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# QapR (PA5506) Represses an Operon That Negatively Affects the *Pseudomonas* Quinolone Signal in *Pseudomonas aeruginosa*

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***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 study, 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 the PQS concentration. We believe that this operon comprises a novel mechanism to regulate PQS concentration in *P. aeruginosa*.**

*Pseudomonas aeruginosa* is a ubiquitous, Gram-negative bacterium that can infect a broad range of hosts, including insects, plants, and animals (1–3). This prevalent opportunistic pathogen is frequently acquired in the nosocomial setting and causes intractable infections in the lungs of cystic fibrosis patients (4–6). 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 (7–10).

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 (7, 9). The *lasRI* signaling system is at the top of the cell-to-cell signaling hierarchy and positively regulates the *rhlRI* and quinolone signaling systems (11). The *las* and *rhl* quorum sensing 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 (12, 13). The other cell-to-cell signaling system functions through the quinolone compound 2-heptyl-3-hydroxy-4-quinolone (the *Pseudomonas* quinolone signal [PQS]) (14). 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 (15). PQS then interacts with and activates PqsR, which positively controls expression of the PQS biosynthetic operon (*pqsABCDE*) in an autoregulatory loop (16). Transcription of the *pqsR* gene is regulated in a complex manner by the *las* and *rhl* quorum sensing systems (16, 17). The quinolone biosynthetic machinery also produces 55 other 4-quinolone compounds, many of which function as antimicrobials (18).

A prior study of factors that control the autolytic phenotype of *P. aeruginosa* identified 10 mutated genes that altered PQS production (19). Subsequent investigations of these genes have yielded a wealth of knowledge about the precursors and biosynthesis of PQS in *P. aeruginosa* (20–23). 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 (24, 25). In this report, we present data demonstrating that QapR is a negative autoregulatory transcription factor that controls a four-gene operon. We also show that the operon led by *qapR* has a negative effect on PQS concentration, thereby providing another layer of regulation for quinolone signaling.

## MATERIALS AND METHODS

**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°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 (26). When required to maintain plasmids, cultures were supplemented with 200 μg/ml of carbenicillin for *P. aeruginosa* and 100 μg/ml 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 also listed in Table 1. To generate an expression plasmid for *qapR*, a 1,078-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 purified PCR fragment was digested with these enzymes and was ligated into vector plasmid pHERD20T (27), which contains a $P_{BAD}$ promoter to control gene expres-

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**TABLE 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* | 42 |
| *P. aeruginosa* strains | | |
| PAO1 | Wild type | 43 |
| 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 |
| PKT-QapR1-07 | *qapR*/PA5507 double deletion mutant derived from PAO1 | This study |
| PKT-QapR1-09 | *qapR*/PA5509 double deletion mutant derived from PKT-QapR1 | This study |
| Plasmids | | |
| pHERD20T | *E. coli/P. aeruginosa* shuttle expression vector | 27 |
| pEX18Ap | Suicide vector for *P. aeruginosa* | 29 |
| pΔqapR | *qapR* deletion suicide vector | This study |
| pqapROE | $P_{BAD}$-*qapR* on pHERD20T | This study |
| pLP170 | Transcriptional fusion vector | 44 |
| pLP0996 | *pqsA′-lacZ* transcriptional fusion | 45 |
| 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 | 46 |
| pTNS2 | Transposase expression vector | 46 |
| pFLP2 | *FLP* recombinase expression vector | 29 |
| pJF-Tn7T-pqsR′Tc | *pqsR′-lacZ* transcriptional fusion vector for chromosomal integration | This study |
| p5507-09OE | $P_{BAD}$-PA5507-PA5509 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 |
| pΔqapR-5507 | PA5506-PA5507 double deletion suicide vector | This study |

sion and a ribosome binding site for translation initiation. This produced the $P_{BAD}$-*qapR* expression plasmid pqapROE.

To generate a PA5507-PA5509 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 with primers engineered to include an NcoI restriction site upstream from the PA5507 start codon and a HindIII site downstream from the PA5509 stop codon. Purified PCR fragments were then digested and ligated into pHERD20T to produce the $P_{BAD}$-PA5507-PA5509 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*. Purified PCR fragment was then digested and ligated into pHERD20T to produce the $P_{BAD}$-*pqsABCD* expression plasmid pPQSsynOE.

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. Primers were engineered to include a HindIII site and an XbaI site upstream and downstream from the fragment, respectively. Purified PCR fragments were digested and ligated in pLP170 to produce the plasmid pLP5506, which 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 with PCR primers engineered to include a PstI site and a HindIII site upstream and downstream from the fragment, respectively. Digested DNA fragment and pUC18-mini-Tn7T-Gm-*lacZ* were digested with PstI and HindIII and ligated to produce the plasmid pJF-Tn7T-pqsR′Tc, which harbors the *pqsR* promoter transcriptional fusion that can be integrated into strains as a chromosomal fusion as described by Choi and Schweizer (28).

All plasmids were confirmed by DNA sequencing of insert DNA. All primers for these studies are listed in Table 2.

**Generation of mutant strains.** Mutant *P. aeruginosa* strains were generated as described previously (29). Mutant alleles were generated using splicing by an overlap extension PCR protocol (30). 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). For the *qapR* PA5507 double mutant, the DNA sequence corresponding to amino acid 30 for *qapR* to amino acid 186 for PA5507 (88% of *qapR* and PA5507) was removed. Oligonucleotide primers used to generate mutant alleles also contained the following restriction sites at each end: BamHI for *qapR* and PA5509 and XbaI for PA5507, PA5508, and *qapR* PA5507. The fragments were digested with the appropriate enzyme and ligated into pEX18Ap, which had been previously digested. This ligation produced the suicide vectors pΔqapR, pΔ5507, pΔ5508, pΔ5509, and pΔqapR-5507. 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 and Schweizer (28). Potential mutants were screened by PCR using appropriate primers, and mutants were further confirmed by sequencing of generated PCR products.

**Assays for PQS and pyocyanin.** To assay for PQS production, bacteria from frozen skim milk stocks were grown on LB agar with antibiotics as

**TABLE 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 | CGTGCCCTGCTCGATGACTACAACGCGTTCAGC AGTCATATT |
| 5506 Down1 | AATATGACTGCTGAACGCGTTGTAGTCATCGAG CAGGGCACG |
| 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 |
| 5510 qRT 1 | GCGACCTGATCATCTACGGG |
| 5510 qRT 2 | GGGCGTAGGAATACACCGAG |
| RplU_rt_1 | GGTGGCAAGCAGCACAAAGTCACCG |
| RplU_rt_2 | GCGGACCTTGTCGTGACGGCCGTGG |
| PA5507-PA5509 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 | ATTCGGCCGCCGGCAGACCACCGTCCTGCAACGC ATCGCC |
| 5507 Down-2 | AAAAATCTAGACTTCGCGGTTGCGCAGTCCC |
| PA5508 mutation | |
| 5508 Up-1 | AAAAATCTAGAGCCAGGCCCAGCACGTGG |
| 5508 Up-2 | CGTCAGCGCCAGTTCGTGGCGCAGGGGCAGCGAA CGCCC |
| 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 |
| *qapR*-PA5507 mutation | |
| 5506-07 Up-1 | AAAAATCTAGAGCCAAGGAAGGCGTGGACCT |
| 5506-07 Up-2 | ATCGCTTCCAGGCAGGCCTGGTAGTCATCGAGCA GGCACGC |
| 5506-07 Down-1 | GCGTGCCTGCTCGATGACTACCAGGCCTGCCTGG AAGCGAT |
| 5506-07 Down-2 | AAAAATCTAGACTTCGCGGTTGCGCAGTCCC |

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

necessary. Isolated colonies were used to inoculate 10-ml LB broth cultures, which were incubated at 37°C with vigorous shaking for 18 h. PQS was extracted as described by Calfee et al. (31), and PQS extracts were visualized as described by D'Argenio et al. (19). 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 described above. Isolated colonies were used to inoculate 10-ml LB broth cultures, which were incubated at 37°C for 18 h with vigorous shaking. Samples of cultures were centrifuged at 16,000 × *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. (32).

**β-Gal assays in *P. aeruginosa*.** Cells from overnight cultures were washed in LB broth and used to inoculate 10-ml LB broth cultures to an optical density at 660 nm ($OD_{660}$) of 0.05. Cultures were incubated at 37°C with vigorous shaking. At 6 and 24 h, aliquots were collected and β-galactosidase (β-Gal) activity was assayed in duplicate. Activity is reported in Miller units as the means ± standard deviations of at least three separate replicates (33).

For experiments including the addition of PQS, cells from an overnight culture were washed in LB broth and used to inoculate 2-ml LB broth 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°C with vigorous shaking. β-Gal activity was assayed at 24 h in duplicate as described above. Activity is reported in Miller units as the means ± standard deviations of at least three independent experiments.

**RNA isolation.** Overnight cultures of strains PAO1 and PKT-QapR1 were washed in LB broth and used to inoculate 10-ml LB broth cultures to an $OD_{660}$ of 0.05. Cultures were incubated at 37°C for 3 h or to an $OD_{660}$ of approximately 1.5 prior to centrifugation at 4°C to harvest bacterial cells. 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 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. RNA concentration was quantified with a NanoDrop ND-1000 spectrophotometer.

**Transcript analysis by RT-PCR.** Purified RNA (50 ng) from strain PAO1 was used as a template for reverse transcriptase PCR (RT-PCR) performed with the Promega Access RT-PCR system by 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°C for 45 min; 95°C for 2 min; 30 cycles of 95°C for 30 s, 55 to 62°C for 30 s (different annealing temperatures were required to optimize different primer sets), and 72°C for 45 s. The final cycle was followed by heating of the samples at 72°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.

**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 deoxynucleoside triphosphates (dNTPs) (USB). Reactions were then heated at 65°C for 5 min, followed by cooling to 4°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°C for 5 min, followed by 50°C for 60 min and then 75°C for 15 min.

**Quantitative real-time PCR.** cDNA and total RNA to be used as the template and negative control, respectively, were diluted 1:200 in nuclease-free water. Oligonucleotide primer pairs for quantitative real-time 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, a 199-bp fragment of PA5509, and a 198-bp fragment of PA5510 as target genes. Quantitative real-time PCR was performed using FastStart SYBR green master mix (Roche Diagnostics) with Bio-Rad CFX96. The following cycle was utilized to amplify and quantify fragments: 95°C for 10 min and then 95°C for 15 s, 55°C for 15 s, and 72°C for 20 s, repeated 40 times. Melt curve data were collected to ensure amplification of one fragment by heating samples from 65°C to 95°C in 0.5°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 (34).

**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°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 L-arabinose. After 5 h of total growth, cells were harvested from cultures by centrifugation at 6,000 × *g* for 10 min at 4°C. Bacterial cell pellets were resuspended in 1 ml of STE buffer (pH 7.5; 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl), and this suspension was passed through a French pressure cell at 16,000 lb/in$^2$ to yield whole-cell lysate. Protein concentration for DNA mobility shift assay was assessed using a Bradford assay (Bio-Rad).

**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 (25). Each reaction mixture contained 0.3 μg of 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 0.5× Tris-borate-EDTA buffer at 4°C. Gels were then exposed to X-ray film to visualize radiolabeled bands.

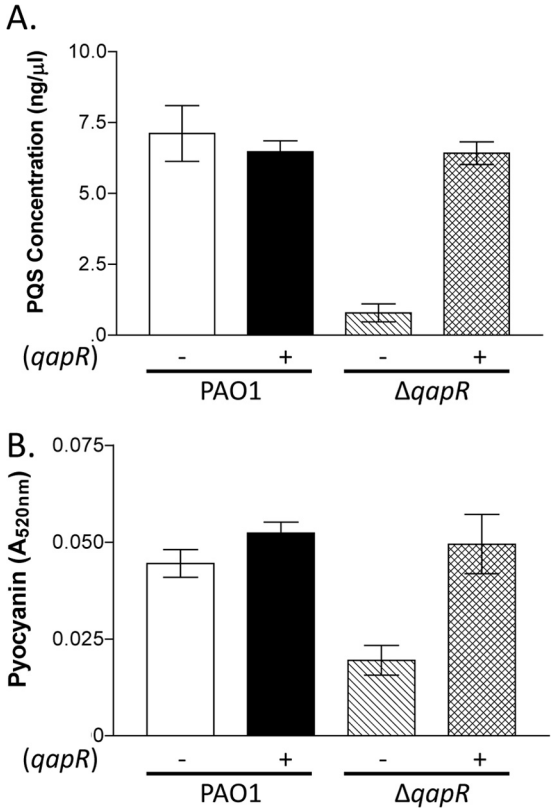


**FIG 1** Mutation of *qapR* decreased PQS concentration in *P. aeruginosa*. Strains are indicated below each bar, and the presence of *qapR* expression plasmid (pqapROE) or control vector (pHERD20T) is indicated with a plus or minus sign, respectively. PAO1 is the wild-type strain, and the Δ*qapR* strain is 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 ± SDs 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 ± SDs of three independent experiments.

## RESULTS

***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 (19). 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. 1A). PQS concentration could be restored to wild-type levels in the *qapR* mutant when *qapR* was provided on a plasmid (Fig. 1A). Expression of *qapR* in strain PAO1 did not affect PQS or pyocyanin (Fig. 1A). In addition, mutation of *qapR* caused a decrease in pyocyanin production in a manner similar to the effect seen for PQS (Fig. 1B). This was expected since pyocyanin biosynthesis is positively controlled by PQS. Taken together, the data of Fig. 1 confirm that PQS concentration is controlled by *qapR*.

**Transcription of the PQS biosynthetic operon, but not *pqsR*, is altered in the *qapR* mutant.** In order to learn more about how *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 compared to the wild-type strain PAO1 (Fig. 2A). Since the *pqsA* promoter is activated by PqsR-PQS (16), 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. 2A, the addition of PQS to cultures restored *pqsA′-lacZ* transcription in the *qapR* mutant strain to the level 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. 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. 2C and D). 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

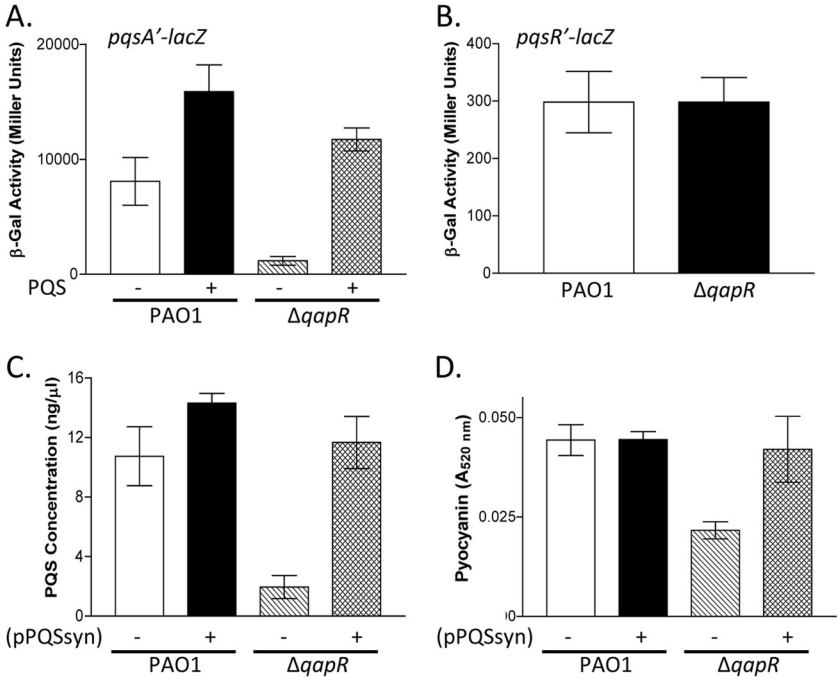


**FIG 2** Supplementation of PQS can restore transcription of the PQS operon in the *qapR* mutant. (A) Strains carrying a *pqsA′-lacZ* fusion on pLP0996 were grown for 24 h with 30 μM PQS where indicated by a plus sign. β-Gal activity was then assayed and is presented in Miller units as the mean ± SDs of results from duplicate assays from three separate experiments. Strains are indicated below each bar: PAO1 is the wild-type strain, and the Δ*qapR* strain is PKT-QapR1. (B) Strains with a *pqsR′-lacZ* fusion integrated into the chromosome were grown for 24 h in LB broth. β-Gal activity produced in each culture was then assayed and is presented in Miller units as the mean ± SDs 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 sign, respectively, were grown in LB broth 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 ± SDs of three independent experiments. (D) Pyocyanin was extracted and quantified from cultures grown as described for panel C. Data are presented as averages ± SDs of three independent experiments.

expression of the quinolone synthetic machinery is affected by *qapR* mutation. Since anthranilate is critical for the production of PQS (21), 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). The results of these experiments implied that expression of the *pqsABCD* operon is affected only by the decreased level of PQS caused by the mutation of *qapR*. This spurred us to look more closely at *qapR* and the genes adjacent to it.

**Characterization of the *qapR*-PA5509 operon.** 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 polycistronic operon which includes the three downstream genes PA5507, PA5508, and PA5509 (35). These genes are illustrated in Fig. 3A, along with gene PA5510. It has been reported that gene PA5508 encodes an enzyme that exhibits γ-glutamyl aromatic monoamine ligase activity (36), while a BLAST search (39) shows that genes PA5507 and PA5509 are predicted to encode proteins with sequence homology to isochorismatases and amidohydrolases, respectively. To analyze the operon structure, we utilized reverse transcriptase PCR from total RNA extracted from strain PAO1. We found that *qapR* is cotranscribed with genes PA5507, PA5508, and PA5509 (Fig. 3B). A link between genes PA5509 and PA5510 was not indicated by our data (Fig. 3B). Since *qapR* is cotranscribed 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

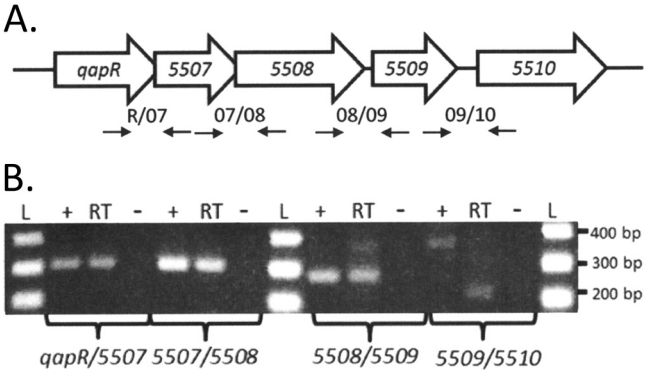


**FIG 3** *qapR* is the first gene of a four-gene operon. (A) Schematic diagram of the predicted *qapR* operon and gene PA5510. Arrows below the schematic indicate the relative positions of primers used to amplify each intergenic region. (B) Agarose gel visualization of products from RT-PCR experiments 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 size ladders (1 Kb Plus Ladder; Invitrogen) are labeled "L," and relevant sizes are indicated next to the image.

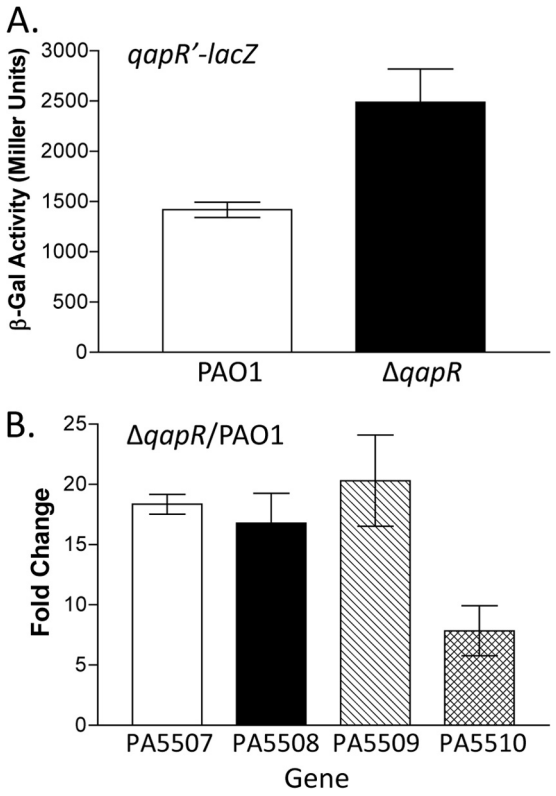


**FIG 4** Transcription of the *qapR* operon is autorepressed. (A) Strains carrying a *qapR′-lacZ* reporter on plasmid pLP5506 were grown for 6 h in LB medium. β-Gal activity produced in each culture was then assayed and is presented in Miller units as the mean ± SDs of results from duplicate assays from three separate experiments. Strains are indicated below each bar: PAO1 is the wild-type strain, and the Δ*qapR* strain is PKT-QapR1. (B) Quantitative real-time PCR was performed on strains PAO1 and PKT-QapR1. Data are presented as fold change ± SD 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.

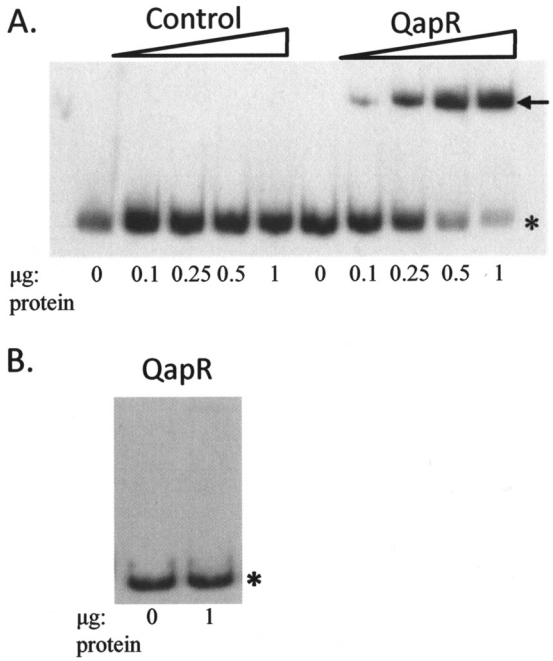


**FIG 5** QapR directly binds the *qapR* promoter. Radiolabeled DNA containing either the *qapR* promoter (A) or the *pqsA* promoter (B) was incubated with *E. coli* cell lysates containing QapR. Total protein added to each reaction is noted below the autoradiograph. A control lysate was prepared from *E. coli* harboring pHERD20T vector. Unbound probe is indicated by the asterisk, and probe bound by QapR is indicated by the arrow. 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.

activity from the *qapR* promoter in strain PAO1 and in the *qapR* mutant strain. Figure 4A shows that transcription from the *qapR* promoter is significantly higher in the *qapR* mutant than in strain PAO1. This was not surprising, since most RpiR homologs have been demonstrated to act as repressors of transcription (24, 25, 37, 38). To further analyze the regulation of the *qapR* operon, we performed quantitative real time-PCR to assess the relative expression of genes PA5507, PA5508, PA5509, and PA5510 in the *qapR* mutant strain. The results of these experiments showed that the PA5507, PA5508, and PA5509 target genes are expressed at a level 15- to 20-fold higher in the *qapR* mutant strain than in the wild-type strain PAO1. These data confirm that the *qapR* operon is derepressed in the *qapR* mutant and suggested that QapR is the repressor. We also saw that the expression of gene PA5510, which encodes a putative amino acid permease, was increased approximately 7-fold in the *qapR* mutant compared to wild-type PAO1. This was a surprise, since our RT-PCR results indicated that PA5510 is not transcriptionally linked to PA5509. Gene PA5510 is 102 nucleotides downstream from PA5509, and this distance, along with the fact that PA5510 is expressed at a level that is less than half that of the other three genes, leads us to conclude that it probably is not transcriptionally linked to PA5509. However, gene PA5510 is obviously repressed by *qapR*, and we plan future studies into its role in PQS regulation.

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 EMSAs with the *qapR* operon promoter. The data showed that lysates containing QapR interacted with the radiolabeled *qapR* operon promoter region, causing a shift in DNA mobility (Fig. 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 amounts of protein were 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. 5A), which suggests that the observed binding was most likely due to the presence of QapR. We also assessed lysate binding to the *pqsA* promoter and observed no interaction (Fig. 5B). This result provided further evidence against the possibility that QapR directly affected *pqsA* transcription. Finally, we found that the addition of exogenous PQS to the QapR-containing lysate had no effect on the interaction of QapR and the *qapR* promoter, which suggests that PQS is not a cofactor for QapR (data not shown). Considering the data in Fig. 5, 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.

**The *qapR* operon decreases PQS concentration.** Since QapR represses transcription of the *qapR* operon, we wanted to deter-

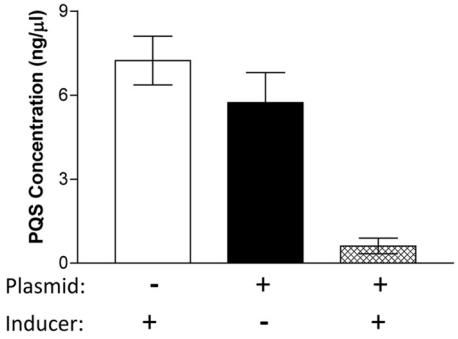


**FIG 6** Expression of PA5507 to PA5509 in *P. aeruginosa* reduces PQS concentration. The genes PA5507, PA5508, and PA5509 were expressed from an inducible promoter on plasmid p5507-09OE in wild-type strain PAO1, indicated by a plus sign below each bar. The control strain containing the parent vector pHERD20T is indicated by a minus sign 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 ± SDs of three independent experiments. The presence or absence of the inducer L-arabinose (0.5%) is indicated below each bar.

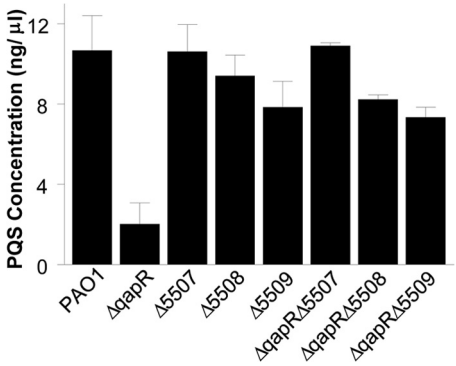


**FIG 7** Mutation of quinolone alteration pathway enzymes abrogates PQS modulation in the Δ*qapR* mutant strain. Cultures were grown for 18 h in LB medium, and PQS was extracted and quantified as described in Materials and Methods. Data are presented as the averages ± SDs of three independent experiments. 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 represented by the designations Δ*qapR*, Δ5507, Δ5508, Δ5509, Δ*qapR*Δ5507, Δ*qapR*Δ5508, and Δ*qapR*Δ5509, respectively.

mine 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 that produced by the *qapR* mutant strain (Fig. 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 enzymes 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. (Note that all of the mutant strains grew in a manner similar to that of the wild-type strain under our conditions.) We found that only the *qapR* mutant strain had a change in PQS concentration compared to the wild-type strain PAO1 (Fig. 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 when QapR is present. Therefore, we constructed double mutant strains in which *qapR* was mutated in combination with gene PA5507, PA5508, or PA5509 and assessed PQS production. Mutation of gene PA5507, PA5508, or PA5509 in the *qapR* mutant strain effectively restored PQS concentration to a level similar to that produced by the wild-type strain PAO1 (Fig. 7). This suggests that genes PA5507, PA5508, and PA5509 each play a role in PQS homeostasis and that QapR effectively represses them in order to prevent a decrease in PQS concentration.

## 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 (19). The original strain in which *qapR* had been mutated also had a lesion in *pqsL*, which caused an overproduction of PQS that led 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. 1). 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 (corresponding to genes 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 mutations in each of these genes in a *qapR* mutant restored PQS concentration to a level similar to that produced by strain PAO1 (Fig. 7). This also explains the results seen with the single PA5507, PA5508, and PA5509 mutants (Fig. 7), because these genes are normally repressed in the wild-type strain and therefore mutating these genes individually would not be expected to produce an effect. This also explains why overexpression of *qapR* in strain PAO1 does not cause a change in PQS concentration. 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 PA5507, PA5508, and/or PA5509 could directly alter PQS. Ladner et al. demonstrated that PA5508 encodes an enzyme that exhibits γ-glutamyl aromatic monoamine ligase activity *in vitro* (36). This enzyme was able to glutamylate several aromatic amine sub-

strates, 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 isochorismatases and amidohydrolases, respectively (39). 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. (40). However, none of the predicted functions of genes PA5507, PA5508, and 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 (22). An additional possibility is that the *qapR* operon enzymes produce a molecule that can inhibit PQS biosynthesis, which would also be very interesting as a mechanism to ensure that 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 (24, 25, 37, 38). 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 (41). This family of regulatory proteins may act as a checkpoint to virulence factor production within the cell. It would be advantageous for a pathogen to link the synthesis of required biosynthetic intermediates with virulence in order to avoid the production of virulence factors at the expense of central metabolism. With this in mind, it is intriguing to speculate that the *qapR* operon may play a role in balancing the flux of molecules routed to virulence factor production or central metabolism.

We believe that 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 treatment with a compound that alters coordinated behavior instead of traditional antibiotic targets.

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