phzO, a Gene for Biosynthesis of 2-Hydroxylated Phenazine Compounds in *Pseudomonas aureofaciens* 30-84

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Certain strains of root-colonizing fluorescent *Pseudomonas* spp. produce phenazines, a class of antifungal metabolites that can provide protection against various soilborne root pathogens. Despite the fact that the phenazine biosynthetic locus is highly conserved among fluorescent *Pseudomonas* spp., individual strains differ in the range of phenazine compounds they produce. This study focuses on the ability of *Pseudomonas aureofaciens* 30-84 to produce 2-hydroxyphenazine-1-carboxylic acid (2-OH-PCA) and 2-hydroxyphenazine from the common phenazine metabolite phenazine-1-carboxylic acid (PCA). *P. aureofaciens* 30-84 contains a novel gene located downstream from the core phenazine operon that encodes a 55-kDa aromatic monooxygenase responsible for the hydroxylation of PCA to produce 2-OH-PCA. Knowledge of the genes responsible for phenazine product specificity could ultimately reveal ways to manipulate organisms to produce multiple phenazines or novel phenazines not previously described.

Certain strains of root-colonizing fluorescent *Pseudomonas* spp. have gained attention in recent years because they produce broad-spectrum antibiotic metabolites that can provide protection against soilborne root diseases (46). One such class of antibiotics, the phenazines, encompasses a large family of heterocyclic nitrogen-containing compounds produced in late exponential and stationary phase. The ability to produce phenazines is limited almost exclusively to bacteria and has been reported in members of the genera *Pseudomonas*, *Streptomyces*, *Nocardia*, *Sorangium*, *Brevibacterium*, and *Burkholderia* (48). There are currently over 50 known phenazine compounds with the same basic structure, differing only in the derivatization of the heterocyclic core. These modifications largely determine the physical properties of phenazines and influence their biological activity against plant and animal pathogens.

The broad-spectrum activity exhibited by phenazine compounds against fungi and other bacteria is not understood. It is thought that they diffuse across the membrane and, once inside the cell, accept a single electron, disrupting respiration by interfering with the normal process of electron transport. This results in the overproduction of O₂^{•−} and H₂O₂, which overwhelm cellular superoxide dismutases and ultimately cause cell death. The cellular superoxide dismutases of *Pseudomonas aeruginosa*, a bacterium which produces the phenazine compound pyocyanin, are more active than those of phenazine-nonproducing bacteria such as *Escherichia coli*, and they provide protection against phenazines (18, 19).

Several studies conducted in the early 1970s revealed tight links between phenazine biosynthesis and the shikimic acid

pathway (48), but the biochemistry and genetic control of phenazine synthesis are still not fully understood. Chorismic acid has long been recognized as the branch point from the shikimic acid pathway to phenazine synthesis (26). Studies with radiolabeled precursors suggest that the phenazine core is formed by the symmetrical condensation of two molecules of chorismic acid (7, 20, 22, 26), while the amide nitrogen of glutamine serves as the immediate source of nitrogen in the heterocyclic nucleus of phenazine compounds (36). Phenazine-1,6-dicarboxylic acid is the first phenazine formed, and it is thought to be converted to phenazine-1-carboxylic acid (PCA), a key intermediate in the synthesis of other phenazines by fluorescent pseudomonads (6, 20, 22, 28).

Genetic studies in fluorescent *Pseudomonas* spp., the only microorganisms for which the genes responsible for the assembly of the heterocyclic phenazine nucleus have been cloned and sequenced, support this model. The phenazine biosynthetic loci from *P. fluorescens* 2-79 (27), *P. aureofaciens* 30-84 (27, 33), *P. aeruginosa* PA01 (D. V. Mavrodi and L. S. Thomashow, unpublished data), and *P. chlororaphis* PCL1391 (T. F. C. Chin-A-Woeng, D. van den Broek, G. de Voer, K. M. G. M. van der Drift, J. E. Thomas-Oates, B. J. J. Lugtenberg, and G. V. Bloemberg, *Pseudomonas* '99: Biotechnology and Pathogenesis, abstr. S48, 1999) are highly conserved. Each contains a seven-gene core operon regulated in a cell density-dependent manner by homologues of LuxI and LuxR (25, 52; D. V. Mavrodi and S. K. Farrand, unpublished data). In *P. fluorescens* 2-79, *P. aureofaciens* 30-84, and *P. chlororaphis* PCL1391, the *phzI/R* genes are found directly upstream from the phenazine core. Phenazine production in *P. aeruginosa* is controlled by two sets of regulatory proteins, *rhII/R* and *lasI/R*, located elsewhere in the genome. The core gene products PhzC, PhzD, and PhzE, which are homologous with PhzF, PhzA, and PhzB in strain 30-84, are similar to enzymes of shikimic and chorismic acid metabolism. Sequence comparisons of PhzD and

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PhzE with other chorismate-modifying enzymes have shed new light on probable intermediates in the PCA pathway, suggesting that phenazine synthesis proceeds via the intermediates aminodeoxyisochorismic acid and 3-hydroxyanthranilate (27) rather than anthranilate, as suggested previously (12).

Although the phenazine biosynthetic loci of fluorescent pseudomonads are highly homologous, individual species typically differ in the range of compounds they produce. Previous work by Pierson et al. (33) suggested that the *phzC* gene of *P. aureofaciens* 30-84, and in particular the last 28 amino acids of the PhzC protein, are essential for the production of 2-hydroxyphenazine-1-carboxylic acid (2-OH-PCA) and 2-hydroxyphenazine (2-OH-PHZ), derivatives that are characteristic of strains previously designated *P. aureofaciens* but now classified as *P. chlororaphis* (24). The purpose of the present study was to determine the genetic basis for the production of these hydroxyphenazines by *P. aureofaciens* 30-84. Two possibilities were considered: first, that product specificity is determined by amino acid substitutions within the core biosynthetic genes, as suggested previously (33); or second, that a core pathway conserved among fluorescent pseudomonads is responsible for the synthesis of PCA, which then can be modified in a strain- or species-specific manner to yield a variety of different phenazine products. Knowledge of the mechanisms responsible for phenazine product specificity ultimately could reveal ways to manipulate organisms to produce multiple phenazines or hybrid phenazine products not previously described. Such compounds may have improved activity against soilborne plant pathogens or may lead to the development of novel pharmaceutical products.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. Rifampin-resistant derivatives of *P. fluorescens* strains 2-79, M4-80R, and Q8r1-96 and *P. chlororaphis* 30-84 (referred to here by its original designation, *P. aureofaciens* 30-84) were used. *Pseudomonas* strains were grown at 28°C in Luria-Bertani (LB) broth, 2× YT broth (37), or M9 minimal medium (2) supplemented with sodium citrate to a final concentration of 40 mM as a carbon source. *E. coli* strains were grown in LB broth or 2× YT broth at 28 or 37°C. To enhance phenazine production, *Pseudomonas* strains were grown in LB broth supplemented with 1.5% glucose. When appropriate, antibiotic supplements were used at the following concentrations: tetracycline, 12.5 µg/ml (*E. coli*) or 25 µg/ml (*Pseudomonas* strains); rifampin, 100 µg/ml; kanamycin, 100 µg/ml; neomycin, 100 µg/ml (*P. fluorescens* 2-79); chloramphenicol, 35 µg/ml; and ampicillin, 100 µg/ml.

DNA manipulations. Standard methods were used for DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, and ligation (2). *Pseudomonas* and *E. coli* cells were transformed by electroporation in a Gene Pulser II system (Bio-Rad, Hercules, Calif.) according to the method of Enderle and Farwell (11) at settings of 25 µF for the capacitor, 200 Ω resistance, and an electric field of 1.8 kV/cm. Genomic DNA was isolated and purified by a cetyltrimethylammonium bromide (CTAB) miniprep procedure (2). For Southern blotting and hybridization, 500 ng of genomic DNA was digested with *Eco*RI and *Pst*I, separated by electrophoresis in an 0.8% agarose gel, and transferred onto a BrightStar-Plus nylon membrane (Ambion, Inc., Austin, Tex.) in 0.4 M NaOH with subsequent cross-linking by exposure to UV irradiation (2). Membranes were prehybridized for 3 h at 60°C in a solution containing 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 4× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 250 mg of denatured salmon sperm DNA/ml. After prehybridization, the membranes were incubated with specific probes overnight under the same conditions and washed with 2× SSC and 0.1% SDS at room temperature, 0.2× SSC and 0.1% SDS at room temperature, 0.2× SSC and 0.1% SDS at 60°C, and 0.1× SSC and 0.1% SDS at 60°C. DNA-DNA hybrids were detected with the BrightStar non-isotopic detection kit (Ambion Inc.) according to the manufacturer's protocol. The 2.1-kb *phzO* probe was amplified by PCR from *P. aureofaciens* 30-84 genomic DNA with the oligonucleotide primers 30-

84XBA (5'-AAG TCC AGA TGC GAA AGA ACG-3') and PHZO10 (5'-AAG TGG CAT GGC TCG AAC AAA G-3'). Amplification was carried out in a 25-µl reaction mixture containing 1× thermophilic DNA polymerase buffer (Promega Corp., Madison, Wis.), 1.5 mM MgCl₂, 5.0% (final concentration) dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.), 200-µM concentrations of dGTP, dATP, dCTP, and dTTP (Perkin-Elmer, Norwalk, Conn.), 20 pM of each primer, and 1.2 U of *Taq* DNA polymerase (Promega Corp.). Amplifications were performed with a PTC-200 thermal cycler (MJ Research Inc., Warrington, Mass.). The cycling program included a 45-s initial denaturation at 94°C followed by 30 cycles of 94°C for 45 s, 51°C for 45 s, and 72°C for 1.5 min. Amplified DNA was labeled with a random primer biotin labeling kit (NEN Life Science Products Inc., Boston, Mass.).

DNA sequencing and analysis. DNA was sequenced by using the ABI Prism Dye Terminator Cycle sequencing kit (Perkin-Elmer), according to the manufacturer's instructions. All custom-designed oligonucleotides came from Operon Technologies Inc. (Alameda, Calif.). Sequence data were compiled and analyzed for open reading frames and codon usage with the Omega version 1.1.3 software package (Oxford Molecular Ltd., Oxford, United Kingdom). A database search for similar protein sequences was carried out with the BLAST (44) and FASTA network servers at the National Center for Biotechnology Information and the European Molecular Biology Laboratory (EMBL), respectively. The probable domain homologies search was performed with PROSITE (EMBL, Heidelberg, Germany) (3) and ISREC ProfileScan (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland [www.isrec.isb-sib.ch/software/PFSCAN_form.html]) computer services. The significance of the similarity of a predicted protein to known proteins was determined by calculating the binary comparison score (Z score). Pairwise alignments were obtained by using the BESTFIT program from the Wisconsin Package (Genetics Computer Group, Madison, Wis.), and the resulting percent identities, percent similarities, alignment scores (A), mean random alignment scores (R), and standard deviations (SD) ($n = 100$) were noted. Z scores were then calculated by the equation $Z = (A - R)/SD$. Multiple sequence alignments were built with Omega's ClustalW and analyzed with the TreeView version 1.5.0 software package (31).

Mating and screening of transconjugants. Plasmids were mobilized from the donor strain *E. coli* S-17 (λ -pir) into *Pseudomonas* recipients by using the filter mating technique described by van Overbeek (49). To counterselect *E. coli* donor cells, mating mixtures were plated on M9 agar supplemented with appropriate antibiotics and sodium citrate as a carbon source. Positive isolates were replated and screened for the presence of phenazine genes by PCR with primers PHZ1 and PHZ2. The oligonucleotide primers PHZ1 (5'-GGC GAC ATG GTC AAC GG-3') and PHZ2 (5'-CGG CTG GCG GCG TAT TC-3') were used as universal phenazine primers to amplify a 1.4-kb fragment containing parts of *phzF* and *phzA* in *P. aureofaciens* 30-84, which correspond to *phzC* and *phzD* in *P. fluorescens* 2-79. The amplification was carried out in a 15-µl reaction mixture. The cycling program included an initial denaturation for 2 min at 94°C followed by 25 cycles of 94°C for 1 min, 56°C for 45 s, 72°C for 1.75 min, and a final extension at 75°C for 1 min. The oligonucleotide primers PHZX (5'-TTT TTT CAT ATG CCT GCT TCG CTT TC-3') and PHZY (5'-TTT GGA TCC TTA AGT TGG AAT GCC TCC G-3') were used to distinguish between the phenazine operons of *P. aureofaciens* 30-84 and *P. fluorescens* 2-79. These primers amplify a 1.1-kb DNA fragment containing parts of *phzX* and *phzY* from strain 30-84 but not from the corresponding, homologous *phzA* and *phzB* sequences of strain 2-79. The program included an initial denaturation at 94°C for 1.5 min followed by 30 cycles of 94°C for 45 s, 58°C for 30 s, 72°C for 1.75 min, and a final extension at 72°C for 1 min.

Protein expression. The *phzO* gene was cloned from *P. aureofaciens* 30-84 and expressed under the control of the *lac* promoter in the plasmid vector pUCP26. *E. coli* JM109 harboring pUCP26, pUCP2.9XP, or pUCP4.5 was grown in LB broth to an optical density at 600 nm of 0.6 and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested 3 h later and total cellular protein was analyzed by electrophoresis in an SDS-10% polyacrylamide gel as described by Copeland (9). Alternatively, *phzO* was amplified by PCR from *P. aureofaciens* 30-84 genomic DNA with the primers PHZOstart (5'-CGA CTC TAG AAC GTT GTC CTT GAC C-3') and PHZO10 in a 30-µl reaction mixture with a cycling program that included a 45-s initial denaturation at 94°C and 29 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 3.25 min. The 1.8-kb reaction product was ligated into pGEM-T Easy (Promega) to give pGEM-PHZO, which was transformed into *E. coli* JM109. The resulting plasmid, pGEM-PHZO, contained the entire *phzO* gene preceded by 88 bp upstream of the start codon and 265 bp downstream of the coding sequence. Expression was induced as described above.

PCA transformation assay. *E. coli* JM109 bearing pUCP26, pUCP2.9XP, pGEM-T Easy, or pGEM-PHZO was grown at 37°C in 2× YT supplemented

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Reference or source
<i>P. aeruginosa</i>		
PAO1	Phz ⁺ , produces pyocyanin	12
ATCC 25007	Phz ⁺ , produces pyocyanin, aeruginosins A and B	40
ATCC 25011	Phz ⁺ , produces aeruginosins A and B	53
KN-1	Phz ⁺ , produces pyocyanin	Laboratory collection
KNP-1	Phz ⁺ , produces pyocyanin	Laboratory collection
KNP-2	Phz ⁺ , produces pyocyanin	Laboratory collection
<i>P. aureofaciens</i>		
30-84	Phz ⁺ , produces PCA, 2-OH-PCA, and 2-OH-PHZ	32
30-84mxO	Phz ⁺ , produces PCA	This study
ATCC 13985	Phz ⁺ , produces PCA, 2-OH-PCA, and 2-OH-PHZ	40
BS 1391	Phz ⁺ , produces PCA, 2-OH-PCA, and 2-OH-PHZ	V. Kotchetkov
BS 1393	Phz ⁺ , produces PCA, 2-OH-PCA, and 2-OH-PHZ	V. Kotchetkov
PGS12	Phz ⁺ , produces PCA, 2-OH-PCA, and 2-OH-PHZ	15
AP-9	Phz ⁺ , produces PCA, 2-OH-PCA, and 2-OH-PHZ	1
TX-1	Phz ⁺ , produces PCA, 2-OH-PCA, and 2-OH-PHZ	EcoSoil Systems
<i>P. chlororaphis</i>		
ATCC 17411	Phz ⁺ , produces chlororaphin	48
ATCC 17809	Phz ⁺ , produces chlororaphin	ATCC
ATCC 9446	Phz ⁺ , produces chlororaphin	40
<i>P. fluorescens</i>		
2-79	Phz ⁺ Rif ^r , produces PCA	50
M4-80R	Phz ⁺ Rif ^r	17
Q8r1-96	Phl ⁺ Rif ^r	35
UQ 112	Phz ⁺ , produces PCA	G. Botelho
UN 15	Phz ⁺ , produces PCA	G. Botelho
UN 4127	Phz ⁺ , produces PCA	G. Botelho
<i>E. coli</i>		
SM17 (λ-pir)	<i>thi pro hsdR hsdM recA rpsL</i> RP4-2 Tet ^r ::Mu Kan ^r ::Tn7	39
JM109	F ⁺ <i>traD36 proA⁺ proB⁺ lacI lacZΔM15/recA1</i>	Promega Corp.
Plasmids		
pALTER-Ex1	ColE1 Tet ^r SP6, <i>tac</i> , and T7 promoters	Promega Corp.
pNOT19	ColE1 <i>bla</i> accessory plasmid	38
pUCP26	pUCP18-derived broad-host-range vector Tet ^r	51
pMOB3	Kan ^r <i>cat sacBR</i>	38
pUCP4.5	pUCP26 containing 4.5 kb- <i>XbaI-EcoRI</i> DNA fragment from <i>P. aureofaciens</i> 30-84; Tet ^r	This study
pUCP2.9XP	pUCP26 containing 2.9-kb <i>XbaI-PstI</i> DNA fragment from <i>P. aureofaciens</i> 30-84; contains <i>phzO</i> , Tet ^r	This study
pGEM-T Easy	Amp ^r , pUC18-derived SP6 and T7 promoters, fl ori, <i>lacZα</i>	Promega Corp.
pGEM-PHZO	pGEM-T Easy containing 1.8-kb <i>phzO</i> fragment amplified from <i>P. aureofaciens</i> 30-84 by PCR	This study
p18Sfi	Amp ^r , pUCP18-derived vector with <i>SfiI-EcoRI-SalI-HindIII-SfiI</i> as multiple cloning site	21
pUT-Km-Tn5	Amp ^r , Tn5-based delivery plasmid with Kan ^r	21
pUT-Km/30-84	pUT-Km-Tn5 with <i>phzXYFABCD</i> from <i>P. aureofaciens</i> 30-84 cloned between <i>SfiI</i> sites	This study
pLAFR3	<i>IncP</i> Tet ^r <i>cos⁺ rlx⁺</i>	41
pLSP259	pLAFR3 containing 20.9-kb DNA fragment from <i>P. aureofaciens</i> 30-84; Tet ^r	32
pLSP282	pLAFR3 containing 24.7-kb DNA fragment from <i>P. aureofaciens</i> 30-84; Tet ^r	32
pLSP282Δ20-9	pLAFR3 containing 15.0-kb DNA fragment from <i>P. aureofaciens</i> 30-84; Tet ^r	32
pLSP282Δ30-8	pLAFR3 containing 11.2-kb DNA fragment from <i>P. aureofaciens</i> 30-84; Tet ^r	32
pNOT2.9T-1	pNOT19 containing 2.9-kb <i>XbaI-PstI</i> DNA fragment from <i>P. aureofaciens</i> 30-84; Tet resistance gene inserted at <i>NcoI</i> in same orientation as <i>phzO</i> ; Tet ^r Amp ^r	This study
pOT-1	2.5-kb <i>EcoRI-NotI</i> DNA fragment from pNOT2.9T-1 in pNOT19; <i>phzO⁺</i> Tet ^r Amp ^r	This study
pOT1-1	pOT-1 containing 5.6-kb <i>sac</i> genes from pMOB3; <i>phzO⁺</i> Tet ^r Amp ^r Chl ^r	This study

^a Phz⁺/- , the strain does (+) or does not (-) produce phenazines; Phl⁺, production of 2,4-diacetylphloroglucinol; *bla*, β-lactamase; *cat*, chloramphenicol acetyltransferase; Amp^r, ampicillin resistance; Chl^r, chloramphenicol resistance; Kan^r, kanamycin resistance; Rif^r, rifampin resistance; Tet^r, tetracycline resistance.

with tetracycline. The cells were harvested, suspended in fresh medium, and induced with 0.5 mM IPTG. PCA was added to a final concentration of 0.3 or 0.5 mg/ml from a 25 mM stock solution in 5% (wt/vol) NaHCO₃. Samples were taken at 3-h intervals, extracted, and analyzed for phenazine composition by reverse-phase high-performance liquid chromatography (RP-HPLC).

Gene replacement mutagenesis of *phzO*. A *phzO* knockout mutant of *P. aureofaciens* 30-84 was generated by gene replacement as described by Schweizer (38). Briefly, a 2.5-kb *PvuII* fragment bearing a tetracycline resistance gene from

pALTER-Ex1 (Promega) was inserted into *phzO* at the *NcoI* site. The interrupted gene was subcloned into pNOT19, yielding pOT1, which was digested with *NotI* and ligated with a 5.3-kb pMOB3 *sacB* cassette. The resulting plasmid, pOT1-1, was mobilized into *P. aureofaciens* 30-84 from *E. coli* S-17 (λ-*pir*), and double crossover progeny were selected as described previously (38).

Analysis of phenazine compounds. Phenazine compounds were extracted according to the method of Bonsall et al. (5). Bacterial strains were cultivated for 72 h in LB broth supplemented with 1.5% glucose. The cultures were acidified

FIG. 1. Physical map of the constructs used in this study and description of the *phz* operon (*phzIRXYFABCD*) and *phzO* region in *P. aureofaciens* 30-84. The phenazine operon has been described previously (33). The *lux* boxes upstream of *phzI* and *phzX* are represented by ■. ► indicates the orientation of the *lac* promoters in pUT-Km and pUCP26. *ggt* indicates the position of an open reading frame with similarity to gamma-glutamyl transpeptidase. The restriction enzymes indicated on the map are B, *Bgl*II; N, *Nco*I; O, *Not*I; P, *Pst*I; R, *Eco*RI; and X, *Xba*I.

with 10% trifluoroacetic acid (TFA) and then extracted twice with ethyl acetate. The organic phase containing the phenazines was evaporated to dryness and suspended in 35% acetonitrile (ACN)–0.1% TFA.

Since phenazine-producing *Pseudomonas* spp. often produce mixtures of phenazine compounds, a generalized HPLC protocol for detection of these metabolites was developed. The protocol utilized a NOVA-PAK C₁₈ reverse-phase Radial-PAK cartridge (4 μ m, 8 by 100 mm) (Waters Corp., Milford, Mass.) and solvent conditions consisting of a 2-min initial wash with 35% ACN–0.1% TFA in H₂O followed by a 25-min linear gradient to 100% ACN–0.1% TFA at a flow rate of 1.0 ml/min. The Waters HPLC system included a 710B WISP, 510 pumps, and a 680 automated gradient controller with a 990 photodiode array detector (Waters Corp.). Phenazine compounds were identified by retention time and UV spectrum. Standards included compounds purified from well-characterized strains (27, 32) and chemically synthesized compounds (PCA, 2-OH-PCA, and 2-OH-PHZ) obtained from Colour Your Enzyme (Bath, Ontario, Canada). Although the protocol allowed simultaneous identification of phenazine compounds including unsubstituted phenazine, PCA, 2-OH-PCA, 2-OH-PHZ, chlororaphin, and 1-OH-PCA, it failed to clearly separate PCA and 2-OH-PCA. 2-OH-PHZ, which is formed by spontaneous decarboxylation of 2-OH-PCA (see below), was therefore used as an indicator of 2-hydroxyphenazine synthesis, and when necessary, the presence of 2-OH-PCA in samples containing PCA was determined by peak purity and spectral analyses using the Waters 991 photodiode array (Waters Corp.).

Fungal inhibition assay. The inhibition of hyphal growth of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas* spp. strains 30-84, 30-84mxO, 2-79, and 2-79 harboring pUCP2.9XP was assayed as described by Ownley et al. (30) using Kanner agar supplemented with potato extract (KMPE), which supports the production of phenazine compounds. Plates were incubated at room temperature in the dark and radial growth of the fungus was measured after 5 days. The experiment was repeated twice with 7 or 8 replicates each time. Inhibition of

mean fungal radial growth by each bacterial strain was analyzed for significance by the Student *t* test at a *P* level of 0.05.

Nucleotide sequence accession number. The nucleotide sequence for the *phzO* gene has been deposited in GenBank under accession number AF230879.

RESULTS

Localization of the 2-hydroxyphenazine gene. Previous studies by Pierson and Thomashow (32) identified two cosmids, pLSP259 and pLSP282, from a genomic library of *P. aureofaciens* 30-84 that were able to restore Phz[−] mutants of 30-84 to production of PCA, 2-OH-PCA, and 2-OH-PHZ. These cosmids contain identical 11.2- and 9.2-kb *Eco*RI fragments, and an additional 3.8-kb *Eco*RI fragment is present in pLSP282 (Fig. 1). To determine whether pLSP259 and pLSP282 are sufficient to enable the synthesis of PCA, the two hydroxyphenazines, or all three products, each cosmid was introduced into *P. fluorescens* strains 2-79, M4-80R, and Q8r1-96 and the cosmid's presence was confirmed by PCR with *phzXY*-specific primers. Phenazine compounds produced by the transformed strains were extracted and analyzed by RP-HPLC. Transformants of all three strains harboring either cosmid produced both PCA and the 2-hydroxyphenazines (Table 2), indicating that pLSP259 and pLSP282 contain the necessary information required for the synthesis of all three compounds.

TABLE 2. Phenazine production as the result of introduction of plasmids into *P. fluorescens* strains

Plasmid	Phenazine compound	Result in recipient <i>P. fluorescens</i> strain:		
		2-79	Q8r1-96	M4-80R
pLSP282	PCA	+	+	+
	2-OH-PHZ	+	+	+
pLSP259	PCA	+	+	+
	2-OH-PHZ	+	+	+
pUT-Km30-84	PCA	+	+	+
	2-OH-PHZ	—	—	—
pLSP282Δ20-9	PCA	+	N/A ^a	N/A
	2-OH-PHZ	—	N/A	N/A
pLSP282Δ30-8	PCA	+	N/A	N/A
	2-OH-PHZ	—	N/A	N/A
pUCP4.5	PCA	+	N/A	N/A
	2-OH-PHZ	—	N/A	N/A
pUCP2.9	PCA	+	N/A	N/A
	2-OH-PHZ	+	N/A	N/A

^a N/A, not analyzed.

We next determined whether the core phenazine operon, *phzXYFABCD*, which was present in both cosmids, was sufficient for the synthesis of the three phenazine products. Previous studies (33) had suggested that in *P. aureofaciens* 30-84, the C-terminal 28 amino acids of PhzC were necessary for the synthesis of 2-OH-PCA and 2-OH-PHZ. PhzC is a 278-amino acid, 30.3-kDa protein with 94% amino acid sequence identity to PhzF from *P. fluorescens* 2-79. These proteins have no common motifs or other similarities with other proteins of known function, but PhzF is absolutely required for the synthesis of PCA in strain 2-79 (27). A pairwise alignment of the terminal 28 amino acids of the two proteins revealed three conservative substitutions: lysine at position 251 in strain 30-84 instead of arginine in strain 2-79; glutamic acid at position 257 instead of aspartic acid; and valine at position 269 instead of isoleucine. To determine the biosynthetic potential of *phzXYFABCD*, the core operon was cloned downstream of a *tac* promoter and transposed from pUT-Km/30-84 into the genomes of *P. fluorescens* 2-79, Q8r1-96, and M4-80R. Transposition in each recipient was confirmed by PCR with *phzXY*-specific primers. RP-HPLC revealed that all three transformed strains produced PCA but not 2-OH-PCA or 2-OH-PHZ (Table 2), indicating that the core genes from strain 30-84 do not contain the information necessary for the synthesis of the 2-hydroxyphenazines.

The regions upstream and downstream of *phzXYFABCD* were next analyzed for genes enabling the conversion of PCA to 2-hydroxyphenazine derivatives. *P. fluorescens* 2-79 harboring either pLSP282Δ20-9, containing the 3.8- and 11.2-kb *EcoRI* fragments 5' to the *phz* operon, or pLSP282Δ30-8, containing the 11.2-kb fragment (32) (Fig. 1), produced only PCA (Table 2), suggesting that the genes required for 2-hydroxyphenazine synthesis do not reside upstream of the core locus. The remaining 4.5-kb fragment downstream of the phenazine operon in pLSP282 was cloned into the broad-host-range vector pUCP26. A smaller 2.9-kb *XbaI-PstI* fragment also was cloned into pUCP26 in the opposite orientation. Plasmid pUCP2.9XP contained the C-terminal region of *phzD* and downstream sequences under the control of the vector's *lac*

promoter. Both plasmids were introduced into *P. fluorescens* 2-79 and the phenazines were extracted for RP-HPLC analysis. Strain 2-79 containing pUCP2.9XP, but not pUCP4.5, produced 2-OH-PHZ in addition to the PCA (Fig. 2), indicating that the 2.9-kb DNA fragment lacked a promoter, was colinear with the phenazine biosynthetic locus, and contained the gene(s) required for the conversion of PCA.

DNA sequence analysis. The 2.9-kb *XbaI-PstI* fragment from *P. aureofaciens* 30-84 was sequenced in both directions and compiled with Omega. Computer analysis revealed a large open reading frame, designated *phzO*, located 271 nucleotides downstream from *phzD* and preceded by a well-conserved ribosome binding site, GAGG. *phzO* encoded a 491-amino acid protein with a calculated molecular mass of 55.1 kDa. Homology searches with the deduced amino acid sequence revealed similarity to bacterial aromatic hydroxylases and monooxygenases (Table 3). Phylogenetic analysis of these aligned protein sequences resulted in the tree shown in Fig. 3. The high bootstrap values (from 1,000 resamplings) showed the robustness of these groups. A second open reading frame with the initiation codon GTG, preceded by the ribosome binding site GGAG and encoding a putative polypeptide with significant similarity (BLAST values of $6.6e^{-43}$) to gamma-glutamyl transpeptidase enzyme precursor proteins, was identified 656 bp downstream of the *phzO* termination codon.

Expression and functional analysis of PhzO. The *phzO* gene from *P. aureofaciens* 30-84 was cloned in pUCP26 under the control of the *lac* promoter and expressed in *E. coli* JM109. Cells from induced cultures expressing PhzO produced a unique band of approximately 55 kDa on SDS-polyacrylamide gels, in good agreement with the size predicted by nucleotide sequence analysis. Induced cultures of *E. coli* expressing PhzO, either in pUCP26 or in pGEM-T Easy, converted PCA (0.3 or 0.5 mg/ml in 5% NaHCO₃) to 2-OH-PCA and 2-OH-PHZ within 3 h, whereas no such conversion occurred in control cultures harboring only the respective vectors (Fig. 2). These results indicate that PhzO, independent of up- and downstream sequences, is sufficient to hydroxylate PCA. To determine whether PhzO is responsible for this reaction in *P. aureofaciens* 30-84, a tetracycline resistance gene was inserted into *phzO* and introduced in the genome by homologous recombination. *P. aureofaciens* 30-84mxO produced PCA but not 2-OH-PCA and 2-OH-PHZ. Finally, to test the hypothesis that the conversion of 2-OH-PCA to 2-OH-PZ occurs spontaneously in the absence of enzymatic activity, as suggested previously (13), solutions of synthetic 2-OH-PCA were incubated for 18 h in 0.1 M sodium phosphate buffer at pHs of 4.0, 6.0, 7.0, and 8.0, extracted, and analyzed by RP-HPLC. At pH 4.0, 2-OH-PZ accounted for only 0.2% of the total phenazine present after 18 h, but at pHs of 6.0, 7.0, and 8.0, 33.3, 74, and 64% of the 2-OH-PCA initially present was converted to 2-OH-PZ.

Conservation of PhzO among phenazine-producing fluorescent *Pseudomonas* spp. A 2.1-kb probe containing the 1.5-kb *phzO* gene and flanking regions was hybridized to total genomic DNA from 20 known phenazine-producing fluorescent pseudomonads to determine whether the gene is unique to producers of 2-hydroxyphenazines or if it also is conserved in other phenazine-producing strains. All seven strains of *P. aureofaciens* contained sequences that hybridized to the probe

FIG. 2. HPLC analyses of phenazine compounds produced by *P. fluorescens* 2-79 harboring the pUCP26 vector (a), *P. fluorescens* 2-79 harboring pUCP2.9XP and containing *phzO* (b), *E. coli* JM109 harboring pUCP26 (c), *E. coli* JM109 harboring pUCP2.9XP and containing *phzO* (d), and peak identity of PCA and 2-OH-PHZ confirmed by spectral analysis (e). Retention times for PCA and 2-OH-PHZ are 14.1 and 11.4 min, respectively. Absorption maxima for PCA are 248 and 371 nm. Absorption maxima for 2-OH-PCA are 257 and 369 nm. Absorption maxima for 2-OH-PHZ are 257, 368, and 387 nm.

(Fig. 4), and each produced 2-OH-PCA and 2-OH-PHZ in addition to PCA, as determined by RP-HPLC. Two additional strains, *P. chlororaphis* 9446 and *P. aeruginosa* 25011, contained a faintly hybridizing band. However, no 2-hydroxylated phenazines were found in the extracts from cultures of these strains (data not shown), which previously were reported to produce chlororaphin and aeruginosins A and B, respec-

tively (24, 54). No hybridization was detected between the *phzO* probe and DNA from *P. chlororaphis* strains ATCC 17411 and ATCC 17809, *P. aeruginosa* strains PA01, PAK-N1, PAK-NP1, PAK-NP2, ATCC 25007, and ATCC 25011, or the PCA-producing *P. fluorescens* strains 2-79, UQ 112, UN 4127, and UN 15 (Fig. 4), even after very heavy overexposure of the films (data not shown).

TABLE 3. Proteins displaying similarity to PhzO

Organism	Protein name	Enzyme	NCBI accession no.	Identity (%) ^a	Similarity (%) ^a	Z score ^a
<i>Bacillus thermoleovorans</i> A2	PheA	Phenol hydroxylase	AAC38324	28.1	37.9	42.1
<i>Klebsiella pneumoniae</i>	HpaA	4-hydroxyphenylacetate-3-hydroxylase	AAC37120	24.8	36.5	44.0
<i>Escherichia coli</i> ATCC 11105	HpaB	4-hydroxyphenylacetate hydroxylase	CAA82321	25.2	36.5	35.8
<i>Ralstonia pickettii</i> DTP0602	HadA	Chlorophenol-4-hydroxylase	BAA13105	26.7	39.9	64.4
<i>Pseudomonas aeruginosa</i> PAO1	PvcC	4-hydroxylphenylacetate hydroxylase	AAC21673	24.5	35.5	61.8
<i>Burkholderia cepacia</i> AC1100	TftD	Chlorophenol-4-monooxygenase	AAC23548	25.1	37.1	65.0
<i>Photorhabdus luminescens</i>	HpaB	4-hydroxylphenylacetate hydroxylase	AAC08739	24.0	35.9	37.0

^a Identities, similarities, and Z scores were determined by the program BESTFIT from the Genetics Computer Group package.

FIG. 3. Phylogenetic relationship between PhzO and various bacterial aromatic monooxygenases. The neighbor-joining tree with bootstrap support was constructed and visualized by using the CLUSTAL W and TreeView version 1.5.0 programs (31), respectively.

Fungal inhibition assays. Assays were conducted in vitro to determine if strains producing hydroxyphenazine compounds inhibited the hyphal growth of *G. graminis* var. *tritici* more than those producing only PCA. The radial growth of the fungus on plates in the presence of strain 30-84 was significantly less than that in the presence of the mutant 30-84mxO, which produced only PCA (18 versus 22 mm, $P \leq 0.05$). Similarly, *P. fluorescens* 2-79(pUCP2.9XP), transformed to hydroxyphenazine production, was more inhibitory than wild-type 2-79 (14 versus 17 mm, $P \leq 0.05$).

DISCUSSION

Results of the current study show clearly that *phzXYFABCD*, the core phenazine biosynthetic operon of strain 30-84, is responsible only for the synthesis of PCA (Fig. 5). When transformed with these genes, the sole phenazine product synthesized by *P. fluorescens* strains Q8r1-96 and M4-80R (which themselves do not produce phenazines) was PCA. A novel gene designated *phzO* was identified immediately downstream of the core biosynthetic operon of strain 30-84. Phenazine-nonproducing strains transformed with *phzXYFABCD* and *phzO*, or *P. fluorescens* 2-79 transformed with *phzO*, synthesized hydroxyphenazine compounds in addition to PCA, and *E. coli* expressing *phzO* rapidly converted exogenously supplied PCA to hydroxyphenazine products. Finally, a mutant of 30-84 inactivated in *phzO* produced only PCA. These results are consistent with those of an earlier study (27) suggesting that minor sequence differences between PhzF of strain 2-79 and PhzC of strain 30-84 are insufficient to account for the differences in the products synthesized by the two strains and implicating additional determinants of phenazine product specificity. This hypothesis is further supported by data reported by Chin-A-Woeng et al. (8), who recently described an aminotransferase gene designated *phzH* located downstream of the phenazine operon in *P. chlororaphis* PCL1391. PhzH was found to be responsible for the conversion of PCA to phenazine-1-carboxamide (chlororaphin), the green phenazine compound characteristic of *P. chlororaphis*. It thus appears that the presence of species-specific phenazine-modifying genes adja-

cent to the core biosynthetic locus may be a common feature among fluorescent *Pseudomonas* spp.

That PhzO belongs to a recently defined (14) family of two-component nonheme flavin-diffusible bacterial aromatic monooxygenases (TC-FDMs) is supported by results of both pairwise comparisons (high Z scores) and multiple sequence alignments (high bootstrap values). These enzymes are NAD(P)H-dependent flavoproteins that lack the defined GXGXXG FAD/NADH binding site typical of aromatic monooxygenases. Instead, they function in concert with a reductase component that uses NAD(P)H to generate a reduced flavin. The flavin then diffuses to the oxygenase, where it serves as a cosubstrate in the oxidation of aromatic compounds by molecular oxygen (14, 53). TC-FDMs hydroxylate aromatic substrates in either the *ortho* or the *para* position and include both dehalogenating (HadA from *Ralstonia pickettii*, PheA from *Bacillus thermoleovorans*, and TftD from *Burkholderia cepacia*) and nondehalogenating (HpaB from *E. coli*, HpaB from *Photorhabdus luminescens*, HpaA from *Klebsiella pneumoniae*, and PvcC from *P. aeruginosa*) enzymes (10, 14, 16, 23, 34, 42, 43). Many require the presence of an additional 19- to 21-kDa "coupling" subunit (16, 23, 34, 43) to provide reduced flavin, but at least for HpaB, the archetype of this family, this requirement can be satisfied with reduced flavin adenine dinucleotide provided exogenously or generated by an alternative flavin reductase (14, 53). This apparently is also the case for PhzO, since the cloned gene in either pUCP2.9XP or pGEM-PHZO (which contained very little flanking sequence from *P. aureofaciens* 30-84) was sufficient to catalyze the conversion of PCA to 2-hydroxyphenazines in *E. coli*. Whereas the genes encoding the oxidase and reductase components of most known TC-FDM enzymes are situated near one other on the chromosome (14), we found no detectable similarity with known flavin reductases in the 1.3-kb DNA segment downstream of *phzO*. Although a functionally "dedicated" reductase may be encoded elsewhere in the genome of strain 30-84, such an enzyme clearly is not required for phenazine 2-hydroxylation in *E. coli*. The apparent absence of a linked reductase gene and the relatively low level of overall homology between PhzO and other members of the TC-FDM family distinguish this phenazine-modifying enzyme from other oxygenases.

Earlier, Flood et al. (13) in a study with deuterated precursors revealed that hydroxylated phenazines are synthesized in *P. aureofaciens* through the formation of a hypothetical arene intermediate in the following order: PCA→2-OH-PCA→2-OH-PHZ (13). The authors also concluded that the hydroxylation of PCA occurred inefficiently, since PCA was more abundant in the extracts than were the hydroxylated derivatives. Based on the results of our study, we speculate that 2-hydroxylation of PCA is carried out in *P. aureofaciens* by a nonheme, flavin-diffusible monooxygenase, PhzO, which adds a hydroxyl group to PCA at the *ortho* position relative to the carboxyl group, resulting in the synthesis of 2-OH-PCA (Fig. 5). The reaction presumably also requires a yet-unidentified, highly active reductase, NAD(P)H, flavin, and O₂. As speculated previously (13), the subsequent decarboxylation of 2-OH-PCA to 2-OH-PHZ occurs spontaneously in the absence of enzymes. Up to 74% of 2-OH-PCA in phosphate buffer at pH 7 was converted to 2-OH-PHZ after 18 h, whereas lesser amounts (33 and 62%) were converted in buffers at pH values

FIG. 4. Southern hybridization of *phzO* probe to total genomic DNA from 20 phenazine-producing *Pseudomonas* strains. Total DNA samples were digested with endonucleases *Pst*I and *Eco*RI.

of 6 and 8, respectively (G. Phillips and L. S. Thomashow, unpublished data).

We screened a collection of phenazine-producing *Pseudomonas* spp. for the presence of *phzO* by Southern hybridization (Fig. 4). Our results indicate that this gene is found almost exclusively in isolates of *P. aureofaciens*. The only two non-*P.*

aureofaciens strains that hybridized with the *phzO* probe were *P. chlororaphis* 9446 and *P. aeruginosa* 25011 (Fig. 4). However, it is possible that these strains do not have the *phzO* homologue, since in both cases the hybridization signal was very weak, the size of the hybridizing fragment was different from that in *P. aureofaciens* strains, and no hydroxylated phenazines

FIG. 5. Proposed mechanism for the production of 2-hydroxyphenazine-1-carboxylic acid and 2-hydroxyphenazine in *P. aureofaciens* 30-84.

were detected in the culture extracts. Based on these findings, we speculate that *phzO* (and probably *phzH*) is a species-specific gene in fluorescent *Pseudomonas* spp. Moreover, the fact that all the tested strains possess a well-conserved core phenazine locus (D. M. Mavrodi and L. S. Thomashow, unpublished observation) may indicate that the acquisition of phenazine-modifying genes by phenazine-producing pseudomonads is a fairly recent event.

Interest in strains of *P. aureofaciens* frequently has centered on their ability to suppress soilborne plant pathogens (4, 32, 40, 45). We used derivatives of strain 30-84 mutated in *phzO* and strain 2-79 transformed with *phzO* to evaluate the importance of hydroxylated phenazines in biological control activity against *G. graminis* var. *tritici* in vitro. For both strains, the ability to produce hydroxyphenazine compounds was correlated with greater antifungal activity than was production of PCA alone. These results are consistent with the findings of Smirnov and Kiprianova (40), who compared the inhibitory effects of PCA, 2-OH-PCA, and 2-OH-PHZ against a variety of bacterial and fungal animal and plant pathogens and found that in all cases the 2-hydroxyphenazines exhibited stronger bacteriostatic and fungistatic activity. We have recently demonstrated that the introduction of the core biosynthetic genes in other biocontrol microorganisms resulted in increased suppression of certain phytopathogenic fungi (46a; Huang et al., unpublished data). The *phzO* gene from *P. aureofaciens* 30-84 is an attractive target for such genetic manipulations because of the wide antimicrobial and antifungal activity of 2-hydroxyphenazines, which, on the other hand, exhibit little or no toxicity to fish, insects, or mammals (29, 47).

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