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Revised Pyocin Typing Method for *Pseudomonas aeruginosa*

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In the Gillies and Govan method of pyocin typing for *Pseudomonas aeruginosa* a cross-streaking technique was used, and 105 main types and 25 subtypes were identified by the patterns of inhibition observed on 13 indicator strains. Disadvantages of the technique included the need to remove test strain growth before application of the indicator strains, the 48-h period needed to obtain a result, and the inability to reliably type mucoid *P. aeruginosa*. Recent studies have enabled us to overcome these disadvantages and significantly improve the speed and application of pyocin typing. Our revised technique utilizes the same 13 indicator strains which are already used internationally. Test strains were rapidly applied to the surface of agar plates with a multiple inoculator. After incubation for 6 h and exposure to chloroform, the indicator strains were applied in agar overlays without prior removal of the test strain growth. After 18 h of incubation, the pyocin type was recognized by inhibition of particular indicator strains. Additionally, the activity of particulate (R and F) and nonparticulate (S) pyocins could be distinguished on the basis of inhibition zone size, which thus allowed further discrimination. The revised technique allows typing within 24 h, increases the number of identifiable types, and can be used to type mucoid strains.

In the last three decades, *Pseudomonas aeruginosa* has assumed an increasingly prominent role as the etiological agent in a variety of serious infections in hospitalized patients (12). At particular risk are patients who have suffered major trauma or burns and are exposed to intensive care units (16). Also at risk are normal individuals exposed to a compromising occupational or recreational environment, e.g., a deep-sea diving bell, in which outbreaks of acute and painful otitis externa due to *P. aeruginosa* have been reported (1), or the jacuzzi, in which *P. aeruginosa* has been implicated in the irritating skin rash known as "hot tub" or jacuzzi syndrome (17).

The increased importance of *P. aeruginosa* as an opportunistic pathogen, together with its well-recognized and characteristic ubiquitous nature, gives rise to many instances in which reliable and discriminating typing or "fingerprinting" of strains is required to investigate outbreaks of nosocomial infection and to aid effective infection control.

Several biological criteria have been assessed for typing *P. aeruginosa*, including pigmentation, antibiograms, and phage sensitivity. However, the two most reliable and generally accepted methods are serotyping and pyocin (aeruginocin) typing (2, 14). A number of pyocin typing methods have been described, but in independent comparative experience and reviews (2, 11) it has been suggested that the most suitable method is that developed in our laboratory (4, 7, 8).

In our pyocin typing technique (7), a cross-streaking method is used which can identify 105 main types on the basis of pyocin production by test strains and the recognition of different inhibition patterns observed against eight indicators, labeled 1 through 8, and further subdivision into 25 possible subtypes with five additional indicator strains, labeled A through E. Thus, the discriminatory potential of the method is good and superior to serotyping. However, in epidemiological studies, as with serotyping, the majority of strains fall into a limited number of types or subtypes. Thus, further discrimination would be an advantage. Other disadvantages of this method are (i) the 48 h required to obtain a result; (ii) the need to remove the producer strain growth before application of the indicator strains, which is a messy and time-consuming procedure; (iii) the inability to reliably type mucoid strains of *P. aeruginosa*, which have become a serious problem in respiratory infections in patients with cystic fibrosis; we have previously described a modified pyocin typing method for mucoid *P. aeruginosa* (18), but the method involves preparation of pyocin-containing extracts from aerated broth cultures, and although simple to perform, it is time consuming and labor intensive; and (iv) the fact that the technique, as first described in 1966 (4), did not distinguish between the different classes of pyocin produced by *P. aeruginosa*, i.e., the particulate R- and F-pyocins (5, 6) and the diffusible S-pyocins (9). It has been suggested (7) that the ability to distinguish between particulate and nonparticulate pyocins on the basis of inhibition zone size would provide additional valuable strain discrimination in pyocin typing.

Our continued studies on the production and detection of individual pyocins, including their production by mucoid *P. aeruginosa*, have led to the reduction or elimination of these disadvantages and, thus, to significant improvements in the speed, sensitivity, and application of pyocin typing for epidemiological purposes.

This paper follows a preliminary report (J. A. M. Fyfe and J. R. W. Govan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C35, p. 317) and describes the development and evaluation of a revised technique for pyocin typing. In the revised technique, the use of the original 13 indicator strains and the inhibition patterns previously described (7) are retained. The main practical modifications to the previously described cross-streaking technique (7) are that the test strains are applied by a spotting method and the indicator strains are incorporated in agar overlays without prior removal of the test strain growth.

MATERIALS AND METHODS

Bacterial strains. Fifty pyocinogenic strains of *P. aeruginosa* were employed initially to compare the pyocin typing results obtained by the standard cross-streaking method and a modified spotting method. Thereafter, an additional 500 clinical isolates of *P. aeruginosa*, including mucoid strains, were used to evaluate the use of the spotting method.

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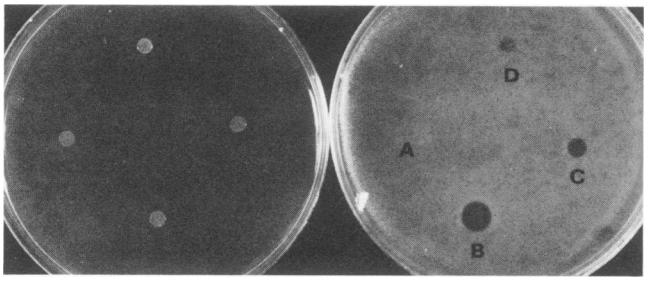

FIG. 1. Examination for pyocin activity against indicator strain 8 in four strains of *P. aeruginosa* by the spotting method. The left plate shows the test strains A, B, C, and D after 6 h of incubation at 30°C and before addition of the indicator strain. The right plate shows the inhibition zones produced by those strains after addition of the indicator strain in an agar overlay and subsequent incubation for 18 h at 37°C. Strain A shows no pyocin activity (−); strain B shows S-pyocin activity (+) characterized by an inhibition zone extending beyond the area of original growth; strain C shows a restricted inhibition zone characteristic of R- and F-pyocins (+); strain D shows a mottled inhibition zone typical of phage activity (-Ø).

Pyocin typing by the cross-streaking method. Pyocin typing by the cross-streaking method was carried out as previously described (7). As suggested in this review, the recognition of S-pyocin activity was incorporated into the typing scheme. Thus, a test strain was allotted to a pyocin type not only on the basis of the pattern of inhibition observed against the 13 standard indicator strains, but it was further characterized by noting the presence of classic S-pyocin activity which causes a zone of inhibition that extends beyond the original growth area of the producer strain. Hence, for example, a strain of pyocin type 1/a producing S-pyocin against indicator strains 7 and B is designated type 1/a ($S_{7,B}$).

Pyocin typing by the spotting method. Strains of *P. aeruginosa* to be typed were streaked for single colonies onto nutrient agar (Columbia agar base; Oxoid Ltd., Basingstoke, London, England) and incubated at 37°C overnight. The single colonies that arose from each test strain were used to prepare a bacterial suspension of 10^8 to 10^9 organisms in 1 ml of sterile physiological saline (absorbance at 550 nm, ≈0.5).

A multipoint inoculator (model A400; Denley Instruments Ltd., Sussex, England), incorporating 21 stainless steel pins (one being a marker pin; diameter of each pin, 2 mm; pins were set 16 mm apart), was used to dispense 1-μl volumes of the bacterial suspensions onto a set of 13 plates (diameter, 90 mm) each containing 10 ml of tryptone soy agar (Oxoid). In this way, 20 test strains could be typed simultaneously against each indicator strain. After the spots dried, usually within a few minutes, the plates were incubated at 30°C for 6 h. Filter paper disks (5 cm; Whatman, Inc., England) were impregnated with chloroform, and the plates were placed over the disks for 15 min to allow the chloroform vapor to kill the bacteria. The plates were then exposed to air for an additional 15 min to eliminate residual chloroform vapor. Cultures of the indicator strains, grown without agitation in nutrient broth (Oxoid no. 2) for 4 h at 37°C to a population size of approximately 10^7 organisms per ml, were applied to the plates by adding 0.1 ml of each bacterial indicator culture to 2.5 ml of molten, semisolid agar (1% peptone; Difco Laboratories, Detroit, Mich., in 0.5% agar; Oxoid L 11) held at 45°C and poured as overlays (NB, a separate indicator strain, was applied to each plate). When the overlays had set, the plates were incubated for 18 h at 37°C, and the pyocin types were determined, as with the cross-streaking method, on the basis of the indicator strains inhibited. The size of the inhibition zones was also taken into account for the purpose of more detailed strain comparison (Fig. 1) and determination of S-pyocin activity incorporated into the typing results as in the cross-streaking method.

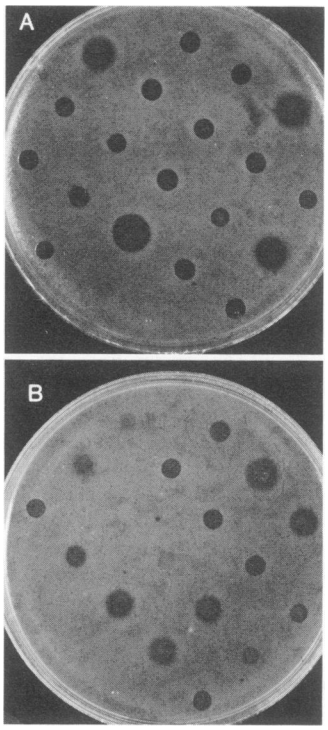

FIG. 2. Typical routine typing plates obtained by the spotting method. (A) Indicator strain 5; (B) indicator strain B.

RESULTS

Detection of pyocin activity by the spotting method. Incubation of the test strains at 30°C on tryptone soy agar for 6 h resulted in detectable pyocin production on the basis of clear zones of inhibition of an indicator strain (Fig. 1). S-type pyocin production could be distinguished from R- and F-types on the basis of zone size. The area of growth after inoculation with a multipoint inoculator had a diameter of 5 mm. Inhibition zones due to R- or F-type pyocins ranged from 5 to 7 mm in diameter and had a sharp edge. S-type pyocins produced zones of 9 to 12 mm in diameter, and these often had a diffuse edge. Phage activity could generally be distinguished from pyocin activity, as the former gave rise to a mottled inhibition zone. Two typing plates on which the same 20 test strains were overlaid with indicator strain 5 (Fig. 2a) and indicator strain B (Fig. 2b) are shown. Comparison of test strains grown on the same set of plates is easier and more meaningful than comparison of those grown on individual plates, as in the cross-streaking method.

Comparison between spotting and cross-streaking methods. For comparative purposes, 50 strains of *P. aeruginosa* were typed by both methods. Twenty-seven strains gave identical results, including the detection of S-type pyocin activity. Nine strains gave the same pyocin type, although the cross-streaking method failed to detect S-pyocin activity against one or more indicators. Fourteen strains gave a different pyocin type by the two methods. In 12 strains, pyocin activity was detected by the spotting method, which failed to

be detected by the cross-streaking method, and in the remaining 2 strains pyocin activity was detected by the cross-streaking but not the spotting method.

Value of S-pyocin production as a further epidemiological discriminator. An additional 500 clinical isolates of *P. aeruginosa* were pyocin typed by the spotting method. The majority of these strains (99%) were typable, and 74% produced S-pyocin activity against 1 or more of the 13 indicator strains. All indicators showed some sensitivity to S-pyocins, and 63 different "S-type patterns" were distinguished. The most common of these were S_5 (11.3% of those strains with S-pyocin activity), $S_{5,B}$ (9.7%), S_7 (8.9%), and $S_{7,B}$ (8.6%).

As an example of the improved discrimination provided by recognition of S-pyocin activity, 34 isolates of *P. aeruginosa*, belonging to the common pyocin type, 1/b, could be divided into 10 distinct groups on the basis of their S-pattern.

Typing of mucoid strains by the spotting method. Thirty mucoid strains of *P. aeruginosa* isolated from the sputa of six patients with cystic fibrosis were pyocin typed by the spotting method. These included multiple isolates from individual sputa. All 30 strains gave clear typing patterns, with 13 strains producing S-pyocins. Multiple isolates from five of the patients were shown to be of the same type (different types for each patient), whereas the sixth patient harbored strains of two distinct types, 29/f(S_5) and 13/k.

DISCUSSION

The revised spotting method of pyocin typing described in this report has advantages over the cross-streaking method previously described (7). (i) The time required to obtain a typing result is reduced from 48 to 24 h. (ii) Up to 20 isolates can be directly compared on the same set of typing plates. This is ideal for comparative typing of multiple colonies from a single specimen to investigate mixed-type infection or to compare isolates from a single epidemic outbreak. (iii) Inhibitory activity due to S-pyocins can be more readily distinguished from R- and F-pyocin activity than when the cross-streaking method is used, thus providing greater discrimination in epidemiological studies. In our study, the epidemiological value of including detection of S-pyocin activity has been emphasized not only by the increased discrimination which it provides but also by the high frequency of S-pyocin production (74%) observed in the 500 test strains examined. This incidence is higher than that reported in a study carried out in India (38%); in the latter survey, however, only 29 strains were examined, and the methods for pyocin production and detection included different cultural conditions and indicator strains (15). (iv) There is no requirement to remove producer strain growth before application of the indicator strains; hence, the method is less tedious and time consuming. Similarly, the application of the indicator strains in agar overlays rather than as cross-streaks is more efficient. (v) Finally, the spotting method is more suitable for typing mucoid *P. aeruginosa*.

An earlier spotting method for pyocin studies described by Kageyama, in which different cultural conditions were used (10), included induction of pyocin production by exposure of the producer strain to UV light. We made a comparative study of six standard reference strains with a range of UV doses and found that under the conditions of the revised typing technique described in this report, no significant advantage was gained for typing purposes by inclusion of an induction stage (unpublished data). In addition, for typing purposes, an induction stage to enhance pyocin production in apparently apyocinogenic strains and thus to reduce the number of untypable strains was unnecessary due to the very low incidence of such strains (1%) found in our survey.

In a wider context, the question remains as to which is the most suitable typing system for epidemiological studies of *P. aeruginosa*, and realistically, is any one system adequate? Despite the improvements in pyocin typing described in this paper, the method still does not match the rapidity of the other most-suitable typing method, serotyping.

P. aeruginosa is serologically heterogeneous, and identification of group-specific heat-stable lipopolysaccharide antigens by agglutination forms the basis of O-serotyping procedures. Several systems have been described and their use reviewed (2, 11, 14). O-serogroup sera are available commercially, but they are expensive and the most widely used system (Difco) requires a set of antigen suspensions for characterizing the sera. In addition, the sera can only be purchased as a complete set of 17 sera.

A major disadvantage of O-serotyping is that the discriminatory power is only fair (3); further discrimination can be provided by detection of H-antigens, but the procedures for H-typing are beyond the scope of many laboratories (14). The typability of *P. aeruginosa* by O-serological typing is usually over 90%, but serotyping is often unsatisfactory for mucoid *P. aeruginosa* in which O-antigens may be masked, typing of colonial dissociants in which serological changes occur within a single culture, and typing polyagglutinable *P. aeruginosa*. The latter, together with mucoid *P. aeruginosa*, forms less than 5% of clinical isolates but is frequently observed in patients with cystic fibrosis (13).

By our use of the revised pyocin typing technique described in this report in combination with O-serotyping it is concluded that neither system provides all the requirements of the ideal typing system for *P. aeruginosa* (unpublished data). We suggest that both systems offer significant contributions to epidemiological studies. O-serotyping provides a rapid indication of antigenic differences when these occur. In an epidemic situation, however, the value of serotyping is limited unless the strains isolated belong to unusual serotypes. Pyocin typing, as described in this paper, requires a period of 24 h to achieve a result but provides adequate discrimination on which to base more confident epidemiological judgment. We suggest that this revised technique provides an improved method for both epidemiological studies of *P. aeruginosa* and for basic studies on the wide range of pyocin activity which can be found in this species.

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