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Biosynthesis of Phenazine Pigments in Mutant and Wild-Type Cultures of *Pseudomonas aeruginosa*

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Pigmentation mutants of *Pseudomonas aeruginosa*, selected by observed visual differences in coloration from the wild-type strain, were examined for altered patterns of phenazine synthesis. Three classes of mutants that were incapable of pyocyanine production were identified. Pigmentation patterns that were found to characterize the various mutant classes implicated precursor-product relationships, and a biochemical scheme covering the terminal reactions of pyocyanine biosynthesis is proposed. Among compounds tested as inhibitors of pigmentation, two effectively inhibited pyocyanine production while allowing cell growth. *p*-Aminobenzoate inhibited total pigmentation; i.e., no other phenazine accumulated. *m*-Aminobenzoate inhibited a presumptive methylation step in pyocyanine biosynthesis, abolishing the formation of pyocyanine and aeruginosin pigments but increasing the yields of phenazine 1-carboxylic acid and oxychlororaphin. D-[2,3,4,5(*n*)-^{14}C]shikimate was most efficiently incorporated into phenazines in the middle to late exponential phase of growth. Label was incorporated predominantly into pyocyanine in the absence of inhibitors and into phenazine 1-carboxylic acid when the organism was grown in the presence of *m*-aminobenzoate.

Many species of *Pseudomonas* produce a variety of extracellular pigments, of which phenazines comprise a significant portion. Pyocyanine is the main phenazine pigment associated with *Pseudomonas aeruginosa*. Defined conditions for the reliable production of phenazines have greatly aided biosynthetic studies (22). Shikimate has been established as a precursor of phenazines (15), and chorismate was identified as the branch point emerging from the aromatic biosynthetic pathway (3, 21). Ring assembly has been shown to take place via a diagonally symmetrical pairing of shikimate derivatives in the case of the biosynthesis of iodinin (1,6-dihydroxyphenazine 5,10-di*N*-oxide) (10, 11, 14). Methionine appears to donate the *N*-methyl groups of pyocyanine (23).

The simultaneous accumulation of more than one phenazine by strains of *P. aeruginosa* has been reported (5, 19, 20), and cultural conditions which differentially affect the fractional composition of phenazine pigments have been described (17). However, the exact relationships between the phenazine compounds produced have not been established. The isolation of pigmentation mutants has been useful in understanding the terminal reactions of iodinin biosynthesis in *P. phenazinium* (1) and has led to the proposal of phenazine 1,6-dicarboxylic acid as a common precursor to all naturally occurring phenazines (1, 2). A procedure for the isolation of pyocyanine mutants in *P. aeruginosa* has been described (4). We report here details of the altered patterns of pigment production of such mutants and experiments aimed at understanding the metabolic relationships of the phenazines formed.

MATERIALS AND METHODS

Microorganisms and media. The wild-type strain of *P. aeruginosa* used in these studies was ATCC 15692 (Holloway strain 1 [13]). Pyocyanine mutants derived from this strain have been briefly described (4). Three media were used. Medium A, a modification of King A medium (18), contained 2% peptone, 1% glycerol, 1% K_2SO_4, 0.14% $MgCl_2$, 0.1 M Tris-hydrochloride (pH 7.5), and 2% Hutners vitamin-free minimal salts (25) (to eliminate production of flourescein); this is a complex, general-purpose medium, used for the isolation of pigmentation mutants. Medium B, a modification of the medium of Frank and DeMoss (7), consisted of 2.5% glycerol, 1% DL-alanine, 0.01% ferric citrate, 0.41% $MgCl_2 \cdot 6H_2O$, 1.42% Na_2SO_4, 0.014% K_2HPO_4, and 0.1 M Tris-hydrochloride, pH 7.5. Medium C, a defined minimal glucose medium (24), was supplemented with 10^{-3} volume of the following stock solution of trace elements containing (per liter of distilled water): $ZnSO_4 \cdot 7H_2O$, 100 mg; $FeCl_3 \cdot 6H_2O$, 20 mg; $CuSO_4 \cdot 5H_2O$, 10 mg; $MnCl_2 \cdot 4H_2O$, 70 mg; $(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$, 40 mg; $Na_2B_4O_7 \cdot 10H_2O$, 90 mg; $CaCl_2$, 500 mg; $CoCl_2 \cdot 6H_2O$, 20 mg. This medium was used to test for auxotrophy. (Pigmentation after growth on

medium C was not observed due to the high phosphate concentration, which has been shown to inhibit pyocyanine production [16].) All additional compounds added to the above media were sterilized by filtration and added separately.

Mutagenesis. Pigmentation mutants were isolated by the method of Carson and Jensen (4).

Growth measurement. Growth was estimated turbidimetrically at 750 nm. At this wavelength, interference by pigments produced by *P. aeruginosa* was minimal.

Separation and examination of phenazines. Phenazines were separated by the method of Chang and Blackwood (5). After extraction of pyocyanine, phenazine 1-carboxylic acid, and oxychlororaphin, the final aqueous layer contained a mixture of aeruginosins A and B (20). Pyocyanine was estimated by the method of Frank and DeMoss (7). Aeruginosins A and B were not routinely separated, and concentrations were estimated together at 520 nm and expressed as optical density units per milliliter of culture supernatant (12). Other phenazines were assayed as previously described (2).

Measurement of radioactivity incorporated into phenazines. The radioactivity of individual phenazines was measured as previously described by liquid scintillation counting (2), using a Packard 3375 liquid scintillation spectrometer.

Inhibitor studies. Pigmentation inhibitors were tested in medium B, either in liquid medium over a range of concentrations (0 to 2 mg/ml) or by addition of 1 mg of solid inhibitor to the surface of solidified medium previously spread with lawns of wild-type *P. aeruginosa* grown on medium B.

Chemicals. All compounds used were reagent grade or of the highest purity available commercially. 2,3-Dihydroxybenzoate and 3,5-dihydroxybenzoate were supplied by Aldrich Chemical Co.; anthranilate and *p*-aminobenzoate were supplied by Calbiochem; *m*-aminobenzoate (MABA), benzoate, dimethylaminobenzaldehyde, *p*-hydroxybenzoate, nicotine, picolinic acid, and pyrrole were supplied by Eastman Organic Chemicals; pyridine was supplied by Fisher Scientific Co.; and nicotinic acid and piperazine were supplied by Sigma Chemical Co. D-[2,3,4,5 (*n*)-^{14}C]-shikimate was obtained from Amersham/Searle, and Aquasol liquid scintillation cocktail was from New England Nuclear Corp.

RESULTS

Analysis of pigmentation variants. The "microtiter" technique (4) was used to isolate pigmentation variants; mutant strains were selected after visual observations of color differences when grown on medium A. They were then tested for prototrophy on minimal glucose medium and analyzed for phenazine pigment levels after growth on both medium A and medium B. Sixty presumptive mutants were studied intensively. Seventy percent of these strains produced the same complement of phenazines as did the wild-type organism, the difference being that the overall quantitive yield was lowered in each mutant. No mutants were isolated which produced more pyocyanine than the parent strain. Three of the strains studied produced no phenazines at all.

Pyocyanine-negative mutants were isolated which produced pigmentation patterns different from that of wild type. Representatives of each of the three groups of mutants obtained are listed in Table 1. None of the mutants produced only one phenazine. Group 1, though unable to synthesize pyocyanine, produced aeruginosins in a slightly increased yield over that of the wild type. Group 2 produced no pyocyanine or aeruginosins and also exhibited lowered yields of phenazine-1-carboxylic acid and oxychlororaphin. Group 3 produced no phenazines. No cross-feeding was observed between any two of thse mutant types. In no mutant was production of phenazine 1,6-dicarboxylic acid observed.

Analysis of the pigmentation mutant groups and a consideration of the structural relationships of the phenazines produced, together with a variety of observations in the literature (see Discussion), led us to the working scheme for terminal reactions of pyocyanine biosynthesis proposed in Fig. 1. Group 1 mutants, blocked in

TABLE 1. *Distribution of phenazines in pigment mutants*

Strain	Phenazine pigment[a]				
	Phenazine 1-carboxylate	Oxychlororaphin	5-Methylphenazinium 1-carboxylate	Pyocyanine	Aeruginosins
Wild type	+++	++	−	++++	+++
Mutants					
Group 1					
PGM12	++	++	−	−	+++
PAC9	+++	++	−	+	+++
PGM-200	+	+	−	−	+++
Group 2					
MC10	+	+	−	−	−
MC22	+	+	−	−	−
PGM31	+	+	−	−	−
Group 3					
MC32	−	−	−	−	−
MC33	−	−	−	−	−
PGM13	−	−	−	−	−

[a] Amounts of phenazines produced by mutant strains were estimated after growth on medium B and are expressed semi-quantitatively based on spectroscopic measurements compared with wild-type concentrations. The amounts of phenazines produced by the wild type were as follows: phenazine 1-carboxylate, 2.1 μmol/ml; oxychlororaphin, 0.5 μmol/ml; 5-methylphenazinium 1-carboxylate, none; pyocyanine, 5.5 μmol/ml; and aeruginosins, 1.36 optical density units at 520 nm.

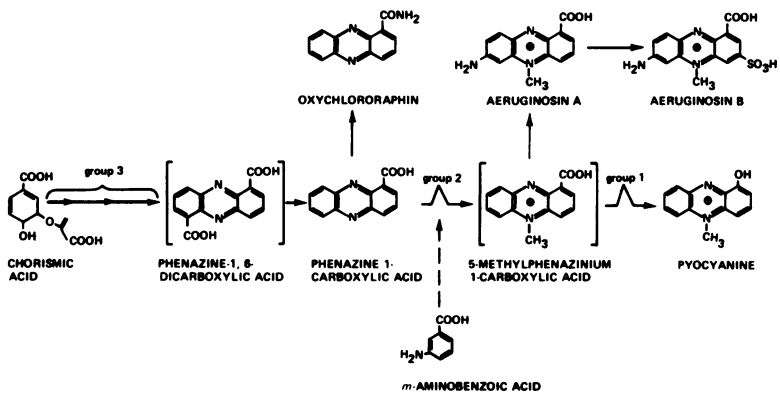

FIG. 1. *Scheme for phenazine biosynthesis in P. aeruginosa. Phenazine 1,6-dicarboxylic acid and 5-methylphenazinium 1-carboxylic acid were not isolated in mutant strains or in cultures grown in the presence of MABA. Their inclusion here is based on alternative considerations (see text). The probable positions of the metabolic lesions in the mutants and the probable site of action of MABA are indicated.*

the terminal step to pyocyanine, did not accumulate the proposed intermediate, 5-methylphenazinium 1-carboxylic acid. However, as a branch point compound, it would be channeled into the "shunt" pathway leading to production of aeruginosins. Sequential mutagenesis of strain PGM12 or PAC9 (group 1 mutants) did not yield derivative mutants producing 5-methylphenazinium 1-carboxylic acid in place of aeruginosins.

Testing of pigmentation inhibitors. Several substances were tested for inhibitory effects on pyocyanine production. The effects of the test compounds were examined in both solid and liquid media (see Materials and Methods). Compounds tested were MABA *p*-aminobenzoate, anthranilate, benzoate, 2,3-dihydroxybenzoate, 3,5-dihydroxybenzoate, dimethylaminobenzaldehyde, *p*-hydroxybenzoate, nicotine, nicotinic acid, picolinic acid, piperazine, pyrrole, and salicylate.

In only two cases did a differential inhibition of pigmentation occur while allowing growth. The production of pyocyanine was inhibited by both MABA and *p*-aminobenzoate. *p*-Aminobenzoate was found to inhibit the formation of all phenazine pigments at concentrations exceeding 0.05%. MABA, although inhibiting pyocyanine and aeruginosin production, allowed the formation of increased amounts of phenazine 1-carboxylic acid and oxychlororaphin. A 50% increase in phenazine 1-carboxylic acid produced was found at 3.4 mM MABA (40 μg/ml). The effect of increasing concentrations of MABA on pigmentation is shown in Fig. 2. A similar pattern of pigmentation in the presence of the inhibitor was observed with the mutant strain PGM12, which was capable of producing aeruginosins but not pyocyanine (Fig. 3).

Time course experiments for growth and pigmentation of wild-type *P. aeruginosa* were carried out in the presence (1 mg/ml) and absence of MABA to assess the effect of MABA on growth and time of onset of pigmentation. The results are shown in Fig. 4 and 5. The time of onset of pigmentation was not altered by MABA, production starting 42 to 45 h after inoculation at an approximate turbidity of 0.7 at 750 nm. Addition of L-methionine (100 μg/ml) or folate (100 μg/ml) or a combination of both did not reverse the inhibition of pyocyanine production caused by the presence of 100 μg of MABA per ml.

Incorporation of [^{14}C]shikimate into phenazines. D-[2,3,4,5 (*n*)-^{14}C]shikimate (0.5 μCi, 10 nmol) was added to 5-ml cultures of *P. aeruginosa* at different stages of growth on medium B. These were then allowed to reach late stationary phase (ca. 144 h after inoculation). Good incorporation into phenazines occurred (Table 2); maximum incorporation into pyocyanine (19%) was observed at the end of the exponential phase of growth at an absorbance of 1.0 at 750 nm, 50 to 55 h after inoculation. Label incorporated into aeruginosins could not be estimated due to contamination with labeled cell constituents. This could not be resolved by the method of Lacoste et al. (20).

In the presence of 1 mg of MABA per ml, label from [^{14}C]shikimate was predominantly present in phenazine 1-carboxylic acid. The time of maximum incorporation was similar to the time of maximum incorporation of label into pyocyanine by cultures without inhibitor (Table 2).

DISCUSSION

The pigmentation mutants obtained and their respective pigment accumulation patterns, together with the inhibitor studies, suggest possi-

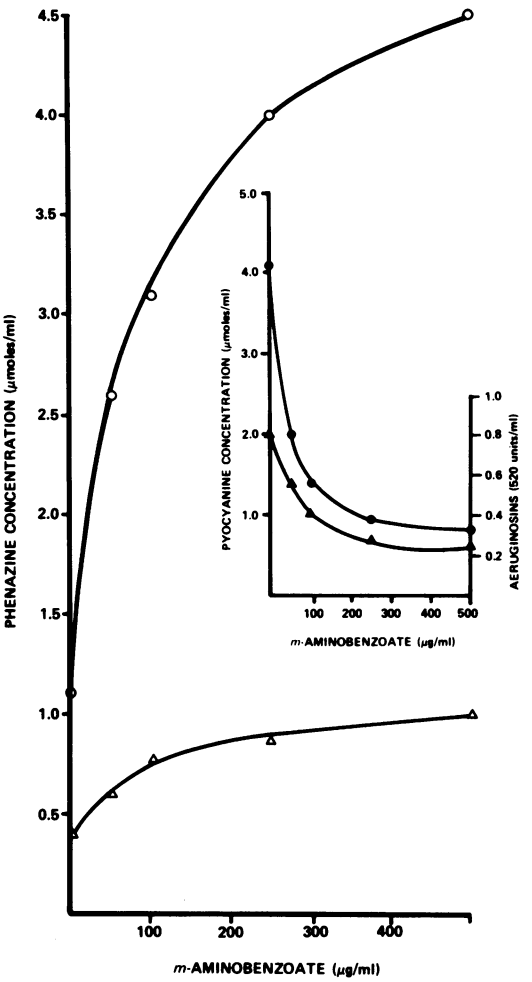

FIG. 2. *Effect of MABA upon pigmentation of wild-type P. aeruginosa. P. aeruginosa was grown on medium B with the indicated concentration of MABA. When cultures reached stationary phase, the pigments were extracted and separated. Pyocyanine (●), phenazine 1-carboxylic acid (○), oxychlororaphin (△), and aeruginosins (▲) were estimated as described in the text.*

ble interrelationships between the phenazines formed, as presented in the scheme proposed (Fig. 1). The lack of cross-feeding between mutant strains is consistent with the following observations. Mutants producing phenazine 1-carboxylic acid, but not pyocyanine or aeruginosin, were noted for their poor production. Furthermore, preliminary experiments with washed-cell suspensions of wild-type *P. aeruginosa* and a mutant blocked before shikimate suggest that the cell is relatively impermeable to phenazine 1-carboxylic acid and that pyocyanine is only formed at high extracellular concentrations (>0.4 mM) of this compound.

Production of 5-methylphenazinium 1-carboxylic acid was not observed in any of the mutants. However, its metabolism to pyocyanine and aeruginosins A and B has been reported (8, 9), thus supporting the proposed role of this compound as a branch point metabolite. Sequential mutagenesis of strains unable to produce pyocyanine but able to produce aeruginosins (group 1 mutants) did not lead to accumulation of this intermediate. This could be due to nonenzymatic formation of aeruginosin A (9). Further evidence supporting the proposed scheme is the conversion of labeled phenazine 1-carboxylic acid to pyocyanine (6).

The decreased production of pyocyanine and aeruginosins caused by MABA suggests that MABA acts by inhibiting the proposed methylating enzyme which converts phenazine 1-car-

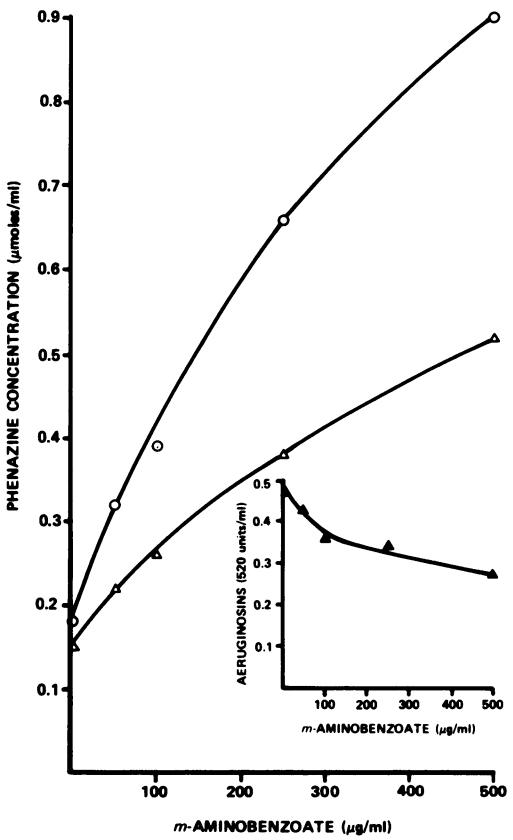

FIG. 3. *Effect of MABA on pigmentation of the pyocyanine mutant PGM12. Strain PGM12, a group 1 mutant which produces aeruginosins but not pyocyanine (see text), was grown under the conditions described in Fig. 2, and amounts of phenazine 1-carboxylic acid (○), oxychlororaphin (△), and aeruginosins (▲) were monitored.*

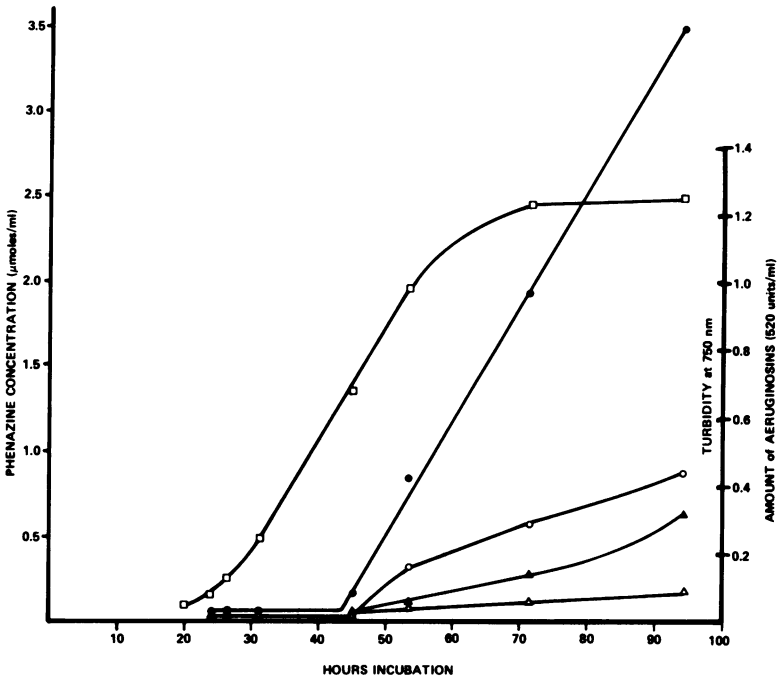

FIG. 4. *Time course of growth and pigmentation. P. aeruginosa was grown on medium B, and aliquots of culture were removed with time. Growth (absorbance at 750 nm)* (□) *and amounts of individual phenazines (pyocyanine* [●], *phenazine 1-carboxylic acid* [○], *oxychlororaphin* [△], *and aeruginosins* [▲]*) were estimated as described in the text.*

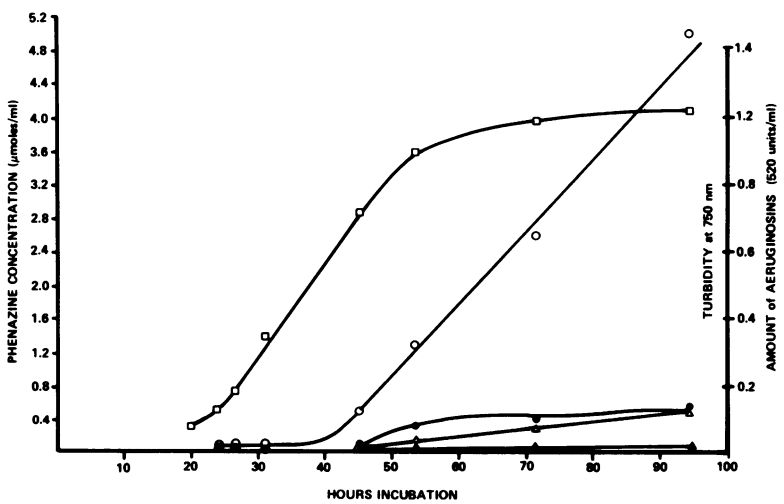

FIG. 5. *Effect of MABA on growth and time of onset of pigmentation. P. aeruginosa was grown in medium B containing 1 mg of MABA per ml. All other conditions were the same as those described in Fig. 4. Symbols:* □, *turbidity at 750 nm;* ●, *pyocyanine;* ○, *phenazine 1-carboxylic acid;* △, *oxychlororaphin;* ▲, *aeruginosins.*

boxylic acid to a common precursor (presumably, 5-methylphenazinium 1-carboxylic acid) of pyocyanine and aeruginosins. MABA exhibits structural similarity to the half-molecule of phenazine 1-carboxylic acid, and perhaps MABA acts as a competitive inhibitor or as a false substrate for the enzyme. Evaluation of this possibility rests on the development of a cell-free assay for the methylating enzyme.

The incorporation of [^{14}C]shikimate into phenazines in *P. aeruginosa* grown with the inhibitor MABA allows the accumulation of la-

TABLE 2. *Incorporation of [^{14}C]shikimate into individual phenazines by P. aeruginosa*[a]

Time (h)	Cell density (absorbance at 750 nm)	Radioactivity recovered (%) in:		
		Pyocyanine	Phenazine 1-carboxylate	Oxychlororaphin
0	No turbidity	10.6 (1.8)	0.07 (17.8)	1.5 (3.0)
24	0.08 (0.13)	14.3 (1.8)	0.08 (15.8)	1.5 (3.5)
45	0.67 (0.71)	17.0 (3.9)	0.05 (23.1)	1.4 (3.0)
53	0.98 (0.88)	19.0 (3.5)	0.07 (24.9)	1.4 (3.1)
73	1.23 (0.98)	16.8 (2.2)	0.04 (21.9)	1.5 (3.0)
95	1.25 (1.01)	10.3 (2.1)	0.09 (16.7)	1.5 (2.6)

[a] D-[2,3,4,5(n)-^{14}C]shikimate (0.05 μCi; 10 nmol) was added to wild-type cultures of *P. aeruginosa* (5 ml) growing on medium B at different times after inoculation. On reaching stationary phase the phenazines were extracted and separated, and radioactivity was measured as described in the text. Times and cell densities are those at the time of label addition. Values in parentheses were obtained for cultures grown in the presence of 1 mg of MABA per ml.

beled phenazine 1-carboxylic acid. This could lead to the development of more sensitive radioactive assays for the study of the enzymology of phenazine metabolism in this organism.

Details of the early stages of pyocyanine biosynthesis leading from chorismate to the proposed terminal reactions are lacking, and the biosynthetic origin of the nitrogen atoms is unknown. Evidence has been presented for a role for phenazine 1,6-dicarboxylic acid as a branch point compound in *P. phenazinium* (1, 2) leading to the formation of iodinin and phenazine 1-carboxylic acid and related compounds. The apparent impermeability of the membrane to this compound in the pseudomonad systems of pigmentation has hampered further investigation. However, incorporation of phenazine 1,6-dicarboxylic acid into a phenazine, lomofungin, produced by *Streptomyces lomodensis* has been reported (8).

None of the mutant strains isolated in this study accumulated phenazine 1,6-dicarboxylic acid, nor was it present when *P. aeruginosa* was grown in the presence of MABA. Although this raises questions as to its potential as a precursor for pyocyanine, the possibility of this compound being a transient or enzyme-bound intermediate must not be ruled out.

Recently, the compound 4,9-dihydroxyphenazine 1,6-dicarboxylic acid dimethylester was isolated from *P. cepacia* (19). This appears to be an intracellular pigment, in contrast to normal phenazine accumulation patterns in other pseudomonads. Whether this is a metabolite of phenazine 1,6-dicarboxylic acid, or an intermediate between chorismate and phenazine 1,6-dicarboxylic acid, remains to be seen. The apparent impermeability of *Pseudomonas* strains to carboxylated phenazines and to compounds like chorismate points to a need for an established cell-free system to study this biosynthetic pathway in these organisms.

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