

## Pattern of Phenazine Pigment Production by a Strain of *Pseudomonas aeruginosa*

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An atypical strain of *Pseudomonas aeruginosa* capable of synthesizing three phenazine pigments was isolated. Cultural conditions, under which the strain forms either chlororaphin, oxychlororaphin, or pyocyanine, are described. This broad spectrum of pigment production, as well as some other characteristics, sets this strain apart from previously described chlororaphin producers.

The fluorescent pseudomonads comprise a bacterial group prominent in the synthesis of phenazine pigments. Traditionally, specific pigments have been associated with certain species, e.g., phenazine-1-carboxylic acid with *Pseudomonas aureofaciens* (3) and pyocyanine with *P. aeruginosa*; but the simultaneous synthesis of more than one phenazine by the same organism is not without precedent (2, 9, 10). We describe here a strain of *P. aeruginosa* that produces not only pyocyanine, its characteristic phenazine pigment, but chlororaphin and oxychlororaphin as well. Each of these three pigments (Fig. 1) is formed under a distinct set of cultural conditions, and optimal conditions for chlororaphin synthesis by this organism differ from those described for other chlororaphin producers.

The *P. aeruginosa* strain was obtained from soil as a contaminant in an enrichment and isolation procedure for acetylene-utilizing microorganisms. The contaminant does not use acetylene, and its isolation from the enrichment appears to be coincidental. Our interest in this organism was initially aroused by its ability to produce, in a glucose-mineral salts medium, bundles of bright green needle-shaped crystals several millimeters in size. Diagnostic procedures according to Stanier et al. (8) identified the isolate as a strain of *P. aeruginosa*.

Pigment production was first observed in a mineral salts medium ( $\text{Na}_2\text{HPO}_4$ , 0.4%;  $\text{KH}_2\text{PO}_4$ , 0.15%;  $\text{NH}_4\text{Cl}$ , 0.1%;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02%;  $\text{FeNH}_4\text{-citrate}$ , 0.0005%) with 1% glucose as a source of carbon and energy. Glucose was sterilized separately in distilled water as a concentrated stock solution and added aseptically to the sterile mineral salts medium. Incubation was carried out at 28°C either under stationary conditions or on a rotary shaker.

Chlororaphin was identified by filtering culture broth through a fine (200-mesh) sieve to retain the crystals. Crystals so collected were washed with 2 N NaOH and water, then dried

at 25°C over  $\text{P}_2\text{O}_5$ . Mass spectrometry by a Hitachi Perkin-Elmer RMU-7 instrument operated at 180°C yielded a molecular ion at 223 and a base peak at 180 (Fig. 2). When crystals were dissolved in chloroform and allowed to stand overnight exposed to air, a yellow crystalline material that melted at 244°C and exhibited UV maxima at 251 and 368 nm was obtained. These yellow crystals (Y) were hydrolyzed in ethanol-10% NaOH (1:1) at 100°C for 2 h, yielding a second yellow crystalline product. The latter material was identified by mixed melting point (authentic standard, 241, to 244°C; mixed melting point undepressed, 241 to 243°C) and TLC ( $\text{CHCl}_3\text{-EtOAc}$  [15:1], Eastman Silica Gel Plates K301R2,  $R_f = 0.6$ ) as phenazine-1-carboxylic acid. The first yellow crystals (Y) were thus phenazine-1-carboxamide (oxychlororaphin), and the green crystals, by extension, were chlororaphin, the charge-transfer complex of phenazine-1-carboxamide and its reduced, dihydro derivative (5).

Confirmation was obtained by resynthesizing chlororaphin directly from oxychlororaphin. This was accomplished by dissolving the oxychlororaphin in ethanol, adding saturated sodium hydrosulfite and then water, and weighing the resultant green crystals. With 25 mg of oxychlororaphin as starting material (dissolved in 22 ml of 95% EtOH, then 5 ml of sodium hydrosulfite was added, and then 200 ml of water was added), 19.4 mg of chlororaphin was collected after 2 h, giving a 77.6% yield. Crystals so obtained were identical to those collected from culture broth in color, macroscopic and microscopic appearance, and solubility characteristics. Under the microscope, both synthesized material and broth crystals were fine green needles that sublimed to form yellow needles (like Y) at 210°C, which then melted at 244 to 246°C (melting point of Y), a phenomenon noted by earlier investigators (5).

Oxychlororaphin was identified by extracting

pigment directly from culture broth using chloroform and comparing it with phenazine-1-carboxamide prepared from chlororaphin. Both the isolated and the reference pigments so prepared behaved identically when compared by thin-layer chromatography, and their UV maxima in  $\text{CHCl}_3$  (368,251) were identical.

Pyocyanine was identified by centrifuging culture broth, passing the supernatant through a column of Amberlite XAD-2 (Chemical Dynamics Corp., South Plainfield, N.J.) and eluting with methanol. The methanol solution was made alkaline and partitioned with chloroform, and the resultant blue  $\text{CHCl}_3$  solution was extracted with 0.2 N HCl. The red acid solution was neutralized with 0.4 M sodium borate buffer (pH 10) and partitioned again with  $\text{CHCl}_3$ . This cycle of extraction with acid and reextraction with

$\text{CHCl}_3$  was repeated once more, and the pigment was crystallized from the final chloroform solution by the addition of petroleum ether in the cold. The obtained melting point (133 to 135°C) and UV maxima (388,278,208 in 0.2 N HCl; 327,310,245 in  $\text{CHCl}_3$ ) were in good agreement with published data (2).

In stationary glucose-mineral salts solution at 28°C, the yellow soluble oxychlororaphin was first synthesized. After 2 days, the green crystals of chlororaphin began to collect in the culture broth. Under similar conditions, but at 37°C, only oxychlororaphin was formed. In shake culture at 37°C, pyocyanine was synthesized. At 28°C in shake culture, none of the above pigments but an unidentified precursor was formed. This precursor was rapidly converted to oxychlororaphin when the culture was removed from the shaker and left stationary.

In addition to temperature and aeration, the added carbon source had a marked influence on pigment production. Of eight monosaccharides, ten amino acids, five organic acids, and several alcohols tested, only glucose, glycerol, and ethanol supported chlororaphin production. Even with glycerol, however, the pattern of pigment production was changed: none of the three phenazines was formed in shake culture at 37°C; whereas fluorescein, the yellow-green fluorescent pigment characterizing the fluorescent pseudomonads was formed in shake culture at 28°C. The results for the glucose-mineral salts medium are displayed in Fig. 3.

An experiment was conducted to determine the best combination of carbon and nitrogen

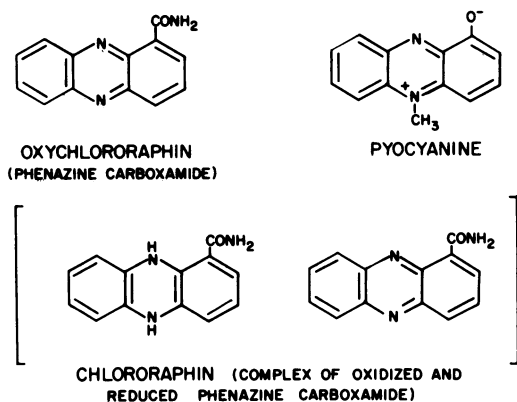


FIG. 1. Structure of the three phenazine pigments.

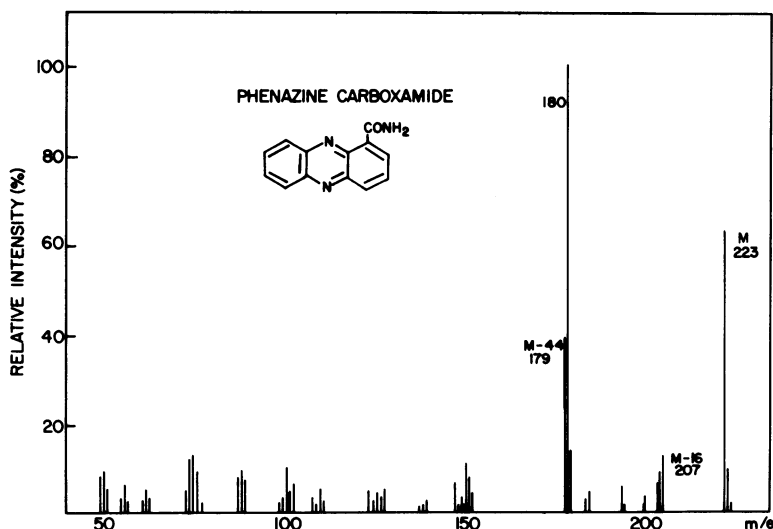


FIG. 2. Mass spectrum of chlororaphin. The spectrum obtained reflects the breakdown pattern of phenazine carboxamide (oxidized form), with a molecular ion peak at 223. The peak at 207 represents loss of the amino group; the peak at 179 represents loss of the carboxamido group. The base peak at 180 reflects the tendency of the  $m/e$  179 moiety to capture a hydrogen to complete the phenazine nucleus.

sources for optimal chlororaphin yields. Glucose and glycerol were the chosen C sources;  $\text{NH}_4^+$ , urea, and asparagine served as N sources. These compounds were chosen because frequently they have been reported to support high yields of chlororaphin and other phenazines as well (2, 5, 7). Experimental conditions were as follows: the organism was cultured in 125-ml Erlenmeyer flasks containing 82 ml of medium, each with 3,990  $\mu\text{g}$  of C and 260  $\mu\text{g}$  of N per ml. Crystals were harvested after 7 days by decanting the pellicle, hydrolyzing residual cell material with hot 2 N NaOH, and collecting the crystals by centrifugation. Crystals were washed three times in distilled water, dried over  $\text{P}_2\text{O}_5$  and weighed (Table 1).

Glucose-ammonia consistently gave the highest chlororaphin yields, closely followed by glucose-asparagine and glucose-urea. Glycerol supported only low yields of chlororaphin. The variations of absolute yields between experiments reflect our difficulties in standardizing fermentation conditions. Minute differences in culture conditions such as new or used foam plugs and the manufacture lot of Erlenmeyer flasks, as well as some unrecognized factors, caused large differences in chlororaphin yields, and a degree of uniformity between replicates could be achieved only by a meticulous matching of culture equipment.

Three types of peptones—bacto-peptone, neo-peptone, and proteose peptone no. 3—were tested as N sources, and all failed to support crystal formation. Consequently, media based on peptones and specially designed to elicit phenazine production, such as King A medium (4), failed to support any crystal formation.

Our organism differs from previously described chlororaphin producers (1, 5, 7) in that we found glucose to be a good carbon source that consistently supported higher crystal yields than glycerol. For production of chlororaphin we also found  $\text{NH}_4^+$  to be a superior nitrogen source to organic sources of nitrogen (urea, asparagine, or peptone) that were favored over  $\text{NH}_4^+$  in earlier reports (1, 5, 6). Screening this organism for production of chlororaphin by us-

TABLE 1. Yield of chlororaphin

Medium	A	B	C
Glucose-ammonia	186.3 (20.2) <sup>a</sup>	166.9 (6.1)	87.6 (22.7)
Glycerol-ammonia	60.5 (22.6)	95.8 (14.4)	23.7 <sup>b</sup>
Glucose-urea	154.9 (15.7)	138.0 (4.8)	71.1 (6.8)
Glucose-asparagine	163.4 (13.7)	125.7 (27.8)	84.5 (6.2)

<sup>a</sup> Standard deviations in parentheses. Each value is the mean of three flasks, expressed in milligrams per liter.

<sup>b</sup> No standard deviation calculated. Because of the low yield, the crystals from three flasks were combined for weighing.

ing King A medium, as performed by Stanier et al. (8), would fail to reveal this strain as a chlororaphin synthesizer, since crystal formation is not supported by the peptone nitrogen source. These results demonstrate the difficulty of designing a standard medium for the elicitation of phenazine pigments. Under the proper conditions, this strain is a vigorous chlororaphin producer; yet cultural parameters must be precisely manipulated to yield the desired pigment. Our isolate is not the only strain of *P. aeruginosa* known to produce chlororaphin (1, 6, 7). Nevertheless, the pattern of carbon source, temperature, and aeration-dependent pigment synthesis displayed by this strain has not been previously reported. Whether this pattern is unique, or whether, as is likely, proper manipulation of cultural conditions using other organisms will reveal similar, if not identical, patterns remains to be seen.

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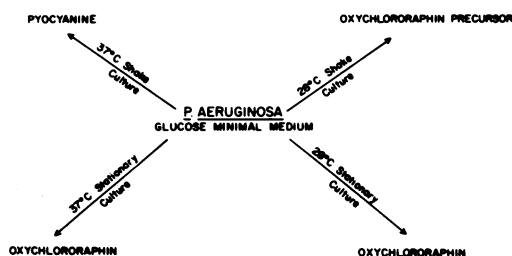


FIG. 3. Pattern of pigment production in the glucose minimal medium.