Phenazine Biosynthesis in *Pseudomonas fluorescens*: Branchpoint from the Primary Shikimate Biosynthetic Pathway and Role of Phenazine-1,6-dicarboxylic Acid

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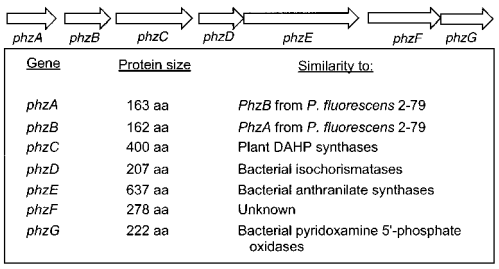
The phenazines number about 80 biologically active (antibacterial, antifungal, antiviral, and antitumor) aromatic natural products synthesized mainly by *Pseudomonas* and *Streptomyces* species.[1] Their biosynthesis has been traced to the shikimate pathway, with two molecules of a monomeric precursor pairing head-to-tail.[2] Based on genetic evidence,[3] chorismic acid has been implicated as a more proximate precursor, but the precise branch point has remained unclear. Crawford and co-workers[4] observed that pyocyanin-producing strains of *P. aeruginosa* contained two sets of anthranilate synthase genes and concluded from gene inactivation and complementation experiments that anthranilate must be a phenazine precursor. Yet, all attempts to demonstrate intact incorporation of labeled anthranilate into phenazines have been unsuccessful.[5] Two very similar phenazine biosynthetic gene clusters from *P. aureofaciens*[6] and *P. fluorescens*[7] also contain anthranilate synthase homologues essential to phenazine formation, and the gene from *P. aureofaciens* complemented a Trp^- mutant of *Escherichia coli* deficient in anthranilate synthase activity. Therefore, it would appear that either anthranilate or an intermediate in its biosynthesis, such as 2-amino-2-deoxyisochorismic acid (ADIC), must be the branchpoint compound.

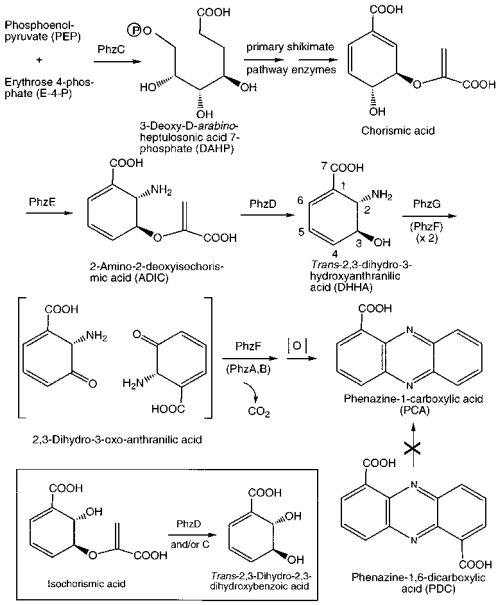
The phenazine biosynthetic gene cluster from *P. fluorescens* strain 2−79 (Figure 1) contains 7 genes, of which five (*phzC-G*) are essential and two others (*phzA* and *B*) substantially enhance the level of synthesis of phenazine-1-carboxylic acid (PCA, Scheme 1), the phenazine produced by this organism.[7] Using *E. coli* transformants expressing all or different subsets of these 7 *phz* genes, we reexamined the point at which phenazine formation branches off from the shikimate pathway. Cell-free extracts of *E. coli* BL21(DE3)pLysS(pT7-6A-G),[7] containing all 7 *phz* gene products, were incubated[8] individually with chorismic acid,[10] ADIC,[11] or anthranilic acid in the presence of ammonium ions as nitrogen source for 3 h at 28 °C. Incubations of the cell-free extract without the substrate and the substrate with heat-denatured extract served as controls.[12] The reaction mixtures were analyzed by HPLC[13] and, following methylation with diazomethane, by GC-MS[14] for substrate utilization and production of PCA or other new compounds. The results (Table 1) showed essentially complete conversion of ADIC to PCA (expt. 2), giving levels at least 4 times those of the cell-free extract background; no significant other new peaks were detected. In contrast, no PCA was formed from anthranilic acid, which remained unchanged



Gene	Protein size	Similarity to:
phzA	163 aa	*PhzB* from *P. fluorescens* 2-79
phzB	162 aa	*PhzA* from *P. fluorescens* 2-79
phzC	400 aa	Plant DAHP synthases
phzD	207 aa	Bacterial isochorismatases
phzE	637 aa	Bacterial anthranilate synthases
phzF	278 aa	Unknown
phzG	222 aa	Bacterial pyridoxamine 5′-phosphate oxidases

Figure 1. The Phenazine (*phz*) biosynthetic gene cluster from *Pseudomonas fluorescens* strain 2−79 and the homology-based tentative functional assignments of its seven genes (according to ref 7).

Scheme 1. Proposed Biosynthetic Pathway for Phenazine-1-carboxylic Acid in *Pseudomonas fluorescens*



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(8) Cells were grown in LBBS[9] medium containing carbenicillin and chloramphenicol at 37 °C and 250 rpm, induced at OD_{600} 0.7 with 0.1 mM IPTG, harvested after 15 h at 30 °C, sonicated in 0.1 M phosphate buffer, pH 7.0, containing 10% glycerol, 0.1 mM EDTA, 1 mM DTT, and 1 mM PMSF (1.5 mL cells from 45 mL of culture) and centrifuged to give the cell-free extract. Incubations contained 0.5 mL of cell-free extract (or a mixture of extracts), 0.2 mL 1 M NH_4HCO_3, pH 8.0, 0.1 mL of 0.1 M $MgCl_2$ and 1 mg of substrate in a total volume of 2 mL.

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(12) The incubations with PhzB and PhzAB indirectly served as additional controls, since no transformations of substrates were observed.

(13) Alltech Ultrasphere C_{18} 5μ 250 mm × 10 mm column (2.5% aqueous acetic acid mobile phase) or on a YMC-Pack ODS-AQ 5μ 250 mm × 10 mm column (0.5% aqueous TFA mobile phase).

(14) HP-5890A series II gas chromatograph with a capillary column coupled to a HP 5971A mass selective detector.

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Table 1. Results from Cell-Free Incubations of *E. coli* BL21 Clones Overexpressing *P. fluorescens* 2−79 Phenazine Biosynthetic Gene Cassettes with Various Substrates

expt. no.	*E. coli* BL21 (DE3)pLysS clone(s) in extract	administered substrate[a]	substrate remaining after incubation	DHHA accum.	PCA prod.
1	T7-6A-G	chorismate[b]	no	0	0
2	T7-6A-G	ADIC	no	0	100%[c]
3	T7-6A-G	anthranilate	**yes**	0	0
4	T7-6A-G	DHHA	no	N/A	100%
5	T7-6B	ADIC	**yes**	0	0
6	T7-6AB	ADIC	**yes**	0	0
7	T7-6CDE	ADIC	no	100%	0
8	T7-6ABCD	ADIC	no	100%	0
9	T7-6AB, T7-6G, T7-6CDE	ADIC	no	100%[d]	0
10	T7-6CDEFG, T7-6AB	ADIC	no	0	100%
11	T7-6CDEFG, T7-6B	ADIC	no	0	25%
12	T7-6CDEFG	ADIC	no	0	13%
13	T7-6A-G	[11-^{13}C]PDC	yes	0	≪1%[e]
14	T7-6A-G	[11-^{13}C]PDC, ADIC	**yes** (PDC), no (ADIC)	0	20%[f]
15	T7-6A-G	3-Hydroxy-anthranilic acid	**yes**	0	0

[a] Substrate concentrations of 0.5 mg/mL were used for all cell-free incubations other than chorismate. [b] Chorismic acid was used at levels of up to 5 mg/mL. [c] PCA production from ADIC was essentially quantitative and was defined as 100%. [d] DHHA production from ADIC was essentially quantitative and was defined as 100%. [e] PCA produced contained 46 atom % excess ^{13}C. [f] PCA produced contained only natural abundance of ^{13}C.

after the incubation (expt. 3). Thus, it is clear that ADIC, not anthranilate, is the phenazine precursor. Surprisingly, chorismate also was not converted to PCA (expt. 1), even at concentrations up to 5 mg/mL, but in this case no substrate remained and new compounds, mostly aromatic acids including prominently protocatechuic acid, were detected. Apparently, chorismic acid is metabolized too rapidly by background enzymes to allow for significant conversion to ADIC.

The same levels of PCA were produced from ADIC when the 7 genes were expressed from a single complete cassette or from two cassettes, one carrying *phzC-G* and the other *phzAB* (expt. 10). However, PCA production decreased 4-fold if the extract containing PhzC-G was supplemented with only PhzB (expt. 11) and 8-fold if both PhzA and -B were omitted (expt. 12). When extracts containing PhzB or PhzAB were incubated with ADIC, the substrate was not utilized and no PCA or other new products were formed (expts. 5 + 6). These results confirm the important but nonessential role of PhzA and PhzB in phenazine biosynthesis, possibly by stabilizing a hypothetical multienzyme complex.[7]

Incubating a mixture of extracts containing all but PhzF with ADIC (expt. 9) resulted in quantitative conversion of the substrate into a new compound that was isolated and identified by NMR[15] and MS as *trans*-2,3-dihydro-3-hydroxyanthranilic acid (DHHA), a compound previously isolated by McCormick et al. from a mutant of the tetracycline producer, *S. aureofaciens*.[16] Incubation of DHHA with the PhzA-G extract (expt. 4) showed complete conversion to PCA, establishing the compound as a bona fide phenazine intermediate. ADIC also was converted quantitatively to DHHA by extracts containing PhzCDE or PhzABCD (expts. 7 + 8). This indicates that (i) PhzA and -B must be involved in steps past the formation of DHHA and (ii) only PhzC and/or -D are necessary for the conversion of ADIC to DHHA. Since PhzC shows strong homology to 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) synthases, the first enzyme of the shikimate pathway, whereas PhzD is similar to bacterial isochorismatases,[7,17] it seems likely that PhzD catalyzes the ADIC to DHHA conversion. Isochorismatase activity was demonstrated for PhzC and/or -D in incubations with PhzA-G, PhzCDE, or PhzA-D, all of which quantitatively converted isochorismate[18] to *trans*-2,3-dihydro-2,3-dihydroxybenzoic acid, whereas PhzAB did not (data not shown).

The data do not reveal the mechanism of the dimerization of DHHA to the phenazine ring system, although they suggest that the process strictly requires only PhzF and -G. It had previously been suggested that 3-hydroxyanthranilic acid might be a phenazine precursor,[7] but this compound was recovered unaltered after incubation with PhzA-G (expt. 15) and no PCA was produced. PhzG is similar to bacterial pyridoxamine 5′-phosphate oxidases, whereas PhzF shows no meaningful sequence homologies. It is therefore tempting to speculate that the dimerization of two molecules of DHHA involves their oxidation, catalyzed by the PhzG protein, to the C-3 ketone. The molecules would then react twice with each other by nucleophilic addition, dehydration, and tautomerization to give a 5,10-dihydrophenazine that subsequently is oxidized to PCA (Scheme 1). Since the postulated intermediate 3-ketone, if free in solution, would spontaneously aromatize by enolization to 3-hydroxyanthranilic acid, it is proposed that the role of PhzF may be to hold this intermediate in an orientation favoring dimerization over enolization. PhzG may either act on free DHHA, with PhzF efficiently binding the released 3-ketone, or it may oxidize DHHA bound to PhzF in a protein complex, perhaps stabilized by PhzAB.

The oxidative dimerization of DHHA would be expected to produce phenazine-1,6-dicarboxylic acid (PDC), which must then be decarboxylated to PCA. The *phzA-G* gene cluster is sufficient for PCA production but contains no obvious candidate for such a decarboxylase, and feeding experiments have left the role of PDC in the formation of phenazines unclear. PDC is incorporated efficiently by Streptomycetes into compounds such as lumofungin,[19] iodinin,[20] and the saphenamycins and esmeraldins,[21] but Pseudomonads incorporated little or no PDC into other phenazines,[2b,22] which has been attributed to their cellular impermeability.[22d,23] To probe this question further, we incubated the PhzA-G extract with [11-$^{13}C_1$]-PDC (preparation[22a]) (expt. 13). GC-MS analysis of the reaction mixture identified miniscule amounts (≪1% conversion), of PCA that, as predicted for the decarboxylation of PDC, contained about 50 atom % ^{13}C, but most of the PDC remained unchanged. When the experiment was repeated with an equal amount of ADIC and [11-$^{13}C_1$]-PDC (expt. 14), the ADIC was completely utilized and produced PCA that contained no detectible excess of ^{13}C, albeit less efficiently than in the absence of PDC. It follows that PDC cannot be an intermediate in the formation of PCA from ADIC; the decarboxylation is probably integral to the dimerization process.

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(15) ^{1}H NMR (D_2O, 500 MHz) δ 7.29 (d, J = 5.56 Hz, 1H, H-6), 6.34 (dd, J = 5.56, 9.88 Hz, 1H, H-5), 6.29 (dd, J = 4.32, 9.88 Hz, 1H, H-4), 4.34 (dd, J = 3.71, 4.32 Hz, 1H, H-3), 4.24 (d, J = 3.71 Hz, 1H, H-2).

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