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Gene PA2449 Is Essential for Glycine Metabolism and Pyocyanin Biosynthesis in *Pseudomonas aeruginosa* PAO1

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Many pseudomonads produce redox active compounds called phenazines that function in a variety of biological processes. Phenazines are well known for their toxicity against non-phenazine-producing organisms, which allows them to serve as crucial biocontrol agents and virulence factors during infection. As for other secondary metabolites, conditions of nutritional stress or limitation stimulate the production of phenazines, but little is known of the molecular details underlying this phenomenon. Using a combination of microarray and metabolite analyses, we demonstrate that the assimilation of glycine as a carbon source and the biosynthesis of pyocyanin in *Pseudomonas aeruginosa* PAO1 are both dependent on the PA2449 gene. The inactivation of the PA2449 gene was found to influence the transcription of a core set of genes encoding a glycine cleavage system, serine hydroxymethyltransferase, and serine dehydratase. PA2449 also affected the transcription of several genes that are integral in cell signaling and pyocyanin biosynthesis in *P. aeruginosa* PAO1. This study sheds light on the unexpected relationship between the utilization of an unfavorable carbon source and the production of pyocyanin. PA2449 is conserved among pseudomonads and might be universally involved in the assimilation of glycine among this metabolically diverse group of bacteria.

Phenazines are a large class of polyaromatic secondary metabolites produced by a variety of soil bacteria, particularly those belonging to the genera *Streptomyces* and *Pseudomonas* (1, 2). The high redox activity associated with phenazines enables them to function in diverse biological processes such as iron assimilation (3, 4), primary energy metabolism (5, 6), and cell-to-cell communication (7). Phenazines also serve as crucial antimicrobial agents, enabling bacteria to kill off competing microorganisms (8–10) and cause disease in a eukaryotic host during infection (11, 12). One of the better-known human pathogens that use phenazines as virulence factors is *Pseudomonas aeruginosa* (13, 14). Indeed, a hallmark feature of *P. aeruginosa* infections in individuals suffering from the genetic disorder cystic fibrosis is the production of the blue phenazine pyocyanin. Pyocyanin accumulates to such high levels (~100 μM) that it gives the sputum of infected individuals a distinctly bluish hue (15).

There is an assortment of phenazine compounds biosynthesized by *P. aeruginosa* (16, 17), and all of them are derived from the common intermediate phenazine-1-carboxylic acid (PCA). In *P. aeruginosa*, PCA is biosynthesized from the central metabolite chorismate through a core group of seven enzymes known as PhzA, -B, -C, -D, -E, -F, and -G (18). PCA is then modified to produce more-complex phenazine derivatives by three tailoring enzymes, PhzH, PhzS, and PhzM (16). PhzH is an ATP-dependent aminotransferase that converts PCA into phenazine-1-carboxamide (PCN). The biosynthesis of pyocyanin consists of an *S*-adenoyslmethionine (SAM)-dependent *N*-methylation of PCA catalyzed by PhzM and then subsequent reduction of the carboxylic acid group via monooxygenase PhzS. PhzS can also oxidize PCA to give 1-hydroxyphenazine as an end product.

Interestingly, *P. aeruginosa* is one of the few human pathogens that generates phenazines via two independent, homologous pathways or gene clusters, *phzA1B1C1D1E1F1G1-phzS* and *phzA2B2C2D2E2F2G2* (16). Whereas the *phzS* gene is in proximity to the *phzA1* cluster, the *phzM* and *phzH* genes are localized elsewhere on the chromosome. Expression of the *phzA1* locus accounts for the majority of phenazines produced by *P. aeruginosa* PAO1 (19). *phzA1* expression is regulated by quorum-sensing (QS) cell signaling mechanisms (19, 20). The QS network in *P. aeruginosa* is composed of three distinct but overlapping systems: the two homoserine lactone (HSL) networks, *N*-3-oxo-dodecanoyl-HSL (3-oxo-C_{12}-HSL) and *N*-butyryl-HSL (C_4-HSL), and 2-akyl-4-quinolone (PQS)-mediated signaling. The autoinducer synthase and transcriptional regulator pairs LasI/LasR and RhlI/RhlR are responsible for the 3-oxo-C_{12}-HSL and C_4-HSL networks, respectively (21–23). Collectively, the 3-oxo-C_{12}-HSL, C_4-HSL, and PQS signaling systems cooperatively regulate the expression of numerous virulence factors, including exotoxins, proteases, rhamnolipids, and phenazines (21, 24).

The biosynthesis of phenazines in *P. aeruginosa* is orchestrated around an extensive network of environmental factors and protein regulatory elements. Classic environmental stresses known to induce phenazine production include phosphate limitation (25–27) and iron availability (28). Starvation of these essential nutrients has been found to positively impact QS, thus enhancing the expression of QS-related genes and phenotypes, including pyocyanin formation.

We have discovered that the metabolism of glycine as a carbon source may serve as an additional nutritional cue for the biosynthesis of pyocyanin in *P. aeruginosa* PAO1. The putative transcrip-

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tional regulator PA2449 gene in *P. aeruginosa* PAO1 was not only necessary for the production of pyocyanin but also essential for the assimilation of glycine as a carbon source. Understanding the relationship between glycine metabolism, pyocyanin biosynthesis, and the PA2449 gene was the main goal of the current study. Importantly, the results of our study provide a foundation for defining the role of PA2449 in the general metabolism and physiology of *Pseudomonas*-related bacteria.

MATERIALS AND METHODS

Media, growth conditions, and chemicals. Bacteria were cultivated in BD Difco Lennox broth (LB) at 37°C unless otherwise stated. Solid bacteriological media were prepared with the addition of BD Bacto agar at 15 g liter^{-1}. For the purposes of the experiments described herein, *P. aeruginosa* strains were grown in 50 ml of peptone broth (PB) in a 500-ml baffled shake flask at 200 rpm at 37°C. PB consisted of (per liter) 1.5 g $MgSO_4$, 10 g K_2SO_4, and 20 g of BD BBL Gelysate peptone (29). Low-phosphate succinate (4), glycerol-alanine (30), nutrient yeast broth (31), and M9 and M63 minimal media (32) were prepared as described previously. M9 minimal medium was supplemented with 5 μM ferrous sulfate. Media were supplemented with kanamycin (50 μg ml^{-1}), tetracycline (10 μg ml^{-1}), and gentamicin (10 μg ml^{-1} for *Escherichia coli* and 30 μg ml^{-1} for *P. aeruginosa*) when needed for plasmid selection.

Chemicals were purchased from Fisher Scientific (Hampton, NJ) and used without further purification. Pyocyanin (catalog no. P0046) and *N*-(3-oxododecanoyl)-L-homoserine lactone (catalog no. O9139) were purchased from Sigma-Aldrich (St. Louis, MO). Purified phenazine-1-carboxylic acid was a kind gift from Linda Thomashow (USDA), and *N*-butyryl-L-homoserine lactone (catalog no. 10007898) was purchased from Cayman Chemical (Ann Arbor, MI).

Bacteria and plasmids. The bacterial strains and plasmids used in this study are given in Tables S1 and S2 of the supplemental material, respectively. Plasmids were maintained in *E. coli* Top10 (Invitrogen, Carlsbad, CA) for cloning purposes. The wild-type *P. aeruginosa* PAO1-UW strain and its transposon mutants were acquired from the *P. aeruginosa* PAO1 transposon mutant library (33). All *P. aeruginosa* PAO1-UW mutants were verified for transposon insertions at proper loci through PCR as recommended by the library curators (University of Washington Department of Genome Sciences). Plasmids were introduced into *P. aeruginosa* by electroporation using established methods (34). Recombinant *P. aeruginosa* strains were selected on LB supplemented with 30 μg ml^{-1} of gentamicin at 37°C for 18 h.

Molecular biology methods. DNA was purified using Promega (Madison, WI) nucleic acid purification kits. Restriction enzymes and ligases were products of New England BioLabs (Ipswich, MA). PrimeStar polymerase (TaKaRa Biosciences, Japan) was used for all PCRs. PCR applications were done by following the recommended protocols for the PrimeStar polymerase and with the oligonucleotides given in Table S3 in the supplemental material. Genomic DNA from *P. aeruginosa* PAO1-UW was used for all PCR applications. All desired PCR products were gel purified and cloned into pCR-Blunt according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Cloned DNA was verified by sequencing (Genewiz, South Plainfield, NJ).

Construction of plasmids pBRL413, pBRL435, and pBRL480. The DNA region containing the putative PA2449 open reading frame (ORF) was amplified by PCR. The gel-purified PA2449 PCR product was cloned into pCR-Blunt to give the plasmid pBRL408. The PA2449 ORF was subcloned from pBRL408 into the NdeI/EcoR I restriction sites of pET28b (Novagen) to yield the plasmid pBRL417. Subsequently, pBRL417 was digested with XbaI and SacI to liberate the PA2449 ORF with a 5′ ribosome binding site (RBS), which was then ligated into the XbaI/SacI sites of pBBR1MCS-5 to generate the plasmid pBRL435. Plasmid pBRL413 was created by subcloning the HindIII *phzA2B2C2D2E2F2* fragment from pUCP-A2G2 (16) into the corresponding HindIII site of pBBR1MCS-5.

The *metE* gene was PCR amplified from genomic DNA of *E. coli* K-12 MG1655. The desired *metE* product was gel purified and cloned into pCR-Blunt to give plasmid pBRL409. The *metE* gene was subcloned from pBRL409 into the KpnI/SacI sites of pBBR1MCS-5 to yield plasmid pBRL480.

Construction of *phzB1*, *phzB2*, *phzM*, and *phzH-lacZ* reporter plasmids. We first constructed a template plasmid for measuring promoter activity in *P. aeruginosa* based on *lacZ* expression. First, the *lacZ* ORF was PCR amplified from *E. coli* K-12 MG1655 genomic DNA with the primers BL393.f and BL393.r (see Table S3 in the supplemental material). The BL393.f primer installed a pET-derived RBS at the 5′ end of the *lacZ* ORF. The gel-purified RBS-*lacZ* product was cloned into pCR-Blunt to give pBRL410. The RBS-*lacZ* fragment was subsequently cloned from pBRL410 into the KpnI/HindIII sites of pBBR1MCS-5 to yield pBRL416. Promoters could then be inserted upstream of the RBS-*lacZ* region via directional cloning with 5′ SphI and 3′ KpnI restriction sites.

Promoter regions for *phzB1*, *phzB2*, *phzM*, and *phzH* were amplified by PCR. The desired PCR-amplified promoter regions were gel purified and cloned into pCR-Blunt (see Table S2 in the supplemental material). Subsequently, the promoter regions of *phzB1*, *phzB2*, *phzM*, and *phzH* were individually subcloned into the SphI/KpnI restriction sites of pBRL416 to give pBRL418, pBRL423, pBRL424, and pBRL425, respectively.

Construction of *gcvP2*, *hcnA*, and *metE lacZ* reporter plasmids. We found that expression of *lacZ* from the *phz* promoters was slightly higher than desired (Miller units were >10,000). This was attributed to the pET-derived RBS. Although this was not a significant problem, we circumvented this concern for the *gcvP2*, *hcnA*, and *metE* promoter-*lacZ* reporter constructs by directly fusing the 5′ promoter region with the *E. coli lacZ* ORF. Thus, *lacZ* translation in these constructs was dictated by the native RBS of the *gcvP2*, *hcnA*, or *metE* promoter region. First, the *lacZ* ORF was PCR amplified from *E. coli* K-12 MG1655 genomic DNA, and the desired *lacZ* product was gel purified. Next, the promoter regions of *gcvP2*, *hcnA*, and *metE* were amplified by PCR with primers that installed a 3′ end homologous region into the *E. coli lacZ* ORF (see Table S3 in the supplemental material). Following gel purification, the *gcvP2*, *hcnA*, and *metE* promoters were individually joined with the *E. coli lacZ* ORF using fusion PCR (35). The desired promoter-*lacZ* fusion constructs were gel purified and subsequently cloned into pCR-Blunt (see Table S2 in the supplemental material). The *gcvP2*-, *hcnA*-, and *metE-lacZ* constructs were subcloned into the KpnI/SphI, KpnI/SphI, and HindIII/SphI restriction sites of pBBR1MCS-5 to give the plasmids pBRL456, pBRL447, and pBRL415, respectively.

Measurement of galactosidase activity. The activity of β-galactosidase was determined for recombinant *P. aeruginosa* PAO1 strains using the Miller assay (36).

***Staphylococcus aureus* killing assays.** In triplicate, *P. aeruginosa* PAO1 and PW5126 were grown in PB to an optical density at 600 nm (OD_{600}) of 0.7. At this time, 2 μl of each culture was spotted onto an LB plate that was freshly swabbed with LB-grown (37°C, 200 rpm, 24 h) *Staphylococcus aureus* ATCC 25923. Spotted plates were grown at 37°C for 24 h.

Spectrophotometric measurement of extracellular pyocyanin. Pyocyanin was measured using reported methods (7). Briefly, cells were removed from cultures by centrifugation (16,000 × *g* for 5 min) and subsequent passage through Acrodisc syringe filters with 0.2-μm nylon membranes. The absorbances of the cell-free samples at 690 nm were measured and converted into pyocyanin concentrations using a molar extinction coefficient of 4,130 M^{-1} cm^{-1} (37).

Growth of *P. aeruginosa* with amino acids serving as the sole carbon or nitrogen source. Analysis of the growth of *P. aeruginosa* with amino acids serving as the sole carbon or nitrogen source was done in triplicate. Both *P. aeruginosa* PAO1 and PW5126 were initially grown on LB at 37°C for 18 h. Individual clones were then patched onto solid minimal salt media (50 mM Na_2HPO_4, 20 mM KH_2PO_4, 10 mM NaCl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 5 μM $FeSO_4$, pH 7.0), which was supplemented with either

20 mM (carbon source) or 5 mM (nitrogen source) L-alanine, L-arginine, L-asparagine, L-aspartate, L-glutamate, L-histidine, L-isoleucine, glycine, L-leucine, L-lysine, L-phenylalanine, proline, L-tryptophan, L-tyrosine, L-serine, and L-valine. When testing amino acids as carbon or nitrogen sources, the minimal media were further supplemented with 20 mM NH_4Cl or 20 mM succinate, respectively. Patched plates were incubated at 37°C for 1 to 5 days.

For liquid cultures, minimal salt medium (given above) was supplemented with either glycine, L-valine, L-serine, or L-glutamine to a final concentration of 20 mM (carbon source) or 10 mM (nitrogen source). This medium was further supplemented with 20 mM NH_4Cl or 20 mM succinate depending on whether the amino acid was supplied as a carbon or nitrogen source, respectively. Strains were inoculated into 50 ml of minimal media to an initial OD_{600} of ~0.1. At 12, 24, 36, 48, 60, 72, and 84 h postinoculation, 2.5 ml of minimal salt medium (does not have a source of carbon or nitrogen) was added to each culture. This addition compensated for volume loss due to both sampling and evaporation. Cultures were grown at 37°C, 200 rpm for 12 to 96 h.

ESI-LC-MS analysis of phenazines. In triplicate, *P. aeruginosa* PAO1 and PW5126 strains were grown in 50 ml PB at 37°C and 200 rpm for 24 h. Cells were removed via centrifugation (5,000 × *g* for 20 min), and the resulting supernatants were extracted with an equal volume (50 ml) of chloroform. Chloroform extracts were evaporated to dryness. The dried-phenazine samples were resuspended in 500 μl acetonitrile and vortexed vigorously for 5 min, and the insoluble material was removed by centrifugation at 5,000 × *g* for 5 min. The resulting supernatants (1 μl) were used for electrospray ionization-liquid chromatography-mass spectrometry (ESI-LC-MS).

ESI-LC-MS analysis was performed on an API2000 LC/tandem MS (LC/MS/MS) system equipped with a TurboIon Spray source interfaced with a Prominence ultrafast liquid chromatograph (UFLC). A reverse-phase BDS Hypersil C_{18} column (100 mm by 2.1-mm inside diameter [ID], 3-μm particle size,) was employed. Mobile phases A (5% acetonitrile:95% water with 0.05% formic acid) and B (95% acetonitrile:5% water with 0.05% formic acid) were used for all experiments. Phenazine separation was performed using a gradient program of 3 min of 100% A, a 40-min linear gradient to 100% B, and 10 min of 100% B at a constant flow rate of 250 μl min^{-1}. Electrospray ionization in the positive mode was performed using the TurboIon Spray source with an ion spray voltage of 5,500 V, a desolvation temperature of 250°C, and gas 1 and gas 2 set at 20 and 30, respectively. Mass spectra were collected over the *m*/*z* range of 150 to 300 with a declustering potential of 22 V, a focusing potential of 400 V, and an entrance potential of 5 V.

ESI-LC-MS analysis of HSLs. In duplicate, *P. aeruginosa* PAO1 and PW5126 were grown in 100 ml of PB at 37°C and 200 rpm to an OD_{600} of ~0.6. Cells were then removed by centrifugation (5,000 × *g* for 20 min), and the resulting supernatants were then extracted three successive times with equal volumes (100 ml) of dichloromethane (DCM). The three 100-ml DCM fractions were combined and evaporated to dryness. The dried residues containing the extracted HSLs were suspended in 500 μl of acetonitrile and then centrifuged at 16,000 × *g* for 10 min to remove insoluble material. The resulting supernatants (1 μl) were used for ESI-LC-MS. For quantification of HSLs present in the samples, standard curves were generated for both authentic C_4-HSL and 3-oxo-C_{12}-HSL. Lower limits of detection for C_4-HSL and 3-oxo-C_{12}-HSL were determined to be 50 pmol and 10 pmol, respectively.

ESI-LC-MS analysis was performed using the same instrumentation and column described above. Mobile phases A (water with 0.05% formic acid) and B (90% methanol:10% water with 0.05% formic acid) were employed. HSL separation was done using an isocratic profile of 10% phase B in phase A for 1 min, a 7-min linear gradient to 100% B, and 4 min of 100% B at a constant flow rate of 0.25 μl min^{-1}. Electrospray ionization in the positive mode was performed using the TurboIon Spray source with an ion spray voltage of 4,500 V, a desolvation temperature of 300°C, and gas 1 and gas 2 set at 20 and 30, respectively. Mass spectra were collected over the *m*/*z* range of 100 to 300 with a declustering potential of 22 V, a focusing potential of 400 V, and an entrance potential of 5 V.

HPLC analysis of free amino acids. In triplicate, *P. aeruginosa* PAO1 and PW5126 were grown in 50 ml PB at 37°C and 200 rpm for 24 h. At 6 and 24 h postinoculation, 5 ml of culture was removed and cleared of cells via centrifugation at 5,000 × *g* for 20 min. The cell-free supernatants were passed through an Amicon 5,000-molecular-weight-cutoff (MWCO) centrifugal filter. The filter was washed with 5 ml of H_2O, and the collected filtrates (10 ml) were lyophilized. Uncultured PB (control) was processed in an identical manner for amino acid analysis. The freeze-dried samples were resuspended in 2 ml of high-pressure LC (HPLC) grade H_2O, vigorously vortexed for 5 min, and then incubated at 37°C for 30 min. Insoluble material was removed by centrifugation at 5,000 × *g* for 5 min.

HPLC was performed using an 1100 series HPLC-diode array detector (HPLC-DAD) system (Agilent Technologies, Santa Clara, CA). The system consisted of a programmable autosampler with a 100-μl built-in loop, a quaternary pump, a column thermostat, a photodiode array detector, and Chemstation software (version B.03.02). Amino acids were derivatized using an automated online method and subsequently separated on a reverse-phase Agilent Zorbax Eclipse amino acid analysis (AAA) column (150 mm by 4.6-mm ID, 3.5-μm particle size) maintained at a temperature of 40°C. Reagents used for derivatization were purchased from Agilent, including *o*-phthaldehyde (OPA) for primary amino acids and 9-fluorenylmethyl chloroformate (FMOC) for secondary amino acids. Detailed information for the setup and execution of this method has been published (38). Chemical separations employed mobile phases A (40 mM Na_2HPO_3, pH 7.8) and B (45% acetonitrile:45% methanol:10% H_2O) in a gradient program consisting of 1.9 min of 100% phase A, a 16.2-min linear gradient to 57% phase B, 4.1 min of 100% phase B, and 2.1 min of 100% phase A at a flow rate of 2 ml min^{-1}. UV detection used a setting of 338 nm, 10 nm bandwidth. Standard curves of glycine and serine were constructed to measure the concentrations of glycine and serine in each individual sample.

cDNA microarray analysis. In quadruplicate, *P. aeruginosa* PAO1 and PW5126 were grown at 37°C in 50 ml of PB in 500-ml baffled shake flasks at 200 rpm to an OD_{600} of 0.7. At this time, 0.5 ml of culture was immediately mixed with 1 ml of RNAprotect bacterial reagent (Qiagen, Valencia, CA). The bacteria in the treated samples were lysed using an enzymatic proteinase K digestion approach as detailed in the RNAprotect bacterial reagent handbook (Qiagen). The RNA was immediately purified using the RNeasy minikit (Qiagen) with an on-column DNase digestion step to remove contaminating DNA. Purified RNA samples were checked for DNA contamination by PCR with the primers BL343.f and BL343.r (see Table S3 in the supplemental material), which were designed to amplify the *rplU* gene (39). Purified RNA samples were also analyzed for quality using a Bioanalyzer (Agilent).

Microarray studies were carried out by the Microarray Core Facility (Upstate Medical University, Syracuse, NY) using GeneChip *P. aeruginosa* PAO1 Affymetrix arrays. Experiments were performed according to the Affymetrix GeneChip expression analysis technical manual (Affymetrix publication 702232, revision 3) and methods established at the State University of New York (SUNY) Upstate (Syracuse, NY). For initial data processing, the Affymetrix software (MicroArray suite 5.0 [MAS5]) was used for quality control (Q/C) analysis, which involved calculating the signal intensities from each perfect match proved on the arrays relative to those for the mismatch probe and determining whether or not the gene was present in the sample. The robust multiarray average (RMA) method was used to normalize the set of arrays in our screen (GeneTraffic software; Stratagene, La Jolla, CA). The MultiExperiment viewer (MeV; v4.6.2) was used for subsequent statistical analysis. To identify genes displaying a significant difference in intensities, a *t* test between subjects was performed using *P* values based on all permutations and an overall alpha value fixed at 0.05. False discoveries were addressed using the standard Bonferroni correction.

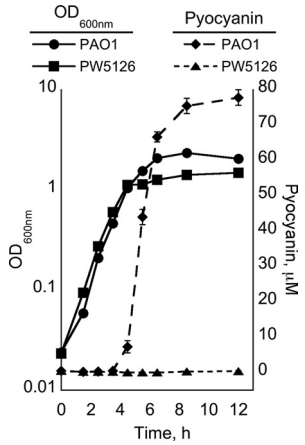

FIG 1 Pyocyanin production from *P. aeruginosa* PAO1 and PW5126 grown in PB. Cultures were assayed for OD_{600} and pyocyanin via spectrophotometry. Individual points represent mean values of triplicate samples, and standard error bars are shown.

Microarray database accession number. Data from the microarray experiments, including all CEL files, were deposited in Gene Expression Omnibus (GEO) database with the accession number GSE39044.

RESULTS

The PA2449 ORF is predicted to encode a transcriptional regulator belonging to the TyrR family. In addition to being a putative TyrR-like protein, the PA2449 protein also possesses homology to transcriptional regulators known as enhancer binding proteins (EBPs) that interact with the alternative sigma factor σ^{54} (RpoN) to activate transcription (40). The RpoN interaction domain is conserved in the PA2449 protein(see Fig. S1 in the supplemental material). In *P. aeruginosa* PAO1 there exists another TyrR-like EBP transcriptional regulator known as PhhR (41). Previous studies found that PhhR functions as an EBP for the transcriptional activation of genes involved in tyrosine and phenylalanine catabolism (42), which consequently enhanced the production of pyocyanin (43). Because the PA2449 protein displays homology to both PhhR (44% identity) of *P. aeruginosa* and TyrR (41% identity) of *E. coli*, we suspected that PA2449 might also be involved in phenazine biosynthesis.

Disruption of the PA2449 gene eliminates pyocyanin production. A *P. aeruginosa* PAO1 strain possessing a transposon insertion within the PA2449 gene was obtained from the *P. aeruginosa* PAO1 transposon mutant library (33). This strain, designated PW5126, was found to be incapable of producing measurable quantities of the blue pigment pyocyanin in a variety of rich and minimal media (Fig. 1). For example, PW5126 did not produce pyocyanin when grown in a common rich medium such as Lennox, Luria-Bertani, or nutrient yeast broth. In addition, pyocyanin was not detected when PW5126 was cultivated in M9 and M63 minimal media supplemented with various carbon sources (20 mM), including glucose, glycerol, succinate, and citrate. Low-phosphate succinate medium (LPSM) and glycerol-alanine (GA) medium are two types of minimal media that stimulate the formation of high levels of pyocyanin (4, 30). However, PW5126 did not generate detectable levels of pyocyanin when grown in either LPSM or GA medium.

Peptone broth (PB) is an established medium for enriching the production of pyocyanin from *P. aeruginosa* (29). PB is the liquid version of *Pseudomonas* isolation agar (PIA), a well-known and classic solid medium used for identifying *P. aeruginosa* based on pyocyanin formation (44). A key formulation of PB that contributes to enhancing pyocyanin production is the use of a peptone containing a high percentage (~20%) of glycine and limiting amounts (<0.2 mM) of phosphate. Either bacteriological or gelatin-based peptones meet these criteria. As with other media tested in our study, PW5126 did not produce detectable quantities of pyocyanin when cultured in gelatin-based PB (Fig. 1 and 2). Therefore, because of the historical importance of PIA, we focused our experiments on defining the relationship between the PA2449 gene and pyocyanin biosynthesis in *P. aeruginosa* PAO1 when grown in gelatin-based PB.

PA2449 is required for pyocyanin production in PB. To verify

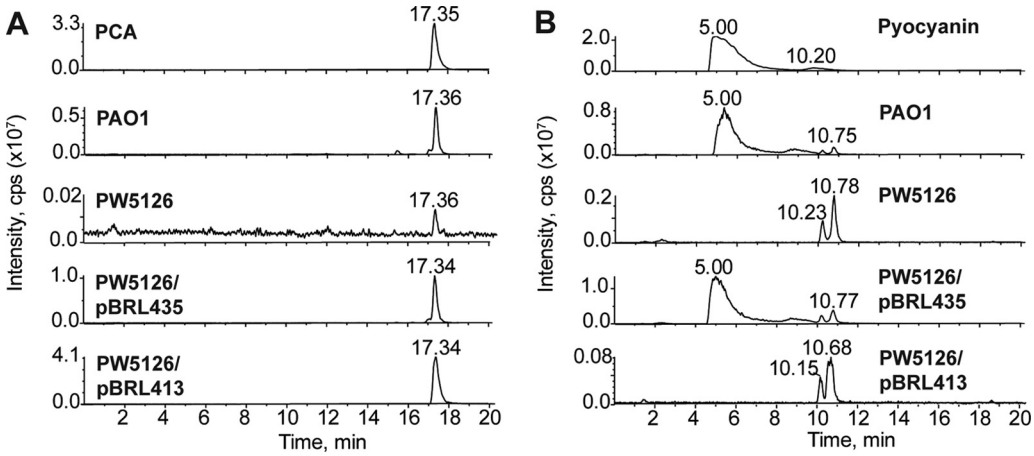

FIG 2 LC-MS analysis of phenazines produced from *P. aeruginosa* PAO1 and PW5126. Strains were grown in PB for 24 h at 37°C. Total phenazines were extracted and subsequently resuspended in water-methanol for LC-MS. Ion extraction of *m/z* 224.75 to 225.5 and *m/z* 210.75 to 211.5 was used to detect the $[M+H]^+$ for PCA (A) and pyocyanin (B), respectively. In contrast to *P. aeruginosa* PAO1, PW5126 did not produce pyocyanin but generated trace quantities of PCA. Expression of the PA2449 ORF from the *lac* promoter of pBBR1MCS-5 (pBRL435) restored PCA and pyocyanin biosynthesis in PW5126. Expression of the *phzA2B2C2D2E2F2G2* cluster from the *lac* promoter of pBBR1MCS-5 (pBRL413) allowed PW5126 to produce PCA but not pyocyanin. These data indicate that production of pyocyanin in *P. aeruginosa* PAO1 requires the PA2449 gene.

that the pyocyanin deficiency of PW5126 was the result of the transposon insertion in the PA2449 gene, the PA2449 ORF of *P. aeruginosa* PAO1 was cloned into the broad-host-range vector pBBR1MCS-5 and the resulting plasmid (pBRL435) was electroporated into PW5126. The recombinant PW5126/pBRL435 strain successfully biosynthesized pyocyanin (Fig. 2), although the levels were ~65% less than those of *P. aeruginosa* PAO1 harboring pBBR1MCS-5. The reduced pyocyanin concentrations could have been due to low expression associated with the *lac* promoter of the pBBR1MCS-5 parent vector. Nonetheless, the sole expression of the PA2449 ORF was sufficient to restore pyocyanin production in PW5126, thus confirming that a functional PA2449 gene is required for the biosynthesis of pyocyanin in *P. aeruginosa* PAO1.

We next determined the overall population and relative amounts of phenazines biosynthesized by PW5126 after 24 h of growth in PB. Total phenazines were extracted from either *P. aeruginosa* PAO1 or PW5126 cultures using chloroform, and the resulting chloroform extracts were analyzed for individual phenazine compounds by LC-MS (Fig. 2). The only phenazine derivative successfully produced by PW5126 was PCA at trace amounts (~0.5% of wild-type levels). This indicated that PCA biosynthesis was still operative in the absence of PA2449, albeit at extremely low levels.

It was initially believed that the absence of the PA2449 gene could disrupt the biosynthesis of key metabolites, e.g., chorismate, that are necessary for the production of phenazines. However, PW5126 readily synthesized PCA when the *phzA2B2C2D2E2F2G2* gene cluster was heterologously expressed from a *lac* promoter (Fig. 2), suggesting that precursor availability was not a concern. We next examined if the PA2449 gene was necessary for activating or upregulating the expression of genes required for phenazine biosynthesis. This was accomplished by measuring the expression of the *phz*-related genes (*phzB1*, *phzB2*, *phzM*, and *phzH*) using β-galactosidase (*lacZ*) reporter assays (Fig. 3). For *P. aeruginosa* PAO1 cultures, there was a significant change in the expression of the *phzB1*-, *phzB2*-, and *phzM-lacZ* constructs, which began approximately 1 h prior to the visible accumulation of pyocyanin in PB. This change in *phzB1*, *phzB2*, and *phzM* expression was not observed for the transposon insertion PA2449 mutant. For PW5126, there was a >95% reduction in *phzB1-lacZ* expression (Fig. 3A), whereas *phzB2*- and *phzM-lacZ* expression decreased by ~60 and 80%, respectively. Expression of the *phzH-lacZ* construct was not affected by the PA2449 mutation (Fig. 3D). These results revealed that the probable cause for the pyocyanin-deficient phenotype of PW5126 was insufficient expression of the *phz* genes that are necessary for the biosynthesis of phenazines in *P. aeruginosa* PAO1.

PA2449 is necessary for the production of C_4-HSL in PB. The signaling molecule C_4-HSL and its cognate receptor, RhlR, positively regulate the expression of the *phzA1* locus (19). Our concern was therefore that the pyocyanin-deficient phenotype of PW5126 was due to low or insufficient levels of HSLs, e.g., C_4-HSL, as a result of the PA2449 mutation. Low levels of HSLs would fail to fully activate expression of the *phz* genes as observed for PW5126 (Fig. 3), so we decided to assay for HSL production by LC-MS (45). Both *P. aeruginosa* PAO1 and PW5126 were grown in PB to an OD_{600} of ~0.7. This cell density represented a crucial point for the analysis of QS in its relation to pyocyanin production because (i) *P. aeruginosa* PAO1 cultures just began to display a distinctive bluish hue indicative of pyocyanin and (ii) it was 1 h after transcriptional activation of the *phz* genes as determined by *lacZ* reporter assays (Fig. 3). Both sets of cultures were cleared of cellular material and subsequently extracted with dichloromethane to isolate total HSLs. The HSL extracts were analyzed by LC-MS for 3-oxo-C_{12}-HSL and C_4-HSL (Fig. 4). Under pyocyanin-producing conditions in PB, *P. aeruginosa* PAO1 produced only C_4-HSL (0.3 ± 0.06 μM) and no detectable quantities of 3-oxo-C_{12}-HSL. In contrast, PW5126 did not produce measurable levels of C_4-HSL

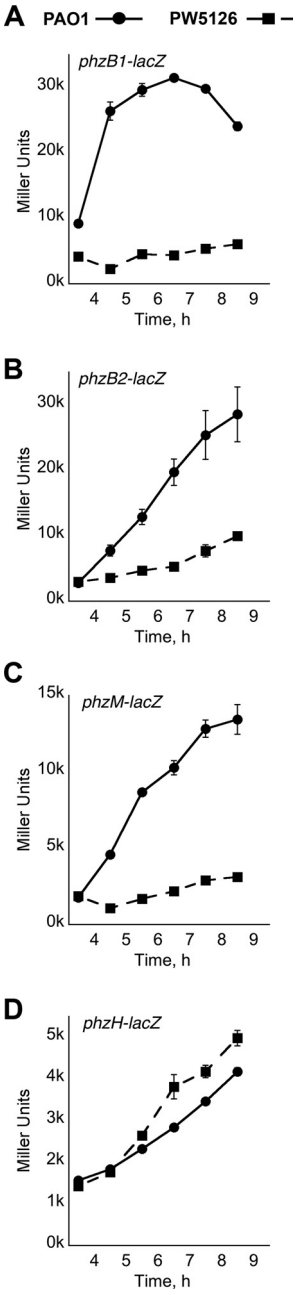

FIG 3 Relative expression levels of the *phzB1*, *phzB2*, *phzM*, and *phzH* genes in *P. aeruginosa* PAO1 and PW5126. The 5′ regulatory DNA regions consisting of either ~1,000 bp (*phzB1* [A], *phzB2* [B], and *phzM* [C]) or 500 bp (*phzH* [D]) of the designated *phz* ORFs were cloned upstream of an *E. coli lacZ* ORF in a promoter-less pBBR1MCS-5 plasmid. *P. aeruginosa* strains harboring the *phz-lacZ* constructs were grown in PB at 37°C and periodically assayed for LacZ activity. Individual points represent mean values of triplicate samples, and standard error bars are shown.

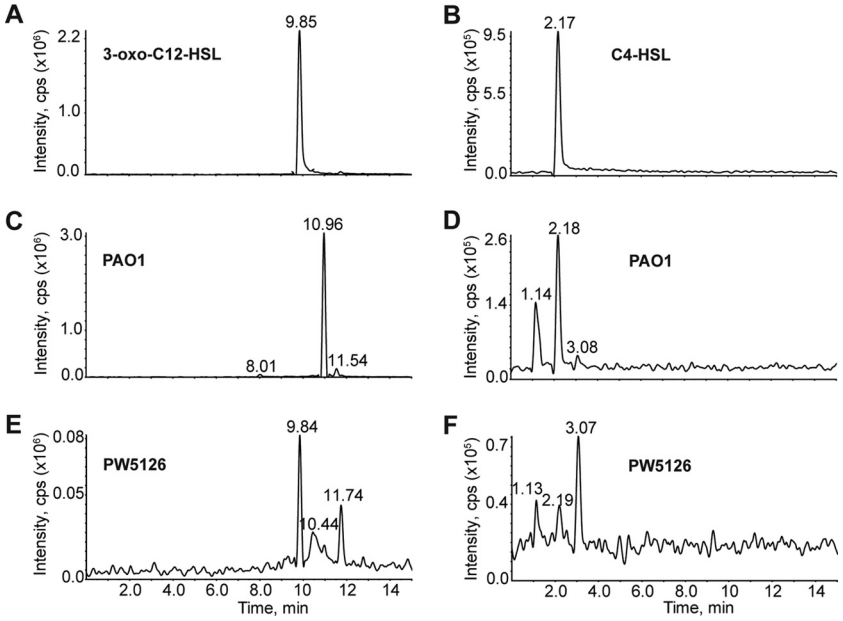

FIG 4 LC-MS analysis of HSLs produced from *P. aeruginosa* PAO1 and PW5126. Strains were grown in PB at 37°C to an OD_{600} of 0.7. Total HSLs were extracted and subsequently resuspended in acetonitrile for LC-MS analysis. HSLs were detected by ion extraction of their respective $[M+H]^+$ *m/z*'s. Chromatograms are shown for authentic 3-oxo-C_{12}-HSL (*m/z* of 298 to 299) (A) and C_4-HSL (*m/z* of 172 to 173) (B). Wild-type *P. aeruginosa* PAO1 did not produce detectable levels of 3-oxo-C_{12}-HSL (C), but C_4-HSL (D) was observed. Conversely, PW5126 produced 3-oxo-C_{12}-HSL (E) and insignificant quantities of C_4-HSL (F).

but small amounts (0.07 ± 0.03 μM) of 3-oxo-C_{12}-HSL were observed. The concentrations of C_4-HSL produced from PW5126 cells, compared to those produced from *P. aeruginosa* PAO1, were not sufficient to activate the expression of the *phz* loci needed for the biosynthesis of pyocyanin (Fig. 3). Additionally, insufficient levels of C_4-HSL and subsequent loss of transcriptional activation of *phz* genes could account for the residual levels of PCA observed from PW5126 cultures (Fig. 2). However, the exogenous addition of HSLs to PW5126 cultures did not restore pyocyanin production, thereby suggesting that involvement of PA2449 is more complex than simply via HSL mechanisms.

For *P. aeruginosa*, 2-alkyl-4-quinolones (PQS) act as signaling molecules and have been shown to facilitate the expression of *rhlI* and *rhlR* required for C_4-HSL biosynthesis (46). If the production of PQS compounds was deficient in PW5126, then this would translate into low levels of C_4-HSL. In addition to their role as signaling molecules in *P. aeruginosa*, PQS and related 4-quinolone compounds are necessary for the killing of several Gram-positive bacteria, including *Staphylococcus aureus*, by *P. aeruginosa* (47–49). We therefore examined (qualitatively) if PW5126 was deficient in the production of quinolones by determining its ability to lyse *S. aureus*. Both *P. aeruginosa* PAO1 and PW5126 were grown in PB to an OD_{600} of ~0.7 and subsequently spotted onto LB plates that had been freshly swabbed with *S. aureus*. After 24 h of incubation at 37°C, we observed that PW5126 produced the same relative level of *S. aureus* killing as *P. aeruginosa* PAO1 (see Fig. S2 in the supplemental material). The inactivation of the PA2449 gene did not appear to have an overall negative effect on quinolone biosynthesis. A more detailed analysis of the spatial and temporal distribution of individual PQS signaling molecules is required to gain an accurate picture on how the disruption of the PA2449 gene affects the PQS network in *P. aeruginosa* PAO1.

Use of glycine as a sole carbon source requires PA2449. As shown in Fig. 1, *P. aeruginosa* PAO1 and PW5126 had similar growth profiles in PB. However, PW5126 displayed a distinct cessation of growth at an OD_{600} of ~1.0, which lasted for approximately 1 h until growth resumed at a slower pace than that of *P. aeruginosa* PAO1. Notably, the cessation of growth of PW5126 occurred slightly after the onset of pyocyanin accumulation in *P. aeruginosa* PAO1 cultures. Because amino acids serve as the only carbon sources in PB (50), we believed that an inability to utilize particular amino acids might be hindering the growth of PW5126. To test this hypothesis, PW5126 was grown on minimal agar plates supplemented with individual amino acids as either the sole carbon or nitrogen source. Glycine, serine, and valine were the only amino acid carbon sources that did not support colony formation of PW5126 even following a prolonged incubation of 5 days at 37°C. Subsequent analysis in liquid culture revealed that both serine and valine were suitable carbon sources for PW5126 (see Fig. S3 in the supplemental material), although overall cell yields on either amino acid were significantly lower (>60%) than those observed for *P. aeruginosa* PAO1. For glycine-grown PW5126, the final cell densities were ~90% lower than those of *P. aeruginosa* PAO1 and did not increase from their initial values for the duration (96 h) of the experiment (see Fig. S3 in the supplemental material). The assimilation of glycine as a carbon source was dependent on the PA2449 gene in *P. aeruginosa* PAO1.

All tested individual amino acids were acceptable nitrogen sources for PW5126 and enabled the formation of colonies within 24 h, with succinate serving as the carbon source. The only exception to these findings was glycine, in which PW5126 took twice as long (48 h) to form visible colonies as *P. aeruginosa* PAO1. This observation was confirmed in liquid culture, where there was a >50% reduction in cell yields of PW5126 when glycine was the nitrogen source (see Fig. S3 in the supplemental material). In con-

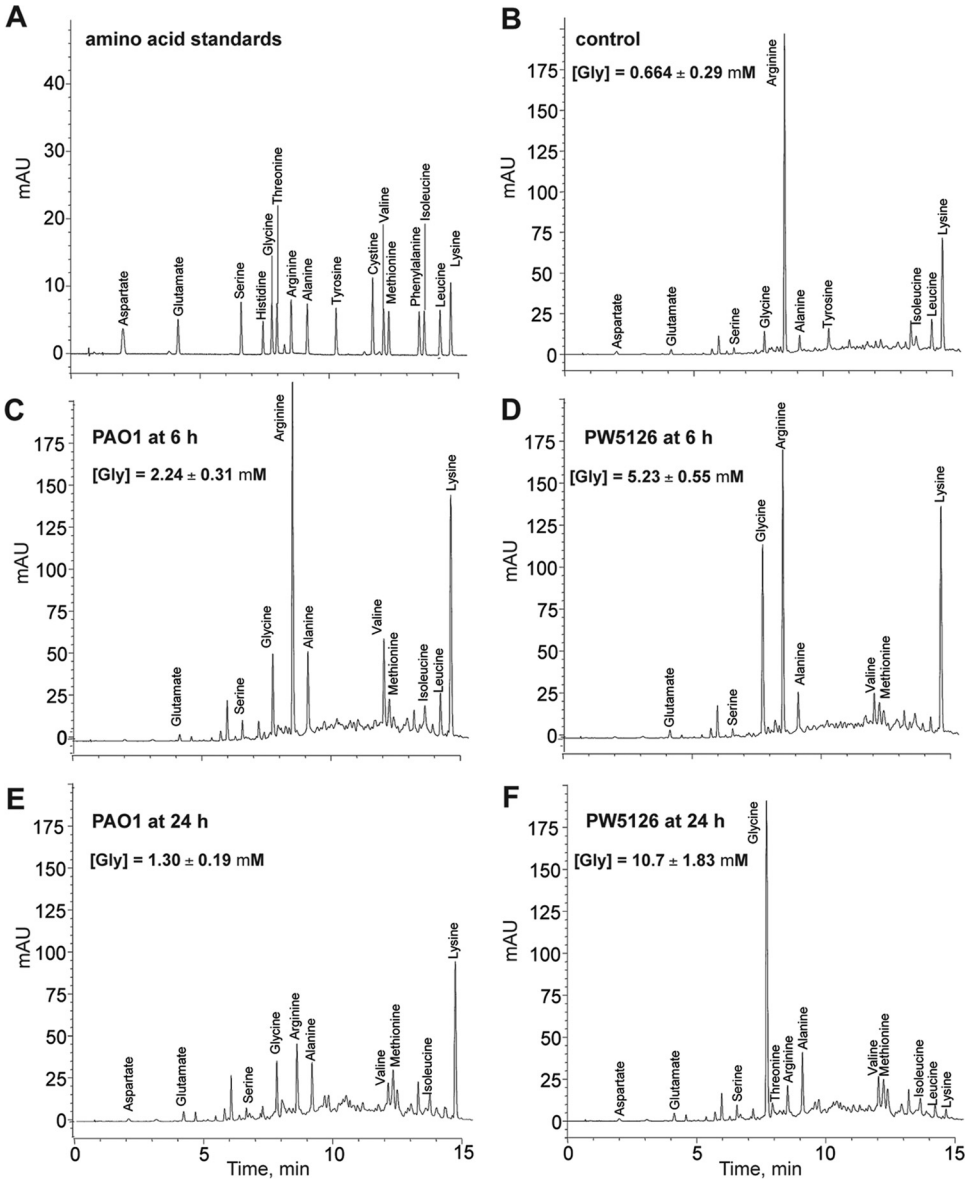

FIG 5 HPLC analysis of free amino acids present in spent PB media of *P. aeruginosa* PAO1 and PW5126. Strains were grown in PB at 37°C. At 6 and 24 h postinoculation, free amino acids present in spent media were derivatized and subsequently separated on a Zorbax Eclipse AAA column. Represented chromatograms are shown for an amino acid mixture (A), uninoculated PB (B), PAO1 at 6 h (C), PW5126 at 6 h (D), PAO1 at 24 h (E), and PW5126 at 24 h (F). Glycine concentrations represent mean values of triplicate samples, and standard deviations are shown.

clusion, the PA2449 gene was essential for the growth of *P. aeruginosa* PAO1 on glycine as a sole carbon but not a nitrogen source.

As stated earlier, the peptone used in PB is composed of ~20% glycine bound in the form of oligopeptides, whereas free glycine is <0.5% (50). These percentages give a total glycine concentration of ~40 mM (free glycine is <1 mM) for the quantity of peptone used in PB medium. The growth arrest observed for PW5126 when cultured in PB might therefore be due to an inadequacy in the assimilation of glycine as it is released from the oligopeptides through the actions of intrinsic proteases/peptidases. To assay for glycine assimilation, spent PB media derived from either *P. aeruginosa* PAO1 or PW5126 cultures were analyzed for free amino acid content at 6 and 24 h postinoculation by HPLC. Overall, the spent PB samples from both sets of cultures displayed similar trends in amino acid composition except for glycine (Fig. 5). Whereas the measured extracellular free glycine remained relatively constant at ~1 mM in *P. aeruginosa* PAO1 cultures, we observed a continuous increase or buildup of free glycine from ~1 to 10 mM for PW5126. The predominant remaining amino acid for PW5126 at 24 h was glycine. The elevated glycine concentrations generated in PW5126 cultures are indicative of a deficiency in glycine assimilation and are consistent with PA2449 being involved in glycine metabolism for *P. aeruginosa* PAO1.

Inactivation of the PA2449 gene affects the transcription of genes involved in glycine metabolism and cell signaling. PA2449 was observed to be essential for both pyocyanin biosynthesis and the utilization of glycine, and its deletion had a deleterious effect on the synthesis of C_4-HSL. To elucidate how these processes are

TABLE 1 Selected list of genes having >2-fold changes in transcript levels in a transposon insertion PA2449 mutant (PW5126) compared to wild-type *P. aeruginosa* PAO1 grown in PB

Function and gene name(s)	Gene ID[a]	Mean fold change ± SD	Biological function of product
Phenazine biosynthesis			
phzA1, *phzA2*	PA1899, PA4210	−24.3 ± 4.69	Biosynthesis of PCA core
phzB1, *phzB2*	PA1900, PA4211	−113 ± 16.8	Biosynthesis of PCA core
phzC1, *phzC2*	PA1901, PA4212	−30.7 ± 9.04	Biosynthesis of PCA core
phzD1, *phzD2*	PA1902, PA4213	−27.5 ± 4.87	Biosynthesis of PCA core
phzE1, *phzE2*	PA1903, PA4214	−45.7 ± 9.59	Biosynthesis of PCA core
phzF1, *phzF2*	PA1904, PA4215	−46.8 ± 12.3	Biosynthesis of PCA core
phzG1, *phzG2*	PA1905, PA4216	−58.6 ± 7.83	Biosynthesis of PCA core
phzS	PA4217	−68.9 ± 8.75	Monooxygenase
phzM	PA4209	−22.7 ± 2.79	Methyltransferase
Glycine metabolism			
hcnA	PA2193	−38.2 ± 5.10	HCN synthase
hcnB	PA2194	−11.9 ± 4.02	HCN synthase
hcnC	PA2195	−9.10 ± 2.19	HCN synthase
gcvT2	PA2442	−12.6 ± 2.34	GCS[b] protein T
sdaA	PA2443	−11.7 ± 1.32	Serine dehydratase
glyA2	PA2444	−54.4 ± 6.04	Serine hydroxymethyltransferase
gcvP2	PA2445	−37.5 ± 4.58	GCS protein P
gcvH2	PA2446	−59.3 ± 25.2	GCS protein H
glyA1	PA5415	−10.4 ± 2.36	Serine hydroxymethyltransferase
QS-related genes			
lasA	PA1871	−9.94 ± 1.45	Protease
lecB	PA3361	−34.5 ± 7.81	Fucose-binding lectin PA-IIL
rhlI	PA3476	−3.59 ± 1.28	C_4-HSL synthase
rhlB	PA3478	−27.4 ± 3.70	Rhamnosyltransferase
rhlA	PA3479	−38.4 ± 6.35	Rhamnosyltransferase
lasB	PA3724	−17.7 ± 2.69	Elastase
Others			
	PA0049	−10.3 ± 3.04	Hypothetical
rahU	PA0122	−29.7 ± 15.5	Aegerolysin
metE	PA1927	−25.7 ± 2.09	Methionine synthase
mexS	PA2491	12.4 ± 4.20	Oxidoreductase
mexE	PA2493	139 ± 16.6	Efflux membrane fusion protein
rahU	PA2494	115 ± 16.5	Multidrug efflux transporter
oprN	PA2495	43.1 ± 8.70	Outer membrane protein
	PA3568	−4.01 ± 0.46	Probable acetyl-CoA synthetase
mmsB	PA3569	−3.42 ± 0.40	3-Hydroxyisobutyrate dehydrogenase
mmsA	PA3570	−2.86 ± 0.42	Methylmalonate-semialdehyde dehydrogenase

[a] ID, GenBank identification.
[b] GCS, glycine cleavage system.

related through the PA2449 gene, we used microarrays to determine the transcriptome of PW5126. It was expected that the resulting transcriptomic data would provide key information on the involvement of PA2449 in both glycine assimilation and the biosynthesis of pyocyanin. In quadruplicate, *P. aeruginosa* PAO1 or PW5126 was grown in PB to an OD_{600} of ~0.7, at which time total RNA was isolated and subsequently processed for analysis with Affymetrix GeneChip *P. aeruginosa* microarrays. The microarray results identified a total of ~300 genes that were statistically different in transcript expression by >2-fold between PW5126 and *P. aeruginosa* PAO1 (see Table S4 in the supplemental material).

As established earlier, disruption of the PA2449 gene significantly reduced the production of C_4-HSL in PB. Expectedly, the majority of genes found to be transcriptionally downregulated in PW5126 were previously reported to under the regulation of C_4-HSL in *P. aeruginosa* PAO1 (Table 1). The most notable members of this group were genes required for phenazine biosynthesis: *phzA1B1C1D1E1F1G1*, *phzA2B2C2D2E2F2G2*, *phzS*, and *phzM*. The *phz* loci were upregulated by >30-fold in *P. aeruginosa* PAO1 compared to PW5126. The failure to activate expression of the *phz* loci, which is supported from both microarray and *lacZ* reporter experiments, would account for the inability of PW5126 to produce pyocyanin. The microarray data also provided some insight into how the disruption of PA2449 may exert its influence on C_4-HSL biosynthesis. The *rhlI* gene, encoding the C_4-HSL synthase, was downregulated 3-fold in PW5126. The *rhlI* gene was the only QS regulatory protein gene downregulated; the *lasI*, *lasR*, *rhlR*, and PQS-related genes displayed no differences in transcript levels.

In sharp contrast to the overabundance of genes regulated by

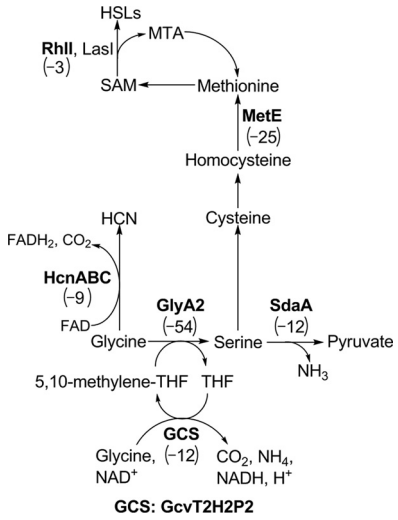

FIG 6 Key pathways of glycine assimilation that might be regulated by PA2449 in *P. aeruginosa* PAO1. Several genes whose products are predicted to be involved in glycine/serine metabolism were observed to be transcriptionally downregulated in the absence of a functional PA2449 gene. The approximate fold changes in the transcription of individual genes of interest (boldface) are given in parentheses.

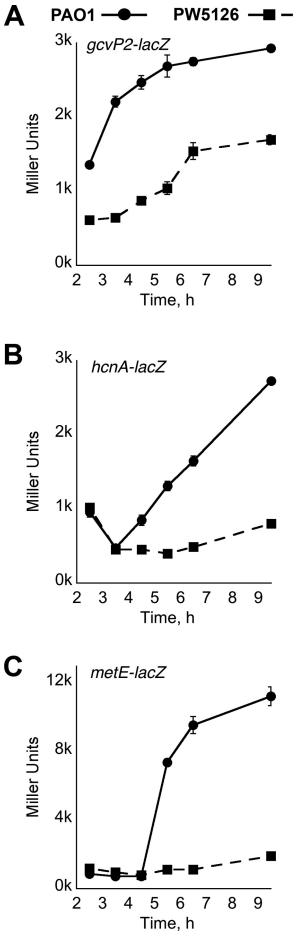

FIG 7 Relative expression levels of the *gcvP2*, *hcnA*, and *metE* genes in *P. aeruginosa* PAO1 and PW5126. The 5′ regulatory DNA regions consisting of ~1,000 bp (*gcvP2* [A]) or 500 bp (*hcnA* [B]and *metE* [C]) upstream of the designated gene ORF were fused with the *E. coli lacZ* ORF. The resulting *lacZ* constructs were cloned into a promoter-less pBBR1MCS-5 plasmid. *P. aeruginosa* strains harboring the *gcvP2*- (A), *hcnA*- (B), and *metE–lacZ* (C) constructs were grown in PB at 37°C and periodically assayed for LacZ activity. Individual points represent mean values of triplicate samples, and standard error bars are shown.

C_4-HSL signaling identified in the microarray experiments, only a limited number of genes represent potential candidates of PA2449 regulation and are restricted to those involved in glycine metabolism (Fig. 6). On the *P. aeruginosa* PAO1 chromosome, the PA2449 gene is localized near a gene cluster that encodes proteins involved in glycine and serine metabolism: *gcvT2* (PA2442), *sdaA* (PA2443), *glyA2* (PA2444), *gcvP2* (PA2445), and *gcvH2* (PA2446). The GcvT2, GcvP2, and GcvH2 proteins constitute a glycine cleavage system used for glycine and single-carbon metabolism (51). The *glyA2* gene product is predicted to be a serine hydroxymethyltransferase, and *sdaA* encodes a serine dehydratase that catalyzes the deamination of serine into pyruvate. All five of these proteins are necessary for glycine and serine utilization, and as shown in Fig. 6, the genes encoding these proteins were transcriptionally downregulated >10-fold in PW5126. Further analysis confirmed that the promoter region of the glycine cleavage gene *gcvP2* was activated >2.5-fold in *P. aeruginosa* PAO1 but not in PW5126 (Fig. 7A). These data and the previous observation that PA2449 was essential for glycine utilization support a probable role for PA2449 in regulating glycine metabolism in *P. aeruginosa* PAO1, possibly through facilitating the transcriptional activation of the *gcvT2*, *sdaA*, *glyA2*, *gcvP2*, and *gcvH2* genes.

Other genes intimately linked to glycine metabolism in *P. aeruginosa* were also found to be differentially transcribed. For example, a metabolic fate of glycine in *P. aeruginosa* is its direct conversion into hydrogen cyanide (HCN) via the HCN synthase encoded by the *hcnABC* locus (52, 53). For PW5126, transcription of the *hcnABC* genes was down by >30-fold. A follow-up *lacZ* reporter assay of the *hcnA* promoter confirmed that its activation was PA2449 dependent (Fig. 7B). The expression of the *hcnABC* locus is complex, involving several different regulators such as ANR, LasR, RhlR, and GacA (53, 54). PA2449 might provide another layer of regulation, which mediates the expression of *hcnABC* in the presence of excess intracellular glycine in *P. aeruginosa* PAO1.

Glycine is also a precursor for sulfur-containing amino acids such as methionine. A gene encoding a methionine synthase, *metE*, was transcriptionally downregulated by 26-fold in PW5126. Examination of the *metE-lacZ* reporter revealed it to be activated only in *P. aeruginosa* PAO1 (Fig. 7C); there was a >90% decrease in expression from the *metE* promoter in the absence of PA2449. It is probable that PA2449 positively regulates the expression of *metE* to increase metabolic flux from glycine to methionine. The increase in methionine biosynthesis could lead to higher levels of *S*-adenoylmethionine (SAM), thereby enhancing pyocyanin production at the PhzM catalytic step and/or the synthesis of C_4-HSL by RhlI. However, the heterologous overexpression of *E. coli metE* or the supplementation of PB with methionine (1 to 10 mM) did not restore pyocyanin biosynthesis in PW5126 cultures. Interestingly, the expression of the *metE-lacZ* fusion was repressed in *P. aeruginosa* PAO1 PB-grown cultures that were supplemented with 1% glycine or serine but not methionine. These findings indicate that the decreased expression of *metE* observed from PW5126 is

due to elevated intracellular concentrations of glycine/serine, which could not be sufficiently metabolized in the absence of PA2449.

In addition to a decreased capacity to consume glycine and serine, PW5126 struggled to use valine as a sole carbon source (see Fig. S3 in the supplemental material). The transcription of genes encoding a 3-hydroxyisobutyrate dehydrogenase (*mmsB*), methylmalonate-semialdehyde dehydrogenase (*mmsA*), and a propionyl-coenzyme A (propionyl-CoA) synthetase (PA3568) were downregulated >3-fold in PW5126 (see Table S4 in the supplemental material). These enzymes function in the distal pathway of valine degradation (55), and a deficiency in their expression would hinder the growth of PW5126 on valine.

Lastly, *mexEF-oprN*, which encodes a resistance nodulation type efflux pump, was transcriptionally upregulated by >50-fold in PW5126. This was a very unexpected result. First, MexEF-OprN overexpression has been previously observed to reduce the production of virulence factors in *P. aeruginosa* (56). MexEF-OprN removes intracellular PQS signaling molecules; this removal subsequently reduces the expression of *rhl*-related genes (57, 58). Overexpression of MexEF-OprN in PW5126 would explain the reduction in transcription of *rhlI* and production of C_4-HSL. Second, an earlier study proposed that MexEF-OprN might cause the efflux of pyocyanin precursors (59). Coordinating the repression of the *mexEF-oprN* locus with the activation of genes whose products function in SAM biosynthesis, e.g., *metE* and *gcvT2H2P2*, via PA2449 could mutually enhance pyocyanin biosynthesis. Whether PA2449 functions in the repression of the *mexEF-oprN* locus to ensure the production of C_4-HSL and pyocyanin requires further exploration.

Serine dehydratase (SdaA) is necessary for the optimal growth of *P. aeruginosa* PAO1 in PB. The results of the microarray revealed several potential genes as having important roles in the production of pyocyanin and/or the survival of *P. aeruginosa* PAO1 in PB. In order to assess some of these genes for functionality, we requested *P. aeruginosa* PAO1 mutants harboring transposon insertions within the candidate genes from the PA Two Allele Library. As shown in Fig. 9, two genes were found to have a significant effect on pyocyanin production. First, disruption of the *mexF* gene resulted in an ~50% increase of extracellular pyocyanin compared to that for *P. aeruginosa* PAO1 (Fig. 8A). This supports the role of MexEF-OprN as an antagonist in pyocyanin production. The second gene observed to impact pyocyanin yields was *sdaA*. There was an ~70% reduction in pyocyanin production in the absence of the serine dehydratase gene *sdaA* compared to that for *P. aeruginosa* PAO1 (Fig. 8A). Subsequent studies showed that the *sdaA* mutation caused a noticeable cessation of growth in PB (Fig. 8B). This trait is similar to that of PW5126 and indicates that deamination of serine into pyruvate is an occurring metabolic process when *P. aeruginosa* PAO1 is grown in PB.

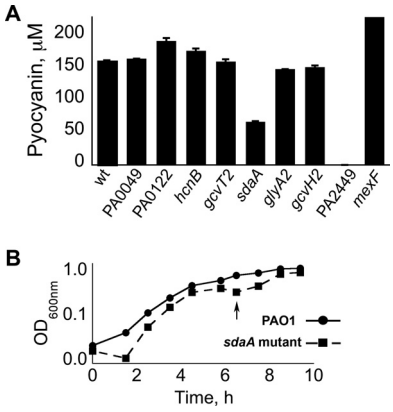

FIG 8 Serine dehydratase (encoded by *sdaA*) is necessary for the optimal growth of *P. aeruginosa* PAO1 in PB. (A) Pyocyanin production from *P. aeruginosa* mutants possessing transposon insertions within genes that are potential candidates of regulation by PA2449. Pyocyanin titers represent mean values from triplicate samples, and standard error bars are shown. wt, wild type. (B) The transposon insertion *sdaA* mutant has a characteristic cessation in growth (arrow) following entry into stationary phase when cultured in PB. Individual points represent mean values derived from triplicate samples, and standard error bars are shown.

DISCUSSION

PA2449 as a regulator in glycine metabolism for pseudomonads. The PA2449 gene, which encodes a putative TyrR-like transcriptional regulator, was essential for *P. aeruginosa* PAO1 to assimilate glycine as a carbon source. Disruption of the PA2449 gene also reduced the growth of *P. aeruginosa* PAO1 on both serine and valine when either one was supplied as a carbon source. The metabolism of glycine and that of serine are interdependent on each other, so it is expected that an inability to assimilate either amino acid causes metabolic strain in the utilization of the other. In contrast, the necessity of the PA2449 gene for optimal growth on valine cannot be readily explained and remains unclear. The PA2449 gene was not required for the utilization of any amino acid, including glycine and serine, as a source of nitrogen. This suggests that a possible function of PA2449 is to aid in the metabolism of glycine as a carbon but not nitrogen source for *P. aeruginosa* PAO1.

Microarray analysis revealed that the disruption of the PA2449 gene affected the transcription of genes encoding proteins of a glycine cleavage system (GcvT2, GcvH2, and GcvP2), a serine hydroxymethyltransferase (GlyA2), and a serine dehydratase (SdaA). This series of five proteins enzymatically converts intracellular glycine into ammonia and pyruvate, which can then enter central metabolism (60). Deamination of serine into pyruvate was discovered to be a metabolic step in the catabolism of glycine, as disruption of the *sdaA* gene hindered the growth of *P. aeruginosa* PAO1 in a glycine-rich medium. The dissimilation of glycine into pyruvate with serine as an intermediate has been documented for other pseudomonads (60–62). Dysregulation of the *glyA2* and *sdaA* genes would limit the metabolic flux of serine into glycine or pyruvate, respectively. *glyA2* and *sdaA* dysregulation could account for the reduced growth of *P. aeruginosa* PAO1 on serine (as a carbon source) in the absence of PA2449.

Whether the PA2449 protein functions as a direct transcriptional activator for any of the genes located within the *gcvT2-sdaA-glyA2-gcvP2-gcvH2* locus remains to be answered. However, the only genes related to glycine metabolism that were transcriptionally downregulated in the absence of PA2449 were *gcvT2-sdaA-glyA2-gcvP2-gcvH2* and *glyA1*. The expression of *glyA1* and *glyA2* has been reported to be regulated by choline catabolism (63, 64), but we suspect that the transcriptional downregulation of *glyA1* in our study was due to a deficiency in QS (65) and not a result of choline catabolism. For example, the transcription of other choline-related catabolic genes (63), including *sdaB* and *soxBDAG*, was unaffected by the disruption of the PA2449 gene.

The transcription of *glyA2* and *gcvT2P2H2* has been shown to be influenced by QS (65) in *P. aeruginosa* PAO1, but neither QS nor choline catabolism has been reported to control the expression of *sdaA* within the *gcvT2-sdaA-glyA2-gcvP2-gcvH2* locus. Lastly, the transcription of genes whose products could participate in glycine metabolism, e.g., *glyA3* and *gcvP1H1T1* (PA5213 to PA5215), was unaffected by the inactivation of the PA2449 gene.

PA2449 may provide another tier of transcriptional regulation of the *gcvT2-sdaA-glyA2-gcvP2-gcvH2* locus. For example, PA2449 might upregulate the transcription of the *gcvT2-sdaA-glyA2-gcvP2-gcvH2* locus to ensure that the resulting GcvP2H2T2-GlyA2-SdaA pathway has sufficient activity to meet the cellular demands associated with having to assimilate glycine as the only available carbon source. The PA2449 gene is conserved among pseudomonads, in which it lies in proximity to genes encoding a serine dehydratase and/or glycine cleavage system. The regulation of glycine and possibly serine catabolism by PA2449 might therefore be a universal phenomenon among *Pseudomonas*-related bacteria.

PA2449 is required for the biosynthesis of pyocyanin. The production of pyocyanin in *P. aeruginosa* PAO1 was completely dependent on the PA2449 gene regardless of environmental conditions. Under a variety of different growth conditions and medium formulations, the PA2449 gene was essential for the production of pyocyanin. Specifically, the PA2449 gene was required for the transcriptional activation of genes, i.e., *phzA1*, *phzA2*, and *phzM*, necessary for the biosynthesis of pyocyanin. Without the PA2449 gene, the basal level of transcription of the *phz* genes was sufficient for allowing the production of PCA at trace quantities. We later found that the cause for the decreased transcription of the *phz* genes in the absence of PA2449 was insufficient levels of C_4-HSL (discussed below), which is involved in *phz* gene activation.

This study characterized the relationship between the PA2449 gene and the production of pyocyanin under nutritionally poor growth conditions, i.e., glycine was the predominant source of carbon, phosphate was in limiting concentrations, and both aromatic and sulfur-containing amino acids were in scarce supply. Phosphate limitation has long been known to be a key determinant in the production of pyocyanin, but our data also implicate glycine (or a glycine-related metabolite) as a possible contributing factor for the biosynthesis of phenazines in *P. aeruginosa* PAO1. Glycine can be considered an unfavorable carbon source, so it is plausible that, when intracellular glycine reaches a threshold concentration under nutrient, e.g., phosphate, limitation, this might be interpreted as a starvation signal. Consequently, this could trigger the production of antimicrobials such as phenazines and HCN to ward off competitors and establish an edge for *P. aeruginosa* PAO1 in the environment.

We have discussed only the utilization of glycine as a carbon substrate in its relation to the production of pyocyanin, but the majority of media used for pyocyanogenic cultures do not use an exogenous source of glycine. However, limiting phosphate availability to $<$0.2 mM is a common formulation for enhancing pyocyanin formation (4, 29, 30). A recent transcriptome analysis of *P. aeruginosa* identified several genes involved in sulfate assimilation, protein biosynthesis, and purine metabolism that were downregulated under phosphate limitation (25). Because glycine (serine) is a biosynthetic precursor to thiols/purines and a product of protein turnover, a reduction in these processes could cause a gradual increase in intracellular glycine (serine) concentrations that is eventually countered by PA2449. Metabolomic analysis of *P. aeruginosa* PAO1 at the onset of pyocyanin production under phosphate limitation is required to validate the existence of such a scenario involving PA2449-directed glycine metabolism.

The role of PA2449 in cell signaling in *P. aeruginosa* PAO1. The inactivation of the PA2449 gene significantly reduced the production of C_4-HSL from *P. aeruginosa* PAO1. The production of pyocyanin was dependent on a C_4-HSL RhlR response, as both Δ*rhlR* and Δ*rhlR* Δ*lasR P. aeruginosa* PAO1 mutants did not generate detectable levels of pyocyanin when grown in PB. These findings indicate that the pyocyanin-null phenotype associated with the disruption of the PA2449 gene is a result of low or insufficient levels of C_4-HSL, which consequently prevent RhlR from transcriptionally activating the *phz* genes required for pyocyanin biosynthesis.

Although we established that the production of C_4-HSL requires a functional PA2449 gene, the reasoning or mechanism(s) underlying their relationship has not yet been determined. The data presented herein show that PA2449 is necessary for the catabolism of glycine as a carbon source, but QS also has also been shown to regulate the transcription of genes involved in glycine metabolism (65). It is possible that glycine metabolism might be transcriptionally regulated only through QS in which PA2449 acts as an intermediary for facilitating the biosynthesis of C_4-HSL in response to glycine availability. However, Δ*rhlR*, Δ*lasR*, and Δ*rhlR* Δ*lasR P. aeruginosa* PAO1 mutants readily grew on minimal media with glycine as the sole carbon source; a trait not shared by the transposon insertion PA2449 mutant. This suggests that glycine metabolism is dependent on PA2449 through mechanisms apart from QS in *P. aeruginosa* PAO1.

The necessity of the PA2449 gene, and possibly glycine metabolism, for the biosynthesis of C_4-HSL is an intriguing question. One possible reason for their connection is that high intracellular levels of glycine might undesirably inhibit the production of C_4-HSL. For example, glycine might be a competitive inhibitor of the RhlI synthase or the RhlR receptor. Furthermore, elevated glycine levels could interfere with the biosynthesis of homoserine and/or SAM, both of which are essential biosynthetic precursors for HSLs. In support of this mode of inhibition, we did observe that the transcription of the methionine synthase gene, *metE*, was repressed either by the inactivation of the PA2449 gene or through the exogenous addition of glycine/serine to *P. aeruginosa* PAO1 cultures. However, the addition of methionine or heterologous expression of the *E. coli metE* gene in the transposon insertion PA2449 mutant did not restore C_4-HSL to levels sufficient for triggering the production of pyocyanin. More in-depth studies are needed to test for the effect of glycine inhibition on such physiological activities.

The reduced production of C_4-HSL in the absence of the PA2449 gene could be attributed to the decreased transcription ($>$3-fold) of the C_4-HSL synthase gene *rhlI*. The role of PA2449 in the transcription of *rhlI* was narrowed down to a few possible factors (Fig. 9). First, we observed elevated transcription ($>$50-fold) of the pump-encoding *mexEF-oprN* genes with the disruption of the PA2449 gene. Overexpression of MexEF-OprN depletes cells of intracellular PQS (56), which is needed to enhance the expression of *rhl*-related genes, including *rhlI* (46). Therefore, PA2449 might function in the repression of the *mexEF-oprN* locus to ensure the accumulation of intracellular PQS for activation of the *rhl* network and subsequent C_4-HSL biosynthesis (Fig. 9A). If

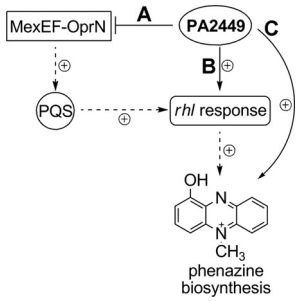

FIG 9 Proposed models for PA2449 in regulating the biosynthesis of C_4-HSL and pyocyanin in *P. aeruginosa* PAO1. (A) Transcription of *mexEF-oprN*, encoding an efflux pump, is repressed by PA2449. This prevents the efflux of intracellular PQS, thus enhancing the expression of *rhl*-related genes and phenotypes, including phenazine production. (B) The PA2449 protein directly activates the transcription of the C_4-HSL synthase gene, *rhlI*, thereby facilitating the expression of the *rhl* network. (C) PA2449 operates independently of the *rhl* network to activate the expression of *phz*-related genes for the biosynthesis of pyocyanin.

indeed this is the mode of regulation by PA2449 of *rhlI* expression, it is expected that deletion of the *mexEF-oprN* locus in the PA2449 mutant (PW5126) will restore intracellular PQS to levels sufficient for mediating C_4-HSL biosynthesis. Closer inspection of a ΔPA2449 Δ*mexEF-oprN P. aeruginosa* PAO1 mutant for these physiological traits is an objective of future studies.

How PA2449 affects transcription of *mexEF-oprN* is unclear. An earlier study reported the direct repression of the *mexEF-oprN* locus from an unknown protein (66). Perhaps the PA2449 protein functions as this "unknown" repressor protein to facilitate the biosynthesis of C_4-HSL under nutrient-poor conditions. In addition, the transcription of *mexS* (PA2493) genes was upregulated by 12-fold in the absence of PA2449. MexS participates in the expression of *mexEF-oprN* in response to perturbations of intracellular redox (67). An additional regulator, MvaT (PA4315), which affects the expression of several QS-related genes such as *mexEF-oprN* (68), was not observed to be differentially transcribed between wild-type cells and the transposon insertion PA2449 mutant. The mechanism by which PA2449 regulates the *mexEF-oprN* locus is currently being investigated.

In addition to influencing the transcription of the MexEF-OprN efflux pump genes, the PA2449 protein might serve as a direct transcriptional activator for the *rhlI* gene (Fig. 9B). The alternative sigma factor RpoN was previously found to contribute to the expression of the *rhlI* gene (69). Transcriptional activation of RpoN-controlled genes requires additional regulators known as enhancer-binding proteins (EBPs). The PA2449 protein is predicted to be an EBP, so it is plausible that it interacts with RpoN to mediate expression of the *rhlI* locus. Further in-depth studies will clarify the role of PA2449 in the production C_4-HSL for *P. aeruginosa* PAO1.

Does the PA2449 protein function as a TyrR regulator or EBP? This study characterizes the physiological and genetic effects of a transposon deletion PA2449 mutant of *P. aeruginosa* PAO1. The PA2449 gene is predicted to encode either a TyrR or EBP type transcriptional regulator. This ambiguity is due to the fact that the PA2449 protein possess homology to both the TyrR protein of *E. coli* and the EBP PhhR of *P. aeruginosa*. However, an alignment between these three proteins shows that the primary sequence of the PA2449 protein is 44% identical to that of PhhR and 41% identical to that of *E. coli* TyrR. Additionally, there is a stretch of ~8 amino acid residues unique to only the PA2449 protein and PhhR. These amino acid residues are located within the predicated RpoN interaction domain, which would explain their absence from the non-EBP TyrR regulator of *E. coli*. Several of the genes that are potentially regulated by the PA2449 protein, including *gcvH2*, *phzB1*, and *rhlI*, possess putative RpoN promoters (http://www.sigma54.ca/promoterdata/Web/data.aspx). We were unable to culture a Δ*rpoN P. aeruginosa* PAO1 strain in peptone broth, stressing the importance RpoN-mediated transcription under nutrient-poor conditions. Additionally, RpoN has been reported to be essential for the utilization of glycine/serine as carbon and nitrogen sources in pseudomonads (70, 71). These data support a probable role for the PA2449 protein as an EBP that interacts with RpoN to mediate transcription under conditions in which glycine is the predominant carbon source.

To biochemically characterize and verify that the PA2449 protein behaves as an EBP, it is imperative to determine the intracellular signal(s) sensed by this protein. Our data clearly show that PA2449 is involved in glycine metabolism, so an obvious candidate for triggering the PA2449 protein into an active state is glycine or possibly its metabolic counterpart serine. Furthermore, excess intracellular concentrations of glycine are expected to have adverse effects on the availability of single-carbon carrier molecules of the tetrahydrofolate (THF) family, e.g., 5,10-methenyl-THF (51). The PA2449 protein may interact with a specific THF derivative, thus activating expression of GcvP2H2T2-GlyA2-SdaA and subsequently restoring balance to the THF pool. PA2449 was involved in C_4-HSL biosynthesis by possibly augmenting the PQS response. Does the PA2449 protein respond to PQS? We are currently examining the biochemical function of the PA2449 protein and attempting to define its *in vivo* targets in *P. aeruginosa* PAO1 when grown under glycine-rich, pyocyanin-producing conditions.

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