A Conserved Suppressor Mutation in a Tryptophan Auxotroph Results in Dysregulation of *Pseudomonas* Quinolone Signal Synthesis

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Pseudomonas aeruginosa is a common nosocomial pathogen that relies on three cell-to-cell signals to regulate multiple virulence factors. The *Pseudomonas* quinolone signal (PQS; 2-heptyl-3-hydroxy-4-quinolone) is one of these signals, and it is known to be important for *P. aeruginosa* pathogenesis. PQS is synthesized in a multistep reaction that condenses anthranilate and a fatty acid. In *P. aeruginosa*, anthranilate is produced via the kynurenine pathway and two separate anthranilate synthases, TrpEG and PhnAB, the latter of which is important for PQS synthesis. Others have previously shown that a *P. aeruginosa* tryptophan auxotroph could grow on tryptophan-depleted medium with a frequency of 10^{-5} to 10^{-6}. These revertants produced more pyocyanin and had increased levels of *phnA* transcript. In this study, we constructed similar tryptophan auxotroph revertants and found that the reversion resulted from a synonymous G-to-A nucleotide mutation within *pqsC*. This change resulted in increased pyocyanin and decreased PQS, along with an increase in the level of the *pqsD*, *pqsE*, and *phnAB* transcripts. Reporter fusion and reverse transcriptase PCR studies indicated that a novel transcript containing *pqsD*, *pqsE*, and *phnAB* occurs in these revertants, and quantitative real-time PCR experiments suggested that the same transcript appears in the wild-type strain under nutrient-limiting conditions. These results imply that the PQS biosynthetic operon can produce an internal transcript that increases anthranilate production and greatly elevates the expression of the PQS signal response protein PqsE. This suggests a novel mechanism to ensure the production of both anthranilate and PQS-controlled virulence factors.

The Gram-negative bacillus *Pseudomonas aeruginosa* is a common nosocomial pathogen that causes devastating opportunistic infections in immunocompromised individuals and chronic infections in cystic fibrosis (CF) patients (1–3). *P. aeruginosa* is notoriously resistant to multiple classes of antibiotics, making many infections difficult to treat. The ability of *P. aeruginosa* to evade the immune system and cause these serious infections can be attributed to the numerous virulence factors employed by the bacteria. Many of these virulence factors are controlled by three known cell-to-cell signals, which compose a complex communication network that ensures precise expression of many genes in *P. aeruginosa*. Two of the cell-to-cell signaling systems are mediated by acyl-homoserine lactone signals that are regulated by their cognate transcriptional regulators, LasR and RhlR (4, 5). Together these two signaling systems regulate 6 to 11% of the *P. aeruginosa* genome (6, 7). The third signal is the *Pseudomonas* quinolone signal (PQS; 2-heptyl-3-hydroxy-4-quinolone) (8). PQS acts as a coinducer for the transcriptional regulator PqsR, which drives transcription of the PQS biosynthetic gene locus and positively regulates multiple virulence factors (8–13). PQS has also been shown to be important for *P. aeruginosa* pathogenesis in *Caenorhabditis elegans*, *Drosophila melanogaster*, lettuce leaf, and mouse infection models (10, 14–17). PQS has been extracted from airway fluid and sputum of infected CF patients, further implying its importance in *P. aeruginosa* infections (18).

With the overwhelming evidence that PQS plays a role in virulence and pathogenesis, we set out to understand both the regulation of the genes required for PQS production and the biosynthesis of PQS. The biosynthetic genes required for PQS synthesis, *pqsABCD* and *pqsH*, are also responsible for the production of at least 56 other 4-hydroxy-2-alkylquinolines (HAQs) (19, 20). The biosynthesis of all HAQs depends on subsequent condensations of anthranilate with malonyl coenzyme A (malonyl-CoA) and then a fatty acid (Fig. 1), the chain length of which determines the side chain length of the HAQ (21, 22). The precursor anthranilate is an important branch point metabolite in *P. aeruginosa* that can be synthesized into either PQS or the amino acid tryptophan, as well as catabolized into tricarboxylic acid cycle intermediates (23). The cellular pool of anthranilate in *P. aeruginosa* is maintained via three separate pathways (24). First, the kynurenine pathway converts tryptophan into anthranilate and is the main source of anthranilate for PQS production when tryptophan is present (24). This pathway is regulated by both LasR and KynR, which induce the pathway genes in the presence of kynurenine (25, 26). Anthranilate is also supplied by the two anthranilate synthases, TrpEG and PhnAB (27), which convert chorismate into anthranilate. TrpEG catalyzes the first step in tryptophan synthesis and is regulated by tryptophan through attenuation and posttranslational competitive inhibition of TrpE (28, 29). Though this enzyme seemingly could provide anthranilate for the cellular pool, TrpEG does not naturally provide anthranilate for PQS production in a *phnA* mutant grown without tryptophan (24). However, overexpression of TrpEG causes PQS production in a *phnAB* mutant grown without tryptophan, thus indicating the importance of *phnAB* regulation for PQS production (30).

The *phnAB* operon is located between the *pqsABCDE* operon (same strand) and *pqsR* (opposite strand), placing it in the center of the PQS synthesis region. Like *pqsABCDE*, *phnAB* is positively controlled by PqsR (31–33). Due to both the location and regula-
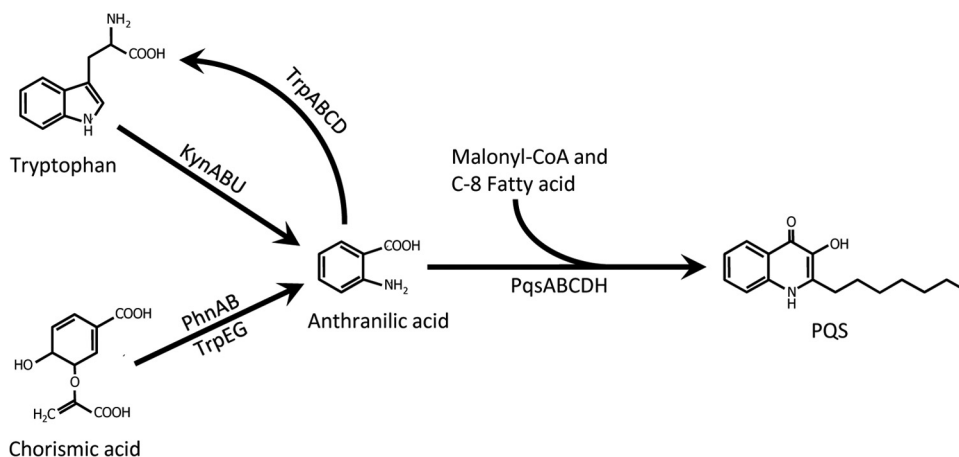
FIG 1 Anthranilate and PQS synthesis in *P. aeruginosa*. In *P. aeruginosa*, anthranilate is made via three separate enzymatic pathways. The kynurenine pathway degrades tryptophan into anthranilate via the KynABU enzymatic pathway, while anthranilate is synthesized from chorismate by two separate anthranilate synthases, PhnAB and TrpEG. Anthranilate can be used as a precursor for many compounds, including tryptophan and 4-quinolones.

tion of *phnAB*, it was assumed that PhnAB provided the majority of anthranilate for PQS synthesis. However, we have shown that PhnAB supplies anthranilate for PQS only under nutrient-limiting conditions (24). With three separate and uniquely regulated pathways to anthranilate, it is clear that the production of anthranilate is a priority for *P. aeruginosa*. This was obvious when the two initial studies describing TrpEG and PhnAB in *P. aeruginosa* revealed an interesting aspect of *phnAB* regulation (27, 34). When a tryptophan auxotroph was plated on tryptophan-depleted media, auxotrophy-negating mutations occurred at a frequency of 10^{-5} to 10^{-6} (27). When one of the revertants was investigated, it was discovered that it had constitutive levels of *phnAB* transcript expression, as opposed to the peak stationary levels exhibited by wild-type *P. aeruginosa* (27). In addition, the revertant had increased levels of pyocyanin, a virulence factor influenced by the PQS signaling system. In this study, we set out to identify the source of the spontaneous mutation that suppresses the tryptophan auxotrophy and discovered a novel regulatory mechanism for *phnAB* and PQS-controlled genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *Escherichia coli* and *P. aeruginosa* strains were freshly plated to begin each experiment and were maintained at $-80°C$ in 30% glycerol and 10% skim milk (Difco), respectively. All strains and plasmids used are listed in Table 1. Bacteria were cultured at 37°C in Luria-Bertani medium (LB) or Vogel-Bonner minimal medium supplemented with 0.5% glycerol (VBG) (35). Growth of bacterial cultures was monitored spectrophotometrically based on the optical density at 600 nm (OD_{600}) for *E. coli* and OD_{660} for *P. aeruginosa*. To maintain plasmids, 100 μg/ml of ampicillin or 200 μg/ml of carbenicillin was added when appropriate. Also, 10 mM L-tryptophan was added to VBG cultures when indicated.

To generate transcriptional fusions for *pqsD*, DNA fragments of various length that were 3′ nested at 58 bp downstream from the *pqsD* start codon (see Fig. 7 for diagram) were amplified by PCR using chromosomal DNA as a template from either strain PAO1 or strain TrpErev1. The oligonucleotide primers (Table 2) used for the amplification contained either a XhoI or a HindIII restriction site. The amplified fragment was digested with XhoI and HindIII, purified from an agarose gel, and ligated into pLP170 digested with the same enzymes. All reporter fusions were sequenced to ensure that mutations did not occur during cloning. Plasmids were transformed into *P. aeruginosa* strains by electroporation (36).

Generation of mutants. A splicing-by-overlap extension protocol was used to generate the mutant allele for the *trpE* deletion strain (37). The mutation was constructed to contain an in-frame deletion in the coding DNA sequence corresponding to amino acids 26 to 445 for *trpE* (85% of protein sequence). Primers were designed to amplify approximately 1 kb of DNA both upstream and downstream from the splice junction, and each primer added a HindIII restriction site to the ends of the PCR product. The PCR fragment and pEX18Ap (suicide vector) were digested with HindIII and ligated together to make the suicide vector p*trpE*del. To generate the PAQC-G1041A mutant, a PCR fragment was created using strain TrpErev1 chromosomal DNA, and it was digested with HindIII and ligated into pEX18AP. The resulting plasmids, p*trpE*del and pPAQC-G1041A, were transformed into strain PAO1 by electroporation (38). Mutants were selected by plating transformants on medium containing carbenicillin and then on medium containing 6% sucrose to select for deletion of the vector sequence (39). PCR was used to screen colonies, and DNA sequencing of PCR products was used to confirm mutations.

Tryptophan auxotroph revertants were made through selective pressure on tryptophan-free media. The tryptophan auxotroph strain PAΔTrpE was grown overnight in VBG supplemented with 10 mM L-tryptophan at 37°C with shaking at approximately 180 rpm. Overnight cultures were washed with fresh, unsupplemented VBG, and 100 μl of washed culture was added to 10 ml of unsupplemented VBG. The culture was grown for 3 h at 37°C with shaking at approximately 180 rpm. After 3 h of growth, cells were harvested by centrifugation at $6,000 \times g$ for 10 min. The cell pellet was resuspended in 1 ml of VBG and then plated onto VBG plates. Individual colonies were then reisolated and stored at $-80°C$ for further analysis.

Whole-genome sequencing. An Illumina GAIIX at the North Carolina State University Gene Sequencing Laboratory was used to sequence wild-type strain PAO1 and three tryptophan auxotroph revertants (TrpErev1, -2, and -3). The sequencing provided between 5.8 and 7.3 million reads for a high-quality map. To determine the location of the mutation(s), the published PAO1 genome (40) was compared to those of the sequenced PAO1 laboratory strain and the tryptophan revertant strains. The Burrows-Wheeler Aligner (BWA) was used to align the raw reads and provided the high-quality map (41). The *trpE* deletion in each of the revertants was confirmed by using the Integrative Genomics Viewer (IGV) (42, 43). Both Joint Genotyper for Inbred Lines (JGIL) and SAMtools and BCFtools were used to identify single nucleotide polymorphisms (SNPs), and the SAMtools and BCFtools were used specifically to identify any insertions and/or deletions (44, 45). The JGIL is designed to look for SNPs in inbred lines but is appropriate

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Reference
Strains		
E. coli DH5α	F'/*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* (*lacZYA-argF*) *U169 deoR* Φ80d*lac*Δ(*lacZ*)*M15recA1*	52
P. aeruginosa		
PAO1	Wild type	
PJF-QR1	*pqsR* deletion mutant derived from strain PAO1	53
PAΔTrpE	*trpE* deletion mutant derived from strain PAO1	48
PJF-QE1	*pqsE* deletion mutant derived from strain PAO1	48
PJF-PhnA	*phnA* deletion mutant derived from strain PAO1	24
PAQC-G1041A	PAO1 derivative with G-to-A mutation in *pqsC* at bp 1041	This study
TrpErev1	Spontaneous mutant of strain PAΔTrpE[a]	This study
TrpErev2	Spontaneous mutant of strain PAΔTrpE[a]	This study
TrpErev3	Spontaneous mutant of strain PAΔTrpE[a]	This study
TrpErev4	Spontaneous mutant of strain PAΔTrpE[a]	This study
TrpErev5	Spontaneous mutant of strain PAΔTrpE[a]	This study
Plasmids		
pLP170	*lacZ* transcriptional fusion vector; Amp[rb]	54
pPqsDwtTcLacZ	*pqsD'-lacZ* transcriptional fusion; Amp[r]	This study
pPqsDG1041ATcLacZ	*pqsDpqsC*G1041A1041'-*lacZ* transcriptional fusion; Amp[r]	This study
pEX18AP	Suicide vector; Amp[r]	39
pPAQC-G1041Adel	G-to-A reversion in *pqsC* suicide vector; Amp[r]	This study
p*trpE*del	*trpE* deletion suicide vector; Amp[r]	This study
pΔ*pqsD*-suc	*pqsD* deletion suicide vector; Amp[r]	This study
pDSW8	*tacp-pqsR* on pEX1.8, Amp[r]	32
pBluescript II SK+	Cloning vector; Amp[r]	Agilent Technologies
pSLM100	*phnA* expression vector; Amp[r]	This study

[a] See Table 3 for the location(s) of mutation(s).
[b] Amp[r], ampicillin resistant.

TABLE 2 Primers used in this study

Primer use and name	Sequence[a]
Mutagenesis	
TrpEF1-HindIII	AAAA**AAGCTT**CCTGATCGATGCCTCCGGTC
TrpEdelF2	TCACCGTTCTTGATCACCGCAGTCGGCAA GGGTCTCGA
TrpEdelR1	TCGAGACCCTTGCCGACTCGCGGTGATCAAG AACGGTGA
TrpER2-HindIII	AAAA**AAGCTT**ACCGGCAACAGCATGGAAC
PqsCUp1	AAAA**AAGCTT**AGATTCCCTCGCACATGCTG
PqsCDown2	AAAA**AAGCTT**ACCTCCTCAGGTTTGCGGTA
Expression plasmids	
pqsClacZfwd	AAAAA**GAATTC**CCGACCGGCGAGGCCAA
pqsClacZrev	AAAA**AAGCTT**TGCTGACCTGGCGTTTCGGC
pqsCLacZfwd-2	AAAAA**CTCGAG**GATTATTTCGTCTTCCACCAG
pqsCLacZfwd-3A	AAAAA**CTCGAG**GCCTGCGCGAGGGCAGAGTCCG
pqsCLacZfwd-3B	AAAAA**CTCGAG**GTATCGTCATGGCCGGCGCAG
pqsCLacZfwd-5	AAAAA**CTCGAG**TTCGCCGCCCAGGTCTGGC
pqsCLacZfwd-6	AAAAA**CTCGAG**AGGTCTGGCAATTGGGTGA
Real-time PCR	
RPLU RT1	GGTGGCAAGCAGCACAAAGTCACCG
RPLU RT2	GCGGACCTTGTCGTGACGGCCGTGG
pqsARTF1	GCCCTTTGCTCGACGATTTCTCG
pqsARTR2	AACCCGAGGTGTATTGCAGGAAACA
pqsCRTF1	CGAGTCCTGGTGGCAATTCT
pqsCRTR1	TCAGCATGTCCACGCTATCC
pqsDRTF1	GTGGATCGGTCTGGGCAACAT
pqsDRTR1	CTCCTCAGGTTTGCGGTACA
pqsERTF1	TGATGACCTGTGCCTGTTGG
pqsERTR1	GTCGTAGTGCTTGTGGGTGA
phnARTF1	ACGTGGACAGCAAGGCTGCG
phnARTR1	GGGCCGGACAGTCCTCGCTC
RT-PCR	
E2	CCAGACTTTCTTCCAGTCGATAGC
F1	TACCGAGTGTCTGCGCCTGT
F2	GGCCAGGTCGAAGCTGAACA

[a] Restriction sites are indicated by bold type.

to use for bacteria due to all strains being derived from the same parent strain.

PQS concentration determination. Washed cells from overnight cultures were used to inoculate 10-ml cultures of LB or VBG to an OD_{660} of 0.05. After 24 h of growth, a 300-μl sample of each culture was extracted with 900 μl of acidified ethyl acetate as previously described (18). One-half of the resulting organic phase was evaporated to dryness at 37°C, and 50 μl of 1:1 acidified ethyl acetate-acetonitrile was used to reconstitute the extract (18). Samples were analyzed by thin-layer chromatography (TLC), visualized by long-wave UV light, and photographed (18). The PQS spots were quantified using Bio-Rad Quantity One software compared to a standard curve of synthetic PQS.

RNA isolation. For all RNA extractions, the cultures were grown at 37°C in either LB medium or VBG until an OD_{660} of 0.2 or 0.6 was reached. At that point, total RNA for reverse transcriptase PCR (RT-PCR) and quantitative real-time RT-PCR (real-time PCR) was purified using the RNeasy mini-spin columns (Qiagen). All RNA samples were treated with RQ1 DNase (Promega) for 2 h at 37°C to remove any contaminating DNA. To remove RQ1 DNase, a phenol-chloroform extraction was performed, followed by an ethanol precipitation. RNA was stored in diethyl pyrocarbonate (DEPC)-treated water at −80°C.

Quantitative real-time PCR. cDNA synthesis was performed on a Mastercycler (Eppendorf) using 2.5 μg of DNA-free RNA and Superscript III (Invitrogen) by following the manufacturer's instructions. Primers to generate cDNA were random primers (Invitrogen) as well as random hexamers, 72% GC (Gene Link). Real-time PCR was performed on a CFX96 (Bio-Rad) using the PerfeCTa SYBR green FastMix for iQ (Quanta Biosystems). The reaction mixtures contained 5 μl of cDNA diluted (1:500) in a 15-μl volume. Relative transcript levels were determined using the Pfaffl method by comparing experimental gene expression (*phnA*, *pqsA*, *pqsC*, *pqsD*, or *pqsE*) to that of the control gene, *rplU* (46). Standard curve and efficiency values were determined by using gene-specific prim-

ers with 10 ng to 0.1 pg of RNA-free genomic DNA from strain PAO1. A melting-curve step was included at the end of each PCR to ensure that primer dimers and nonspecific variation between samples did not occur. Each real-time PCR was performed on at least three biological replicates of each RNA sample, with technical replicates performed in duplicate.

RT-PCR. For reverse transcriptase PCR, the AccessQuick kit from Promega was used. PAO1 chromosomal DNA (100 ng) was used as a positive control, and 300 ng of RNA was used in all experimental samples. Two biological RNA samples per strain were used to verify results.

β-Gal assays in *P. aeruginosa*. Cells from overnight cultures of *P. aeruginosa* grown in VBG were washed and resuspended in fresh medium to an OD_{660} of 0.05 and incubated at 37°C with shaking at approximately 180 rpm until the OD_{660} had reached 0.2. At that time, cultures were assayed for β-galactosidase (β-Gal) activity in duplicate. Data are presented in Miller units as the mean ± the standard deviation (σ^{n-1}) of at least three separate experiments.

RESULTS

Tryptophan starvation reverts tryptophan auxotrophs by permanently increasing *phnAB* expression. *P. aeruginosa* possesses two anthranilate synthases, TrpEG and PhnAB. The existence of two anthranilate synthases and the kynurenine pathway ensures that the cellular pool of anthranilate can be maintained for tryptophan synthesis and PQS production. In two studies describing both TrpEG and PhnAB, Essar et al. discovered that a *trpE* mutant is a tryptophan auxotroph that will revert to tryptophan prototrophy at a high frequency (10^{-5} to 10^{-6}) when grown on tryptophan-depleted medium (27, 34). The tryptophan auxotroph revertant exhibited increased pyocyanin levels and a constitutive high induction of the *phnAB* transcript, which apparently complemented the loss of anthranilate production caused by the mutation of *trpE* (27). These strains are no longer available (D. Essar, personal communication), so our laboratory selected revertants in the same manner and set out to identify the mutation(s) responsible for the loss of tryptophan auxotrophy. In order to do this, we constructed a *trpE* mutant (PAΔTrpE) and grew it on minimal media depleted of tryptophan to select for a revertant. This was repeated three times and we chose to analyze five colonies that grew well, indicating that they were tryptophan auxotroph revertants. All five revertants showed increased pyocyanin levels, which was also reported by Essar et al. (27) (Fig. 2A). In addition, the auxotrophy revertants all produced a greatly decreased amount of PQS (Fig. 2B), which was unexpected because PQS positively controls pyocyanin production (10). (This phenomenon is addressed below.) Quantitative real-time PCR analysis of *phnA* showed that all five revertants had at least a 3.5-fold increase in *phnA* expression compared to strain PAO1 when grown in either rich medium (Fig. 3A) or minimal medium without tryptophan (Fig. 3B). This is similar to data reported previously (27), and taken together, our data lead us to believe that we were studying the same phenomenon as first reported by Essar et al. (27) and that an unknown regulatory event occurs when cells are starved for tryptophan.

Discovery of a conserved point mutation in tryptophan auxotroph revertants. To understand the mechanism of change in the revertant strains, the full genome sequences of three tryptophan auxotroph revertants (TrpErev1, -2, and -3) were determined. This experiment identified two locations that had a single nucleotide change. One change was found in all three strains, and the other was found in just two strains, which were derived from the same selection experiment and likely were clones (Table 3). No other substitution, insertion, or deletion mutations were found.
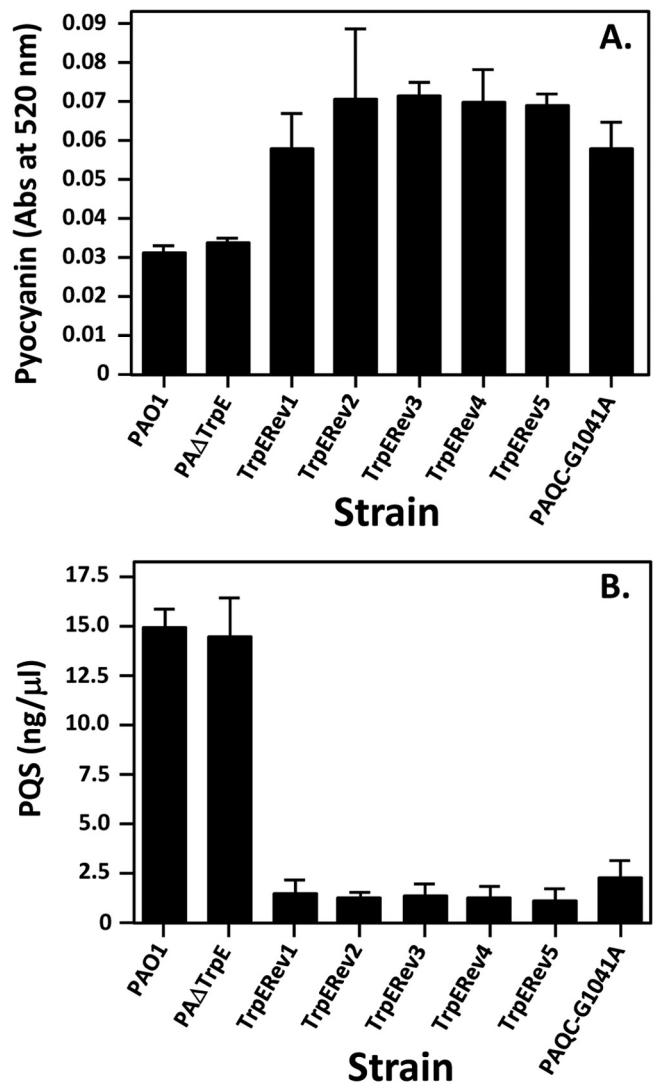
FIG 2 Pyocyanin and PQS production by tryptophan auxotroph revertants. (A) Pyocyanin assays were performed after 18 h of growth in LB broth. Results are shown as light absorbance at 520 nm and are the mean ± σ^{n-1} of results from duplicate assays from at least three separate experiments. (B) Ethyl acetate extracts from cultures grown in LB broth for 24 h were analyzed by TLC. Equal volumes of extracts were resolved in each lane. The TLC plates were photographed under UV light, and densitometric readings of PQS spots were compared to those from a standard curve of synthetic PQS in order to determine the PQS concentration in extracts. Results shown are the average ± σ^{n-1} of results from at least three separate experiments.

One mutation was a G-to-A change at nucleotide 1041 of *pqsC* that did not cause a change in the encoded amino acid. The other mutation was in the *ntrC* gene (nucleotide 1227), and it also did not cause an amino acid change. Once these mutations were identified, the same regions of two other revertant strains (TrpErev4 and TrpErev5) were amplified by PCR and sequenced. These other two strains were derived from independent experiments; both had the same *pqsC* mutation as described above (referred to henceforth as G1041A), and neither had a mutation in *ntrC* (Table 3). These data led us to further examine the G1041A mutation.

The G1041A mutation is two codons from the *pqsC* stop codon and is in the third base of a valine codon, resulting in a codon that
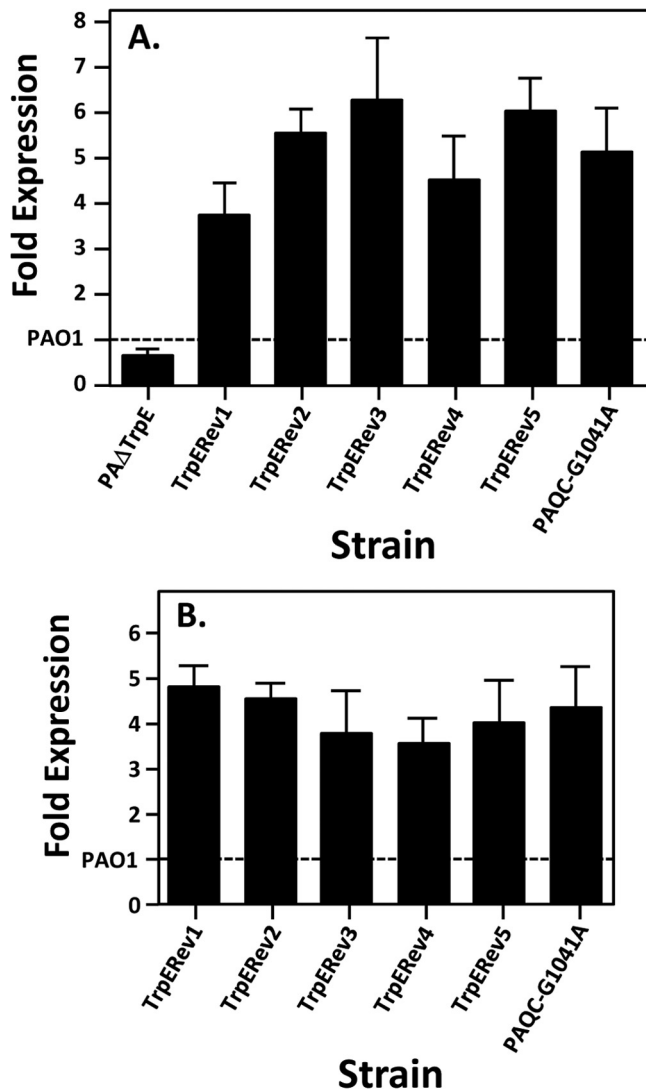
FIG 3 Tryptophan auxotroph revertants have increased *phnA* transcript levels. Quantitative real-time PCR was used to measure the expression of *phnA* mRNA from the indicated strains grown at 37°C in LB medium (A) and VBG (B). Wild-type strain PAO1 transcript levels are set at 1, which is indicated by the dashed line. Strain PAΔTrpE was not included in panel B because it will not grow in VBG medium without tryptophan supplementation. Error bars indicate the standard deviations of three independent experiments performed in duplicate.

still encodes valine and therefore does not alter PqsC. There is a 43-bp intergenic region between the end of the *pqsC* coding sequence and the start codon of *pqsD*, so this change does not affect the PqsD protein (see the asterisk in Fig. 5 for a visual representation of the location of this mutated nucleotide). To ensure that the G1041A mutation in *pqsC* resulted in the effects observed in the tryptophan auxotroph revertants, we constructed a mutant using wild-type strain PAO1, which contained only the G1041A mutation. Alteration of this single base in strain PAO1 resulted in the same phenotype as in the revertant strains: pyocyanin increased (Fig. 2A), PQS decreased (Fig. 2B), and the *phnA* transcript increased (Fig. 3). This confirmed that a single base change at nucleotide 1041 of *pqsC* caused the observed results and is a mechanism by which *P. aeruginosa* can survive when starved for

tryptophan (and probably anthranilate). Our next goal was to try to understand the mechanism through which this single base change brought about the observed effects.

A synonymous mutation in *pqsC* results in differential expression of a novel transcript within the PQS biosynthetic operon. The G1041A mutation in *pqsC* resulted in increased pyocyanin and decreased PQS, which seems contradictory since PQS is required for the production of pyocyanin (10). However, PQS has not been directly linked to pyocyanin control, and it has been shown that the last gene of the *pqsABCDE* operon, *pqsE*, can independently activate pyocyanin production (47, 48). In addition, it is known that the overexpression of *pqsE* causes PQS production to stop (49). This led us to speculate that the G1041A mutation was causing *pqsE* expression to increase. We therefore utilized quantitative real-time PCR to compare transcript levels of genes within the *pqsABCDE* and *phnAB* operons. Analysis of RNA from a *trpE* auxotroph revertant (Fig. 4A) and our constructed G1041A mutant (Fig. 4B) showed that both strains have a decreased level of *pqsA* and *pqsC* transcript compared to the wild-type strain, PAO1. These decreases are most likely due to the lower PQS levels described above, which would result in less coinducer for the transcriptional regulator PqsR to drive transcription of the *pqsABCDE* operon (32). Most interestingly, levels of the *pqsD*, *pqsE*, and *phnA* mRNA were increased at least 3-fold in both strain TrpErev1 and strain PAQC-G1041A compared to the wild-type strain (Fig. 4). This result was unexpected because previous data suggested that the PQS biosynthetic operon transcript is derived from the *pqsA* promoter and that the transcript included only *pqsABCDE* (20). However, the data of Fig. 4 led us hypothesize that the G1041A mutation caused the induction of a transcript within *pqsC* and that this transcript contains at least *pqsD*, *pqsE*, and *phnA*. To investigate this possibility, we analyzed transcripts by reverse transcriptase PCR (RT-PCR). The oligonucleotides used for RT-PCR are illustrated in Fig. 5A and included a set used by McGrath et al. (primer set F1 and F2) when they suggested that the PQS biosynthetic operon included only *pqsABCDE* and not *phnAB* (20). We obtained results similar to those we reported previously (20) when mRNA derived from a wild-type culture grown in LB medium was used in the RT-PCR experiments. The wild-type strain PAO1 mRNA (Fig. 5B, lane 3) did not produce a product that would indicate mRNA continuity between *pqsE* and *phnA*. However, an RT-PCR product indicative of a continuous transcript was evident with both primer sets from the analysis of G1041A mutant mRNA (Fig. 5B, lane 5). (The E1-F2 primer set starts and ends, respectively, 556 nucleotides upstream from and 468 nucleotides downstream from the *phnA* ATG start codon.) This suggested that the G1041A mutant strain does express a novel transcript that includes at least part of *pqsE* and *phnA*. Most inter-

TABLE 3 Location(s) of the mutation(s) in tryptophan auxotroph revertants

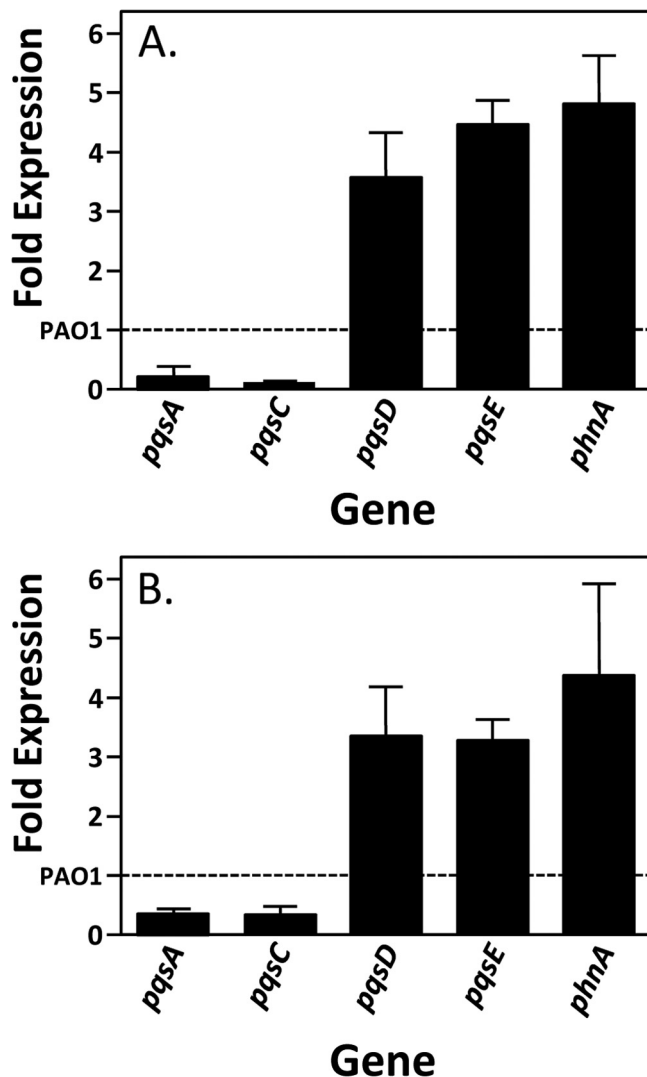| Revertant strain | Mutation | |
	pqsC	*ntrC*
TrpErev1	Base 1041, G to A	No change
TrpErev2	Base 1041, G to A	Base 1227, G to A
TrpErev3	Base 1041, G to A	Base 1227, G to A
TrpErev4	Base 1041, G to A	No change
TrpErev5	Base 1041, G to A	No change

FIG 4 A single nucleotide mutation in *pqsC* induces *pqsD*, *pqsE*, and *phnA*. Quantitative real-time PCR was used to measure the expression of *pqsA*, *pqsC*, *pqsD*, *pqsE*, and *phnA* mRNA from strains TrpErev1 (A) and PAQC-G1041A (B) grown at 37°C in VBG to an OD_{660} of 0.2 (mid-log phase). Wild-type strain PAO1 transcript levels are set at 1, which is indicated by the dashed line. Error bars indicate the standard deviations of three independent experiments performed in duplicate.

estingly, the same products appeared in the analysis of either the wild-type strain PAO1 or mutant G1041A mRNA from cultures grown in minimal medium (Fig. 5C, lanes 3 and 5, respectively). Considering these data along with those in Fig. 4, it appears that under nutrient-limiting conditions, a novel transcript that includes *pqsD*, *pqsE*, and *phnA* is induced in the wild-type strain and that this transcript is constitutively induced in the G1041A mutant.

Evidence for a novel transcript. In order to confirm that the putative transcript described above was present in wild-type strain PAO1, we used quantitative real-time PCR to compare *pqsA* and *pqsD* expression in minimal and rich media. The data showed that the ratio of expression for *pqsA* and our control gene (*rplU*) was approximately 1:1 in minimal versus rich medium, indicating no change in relative expression (Fig. 6). However, we found that

pqsD expression was over 2-fold higher in minimal medium than in rich medium (Fig. 6). These data confirmed the data of Fig. 5 and indicated that in wild-type strain PAO1, the *pqsA* and *pqsD* transcripts are differentially regulated and therefore most likely on two differentially expressed mRNAs.

Despite using multiple different protocols and both natural and enriched mRNA, repeated attempts to map the 5′ end of this novel transcript did not succeed in reproducibly identifying a transcriptional start site (data not shown). Therefore, to understand the sequence elements required to control this transcript, we constructed a series of *lacZ* reporter fusions that were nested within the 5′ region of *pqsD* and contained the *pqsC* G1041A mutation and progressive deletions of portions of *pqsC* (Fig. 7). This experiment showed that the region of *pqsC* that is upstream from nucleotide −87 relative to the *pqsD* start codon was required for transcription. Deleting an additional 11 bp from this sequence produced the fusion that ended at nucleotide −76 relative to the *pqsD* start codon and caused a complete loss of transcriptional activity. This suggests that a promoter element is located between 76 and 87 bp upstream from the *pqsD* start codon. The G1041A mutation is at position −48 relative to the *pqsD* start codon, which places the mutation close to the location where one would expect to find a transcriptional start site if a promoter begins just inside position −87. This allows us to speculate that the G1041A mutation is either at or near the transcriptional start site or is within a −10 region of a promoter. The latter option seems more likely, as the large positive effect on transcription would be more simply explained by the mutation leading to the creation of an optimal promoter sequence. It is also interesting that as the 5′ region of the fusions was removed, a progressive decrease in transcriptional activity occurred from a maximum of 26,230 ± 2,015 down to 8,366 ± 925 U of β-Gal activity, indicating that the promoter of the novel transcript is modulated by elements well upstream from the −35 region. Nonetheless, the promoter was still highly induced when only 87 nucleotides upstream from the *pqsD* start codon were included. Taken together, the data suggest that a conserved synonymous mutation near the end of *pqsC* is selected for when a tryptophan auxotroph is grown under tryptophan-limiting conditions. This mutation leads to the overexpression of a transcript that rescues the tryptophan auxotroph due to the induction of *phnAB*, and to the induction of *pqsE*, which induces PQS-controlled genes in the absence of PQS.

DISCUSSION

The data we present here point to a mechanism that explains how tryptophan auxotroph revertants alter their ability to produce PQS-controlled virulence factors. The intriguing results reported in 1990 by Essar et al. (27) on the link between *phnA* dysregulation and phenazine production led us to investigate this natural phenomenon. Our isolation of five tryptophan auxotroph revertants, which mirrored the prior results (27), suggested that we had recreated the mutation that allowed for survival of a tryptophan auxotroph on tryptophan-depleted medium (Fig. 2 and 3). Full-genome sequencing of the revertants revealed a point mutation in *pqsC* at nucleotide 1041 that occurred in all five strains. The single nucleotide *pqsC* G1041A mutation was interesting because it was within the PQS biosynthetic operon upstream from *phnAB*, causing us to focus on it as a likely cause of the observed phenotype. The construction of a G1041A mutant strain (PAQC-G1041A) then confirmed that the single nucleotide change in *pqsC* was re-
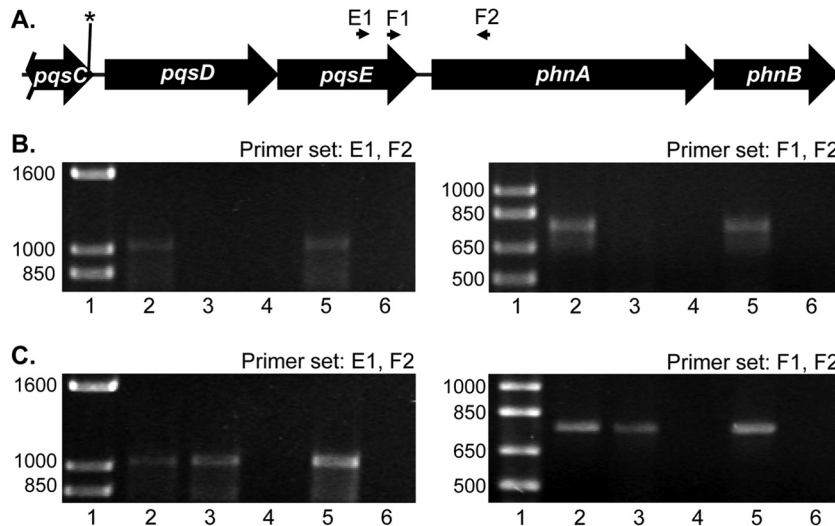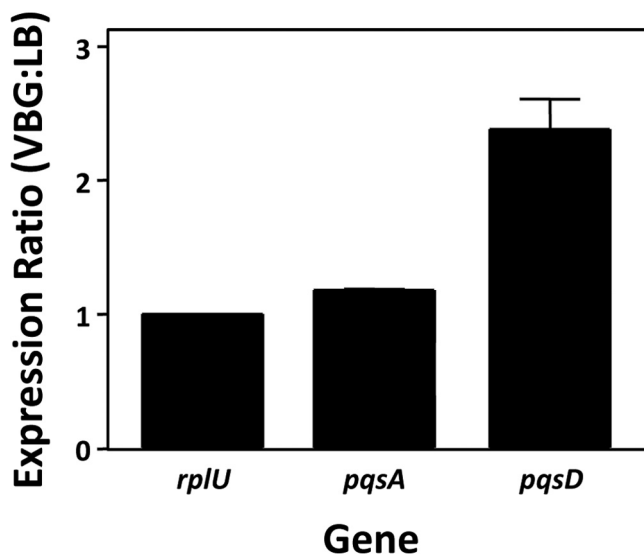
FIG 5 Analysis of the *pqsE* and *phnA* transcripts by RT-PCR. (A) Diagram of the *pqsD* through *phnB* genetic region with location of the primers used for RT-PCR experiments. The asterisk indicates the location of the G1041A mutation in *pqsC*, of which only the 3′ region is shown. (B and C) Agarose gel with products from RT-PCR experiments performed with the indicated oligonucleotide primer pairs and RNA isolated from cultures grown in LB medium (B) or VBG (C). Lane 1 contains molecular size standards (1 Kb Plus DNA ladder; Invitrogen); size, in base pairs, is marked on the left side of each panel. Lane 2 contains reactions performed with chromosomal DNA as a template for positive-control reactions. Lanes 3 and 4 contain total RNA from wild-type strain PAO1, and lanes 5 and 6 contain total RNA from strain PAQC-G1041A. RNA was isolated from VBG at an OD_{660} of 0.2 and was used as a template for experimental reactions (lanes 3 and 5). For negative controls, reverse transcriptase was omitted from reactions shown in lanes 4 and 6.

sponsible for the increase in pyocyanin production and *phnA* transcription exhibited by our revertants (Fig. 2 and 3) and likely those originally reported by Essar et al. (27).

Further investigation of the G1041A mutation in *pqsC* led us to discover that not only was the single nucleotide change responsible for elevated levels of pyocyanin production and the *phnA* tran-

FIG 6 The *pqsA* and *pqsD* genes are differentially regulated in wild-type strain PAO1 when grown in rich (LB) or nutrient-limiting (VBG) medium. Cultures of strain PAO1 were grown to mid-log phase, at which time RNA was purified. Quantitative real-time PCR was used to measure expression of the indicated genes, and the ratio of expression in VBG versus LB was determined. The expression of the housekeeping gene *rplU* was measured as a control and set to a value of 1 for the purpose of comparison. Error bars indicate the standard deviations of three independent experiments performed in duplicate.

script (Fig. 2 and 3) but also it resulted in dysregulation of the PQS biosynthetic operon (Fig. 4). The G1014A mutation resulted in decreased levels of *pqsA* and *pqsC* transcripts, which is not surprising, as they are likely due to decreased coinducer (PQS) (Fig. 2) for PqsR, the transcriptional regulator of the PQS biosynthetic operon (32). What was perplexing was that the G1041A mutant had elevated transcript levels of the last two genes in the PQS operon, *pqsD* and *pqsE* (Fig. 4). Though these results were unexpected, they provided an explanation for the phenotypic results we had obtained (Fig. 2). The significant increase of the *pqsE* transcript would explain the observed increase in pyocyanin (Fig. 2), as this effect has been shown previously (10, 48). Furthermore, the elevated transcription of *pqsE*, and likely overproduction of the PqsE protein, may also account for the decrease in PQS production in the revertants (Fig. 2), as overproduction of PqsE results in decreased levels of PQS (49, 50). Though the overall phenotypic results were explained by the increase in *pqsE* transcript overexpression, we were still uncertain of what was causing the elevation of the transcript and how *pqsD*, *pqsE*, and *phnA* are linked.

In order to identify the possible causes for the elevation of the transcript, we first focused on the link between *pqsDE* and *phnA*. Northern blot analysis of these transcripts were inconclusive, so we utilized RT-PCR to determine whether there was a transcriptional link between *pqsE* and *phnAB*, which had been previously reported to be separate (20). We confirmed that in rich medium, there is no transcriptional link between *pqsE* and *phnA* in the wild-type strain, PAO1, and that there is a link in the G1041A mutant (Fig. 5). Most interestingly, this link was present in both the wild-type and mutant strains grown under nutrient-limiting conditions (Fig. 5). These data suggested that an alternate transcript is activated under nutrient-limiting conditions and that tryptophan starvation causes a mutation that greatly induces this transcript. This was demonstrated by showing that in the wild-
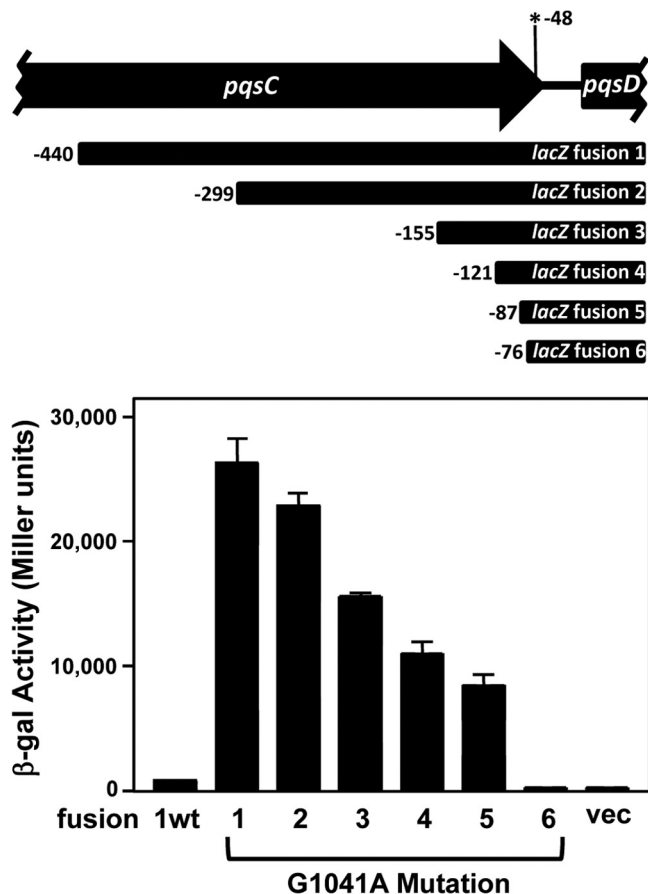
FIG 7 The G1041A mutation results in the expression of a new transcript upstream from *pqsD*. The *P. aeruginosa* wild-type strain PAO1 containing a reporter plasmid with either the wild-type sequence (p*pqsD*wtTcZ, indicated as "1wt") or nested deletion plasmids with the *pqsC* point mutation (p*pqsD*G1041ATcZ 1 through 6) was grown in VBG until an OD$_{660}$ of 0.2 was reached. The cultures were then assayed for β-Gal activity, which is presented in Miller units as the mean $\pm\ \sigma^{n-1}$ of results from at least three separate experiments. The numbers on the fusion diagram indicate the distance of the 5′ end of each fusion from the *pqsD* start codon. The asterisk indicates the location of the G1041A mutation.

type strain, a transcript containing *pqsD* was 2.5-fold higher in cultures grown under nutrient-limiting conditions than in one grown in rich medium (Fig. 6). These results suggest that the transcript between *pqsD*, *pqsE*, and *phnA* is likely expressed only under nutritionally stressed conditions and further explained our previous results which indicated that PhnA could restore PQS to a kynurenine pathway mutant only under nutrient-limiting conditions (24). These data also nicely corroborate those of Palmer et al. (30), who showed that overexpression of *phnAB* complements tryptophan auxotrophy and PQS production in a *trpE* mutant. Considering all of this, we believe that we have identified a unique natural transcript that allows for differential expression of *phnAB* when grown under different conditions. An alternative explanation for our data is that the G1041A mutation could change mRNA stability or result in antitermination of the *pqsABCDE* transcript. This seems unlikely, as the novel transcript we describe which connects *pqsE* and *phnA* was observed in the wild-type strain under minimal-medium growth conditions, but a change in message stability and/or termination in the mutant is possible.

Nevertheless, it is clear that the mutation does provide for a significant increase in the transcript, and the exact mechanism through which this happens is unknown.

We attempted to directly locate the start site of the novel transcript but did not succeed and had to resort to a reporter deletion analysis to roughly map the minimal transcriptional control element. These data showed that the positive regulatory element was at least 87 bp upstream from the *pqsD* start codon, which causes us to speculate that the G1041A mutation is probably in a −10 region of a promoter (Fig. 7). The promoter is apparently naturally responsive to low-nutrient conditions (Fig. 5) and selective pressure, such as tryptophan auxotrophy, will cause it to be mutated so that it is highly induced. It should also be noted here that a previous transcriptome study on *P. aeruginosa* strain PA14 suggested that there is a potential transcriptional start near the stop codon of *pqsC*, which would be very close to the G1041A mutation, but this start site was not confirmed (51).

Our data clearly show that the G1041A mutation resulted in the upregulation of an alternate transcript that includes *pqsD*, *pqsE*, and *phnA*. A large increase in PqsE would cause PQS production to stop while activating PQS-controlled genes in the absence of PQS (47–49). At the same time, an increase in PhnAB will complement the loss of TrpE. The lack of 4-quinolone production would also ensure that anthranilate, for which the cell is starved, can be utilized in pathways that are important for survival. In general, these interesting findings indicate that anthranilate production is highly important to *P. aeruginosa* and that multiple layers of regulatory mechanisms exist to ensure the production of this metabolite.

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