

THE INFLUENCE OF CARBON SOURCES ON PIGMENTS
PRODUCED BY PSEUDOMONAS AERUGINOSA

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ABSTRACT: Conditions and media for production of red and crystalline pigments by Pseudomonas aeruginosa were described. With these methods 57.3% of 199 cultures formed a red pigment and 56.6% of 122 cultures formed crystalline pigments. The cultures were all capable of producing pyocyanin. Variation of the carbon source in minimal salts medium caused considerable differences in the pigments produced.

Production of water soluble, diffusible pigments is a characteristic used in the identification of the various pseudomonads. Pyocyanin, the blue, water-soluble phenazine pigment is characteristic of Pseudomonas aeruginosa. Red water-soluble pigments are also formed by P. aeruginosa (Meader et al., 1925; Holliman, 1957 and Labeyrie et al., 1968), however, there is no information as to the number of isolates capable of forming a red pigment. A few strains of P. aeruginosa were shown to produce crystalline pigments (Haynes and Rhodes, 1962; Chang and Blackwood, 1968), which are characteristics of P. chlororaphis (Haynes and Rhodes, 1962) and P. aureofaciens (Haynes et al., 1956). The present study shows how the carbon source influences the formation of pigments by isolates of P. aeruginosa which are also capable of producing pyocyanin.

MATERIALS AND METHODS

Cultures of P. aeruginosa were isolated from a variety of pathological and non-pathological sources and identified by methods described by Wahba and Darrell (1965). All cultures were examined with the pyocin typing method of Kocka and Harris (1973). This procedure by definition had diagnostic value as well as indicating a difference among strains. Strains of P. aeruginosa capable of forming oxychlororaphin and P. aureofaciens cultures that produced phenazine-1-carboxylic acid were obtained from W. C. Haynes (Northern Regional Research and Development Division, Peoria, Illinois 61604, USA). Cultures were incubated at 30°C unless otherwise noted.

The defined mineral salts base contained: 14.4 g K_2HPO_4 , 5.6 g KH_2PO_4 , 1.6 g $MgSO_4 \cdot 7H_2O$, 1.6 g NaCl, 1.6 ml of 10% $CaCl_2$, 1.6 ml 10% $FeCl_3$ and 4 liters of distilled water. Before the medium was autoclaved, 0.5% $(NH_4)_2SO_4$ and the carbon source were added. The carbon source was either 0.2% (w/v) or 2.0% (w/v) as noted. The pH was then adjusted to 6.8. When a solid medium was desired, 1.0% Ionagar 2S (Wilson Diagnostics, Inc., Glenwood, Illinois USA) was used.

Sierra's medium (Sierra, 1957) and Gessard's medium (Harris, 1950) were used to demonstrate pyocyanin formation. Pyocyanin was extracted from the culture medium with chloroform. When acidified the pyocyanin moved to the aqueous layer and turned this layer red. This test has been considered presumptive for pyocyanin (Young, 1947).

RESULTS

During the process of screening P. aeruginosa cultures for the ability to utilize glycine as a sole carbon and energy source, a red pigment was formed by a majority of the isolates (Table 1). This pigment was produced after 5 to 7 days growth on 0.2% (w/v) glycine-mineral salts agar at 30°C. When grown in glycine-mineral salts liquid culture with slow shaking, the maximum amount of pigment was formed after

6 days growth at 30°C. Just prior to the maximum pigments production, the population started to decline, and the pH reached its highest value of 8.1. Some cultures that made pyocyanin on the glycine-mineral salts medium also produced the red pigment (Table 2). Pyocyanin was generally formed after 2 days growth at 30°C and masked the red pigment which was formed at a later time. The red pigment was seen after extracting the pyocyanin from the medium with chloroform.

Alanine, glutamate or asparagine as sole carbon sources also supported the formation of the red pigment, but other pigments also formed obscured its appearance.

When 0.2% glycerol and 0.2% glycylglycine together replaced glycine in the mineral salts agar as the carbon source, the cultures first turned yellow. Then occasionally the iridescent material and a red water-soluble pigment were formed. After 5 to 7 days at 30°C, two crystal types were present in the subsurface growth of many cultures. Large ray-like crystals, sometimes several mm long were yellow or yellow-green. Application of 1% sodium dithionite to the surface growth caused the crystals to be reduced to a dark green color. The second crystal type was irregular in shape and generally smaller than 1 mm in its largest dimension. These crystals were yellow to orange in color and turned brilliant orange when treated with sodium dithionite solution. The strains of *P. aeruginosa* capable of producing oxchlororaphin formed crystals similar to the large form and *P. aureofaciens* formed crystals identical in appearance to the smaller form. Both reacted in the expected manner to sodium dithionite solution.

The glycylglycine-glycerol medium was found to be the most consistent medium for the production of these crystals. Crystals were also formed by some strains with leucylglycine-glycerol, glycylserine-glycerol and glycine-glycerol as a carbon source. The organisms required ammonium sulfate or a nitrogen source for crystal production, however, the organisms were capable of growing with only the amino acid as the nitrogen

source. The most favorable temperature for crystal formation was 30°C. Crystals were seldom formed at 37°C although, growth was luxuriant.

In liquid culture of glycylglycine-glycerol at 30°C, the pH of the medium went to 6.5 in a one week period. Crystals were generally seen 6 to 10 days after inoculation at 30°C. Aeration hindered the formation of crystals.

Of 122 strains of P. aeruginosa examined on the glycylglycine-glycerol medium, 8 strains produced both crystalline pigments simultaneously, 25 produced only the large type crystal, 36 produced only the small type crystal and 53 did not form crystals on this medium. Each strain was tested several times on the medium and consistent results were obtained. No pyocyanin was formed by any culture on this synthetic medium although all did so on the more complex Sierra or Gessard media. When the glycerol content of the medium below 0.1% the red water-soluble pigment was formed in place of the crystals.

DISCUSSION

We have presented evidence that pigments other than pyocyanin are commonly formed by many strains of P. aeruginosa. The probable reasons these pigments were not commonly seen are because they were not formed on routine laboratory media, they were masked by pyocyanin, the cultures were incubated at too high temperatures or not-incubated long enough.

Pyocyanin was generally formed when alanine, glutamate or pyruvate was used as a sole carbon source. However, the crystalline pigments were not formed by P. aeruginosa with these carbon sources which corresponds with the data obtained by Korth (1973) for P. aureofaciens and P. chlororaphis. This shows a difference in metabolism of the phenazine pigments by P. aeruginosa.

Haynes and Rhodes (1962) reported that they unable to devise a medium that supported consistent production of crystals from P. aeruginosa. The glycylglycine-glycerol medium did support repeatable crystalline pigment production by over 50% of all isolates.

This data indicated that crystalline production should not be considered unique to P. chlororaphis and P. aureofaciens.

The red pigment was not chemically characterized in this study; however, the medium used for its production was similar to that used for the production of the aeruginosins (Holliman, 1969) with the exception of leucine and mannitol. Addition of these substances to our medium caused pyocyanin production by our cultures.

TABLE 1 Production of pigment on glycine mineral salts agar by
Pseudomonas aeruginosa

Culture growing on glycine	PIGMENT PRODUCED			
	red	pyocyanin	brown	none
199	114	25	1	59
%100	57.3	12.6	0.5	29.6

TABLE 2 The effect of carbon sources on the production of the iridescent material and pigments by selected strains of Pseudomonas aeruginosa.

CARBON SOURCE ^a	STRAIN ^b					
	5	12	19	43	54	P17
glucose	I	-	-	-	-	-
glycerol	I	-	-	-	-	-
glycine	R	-	R	R	PR	P
alanine	PR	PRI	PR	PR	P	PRI
acetate	P	I	I	P	I	I
asparagine	PR	I	PI	P	PRI	I
pyruvate	P	I	I	P	PI	I
glutamate	P	I	PI	PR	PI	I
malate	P	-	-	P	P	-
citrate	-	-	-	P	-	-
1% glycerol	I	I	-	-	I	I
tryptophan	-	-	-	-	-	-
tryptophan-glycerol	I	I	I	-	I	I
anthranilate	I	I	I	P	PI	I
glycylglycine-glycerol	IC	IC	IRC	RC	IRC	IC
nutrient agar	P	I	PI	P	I	PI
Sierra's agar	P	P	P	P	P	P

a. All carbon sources were 0.2% unless noted in mineral salts agar. Cultures were held at 30°C for 14 days.

b. R represents red pigment; P- pyocyanin; I-iridescent material; C- crystalline pigment and - no pigment.

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