

A Seven-Gene Locus for Synthesis of Phenazine-1-Carboxylic Acid by *Pseudomonas fluorescens* 2-79

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Pseudomonas fluorescens 2-79 produces the broad-spectrum antibiotic phenazine-1-carboxylic acid (PCA), which is active against a variety of fungal root pathogens. In this study, seven genes designated *phzABCDEFGHI* that are sufficient for synthesis of PCA were localized within a 6.8-kb *BglIII-XbaI* fragment from the phenazine biosynthesis locus of strain 2-79. Polypeptides corresponding to all *phz* genes were identified by analysis of recombinant plasmids in a T7 promoter/polymerase expression system. Products of the *phzC*, *phzD*, and *phzE* genes have similarities to enzymes of shikimic acid and chorismic acid metabolism and, together with PhzF, are absolutely necessary for PCA production. PhzG is similar to pyridoxamine-5'-phosphate oxidases and probably is a source of cofactor for the PCA-synthesizing enzyme(s). Products of the *phzA* and *phzB* genes are highly homologous to each other and may be involved in stabilization of a putative PCA-synthesizing multienzyme complex. Two new genes, *phzX* and *phzY*, that are homologous to *phzA* and *phzB*, respectively, were cloned and sequenced from *P. aureofaciens* 30-84, which produces PCA, 2-hydroxyphenazine-1-carboxylic acid, and 2-hydroxyphenazine. Based on functional analysis of the *phz* genes from strains 2-79 and 30-84, we postulate that different species of fluorescent pseudomonads have similar genetic systems that confer the ability to synthesize PCA.

Certain members of the genus *Pseudomonas* produce diverse low-molecular-weight ("secondary") metabolites including nitrogen-containing heterocyclic pigments known as phenazine compounds (5, 19). Phenazines are synthesized by a limited number of bacterial genera including *Pseudomonas*, *Burkholderia*, *Brevibacterium*, and *Streptomyces* (38). Almost all phenazines exhibit broad-spectrum activity against various species of bacteria and fungi (32). This activity is connected with the ability of phenazine compounds to undergo oxidation-reduction transformations and thus cause the accumulation of toxic superoxide radicals in the target cells (15). Some phenazine compounds can act as bacterial virulence factors. For example, pyocyanin, produced by the opportunistic pathogen *Pseudomonas aeruginosa* during cystic fibrosis, has been shown to inhibit the ciliary function of respiratory epithelial cells (40). Phenazine antibiotics produced by the biocontrol strains *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 are major factors in the ability of these strains to inhibit the growth of fungal root pathogens. Moreover, studies involving phenazine-deficient mutants have clearly demonstrated that antibiotic production in natural habitats plays an important role in the ecological competence and long-term survival of these strains in the environment (21). Over 50 naturally occurring phenazine compounds have been described, and certain bacterial producers are able to synthesize mixtures of as many as 10 different phenazine derivatives at one time (32, 38). Growth conditions

also may influence the number and types of phenazines synthesized by an individual strain (38).

Early studies with radiolabeled precursors revealed tight links in several microorganisms between biosynthesis of phenazine compounds and the shikimic acid pathway (38). Phenazine-1,6-dicarboxylic acid is believed to be the first phenazine formed and to be the one from which others are derived (19). It also was proposed that the phenazine nucleus is formed by the symmetrical condensation of two molecules of chorismic acid and that enzymes involved in this conversion must have many features in common with anthranilate synthases (17). Despite intensive biochemical studies, the biosynthetic intermediates have not been identified and little is known about the genetics of phenazine synthesis (38). To date, the best-studied phenazine genes are those cloned from *P. aureofaciens* 30-84 (26, 27). The products of the *phz* structural genes from strain 30-84 are similar to enzymes from the shikimic acid and tryptophan biosynthetic pathways, confirming predictions from earlier biochemical analyses. Two other genes, *phzI* and *phzR*, encode parts of a quorum-sensing circuit that regulates phenazine production in *P. aureofaciens* 30-84 in a cell density-dependent manner (26).

In this paper, we present organizational and functional analyses of the complete genetic locus for phenazine-1-carboxylic acid biosynthesis from *P. fluorescens* 2-79. Two new genes from the homologous locus of *P. aureofaciens* 30-84 also are described, and the structure and function of the biosynthetic gene clusters from the two strains are compared. Results of this study suggest that the mechanism of phenazine biosynthesis is highly conserved among fluorescent *Pseudomonas* species.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. A spontaneous rifampin-resistant derivative of *P. fluorescens* 2-79 was used in all studies. *P. fluorescens* 2-79 and *P. aureofaciens*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>P. fluorescens</i> 2-79	Phz ⁺ wild type	36
<i>P. fluorescens</i> 2-79RN ₁₀	Phz ⁺ Rif ^r Nal ^r	36
<i>P. fluorescens</i> 2-79MXC	Phz ⁻ Rif ^r <i>phzC</i> ::Kan ^r	This study
<i>P. fluorescens</i> 2-79MXD	Phz ⁻ Rif ^r <i>phzD</i> ::Kan ^r	This study
<i>P. fluorescens</i> 2-79MXE	Phz ⁻ Rif ^r <i>phzE</i> ::Kan ^r	This study
<i>P. fluorescens</i> 2-79MXG	Phz ⁺ Rif ^r <i>phzG</i> ::Kan ^r	This study
<i>P. fluorescens</i> 2-79.8A	Phz ⁻ Rif ^r <i>phzE</i> ::Tn5	37
<i>P. aureofaciens</i> 30-84	Phz ⁺ wild type	25
<i>E. coli</i> JM109	F' <i>traD36 proA</i> ⁺ <i>proB</i> ⁺ <i>lacI</i> ^q <i>lacZΔM15/recA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB) mcrA</i>	Promega
<i>E. coli</i> HB101	Δ(<i>gpt-proA</i>)62 <i>leuB6 thi-1 lacY1 hsdS_B20 recA rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44 mcrB_B</i>	1
<i>E. coli</i> S17-1	<i>thi pro hsdR hsdM recA rpsL RP4-2 (Tet^r::Mu) (Kan^r::Tn7)</i>	31
<i>E. coli</i> BL21	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm</i>	35
<i>E. coli</i> BL21(DE3)	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm (λcIts857 ind1 Sam7nin5 lacUV5-T7gene1)</i>	35
<i>E. coli</i> BL21(DE3)/pLysS	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm (λcIts857 ind1 Sam7nin5 lacUV5-T7gene1)pLysS</i>	23
<i>E. coli</i> C2110	K-12 <i>gyrA polA1 his rha</i>	34
Plasmids		
pBluescript II KS(SK)	ColE1 f1(+/-) <i>bla</i>	Stratagene
pT7-5, pT7-6	T7 promoter ColE1 <i>bla</i>	1
pET-3a	T7 promoter ColE1 <i>bla</i>	35
pGP1-2	Source of T7 RNA polymerase p15A	1
pUC4K	Source of Kan ^r cassette ColE1 <i>bla</i>	Pharmacia
pJQ200SK	p15A <i>aacC1 Mob</i> ⁺ <i>sacB</i>	29
pHoHo	Tn3 delivery vector <i>lacIZYA</i> ⁺ <i>tnpA bla</i>	34
pSShe	Helper plasmid for Tn3 mutagenesis; <i>tnpA cat</i>	34
pRK2013	Helper plasmid; ColE1 Kan ^r Mob ⁺ Tra ⁺	9
pPHZ108A	pLAFR3 containing <i>P. fluorescens</i> 2-79 genomic DNA, Phz ⁺	37
pT7-5FABCD	pT7-5 containing 5.7-kb <i>EcoRI-HindIII</i> fragment with <i>phzFABCD</i> genes from <i>P. aureofaciens</i> 30-84	This study
pT7-5X-D	pT7-5 containing 6.9-kb <i>PstI-HindIII</i> fragment with <i>phzXYFABCD</i> genes from <i>P. aureofaciens</i> 30-84	This study
pT7-6A-G	pT7-6 containing 6.9-kb <i>BglII-XbaI</i> fragment from pPHZ108A with <i>phzABCDEFGF</i> genes	This study
pT7-6ABCD	pT7-6 containing 4.5-kb <i>BglII-PstI</i> fragment from pPHZ108A with <i>phzABCD</i> genes	This study
pT7-6AB	pT7-6 containing 1.3-kb <i>BglII-EcoRV</i> fragment from pPHZ108A with <i>phzAB</i> genes	This study
pT7-6B	pT7-6 containing 1.4-kb <i>XhoI-EcoRV</i> fragment from pPHZ108A with <i>phzB</i> gene	This study
pT7-5CD	pT7-5 containing 2.0-kb <i>EcoRI-KpnI</i> fragment from pPHZ108A with <i>phzCD</i> genes	This study
pT7-5CDE	pT7-5 containing 4.3-kb <i>EcoRI-BglII</i> fragment from pPHZ108A with <i>phzCDE</i> genes	This study
pT7-6CDEFG	pT7-6 containing 5.8-kb <i>EcoRI-XbaI</i> fragment from pPHZ108A with <i>phzCDEFG</i> genes	This study
pT7-5G	pT7-5 containing 1.5-kb <i>BglII-XbaI</i> fragment from pPHZ108A with <i>phzG</i> gene	This study
pET-3XY	pET-3a containing <i>phzX</i> and <i>phzY</i> genes	This study

^a *bla*, β-lactamase; *aacC1*, gentamicin acetyltransferase-3-1; *cat*, chloramphenicol acetyltransferase; Kan^r, kanamycin resistance; Rif^r, rifampin resistance; Nal^r, nalidixic acid resistance.

30-84 were grown at 28°C in Luria-Bertani (LB) broth (1). *E. coli* strains were grown at 28 or 37°C in LB or M9 minimal medium (1). Antibiotic supplements were used at the following concentrations: ampicillin, 80 μg/ml; carbenicillin, 80 μg/ml; rifampin, 75 μg/ml; kanamycin, 30 or 150 μg/ml; tetracycline, 12.5 μg/ml; gentamicin, 10 μg/ml.

Transposon Tn3-*lacZ* mutagenesis. Tn3-*lacZ* insertions were made by using the transposon system described by Stachel et al. (34). The target cosmid, pPHZ108A, was introduced into *E. coli* HB101(pHoHo1, pSShe). To select pPHZ108A::Tn3-*lacZ* derivatives, the strain harboring pPHZ108A, pHoHo1, and pSShe was mated with recipient strain *E. coli* C2110, using *E. coli* HB101(pRK2013) as a helper. The site and orientation of insertions into pPHZ108A were analyzed by restriction mapping. The plasmids were then introduced into the phenazine-1-carboxylic acid (PCA)-nonproducing Tn5 mutant *P. fluorescens* 2-79.8A by triparental matings to test the effect of each Tn3-*lacZ* insertion on PCA production. Expression in transconjugants of the *lacZ* reporter gene, an indicator of transcriptional activity, was detected on LB agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal).

DNA manipulations. Standard methods were used for DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation (1). Total DNA from *P. fluorescens* 2-79RN₁₀ was isolated and purified by a cetyltrimethylammonium bromide miniprep procedure (1). For Southern blotting and hybridization, total DNA samples were digested with restriction endonucleases, separated by electrophoresis in a 0.8% agarose gel, transferred onto a BrightStar-Plus nylon membrane (Ambion, Inc., Austin, Tex.), and hybridized

with a specific DNA probe labeled with the DECAprime-Biotin random priming kit (Ambion, Inc.). DNA-DNA hybrids were detected with the BrightStar nonisotopic detection kit (Ambion, Inc.) as specified by the manufacturer.

Cloning of *phzX* and *phzY* genes from *P. aureofaciens* 30-84. A DNA fragment containing *phzX* and *phzY* genes was amplified with *P. aureofaciens* 30-84 genomic DNA as a template and with oligonucleotide primers 30-84/1 (5'-CAG TTCATCCGGCGGGCTGCAG-3') and 30-84/2 (5'-CCCCTTTCAGTAAGTC TTCCATGATGCG-3'). Target DNA was amplified with Vent DNA polymerase (New England Biolabs, Beverly, Mass.) and the following cycling program: 94°C for 1 min, 64°C for 45 s, and 72°C for 1 min (30 cycles). The 1.2-kb PCR product was cloned into the *SmaI* site of pBluescript II KS, and the resulting plasmid was used to determine the nucleotide sequence.

DNA sequencing and analysis. DNA was sequenced by the dideoxy chain termination method with Sequenase 2.0 (Amersham International, Little Chalfont, United Kingdom). Exonuclease III deletion derivatives were constructed with enzymes supplied by Amersham, as specified by the manufacturer. Sequence data were compiled and analyzed with the GCG package (13). DNA sequences were compiled with GELASSEMBLE, and open reading frames (ORFs) and codon usage were analyzed with MAP and FRAMES. A database search for similar protein sequences was carried out with the BLAST and FASTA network servers at the National Center for Biotechnology Information and the European Molecular Biology Laboratory, respectively. The probable domain homology search was performed with the PROSITE (European Molecular Biology Laboratory, Heidelberg, Germany) and SBASE (International Cen-

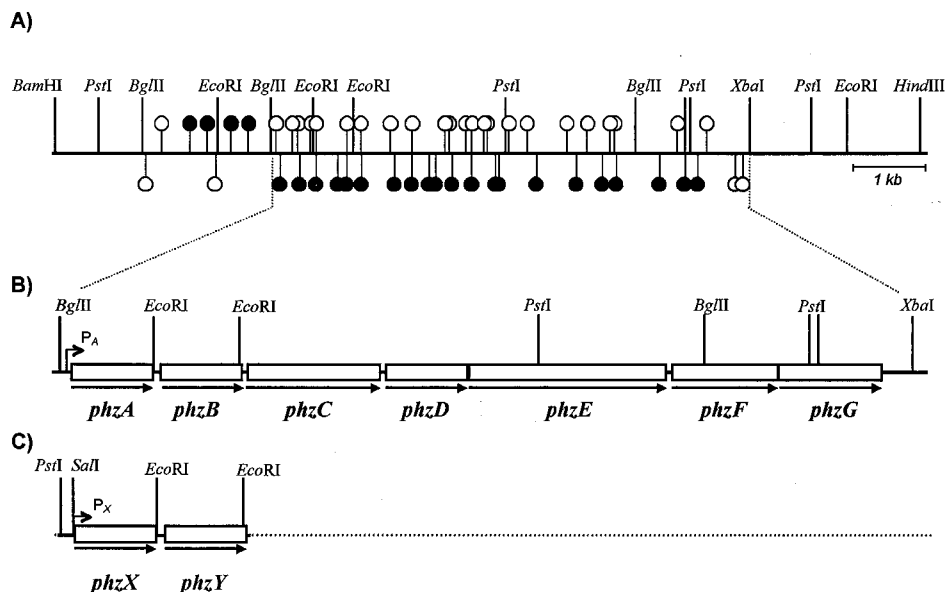


FIG. 1. Physical maps of cosmid clone pPHZ108 (A), the DNA region from *P. fluorescens* 2-79 encoding genes involved in the production of PCA (B), and a portion of the homologous genetic locus from *P. aureofaciens* 30-84 (C). Open boxes indicate genes encoding phenazine biosynthesis enzymes. The direction of the gene transcription is shown by an arrow. The symbols P_A and P_X represent the position and orientation of corresponding promoters. Insertions of Tn3-*lacZ* that interfered with phenazine production are marked on the map of pPHZ108A as \uparrow (Lac^+ phenotype) and \downarrow (Lac^- phenotype).

ter for Genetic Engineering and Biotechnology, Trieste, Italy) computer servers (3, 12). Pairwise alignments were obtained with the GCG GAP program (gap weight = 4).

Analyses of polypeptide gene products. The *P. fluorescens* 2-79 structural *phz* genes were expressed under the control of the T7 promoter in plasmid vectors pT7-5 and pT7-6 in *E. coli* BL21 with pGP1-2 as a source of T7 RNA polymerase and with [^{35}S]methionine for selective labeling of target proteins (1). Labeled proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography.

For expression studies of *phzX* and *phzY* from *P. aureofaciens* 30-84, the genes were cloned into the *NdeI*-*Bam*HI sites of pET-3a vector (35) after amplification with the oligonucleotide primers *phzXNdeI* (5'-TTTTTTCATATGCCTTGCTTCGCTTC-3') and *phzYBamHI* (5'-TTTGGATCCTTAAGTTGGAATGCCTTCG-3') and subsequent cleavage of the PCR product with the corresponding restriction endonucleases. Expression of the *phzX* and *phzY* genes was carried out in *E. coli* BL21(DE3)/pLysS (23). Total cellular protein was separated by SDS-PAGE and stained with Coomassie brilliant blue as described elsewhere (7).

Gene replacement experiments in *P. fluorescens* 2-79. A 1.4-kb Km^r cassette (Pharmacia Biotech, Inc., Uppsala, Sweden) containing the aminoglycoside 3'-phosphotransferase gene was used in gene replacement experiments. Plasmids containing individual *phz* genes that were insertionally inactivated by the antibiotic resistance gene were constructed. Target DNA fragments were then subcloned into pJQ200SK, a gene replacement vector harboring the *sacB* gene as a counterselectable marker (29). The resulting plasmids were transferred into *P. fluorescens* 2-79 via biparental matings with *E. coli* S17-1, followed by selection for the plasmid resistance marker. Subsequent selection was performed on LB agar containing 5% sucrose and kanamycin. Introduced mutations were verified at the DNA level by PCR screening and Southern hybridization.

Extraction and detection of phenazine compounds. Prior to extraction of phenazine compounds, *Pseudomonas* strains were cultivated in LB medium for 3 days at 28°C. *E. coli* BL21(DE3) clones harboring *phz* genes under control of the T7 promoter were grown in LB medium at 28°C without addition of isopropyl- β -D-thiogalactopyranoside (IPTG). The "leaky" *lac* promoter of the λ DE3 lysogen maintained high enough levels of T7 RNA polymerase in the cell to enable the production of detectable amounts of phenazines.

Phenazine compounds were extracted from 3-day-old cultures of *E. coli* and *Pseudomonas* strains with ethyl acetate by the method of Bonsall et al. (4). Filtered crude extracts were subjected to C₁₈ reverse-phase high-performance liquid chromatography (HPLC) (Waters, Symmetry C₁₈; 5- μ m particles of packing material; 3.0 by 150 mm) with a 30- μ l injection volume. The Waters HPLC Integrity System consisted of an Alliance 2690 separation module, a 996 photodiode array detector, and a Thermobeam mass spectrometry detector. Solvent conditions included a flow rate of 350 μ l/min with a 2-min initial condition of 10% acetonitrile–2% acetic acid followed by a 20-min linear gradient to 100% acetonitrile–2% acetic acid. HPLC gradient profiles were monitored at the spec-

tral peak maxima (247.6 and 368.2 nm) that are characteristic of PCA in the designated solvent system. Mass spectrometry conditions included an ion source temperature of 220°C, an expansion region temperature of 80°C, a nebulizer temperature of 84°C, and a helium flow at 15 lb/in².

Nucleotide sequence accession numbers. The GenBank accession numbers for the nucleotide sequence data for *phz* genes from *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 are L48616 and AF007801, respectively.

RESULTS

Mutagenesis of *P. fluorescens* 2-79 with Tn3-*lacZ*. The phenazine biosynthetic locus from *P. fluorescens* 2-79 was cloned previously as a 12-kb *Hind*III-*Bam*HI DNA fragment within plasmid pPHZ108A and characterized by restriction mapping (37). Mutagenesis of pPHZ108A with Tn3-*lacZ*, which can generate fusions in which expression of the *lacZ* gene is regulated by the promoter of the gene bearing the insertion, yielded 57 unique transposon insertions that interfered with phenazine production. These insertions identified two adjacent, divergently transcribed units of approximately 6 and 0.75 kb that were strongly and weakly expressed, respectively, under conditions favorable to the production of PCA (data not shown). Most of the large transcriptional unit was contained within a 5.4-kb *Bgl*II fragment, with the remainder localized to the adjacent 2.0-kb *Bgl*II-*Xba*I DNA fragment (Fig. 1). Consistent with results from Tn3-*lacZ* mutagenesis, the *Bgl*II fragment alone did not enable PCA production in *P. fluorescens* 2-79.8A, suggesting a requirement for additional downstream sequences.

DNA sequence analysis. The overlapping 5.7-kb *Eco*RI-*Xba*I and 5.4-kb *Bgl*II fragments and the adjacent 1.8-kb *Bgl*II fragment were subcloned into pBluescript II KS and SK cloning vectors and used to determine the nucleotide sequence of an 8,505-bp DNA segment from pPHZ108A. Computer analysis of the DNA sequence within the large transcriptional unit revealed seven ORFs with high coding probability, designated *phzABCDEFGHI*. Each of these genes is preceded by a well-conserved ribosome binding site. In the *phzD*-*phzE* and *phzF*-

TABLE 2. ORFs in the *phz* loci of *P. fluorescens* and *P. aureofaciens*

Gene	Protein length (residues)	Protein mol mass (kDa)	Similarity to:
<i>phzA</i>	163	18.7	PhzB from <i>P. fluorescens</i> 2-79; PhzX and PhzY from <i>P. aureofaciens</i> 30-84
<i>phzB</i>	162	18.8	PhzA from <i>P. fluorescens</i> 2-79; PhzX and PhzY from <i>P. aureofaciens</i> 30-84
<i>phzC</i>	400	44.0	PhzF from <i>P. aureofaciens</i> 30-84; plant phospho-2-dehydro-3-deoxyheptonate aldolases (DAHP synthases)
<i>phzD</i>	207	23.0	PhzA from <i>P. aureofaciens</i> 30-84; bacterial isochorismatases
<i>phzE</i>	637	69.9	PhzB from <i>P. aureofaciens</i> 30-84; bacterial class I glutamine amidotransferases
<i>phzF</i>	278	30.0	PhzC from <i>P. aureofaciens</i> 30-84; hypothetical proteins ORF o276#3 from <i>E. coli</i> and ORF slr1019 from <i>Synechocystis</i> sp.
<i>phzG</i>	222	24.9	PhzD from <i>P. aureofaciens</i> 30-84; bacterial pyridoxamine-5'-phosphate oxidases
<i>phzX</i>	166	19.2	PhzY from <i>P. aureofaciens</i> 30-84; PhzA and PhzB from <i>P. fluorescens</i> 2-79
<i>phzY</i>	163	18.8	PhzX from <i>P. aureofaciens</i> 30-84; PhzA and PhzB from <i>P. fluorescens</i> 2-79

phzG pairs, the stop (UGA) and start (AUG) codons of the adjacent genes overlap, possibly reflecting their translational coupling. Two additional genes homologous to *phzI* and *phzR* also were identified upstream of the *phzABCDEFG* cluster (20).

Homology searches of deduced amino acid sequences against the Swiss-Prot, GenPept, and PIR protein databases identified a number of proteins with significant similarities to the predicted protein products of the *phzABCDEFG* genes (Table 2). Apart from the results listed and discussed below, products of the *P. fluorescens* 2-79 *phzCDEFG* genes also were highly homologous (above 90% identity and similarity) to the products of the *phzFABCD* genes, respectively, from *P. aureofaciens* 30-84 (27).

No similarities for the PhzA and PhzB proteins, or for the homologous proteins PhzX and PhzY from *P. aureofaciens* 30-84, were identified in database searches. On the other hand, all four polypeptides were remarkably homologous, with similarity increasing toward the C-terminal part of the proteins (62% overall identity and 24.7% similarity) (Fig. 2).

The 400-amino-acid PhzC protein showed 39.1% identity and 46.1% similarity to the AroG 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase from *Lycopersicon esculentum* chloroplasts, as well as to other plant DAHP synthases (Swiss-Prot accession no. P37216, P21357, and P27608). DAHP synthase is the first enzyme of the shikimate pathway and catalyzes the condensation of phosphoenolpyruvate and erythrose-4-phosphate. In contrast to bacterial DAHP synthase isoenzymes, which are feedback inhibited by one of the aromatic amino acids, plant DAHP synthases are not inhibited by aromatic acids but are activated by tryptophan (10, 28).

The *phzD* gene encodes a small protein of 207 amino acid residues that is homologous (46.9% identity and 59.9% similarity) to the 285-amino-acid isochorismatase EntB from *E. coli* (11). Isochorismatase is an enzyme from the biosynthetic pathway of enterobactin—an iron-chelating product derived from chorismic acid and involved in the transport of iron from the bacterial environment into the cell cytoplasm.

The results of BLAST search analyses revealed that PhzE was similar to a large group of enzymes including anthranilate synthase from *Streptomyces venezuelae* (45.9% identity and 33.2% similarity), other bacterial anthranilate synthases, *p*-aminobenzoate synthases, and menaquinone-specific isochorismatase synthases. According to the PROSITE database search results, the C-terminal part of PhzE (amino acid residues 438 to 637) resembles a class I glutamine amidotransferase (GATase) containing a well-conserved putative active site **PFLAVCLSHQVL** (letters in boldface type indicate highly conserved residues, and the essential cysteine is underlined).

GATases are a large group of biosynthetic enzymes able to catalyze the removal and transfer of the ammonia group from glutamine to various substrates, forming a new carbon-nitrogen bond. The GATase domain exists either as a separate polypeptide subunit or as part of a larger polypeptide fused in different ways to a synthase domain (28).

The product of the *phzF* gene shows weak similarity to a number of hypothetical proteins of unknown function listed in the databases. The latter include ORF o276#3 from *Escherichia coli* (GenBank accession no. D90786), ORF slr1019 from *Synechocystis* sp. (GenBank accession no. D90904), and the hypothetical 32.6-kDa protein YHI9 from *Saccharomyces cerevisiae* (Swiss-Prot accession no. P38765).

PhzG shows 29.3% identity and 38.1% similarity to the PdxH pyridoxamine-5'-phosphate oxidase from *E. coli* and to the similar protein from *H. influenzae* (Swiss-Prot accession no.

PhzA	M---PGLSLSGGFNDHLELRKRNATVDQYMRNNGEDLRRRHELFTPDGS	47
PhzX	MLPMPASLSPSGFNDHLELRQKRNATVEQYMRNNGKDLRRRHELFTQDGS	50
PhzB	M-PDSTVQPPIIT-DDT-ELRRKRNATVEQYMRKQGDLRRRHELFTEDGS	47
PhzY	M-SNSAAQLTA-NDTTELRRKRNATVEQYMRKQGDLRRRHELFTEDGT	48
	* .. . * * * * * * * * * * * * * * * * * *	
PhzA	GGSWNTETGEPLVFKGHAKLAALGVWLHQCFFDQWHNVRFVETDNPNHF	97
PhzX	GGSWNTETGKPLVFKGHTKLAALGVWLEKCFDQWHNVRFVETDNPNHF	100
PhzB	GGLWTTDTGAPIVISGKAKLAEHAVVSLKCFDQWVWYVNVKVFETDDPNHI	97
PhzY	GGLWTTDTGAPIVISGKAKLAEHAVVSLKCFDQWVWYVNVKVFETDDPNHI	98
	** *	
PhzA	WVESDGRGTRVPGYPEGYCENHYIHSFELDNGKITQNRREFMNPFEQLRA	147
PhzX	WVESERRGKTLVPGYPEGYCENHYIHSFELDDGKITQSRREFMNPFEQLRA	150
PhzB	WVECDGHGKILFPGYPEGYENHFLHSFELQDGKVKRNREFMNVFQQLRA	147
PhzY	WVECDGHGKILFPGYPEGYENHFLHSFELEDGKVKRNREFMNVFQQLRA	148
	* *	
PhzA	LGIPVPKIKREGIPAS	163
PhzX	LGIPVPRIKREGIPAS	166
PhzB	LGIPVPHIKREGIPA-	162
PhzY	LGIPVPQIKREGIPT-	163
	* *	

FIG. 2. Alignment of the deduced amino acid sequences of the PhzA (*P. fluorescens* 2-79), PhzX (*P. aureofaciens* 30-84), PhzB (*P. fluorescens* 2-79), and PhzY (*P. aureofaciens* 30-84) proteins. Identical (*) and similar (·) amino acid sequences are indicated. Dashes represent gaps inserted in the sequences to improve the alignment.

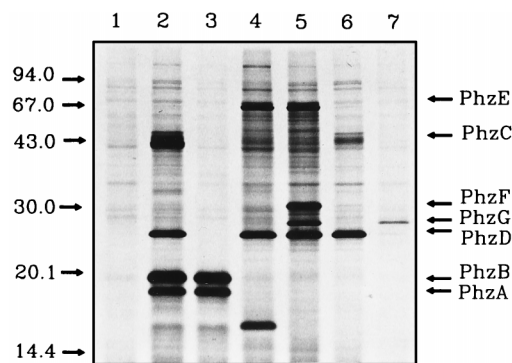


FIG. 3. Autoradiograph of polypeptides labeled with [^{35}S]methionine and resolved by electrophoresis on an SDS-12% polyacrylamide gel. Samples were prepared from *E. coli* BL21 containing pGP1-2 plus the indicated plasmids. The location of DNA fragments inserted in pT7-5 and pT7-6 vectors for gene expression is shown in Fig. 4. Lanes: 1, pT7-6; 2, pT7-6ABCD; 3, pT7-6AB; 4, pT7-6CDE; 5, pT7-6CDEFG; 6, pT7-5CD; 7, pT7-5G. An intense 16-kDa band in lane 4 is a product of a truncated *phzF* gene, and an intense protein band almost overlapping with PhzC in lane 2 is a product of a truncated *phzE* gene. Positions of molecular size markers are given on the left in kilodaltons.

P28225 and P44909). These enzymes are involved in de novo synthesis of pyridoxine (vitamin B₆) and pyridoxal phosphate in bacterial cells, and they convert pyridoxamine-5'-phosphate into pyridoxal-5'-phosphate (18). Moreover, the results of a PROSITE database search revealed that part of PhzG (amino acid residues 192 to 205) possesses an amino acid sequence motif, **LEFWGNGQERLHER** (letters in boldface indicate highly conserved residues), characteristic of all bacterial pyridoxamine-5'-phosphate oxidases studied to date.

Identification of proteins encoded by *phzABCDEFGHI*, *phzX*, and *phzY*. Using a pair of pT7-5 and pT7-6 transcriptional vectors and the two-plasmid expression system of Tabor, we identified products for all of the *phzABCDEFGHI* genes (Fig. 3). The rather high level of expression of all seven genes reflects efficient utilization of the *Pseudomonas* translational signals by the *E. coli* protein-synthesizing machinery.

Despite the very similar sizes of the PhzA and PhzB proteins (18.7 and 18.8 kDa, respectively), they could be resolved by SDS-PAGE (Fig. 3, lanes 2 and 3). The single PhzB polypeptide also was identified by expression from pT7-6B (data not shown). Expression of the *phzC* gene was confirmed with several different plasmids, and in most cases a faint protein band of about 44 kDa was detected (lane 6). The unusual appearance of this protein in the gels probably is a result of partial proteolysis of the PhzC polypeptide in *E. coli* cells. The *phzD*, *phzE*, *phzF*, and *phzG* genes were well expressed, and the sizes of their products estimated by SDS-PAGE were in agreement with those predicted from nucleotide sequences (lanes 2, 4, 5, and 6). The comparatively poor expression of *phzE* (data not shown) and *phzG* (lane 7) genes alone is consistent with their likely translational coupling with the *phzD* and *phzF* genes, respectively. According to results from Northern blotting experiments, the *phzABCDEFGHI* genes form a distinct operon in *P. fluorescens* 2-79 and are transcribed as a single mRNA (20).

The *phzX* and *phzY* genes from *P. aureofaciens* 30-84 were expressed in *E. coli* BL21(DE3)/pLysS with a pET3a translational vector (data not shown). The level of expression was very high, and the PhzX and PhzY proteins (19.2 and 18.8 kDa, respectively) were easily detected after SDS-PAGE by a Coomassie blue staining procedure.

Functional analysis of the *phz* genes. To determine which genes in the phenazine biosynthetic cluster are essential for the

production of PCA, the Kan^r cassette was inserted into *phzC*, *phzD*, *phzE*, and *phzG* and each disrupted gene was introduced into the genome of strain 2-79 by homologous recombination. For unknown reasons, it was not possible to recover *phzA*, *phzB*, or *phzF* recombinants. Insertions of the Kan^r cassette in *phzC*, *phzD*, and *phzE* interfered with phenazine production, and the corresponding mutant strains were completely deficient in PCA biosynthesis (Fig. 4A). *P. fluorescens* 2-79 MXG, bearing a Kan^r insertion within *phzG*, was able to produce PCA, although the yield was only about 1.3% of that of wild-type strain 2-79.

Combinations of genes from the 2-79 phenazine gene cluster also were cloned under control of a T7 promoter and tested in *E. coli* for the ability to enable phenazine production. Products from strain *E. coli* BL21(DE3) expressing pT7-6A-G, which contains the entire biosynthetic gene cluster, consisted almost entirely of PCA, and the yield was nearly equal to that from wild-type *P. fluorescens* 2-79 (Fig. 4B). In contrast, strain BL21(DE3) expressing pT7-6CDEFG, which lacks *phzA* and *phzB*, produced a heterogeneous mixture of nitrogen-containing aromatic and heterocyclic compounds. Analysis of these compounds by mass spectrometry confirmed the presence of smaller amounts of PCA as well as of a mixture of heterocyclic, nitrogen-containing compounds including unsubstituted phenazine. Expression of other combinations of *phz* genes, including *phzAB*, *phzABCD*, *phzCD*, *phzCDE*, and *phzB* or *phzG*, yielded no detectable phenazine products (Fig. 4).

The *phzX* and *phzY* genes occupy the same relative position in the phenazine biosynthetic cluster of *P. aureofaciens* 30-84 as do *phzA* and *phzB* in the 2-79 gene cluster (Fig. 4C), and all four gene products are highly conserved (Fig. 2). To determine whether *phzXY* influences the quality and quantity of compounds produced from the phenazine biosynthetic cluster of strain 30-84, plasmids pT7-5X-D, containing the entire 30-84 biosynthetic cluster, and pT7-5FABCD, containing only the *phzFABCD* genes (Fig. 4C), were expressed in *E. coli* BL21(DE3). As with the cloned genes from 2-79, the complete gene cluster in pT-7X-D enabled the almost exclusive production of PCA. Plasmid pT7-5FABCD, lacking *phzXY*, synthesized smaller amounts of PCA and a mixture of other nitrogen-containing aromatic compounds.

DISCUSSION

Results of structural and functional analyses presented here show that products of the *phzABCDEFGHI* gene cluster are responsible for synthesis of PCA by *P. fluorescens* 2-79. Phenazines are products of the bacterial common aromatic amino acid pathway, with chorismate as the probable branch point intermediate (38), but the precise mechanism of synthesis and the identity of the biosynthetic intermediates remain unknown. Our evidence that the products of *phzC*, *phzD*, and *phzE* have significant homology to well-characterized enzymes of the shikimate and tryptophan biosynthetic pathways is consistent with previous findings (19, 38) and provides new insight into the phenazine biosynthetic pathway in fluorescent *Pseudomonas* spp.

PhzC shows a high degree of similarity to plant DAHP synthases that catalyze the first step of the shikimate pathway. In bacteria, DAHP synthase isoenzymes are regulated transcriptionally as well as through feedback inhibition by specific amino acids or pathway intermediates and thus represent a key control point regulating carbon flow into the shikimate pathway (16). All known DAHP synthases recently were grouped into two distinct classes based on protein sequence similarity. Our sequence data indicate that the PhzC protein from *P.*

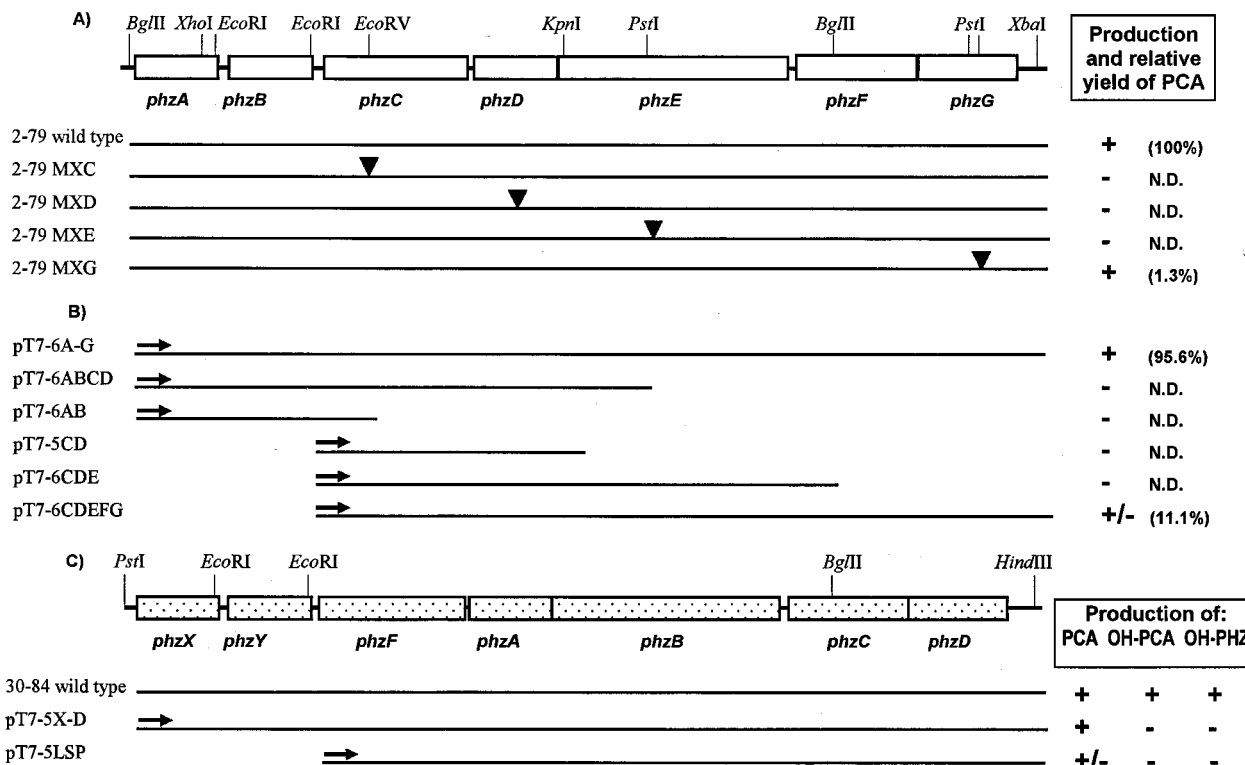


FIG. 4. Analysis of phenazine biosynthesis in *P. fluorescens* 2-79 gene replacement mutants (A) and by *E. coli* BL21(DE3) clones harboring plasmids with different sets of *phz* genes from *P. fluorescens* 2-79 (B) or *P. aureofaciens* 30-84 (C) cloned under the control of a T7 promoter. Restriction maps, locations of individual *phz* genes, and DNA fragments contained within plasmids used in the study are shown. Arrows indicate the position and orientation of the T7 promoter. Solid triangles denote locations of the Kan^r cassette insertions in the chromosome of *P. fluorescens* 2-79 mutants. N.D., not detected. OH-PCA, 2-hydroxyphenazine-1-carboxylic acid; OH-PHZ, 2-hydroxyphenazine.

fluorescens 2-79 is a typical type II enzyme, together with *P. aureofaciens* PhzF and the recently studied DAHP synthases from *Streptomyces coelicolor* and *S. rimosus* (39). Being expressed late in growth, PhzC could function to divert common carbon metabolites into the shikimate pathway, providing the high levels of chorismic acid needed to support the synthesis of PCA, which can accumulate in culture media at concentrations of up to 1 g/liter. The remainder of the enzymatic activities needed for chorismate biosynthesis probably are provided by shikimic acid pathway enzymes encoded by *aroD*, *aroB*, *aroE*, *aroL*, *aroA*, and *aroC*, since these genes are known to be expressed constitutively in pseudomonads (24).

Based on their sequence, it is likely that products of the *phzD* and *phzE* genes act to modify chorismate prior to the condensation reaction resulting in formation of the phenazine nucleus. The first two-thirds of the PhzE protein display relatively weak similarity to component I of bacterial anthranilate synthases. The C-terminal part (amino acid residues 438 to 637) exhibits strong homology to members of class I GATase enzymes, which comprise component II of anthranilate synthases. The PhzE protein is most closely related to a small subset of anthranilate synthases of unusual structure (TrpE from *Streptomyces venezuelae*, TrpE from *Rhizobium meliloti*, and TrpE from *Azospirillum brasilense*) that have evolved as a fusion of genes encoding anthranilate synthase components I and II (2, 8). Component I of anthranilate synthase is a bifunctional enzyme that catalyzes the formation of the aromatic product anthranilate from chorismic acid in two discrete steps (22). The first step is catalyzed by aminodeoxyisochorismate (ADIC) synthase and is thought to involve amination of cho-

risemic acid to ADIC. The second step, which is catalyzed by ADIC lyase, involves elimination of pyruvate and aromatization to form anthranilate. ADIC remains enzyme bound, but it has been demonstrated that a substitution of a single amino acid residue within component I of anthranilate synthase is sufficient to uncouple ADIC synthase and ADIC lyase activity (22). Interestingly, it previously was postulated that ADIC could be a potential precursor for the phenazine compounds iodinin and aminophenoxazinone, produced by cultures of *Brevibacterium iodinum* (30). Collectively, these observations and the rather weak similarity between the PhzE protein and most bacterial anthranilate synthases may indicate that PhzE functions specifically as an ADIC synthase (Fig. 5). The GATase domain might confer upon PhzE the ability to use glutamine as a source for the amination of chorismate. This is consistent with the data of Römer and Herbert, who demonstrated that the amide nitrogen of glutamine serves as the immediate source of nitrogen in the heterocyclic nucleus of phenazine compounds (30).

The product of the *phzD* gene shows a high degree of similarity to bacterial 2,3-dihydro-2,3-dihydroxybenzoate synthases (isochorismatases). The best-studied isochorismatase, EntB from *E. coli*, has a predicted M_r of 32,500 and is active as a pentamer in the isochorismatase reaction, catalyzing the hydrolysis of pyruvate from isochorismic acid (11). The presence of an isochorismatase analog in the *phz* locus might reflect the need for pyruvate hydrolase activity, rather than an ADIC lyase activity (which could be provided by anthranilate synthase), for modification of the immediate precursor of the phenazine nucleus. EntB actually is a bifunctional enzyme (of-

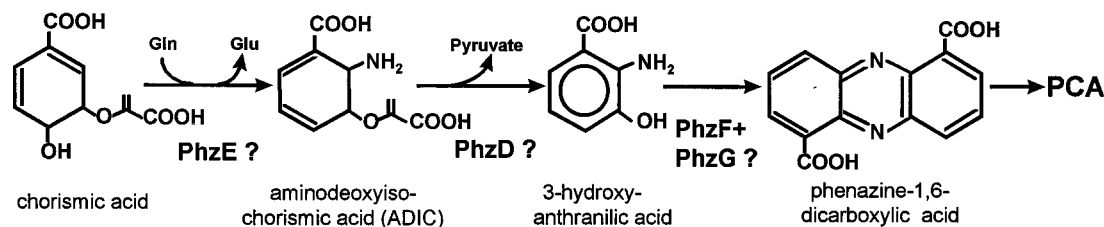


FIG. 5. Proposed action of PhzC, PhzD, PhzE, PhzF, and PhzG in the biosynthesis of PCA.

ten called EntB/G), with EntG activity encoded by the *entB* 3' terminus (33). EntG functions as a part of the EntDEFG enterobactin synthetase multienzyme complex that catalyzes the last step of enterobactin biosynthesis. Interestingly, the PhzD protein resembles a truncated EntB, which lacks 78 amino acid residues at the C terminus. It therefore is probable that PhzD has only isochorismatase activity, which could function to remove the pyruvate side chain from ADIC to yield the putative phenazine precursor 3-hydroxyanthranilate (Fig. 5).

It seems likely that the products of *phzF* and *phzG* function in the condensation of two molecules of 3-hydroxyanthranilate, or a similar precursor, to generate the phenazine nucleus. Sequence analysis of PhzF revealed no motifs or similarities to other proteins of known function. However, PhzG resembles bacterial pyridoxamine-5'-phosphate oxidases that function in the de novo synthesis of pyridoxine (vitamin B₆) and in the conversion of pyridoxamine-5'-phosphate to pyridoxal phosphate, a cofactor for numerous transamination reactions (18). Interestingly, pyridoxal phosphate is required for aminodeoxychorismate lyase (PabC) activity in *E. coli* (14), where the cofactor is proposed to bind via an imine linkage to the 4-amino position of 4-amino-4-deoxychorismate during the synthesis of *p*-aminobenzoic acid (22). While it is tempting to speculate that pyridoxal phosphate may play a similar role in phenazine synthesis, binding at the 2-amino position of the hypothetical precursor 3-hydroxyanthranilate (Fig. 5), no conserved pyridoxal phosphate-binding motifs were identified in PhzF or, indeed, in any of the other *phz* gene products. Thus, the step(s) involved in this final condensation reaction remains obscure.

We also were unable to identify any motifs or similarity between products of *phzA* and *phzB* and protein sequences listed in various databases. However, PhzA and PhzB are remarkably similar to each other (Fig. 2), which strongly suggests that their cognate genes evolved as the result of a duplication event. Homologs of *phzA* and *phzB* also were identified in the phenazine biosynthetic locus from *P. aureofaciens* 30-84. Functional analysis indicated that in both 2-79 and 30-84, these genes influence the kinds and relative amounts of aromatic and heterocyclic compounds synthesized as a result of *phz* gene expression. Thus, *E. coli* expressing the complete *phz* locus synthesized large amounts exclusively of PCA whereas the same host expressing the *phzCDEFG* genes produced large quantities of a mixture of aromatic and heterocyclic nitrogen-containing compounds that included only minor amounts of PCA. PhzA and PhzB may stabilize a multienzyme phenazine biosynthetic complex, whose existence has been postulated previously (6). In their absence, the biosynthetic system still functions, but the specificity and perhaps also the efficiency decrease dramatically.

Results of this study indicate that the phenazine biosynthetic gene cluster from *P. fluorescens* 2-79 is remarkably conserved relative to that previously described from *P. aureofaciens*. In related work, we recently have shown that a portion of the

pyocyanin biosynthetic locus from *P. aureofaciens* PAO1 retains certain of these same organizational and structural features (20). It thus appears that different fluorescent *Pseudomonas* species may have a common pathway, which confers upon them the ability to synthesize the phenazine nucleus.

How, then, can the specific array of phenazine compounds produced by individual species of fluorescent pseudomonads be explained? *P. fluorescens* 2-79 produces only PCA, whereas *P. aureofaciens* 30-84 produces, in addition to PCA, lesser amounts of 2-hydroxyphenazine-1-carboxylic acid and (probably by spontaneous, nonenzymatic decarboxylation) small quantities of 2-hydroxyphenazine (25, 27). The conversion of PCA to 2-hydroxyphenazine-1-carboxylic acid in strain 30-84 previously was attributed to the product of *phzC* (27), which has 93.5% identity and 95.3% similarity to the corresponding PhzF protein from *P. fluorescens* 2-79. Although small differences in sequence between these proteins cannot formally be ruled out as contributing to product specificity, the sequence data do not provide a useful insight into how *phzC* might function in derivatization of the phenazine nucleus, nor is the location of the gene within a cluster of core biosynthetic genes consistent with modification of the key product. Moreover, our observations that expression in *E. coli* of incomplete loci lacking *phzAB* or *phzXY* yielded mixtures of compounds including PCA and unsubstituted phenazine, whereas expression of the complete locus from either strain enabled the synthesis of large amounts of essentially homogeneous PCA, argue that the compounds detected in earlier expression studies with strain 30-84 that included only *phzFABCD* are products of inefficient or nonspecific synthesis that do not accurately reflect the biosynthetic potential of the intact locus. Our results obtained by HPLC, UV-visible spectral analyses, and mass spectrometry support the idea that the *phzF* product from strain 2-79 and its homolog from strain 30-84 participate directly in the condensation reactions leading to production of phenazine-1,6-dicarboxylic acid, the postulated precursor of other phenazines including PCA (19). We further hypothesize that enzymes encoded by other genes which may or may not be physically linked to the *phz* loci, as well as spontaneous chemical reactions, are responsible for modification of phenazine-1,6-dicarboxylic acid or PCA to form the various phenazine compounds characteristic of different species of fluorescent pseudomonads.

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