PYOCIN TYPING: A METHOD OF CATEGORIZING *PSEUDOMONAS AERUGINOSA* CULTURES

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ABSTRACT: A set of pyocin typing—indicator strains of *Pseudomonas aeruginosa* were identified and used to type 200 cultures of this organism that were isolated from a variety of sources. These results were compared with similar data derived with a set of indicator strains previously used by other investigators. This method of bacteriocin typing is outlined and discussed.

Due to the seriousness of antibiotic-resistant *Pseudomonas aeruginosa* infections and the strong indication that man and domestical animals are the natural reservoirs of this organism (Hoadley et al., 1968; Gorgan, 1966), epidemiological methods are needed, particularly in hospital environments. In addition, investigators, who isolate these organisms from soil, water and other natural sources, also desire a means of categorizing their isolates with a simple, rapid method. Phage typing and serological methods have been used, but these are often costly, variable and time consuming. Holloway (1960) suggested that the bacteriocins produced by *P. aeruginosa* (pyocins) might be used in a typing scheme. Darrell and Wahba (1964), Gillies and Govan (1966), Osman (1965) and Zabransky and Day (1969) have worked with such systems and found approximately 90% of the cultures examined produced pyocins against their indicator strains.

We, too, examined this type of system by isolating a set of indicator strains and then compared the results of this set with the results of the set used by Darrell and Wahba (1964).

Materials and Methods

The organisms used in this study were isolated from various pathological and non-pathological sources including: human and animal infections, human fecal material, veterinary autopsy specimens, contaminated tissue culture, soil, water, sewage, food stuffs, grain and plankton.

All the organisms were examined for: growth on 1% (w/w) triphenyltetrazolium chloride in nutrient agar (Wahba and Darrell, 1965); liquefication of gelatin; pyocyanin production; growth at 41C utilization of glycine and hexadecane as a sole carbon source; the inability to use m-inositol, trehalose and mannose as sole carbon sources (Stanier et al., 1966). The results of these tests and the preliminary screening tests of motility, the gram stain, the oxidase reactron, oxidation of glucose in Hugh and Leifsons medium (1953), and colonial characteristics were used to distinguish *P. aeruginosa* from *P. fluorescens* and *P. putida.*

The pyocin indicator strains used by Darrell and Wahba (1964) and their most recent typing scheme were obtained from Dr. M. T. Parker, Central Public Health Laboratory, Colindale Avenue, London England.

All other cultures were Kansas State University stock cultures. Cultures were maintained on duplicate, refrigerated nutrient agar slants and transferred monthly.

PYOCIN TYPING

The method of pyocin typing was similar to those described by Wahba (1963) and Gillies and Govan (1966). Nutrient agar (Fishers) supplemented with 0.1% (w/w) sodium citrate, 0.1% (w/v) dipotassium hydrogen phosphate and 0.001% (w/v) $FeCl_3.6H_2O$ was the medium used for pyocin typing. The ferric chloride was added to allow uniform growth with all strains since added iron inhibits the iridescent phenomenon often associated with *P. aeruginosa* (Wensinck et al., 1967).

The typing procedure was as follows:

1. A heavy streak, 2 cm wide of the organism to be examined was smeared on the agar surface with a sterile cotton tipped applicator and incubated at 30C for 16 hr. Glass petri dishes, 15 cm dia were used.

2. The dishes were then inverted, filter paper soaked in chloroform was placed in the lids of the dishes and the chloroform vapors were exposed to the growth for 15 min.

3. A clean glass microscope slide was used to remove the growth from the surface of the agar and then the agar surface was again exposed to chloroform for another 15 min.

4. The filter paper was removed from the lids of the dishes and the agar surface was exposed to the air for 5 min. Then the plates were covered and allowed to remain at room temperature for 1 hr.

5. Cross streaks of the indicator strains were made perpendicular to the area of the original growth and incubated 12 to 14 hr at 30C. Indicator strains were grown prior to inoculation in nutrient broth to approximately 10^8 cells per ml.

6. Three consistent typings were required before a type was assigned to a particular culture.

Demonstration of a Plaque Forming Agent

A culture of the organism to be tested was grown in nutrient broth for 18 hrs at 30C on a rotary shaker. The culture was centrifuged, filtered through a 0.46 μ membrane filter, a few drops of chloroform were added to the supernatant and then the supernatant was diluted and spotted on a nutrient agar plate smeared with the indicator strain. After overnight incubation at 30C, the clear areas were picked and added to 0.5 ml nutrient broth with a drop of chloroform. This suspension was diluted and spotted on lawns of the indicator strain. If the agent was transferable three times and formed discrete plaques it was considered to be a bacteriophage.

Demonstration of an Inhibitory Agent Permeable to Dialysis Tubing:

Sterile membrane filters (0.46 μ) and pieces of dialysis tubing were aseptically layered on nutrient agar plates. A wide streak of the test organism was smeared on the filter or dialysis tubing and incubated 18 hr. at 30C. After incubation the tubing or filter with the growth was pealed off the agar and the pyocin typing procedure was followed.

RESULTS

The incubation time and temperature of the initial inoculum was determined by trial and error to be optimal for typing at 30C and 16 hr. Longer incubation times or higher temperatures apparently inactivated or destroyed pyocins.

Eight indicator strains were selected from over 200 isolates by

means of pyocin typing the isolates against one another and then those with varied patterns were chosen as the indicator strains. Table 1 lists the indicator strains selected for this study. With these strains, 15 pyocin patterns were obtained from the 200 isolates (Table 2). Seven isolates did not produce any reaction to our indicator strains.

When the same 200 cultures were examined using the Darrell and Wahba indicator strains, the major types listed by Darrell and Wahba were present, but also many other patterns. Only 4 cultures could not be typed. The same 4 could not be typed with our indicators. Zabransky and Day (1969) also noted several other patterns and tried to resolve this problem by denoting them as subtypes. Even with these subtypes, some cultures did not fit this typing scheme. Instead of attempting to use a typing scheme, the organisms were noted by the indicator

TABLE 1. "K" Pyocin Indicator Strains.

STRAIN	SOURCE
K4	soil-dodecane enrichment
K5	tissue culture contaminant
K9	porcine autopsy material
K11	bovine brain
K12	chicken meat
K15	porcine autopsy material
K17	milk
K19	equine autopsy material

TABLE 2. Pyocin Patterns of *Pseudomonas aeruginosa* Isolates With "K" Indicator Strains.

Pattern of Strains Inhibited	Number of Isolates With Pattern
K4	14
K12	18
K4, K12	1
K4, K12, K15	7
K4, K11, K15	22
K4, K11, K19	1
K11, K12, K15	1
K4, K11, K12, K19	43
K4, K12, K15, K17	1
K4, K5, K12, K15	3
K4, K11, K12, K15, K19	59
K4, K9, K11, K12, K19	2
K4, K11, K12, K15, K17, K19	14
K4, K5, K11, K12, K15, K19	1
K4, K5, K9, K11, K12, K15, K19	27
none	7
	200

strains they inhibited, a method generally used in phage typing schemes.

There was a correlation between some of our pyocin indicator strains and the Darrell and Wahba set. Strain K4 of our set corresponded to 8/39; K12 corresponded to 577 and K15 corresponded to B10, although a few exceptions did occur. This correlation was only the relationship of a similar inhibition pattern. Pyocyanin production, the formation of the iridescent material and colonial characteristics of these strains were not similar.

Twenty strains were pyocin typed every six months during a two year period and no variations were noted in the pyocin patterns of these strains.

The inhibition zones produced during pyocin typing were examined in detail and often found to contain a transferable lytic agent. Pyocins, by definition fade on dilution instead of forming discrete plaques and are unable to multiply within cells. However, agents were isolated which formed plaques and were transferable. For example, K19 produced pyocins against strains M8 and B26 but carried a phage for strains 577 and K12.

Staphylococcus aureus, Micrococcus lysodeikticus, Serratia marcescens, Bacillus subtilis, Alkaligenes viscolatis, Escherichia coli 3968 and 3969 (colicin producers), *Chromobacterium* sp. and three cultures of *P. fluorescens,* did not inhibit either set of indicator strains and therefore were considered unable to produce phage or bacteriocins against *P. aeruginosa.*

When these organisms were used as indicator strains, they were often inhibited by *P. aeruginosa* (Table 3). This was true only if the *P.* aeruginosa was a pyocyanin producing culture. With dialysis tubing and membrane filters, the agent inhibiting organisms other than *P. aeruginosa* was shown to pass through both dialysis tubing and membrane filters, whereas, pyocins and phage could only permeate the membrane filter.

TABLE 3. Growth Inhibition by *Pseudomonas aeruginosa* towards Unrelated Organisms.

Organism tested	*P. aeruginosa* strains					pyocyanin** only
	K11*	K17	K5*	K12*	K14*	
Staphylococcus aureus	+	—	+	+	+	+
Micrococcus lysodeikticus	+	—	+	+	+	+
Serratia marcescens	—	—	—	—	—	—
Bacillus subtillis	+	—	+	+	+	+
Alkaligenes viscolatis	+	—	+	+	+	+
Chromobacterium sp.	—	—	+	—	+	+
Escherichia coli	+	—	+	+	+	+

* pyocyanin producing strains
** pyocyanin 30 ug/ml in nutrient broth
\+ refers to growth inhibiton
— refers to no change

DISCUSSION

Pyocin typing was clearly applicable to our purpose of distinquishing between isolates of *P. aeruginosa*. During these investigations this technique was also used: to show that the organisms were capable of bacteriocin production as a diagnostic method for the identification of *P. aeruginosa* (Wahba and Darrell, 1965); as a method to determine if two isolates from the same specimen were different; and to determine if a stock culture was contaminated or formed dissociates if there was a visible change in culture characteristics.

Since agar that had supported the growth of the culture was also used for indicator strains, the metabolic products produced by the original culture must be taken into account in the technique pyocin typing.

This study has shown the pyocin typing often involved agents in the agar other than bacteriocins and were probably bacteriophages. Pigments of *P. aeruginosa* were apparently not toxic to *P. aeruginosa*. The term pyocin typing has been widely accepted, however, this investigation indicated that one should be aware of agents other than bacteriocins causing growth inhibition.

This technique, itself, was inexpensive and required little work time, but the results were not available until two days after the procedure was begun. Reliability was attested to by the fact that 20 strains retained the same pyocin types for two years and there was very little variability in the other cultures. Shionoya and Homma (1968) reported that dissociated strains often showed different susceptibilities than the parent strain to pyocins. The indicator strains used in our study did not form notable dissociates during the period of this investigation. Use of organisms other than *P. aeruginosa* as indicator showed the need that indicator strains must be *P. aeruginosa* otherwise false results would be likely. Organisms other than *P. aeruginosa* did not produce agents that inhibited the growth of *P. aeruginosa*. Only a few known cultures were reported in this study, however, many organisms other than *P. aeruginosa* were encountered during the progress of this work.

The use of the Darrell and Wahba indicator strains as a control were important because this set has frequently been used by other investigators and therefore should serve as a standard until more defined standards are formed. The time and temperature of pyocin typing has been disputed by most investigators in this field. Time and temperatures longer than those used in this study apparently cause inactivation of some pyocins and therefore the lower temperature was used to give the maximum inhibition. Gillies and Govan (1966) recommended the use of a lower growth temperature however, Wahba (1963) and Zabransky and Day (1969) used 37C temperature. With such discrepancies in technique, standaridized procedures are not likely to be obtained.

Bacteriocin producting by organisms other than *P. aeruginosa* has been noted in the literature (Reeves, 1965) and used in the classification of these organisms (Farmer, 1972; Slopek and Maresz-Babczyszin, 1967). Therefore this technique can be easily and cheaply applied for classifying bacteriocin-producing organisms obtained in survey studies.

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