The Branch Point Metabolite for Pyocyanine Biosynthesis in *Pseudomonas aeruginosa*

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The *Pseudomonas* pigments are conveniently classified into the fluorescent water-soluble pigments and the chloroform-soluble phenazine derivatives. The structures of the fluorescent pigments remain largely uncharacterized, though one may be a pteridine derivative (Chakrabarty & Roy, 1964). They are probably synthesized through a biochemical route independent of the pathway of synthesis of the phenazine compounds. The phenazine pigments are better characterized because growth media exist which reliably promote their formation.

Ingledew & Campbell (1969) and MacDonald (1963) did precursor-product studies with ^{14}C radioisotopes to demonstrate that growing cells preferentially utilize exogenous shikimate as a precursor of pyocyanine. Shikimate was found to account for all of the carbon atoms in the ring position of the pyocyanine molecule. Similar procedures eliminated phenylalanine, tyrosine, tryptophan, and anthranilic acid as precursor molecules. The methyl group on N-5 of pyocyanine is derived from the S-methyl of methionine (Kurachi, 1969; Sheikh & MacDonald, 1964).

Since shikimate formation involves several reversible steps (the interconversion of shikimate with 5-dehydroshikimate, and of 5-dehydroshikimate with 5-dehydroquinone), 5-dehydroshikimate and 5-dehydroquinone qualify *a priori* as candidates for the branch point metabolite. Three additional common precursors follow shikimate in the branched biosynthetic pathway leading to aromatic amino acids and vitamins. Therefore, although the labelling experiments established shikimate as a pyocyanine precursor, they did not pinpoint what particular intermediate between 5-dehydroquinone and chorismate is the branch point metabolite. Unfortunately, most of the common aromatic intermediates are unstable and/or do not penetrate the cell membrane. Thus, the labelling experiments that were suitable with shikimate, which is stable and penetrates into bacteria, could not be used to identify the branch point intermediate. The approach of using mutants blocked in the various enzyme reactions of aromatic synthesis has not been used previously. We examined the ability of such mutants to synthesize pyocyanine in the presence and the absence of shikimate.

Prototrophic mutants unable to degrade shikimate and auxotrophs (Table 1) were isolated by the methods previously described (Calhoun & Feary, 1969; Calhoun & Jensen, 1972). Pyocyanine production was tested in the 'A' medium of King, Ward & Raney (1954), a glycerol – 2% (w/v) peptone medium. The pH of the minimal salts solution was adjusted to 7.2 before autoclaving. A 4 to 5 h culture was grown at 37 °C with shaking in 2 ml of trypticase soy broth (Difco) in a 15 × 100 mm test tube. The culture was centrifuged, and the pellet was resuspended in 2 ml of sterile water. Resuspended cells were spread evenly on the surface of a King agar plate in 0.2 ml volumes and incubated at 37 °C for 5 days. Pigment production was visible after 6 to 8 h for most strains, and was uniformly obvious after 24 to 36 h.

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Table 1. *Pyocyanine production by mutants of Pseudomonas aeruginosa*

The tryptophan auxotrophs UAB0426, UAB 75, UAB 135, UAB 308, BCM 1 and BCM 13 correspond to the following blocks: anthranilate synthetase; phosphoribosyl transferase; phosphoribosyl anthranilate isomerase; indole glycerol phosphate synthetase; tryptophan synthetase A; tryptophan synthetase B, respectively.

Isolate no.	Nutritional phenotype*	Pyocyanine formation†	
		Without shikimate	With 0.1 % shikimate
Wild-type		+	+
UAB 446	Leucine	+	NT
UAB 461	Methionine	+	NT
UAB 421	Histidine	+	NT
UAB 429	Arginine	+	+
UAB 445	Proline	+	NT
UAB 401	Adenine	+	+
UAB0426	Tryptophan/ind/ant	+	NT
UAB 75	Tryptophan/ind	+	NT
UAB 135	Tryptophan/ind	+	+
UAB 308	Tryptophan/ind	+	NT
BCM 1	Tryptophan/ind	+	+
BCM 13	Tryptophan	+	NT
BCM 4	Phenylalanine/phenylpyruvate	+	+
BCM 20	Shikimate degradation	+	+
BCM N8	Aro/shikimate/quinat	—	+
MC 36	Aro/shikimate/quinat	—	+
BCM N7	Aro/shikimate/quinat	—	+
BCM 40	Aro/shikimate/quinat	—	+
BCM N6	Aro/shikimate/quinat	—	+
BCM BBI	Aro/shikimate/quinat	—	+
BCM N5	Aro/shikimate	—	+
BCM NI4	Aro	—	—
MC 42	Aro	—	—
BCM A2	Aro	—	—
MC 37	Aro	—	—
MC 38	Aro	—	—

* Abbreviations used: aro refers to the combination of tryptophan, tryptidine and phenylalanine; ind, indole; and ant, anthranilate.

† Notations: NT, not tested; +, normal pyocyanine production; —, no pyocyanine formed. The solid media used for qualitative scoring of pyocyanine production was that described by King *et al.* (1954).

Mutants unable to degrade shikimate as a sole source of carbon retained the ability to synthesize pyocyanine. Pigment production was also normal in a variety of amino acid auxotrophs (Table 1). Pyocyanine was excreted into the agar by these mutant isolates as an intensely green to blue-green pigment. In contrast, none of the mutants requiring all three aromatic amino acids produced pyocyanine. These pyocyanine-less mutants produced only a pale yellow chromophore, even after 5 days. All of the mutants tested produced normal levels of fluorescent pigment (as judged by inspection under ultraviolet light).

Pyocyanine production was restored consistently by 0.1 % (w/v) shikimate (Sigma Chemical Co.) in mutants blocked before shikimate (phenotype: alternative growth response either to shikimate or to combination of the three aromatic amino acids). On the other hand, pyocyanine synthesis was not restored by shikimate in mutants blocked after shikimate (phenotype: growth with aromatic amino acids; no growth with shikimate). Pyocyanine formation in liquid culture at 37 °C was measured by the method of Frank & DeMoss

(1959). When wild-type MC36 and MC42 were grown in the presence of aromatic end products in the absence of shikimate, 65, 0 and 0 nmol pyocyanine/ml were formed, respectively, after 8 h at 37 °C (bacterial concentration, measured by E_{625} nm, was 0.846 to 1.128 mg, dry wt, of cells/ml). In the presence of shikimate 130, 130 and 0 nmol of pyocyanine were formed, respectively, under the same conditions of growth.

These results allow the conclusion that shikimate or any biosynthetic precursor of shikimate can be eliminated as the branch point compound. Three enzymatic reactions convert shikimate to chorismate. Hence, the remaining branch point possibilities are shikimate-5-phosphate, enolpyruvylshikimate-5-phosphate and chorismate. Although the mutant blocks have not been identified by enzyme assay, it seems likely that the five mutants that cannot convert shikimate to chorismate represent more than one mutant class. Shikimate-5-phosphate can probably be eliminated since mutants lacking shikimate kinase are known to be uncommon. None of the five mutants produced pigment following starvation for aromatic end products, as might be expected if a pyocyanine precursor accumulated behind an enzyme block. The results implicate chorismate as the probable branch point metabolite for pyocyanine.

Aromatic auxotrophs that grow on shikimate cannot use chorismate to satisfy their growth requirements, indicating that significant amounts of chorismate are not transported into the cells. Accordingly, previous results (Ingledew & Campbell, 1969) showing that chorismate did not compete with the incorporation of labelled shikimate into pyocyanine do not rule out chorismate as a precursor of pyocyanine.

Unquestionably pyocyanine represents a substantial proportion of the total output of the aromatic pathway. Hence, studies concerning the control of the regulatory enzymes in the pathway by end products must deal with the role of pyocyanine as an aromatic end product before the understanding of the physiological pattern of control can be complete.

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