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Characterization of the *Pseudomonas aeruginosa* Transcriptional Response to Phenylalanine and Tyrosine▽

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Pseudomonas aeruginosa **is a Gram-negative opportunistic pathogen often associated with chronic infections in the lungs of individuals with the heritable disease cystic fibrosis (CF). Previous work from our laboratory demonstrated that aromatic amino acids within CF lung secretions (sputum) not only serve as carbon and energy sources but also enhance synthesis of the cell signaling molecule *Pseudomonas* quinolone signal (PQS). The present study investigates the role of the aromatic amino acid-responsive regulator PhhR in mediating these phenotypes. Transcriptome analysis revealed that PhhR controls four putative transcriptional units (*phhA*, *hpd*, *hmgA*, and *dhcA*) involved in aromatic amino acid catabolism; however, genes involved in PQS biosynthesis were unaffected. The *phhA*, *hpd*, *hmgA*, and *dhcA* promoters were mapped by primer extension, and purified His_6-PhhR was shown to bind the *phhA*, *hpd*, and *dhcA* promoters *in vitro* by use of electrophoretic mobility shift assays. Our work characterizes a transcriptional regulator of catabolic genes induced during *P. aeruginosa* growth in CF sputum.**

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen commonly found in soil and water. It is notorious for causing persistent, chronic lung infections in individuals with the genetic disease cystic fibrosis (CF). A critical symptom of CF is a buildup of thick mucus (sputum) in the lungs, which inhibits the ability to clear invading pathogens (6). Additionally, sputum represents an excellent growth substrate for several bacteria, including *P. aeruginosa* (10). Due to natural resistance to most conventional antimicrobials, *P. aeruginosa* infections are particularly difficult to treat and are the leading cause of morbidity and mortality in individuals with CF (7).

To better understand the physiology of *P. aeruginosa* during growth in the CF lung, we previously developed a defined synthetic CF sputum medium (SCFM) that mimics the nutritional environment of CF sputum (19). Consistent with growth in authentic CF sputum, *P. aeruginosa* produces higher levels of the cell signaling molecule 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal [PQS]) in SCFM (19, 21). PQS is a quorum sensing signal (23) essential for production of a range of secreted virulence factors, including phenazines and hydrogen cyanide (9). Interestingly, removal of aromatic amino acids from SCFM decreased PQS production approximately 5-fold, implicating these amino acids as key mediators of enhanced PQS production in CF sputum (19). Further study revealed that the aromatic amino acids phenylalanine and tyrosine, but not tryptophan, were the primary inducers of PQS biosynthesis in SCFM (19, 21). While tryptophan was recently reported to enhance production of PQS (8), this amino acid is likely not important in CF sputum, as it is present at extremely low levels (~10 μM) (19).

In addition to enhancing PQS production, phenylalanine and tyrosine also serve as important carbon sources for *P. aeruginosa* in CF sputum (19). In *Pseudomonas putida*, the transcriptional regulator PhhR is required to induce genes encoding enzymes critical for catabolism of these amino acids (12, 13). Beyond regulation of genes involved in aromatic amino acid catabolism, PhhR regulates several other classes of genes in *P. putida* and has thus been described as a global transcriptional regulator (11). *P. aeruginosa* possesses a PhhR homolog (PA0873 in strain PAO1 and PA14_52980 in strain PA14) with 88% identity to *P. putida* PhhR that has been proposed to be critical for induction of phenylalanine catabolic genes (27).

The present study investigates the role of PhhR in mediating two important *P. aeruginosa* phenotypes observed during growth in CF sputum: catabolism of phenylalanine/tyrosine and phenylalanine/tyrosine-mediated induction of PQS. Transcriptome analysis revealed that PhhR controls four putative transcriptional units involved in aromatic amino acid catabolism; however, genes involved in PQS biosynthesis were unaffected. Promoters for several identified genes were mapped, putative binding sites for PhhR were identified using *in silico* analysis, and the ability to specifically bind these promoter regions was determined using electrophoretic mobility shift assays (EMSAs).

MATERIALS AND METHODS

Bacterial strains and media. *P. aeruginosa* strain PA14 and the isogenic *phhR* mutant were obtained from the PA14 nonredundant transposon mutant library (15; http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi). The transposon insertion site in *phhR* was confirmed by PCR. *P. aeruginosa* was routinely cultured on tryptic soy agar (14). *Escherichia coli* DH5α was used as the recipient for transformation and was cultured on LB Miller broth/agar (Fisher Scientific). Cultures were grown at 37°C with shaking at 250 rpm. Antibiotics were used at the following concentrations, unless otherwise noted: ampicillin, 50 μg/ml for *E.*

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coli; and carbenicillin, 300 μg/ml for plasmid selection and 25 μg/ml for plasmid maintenance in *P. aeruginosa*.

P. aeruginosa was also grown in defined SCFM (19). SCFM normally contains 0.8 mM tyrosine, 0.5 mM phenylalanine, and 10 μM tryptophan; however, when SCFM without aromatic amino acids was used, equimolar serine was added in place of these amino acids. Serine was chosen because it has been shown previously to not affect PQS production (19, 21). To evaluate growth of *P. aeruginosa* with tyrosine or phenylalanine as a carbon and energy source, a MOPS (morpholinepropanesulfonic acid)-buffered salts base (50 mM MOPS, pH 7.2, 93 mM NH_4Cl, 43 mM NaCl, 3.7 mM KH_2PO_4, 1 mM $MgSO_4$, 3.5 μM $FeSO_4 \cdot 7H_2O$, 2 mM proline) was supplemented with 10 mM tyrosine or 10 mM phenylalanine. Proline was added to reduce the considerable lag in growth commonly observed when phenylalanine/tyrosine is used as a sole carbon and energy source.

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

PQS extraction. Overnight cultures of *P. aeruginosa* PA14 or the *phhR* mutant grown in SCFM were subcultured into fresh SCFM to an optical density at 600 nm (OD_{600}) of 0.05. Cells were grown to exponential phase (OD_{600} of 0.45 to 0.55), pelleted by centrifugation at 9,000 × *g* for 5 min, washed twice with carbon-free SCFM, and starved for 2 h in carbon-free SCFM. Starved cultures were used to inoculate SCFM or SCFM without aromatic amino acids to an OD_{600} of 0.01. Cultures were allowed to reach near-maximum growth yields (OD_{600} of ~3) and were extracted twice with an equal volume of acidified ethyl acetate (150 μl acetic acid per liter ethyl acetate; Fisher). Extracts were dried under a constant stream of N_2 gas, resuspended in methanol (Fisher), and analyzed by thin-layer chromatography (TLC) as described previously (18, 19, 23). PQS was identified by comigration with 500 ng synthetic PQS standard. PQS on TLC plates was visualized by fluorescence after excitation with long-wave UV light, using a G:Box gel imager (Syngene).

Global expression profiling. For Affymetrix GeneChip analysis, *P. aeruginosa* PA14 was grown in SCFM or SCFM without aromatic amino acids, and the *phhR* mutant was grown in SCFM. To prepare cells for RNA extraction, exponentially growing cells were diluted to an OD_{600} of 0.001 and allowed to grow to an OD_{600} of 0.35 to 0.45. Cultures were mixed 1:1 with RNALater (Ambion), and total RNA was isolated. DNA contamination within RNA samples was removed by DNase treatment (Promega) and monitored by PCR amplification of the *rplU* gene as previously described (17, 19–21, 25). RNA integrity was monitored by agarose gel electrophoresis. cDNA synthesis from total RNA and cDNA fragmentation and labeling were performed as previously described (17, 19–21, 25). Processing of Affymetrix *P. aeruginosa* GeneChips was performed at the University of Iowa DNA Facility. All experiments were performed in duplicate, and data were analyzed using GeneChip operating software, version 1.4. Differentially regulated genes were identified by pairwise comparisons (4 or 6 total) of all GeneChips ($P \leq 0.05$).

Complementation of the *P. aeruginosa phhR* mutant. The *phhR* gene was PCR amplified from *P. aeruginosa* PA14 chromosomal DNA by using the primers *phhR*-comp-for (5′-TCCCCCGGGGAACGACAACACNNNNNCACGCC-3′) and *phhR*-comp-rev (5′-TCCCCCGGGCCGCCGTTTCTTTCCCAGCCTG-3′). The *phhR*-comp-for primer was designed such that the native Shine-Dalgarno sequence of *phhR* was replaced with N_5. The resulting 1,628-bp fragments were cloned into the pGEM-T Easy vector (Promega) per the manufacturer's instructions. Plasmids were isolated from 10 pooled white colonies and digested with SmaI to excise *phhR*. The *phhR* DNA fragments were gel purified and then ligated into SmaI-digested pUCP18 (29). Resulting pUCP18 plasmids containing *phhR* were purified and transformed into the *P. aeruginosa phhR* mutant by $MgCl_2$ transformation (30). Transformants were screened for the ability to grow with tyrosine as a carbon source. One plasmid that restored growth with tyrosine was identified (pKP-phhR). The sequence of the *phhR* gene in pKP-phhR was confirmed by DNA sequencing at the University of Texas Institute for Cell and Molecular Biology DNA Facility. In pKP-phhR, *phhR* expression is controlled by a constitutive *lac* promoter.

Primer extension analysis. To obtain RNA for primer extension analyses, *P. aeruginosa* was grown to late exponential phase in SCFM (OD_{600} of 1.3) and mixed 1:1 with RNALater (Ambion). Total RNA was isolated using an RNeasy Mini kit (Qiagen), and DNA contamination and RNA integrity were monitored as described above for Affymetrix GeneChip analysis. Primer extension was performed using fluorescently 6-carboxyfluorescein (FAM)-labeled primers as previously described (4, 16). Briefly, 1 μl of a 0.4 μM 5′-FAM-labeled primer was added to 20 to 30 μg total RNA in a final volume of 20 μl and incubated at 70°C for 10 min. Reaction mixtures were quickly chilled in an ice-water bath and then incubated at 65°C for 20 min. The temperature was shifted to 42°C, and reagents for cDNA synthesis (SuperScript II system [Invitrogen]; 8 μl 5× buffer, 4 μl 0.1 M dithiothreitol [DTT], 4 μl 10 mM deoxyribonucleotides, 4 μl SuperScript II enzyme) were added. Reaction mixtures were incubated at 42°C for 2 h, ethanol precipitated, and submitted for DNA sizing analysis at the University of Oklahoma Health Sciences Center Laboratory for Genomics and Bioinformatics. Some reaction mixtures were treated with 1 μl RNase H (Invitrogen) at 37°C for 20 min prior to precipitation. Primers used for primer extension are shown in Fig. 2. When more than one fluorescent peak was present, the highest peak, which corresponds to the major primer extension product, was reported. Primer extension analysis of each gene was performed at least twice.

PhhR purification. The *phhR* gene was PCR amplified from *P. aeruginosa* PA14 chromosomal DNA by using the primers *phhR*-for-NdeI (5′-GGAATTCCATATGCGTATCAAAGTGCACTGCCAG-3′) and *phhR*-rev-XhoI (5′-CCGCTCGAGTCAGCCCTCGCCTTGCCCCAC-3′). The resulting 1,560-bp fragment was digested with NdeI/XhoI and ligated into pET15b (Novagen) to generate pKP501. This construct adds a six-histidine tag (His_6) to the N terminus of PhhR. For *E. coli* transformations, 1% glucose was added to the growth medium to suppress transcription of *phhR*. The sequence of the pKP501 *phhR* gene was confirmed at the University of Texas Institute for Cell and Molecular Biology DNA Facility. For overexpression of His_6-PhhR, the *E. coli* host strain BL21(DE3) (Novagen) was used. BL21(DE3) carrying pKP501 was subcultured from LB Miller broth supplemented with 100 mM glucose and ampicillin into fresh LB Miller broth with ampicillin. During exponential growth (OD_{600} of 0.65 to 0.85), 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce expression of His_6-*phhR*. Cultures were harvested for protein purification after 1 h of incubation with IPTG.

To purify His_6-PhhR, cells were pelleted by centrifugation at 10,000 × *g* for 5 min and resuspended in 3 ml cold buffer A (25 mM potassium phosphate buffer, pH 7.4; 0.5 M NaCl; 5 mM DTT; 20 mM imidazole). Cells were lysed by two passes through a French press at ~20,000 lb/in^2 at 4°C, and the resulting lysate was centrifuged at 15,600 × *g* for 20 min to pellet insoluble material. The supernatant was then passed over a 1-ml His-Trap HP column (GE Healthcare) equilibrated with cold buffer A. The column was washed twice with 3 ml cold buffer A, and protein bound to the column was eluted with 3 ml cold buffer B (25 mM potassium phosphate buffer, pH 7.4; 0.5 M NaCl; 5 mM DTT; 500 mM imidazole). All fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. Western blotting on nitrocellulose membranes with an alkaline phosphatase-conjugated monoclonal anti-polyhistidine clone His-1 antibody (Sigma) and Western Blue stabilized substrate for alkaline phosphatase (Promega) was used to confirm the presence of His_6-PhhR in eluted fractions. Purified His_6-PhhR was desalted with an Amicon Ultra-4 centrifugal filter device (10-kDa cutoff) by successive concentration and dilution in storage buffer (50 mM Tris-HCl, pH 7.4; 100 mM NaCl; 5 mM DTT; 10% glycerol) until the imidazole concentration was ≤10 mM. Protein concentrations were quantified with the Bio-Rad protein assay (Bio-Rad). Purified His_6-PhhR was stored in storage buffer at 4°C and −80°C.

EMSA. Primers used to generate probes for EMSA are shown in Table 1. Probes were generated by PCR and gel purified. Probes (5 to 10 pmol each) were labeled with [γ-^{32}P]ATP (Sigma-Aldrich), using a KinaseMax kit (Ambion) per the manufacturer's instructions. Unincorporated radiolabeled nucleotides were removed using NucAway spin columns (Ambion). Unlabeled probes targeting intragenic regions of relevant genes were used as cold competitors in EMSAs (see below).

For EMSA, probes (10^4 cpm) were incubated with various concentrations of His_6-PhhR (0, 100, 250, and 500 nM) in 1× DNA binding buffer [20 mM Tris-HCl, pH 7.5; 50 mM KCl; 1 mM EDTA; 1 mM dithiothreitol; 2% glycerol; 100 μg/ml bovine serum albumin; 10 μg/ml poly(dI-dC) (modified from reference 26)]. For each cold competition reaction, a 20 (*hpd* and *dhcA*)- or 50 (*hmgA* and *phhA*)-fold molar excess of unlabeled probe was added to the binding reaction mix. Competitions were performed with 100 (*hpd*), 250 (*dhcA*), or 500 (*phhA* and *hmgA*) nM His_6-PhhR. The *hmgA* promoter binding reaction mixture included 100 μM (each) phenylalanine and tyrosine; all other reaction mixtures did not contain free amino acids. EMSA reaction mixtures were incubated at 30°C for 30 min prior to separation in 5% native polyacrylamide gels. Gels were prerun at 80 V for 1 h prior to loading EMSA reaction mixtures. Gels were dried and exposed to phosphorimager screens overnight, and ^{32}P-labeled bands were visualized with a personal molecular imager (Bio-Rad) or Storm 860 imaging system (GE Healthcare Life Sciences).

TABLE 1. Primers used for EMSA

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

[a] Primers used to amplify promoter probes for EMSA are shown in bold; primers used as nonspecific competitor probes from intragenic regions are shown in plain text. Primers used to amplify the *phhA* promoter contained recognition sites for restriction endonucleases (underlined).

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

RESULTS AND DISCUSSION

Previous work from our laboratory demonstrated that aromatic amino acids within CF sputum not only serve as carbon and energy sources but also enhance synthesis of the cell signaling molecule PQS (19, 21). Although these studies provided new insight into the *P. aeruginosa* response to nutritional cues, it was not clear if phenylalanine and tyrosine provoke other phenotypic responses. To begin to address this question, the global transcriptional response of *P. aeruginosa* to aromatic amino acids was assessed using Affymetrix GeneChips. For these experiments, *P. aeruginosa* was grown in a synthetic CF sputum medium (SCFM) designed to mimic the nutritional conditions of sputum from the CF lung. Twenty-two genes were differentially expressed >4-fold when cells were grown in SCFM compared to SCFM in which the aromatic amino acids had been removed (Table 2). As expected, genes involved in biosynthesis and response to PQS as well as genes important for catabolism of phenylalanine/tyrosine were highly induced in the presence of aromatic amino acids.

In addition to these genes, *phhR* was also induced in the

TABLE 2. Gene expression with aromatic amino acids and in the *phhR* mutant

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

[a] From the *Pseudomonas* Genome Database (www.pseudomonas.com).

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

[c] Fold regulation of genes differentially expressed in wt *P. aeruginosa* compared to the isogenic *phhR* mutant. Bacteria were grown in SCFM, and a negative number indicates a downregulation of the gene in the *phhR* mutant. NC, no change in mRNA level, as determined by GeneChip operating software, version 1.4; NT, the gene could not be tested due to insertion of the MAR2×T7 mariner transposon in *phhR*. The generation times of wt *P. aeruginosa* and the *phhR* mutant in SCFM were similar (data not shown).

presence of aromatic amino acids (Table 2). PhhR has been implicated as a phenylalanine/tyrosine-responsive transcriptional regulator critical for induction of phenylalanine/tyrosine catabolic genes (27). Based on these data, we hypothesized that PhhR was the transcriptional regulator mediating differential expression of the aromatic amino acid-responsive genes observed in Table 2. To test this hypothesis, global expression profiling of wild-type (wt) *P. aeruginosa* and the isogenic *phhR* mutant was performed in SCFM. Twelve genes were downregulated >4-fold in the *phhR* mutant compared to the wild-type strain (Table 2). All of the genes downregulated in the *phhR* mutant are putatively involved in phenylalanine/tyrosine degradation to fumarate and acetyl-coenzyme A (acetyl-CoA) (Fig. 1A) or in amino acid transport. Of note, we propose that DhcA and DhcB constitute the acetoacetyl-CoA transferase (Fig. 1A), as these proteins share high similarity with the *E. coli* subunits of this enzyme, namely, AtoD (64% similarity; E value of $<10^{-43}$ by BLASTp) and AtoA (62% similarity; E value of $<10^{-48}$ by BLASTp), respectively. Recent observations that *dhcA* and *dhcB* likely encode products comprising a 3-dehydrocarnitine-CoA transferase further support this designation (28). Acetoacetate and 3-dehydrocarnitine are structurally similar, with both being 3-ketoacids, differing only by the presence of a trimethylated amine on carbon four of 3-dehydrocarnitine. In addition, isogenic *P. aeruginosa dhcA* and *dhcB* mutants displayed reduced growth when phenylalanine was supplied as the major carbon and energy source (data not shown).

Interestingly, genes involved in PQS biosynthesis were not differentially regulated in the *phhR* mutant (Table 2), supporting the hypothesis that while PhhR is likely important for catabolism of phenylalanine/tyrosine, it is not responsible for aromatic amino acid-mediated increases in PQS production. To test this hypothesis, the abilities of aromatic amino acids to support growth and to stimulate PQS production were assessed for wt *P. aeruginosa* and the *phhR* mutant. As expected, the *P. aeruginosa phhR* mutant demonstrated poor growth with phenylalanine and tyrosine as the major carbon and energy source (Fig. 1B); however growth with tryptophan was unaffected (data not shown). Similar to wt *P. aeruginosa*, the *phhR* mutant demonstrated increased PQS production during growth with aromatic amino acids (Fig. 1C). These results indicate that PhhR is an aromatic amino acid-responsive transcriptional regulator that controls—either directly or indirectly—genes involved in phenylalanine/tyrosine catabolism but not PQS production.

Initial attempts to genetically complement the *phhR* mutant by a variety of methods, including the use of heterologous inducible promoters, were unsuccessful. We reasoned that this may be due to improper expression of PhhR. To overcome this problem, the *phhR* Shine-Dalgarno sequence (ribosome binding site) was randomized during PCR amplification by incorporating N_5 into the 5′ amplification primer in place of the native *phhR* Shine-Dalgarno sequence, CGGGC. Amplicons were ligated into the multicopy plasmid pUCP18 and transformed into *E. coli*. Plasmids were then pooled from the resulting *E. coli* transformants and transformed into the *P. aeruginosa phhR* mutant. Plasmids from *P. aeruginosa phhR* transformants that grew with tyrosine as a carbon source were selected for DNA sequencing. This approach yielded a plasmid

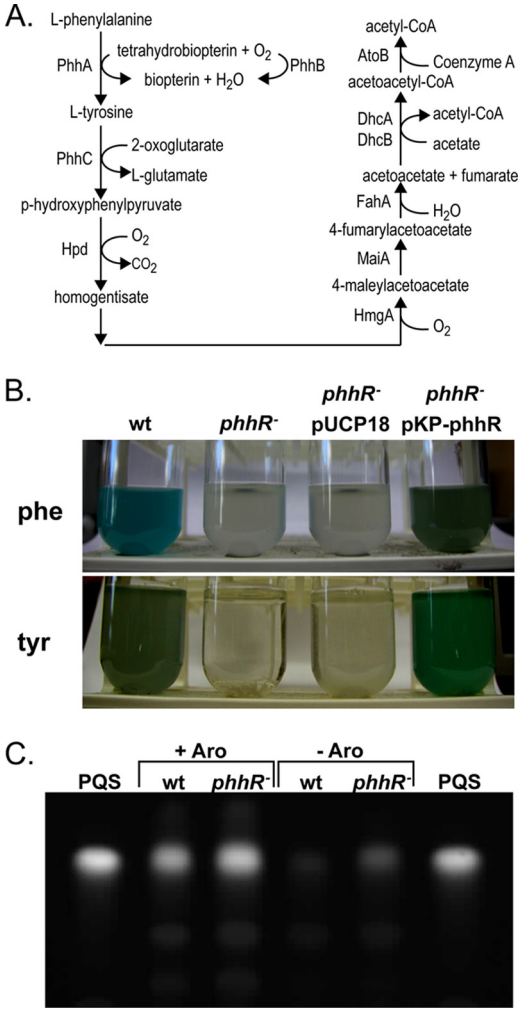

FIG. 1. PhhR is critical for tyrosine/phenylalanine catabolism. (A) Predicted pathway of phenylalanine and tyrosine catabolism in *P. aeruginosa* (from the Pseudomonas Genome Database [www.pseudomonas.com]). The acetoacetate catabolic pathway is from the Encyclopedia of *Escherichia coli* K-12 Genes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

(pKP-phhR) containing *phhR* with a Shine-Dalgarno sequence of GTGCT. The new sequence is likely less favorable to ribosome binding, resulting in tolerable levels of PhhR in the cells. Introduction of pKP-phhR into the *P. aeruginosa phhR* mutant

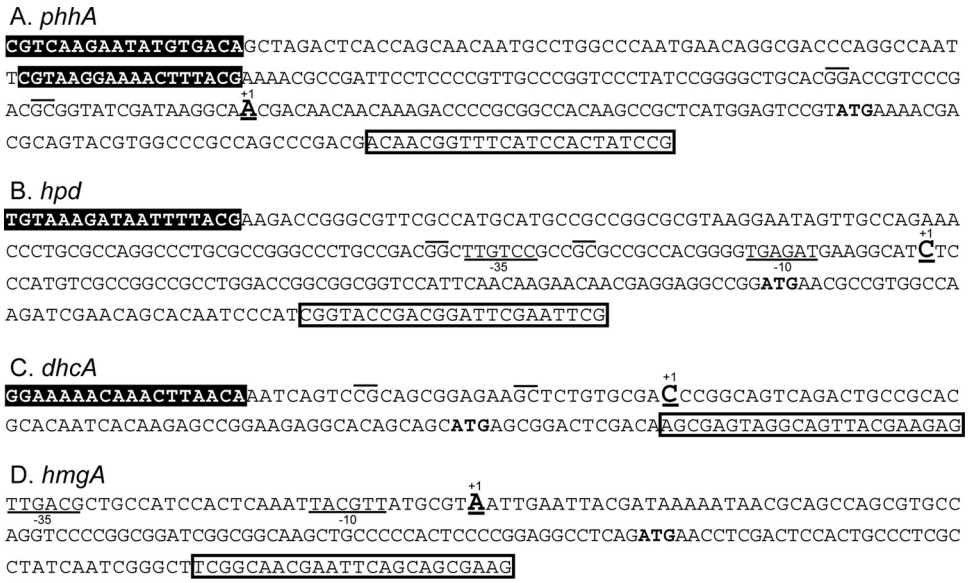

FIG. 2. Promoter architecture of PhhR-controlled genes. Primer extension analysis was used to map transcriptional start sites (Table 2). Shown are the promoter sequences for four PhhR-dependent transcriptional units: *phhA* (A), *hpd* (B), *dhcA* (C), and *hmgA* (D). The target DNA sequence of the primer extension oligonucleotide primer is boxed. Transcriptional start sites are indicated in large, bold, underlined letters (+1). Putative σ^{70} promoters containing homology to the canonical −10 and −35 sequences are indicated by single underlines. Putative σ^{54} promoters are indicated by overlines. Proposed PhhR binding sequences are included within black boxes.

restored growth with phenylalanine and tyrosine as the primary carbon source (Fig. 1B).

In silico analysis revealed that the PhhR-regulated phenylalanine/tyrosine catabolic genes are arranged into four operons. To identify important transcriptional regulatory DNA elements, promoter regions from these four transcriptional units were mapped using primer extension (Fig. 2 and Table 3). To map the transcriptional start sites, a nonradioactive primer extension assay previously used to map *Helicobacter pylori* and *P. aeruginosa* transcriptional start sites was employed (4, 16). Briefly, a fluorescently labeled primer homologous to the gene of interest was used to generate cDNA from total cellular RNA. The size and quantity of the cDNA product were determined using a standard DNA sequencer and used to map the transcriptional start site of the gene of interest (Table 3). This analysis revealed that *phhA* possesses a transcriptional start site located 48 bp upstream of the translational start codon (Fig. 2A and Table 3), *hpd* possesses a transcriptional start site located 64 bp upstream of the translational start codon (Fig. 2B and Table 3), *dhcA* possesses a transcriptional start site located 56 bp upstream of the translational start codon (Fig. 2C and Table 3), and *hmgA* possesses a transcriptional start site located 88 bp upstream of the translational start codon (Fig. 2D and Table 3).

TABLE 3. Summary of primer extension data

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

[a] Fluorescent peaks were identified using Peak Scanner software (Applied Biosystems).

Promoter regions of *phhA*, *hpd*, *hmgA*, and *dhcA* were subjected to *in silico* analyses to identify putative regulatory DNA sequences. A consensus DNA binding site has been proposed for *P. putida* PhhR (TGTAAAGATAGTTTTACA) (11) and the *E. coli* PhhR homolog TyrR (TGTAAA-N_6-TTTACA) (24). In addition, two potential PhhR binding sites were previously mapped to the *phhA-phhR* intergenic region of a non-PA14 *P. aeruginosa* strain (27). Examination of the *phhA* promoter region in *P. aeruginosa* PA14 revealed that these two sites are conserved in this strain, centered at −86 and −159 bp respective to the *phhA* transcriptional start site (Fig. 2A). Putative PhhR binding sites were also identified upstream of the *hpd* (−138 bp) (22) and *dhcA* (−40 bp) transcriptional start sites (Fig. 2B and C); however, unlike the case in *P. putida* (11), a DNA sequence similar to the consensus PhhR and TyrR binding sites was not identified in the *hmgA* promoter (Fig. 2D).

PhhR has been proposed to modulate transcription from σ^{54}- and σ^{70}-dependent promoters (12, 13, 27). Both σ^{54} and σ^{70} DNA binding sites have been identified between *phhR* and *phhA* on the *P. aeruginosa* chromosome (27), and PhhA production is σ^{54} dependent in *P. aeruginosa* (27). σ^{54} binding sites in promoters of interest can be difficult to identify *in silico*. σ^{54}-dependent genes generally possess a conserved GG-N_{10}-GC sequence centered from −24 to −12 bp relative to their transcriptional start sites, although this may vary between promoters (3). For some σ^{54}-dependent promoters, the conserved GG-N_{10}-GC sequence is not centered at positions −24 to −12 but is instead shifted several bases upstream or downstream (3). Of particular note is the *algD* promoter of *P. aeruginosa*, which has a GG-N_{10}-GC sequence centered at positions −34 to −22 (31). In addition, while the GG and GC

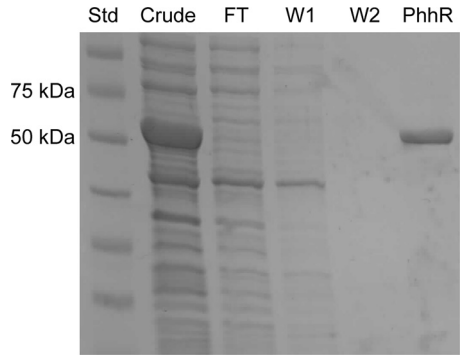

FIG. 3. Purification of His_6-PhhR. His_6-PhhR was purified using a HisTrap nickel column, separated in a 10% SDS-PAGE gel, and visualized after staining with Coomassie blue. The lanes contained molecular size markers (Std), soluble crude extract (crude), column flowthrough after soluble cell extract addition (FT), the first wash with buffer A containing 20 mM imidazole (W1), the second wash with buffer A containing 20 mM imidazole (W2), and elution of His_6-PhhR with buffer B containing 500 mM imidazole (PhhR). Protein eluted from the buffer B wash (PhhR) was desalted and used for EMSA.

sequences are the most highly conserved sites in the consensus σ^{54} binding sequence (3), these sites can be variable. Examination of the promoter sequences revealed putative σ^{54} binding sites upstream of *phhA*, *hpd*, and *dchA* but not *hmgA* (Fig. 2A to D).

To examine if PhhR binds directly to the promoter regions of PhhR-regulated genes, *P. aeruginosa phhR* was cloned into an expression vector (pET15b) to create N-terminally His_6-tagged PhhR. Affinity purification using a nickel column resulted in nearly pure His_6-PhhR, with a prominent band at approximately 58 kDa on Coomassie-stained SDS-PAGE gels (Fig. 3). Binding of His_6-PhhR to the *phhA*, *hpd*, *hmgA*, and *dhcA* promoters was characterized by EMSA. DNA fragments containing promoter regions were generated by PCR (Fig. 4A to D) and 5′-end labeled with ^{32}P for use as EMSA probes. Each probe was incubated with increasing concentrations of PhhR and was submitted to competition with nonradioactive (cold) excess specific and nonspecific competitor DNAs to confirm the specificity of PhhR binding.

Purified His_6-PhhR bound to and retarded mobility of the *phhA*, *hpd*, and *dhcA* promoters in the absence of aromatic amino acids, and the addition of specific, but not nonspecific, cold competitor DNA eliminated this binding (Fig. 4A to C). These results suggest that PhhR is a direct regulator of the *phhA*, *hpd*, and *dhcA* operons. The observation that purified His_6-PhhR bound to the *phhA*, *hpd*, and *dhcA* promoters in the absence of aromatic amino acid ligands was not unexpected, as *P. putida* PhhR binds target promoters *in vitro* in the absence of amino acid inducers (11). Notably, regulation of the *dhcA* operon may be more complex, as it was recently reported to also be under the control of the divergently transcribed transcriptional regulator DhcR, although direct binding of this regulator to the *dhcA* promoter was not examined (28).

We could not detect binding of His_6-PhhR to the *hmgA* promoter (Fig. 4D), even upon addition of phenylalanine and tyrosine to the binding reaction mix. The *P. aeruginosa hmgA* promoter does not possess a DNA sequence with high homology to a PhhR binding sequence (Fig. 2). This is in contrast to

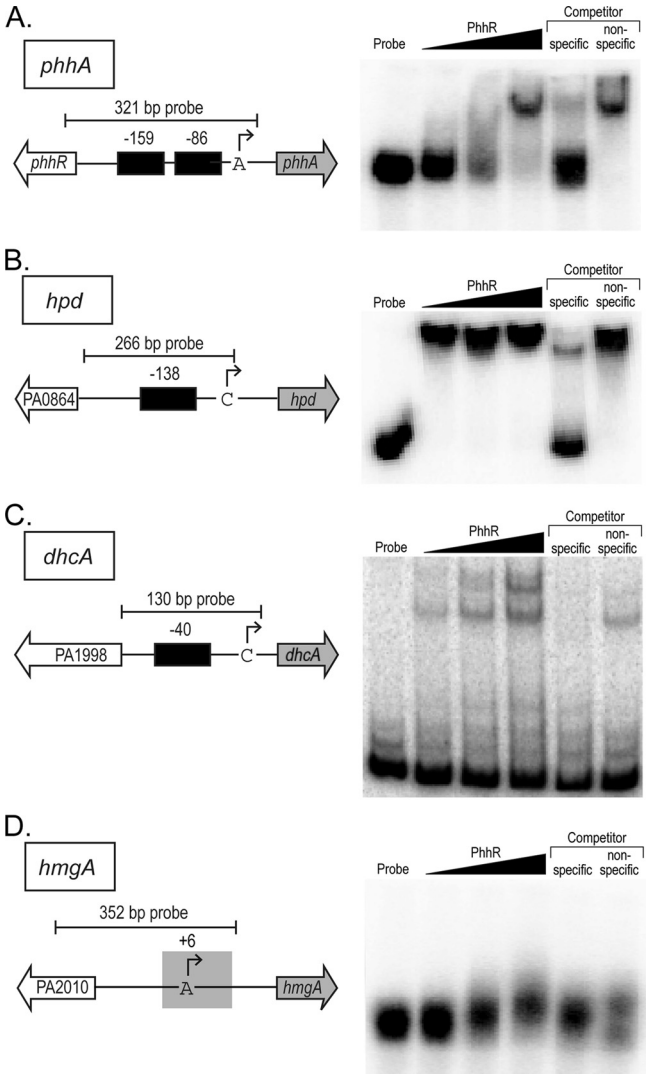

FIG. 4. His_6-PhhR binds to the promoter regions of phenylalanine/tyrosine catabolic genes. (Left) Diagrammatic representation of the *phhA* (A), *hpd* (B), *dhcA* (C), and *hmgA* (D) promoters. Line arrows indicate transcriptional start sites (Table 2), and block arrows represent open reading frames. Putative PhhR binding sites are shown as black boxes, and the gray box in panel D represents a putative HmgR binding site. Numbers above putative binding sites represent the location of the center of the binding site relative to the transcriptional start site. The locations of promoter probes used for EMSA are also shown. The figure is not drawn to scale. (Right) EMSA of His_6-PhhR binding to *phhA* (A), *hpd* (B), *dhcA* (C), and *hmgA* (D). The lanes contained ^{32}P-labeled promoter probe alone (probe); ^{32}P-labeled probe with 100 nM (lanes 2), 250 nM (lanes 3), or 500 nM His_6-PhhR (lanes 4); ^{32}P-labeled probe with 100 to 500 nM His_6-PhhR and a 20- to 50-fold molar excess of unlabeled probe (specific); and ^{32}P-labeled probe with 100 to 500 nM His_6-PhhR and a 20- to 50-fold molar excess of unlabeled nonspecific DNA competitor (nonspecific).

the *hmgA* promoter in *P. putida*, which contains a functional PhhR binding sequence (11). How could *P. aeruginosa* PhhR regulate *hmgA* transcription without binding the promoter? Clues were provided by a recent study demonstrating that the transcriptional regulator HmgR also controls *hmgA* transcription in *P. putida* (1). HmgR binds and represses transcription of the *hmgA* promoter unless it is bound to its ligand,

homogentisate. Homogentisate is produced during catabolism of phenylalanine/tyrosine by the enzyme Hpd (Fig. 1A). The *P. aeruginosa phhR* mutant produced significantly less *hpd* mRNA (Table 2), suggesting that this mutant likely produces very little intracellular homogentisate, even in the presence of phenylalanine/tyrosine. The low levels of homogentisate would not allow derepression of HmgR, thereby resulting in lower levels of *hmgA* mRNA in the *phhR* mutant. In support of this hypothesis, *P. aeruginosa* possesses an HmgR gene homolog (PA2010 in strain PAO1 and PA14_38500 in strain PA14) immediately upstream of *hmgA*, and the *hmgA* promoter possesses a putative HmgR binding site (1).

The goal of this study was to expand on previous work from our laboratory demonstrating that aromatic amino acids within CF lung secretions (sputum) not only serve as carbon and energy sources but also enhance PQS synthesis. This study provides evidence that PhhR controls genes important for catabolism of phenylalanine and tyrosine. The role of PhhR in regulation of phenylalanine catabolic genes is reminiscent of *P. putida* PhhR (11); however, it is intriguing that in contrast to *P. putida* PhhR, *P. aeruginosa* PhhR does not control expression of aromatic amino acid biosynthesis genes (11). This study also provides evidence that PhhR is not critical for induction of PQS biosynthetic genes. Instead, the data support a previous model of enhanced PQS production in which the presence of phenylalanine/tyrosine allows increased flux of the common biosynthetic precursor chorismate away from aromatic amino acid production and into PQS biosynthesis (19).

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