THE IRIDESCENT MATERIAL AND PYOCYANIN OF PSEUDOMONAS AERUGINOSA

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ABSTRACT.—Strains of *Pseudomonas aeruginosa* producing an entire film of iridescent material over the colony did not produce pyocyanin on nutrient agar or on 2 per cent glycerol-nutrient agar unless additional iron was incorporated into the medium. Iridescent material was produced on 51 of the 205 strains isolated in this study and 3 strains that produced the iridescent material were unable to form pyocyanin. There was no apparent relationship between pyocin types and the production of pyocyanin or the iridescent material.The majority of cells in iridescent colonies were viable. The strains used in this study were not "cured" of pyocin types or the iridescent phenomenon by the methods used to cure other organisms of episomal elements. Non-iridescent cultures were isolated from iridescent strains when grown on demethylchloretetracycline-nutrient agar. These organisms retained the same pyocin types as the parent strain. Single carbon source studies indicated that the most likely precursor to the iridescent material was anthranilate.

Since its discovery, the iridescent phenomenon has been considered to be caused by a lytic event. Hadley (1924) showed that an organism capable of producing the iridescent material carried a phage for non-iridescent strains, but the iridescent material was not produced in the plaques on the non-iridescent strains. More recent studies (Warner, 1950; Don and Van Den Ende, 1950; Sierra and Zagt, 1960; Berk, 1963, 1966; Wensinck et al., 1967 and Zierdt, 1971) indicated that there was little evidence in favor of phage mediated lysis. Berk (1963) refered to the iridescent material as "unlysable remnants of the lysed cells:, however, Wensinck et al. (1967) identified this material as a 2-alkyl-4-quinolinol. The production of these substances by *P. aeruginosa* has been reported since 1952 (Wells, 1952) but it was not until the work of Wensinck et al., (1967) that this material was related to the iridescent phenomenon.

Warner (1950) noted the iridescent material was generally associated with those cultures that produced pyocyanin in abundance. This relationship, however, was not expounded. Both pigmentation and iridescence were stimulated by glycerol (MacDonald, 1963; Wensinck et al., 1967), while addition of iron (Wensinck et al., 1967) inhibited the formation of the iridescent material. Hellinger (1951), using only one strain of *P. aeruginosa,* concluded that iron was not essential for the production of pyocyanin, whereas Burton et al. (1948) reported iron essential for pyocyanin production. The requirement of iron for pyocyanin production may be a moot point, but it bears consideration since the pigment has value as a diagnostic tool and its function remains unknown. We noted an obvious relationship between pyocyanin formation and the iridescent phenomenon while examining the different colonial forms of *P. aeruginosa* on media with and without added iron. This paper also reports on viability on the iridescent colonies.

Materials and Methods

Organisms. Two hundred and five strains of *P. aeruginosa* were isolated from a variety of sources includ-

ing soil, water, and human and animal pathological specimens. The organisms were identified as *P. aeruginosa* by the methods described by Wahba and Darrell (1965). All strains were pyocin typed with the strains used by Darrell and Wahba (1964) following the methods of Gillies and Govan (1966). Pyocin tests strains were obtained from Dr. M. T. Parker of the Central Public Health Laboratory, London, England.

Media. Gessard's medium was used for the production of pyocyanin in liquid culture since it has shown to stimulate pigment production (Burton et al., 1948). This medium consisted of: 5 percent glycerol, 2 percent peptone, 2 percent $MgSO_4 \cdot 7H_2O$, 0.04 percent K_2HPO_4 in distilled water. Ferric sulfate was added at a concentration of 0.001 percent wherever "iron" was used. Nutrient agar (Biocert, Fisher Scientific Company, Fairlawn, New Jersey), and 2 percent (w/v) glycerol-nutrient agar were prepared as directed by the manufacturer. A mineral salts agar base (Burns and Harris, 1953) with 0.2% (w/v) of either glucose glycerol, glycine, alanine, trypotophan, sodium acetate, sodium pyruvate, asparagine, sodium glutamate, sodium malate, sodium citrate or sodium anthranilate as sole carbon source was used for the determination of the nutritional requirements for the iridescent material. All glassware was washed with acid and rinsed with distilled water.

Curing. Methods described to "cure" bacteria other than *P. aeruginosa* of episomal elements were used in attempts to alter either the pyocin type or the iridescent phenomenon. These methods included cobalt chloride (Hirota, 1957) and acridine orange (Hirota, 1960) as used on *Escherichia coli:* the acridine orange treatment as used on *Proteus morganii* (Smit et al., (1968) and the mitomycin acridine treatment as used on *Bacillus subtilis* (*Seaman* et al., 1964).

Viable Cell counts. Equal size colonies of a strain growing on nutrient agar and on nutrient agar supplemented with 0.001% $FeCl_3 \cdot 6H_20$ were aseptically cut from the agar and shaken in 100 ml of nutrient broth and plated on nutrient agar.

Culture Viability. Each of ten nutrient agar slants in screw cap tubes were inoculated with 0.1 ml of a 10^8 cell/ml suspension of bacteria. The cultures were then incubated continuously at 30C and every five days, one slant was removed from the incubator and streaked for viability on nutrient agar.

Pyocyanin Assay. Cells were inoculated in 10 ml of Gessard's medium in a 50 ml Erlenmeyer flask and were incubated with and without shaking for 3 days at 30C or 1 day at 37C as noted. The suspensions were acidified with dilute acid and centrifuged. The clarified supernatant was neutralized with dilute NaOH, extracted with chloroform and read at 685 nm in a Spectronic 20 spectrophotometer.

RESULTS

While examining many isolates it was noted that strains showed different types of iridescent phenomena. Some strains formed colonies with central pits that contained the iridescent material (Fig. 1). In general the iridescent material was confined to these pits and did not spread over the colony. These strains produced pyocyanin on nutrient agar without added iron. Other strains produced a continuous film of iridescent material which covered the entire colony (Fig. 2). This group

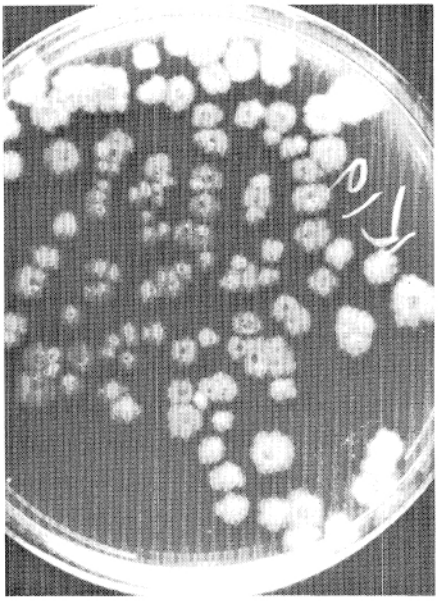

FIGURE 1. Iridescent material in pits of colonies of *Pseudomonas aeruginosa*. K19G.

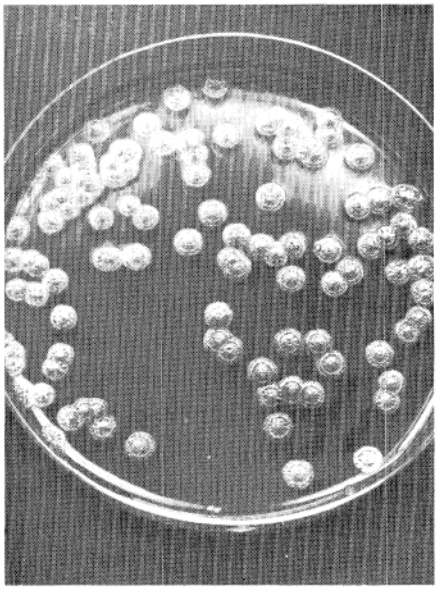

FIGURE 2. Iridescent material completely covering colonies of *Pseudomonas aeruginosa*. K12G.

did not produce pyocyanin on nutrient agar or on 2 percent glycerol-nutrient agar but did form the pigment if iron was incorporated into these media. There were three strains that produced the iridescent material but did not produce pyocyanin on any of the media used even when the production of the iridescent material was suppressed. Other strains required the presence of glycerol to stimulate iridescence and some strains did not produce iridescence under the conditions employed in this study. The iridescent material was produced by 51 of the 205 strains isolated in this study.

The requirement of iron for the production of pyocyanin by the iridescent strains of *P. aeruginosa* was verified in Gessard's liquid culture medium (Table 1). Ferric nitrate, ferric chloride of ferrous chloride could be substituted in these studies. Table 2 shows the relationship between the iridescent material and pyocyanin of 25 selected cultures.

The pyocin types of these organisms were not affected by the incorporation of iron in the culture medium and the types could not be correlated with the formation of pyocyanin or the iridescent material (Table 3).

Purified iridescent material from two strains grown on nutrient agar or on acetate-mineral salts agar had similar ultraviolet spectra identical to the spectrum reported by Wensinck et al. (1967) for the 2-alkyl-4-quinolinol compounds.

Viable Cell Counts. The number of viable cells in colonies expressing the iridescent phenomenon and in colonies of the same strain grown on a medium that inhibited the iridescent material were examined (Table 4). Two strains were used for comparisons in these experiments. Colonies of strain K12G were completely covered with the iridescent material

TABLE 1. Production of Pyocyanin in Gessard's Medium.

Strain	O. D. 685 mμ[2]	
Stationary cultures[1]	With iron	Without iron
K12G	> 2.0	0.2
K19G	> 2.0	0.5
Shaken cultures[1]		
K12G	0.01	0.04
K19G	0.04	0.05

1. Cells were inoculated in 10 ml of Gessard's medium in 50 ml Erlenmeyer flasks and incubated 3 days at 30C.
2. Optical density of supernatant fluid read at 685 mμ in a Spectronic 20 spectrophotometer.

TABLE 2. Production of Pyocyanin and Iridescent Material by *Pseudomonas aeruginosa*

Culture	*Pyocyanin*[1]	*Iridescent*[2]	*OD*[3] *685nm*
G-1	+	—	.15
G-2	+	—	.03
G-3	+	—	.15
G-4	+++	+	.38
G-5	+	+	.37
G-6	+	—	.05
G-7	+	—	.03
G-8	+	—	.18
G-9	+	—	.15
G-10	+	—	.06
G-11	+++	+	.36
G-12	++	+[4]	.18
G-13	+	—	.12
G-14	+	—	.20
G-15	+	—	.22
G-16	+	+	.27
G-17	+	—	.13
G-18	+	—	.03
G-19	+++	+	.23
G-20	+++	+	.25
G-21	+++	+	.50
G-22	++	+[4]	.35
G-23	+++	+	.25
G-24	++	+[4]	.15
G-25	+++	+	.50

1. Qualitative estimation of pyocyanin in Sierra medium.
2. Iridescent material formed on 1% (w/v) glycerol-nutrient agar.
3. Optical density of chloroform extract of pyocyanin from overnight culture in Gessard's liquid medium with iron.
4. Iridescent material localized in pits on colony.

TABLE 3. Iridescence, Pyocyanin, and Pyocin Types of Selected Strains of *Pseudomonas aeruginosa*.

Source	Strain	Iridescence[1]	Pyocyanin[2] Production	Pyocyanin Production (iron)	Pyocin Type[4]
KSU lab stock	K24G	stimulated	++	++	8-17-39-577 8/39
porcine autopsy	K15G	none	+	+	8-17-39-577 8/39
chicken	K12G	entire	—	+++	8-39-8/39
bovine intestine	K110G	none	++	++	8-39-8/39
equine autopsy	K19G	pitted	+++	+++	8-26-577
bovine brain	K11G	none	++	++	8-26-577
procine autopsy	K18G	entire	—	+++	8-26-39-52 283-577-8/39
canine urine	K56G	entire	—	+++	8-10-39-52-283-577-584-593-8/39
canine ear exudate	K50G	entire	—	—	8-10-39-52 577-8/39

[1]Entire iridescence refers to a film of iridescent material over the whole colony. Pitted refers to iridescence in pits on the colony. Stimulated refers to iridescence formed when grown in the presence of glycerol.

[2]Pyocyanin formed on 2 percent glycerol-nutrient agar.

[3]Pyocyanin formed on 2 percent glycerol-nutrient agar with 0.001 percent ferric sulfate.

[4]Pyocin test strains used by Darrell and Wahba (6).

TABLE 4. The number of cells in colonies with and without the iridescent material.

Strain	colony age (hr)	iridescent[1] culture	% viable	non-iridescent[1] culture
K12G	60	156×10^7	82	185×10^7
	72	213×10^7	83	259×10^7
K19G	48	90×10^7	71	127×10^7
	72	202×10^7	90	222×10^7

[1]Average values of two trials.

whereas strain K19G formed the iridescent material only in pits within the colonies. These data showed that if the iridescent material was the result of lysis, it was less than ¼ of the population, when compared with the same culture grown on media that inhibited this substance. It should be noted that the viable count increased with the age of the colonies (Table 3), indicating that the cells were still multiplying and not totally lysed. Colonies isolated from these iridescent cultures displayed the iridescent material and had the same pyocin type as the parent culture.

Culture Viability. Four different strains on nutrient agar slants were examined during a 50 day period at 30C for viability. Two cultures were iridescent strains and two cultures were non-iridescent strains. All cultures were viable after the 50 day period, all retained the same pyocin types and all retained their ability or inability to form the iridescent material. From these results it was clear that non-iridescent variants did not take over the iridescent cultures and therefore the cells capable of producing the iridescent material were viable.

Isolation of Non-iridescent Cultures from Iridescent Strains. Methods used to cure bacterial cells other than *P. aeruginosa* of episomal elements failed after several trials to cure strains K12G or K19G of their pyocin types or the iridescent phenomenon.

Although non-iridescent colonies of the iridescent strains were isolated by streaking the cultures on nutrient agar with 10 mcg/ml demethylchlortetracycline (Lederle Laboratories, Pearl River, New Jersey), these were probably variants in the parent culture. These isolates retained the same pyocin type as the parent culture and did not revert to the iridescent form even after 1 year continuous transfer on nutrient agar slants.

Nutritional Requirements for the Iridescent Material. Fourteen selected strains were heavily inoculated on a mineral salts agar base which included adequate iron to permit growth and a useable carbon source. One strain, K50G would form the iridescent material only if the medium contained protein, anthranilate or tryptophan and glycerol. This organism was not a mutant requiring tryptophan or anthranilate for growth. With the exception of this strain, the organisms that produced the iridescent material formed this material on the same media as non-iridescent cultures producing pyocyanin. Sodium acetate, sodium pyruvate, sodium glutamate and alanine usually supported the production of the iridescent material.

DISCUSSION

Wensinck et al., (1967) showed that the iridescent material was essentially a pure substance that could be controlled by the iron concentration of the medium. Iron added to aerated liquid culture media is needed by some strains of *P. aeruginosa* for longevity of these cultures (Weinburg and Goodnight, 1970). Our results of the iridescent colonies grown on solid media indicated that the majority of the cells in colonies were alive and these colonies were capable of increasing in size with time. All colonies that were formed from cells of the iridescent colonies also displayed the iridescent phenomenon and had the same pyocin type as the parental culture. Even though the colonies of one strain were completely covered with the iridescent material, the cells under this substance were still capable of forming colonies. The strains of *P. aeruginosa* used in this particular experiment were chosen at random,

however, all iridescent cultures of this organism may not have the same survival patterns because *P. aeruginosa* cultures are often lysogenic (Paterson, 1965). The purpose of this study was to show that the iridescent material covering a colony did not mean the culture had totally lysed.

Pyocyanin appears to be an alternative secondary metabolite to the iridescent material when iron is present in the appropriate medium. Organisms that formed the iridescent material did so on the same medium as the non-iridescent cultures produced pyocyanin. Strains that produced large amounts of pyocyanin were invariably capable of forming the iridescent material, however, a few strains produced only the iridescent material and were apyocyanogenic.

In addition to the iridescent material and pyocyanin, *P. aeruginosa* produces other secondary metabolies including oxychlororaphin, phenazine-1-carboxylic acid and the aeruginosins (Holliman, 1961: Chang and Blackwood, 1968; Kocka, 1969). The formation of these substances depend upon several variables: aeration (Holliman, 1969), pH (Zierdt, 1971), temperature (Kocka, 1969), iron concentration (Wensinck et al, 1967), the stage of growth; Kocka, 1969) and the carbon source (Berk, 1963; Wensinck et al., 1967; Kocka, 1969).

The function of these substances has not conclusively been determined, although Mann (1970) and Korth (1971) indicated that pyocyanin and phenazine-1-carboxylic acid function as redox agents. It is notable that the formation of these secondary metabolites is favored in non-aerated or static conditions and that the carbon source is a determining factor as to which secondary metabolite will be formed.

Since a single strain of *P. aeruginosa* is capable of forming all or several of these secondary metablolites previously mentioned (Chang and Blackwood, 1968; Kocka, 1969) depending upon the cultural conditions, these substances may act as a detoxification mechanism in the phases of growth after logarithmic growth.

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