursors for PQS production are present and that expression of the quinolone synthetic machinery is affected by *qapR* mutation. Since anthranilate is critical for the production of PQS [24], we supplemented cultures with anthranilate to determine that this precursor was not depleted in the *qapR* mutant.

We found that supplementation of culture media with anthranilic acid could not restore PQS concentration in the *qapR* mutant strain (data not shown). Results of these experiments implied that expression of the *pqsABCD* operon is affected only by decreased PQS level due to mutation of *qapR*. This spurred us to look more closely at *qapR* and the genes adjacent to it.

2.4.3 Characterization of the *qapR*-PA5509 operon.

In order to understand the role of *qapR*, it was necessary to analyze the putative operon that it leads. The *qapR* gene is predicted to be at the 5’ end of a four gene

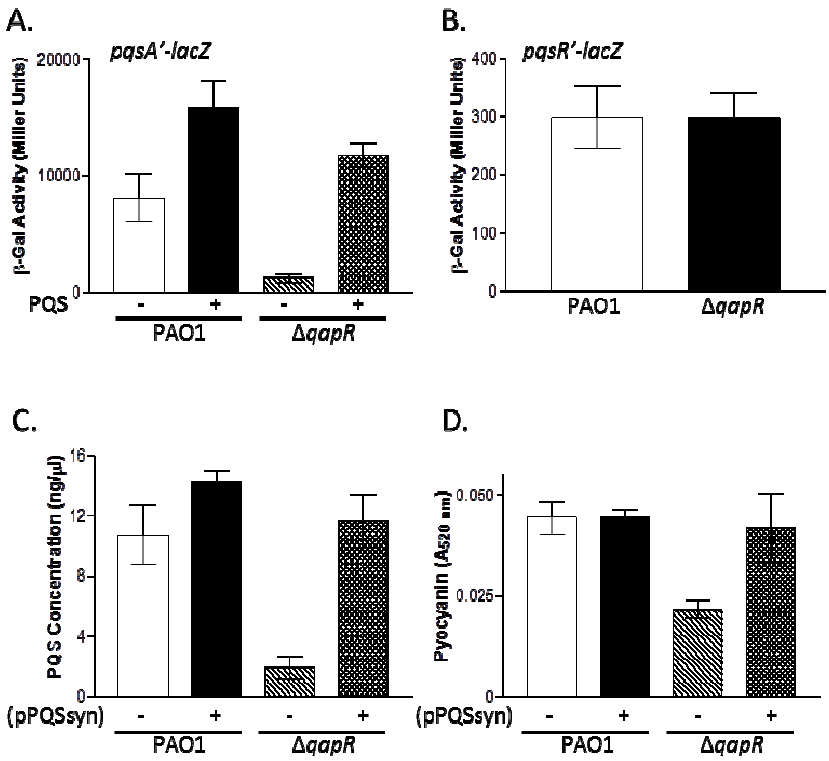

Figure 2.2. Supplementation of PQS can restore transcription of the PQS operon in the *qapR* mutant. (A.) Strains carrying a *pqsA*'-*lacZ* fusion on pLP0996 were grown for 24 h with 30 μM PQS where indicated by a plus symbol (+). β-Gal activity was then assayed and is presented in Miller units as the mean +/- SD of results from duplicate assays from three separate experiments. Strains are indicated below each bar: PAO1 is the wild type strain and Δ*qapR* is strain PKT-QapR1. (B.) Strains with a *pqsR*'-*lacZ* fusion integrated into the chromosome were grown for 24 h in LB medium. β-Gal activity produced in each culture was then assayed and is presented in Miller units as the mean +/- SD of results from duplicate assays from three separate experiments. (C.) Strains carrying the PQS biosynthetic operon (pPQSsyn) or control vector (pHERD20T) indicated by a plus (+) or minus (-), respectively, were grown in LB medium for 18 h supplemented with 1% $_{L}$-arabinose. PQS was extracted and quantified as described in Materials and Methods. Data are presented as the averages +/- SD of three independent experiments. (D.) Pyocyanin was extracted and quantified from cultures grown as described in (C.). Data are presented as averages +/- SD of three independent experiments.

polycistronic operon which includes the three downstream genes, *PA5507-5508-5509* [64].These genes are illustrated in Fig. 2.3A, along with the gene *PA5510*. It has been reported that gene *PA5508* encodes an enzyme that exhibits γ-glutamyl aromatic monoamine ligase activity while a BLAST search shows that genes *PA5507* and *PA5509* are predicted to encode proteins with sequence homology to iso-chorismatases and amidohydrolases, respectively [50]. To analyze the operon structure, we utilized reverse transcriptase PCR from total RNA extracted from strain PAO1. We found that *qapR* is co-transcribed with genes *PA5507*, *PA5508*, and *PA5509* (Fig. 2.3B). A link between genes *PA5509* and *PA5510* was not indicated by our data (Fig. 2.3B). Since *qapR* is co-transcribed with three downstream genes, and other RpiR homologs have been shown to be autoregulatory, we examined the role of QapR in the control of this four gene operon.

To investigate regulation of the *qapR* operon, a *qapR'-lacZ* reporter fusion was constructed and we assessed transcriptional activity from the *qapR* promoter in strain PAO1 and in the *qapR* mutant strain. Fig. 2.4A shows that transcription from the *qapR* promoter is significantly higher in the *qapR* mutant compared to strain PAO1. This was not surprising since most RpiR homologs have been demonstrated to act as repressors of transcription [15, 39, 47, 94]. To further analyze the regulation of the *qapR* operon, we performed quantitative real time-PCR (qRT-PCR) to assess the relative expression of genes *PA5507*, *PA5508* and *PA5509* in the *qapR* mutant strain. The results of these experiments showed that each target gene is expressed at a level 15 to 20 fold higher in the *qapR* mutant strain when compared to the wild type strain PAO1. These data

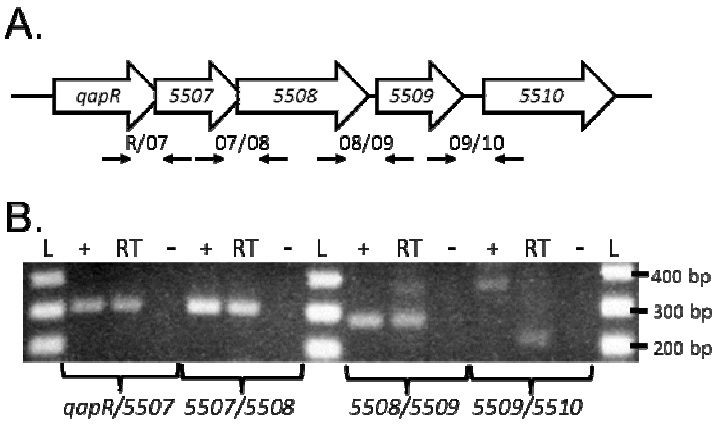

Figure 2.3. qapR is the first gene of a four gene operon. (A.) Schematic diagram of the predicted *qapR* operon and gene *PA5510*. Arrows below the schematic indicate the relative position of primers used to amplify each intergenic region. (B.) Agarose gel visualization of products from RT-PCR experiments performed with oligonucleotide primers specific for each intergenic region of the *qapR* operon. Reactions are labeled 'RT' for reverse transcriptase, '+' for positive control and '-' for negative control. Chromosomal DNA was used as a template for positive controls, and for negative controls, reverse transcriptase was omitted from reactions. Molecular mass ladders (1 Kb Plus Ladder, Invitrogen) are labeled 'L' and relevant masses (noted as bp) are indicated next to the image.

confirm that the *qapR* operon is derepressed in the *qapR* mutant and suggested that QapR is the repressor.

To test if QapR directly regulates the *qapR* operon, we assessed direct binding of the protein to the promoter by electrophoretic mobility shift assay (EMSA). To investigate this, *E. coli* cell lysates containing QapR were utilized in EMSA experiments with the *qapR* operon. The data showed that lysates containing QapR interacted with the radiolabeled *qapR* operon promoter region causing a shift in DNA mobility (Fig. 2.5A). A minimal amount of protein (0.1 µg of cell lysate) was able to alter the mobility of the

qapR promoter probe and this effect intensified as increasing protein was added (up to 1 µg). The mobility of the *qapR* fragment was unaffected by a control lysate prepared using *E. coli* that harbored the parent expression vector (Fig. 2.5A), indicating that the observed binding is due to the presence of QapR. We also assessed lysate binding to the *pqsA* promoter and observed no interaction (Fig. 2.5B). This result provided further evidence against the possibility that QapR is directly affecting *pqsA* transcription. Considering these results, we decided to investigate the QapR-controlled PA5507, PA5508, and PA5509 genes to see if they were the cause of decreased PQS concentration in the *qapR* mutant strain.

2.4.4 The *qapR* operon decreases PQS concentration.

Since QapR is repressing transcription of the *qapR* operon, we wanted to determine the role played by the PA5507, PA5508, and PA5509 proteins in influencing PQS concentration. When we expressed these three genes from an inducible promoter in strain PAO1 they caused a decrease in PQS concentration to a level very similar to

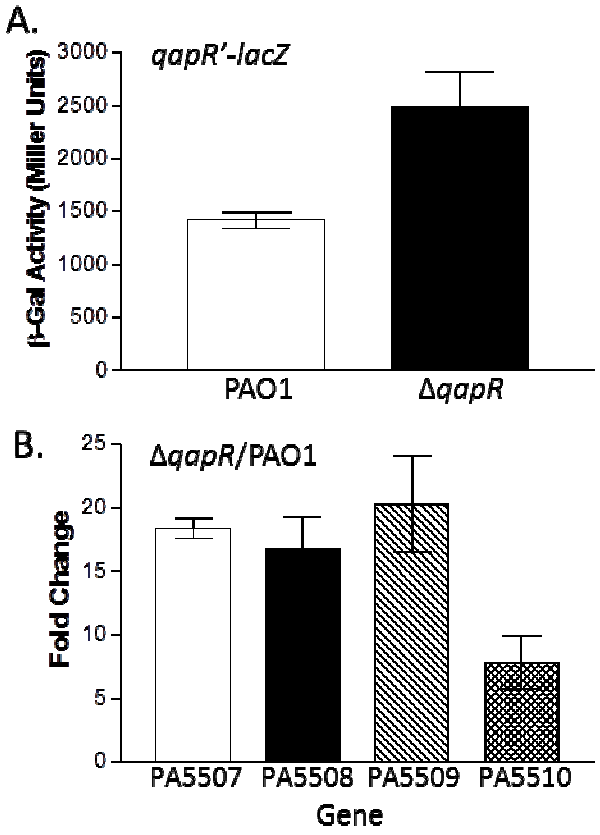

Figure 2.4. Transcription of the qapR operon is autorepressed. (A.) Strains carrying a *qapR*'-*lacZ* reporter on plasmid pLP5506 were grown for 6 h in LB medium. β-Gal activity produced in each culture was then assayed and is presented in Miller units as the mean +/- SD of results from duplicate assays from three separate experiments. Strains are indicated below each bar: PAO1 is the wild type strain and Δ*qapR* is strain PKT-QapR1. (B.) Quantitative real time-PCR was performed on strains PAO1 and PKT-QapR1. Data are presented as fold change +/- SD as compared to the housekeeping control gene *rplU* (set to a value of 1) from three separate biological samples. Target genes are indicated below each bar.

that produced by the *qapR* mutant strain (Fig. 2.6). These data suggest that at least one of the enzymes encoded by *PA5507*, *PA5508*, or *PA5509* is responsible for the decrease in PQS seen in the *qapR* mutant strain.

To confirm that the genes encoded by PA5507, PA5508, and PA5509 are responsible for decreasing PQS concentration, we constructed isogenic, in-frame deletion mutants for all genes of the *qapR* operon (*qapR*, PA5507, PA5508, and PA5509) and assessed PQS production by each strain. We found that only the *qapR* mutant strain had a change in PQS concentration when compared to the wild type strain PAO1 (Fig. 2.7). This result was logical since QapR represses transcription of the operon, and therefore effects of these mutations would be masked due to the fact that they are not normally expressed under repression conditions. Therefore, we constructed a *qapR*/5508 double mutant strain and assessed PQS production. Mutation of PA5508 in the *qapR* mutant strain effectively restored PQS concentration to a level of that produced by the wild type strain PAO1 (Fig. 2.7). These data support the conclusion that mutation of *qapR* derepresses a pathway that causes a decrease in PQS concentration in *P. aeruginosa*.

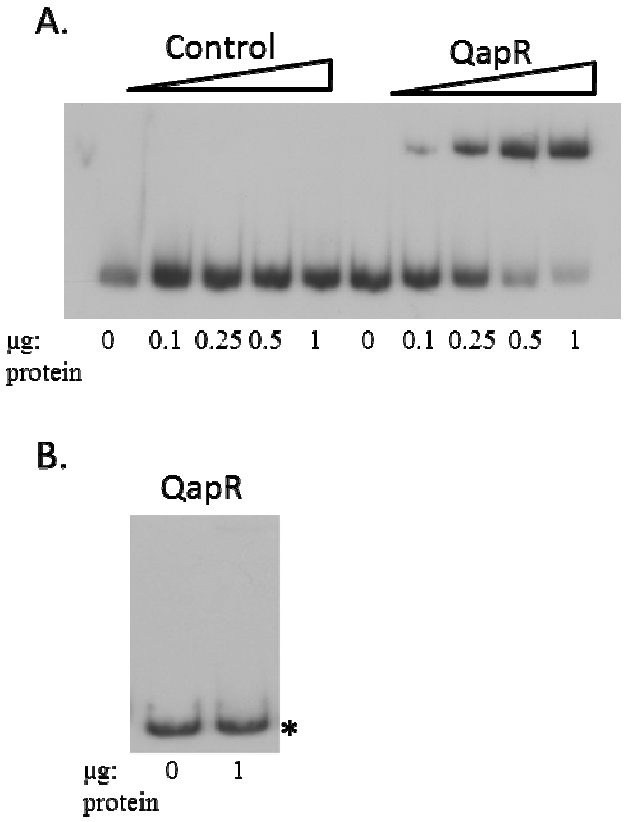

Figure 2.5. QapR directly binds the qapR promoter. Radiolabeled DNA containing either the *qapR* promoter (A.) or the *pqsA* promoter (B.) was incubated with *E. coli* cell lysates containing QapR. Total protein added to each reaction is noted below the autoradiograph. A control lysate was prepared from *E. coli* harboring pHERD20T vector. Unbound probe is indicated by the '*' symbol and probe bound by QapR is denoted by the '←' symbol. Total binding reaction mixtures were separated on nondenaturing polyacrylamide gels which were dried and overlaid with X-ray film. Films were developed after approximately 18 h of exposure. The autoradiographs depicted are representative of at least three independent experiments.

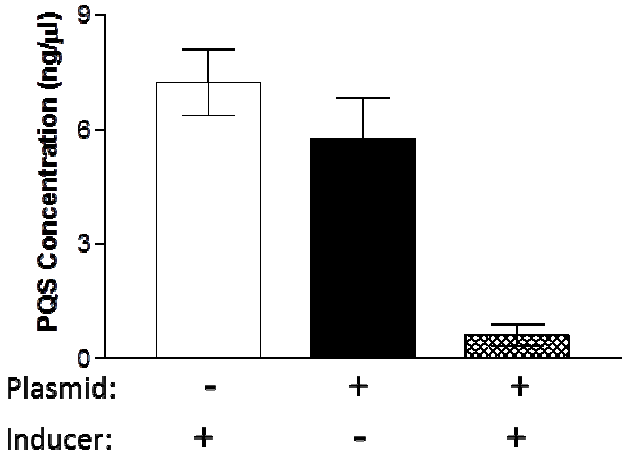

Figure 2.6. Expression of *PA5507-5509* in *P. aeruginosa* reduces PQS concentration. The genes *PA5507-PA5508-PA5509* were expressed from an inducible promoter on plasmid p5507-09OE in wild type strain PAO1 indicated by a plus (+) below each bar. The control strain containing the parent vector pHERD20T is indicated by a minus (-) in the plasmid lane. After 6 h of growth in LB medium, PQS was extracted and quantified as described in Materials and Methods. Data are presented as the averages +/- SD of three independent experiments. The presence or absence of the inducer $_{L}$-arabinose (0.5 %) is indicated below each bar of the figure.

2.5 Discussion

The studies reported here are an interesting extension of the discovery by D'Argenio *et al*. that the mutation of gene PA5506 (*qapR*) caused *P. aeruginosa* cultures to have a decreased level of PQS (8). The original strain in which *qapR* had been mutated also had a lesion in *pqsL*, which caused an overproduction of PQS that lead to an autolytic phenotype. In this report, we show that mutation of *qapR* alone, which encodes an RpiR-family transcriptional regulator homolog, resulted in greatly decreased levels of PQS produced in this strain (Fig. 1A). Transcription of the PQS biosynthetic operon was also much lower in the *qapR* mutant, while transcription of *pqsR* was unaffected (Fig. 2). The PQS biosynthetic operon transcriptional activity could be restored in the *qapR* mutant by addition of PQS which suggests that PQS deficiency is the limiting factor (Fig. 2). In support of this theory, expression of *pqsABCD* restored PQS concentration in the *qapR* mutant strain (Fig. 2), while the addition of anthranilate did not. Taken together, these data led us to conclude that PQS precursor availability is not affected by the *qapR* mutation.

We have shown that *qapR* is transcribed as the first gene in a polycistronic operon along with three downstream genes (Fig. 3). Our data show that transcription of this operon is directly repressed by QapR (Fig. 4 and 5) and that overexpression of the *qapR* operon enzymes (PA5507, PA5508, and PA5509) caused a significant decrease in PQS concentration in *P. aeruginosa* (Fig. 6). Together, these results suggest that our *qapR* mutant acted indirectly to lower PQS levels by derepressing genes PA5507, PA5508, and PA5509. This implication was confirmed when our *qapR*/PA5508 double mutant restored PQS concentration back to a level similar to that produced by strain

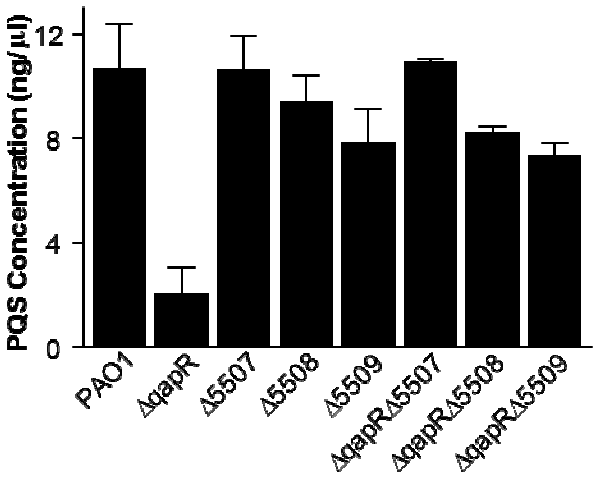

Figure 2.7. Mutation of quinolone alteration pathway enzymes abrogates PQS modulation in the Δ*qapR* mutant strain. Cultures were grown for 18 h in LB medium and PQS was extracted and quantified as described in Materials and Methods. Data are presented as the average +/- SD of three independent experiments. Strains are indicated below each bar. Strains PKT-QapR1, PKT-07, PKT-08, PKT-09, PKT-QapR1-07, PKT-QapR1-08, and PKT-QapR1-09 are written as Δ*qapR*, Δ*5507*, Δ*5508*, Δ*5509*, Δ*qapR* Δ*5507*, Δ*qapR* Δ*5508*, and Δ*qapR* Δ*5509*, respectively.

PAO1 (Fig. 2.7). This also explains the results seen with the single PA5507, PA5508, and PA5509 mutants (Fig. 2.7) because these genes are normally repressed in the wild type strain and therefore mutation of these genes would not change expression. The mechanism through which these genes affect PQS concentration is not known, but there are several obvious possibilities to consider. We propose that the *qapR* operon has a low, basal level of transcription as it is repressed by QapR. Derepression of the operon leads to expression of the PA5507, PA5508, and PA5509 genes and their encoded proteins which interact either directly with PQS, or with a PQS precursor that is downstream from anthranilate. Alternatively, the proteins encoded by genes PA5507, PA5508, and/or PA5509 could be directly altering PQS. Ladner *et al.* demonstrated that PA5508 encodes an enzyme that exhibits γ-glutamyl aromatic monoamine ligase activity *in vitro* [50]. This enzyme was able to glutamylate several aromatic amine substrates including tyramine, serotonin, and norepinephrine while tyrosine and tryptophan were nonsubstrates. These data can be used to support either of our above theories. The genes PA5507 and PA5509 are predicted to encode proteins with sequence homology to iso-chorismatase and amidohydrolase activity, respectively [2]. These putative functions do not immediately suggest a possible reaction directly involving quinolones because of the absence of a reactive primary amino group. Nonetheless, there are other possible mechanisms through which PQS production could be attenuated by the *qapR* operon enzymes. Degradation of PQS has been demonstrated by dioxygenolytic cleavage catalyzed by the enzyme Hod (3-Hydroxy-2-methyl-4(1H)-quinolone 2,4-dioxygenase) from *Arthrobacter* spp. [84]. However, none of the predicted functions of genes PA5507, PA5508, or PA5509 were analogous to Hod

activity. Therefore, it is more likely these enzymes affect PQS concentration by modification of PQS or a precursor, rather than degradation of the quinolone ring. It is possible that the direct PQS precursor HHQ (2-heptyl-4-quinolone) is modified by these enzymes preventing PqsH from converting HHQ into PQS [90]. An additional possibility is that the *qapR* operon enzymes are producing a molecule that can inhibit PQS biosynthesis which would also be very interesting as a mechanism to ensure PQS or quinolones in general are not overproduced to the detriment of the cell.

The results of this study detail a transcriptional regulator from a characterized family of regulators known to control genes involved in central metabolism. We have not investigated regulation of central metabolic genes by QapR but the possibility for it to regulate other genes exists. Other members of the RpiR-family of transcriptional regulators are most commonly associated with the repression of carbohydrate metabolism genes [15, 39, 47, 94]. Two RpiR-family homologs in *Staphylococcus aureus* have been demonstrated to regulate pentose phosphate pathway enzymes in addition to virulence genes via regulation of RNAIII synthesis [109]. This family of regulatory proteins may be acting as a checkpoint to virulence factor production within the cell. It would be advantageous for a pathogen to link synthesis of required biosynthetic intermediates with virulence to avoid production of virulence factors at the expense of central metabolism. And the *qapR* operon may be playing a role in balancing the flux of molecules routed to virulence factor production or central metabolism.

We believe the knowledge gained from this study and continued investigation of the *qapR* operon enzymes could provide a new route to fighting *P. aeruginosa* infection.

Since the *qapR* operon can decrease PQS concentration, this implies that a novel mechanism to disrupt quinolone signaling exists within *P. aeruginosa*. If a means of derepressing the *qapR* operon *in vivo* is discovered, then *P. aeruginosa* infection severity may be decreased by treating with a compound that alters coordinated behavior instead of traditional antibiotic targets.

Table 2.1 Bacterial strains and plasmids used in this study.

Strain or Plasmid	Relevant genotype or phenotype	Reference or source
E. coli DH5α	λ^- φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) *U196 recA1 endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	[106]
P. aeruginosa strains		
PAO1	Wild type	[36]
PKT-QapR1	*qapR* deletion mutant derived from PAO1	This study
$PAO1_{pqsR'\text{-}lacZ}$	Wild type harboring chromosomal *pqsR'-lacZ*	This study
$PKT\text{-}QapR1_{pqsR'\text{-}lacZ}$	*qapR* deletion mutant harboring chromosomal *pqsR'-lacZ*	This study
PKT-07	PA5507 deletion mutant derived from PAO1	This study
PKT-08	PA5508 deletion mutant derived from PAO1	This study
PKT-09	PA5509 deletion mutant derived from PAO1	This study
PKT-QapR1-08	*qapR*/PA5508 double deletion mutant derived from PKT-QapR1	This study
Plasmids		
pHERD20T	*E. coli/P. aeruginosa* shuttle expression vector	[85]
pEX18Ap	Suicide vector for *P. aeruginosa*	[34]
pΔqapR	*qapR* deletion suicide vector	This study
pqapROE	P_{BAD}-*qapR* on pHERD20T	This study
pLP170	Transcriptional fusion vector	[83]
pLP0996	*pqsA'-lacZ* transcriptional fusion	[65]
pLP5506	*qapR'-lacZ* transcriptional fusion	This study

pPQSsynOE	P_{BAD}-*pqsABCD* on pHERD20T	This study
pUC18-mini-Tn7T-Gm-*lacZ*	Chromosomal *lacZ* transcriptional fusion vector	[10]
pTNS2	Transposase expression vector	[10]
pFLP2	*FLP* recombinase expression vector	[34]
pJF-Tn7T-pqsR'Tc	*pqsR'-lacZ* transcriptional fusion vector for chromosomal integration	This study
p5507-09OE	P_{BAD}-*PA5507-5509* on pHERD20T	This study
pΔ5507	PA5507 deletion suicide vector	This study
pΔ5508	PA5508 deletion suicide vector	This study
pΔ5509	PA5509 deletion suicide vector	This study

Table 2.2 Primers used in this study.

Primer	Sequence (*5'→3'*)[a]
qapR Expression	
5506OE Up1	AAATCTAGAATGCAAGAACTAAAACAACGC
5506OE Down1	AAAAAGCTTGTTCTCGTCCACTGCGCGCAT
qapR Mutation	
5506 Up1	AAAGGATCCGCTGATCCTCGTCCAGATTC
5506 Up2	CGTGCCCTGCTCGATGACTACAACGCGTTCAGCAGTCATATT
5506 Down1	AATATGACTGCTGAACGCGTTGTAGTCATCGAGCAGGGCACG
5506 Down2	AAAGGATCCTGCTCTCGTCGATCAGGTC
qapR Operon RT-PCR	
5506-1	AATCCGCCTCGCCCTTCGAC
5507-1	GTTCTGCACGCGCTCGTAGAA
5507-2	TGCCGCCGACCGCGGTTA
5508-1	AGGTCCTGCGGGGTCAGCGA
5508-2	GGACACCTACCTCGCCATGA
5509-1	AAAGCTCTTCGGCCAGCGC
5509-2	GTTCCGGTGCATGGCGACGC
5510-1	TGCCCACCAGGTAGGCCATC
EMSA Probes	
5506 EMSA 1	AAGCCGCGTAACCGCCCC
5506 EMSA 2	GGGGCATCCTCGAAGGTGGG
pqsA EMSA 1	TGTAACGGTTTTTGTCTGGC
pqsA EMSA 2	GACAGAACGTTCCCTCTTCA
PQS Operon Expression	
PQS syn 1	AAAAACCATGGATGTCCACATTGGCCAACCT
PQS syn 2	AAAAATCTAGAACCGAGACGCAGCAGGAA
qapR'-lacZ Reporter	
5506placZ 1	AAAAAAAGCTTCAAAGGCGCGGTGGTACCGG
5506placZ 2	AAAAATCTAGAGCACCACCTTGCGCTCCG
Quantitative Real Time-PCR	
5507 qRT 1	GTTCAGCCTGCCCCACCACT
5507 qRT 2	TTCTCGTCCACTGCGCGCAT
5508 qRT 1.2	CTGGGCAAGCAGTACAACCT

5508 qRT 2.2	GGATCGACATCGGCCAGG
5509 qRT 1	GCGGCGGAAGAACACATCGC
5509 qRT 2	TCTCGGACAGCTCGCGGTTG
RplU_rt_1	GGTGGCAAGCAGCACAAAGTCACCG
RplU_rt_2	GCGGACCTTGTCGTGACGGCCGTGG
PA5507-5509	
Expression	
PA5507-09 Ex 1	AAAAACCATGGATGTTCAGCCTGCCCCACCA
PA5507-09 Ex 2	AAAAAAAGCTTACGGGCTCCTCGGGAAAAGG
pqsR'-lacZ	
Chromosomal	
Reporter	
pqsR lacZ Tc 1	AAAAACTGCAGACCAGGAGTCGTTTCGGAAAT
pqsR lacZ Tc 2	AAAAAAAGCTTGCAGCGGAGGAAATCGAACCG
PA5507 Mutation	
5507 Up-1	AAAAATCTAGACGTCTGCCTGCCGTGCCACG
5507 Up-2	GGCGATGCGTTGCAGGACGGTGGTCTGCCGGCGGCCGAAT
5507 Down-1	ATTCGGCCGCCGGCAGACCACCGTCCTGCAACGCATCGCC
5507 Down-2	AAAAATCTAGACTTCGCGGTTGCGCAGTCCC
PA5508 Mutation	
5508 Up-1	AAAAATCTAGAGCCAGGCCCAGCACGTGG
5508 Up-2	CGTCAGCGCCAGTTCGTGGCGCAGGGGCAGCGAACGCCC
5508 Down-1	GGGCGTTCGCTGCCCCTGCGCCACGAACTGGCGCTGACG
5508 Down-2	AAAAATCTAGACAGCCGTAGACCCCGAACGG
PA5509 Mutation	
5509 Up-1	AAAGGATCCGTTCGAACACGAATTCAGCC
5509 Up-2	TCCATCGTTGCGGATCTCCCAGGCGATGTGTTCTTC
5509 Down-1	GAAGAACACATCGCCTGGGAGATCCGCAACGATGGA
5509 Down-2	AAAGGATCCAGTGGTTGATCACGCTCA

[a]Underlined sequence denotes restriction site utilized for cloning.

CHAPTER THREE: TRANSLATIONAL COUPLING PROTECTS *PSEUDOMONAS* QUINOLONE SIGNAL PRODUCTION IN *PSEUDOMONAS AERUGINOSA*

3.1. Summary

Pseudomonas aeruginosa can sense and respond to a myriad of environmental signals and utilizes a system of small diffusible molecules to communicate through intercellular signaling. The small molecule 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* Quinolone Signal [PQS]) is one of these signals and its synthesis is important for virulence. Previously, we identified an RpiR-type transcriptional regulator, QapR (*PA5506*), that modulates PQS production by repressing the *qapR* operon, which is capable of reducing PQS concentration through an unknown mechanism. In this study, we report that QapR translation is linked to the downstream gene *PA5507*, which provides another layer of regulation to protect quinolone production. A point mutation in *qapR* that introduced a premature stop codon alleviated transcriptional repression of the *qapR* operon but did not negatively affect PQS concentration as expected. This was investigated with a series of *lacZ* reporter fusions which showed that translation of QapR must terminate at, or close to, the natural *qapR* stop codon in order for translation of PA5507 to occur. This regulation was partially alleviated by altering the *PA5507* ribosome binding site, which supports the hypothesis that PA5507 is not independently translated. Considering these findings, we propose that an inverse correlation between PA5507 translation and PQS concentration exists.

3.2 Introduction

The gram negative bacterium *Pseudomonas aeruginosa* is a well-known opportunistic pathogen of humans that can infect a broad range of hosts [16, 87, 97]. This organism can cause significant morbidity and mortality in immunocompromised individuals as well as those afflicted with cystic fibrosis where *P. aeruginosa* can establish intractable infections in the lungs [21, 31]. *P. aeruginosa* is able to adapt to a wide range of niches due to its ability to respond to environmental stimuli through a network of transcription factors, two-component systems, and intercellular signaling networks [3].

In *P. aeruginosa*, three interconnected signaling systems regulate a battery of virulence factors and other cellular functions [74, 75, 79]. The *las* and *rhl* systems produce and respond to homoserine lactone signaling molecules and are true quorum sensing systems [18, 26]. The third signaling system produces and responds to the small molecule 2-heptyl-3-hydroxy-4-quinolone (the *Pseudomonas* quinolone signal [PQS]) [79]. This signaling system is positively regulated by the *las* quorum sensing system and negatively regulated by the *rhl* quorum sensing system [6, 30, 65]. PQS is produced from the precursor molecules anthranilate, malonyl-CoA, and octanoyl-CoA through the action of enzymes encoded by the genes *pqsABCD* and *pqsH* [8, 14, 22, 90]. PQS, one of 56 quinolone molecules produced by *P. aeruginosa* [55], then interacts with the transcriptional regulator PqsR, which activates transcription of the PQS biosynthetic operon (*pqsABCDE*) in a positive autoregulatory loop [99]. The last gene of the biosynthetic operon, *pqsE*, is required for producing the response to PQS,

which includes increased production of the virulence factors pyocyanin, rhamnolipid, and elastase [25].

Our previous study detailed a transcriptional regulator that represses an operon that negatively affects PQS concentration in *P. aeruginosa* [98]. QapR encodes an RpiR-type regulator that is the first gene of a four gene operon containing itself, *PA5507, PA5508,* and *PA5509* (PA5507-PA5509 are required to reduce PQS concentration). The mechanism for this reduction is unknown, but *PA5507* has sequence homology to isochorismateses, *PA5508* has homology to glutamine synthetases but functions *in-vitro* as a monoamine ligase [50], and *PA5509* has sequence homology to amidohydrolases. Unfortunately, these putative functions do not indicate an obvious reaction that would directly affect quinolones. Our initial studies indicated transcriptional repression of the *qapR*-led operon but we suspected more intricate regulation due to the disparity in PQS concentration produced by two different *qapR* transposon mutants. The *qapR* insertion mutant from D'Argenio *et al*. [14] has an insertion in the 3' half of the gene and yielded significantly reduced PQS concentration while the *qapR* transposon mutant from the PA14 Non-Redundant library had a transposon inserted into the 5' half of the gene, which had no effect on PQS concentration [57].

One mechanism of controlling the expression of genes within polycistronic operons is translational coupling. This occurs when translation of a preceding gene affects translation of a downstream gene, as has been shown for *trpE-trpD* in *Escherichia coli* [72]. These two genes are transcribed as part of a polycistronic operon and premature termination of TrpE translation has a significant negative effect on TrpD

translation. Several other studies have shown that translational coupling can serve as a powerful regulatory mechanism for controlling protein stoichiometry of polycistronic transcripts in prokaryotes and bacteriophage [38, 91]. In this report, we present data which shows that *qapR* is translationally coupled to *PA5507* and this coupling provides a secondary layer of regulation to protect PQS signaling in *P. aeruginosa*.

3.3 Experimental Procedures

3.3.1 Bacterial strains, plasmids, and growth conditions.

Bacterial strains used in this study are listed in Table 1. Strains of *P. aeruginosa* and *Escherichia coli* were maintained at -70^{o}C in 10% skim milk (Becton, Dickinson) and 15% glycerol, respectively. Bacteria were freshly plated from frozen stocks to begin each experiment and were grown in Luria-Bertani (LB) medium as noted below [89]. When required to maintain plasmids, cultures were supplemented with 200 µg ml^{-1} of carbenicillin for *P. aeruginosa* and 100 µg ml^{-1} of ampicillin for *E. coli*. L-Arabinose (Sigma-Aldrich) was added to cultures to induce expression of genes under the control of the P_{BAD} promoter where indicated.

Plasmids used in this study are listed in Table 1. To generate an expression plasmid for *PA5507*, a 688 bp DNA fragment, which began at the *PA5507* start codon and ended 34 bp downstream from the stop codon, was amplified by PCR using chromosomal DNA from strain PAO1 as a template. The oligonucleotide primers used for PCR were engineered to include an NcoI site upstream from the start codon and an XbaI site downstream from the stop codon. The purified PCR fragment was digested with these enzymes and was ligated into vector plasmid pHERD20T [85], which contains a P_{BAD} promoter to control gene expression and a ribosome binding site for translation initiation. This produced the P_{BAD}-*PA5507* expression plasmid p5507Exp. A similar strategy was used to generate expression plasmids for *PA5508* and *PA5509*. For *PA5508*, a 1,438-bp DNA fragment, which began at the *PA5508* start codon and ended 106 bp downstream from the stop codon, was amplified by PCR with primers engineered to include an NcoI site upstream from the start codon and a HindIII site

downstream from the stop codon. The purified fragment was digested and ligated into pHERD20T. This produced the P_{BAD}-*PA5508* expression plasmid p5508Exp. For *PA5509*, a 769 bp DNA fragment, which began at the *PA5509* start codon and ended 100 bp downstream from the stop codon, was amplified by PCR with primers engineered to include an NcoI site upstream from the start codon and a HindIII site downstream from the stop codon. The purified fragment was digested and ligated in pHERD20T. This produced the P_{BAD}-*PA5509* expression plasmid p5509Exp.

To generate a *qapR-07'*(C90G)-*lacZ* reporter plasmid, a 1,178-bp DNA fragment corresponding to bp -230 from the *qapR* start codon to bp +86 relative to the *PA5507* start codon was amplified via PCR using chromosomal DNA from strain PKT-QapR-C90G as a template. Primers were engineered to include an EcoRI site and a BamHI site upstream and downstream from the fragment, respectively. Purified PCR fragments were digested and ligated into pSW205 [102] to produce the plasmid pqapR-07' (C90G)/SW205, which harbors the natural *qapR* promoter, the *qapR*-C90G point mutant gene and a fragment of *PA5507* translationally fused to the *lacZ* gene. The Δ*qapR-07'-lacZ* reporter plasmid was generated using the same primers as the point mutant reporter but with Δ*qapR* chromosomal DNA as template. This plasmid contains a 443-bp DNA fragment corresponding to bp -230 from the *qapR* start codon to +86 bp relative to the *PA5507* start codon. The purified and digested fragment was ligated into pSW205 to produce plasmid pΔqapR-07'/SW205, which harbors the natural *qapR* promoter, the Δ*qapR* deletion mutant gene and a fragment of *PA5507* translationally fused to the *lacZ* gene.

To generate a *qapR*'-*lacZ* reporter plasmid controlled by the foreign P_{lacUV5} promoter, an 85-bp DNA fragment corresponding to bp -17 to bp +68 relative to the *qapR* start codon was amplified via PCR using chromosomal DNA from strain PAO1 as a template. Oligonucleotide primers were engineered to include an EcoRI site and the P_{lacUV5} promoter (40 bp comprising -35 and -10 sequences) upstream from the transcriptional start site and a BamHI site downstream from the fragment. The purified PCR product was digested with these enzymes and was ligated into pSW205 to produce the plasmid pqapR'lacUV5/SW205. This plasmid harbors the foreign P_{lacUV5} promoter fused to the *qapR* transcriptional start site and a fragment of *qapR* translationally fused to the *lacZ* gene.

To generate a *qapR-07'-lacZ* reporter plasmid controlled by the foreign P_{lacUV5}, a 965 bp DNA fragment corresponding to bp -17 relative to the *qapR* start codon to bp +86 relative to the *PA5507* start codon was amplified via PCR using chromosomal DNA from strain PAO1 as template. Oligonucleotide primers were engineered to include an EcoRI site and the P_{lacUV5} promoter upstream from the *qapR* transcriptional start site and a BamHI site downstream from the fragment. The purified PCR product was digested with these enzymes and was ligated into pSW205 to produce the plasmid pqapR-07'lacUV5/SW205. This plasmid harbors the foreign P_{lacUV5} promoter fused to the *qapR* transcriptional start site, the *qapR* gene, and a fragment of *PA5507* translationally fused to the *lacZ* gene. A similar plasmid, pqapR-07' (C90G)lacUV5/SW205, was generated via PCR using the same primers as above and chromosomal DNA from the *qapR*-C90G point mutant as template. This plasmid

contains the foreign P_{lacUV5} promoter fused to the *qapR* transcriptional start site, the *qapR*-C90G gene, and a fragment of *PA5507* translationally fused to the *lacZ* gene.

To generate a *qapR-07'*(C651G)-*lacZ* reporter plasmid controlled by the foreign P_{lacUV5} promoter, a 965 bp DNA fragment corresponding to bp -17 relative to the *qapR* start codon to bp +86 relative to the *PA5507* start codon was amplified via PCR with chromosomal DNA from strain PAO1 as template. To introduce the C651G mutation, two fragments of 668 bp and 297 bp were amplified via PCR with primers that were engineered to contain an EcoRI site and the P_{lacUV5} promoter and the C651G base mutated upstream and downstream from the 668 bp fragment, respectively. The 297 bp fragment was amplified with primers engineered to contain a BamHI site downstream from the fragment. Purified PCR product was ligated to produce the full-length fragment and was gel purified. This purified fragment was then digested with BamHI/EcoRI and ligated into pSW205 to produce plasmid pqapR-07' (C651G)lacUV5/SW205. This plasmid harbors the foreign P_{lacUV5} promoter fused to the transcriptional start site of *qapR*, the *qapR*-C651G gene, and a fragment of *PA5507* translationally fused to the *lacZ* gene. A similar plasmid, pqapR-07'(G805T)lacUV5/SW205, was generated via a similar technique as above. Briefly, two fragments of 821 bp and 144 bp were amplified via PCR with primers engineered to include an EcoRI site and the P_{lacUV5} promoter upstream from the 821 bp fragment. The 144 bp fragment was amplified with primers engineered to contain the G805T mutation and a BamHI site upstream and downstream from the fragment, respectively. This plasmid harbors the P_{lacUV5} promoter fused to the *qapR* transcriptional start site, the *qapR*-G805T gene and a fragment of *PA5507* translationally fused to the *lacZ* gene.

To generate a *qapR-07'*(RBS mutation)-*lacZ* reporter plasmid controlled by the P_{lacUV5} promoter, a 965 bp DNA fragment corresponding to bp -17 relative to the *qapR* start codon to bp +86 relative to the *PA5507* start codon was amplified via PCR using chromosomal DNA from strain PAO1 as template. To introduce the RBS mutation, two fragments of 867 bp and 98 bp were amplified using oligonucleotide primers engineered to include an EcoRI site and the P_{lacUV5} promoter upstream from the 867 bp fragment. The 98 bp fragment was amplified by primers that were engineered to mutate the putative *PA5507* RBS sequence of AGGAA to TTTTT upstream from the fragment and a BamHI site downstream from the fragment. These fragments were purified and ligated to produce the full length fragment. Full length fragment was digested and ligated into pSW205 to produce the plasmid pqapR-07'(RBS-mut)lacUV5/SW205 which harbors the P_{lacUV5} promoter fused to the *qapR* transcriptional start site, the *qapR* gene, and gene *PA5507* that has a mutated RBS translationally fused to the *lacZ* gene.

All plasmids were transformed into *P. aeruginosa* by electroporation as described by Choi and Schweizer [11]. All plasmids and constructed fusions were confirmed by DNA sequencing.

3.3.2 Generation of mutants.

Mutant *P. aeruginosa* strains were generated as described previously by Hoang *et al*. [34]. The *qapR*-C90G point mutant allele was constructed by amplification of a 2812 bp DNA fragment including flanking sequence (~1000 bp upstream and downstream from *qapR*) using chromosomal DNA from strain PAO1. Oligonucleotide primers were engineered to include HindIII sites up and downstream from the fragment.

Purified fragment was digested with HindIII and ligated into pEX18Ap which had been digested with HindIII to produce pqapR-large frag. This plasmid was purified and utilized as template for inverse PCR with 5'-phosphorylated primers to mutate *qapR* bp +90 from C to G (which will convert codon 30 (TAC) from tyrosine to a stop codon (TAG)). Inverse PCR product was purified and self-ligated to produce the suicide vector pqapR-C90G. The Δ*qapR*-RBS-mut allele was constructed by amplification of a 2077 bp DNA fragment from Δ*qapR* chromosomal DNA with primers engineered to include HindIII sites up and downstream from the fragment. To introduce the RBS mutation, two fragments of 1243 bp and 834 bp were amplified via PCR with primers engineered to mutate the putative *PA5507* RBS from AGGAA to TTTTT. These two fragments were gel purified and ligated to produce the 2077 bp fragment Δ*qapR*-RBS-mut allele which was digested and ligated into pEX18Ap that had also been digested to produce pΔqapR-RBS-mut. The Δ*qapR*-C651G allele was constructed by amplification of a 2,256 bp DNA fragment using chromosomal DNA from strain PAO1. Oligonucleotide primers were engineered to include HindIII sites up and downstream from the fragment. To introduce the C651G mutation, two fragments of 1,218 bp and 1,038 bp were amplified with primers engineered to introduce the C651G mutation upstream from the 1,038 bp fragment. Purified fragments were ligated to produce the full length Δ*qapR*-C651G mutant allele. This fragment was digested with HindIII and ligated into pEX18Ap that had also been digested to produce plasmid pΔqapR-C651G. The Δ*qapR*-G805T allele was constructed by amplification of a 2,097 bp DNA fragment using chromosomal DNA from strain PAO1. Oligonucleotide primers were engineered to include HindIII sites up and downstream from the fragment. To introduce the G805T mutation, two

fragments of 1,218 bp and 879 bp were amplified with primers engineered to introduce the G805T mutation upstream from the 879 bp fragment. Purified fragments were ligated to produce the full length Δ*qapR*-G805T mutant allele. This fragment was digested with HindIII and ligated into pEX18Ap that had also been digested to produce plasmid pΔqapR-G805T. To transfer the mutant alleles onto the *P. aeruginosa* PAO1 chromosome, the plasmid was electroporated into cells and integrants were selected as described by Choi and Schweizer (2006). Potential mutants were screened by PCR using appropriate primers and DNA sequencing to ensure that the mutations had been transferred to the chromosome.

3.3.3 Assay for PQS quantification.

To assay for PQS production, bacteria from frozen milk stocks were grown on LB agar with antibiotics as necessary. Isolated colonies were used to inoculate 2 ml LB broth cultures supplemented with antibiotics and $_{L}$-arabinose where noted and were incubated at 37^{o}C with vigorous shaking for 5 or 18 h, as noted in figure legends. PQS was extracted as described by Calfee, Coleman, and Pesci [8] and PQS extracts were visualized under long-wave UV light and photographed as described by D'Argenio *et al*. [14]. Images were then analyzed using QuantityOne software (Bio-Rad) to quantify PQS by densitometry.

3.3.4 β-Gal assays in P. aeruginosa.

Cells from overnight cultures were washed in LB broth and used to inoculate 2 ml LB broth cultures supplemented with appropriate antibiotics to an optical density at 660

nm (OD_{660}) of 0.05. Cultures were incubated at 37^{o}C with vigorous shaking. At 6 h, aliquots were collected and β-galactosidase (β-Gal) activity was assayed in duplicate. Activity is reported in Miller units as the mean ± standard deviation of at least three separate experiments [66].

3.3.5 RNA isolation.

Washed cells from overnight cultures of strains PAO1, PKT-QapR-C90G, and PKT-QapR1 pΔqapR-07'/SW205 were used to inoculate 10 ml LB broth cultures, supplemented with antibiotics as necessary, to an OD_{660} 0.05. Cultures were incubated at 37^{o}C with shaking for 3 h (OD_{660} ~1.5) for strains PAO1 and PKT-QapR-C90G or for 6 h (OD_{660} >~3) for strain PKT-QapR1 (pΔqapR-07'/SW205) prior to treatment with RNAprotect (Qiagen) following the manufacturer's protocol. Cells were collected by centrifugation at 6,000 x g for 10 min at 4^{o}C, and total cellular RNA was isolated from *P. aeruginosa* cells using the RNeasy Midiprep kit according to the manufacturer's protocol (Qiagen). Contaminating DNA was removed by treatment of RNA samples with RQ1 RNase-Free DNase according to the manufacturer's protocol (Promega). RNA was then extracted with 1:1 phenol-chloroform and collected by ethanol precipitation. Purified RNA was resuspended in nuclease-free water and was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo-Scientific).

3.3.6 cDNA synthesis for quantitative real-time PCR.

Total RNA from strains PAO1 and PKT-QapR-C90G were isolated and purified as above. cDNA was synthesized in a 21 µl reaction volume from 5 µg of total RNA

using a 1:1 mixture of GC-rich hexamers (Gene Link) and random hexamers (Invitrogen) for priming with 40 µM deoxynucleoside triphosphates (dNTPs)(USB). Reactions were then heated to 65^{o}C for 5 min, followed by cooling to 4^{o}C for 1 min. Following this step 200 U of SuperScript III reverse transcriptase in First Strand Buffer (Invitrogen) with dithiothreitol and RNase Out RNase Inhibitor (Invitrogen) was added to each reaction mixture, yielding a final volume of 30 µl. Reaction mixtures were then heated to 25^{o}C for 5 min, followed by 50^{o}C for 60 min and then 75^{o}C for 15 min.

3.3.7 Quantitative real-time PCR.

cDNA and total RNA to be used as the template and negative control, respectively, were diluted 1:50 in nuclease-free water. Oligonucleotide primer pairs for qRT-PCR were generated by the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were designed to amplify a 200 bp fragment of *rplU* (control), a 213 bp fragment of *PA5507*, a 140 bp fragment of *PA5508*, and a 199 bp fragment of *PA5509* as target genes. qRT-PCR was performed using FastStart SYBR green master mix (Roche Diagnostics) with a Bio-Rad CFX96 cycler. The following cycle was utilized to amplify and quantify fragments: 95^{o}C for 10 min and then 95^{o}C for 15 s, 55^{o}C for 15 s, and 72^{o}C for 20 s, repeated 40 times. Melt curve data were collected to ensure amplification of one fragment by heating samples from 65^{o}C to 95^{o}C in 0.5^{o}C increments. Data were generated from three separate RNA and cDNA preparations and at least two technical replicates for each primer set. Relative expression of each gene was determined by comparing target genes with the control gene (*rplU*) using the Pfaffl method [81].

3.3.8 Primer extension.

RNA was extracted and purified from strain PKT-QapR1 pΔqapR-07'/SW205 as described above. Plasmid pΔqapR-07'/SW205 was included in order to improve the extension process by increasing the number of copies of *qapR* mRNA present. Two primers were used in separate primer extension experiments: 5'-GGCGTCAGTTCGGCGGGCGG-3' corresponding to bp +31 to +50 relative to the *qapR* start codon was annealed at 55^{o}C, and 5'-TAGTCATCGAGCAGGGCACGCAC-3' corresponding to bp +67 to +89 relative to the *qapR* start codon was annealed at 45^{o}C. These primers were radioactively labeled using [γ-^{32}P]ATP (Perkin-Elmer) and T4 Polynucleotide kinase (New England Biolabs). Primer extension reactions were carried out as described previously by Pearson *et al*. [76] using 40 µg RNA and SuperScript III reverse transcriptase (Invitrogen). DNA sequencing ladders were prepared using the ThermoSequenase Cycle Sequencing kit (USB) using oligonucleotide primers utilized for extension reactions and pΔqapR-07'/SW205 as template. Extension and sequencing reactions were resolved on 8M urea, 8% polyacrylamide gels. Dried gels were visualized by autoradiography.

3.4 Results

3.4.1 A premature stop codon in qapR does not affect PQS concentration.

Previously, we showed that deletion of the transcriptional regulator gene *qapR* from *P. aeruginosa* had a negative effect on PQS concentration [98]. We found that deletion of *qapR* prevented transcriptional auto-repression of the *qapR* operon and allowed for overexpression of the downstream genes *PA5507, PA5508,* and *PA5509*, which led to decreased PQS concentration by an unknown mechanism. Since PQS is important for virulence, and modulation of its production could be beneficial to reducing infection severity, we wanted to further investigate the regulatory mechanism controlling the *qapR* operon. We initially hypothesized that the *qapR* ORF encoded a negative regulatory element, which was deleted in the Δ*qapR* mutant. This idea was developed while considering the PQS concentration produced by two different *qapR* transposon mutants. The transposon mutant 64R2 was found to suppress autolysis in a PQS overproducing mutant [14]. This mutant contained an insertion 649 bp downstream from the *qapR* translational start and displayed reduced PQS concentration. The other *qapR* transposon mutant was obtained from the PA14 Non-Redundant Insertion Mutant Library [57] and has an insertion 11 bp downstream from the *qapR* translational start codon. No reduction in PQS concentration was seen with this insertion. This led us to speculate that there existed a regulatory element within *qapR* that modulated expression of the downstream genes of the *qapR* operon.

In order to investigate this hypothesis, we constructed a *P. aeruginosa* strain that carried a point mutation which introduced a premature stop codon in *qapR* (referred to as strain PKT-QapR-C90G). This mutation altered base 90 of the *qapR* coding

sequence which converted a tyrosine codon (TAC; amino acid 30 out of 286) to a stop codon (TAG) while preserving the remainder of the *qapR* coding sequence. As expected, this mutant was similar to the Δ*qapR* deletion mutant with regard to transcriptional activity from the *qapR* promoter. β-gal activity from the *qapR'-lacZ* reporter fusion was increased in both mutant strains compared to the wild type strain PAO1 (Fig. 1A). This derepression was confirmed with quantitative real-time PCR that assessed the relative transcript level of the genes downstream from *qapR* in the *qapR* operon. We found, similar to the Δ*qapR* deletion mutant, that all three genes of the *qapR* operon were expressed several fold higher in the *qapR*-C90G point mutant compared to the wild type strain PAO1 (Fig. 1B). Because of this, we expected that PQS concentration from strain *qapR*-C90G would be decreased, but this was not the case. The point mutant showed no reduction in PQS concentration when compared to strain PAO1 (Fig. 2). Both the *qapR*-C90G point mutant and strain PAO1 produce approximately 3 times more PQS than the Δ*qapR* deletion mutant strain. These data indicated that a negative regulatory element was not present but that regulation of this operon was more complex than simple transcriptional repression by QapR.

3.4.2 PA5507 expression is required to decrease PQS concentration.

Since PQS concentration was unaffected by premature termination of *qapR* translation, but transcription of the *qapR* operon was increased, we wanted to investigate possible deficiencies in the expression of the functional enzymes of the *qapR* operon (PA5507, PA5508, and PA5509). Previously, it was shown that overexpression of PA5507, PA5508, and PA5509 together from an inducible promoter

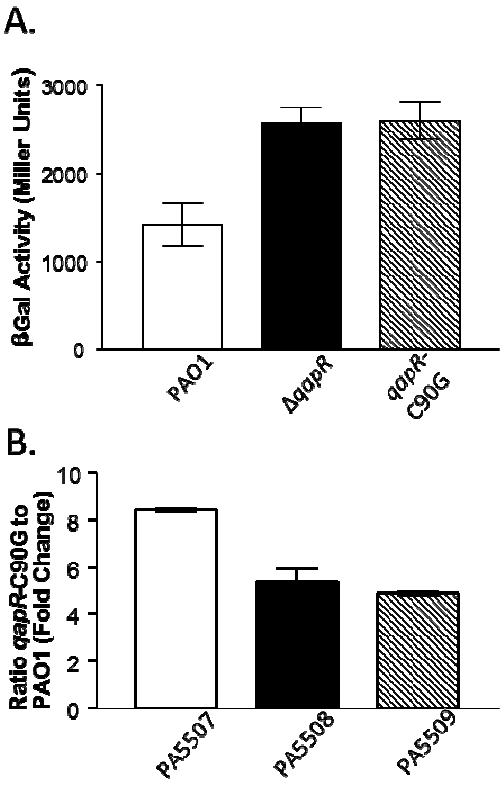

Fig. 3.1. Premature termination of *qapR* translation derepresses the *qapR* operon. (A) Strains carrying a *qapR*'-*lacZ* transcriptional fusion on pLP5506 were grown for 6 h in LB medium. β-gal activity was then assayed and is presented in Miller units as the mean ± SD of results from duplicate assays from three separate experiments. Strains are indicated below each bar. (B) Quantitative real-time PCR was performed on strains PAO1 (wild-type) and PKT-QapR-C90G. Data are presented as the ratio of expression in strain PKT-QapR-C90G to the expression in strain PAO1 ± SD from three separate biological samples. Target genes are indicated below each bar.

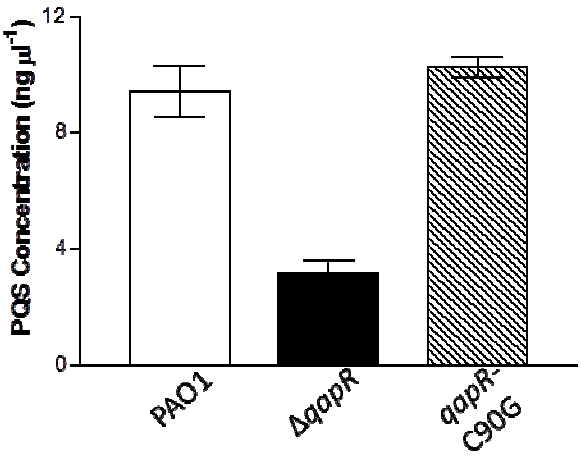

Fig. 3.2. Point mutation of *qapR* does not affect PQS concentration. Cultures were grown for 18 h in LB medium and PQS was extracted and quantified as described in Experimental Procedures. Data are presented as the mean ± SD of three independent experiments. Strains are indicated below each bar.

could reduce PQS concentration in strain PAO1 [98]. To determine the effects of individual genes, we constructed expression vectors with each gene controlled by an inducible promoter. This allowed us to assess the effects of overexpression of each protein on PQS concentration in the *qapR*-C90G point mutant. We utilized this mutant because the *qapR* operon is overexpressed at the transcriptional level but PQS is unaffected which indicates a deficiency in the potential pathway catalyzed by PA5507, PA5508, and PA5509 (Fig. 1). We found that expression of only PA5507 was necessary for reducing PQS concentration in the *qapR*-C90G point mutant, while expression of PA5508 or PA5509 had no effect on PQS concentration (Fig. 3). This result indicates that PA5507 expression may be the limiting factor with regards to reducing PQS concentration in the *qapR*-C90G point mutant.

3.4.3 Translation of gene PA5507 is coupled to qapR translation.

In order to investigate translation of PA5507, we constructed reporter fusions that included the natural *qapR* promoter and contained either the *qapR*-C90G point mutant or the Δ*qapR* deletion mutant allele, with the *PA5507* coding sequence translationally fused to the *lacZ* gene (see diagram in Fig. 4). The Δ*qapR* deletion mutant fusion contains the natural start and stop codons for *qapR* while the *qapR*-C90G fusion has the natural start codon and a premature stop codon upstream from the natural stop codon in *qapR*. This allowed us to analyze the effect of premature QapR translational termination on PA5507 translation. As can be seen in Figure 4, translation of PA5507 is approximately 7-fold higher with the Δ*qapR* deletion mutant allele present compared to the *qapR*-C90G point mutant allele fusion. These data indicated that prematurely

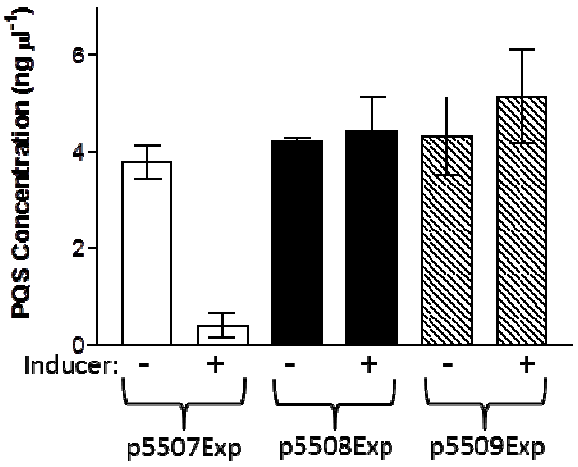

Fig. 3.3. Expression of gene *PA5507* is required to reduce PQS concentration when *qapR* translation is terminated prematurely. The genes PA5507, PA5508, or PA5509 were expressed from an inducible promoter on plasmids p5507Exp, p5508Exp, or p5509Exp in strain PKT-QapR-C90G. Cultures were grown for 5 h in LB medium with (+) or without (-) 1% $_{L}$-arabinose. PQS was extracted and quantified as described in Experimental Procedures. Data are presented as the mean ± SD of three independent experiments.

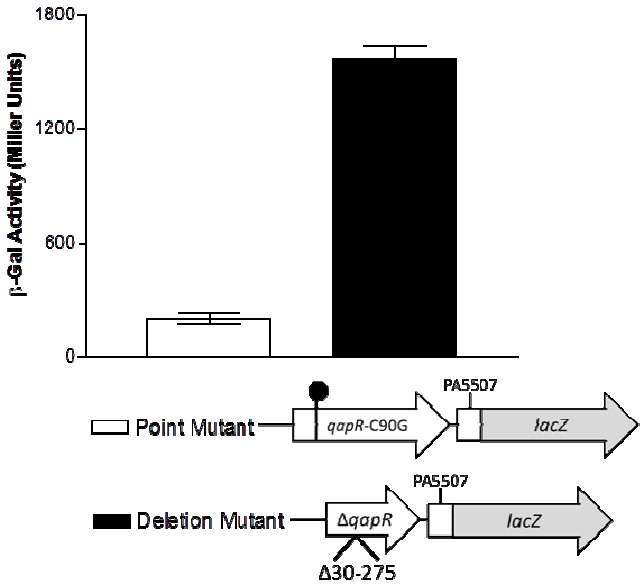

Fig. 3.4. Translation to the natural *qapR* stop codon increases *PA5507* translation. Wild-type strain PAO1 carrying either a *qapR*-C90G'-*lacZ* (noted as Point Mutant) or a Δ*qapR*'-*lacZ* (noted as Deletion Mutant) translational fusion were grown in LB medium for 6 h. Reporter fusions included the natural *qapR* promoter. Amino acids removed from *qapR* coding sequence are indicated on the deletion mutant. The engineered premature stop codon is indicated by a black octagon. β-gal activity was then assayed and is presented in Miller units as the mean ± SDs of results from duplicate assays from three separate experiments.

stopping QapR translation causes a large decrease in PA5507 translation. These assays were performed in strain PAO1 and the fusions should have the same relative rate of transcription based on being driven by the natural promoter. However, since mutations in QapR also affect regulation of transcription from the *qapR* promoter, we decided to investigate translation of PA5507 from a foreign promoter to reduce any effects of transcriptional variation.

In order to examine translation of PA5507 without transcriptional variation, we first identified the transcriptional start site for the *qapR* operon using primer extension analysis. These experiments showed a single transcriptional start site for the *qapR* operon (Fig. 5A). This site was located at -17 bp relative to the *qapR* translational start codon and was confirmed by repeating the experiment with a second primer in a separate extension reaction and obtaining the same result (data not shown). With this information, we constructed a series of translational fusion reporters driven transcriptionally by the *lacUV5* promoter [17]. The *lac* operator sequence was not included since *P. aeruginosa* does not encode *lacI* and we were not concerned with inducible transcription.

We found introduction of the *qapR*-C90G mutation reduced PA5507 translation by approximately 80% and a similar reduction was evident when the stop codon was moved further downstream into the *qapR* coding sequence with the C651G mutation (Fig. 5B). This mutation converted a tyrosine codon (TAC) to a stop codon (TAG) and is approximately 75% into the *qapR* coding sequence. However, when we introduced a stop codon that allowed for the translation of greater than 90% of the *qapR* coding sequence (G805T), we saw a reduction in PA5507 translation of less than 15%.

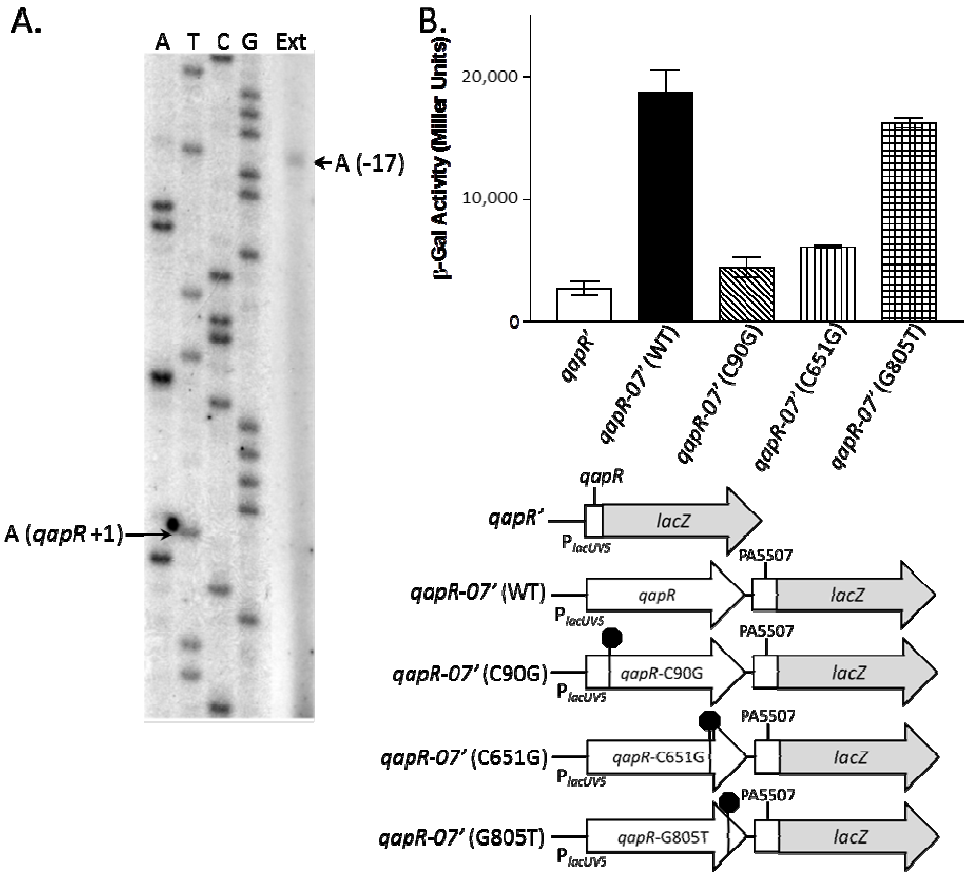

Fig. 3.5. Translation of gene *PA5507* is coupled to *qapR* translation. (A) Primer extension analysis of *qapR* operon transcript. Sequencing reaction mixtures are labeled according to nucleotide (A, T, C, G) and lane 'Ext' contains the mixture for the primer extension reaction performed with RNA harvested from a 6 h culture of strain Δ*qapR* harboring a Δ*qapR*'-*lacZ* translational fusion plasmid. The *qapR* translational start site is denoted as 'A (*qapR* +1)'. Sequencing reactions were exposed for 18 h and primer extension products were exposed for 1 week. (B) Wild-type strain PAO1 carrying translational fusion plasmids driven by the foreign *lacUV5* promoter were grown in LB medium for 6 h. Reporter fusions are indicated below each bar and depicted schematically below the figure. Engineered stop codons are indicated by black octagons. Data are represented as the mean ± SD of results from duplicate assays from three separate experiments.

Together, these results indicated that translation of PA5507 is coupled to the translation of the upstream protein QapR. They also suggest that when the ribosome complex approaches the *qapR* stop codon, translation of PA5507 is permitted.

3.4.4 Premature termination of qapR translation protects PQS concentration.

On the *P. aeruginosa* PAO1 chromosome, *qapR* and *PA5507* are separated by a 4-bp intergenic region (Fig. 6A). A putative ribosome binding site (RBS) AGGAA is located 7 bp upstream from the *PA5507* start codon [93]. We examined the effect of mutating the RBS on PA5507 translation by changing the site from AGGAA to TTTTT within the PA5507 translational *lacZ* fusion driven by the *lacUV5* promoter. This mutation reduced PA5507 translation by approximately 90% which indicates that this is a functional RBS for PA5507 translation (Fig. 6B). Although this mutation reduced PA5507 translation significantly, activity was not completely abolished as would be expected if the RBS was the only source of translation initiation for *PA5507*. This suggests that there is a small amount of ribosomal re-initiation from ribosomes that have been terminated at the *qapR* stop codon. It has been shown that ribosomes may remain bound to mRNA following translational termination to scan for nearby translational start sites [1]. Therefore, since *qapR* and *PA5507* have their respective stop and start codons in close proximity, re-initiation could explain the low-level PA5507 translation of the RBS mutant in Fig. 6B.

We sought to confirm the results of reporter fusion experiments *in vivo* by introducing premature stop and RBS mutations into the *P. aeruginosa* chromosome. We then assessed the effects that these mutations would have on PQS concentration. The

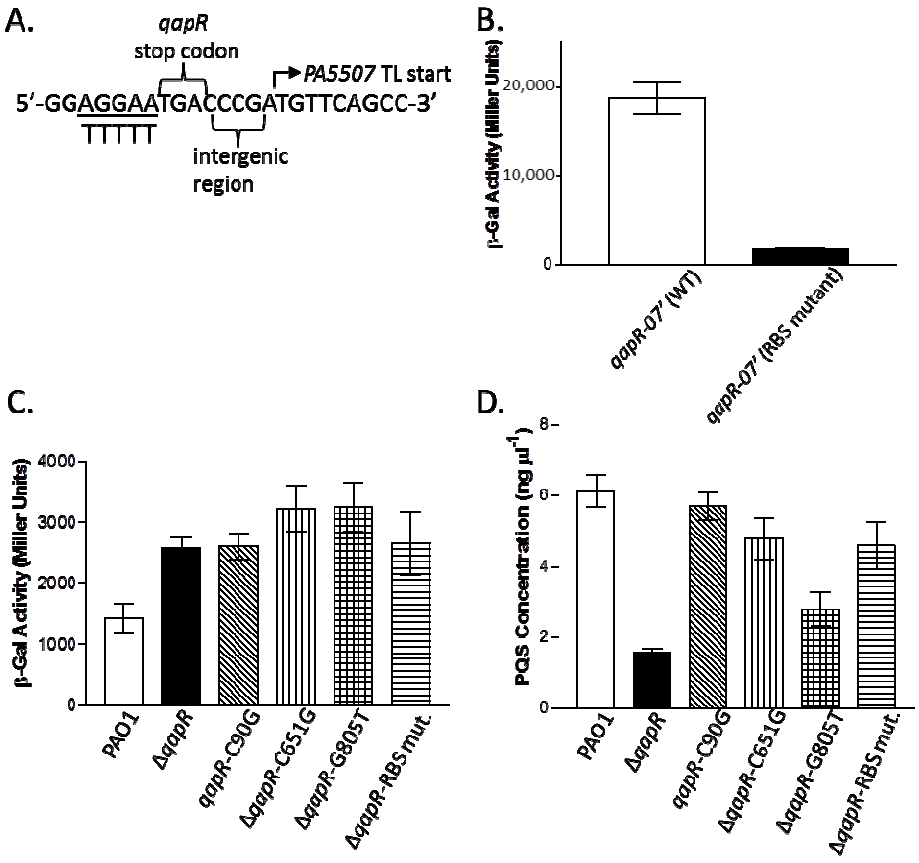

Fig. 3.6. Premature termination of *qapR* translation protects PQS concentration. (A) Intergenic region between *qapR* and *PA5507*. The putative RBS for *PA5507* is underlined. Mutated RBS nucleotides are shown below the putative RBS. The *qapR* stop codon nucleotides and the *PA5507* start codon nucleotides are indicated. (B) Wild-type strain PAO1 carrying translational fusion plasmids driven by the foreign *lacUV5* promoter were grown in LB medium for 6 h. Reporter fusions are indicated below each bar and data are represented as the mean ± SDs of results from duplicate assays from three separate experiments. (C) Strains carrying a *qapR*'-*lacZ* transcriptional fusion on pLP5506 were grown for 6 h in LB medium. β-gal activity was then assayed and is presented in Miller units as the mean ± SD of results from duplicate assays from three separate experiments. Strains are indicated below each bar. (D) Cultures were grown for 6 h in LB medium and PQS was extracted and quantified as described in Experimental Procedures. Data are presented as the mean ± SD of three independent experiments. Strains are indicated below each bar.

premature stop codon mutations and the RBS mutation were added to the chromosome of the Δ*qapR* deletion mutant allele to ensure that the *qapR* operon would be overexpressed to mimic the Δ*qapR* deletion mutant, which overexpresses the *qapR* operon. Activity from the *qapR* promoter is increased in all mutant strains compared to strain PAO1 which indicates that the *qapR* operon is overexpressed in all mutant strains (Fig. 6C). Figure 6D shows that mutation of the *PA5507* RBS or premature termination of QapR translation at codon 217 (C651G), distal to the *PA5507* start codon, only reduced PQS concentration slightly compared to strain PAO1. However, when QapR translation was terminated proximally to the *PA5507* translational start at codon 269 of QapR (G805T) PQS concentration was reduced to approximately 50% of that seen from strain PAO1 (Fig. 6C). These data confirm that PA5507 translation is intimately linked to translation of the regulator QapR in *P. aeruginosa*.

3.5 Discussion

The studies reported here are a continuation of our work detailing the effects of mutating the transcriptional regulator *qapR* on PQS concentration in *P. aeruginosa* [98]. In this report, we show that transcriptional repression by QapR is not the sole regulation mechanism for the genes encoded downstream from *qapR* in the *qapR* operon (*PA5507*, *PA5508*, and *PA5509*). When translation of QapR was prematurely terminated by the introduction of a stop codon, PQS concentration was unaffected even though transcription of the *qapR* operon was increased (Fig. 1 & 2). This led us to assess translation of PA5507, PA5508, and PA5509 since we had previously shown the necessity of expressing these genes for reducing PQS concentration. Overexpression of PA5507 in the prematurely terminated *qapR* mutant (*qapR*-C90G) was sufficient to reduce PQS concentration by approximately 90% while expressing PA5508 or PA5509 had no effect on PQS concentration (Fig. 3). A reporter fusion driven by the natural *qapR* promoter was then utilized to determine that translation of the enzyme encoded by *PA5507* was significantly lower in the premature termination mutant (*qapR*-C90G) compared to the deletion mutant (Δ*qapR*) (Fig. 4). This suggested that *qapR* and *PA5507* were coupled at the translational level.

To ensure that this result was not due to discrepancies in transcription, the reporter fusions were placed under the control of the constitutive *lacUV5* promoter. These constructs also showed that PA5507 translation was significantly reduced when QapR translation is prematurely terminated early in the polypeptide (Fig. 5). Translational activity was mostly restored to PA5507 when the premature stop codon was moved downstream to within 60 nucleotides from the *PA5507* start codon (Δ*qapR*-

G805T) (Fig. 5). This result confirmed that QapR and PA5507 translation is coupled. We have shown that mutation of the putative ribosome binding site upstream (RBS) from *PA5507* in the *qapR* coding region greatly reduces translation of PA5507 (Fig. 6). However, this mutation does not completely abolish PA5507 translation (similar to premature termination of QapR) leading us to conclude that *de novo* initiation at the RBS of *PA5507* is likely to drive most PA5507 expression but re-initiation from ribosomes terminated at the QapR stop codon cannot be ruled out. These findings were correlated to PQS concentration by showing that the Δ*qapR*-C651G and Δ*qapR*-RBS-mut mutations had only a minimal effect on PQS concentration (Fig. 6). However, premature termination of QapR translation with the Δ*qapR*-G805T mutation greatly reduced PQS concentration (Fig. 6).

To help understand what these data mean, a potential model of *qapR* operon regulation is presented in Figure 7. This model shows the QapR regulator repressing transcription of the *qapR* operon and this repression is required for *P. aeruginosa* to produce a high concentration of the signaling molecule PQS. The inset depicts the effects of QapR termination either proximal or distal to the *PA5507* translational start codon. When QapR translation is terminated distal from *PA5507*, PQS concentration is protected due to low translation of PA5507 even if the *qapR-PA5509* operon is overexpressed at the transcriptional level. This regulation scheme ensures that a high concentration of PQS will be present even if a lesion in the *qapR* coding region has been acquired since translation of the regulator is coupled to the downstream gene. The *qapR* operon is regulated at multiple levels in *P. aeruginosa* which may indicate its role in reducing quinolone production under a specific growth condition.

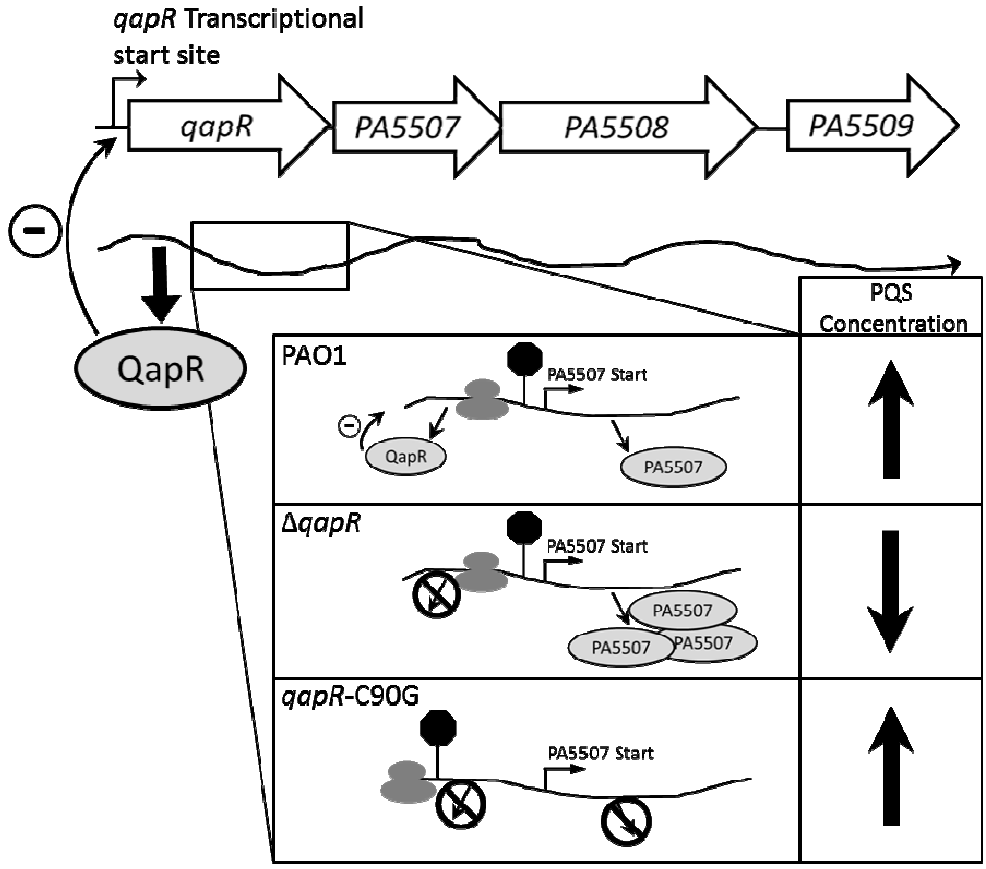

Fig. 3.7. Model of *qapR* operon regulation. Depicted is the *qapR* operon comprised of genes *qapR*, *PA5507*, *PA5508*, and *PA5509*. These four genes are transcribed as a single polycistronic mRNA whose transcription is repressed by QapR. The inset box highlights the *qapR-PA5507* intergenic region. We have implicated at least two factors that are required for *qapR* operon enzymes (PA5507, PA5508, and PA5509) to affect PQS concentration in *P. aeruginosa*. First, termination of QapR translation proximal to the *PA5507* start codon (up to 60 bp away) allows for full translational activity while QapR termination distal to *PA5507* (greater than 200 bp) prevents full translational activity. Second, QapR is an auto-regulatory repressor which maintains transcription of the *qapR* operon at a low level in PAO1. This repression is alleviated in the mutated strains. The combination of high *qap* operon transcription and high PA5507 translation then yields a low PQS phenotype.

The *qapR* operon is encoded by all sequenced strains of *P. aeruginosa* listed on the *Pseudomonas* Genome Database [105]. Six other *Pseudomonas* species (*P. brassicacearum*, *P. chlororaphis*, *P. fluroescens*, *P. protegens*, *P. mandelii*, and *P. knackmussi*) were also found to encode the intact *qapR* operon. The majority of *Pseudomonas* strains encode homologs of *qapR* operon genes but the organization and location of the operon is not conserved. This information does not allow us to propose a function for the *qapR* operon other than its role in reducing PQS concentration in *P. aeruginosa*. This operon likely provides a catabolic pathway for a nutrient because homologous operons are also found outside of the *Pseudomonas* genus. The *qapR* operon structure is conserved in many sequenced members of the Proteobacteria phylum including several soil/rhizosphere α-Proteobacteria such as *Ochrobactrum anthropic*, *Ensifer adhaerens*, and *Mesorhizobium* spp. These organisms can be free-living in the environment (soil or water) or associated with plants as pathogens or rhizosphere symbionts. Other Proteobacteria encode all genes of the operon but with scrambled organization including the α-proteobacteria *Sinorhizobium meliloti* and *S. medicae*, the β-proteobacterium *Burkholderia oklahomensis*, and the γ-proteobacterium *Klebsiella pneumonia*. This vast distribution among an entire phylum of bacteria indicates that the *qapR* operon is utilized in some way by all of these organisms whether in a pathogenic or free-living niche but does not allow for speculation of function.

The organisms that retain the *qapR* operon genes in scrambled order are not likely to exhibit the same regulation scheme as *P. aeruginosa*. Transcriptional regulation may be similar due to the presence of an RpiR-type homolog in each organism but

translational coupling of this regulator to a functional enzyme as seen in *P. aeruginosa* is not likely to be conserved. In most cases of coupling, translation of an upstream open reading frame positively influences translation of a downstream open reading frame on the same polycistronic mRNA. In the plant pathogen *Agrobacterium tumefaciens*, *virB7-virB8* encode components of the secretion apparatus complex. These two genes overlap by 8 nucleotides and are translationally coupled to ensure proper formation of the T-pilus for injection of tumor-inducing DNA into host plant cells [68]. Coupling of *virB7-virB8* is similar to *qapR-PA5507* coupling but the *qapR-PA5507* coding sequences do not overlap. In *Salmonella typhimurium*, the *ompR-envZ* two-component system is translationally coupled to ensure that a proper intracellular concentration of both proteins is produced for appropriate signal transduction [58]. Coupling of this two-component system is similar to that of *qapR-PA5507* in that a transcriptional regulator is coupled to a functional protein. However, *ompR-envZ* share an overlapping start/stop codon (ATGA) and *envZ* has no ribosome binding site for *de novo* translation initiation which is not similar to *qapR-PA5507* coupling where a 4 bp intergenic region separates the genes and a strong RBS controls PA5507 translation. Conversely, open reading frames in polycistronic operons do not have to be translationally coupled as is the case with the *pvuIIC-pvuIIR* restriction-modification genes from *Proteus vulgaris*. These two genes overlap by 20 nucleotides but are not coupled at the translational level [43]. These slight differences contribute to the difficulty of predicting translational coupling which is likely an under-appreciated regulation mechanism in prokaryotes.

In *P. aeruginosa*, translational coupling of *qapR* to *PA5507* provides for multiple levels of regulation of an enzyme that can negatively affect PQS concentration. This

regulation is likely important not only for virulence in acute infections but also for biofilm formation in chronic infections, both of which require PQS signaling [20]. QapR represses transcription of the *qapR* operon to protect PQS production *in vivo*. This regulator should respond to an as-of-yet undiscovered molecule to alleviate transcriptional repression of the operon similar to homologous RpiR-type regulators [15]. The QapR ligand molecule could be produced or accumulate in response to a stressor in the environment and would cause the cells to reduce quinolone production, possibly due to quinolone pro-oxidant characteristics [32]. However, if a mutation was accumulated in *qapR* that prematurely terminates translation of the regulator then PA5507 would not be efficiently translated even though it would be transcribed at a high level. These systems appear to be in place to regulate the concentration of quinolone present at any given time during the course of growth (whether environmental or pathogenic) to contribute to the vast continuum of signals to which *P. aeruginosa* can sense and respond.

Table 3.1 Bacterial strains and plasmids used in this study.

Strain or Plasmid	Relevant genotype or phenotype	Reference or source
E. coli DH5α	λ⁻φ80d*lacZ*ΔM15 Δ(*lacZYA-argF) U196 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	[106]
P. aeruginosa strains		
PAO1	Wild-type	[36]
PKT-QapR1	*qapR* deletion mutant derived from PAO1	[98]
PKT-QapR-C90G	Point mutant of *qapR* derived from PAO1	This study
PKT-QapR1-RBS mut.	*qapR* deletion mutant with *PA5507* RBS mutation derived from PAO1	This study
PKT-QapR1-C651G	*qapR* deletion mutant with point mutation at nucleotide 651 derived from PAO1	This study
PKT-QapR1-G805T	*qapR* deletion mutant with point mutation at nucleotide 805 derived from PAO1	This study
Plasmids		
pHERD20T	*E. coli*/*P. aeruginosa* shuttle expression vector	[85]
pSW205	Translational *lacZ* fusion vector	[102]
pLP5506	*qapR'-lacZ* transcriptional fusion	[98]
pΔqapR	Δ*qapR* suicide vector on pEX18Ap	[98]
pEX18Ap	Suicide vector for *P. aeruginosa*	[34]
pqapR-large frag.	*qapR* (with flanking sequence) suicide vector on pEX18Ap	This study
pqapR-C90G	*qapR* point mutant suicide vector on pEX18Ap	This study
pΔqapR-RBS-mut	Δ*qapR*-RBS-mut mutant suicide vector on pEX18Ap	This study

pΔqapR-C651G	Δ*qapR*-C651G mutant suicide vector on pEX18Ap	This study
pΔqapR-G805T	Δ*qapR*-G805T mutant suicide vector on pEX18Ap	This study
P5507Exp	P_{BAD}-*PA5507* on pHERD20T	This study
P5508Exp	P_{BAD}-*PA5508* on pHERD20T	This study
P5509Exp	P_{BAD}-*PA5509* on pHERD20T	This study
pqapR-07'(C90G)	*qapR-07*'(C90G)-*lacZ* translational fusion	This study
pΔqapR-07'	Δ*qapR-07*'-*lacZ* translational fusion	This study
pqapR'lacUV5	P_{lacUV5}-*qapR'-lacZ* translational fusion	This study
pqapR-07'lacUV5	P_{lacUV5}-*qapR-07'-lacZ* translational fusion	This study
pqapR-07'(C90G)lacUV5	P_{lacUV5}-*qapR-07'*(C90G)-*lacZ* translational fusion	This study
pqapR-07'(C651G)lacUV5	P_{lacUV5}-*qapR-07'*(C651G)-*lacZ* translational fusion	This study
pqapR-07'(G805T)lacUV5	P_{lacUV5}-*qapR-07'*(G805T)-*lacZ* translational fusion	This study
pqapR-07'(RBS mut.)lacUV5	P_{lacUV5}-*qapR-07'*(RBS mut.)-*lacZ* translational fusion	This study

Table 3.2 Primers used in this study.

Primer	Sequence (5'→3')[a,b,c]
PA5507 expression	
PA5507-09 Ex 1	AAAAACCATGGATGTTCAGCCTGCCCCACCA
5507 Ex 2.1	AAAAAAAGCTTACGAAGCTGACCAGGCGTACC
PA5508 expression	
5508 Ex 1	AAAAACCATGGGTGAACCGCCTGCAGCCGGT
5508 Ex 2	AAAAAAAGCTTCGGAATGTGCCGGCTGGCATG
PA5509 expresssion	
5509 Ex 1	AAAAACCATGGGTGGTCCTGGTCTGCGAGCA
PA5507-09 Ex 2	AAAAAAAGCTTACGGGCTCCTCGGGAAAAGG
qapR-07'(C90G)/ ΔqapR-07' TL fusion	
5506-lacZ1 TL	AAAAAGAATTCCAAAGGCGCGGTGGTACCGG
5507-lacZ2.1 TL	AAAAAGGATCCGCACGCTGCATGTCGACGA
qapR' lacUV5 TL fusion	
5506-lacZTL-UV5Up	AAAAAGAATTC*AGGCTTTACACTTTATGCTTCCGGCTCGTATAATGTGTGG*ACCTTCGAGGATGCCCCATGCAAGAACTA
5506-lacZ2.1 TL	AAAAAGGATCCACCACCTTGCGCTCCGCG
qapR-07' lacUV5/ qapR-07'(C90G) lacUV5 TL fusion	
5506-lacZTL-UV5Up	See above
5507-lacZTL-UV5Dn	AAAAAGGATCCGCACGCTGCATGTCGACGAACAGG
qapR-07'(C651G) lacUV5 TL fusion	
5506-lacZTL-UV5Up	See above
C651GInv. 2	CTAGATGTCGGTGAACAGCACCAGCCG
C651GInv. 1	**G**CCTCGCCGCTGCGCGAA
5507-lacZTL-UV5Dn	See above
qapR-07'(G805T) lacUV5 TL fusion	
5506-lacZTL-UV5Up	See above
G805TInv. 2	CAGGCGTTCGTCGAGCGGC
G805TInv. 1	**T**AGGGCATCGACCAGCTCCGCAAC
5507-lacZTL-UV5Dn	See above

qapR-07'(RBS mut.) lacUV5 TL fusion	
5506-lacZTL-UV5Up	See above
5507-mut.RBS 2	CCAGAATATGACTGCTGAACGCGTTGCG
5507-mut.RBS 1	**TTTTT**TGACCCGATGTTCAGCCTGCCC
5507-lacZTL-UV5Dn	See above
qapR large frag.	
5506 Up 1	AAAAAAAGCTTGCTGATCCTCGTCCAGATTC
5506 Down 2	AAAAAAAGCTTTGCTCTCGTCGATCAGGTC
qapR-C90G	
5506-C90GInv 1	**G**CCGCGCCTCGGCCTCG
5506-C90GInv 2	TAGTCATCGAGCAGGGCACGCAC
ΔqapR-RBS-mut	
5506 Up 1	See above
5507-mut.RBS 2	See above
5507-mut.RBS 1	See above
5506 Down 2	See above
ΔqapR-C651G	
5506 Up 1	See above
5506-90 rev	GTAGTCATCGAGCAGGGCAC
C561Gforward.2	TA**G**GCCTCGCCGCTGCGC
5506 Down 2	See above
ΔqapR-G805T	
5506 Up 1	See above
5506-90 rev	See above
G805Tforward	**T**AGGGCATCGACCAGCTC
5506 Down 2	See above

[a]Underlined sequence denotes restriction site utilized for cloning.
[b]Italicized sequence denotes P_{lacUV5} promoter sequence.
[c]Bold sequence denotes bases that have been mutated for this study.

CHAPTER FOUR: GENERAL SUMMARY

The goal of these studies was to investigate an operon that can modulate PQS concentration through an unknown mechanism in *P. aeruginosa*. Since PQS is an important molecule for virulence, mechanisms that abrogate or dysregulate PQS production could be utilized as targets for anti-infective drugs. The goal of studying these mechanisms is to find a means to reduce virulence without placing significant selective pressure on infecting organisms as traditional antibiotics do since they are directed at required physiological processes (i.e.- transcription/translation). We live in the age of antibiotic resistance and any tactic of reducing virulence of microorganisms should be investigated vigorously.

We initiated these studies with the knowledge that a mechanism of reducing the PQS produced by *P. aeruginosa* is encoded on its genome. D'Argenio *et al.* identified a transcriptional regulator (gene *PA5506*) that, when mutated, reverted autolytic colony morphology mutants back to the wild type PAO1 colony morphology [14]. The autolytic phenotype identified by D'Argenio was found to correlate with overproduction of PQS and autolysis suppressor mutants reduced the concentration of PQS. Insertion in *PA5506* in the Δ*pqsL* background reduced PQS concentration to a level below that produced by the wild type strain PAO1.

An isogenic, in-frame deletion of gene *PA5506* (renamed *qapR*) was constructed and this mutant was assessed for PQS and pyocyanin production. Fig. 2.1 shows that deletion of *qapR* reduces PQS and pyocyanin production significantly compared to the wild type strain PAO1. PQS and pyocyanin levels can be restored to the wild type level by providing *qapR* on a plasmid under the control of an inducible promoter

(Fig. 2.1). This effect is due to QapR binding the *qapR* promoter which represses transcription of the *qapR* operon and leads to reduced expression of the enzymes of the *qapR* operon. We could not immediately infer a role for *qapR,* which is homologous to regulators of carbohydrate metabolism genes, in controlling PQS concentration so we investigated transcription of the PQS biosynthetic operon. We found that transcription from a *pqsA*'-*lacZ* reporter fusion was significantly decreased in the Δ*qapR* mutant compared to strain PAO1 (Fig. 2.2A). This was expected due to the reduced PQS concentration produced by the Δ*qapR* mutant since PqsR requires PQS to positively regulate transcription of *pqsA* (the first gene of the PQS biosynthetic operon). Addition of exogeneous PQS to cultures restored *pqsA*'-*lacZ* activity in the Δ*qapR* mutant to nearly a wild type level which suggested that PqsR was present to bind PQS and activate transcription of the PQS biosynthetic operon (Fig. 2.2A). Transcription from the *pqsR* promoter was found to be unaffected by mutation of *qapR* further confirming that PqsR is present and functional in the Δ*qapR* mutant (Fig. 2.2B). We also found that addition of the precursor anthranilate could not restore PQS production to the Δ*qapR* mutant (Fig. 4.1) but overexpression of PqsABCD could restore PQS and pyocyanin production to the Δ*qapR* mutant (Fig. 2.2C & D). These results led us to conclude that precursor pools for quinolone production are unaffected by *qapR* mutation. The regulatory gene *qapR* was predicted to lead an operon of four genes and we assessed transcript structure with reverse transcriptase-PCR. Fig. 2.3 shows that *qapR* is co-transcribed with the three downstream genes *PA5507, PA5508,* and *PA5509*. This lead us to investigate the regulation of this operon by constructing a *qapR*'-*lacZ* reporter

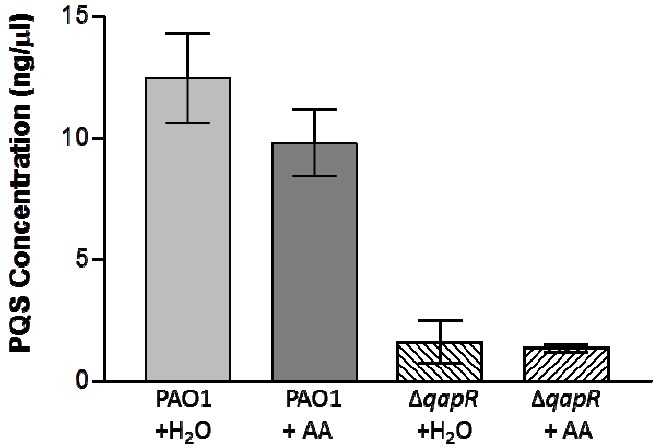

Figure 4.1. Anthranilic acid supplementation cannot restore PQS concentration in the Δ*qapR* mutant. Strains are noted below each bar, PAO1 is the wild type strain and Δ*qapR* is the *qapR* deletion mutant. Cultures were grown for 18h in LB medium supplemented with dH_2O or 0.5 mM anthranilic acid in dH_2O. Cultures were extracted with acidified ethyl acetate and PQS was quantified via TLC and densitometry [8, 14].

fusion. We found that transcription of the *qapR* operon was increased in the Δ*qapR* mutant compared to wild type strain PAO1 (Fig. 2.4A). We confirmed this derepression of transcription by utilizing quantitative real-time PCR and found that genes *PA5507, PA5508,* and *PA5509* are transcribed more than 15-fold higher in the Δ*qapR* mutant compared to wild type strain PAO1 (Fig. 2.4B). We found that QapR is an autoregulatory transcription factor through electrophoretic mobility shift assays with radiolabeled *qapR* promoter. Fig. 2.5A shows that QapR-containing lysate could interact with the *qapR* promoter region independently of any added ligand. QapR was unable to interact with a control promoter (*pqsA*) under the same binding conditions as those used for the *qapR* promoter which indicates that the binding is specific to a sequence within the *qapR* promoter (Fig. 2.5B). This led us to assess the effect of overexpression of the functional genes of the *qapR* operon. We constructed an expression vector containing *PA5507-PA5508-PA5509* under the control of an inducible promoter and expression of these three genes was sufficient to significantly reduce PQS concentration in the wild type strain PAO1 (Fig. 2.6). We then constructed mutants of *qapR* operon enzyme genes individually and in combination with the *qapR* regulator. Fig. 2.7 shows that deletion of any of the *qapR* operon enzymes (PA5507*,* PA5508*,* or PA5509) in combination with *qapR* can restore high PQS concentration to the Δ*qapR* mutant, similar to the wild type strain PAO1. This result confirms that the *qapR* operon is responsible for reducing PQS concentration in the Δ*qapR* mutant.

Further investigation of two different *qapR* transposon mutants led us to suspect that a post-transcriptional regulatory element existed in the *qapR* coding region. In these mutants, the *qapR* coding sequence was interrupted but differential PQS

phenotypes were obtained (Fig 4.2). The transposon mutant from D'Argenio yielded greatly reduced PQS concentration while the *qapR* transposon from the PA14 Non-Redundant library produced a concentration of PQS similar to the wild type strain. We set out to investigate this by constructing a *P. aeruginosa* strain that carried a point mutation which introduced a premature stop codon in *qapR*. This mutation prevented QapR translation while preserving the remaining *qapR* coding sequence. This mutant, *qapR*-C90G, displayed increased transcription of the *qapR* operon as expected from a *qapR* mutant (Fig. 3.1). Surprisingly, this mutant did not exhibit reduced PQS concentration as would be expected from a *qapR* mutant (Fig. 3.2). This result suggested that one of the *qapR* operon enzymes may not be efficiently translated since elevated transcription of the *qapR* operon would normally give correspondingly higher protein expression and subsequently low PQS concentrations. We constructed expression vectors for the enzymes of the *qapR* operon, individually. Fig. 3.3 shows that expression of only PA5507 in the *qapR*-C90G point mutant is required to reduce PQS concentration. This result suggests that the other enzymes of the operon (PA5508 and PA5509) are present at a sufficient level for the pathway to reduce PQS concentration when PA5507 is provided *in trans*. In order to investigate the requirements for PA5507 translation, we constructed *lacZ* reporter fusions that contained either the Δ*qapR* mutant allele or the *qapR*-C90G mutant allele with *PA5507* fused to *lacZ*. Fig. 3.4 shows that PA5507 translation is significantly reduced when comparing the activity of the deletion mutant construct to the activity of the point mutant. We then located the transcriptional start site for the *qapR* operon in order to construct *lacZ* fusions that are transcriptionally driven by a foreign promoter. In these fusion constructs, the *lacUV5* promoter drives

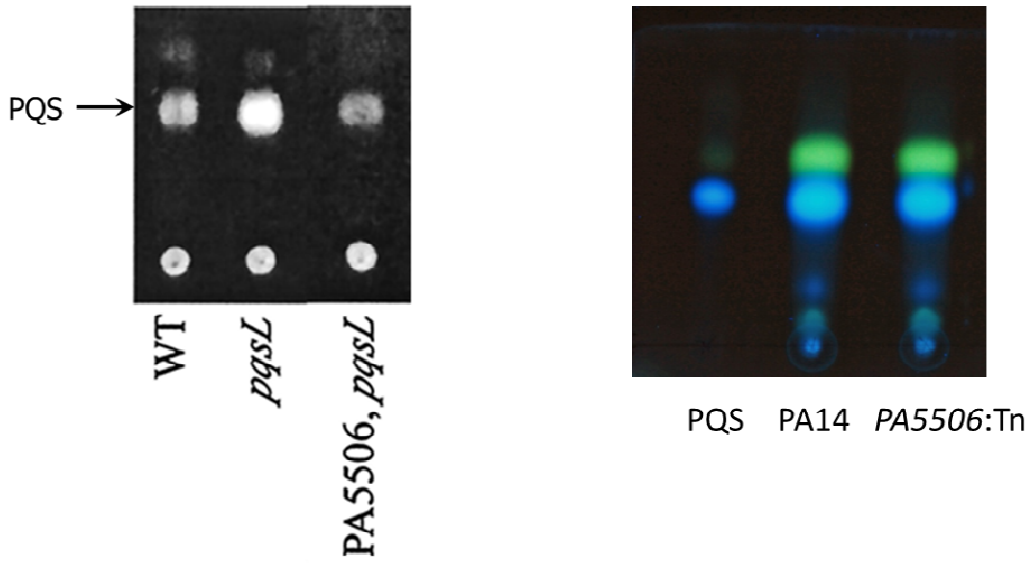

Figure 4.2. L64R2 and PA14 transposon mutant PQS visualized via thin layer chromatography. PQS was extracted from *P. aeruginosa* cultures, separated on TLC plates, and visualized under UV light. (Left panel) Transposon mutants in *pqsL* and *PA5506*, *pqsL* show PQS overproduction in the *pqsL* mutant which is reduced upon introduction of a lesion into the *PA5506* coding sequence [14]. (Right panel) Transposon mutant in the PA14 strain shows that insertion in *PA5506* has no effect on PQS concentration.

transcription of *qapR* carrying the wild type allele or one of the following point mutant alleles: *qapR*-C90G, *qapR*-C651G, or *qapR*-G805T with *PA5507* fused to *lacZ*. These fusions were designed to investigate the effect of QapR translation on translation of PA5507. Fig. 3.5 shows that termination of QapR translation distal to the start codon of *PA5507* has a negative effect on translational activity. This effect is negated when QapR translation is terminated proximal (within 60 bp) to the *PA5507* start codon. Since translation of PA5507 is dependent upon translation of the upstream gene *qapR*, we concluded that QapR and PA5507 are translationally coupled. We then investigated the putative ribosome binding site (RBS) located upstream from *PA5507* within the *qapR* coding sequence. When this RBS was mutated, PA5507 translation was dramatically decreased based on *lacZ* reporter fusion activity (Fig. 3.6B). This led us to conclude that the RBS upstream from *PA5507* is functional for initiating translation but may be occluded when QapR translation is prematurely terminated. We then sought to correlate the effects of these mutations on PA5507 translation as it relates to PQS concentration. Fig. 3.6D shows that premature termination of QapR translation further than 60 nucleotides upstream from the *PA5507* start codon or mutation of the *PA5507* RBS does not significantly affect PQS concentration. Based on these findings, we concluded that PQS concentration is inversely correlated to PA5507 translation. We can also suggest that these regulatory mechanisms are in place to prevent PA5507 from dampening PQS-mediated signaling in *P. aeruginosa*. If expression of PA5507 was not tightly controlled, *P. aeruginosa* would not be able to produce sufficient concentrations of PQS for proper signaling. This regulation may also comprise a mechanism to prevent

P. aeruginosa from overproducing PQS to the detriment of the cell since derepression of the *qapR* operon can effectively reduce PQS concentration *in vivo*.

In this regulatory network, QapR represses transcription of the *qapR* operon and likely allows for a basal level of expression of the enzymes of the operon. We speculate that transcriptional repression would only be alleviated after the unknown cofactor of QapR reached a sufficient concentration within the cell for QapR to bind it and cause its release from the *qapR* promoter. As long as the QapR cofactor was at a high concentration, transcription of the *qapR* operon would proceed uninhibited and would lead to elevated transcription of the *qapR* operon. If translation of QapR has not been prematurely interrupted by mutation, then PA5507 will be translated efficiently and will be expressed at a level sufficient to reduce PQS concentration with the enzymatic actions of PA5508 and PA5509. However, if QapR has incurred a mutation that would negatively affect translation, then PA5507 would not be translated efficiently and PQS concentration would be unaffected. Likewise, if the QapR cofactor concentration decreases then transcription would be repressed by QapR without the cofactor. This suggests that the *qapR* operon is intricately regulated by transcriptional repression and translational coupling to the regulatory protein that controls transcription of the operon.

There are several interesting facets of this operon and its regulation that have yet to be explored. In the future, identifying the QapR co-factor is a very important goal. It has been shown that members of the RpiR-family of transcriptional regulators bind intermediates of pathways they regulate to alter transcription of target genes [15]. If the co-factor is a soluble, non-toxic compound, then fighting *P. aeruginosa* virulence from within by reducing PQS concentration through the action of the *qapR* operon may be

possible. Defining the QapR regulon is also a worthwhile study that should establish the gene/s regulated directly by QapR. It is possible that QapR serves only to regulate the *qapR* operon, but this seems unlikely considering the integration of regulatory networks within *P. aeruginosa*.

The main challenge for future studies of the *qapR* operon will be to define the mechanism by which the operon enzymes reduce PQS concentration in *P. aeruginosa*. The PA5507 enzyme has sequence homology to isochorismatases which are predicted to catalyze the breakdown of isochorismate to pyruvate and (2S,3S)-2,3-dihydroxy-2,3-dihydrobenzoate. PA5508 has sequence homology to glutamine synthetases and Ladner *et al.* demonstrated that the enzyme exhibits γ-glutamyl monoamine ligase activity *in vitro* [51]. PA5509 has sequence homology to amidohydrolases which are predicted to catalyze the conversion of *N*-formyl-L-glutamate to formate and L-glutamate. The homologies of PA5507 and PA5509 and the established activity of PA5508 *in vitro* do not yield an obvious pathway that would involve quinolones directly. However, there are a few possible routes through which the *qapR* operon enzymes could be affecting PQS concentration in *P. aeruginosa*. The *qapR* operon could be altering PQS directly by addition or subtraction of a functional group (i.e.- amine group or acyl chain) which would reduce the bioactivity of the PQS molecule. This alteration could prevent PqsR from binding altered PQS and block the positive effect that PqsR-PQS exerts on the *pqsABCDE* operon (see Fig. 4.3A for a potential model). Another possible mechanism for the *qapR* operon to reduce PQS concentration would be through alteration of a precursor molecule required for

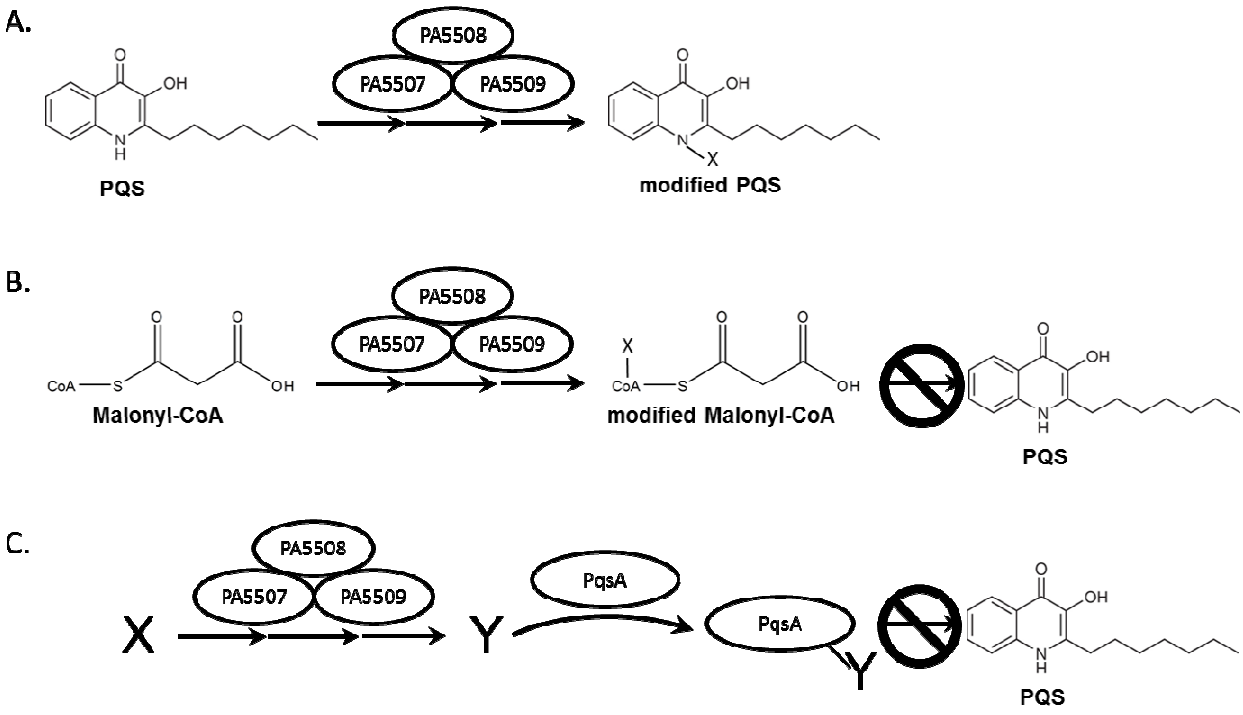

Figure 4.3. Schematic representation of putative *qapR* operon enzyme function. (A) Direct modification of PQS by the *qapR* operon enzymes would reduce PQS bioactivity by preventing proper binding of the signal by the regulator PqsR. (B) Malonyl-CoA is a precursor of PQS biosynthesis and modification of this molecule could prevent PQS biosynthesis by perturbing the malonyl-CoA pool. (C) A molecule may be converted to an unknown metabolite by the *qapR* operon enzymes. This metabolite could inhibit the activity of one of the PQS biosynthetic enzymes (PqsA is shown as an example).

quinolone biosynthesis. This action would prevent the quinolone biosynthetic machinery from producing PQS due to deficient precursor pools (Fig. 4.3B). We speculated that anthranilate was the precursor affected by the *qapR* operon but supplementing cultures with anthranilate did not restore PQS concentration to the Δ*qapR* mutant (Fig. 4.1). Quinolone biosynthesis requires the precursors anthraniloyl-CoA, malonyl-CoA, and octanoyl-CoA so perhaps one of the other precursors is affected by the *qapR* operon. Finally, the *qapR* operon could be producing a metabolite that has a negative effect on PQS production in *P. aeruginosa.* This metabolite would likely be structurally similar to a quinolone precursor molecule that could inhibit an enzyme required for PQS production (Fig. 4.3C). Coleman *et al.* showed that analogs of anthranilate could reduce PQS production by inhibiting the activity of PqsA *in vitro* and *in vivo* [12]. One molecule found to inhibit PqsA activity was salicylate which is similar to (2S,3S)-2,3-dihydroxy-2,3-dihydrobenzoate molecule which is a product of the putative isochorismate reaction. The discovery of the true reaction mechanism catalyzed by the *qapR* operon could one day open new avenues of research and allow for the development of anti-infective compounds aimed at reducing *P. aeruginosa* virulence.

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