Pyocin Typing of *Pseudomonas aeruginosa*: a Simplified Method

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A simplified method has been devised for typing *Pseudomonas aeruginosa* by pyocin production. Pyocins are produced as strains grow overnight in Trypticase soy broth (without glucose) plus 1% potassium nitrate. Because *P. aeruginosa* can use nitrate instead of oxygen as a terminal electron acceptor, mechanical shaking is not necessary, nor is induction by mitomycin C. Pyocins can now be produced in screw-cap tubes in a water bath or incubator. A total of 250 strains were tested as possible pyocin indicators, which included 60 strains already used in pyocin-typing systems. The final set contained 18 indicators which were chosen because (i) they had clear positive or clear negative reactions, thus eliminating reactions difficult to read, (ii) they had few zones due to bacteriophage lysis, and (iii) they were most sensitive in differentiating clinical isolates of *P. aeruginosa*. The final typing method was tested in several studies and the results were clear; thus definitive epidemiological conclusions could be made. Because it is simple to perform and easily automated, the new method should have application in many hospitals; however, it should be used only in carefully planned epidemiological studies. The method and its application are described in detail, and some pitfalls are discussed.

Nosocomial infections caused by *Pseudomonas aeruginosa* continue to be a problem in many hospitals (3, 5, 8, 12, 16, 23). Within any institution there can be hundreds of different strains because it is often found in the human gut, and because it can survive and multiply in moist environments of the hospital. In any epidemiological study, a sensitive technique is required to compare these numerous strains.

A number of methods have been used to differentiate *P. aeruginosa* in epidemiological studies. These epidemiological markers include serological typing (14, 17, 18, 21, 33, 35), bacteriophage typing (11, 20, 30), pyocin production (6, 7, 11, 15, 16, 38), pyocin sensitivity (11, 24), antibiotic susceptibility (3), and gross phenotypic properties (3). Some of these methods have been useful, but others have given misleading results (6, 39).

Pyocin production appears to be one of the best and most stable markers (6, 11, 16, 34, 38). Pyocins have been produced on solid media in the "scrape and streak" method (15, 34, 38), but results have not always been satisfactory (7).

Pyocins have also been produced in broth with mechanical agitation and induction by mitomycin C (11). Although results were more reproducible, this latter method required special equipment and could not be used in many clinical laboratories.

We recently described a simplified method of producing pyocins (22) which should be applicable in most laboratories. Although the new method worked with all of the sets of pyocin indicators currently in use, some gave much clearer reactions than others. The purpose of this study was to establish a set of pyocin indicators which would work with the new method of pyocin production and to propose a simplified method of pyocin typing.

**MATERIALS AND METHODS**

**Media.** Trypticase soy agar (TSA) was from Baltimore Biological Laboratories (BBL), Cockeysville, Md., and was prepared according to the manufacturer's instruction. Medium 81 contained Trypticase soy broth without glucose (BBL), 27.5 g; potassium nitrate, 10 g; and distilled water, 1,000 ml. The medium was dispensed in 10-ml amounts in screw-cap tubes (125 by 16 mm) and 4-ml amounts in tubes (100 by 13 mm). Skim milk medium contained 100 g of skim milk powder in 900 ml of distilled water. All media...
were autoclaved at 121°C for 10 min (skim milk medium) or 15 min (all others).

Strains of P. aeruginosa. The 24 standard pyocin-producer strains designated "NIH A-NIH X" and the 27 pyocin-indicator strains designated "NIH 1 to NIH 27" were described by Farmer and Herman (11). Thirteen pyocin-indicator strains designated "Gillies T 1 to T 13" and five pyocin-producing strains were obtained from R. R. Gillies, University of Edinburg, Scotland (15, 16). Twelve pyocin indicators (M8, B10, B39, S17, B26, A52, 10/55, 283, 577, 594, 593, and 8/39) designated "Mayo 1 to Mayo 12" were obtained from R. J. Zahransky, Mayo Clinic, Rochester, Minn. (38). None of the 12 strains were those used by Darrel and Wahba (6). Eight pyocin indicators designated "Griffith 1 to Griffith 8" were obtained from L. J. Griffith, Veterans Administration Hospital, Wilming- ton, Del. Seventy-four strains designated "Brazil isolates" were from patients and the environment and had been submitted for an epidemiological study by G. A. Sole Vernin, University of São Paulo, Brazil. Ninety-six strains designated "Bethesda isolates" were from patients at the Clinical Center, National Institutes of Health, Bethesda, Md. Fifty-eight strains designated "SH isolates" were from patients at a 500-bed community hospital. Four strains designated as "Brooke isolates" were from patients at Brooke General Hospital, Fort Sam Houston, Tex. The isolates were identified as P. aeruginosa by colonial morphology, oxidase reaction, oxidative utilization of D-glucose, oxidation of D-glucosamine to 2-ketoglucose, growth on Pseudosel Agar (BBL), pyocycin production on Pseudomonas agar P (Difco), and fluorescein production on Pseudomonas agar F (Difco).

Stock and working cultures. Each culture was streaked on TSA and incubated at 37°C for 24 h. The plates were examined with a dissecting microscope (oblique lighting), and colony types were described and picked for stock culture. If multiple colony types were present, at least one of each type was picked. When there was evidence of colonial dissociation (one colony type giving rise to another colony type), each type was picked with an inoculating needle and re-streaked until it was isolated. Each colony selected for stock culture was inoculated in a circle about 10 mm in diameter on a fresh TSA plate and incubated at 37°C for 24 h. Part of the growth was removed into 5 ml of skim milk medium and stored in the dark at 4°C as the working culture. The remainder of the growth was removed into 2 ml of skim milk medium, frozen in a dry ice-acetone bath, and stored at -20°C (-70°C is actually better) as the permanent stock.

Pyocin production. Pyocins were produced in pyocin production medium 81 by the simplified method recently described (22). Briefly, the procedure was as follows: The strain to be typed was picked and transferred into 10 ml of pyocin medium 81 and grown at 32°C for 18 h. Pyocins were produced during this time. Chloroform (1.0 ml) was added to each tube, and the mixture was shaken vigorously for 10 s, allowed to stand at room temperature for 10 min, and then shaken again. Residual chloroform was evaporated by leaving the caps loose overnight in the refrigerator. Centrifugation will remove the cellular debris, but it is not necessary. These pyocin lysates were then dropped onto lawns of the indicator strains. An un inoculated tube of medium 81 was carried through each step of the procedure as a control. Pyocin production from all strains to be compared was done on the same day.

Pyocin lysates can be applied manually with disposable tuberculin syringes or with the accu-drop bacteriophage-bacteriocin applicator (Sylvania Corp., Milburn, N. J.). The template supplied with this apparatus was designed for the simultaneous addition of 24 lysates; however, a new template was designed to apply 59 lysates to a lawn on a petri plate (150 by 15 mm).

Preparation of indicator lawns. Each strain of P. aeruginosa to be evaluated as an indicator was inoculated into 4 ml of medium 81 and incubated 24 h at 32°C. A 0.01-ml amount of this 24-h culture was measured with a standard 0.01-ml loop (Arthur H. Thomas Co., Philadelphia, Pa.), mixed with 4 ml of medium 81, and poured onto the surface of a very dry TSA plate (50 ml of TSA in a plate 150 by 15 mm or 20 ml in a plate 100 by 15 mm). All liquid was removed with an automatic pipettor. The top of the dish was removed, and the plate was allowed to dry 1 h at room temperature. One drop of each pyocin lysate was applied to the indicator lawns and to a control plate. After the drops had dried, the plates were incubated at 37°C for 24 h.

Reading results. After incubation, the plates were read with a bactronic colony counter (New Brunswick Scientific Co. Inc., New Brunswick, N. J.). Any inhibition greater than the control was defined as positive. Although all reactions were expressed as + or -, the degree of inhibition and any distinctive appearance of the positive zones was also recorded. Some zones were due to bacteriophage as evidenced by definite plaques or residual cell debris within the zone. Phage activity can be confirmed by replica plating onto a second lawn of the indicator strain; pyocin activity is not replicable (11).

Recording pyocin production patterns. After all of the pyocin reactions had been recorded and converted to "+" or "-", Table 1 was consulted, and the 18 reactions were converted to a six-digit number as explained in the table. To have a small number of types, we have defined the first two digits as the strain "type". Thus, there are 64 possible types, the first being type 11 and the last type 88. The term "pattern" was defined to be all six digits which code for 18 pyocin reactions. For example, in Table 4 the strain NIH K would be "type 38" and "pattern 387412." Different strains will be included in the same type; however, they can usually be differentiated by considering the complete pattern.

Selection of the new indicator set. Of 250 strains of P. aeruginosa screened as possible indicators, 172 were eliminated because they (i) had too many zones of weak inhibition, (ii) had too many zones due to phage lysis, (iii) had no zones of inhibition, or too few zones, (iv) had irregular lawns, (v) had autoxploras, or (vi) gave the same information about the producer strains given by other indicators. The remaining 78
Table 1. Simplified method for reporting pyocin production patterns

| Three pyocin reactions | Representation |
|------------------------|----------------|
| ++ +                   | 1              |
| ++ -                   | 2              |
| + - +                  | 3              |
| - + +                  | 4              |
| - - +                  | 5              |
| - - -                  | 6              |
| - - -                  | 7              |
| - - -                  | 8              |

*A strain with bacteriocin production pattern ++ + ++ + ++ + ++ ++ would be coded 61 4813 (++ = 6, ++ + = 1, ++ = 4, ++ = 8, +++ = 1, ++ + = 3). If the number of reactions is not evenly divisible by 3, a second (+ + = A, ++ = B, ++ + = C, and ++ = D) and third code (+ = E, ++ = F) can be used.*

Indicators were then tested against 100 different strains of *P. aeruginosa* isolated from hospital patients or the hospital environment. The 7,800 reactions were analyzed with the aid of a computer program (10) written to select the most useful indicators for differentiating the hospital strains.

**RESULTS**

Eighteen ALA indicators. Table 2 shows the 18 pyocin indicator strains that were chosen, their former designation, and the reactions of 100 different strains against them. Of 1,800 test results, 96.5% were clearly positive (pyocin activity) or clearly negative. Only 1.7% were reactions classified as questionable, and only 1.8% were due phage activity. The new set of indicator strains includes 19 strains of *P. aeruginosa* which had been used in other indicator sets.

Classification of positive reactions. Zones of inhibition varied in size, shape, appearance, and degree. Positive reactions were scored as follows: (i) a zone of complete inhibition with no resistant colonies in it—designated “+”; (ii) completely cleared as above but the zone of inhibition was larger than the size of the original drop which indicates diffusion of the pyocin—designated “+d”; (iii) clear zone with a countable number of small colonies in the zone of inhibition—designated “+50” where 50 would be replaced by the actual number of colonies; (iv) weak zone, with some inhibition of the lawn but the zone is overgrown with colonies—designated “+w”; (v) confluent lysis by bacteriophage—designated “φcF”; (vi) phage lysis but a small number of plaques—designated “φ20” where 20 would be replaced by the actual number of plaques. In addition, any of the above zones can be surrounded by an area of partial clearing (perhaps due to lysozyme or other cell wall digesting enzymes) which was defined to be a metabolic effect, and the symbol “M” was placed after the original designation. For example, a zone with 50 colonies in it, but also surrounded by this partial clearing, would be designated as “+50M.” These symbols are tabulated in Table 3. When the same strain was tested 10 times in the same experiment, the

Table 2. Reaction of the 18 ALA indicator strains against 100 hospital strains of *P. aeruginosa*

| ALA indicator | Former designation | Reaction for each indicator that were: |
|---------------|--------------------|--------------------------------------|
|               |                    | Clearly positive | Clearly negative | Questionable* (+ or -) | Due to phage activity |
| 1             | NIH 7              | 42               | 52              | 1                          | 5                        |
| 2             | NIH 14             | 50               | 46              | 0                          | 4                        |
| 3             | NIH 15             | 47               | 49              | 2                          | 2                        |
| 4             | NIH 20             | 52               | 47              | 0                          | 1                        |
| 5             | Gillies T,         | 58               | 33              | 2                          | 7                        |
| 6             | Brazil isolate     | 42               | 55              | 2                          | 1                        |
| 7             | Brazil isolate     | 49               | 49              | 0                          | 2                        |
| 8             | Mayo 2             | 56               | 40              | 2                          | 2                        |
| 9             | Mayo 9             | 77               | 29              | 0                          | 2                        |
| 10            | Griffith 5         | 36               | 60              | 2                          | 0                        |
| 11            | Griffith 7         | 73               | 22              | 5                          | 0                        |
| 12            | Bethesda isolate   | 60               | 37              | 0                          | 3                        |
| 13            | Bethesda isolate   | 72               | 23              | 1                          | 4                        |
| 14            | Bethesda isolate   | 54               | 42              | 4                          | 0                        |
| 15            | SH isolate         | 46               | 52              | 2                          | 0                        |
| 16            | Gillies T,         | 48               | 50              | 2                          | 0                        |
| 17            | Mayo 5             | 74               | 26              | 0                          | 0                        |
| 18            | Bethesda isolate   | 51               | 48              | 1                          | 0                        |

*These reactions are the ones most likely to lead to misreading errors.*

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results were identical even with respect to the size and shape of the zones.

**Differentiation of 100 hospital strains by the new set of indicators.** When the reactions of the 100 strains were converted to six-digit codes, 92 of the strains had codes different from any other strain. Table 4 shows that two strains can be differentiated even if all 18 bacteriocin reactions (scored only as ‘+’ or ‘-’) are the same. If differences are apparent in the size, shape, or degree of inhibition of the zone, it is a very good indication that the strains are genetically and epidemiologically distinct. The two isolates in Table 4 were isolated from different patients in hospitals separated by over 1,000 miles, so they are epidemiologically distinct. Careful examination of the individual zones of inhibition showed that there were major differences between the two strains. These differences are good epidemiological markers for strains because they are usually stable. Table 5 shows that all of the strains with the same code could be differentiated, except for group III. These two strains are epidemiologically distinct (from hospitals 3,000 miles apart) and appear to have identical reactions due to chance alone. Thus, the new method was 99% accurate in differentiating different strains.

**Problem of bacteriophage.** The new procedure was designed to minimize the production of bacteriophage, but Table 1 shows that some bacteriophage activity was still present. About 75% of the hospital isolates produced phage against at least one trial indicator, but by eliminating those indicators more sensitive to phage, we have reduced the phage reactions to 1.8%.

**Use in epidemiological studies.** The new typing method was used to compare 23 isolates of *P. aeruginosa* from a nursery outbreak, which had previously been studied and reported on (3). The new typing method gave results which allowed identical epidemiological conclusions to be made. Other studies are still in progress, but the preliminary results indicate that this procedure and set of indicators are very sensitive for determining epidemiological relationships among patient and environmental strains of *P. aeruginosa*.

**Comparison of indicator strains from different typing systems.** Table 6 shows the results when 100 different strains were typed against five different sets of indicator strains. There was a considerable difference in their ability to differentiate the 100 strains. The set of 18 ALA indicators was by far the most sensitive, which is not surprising because 11 of these 18 were chosen as being the best ones from the other four sets. Regardless of the number of indicators in a set, the percentage of typable strains was high. It should be pointed out that the sets used by Gillies and Govan, Zabransky and Day, and Griffith were intended for the scrape and streak method (bacteriocin production on an agar surface), not for bacteriocin

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**Table 3. Examples of symbols used in representing different zones of inhibition**

| Symbol | Meaning                      |
|--------|------------------------------|
| +      | Complete clearing due to pyocin |
| ++     | Clear zone with 50 colonies in it |
| + d    | Clear zone, but larger than original drop (d is for diffusion) |
| + w    | Weak inhibition, completely overgrown with colonies |
| φcf    | Confluent lysis by bacteriophage |
| φ15    | 15 bacteriophage plaques |

**Table 4. Use of differences in zone size and shape in differentiating two strains with the same 18 reactions**

| Isolate | Pyocin production pattern | Reaction against indicator: |
|---------|---------------------------|------------------------------|
|         | ALA 9 | ALA 10 | ALA 12 | ALA 16 |
| Brooke 1 SH 1 | 621111 | φcf | + | + | + | d |
|           | 621111 | + | + | + | + | + |

**Table 5. Differentiation of strains with the same 18 reactions**

| Group | Strains* | Pyocin production pattern | Conclusion when zones were compared |
|-------|----------|---------------------------|-------------------------------------|
| I     | NIH K    | 387412                    | Different                           |
|       | Brazil isolate | 387412                    |                                     |
|       | Bethesda isolate | 387412                    |                                     |
| II    | NIH M    | 377111                    | Same                               |
|       | Brazil isolate | 377111                    |                                     |
| III   | NIH B    | 611224                    | Different                           |
|       | Brazil isolate | 611224                    |                                     |
| IV    | Brooke isolate | 621111                    | Different                           |
|       | SH isolate | 621111                    |                                     |
| V     | Brazil isolate | 613124                    | Different                           |
|       | Bethesda isolate | 613124                    |                                     |
| VI    | Brooke isolate | 621724                    | Different                           |
|       | SH isolate | 621724                    |                                     |
|       | Brazil isolate | 621724                    |                                     |

* All strains were epidemiologically distinct; therefore, there should be differences among them.
production in broth. Table 6 must be interpreted with this in mind. However, the results are similar to those reported by the workers who used the indicator strains as they were originally described.

**Reproducibility.** Two different types of reproducibility must be considered—"same day reproducibility" and "different day reproducibility." Multiple typings of a strain done on the same day have always given identical results, even to the size and shape of the zones of inhibition. This was best illustrated when 21 different isolates of the same outbreak strain were typed on the same day. This strain had passed from patient to environment, then back to new patients, but the pyocin production patterns were identical. Thus, the same day reproducibility of the new typing method was excellent. When strains that had been typed previously were repeated, the results were sometimes slightly different. This difference was most apparent in the very weak reactions: the conversion of a "÷w" to a "÷" or a "÷" to a "÷÷÷." Strong reactions never changed. Thus, different day reproducibility of the same strain is not as good, and we urge that if there is any doubt about two isolates being the same, they should be compared at the same point in time.

**DISCUSSION**

Recent studies have shown that infections with gram-negative bacilli are more often the result of hospitalization rather than the cause for it (13, 27). Furthermore, the risk of serious infections, particularly septicemias, increases with extended hospitalization (23). Because of its antibiotic resistance and ubiquitous distribu-

| Pyocin indicator set | No. of indicators in set | No. of different pyocin production patterns | No. of strains in most frequent patterns | No. of untypable strains |
|----------------------|--------------------------|-------------------------------------------|----------------------------------------|-------------------------|
| Gillies and Govan    | 8                        | 39                                        | 37                                     | 3                       |
| Griffith             | 8                        | 18                                        | 20                                     | 19                      |
| Zabransky and Day    | 12                       | 41                                        | 14                                     | 9                       |
| Farmer and Herman    | 27                       | 82                                        | 4                                      | 1                       |
| ALA (this study)     | 18                       | 92                                        | 3                                      | 0                       |

*The bacteriocins were produced in medium 81 as described in this paper.

* Differences in zone size and shape would result in further differentiation but are not considered in this table.

production in the hospital environment, *P. aeruginosa* is one of the most dangerous opportunistic pathogens (3, 23, 26, 31, 32). The need for a simple, reliable technique for comparing all "in-house" isolates of *P. aeruginosa* is evident. If this can be done accurately the epidemiology of *P. aeruginosa* infections in a given institution should be apparent.

During our epidemiological studies on *P. aeruginosa* outbreaks, we have evaluated a number of epidemiological markers (3). Techniques based on antibiotic susceptibility, bacteriophage susceptibility, and pyocin sensitivity all have as a common factor the cell surface of the unknown strain. The phenomenon of dissociation in *P. aeruginosa* is well known (19, 39), and dissociation can bring about surface changes in the dissociated strains (20) which affect their receptivity to any test substances. Dissociation also affects gross phenotypic properties (3, 39). Pyocin production and serological typing have been the best and most stable markers, and for this reason we have sought to improve pyocin production. Antisera for typing *P. aeruginosa* have not been available, but recently the Pasteur Institute has distributed antisera for the 13 Habs "O types" to hospital laboratories in France. In the next few years, antisera should be available from a commercial source(s) in the United States. Determination of the O antigen is certainly the logical first step in any typing system for *P. aeruginosa*. However, many institutions will need a more sensitive marker to differentiate strains with the same O-type, and at this time, pyocin production appears to be one of the best supplementary markers.

The procedure described in this paper was designed to be as simple as possible, but also to be extremely sensitive in differentiating strains. Pyocins are produced in broth because they can be added quickly to plates of indicator strains. This can be done by hand with sterile disposable tuberculin syringes, or 24 pyocins can be added simultaneously with a dropper which is commercially available. As many as 59 drops can be dropped simultaneously with a modification of the dropper. Zones of inhibition are more easily compared if pyocins are produced in broth. Often all strains in a run can be differentiated by their reactions against the first six indicator strains. This part of the procedure could be done first. After the results are read and analyzed, the strains can be compared against the remaining 12 indicators if more sensitivity is required.

The new set of 18 indicator strains was chosen from 250 strains which were evaluated. Eleven
indicators in the new set had been used previously as pyocin indicators. All indicators were chosen with the aid of a computer program which analyzes pyocin reactions and chooses only the best ones for routine typing. There are 2^18 or 262,144 possible patterns when 18 indicator strains are used. Each of these patterns has been assigned a number; 11 111 is the first, 88 8888 is the last. This simplified notation should facilitate recording of results. The first two digits of this six-digit number have been defined as a strain's "pyocin type." This notation should make it easier to refer to certain strains, but it should be remembered that further differentiation is usually possible.

The methods described in this paper have been presented in great detail but should not be considered as a final standardized method. We were tempted at first to propose it as a standardized method, but because there will be improvements with time, we have resisted this temptation. Other more useful indicator strains will be discovered which can be incorporated into a standardized system. After antisera become commercially available for typing P. aeruginosa, the roles of serological typing and pyocin production can be defined better. Because there are already a number of methods for pyocin typing, we propose the following nomenclature for differentiating among them. If results are obtained by the Gillies and Govan method (15), then they can be reported "GG pyocin type ______." If the results are from our procedure, then they can be reported "ALA pyocin type ______." As other provisional methods become available, similar initials can be proposed to define the results.

Who should do pyocin typing? The answer should be "everyone who is concerned about P. aeruginosa infections." Unfortunately, many laboratories cannot spare the personnel time that is required for these procedures, and to do an acceptable job of typing does require significant time. The ideal solution to this dilemma would be for state health departments to offer serological or pyocin typing, or both, as a service. When a hospital suspects a cross-infection problem or outbreak, it could submit the strains for typing and thus determine the epidemiological situation. Until typing procedures are implemented at the state level, hospitals have no recourse but to initiate the procedures for themselves, and unfortunately only a small minority have enough resources to do this. At this stage pyocin typing should be considered an "in-house" technique for comparing strains of P. aeruginosa.

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