FURTHER STUDIES IN THE PYOCINE TYPING OF *PSEUDOMONAS PYOCYANEA*

J. R. W. GOVAN AND R. R. GILLIES

Department of Bacteriology, University of Edinburgh Medical School

PLATE III

WE have previously reported our method of typing strains of *Pseudomonas pyocyanea* (*Ps. aeruginosa*) by their ability to produce pyocines that give characteristic patterns of inhibition on a set of eight indicator strains (Gillies and Govan, 1966). We also noted that unless incubation of the strain to be typed was undertaken with fairly strict limits on the temperature and period of incubation, a significant proportion of strains gave aberrant results. Our method, although employing fewer indicator strains, was more sensitive than that of Darrell and Wahba (1964) and allowed the recognition of a greater number of pyocine types of *Ps. pyocyanea*.

In the present paper we report more fully on the main typing procedure and outline a method of subdividing strains of pyocine type 1, the commonest type in our series. We also present evidence in support of a hypothesis made in our earlier paper, namely that the finding of strains of more than one type in a patient's lesion is due to infection with strains of different types, and not to instability and variation of type in a single infecting strain.

MATERIALS AND METHODS

Producer strains. During the period 1961–1967, 5690 strains of *Pseudomonas pyocyanea* were typed by their pyocine-producing abilities. These strains were received from numerous diagnostic laboratories either as subcultures of single colonies or as primary diagnostic plate cultures, from which several colonies could be typed. The majority, 91 per cent., of the strains had been isolated from a wide variety of lesions: 4844 strains from lesions in patients being treated in hospital and 349 strains from lesions in patients being treated at home. The remaining 497 strains had been isolated from fomites, dust, surgical instruments, respirators and other apparatus employed in the care of patients in hospitals.

Indicator strains. The indicator strains used in the main pyocine typing procedure were the eight strains (no. 1–8) employed in our previous study. Five additional strains (A–E) of *Ps. pyocyanea* were used in the subtyping of pyocine type-1 strains. These two sets of indicator strains have been in use for more than 4 yr and 2 yr respectively.

Culture media. Tryptone soya agar (Oxoid) was prepared in accordance with the maker's instructions and 5 per cent. of horse blood was added. This tryptone soya blood agar (TSBA) was used for growing producer strains. Infusion broth (Cruickshank, 1965) was used to grow the indicator strains for 4 hr at 37°C before applying them to the processed test plate.

Pyocine typing techniques. The main typing procedure was unchanged from that previously reported (Gillies and Govan) and may be summarised as follows. The strain of

Received 6 May 1968; accepted 3 July 1968.

Ps. pyocyanea to be typed (a potential producer strain) is streaked across the surface of a plate of TSBA so that the width of the inoculum is about 1 cm. The plate is then incubated at 32°C for 14 hr. Thereafter the macroscopic growth is removed with a glass slide and microscopic remnants of growth are killed by pouring 3–5 ml CHCl_3 into the lid of the petri dish and replacing the medium-containing portion.

TABLE I
Patterns of inhibition produced by the 37 different pyocine types
of *Pseudomonas pyocyanea*

Pyocine type of producer strain no.	Inhibition of indicator strain no.							
	1	2	3	4	5	6	7	8
1	+	+	+	+	+	—	+	+
2	—	+	—	—	—	—	—	—
3	+	+	+	—	+	—	+	—
4	+	+	+	+	+	—	—	+
5	—	—	—	—	+	—	—	—
6	+	+	+	+	+	—	+	—
7	+	+	+	—	—	—	+	+
8	—	+	+	+	—	—	+	—
9	—	—	—	—	+	—	+	—
10	+	+	+	+	+	+	+	+
11	+	+	+	—	—	—	+	—
12	+	+	—	+	+	—	—	+
13	—	—	—	+	—	—	—	+
14	—	—	+	—	+	—	+	—
15	—	+	—	—	+	—	+	—
16	+	—	+	+	—	—	+	+
17	—	—	+	—	—	—	+	—
18	+	—	+	+	+	—	+	+
19	—	—	+	+	—	—	+	—
20	—	—	—	—	+	+	—	—
21	—	+	—	+	+	—	—	—
22	+	+	+	—	+	+	+	—
23	+	—	—	—	+	—	+	—
24	—	—	+	+	+	—	+	+
25	+	—	+	—	—	—	+	—
26	+	—	—	—	—	—	+	—
27	+	—	+	—	+	—	+	—
28	—	—	—	+	—	—	+	—
29	—	+	—	—	+	—	—	—
30	—	+	+	—	—	—	—	—
31	—	—	—	—	—	—	+	—
32	—	—	—	+	+	—	—	+
33	+	+	+	+	+	+	+	—
34	—	—	—	—	—	—	—	+
35	+	+	—	—	+	—	+	—
36	—	+	—	+	—	—	—	+
37	—	+	+	+	+	—	+	—

+ = Inhibition; — = no inhibition.

After 15 min. the plate is opened and residual CHCl_3 is decanted. Traces of CHCl_3 vapour are eliminated from the culture plate by exposing it to the air for a few minutes. The eight indicator strains are then applied at right-angles to the original inoculum line. Any pyocines produced by the original inoculum will have diffused into the medium and can then exert their inhibitory activity on the indicator strains during the second period of incubation, which is at 37°C for 8–18 hr. From the patterns of inhibition on the indicator strains, 37 pyocine types of *Ps. pyocyanea* are recognised (table I).

PROGRESS IN PYOCINE TYPING

FIG. 1.—Pattern of inhibition given by a producer strain of pyocine type 1b incubated for 14 hr at 32°C during growth of the producer strain. All the indicator strains except A (top left) are inhibited.

FIG. 2.—The pattern of inhibition obtained in a test identical to that outlined in fig. 1 except that the producer strain was incubated for 14 hr at 35°C. Although the same pattern of inhibition is discernible, the amount of resistant growth in the zones of inhibition has increased.

FIG. 3.—Pattern of inhibition obtained in a test identical to those in figs. 1 and 2, except that the producer strain was incubated for 14 hr at 37°C. None of the indicator strains was inhibited. The difficulty in removing all the growth of the producer strain after incubation at 37°C is apparent.

The technique for subdividing pyocine type-1 strains is identical to that in the main typing procedure except that five additional indicator strains are used to recognise eight subtype patterns (table II).

TABLE II

Patterns of inhibition produced by the 8 subtypes of pyocine type 1 of Pseudomonas pyocyanea

Pyocine subtype of type-1 producer strain *	Inhibition of indicator strain				
	A	B	C	D	E
a	+	+	+	+	+
b	-	+	+	+	+
c	-	-	+	+	+
d	+	-	+	+	+
e	-	+	+	-	+
f	-	-	-	-	-
g	-	-	+	-	+
h	-	+	-	+	+

* Strains in these subtypes are designated as "type 1a", "type 1b", etc.

TABLE III

Constancy of pyocine type in sets of replicate isolates of Pseudomonas pyocyanea obtained on different occasions from the same site in the same patient. Results from 626 sets of isolates from 511 patients

Number of replicate isolates obtained from site *	Number of sets where the isolates were	
	all of one type	of more than one type
1	292	33
2	98	19
3	53	2
4	24	5
5	22	1
6	13	1
7	11	2
8	10	0
9	2	1
10	6	0
11	4	1
> 11†	22	4

* Excluding the first strain isolated from the site.

† Number of isolates from sites where all isolates were of the same type were 13, 15, 15, 16, 16, 16, 17, 17, 18, 19, 20, 21, 25, 25, 27, 29, 29, 30, 37, 43, 44 and 54. Number of isolates from sites that yielded strains of more than one type were 13, 14, 17 and 18.

RESULTS

Results obtained with the main typing method

We have continued to collect evidence on the validity of the typing method by checking the stability of pyocine production in strains of *Pseudomonas pyocyanea* stored on nutrient agar at room temperature (*c.* 21°C) for periods varying from 3 mth to 4 yr. Tests were made on 260 strains in 22 pyocine types, and 15 (5.7 per cent.) of the strains were found to produce patterns of inhibition

TABLE IV

*Distribution of pyocine types in 2396 strains of Pseudomonas pyocyanea isolated during the years 1961-1967 from patients in hospital and patients in the open community**

Pyocine type	Percentage of strains belonging to stated pyocine type in patients † in		
	hospital (1945 strains)	the open community (451 strains)	either hospital or the open community (2396 strains)
1	32.6	34.4	34.2
3	28.0	18.8	25.2
5	5.3	7.1	5.7
10	2.5	3.1	2.9
35	2.1	2.7	2.9
29	1.6	2.0	2.1
27	3.3	0.9	2.0
16	0.9	3.8	1.7
11	2.2	1.3	1.6
9	2.7	0.4	1.3
u/t	6.9	10.2	7.6
u/c	6.2	3.1	5.5
Other types	5.7 (17 types)	12.2 (14 types)	7.3 (24 types)

* The data include those given in table III of our previous paper (Gillies and Govan).

† Only one strain from each patient is represented. Strains from cases from which more than one type was isolated are not included. Only one strain from each epidemic is represented.

u/t = Untypable strains, i.e., not producing pyocines detectable with our indicators.

u/c = Unclassifiable strains, i.e., solitary strains giving patterns other than those shown in table I.

that differed from those given on initial typing. Thirteen of the 15 strains had been stored for more than 6 mth and the remaining two strains showed loss of activity to only one of the eight indicator strains. Six producer strains, used as controls, have been tested at least twice weekly for more than 4 yr and another two producer strains have been tested similarly for more than 2 yr. These eight producer strains have been entirely consistent in their production of pyocines as judged by the patterns of inhibition they produced on the set of indicator strains.

To determine the stability of pyocine production in bacteria living in their natural habitats we have noted whether there is uniformity of pyocine type

among strains of *Ps. pyocyanea* isolated from different cases in the same epidemic. During the last 4 yr we investigated 20 different epidemics, each involving between 5 and 90 patients, and found that every strain in each epidemic gave the same typing pattern. As another index of the stability of pyocine production *in vivo* we determined the constancy of pyocine type in replicate isolates of *Ps. pyocyanea* obtained from the same site in the same patient. The results are given in table III; they show that not all replicate isolates were of the same pyocine type as the original isolate from the same site. The explanation of this finding will be discussed below.

TABLE V

Constancy of subtype in sets of replicate isolates of pyocine type-1 strains of Pseudomonas pyocyanea obtained on different occasions from the same site in the same patient. Results from 190 sets of isolates from 162 patients

Number of replicate isolates obtained from site *	Number of sets where the replicate isolates were	
	all of one subtype	of more than one subtype
1	95	4
2	23	0
3	12	5
4	10	0
5	8	3
6	4	0
7	3	0
8	3	1
> 8 †	16	3

* Excluding the first strain isolated from the site.

† Number of isolates from sites where all isolates were of the same subtype were 10, 10, 10, 11, 11, 11, 13, 14, 15, 16, 17, 17, 19, 22, 44 and 55. Number of isolates from sites that yielded strains of more than one subtype were 10, 25 and 25.

Subdivision of pyocine type-1

The distribution of pyocine types of *Ps. pyocyanea* collected between 1961 and 1967 is given in table IV. Type-1 strains were the commonest and accounted for more than one-third of all strains examined. As already stated, the method of subdividing pyocine type-1 strains is identical with that employed in the main typing procedure except that five additional indicator strains are used. By this modified method we have so far recognised eight distinct subtypes (a-h) of pyocine type 1 (table II). Strains in these subtypes are designated "type 1a", "type 1b", etc. As in the main pyocine typing procedure, subtyping is temperature-dependent, and the incubation of the producer strain must be done at 32°C for 14 hr, and certainly for not more than 18 hr, otherwise aberrant patterns of inhibition are obtained and some strains do not appear to produce pyocines (figs. 1-3).

The eight subtypes of pyocine type 1 are apparently stable in their pyocine-producing abilities when they are cultured and stored *in vitro*, since there was no alteration, either quantitative or qualitative, in the patterns of inhibition when strains were retested after several months' storage on nutrient agar at room temperature (c. 21°C). The *in-vivo* stability of pyocine production in the eight subtypes was determined by observation of the constancy of pyocine subtype in replicate isolates obtained from the same site in the same patient. The results are summarised in table V. As in the observations on the main series of types (table III), not all replicate isolates were found to be of the same pyocine subtype as the original isolate from the same site.

The distribution of subtypes of pyocine type-1 strains is given in table VI.

TABLE VI

*Distribution of subtypes in 795 pyocine type-1 strains of Pseudomonas pyocyanea isolated from patients in hospital and patients in the open community **

Pyocine subtype of type-1 strains	Percentage of strains belonging to stated pyocine subtype in patients † in		
	hospital (662 strains)	the open community (133 strains)	either hospital or the open community (795 strains)
a	5.7	2.3	5.7
b	29.6	36.2	30.3
c	30.2	17.3	26.0
d	18.1	15.0	17.4
e	2.7	1.5	2.6
f	4.4	12.0	5.7
g	2.4	2.3	2.9
h	4.8	6.7	5.9
u/c	2.1	6.7	3.5

* Strains collected during the period 1961-1967.

† Only one strain from each patient is represented. Strains from cases where more than one subtype was isolated are not included. Only one strain from each epidemic is represented.

u/c = Unclassifiable strains, i.e., solitary strains giving patterns other than those shown in table II.

*Occurrence of more than one pyocine type or subtype of
Pseudomonas pyocyanea in the same lesion*

We have previously suggested (Gillies and Govan) that when a series of replicate isolates of *Ps. pyocyanea* from the same site in the same patient includes one or more of the isolates differing in pyocine type from that of the original isolate, it is possible that the patient is yielding different strains with which he had been separately infected. This seems as likely an explanation of the findings as is the supposition that strains are variable in their pattern of pyocine production.

We have attempted to collect evidence in support of our hypothesis by typing numerous colonies of *Ps. pyocyanea* from individual diagnostic plates. The results are given in table VII. An average of six (range 2-33) colonies per plate were typed and the results are given separately for strains isolated from

patients sick at home (home series) and those in hospital (hospital series). It was found that the frequency with which more than one pyocine type or subtype was present in a plating was more than twice as great in platings from specimens in the hospital series (1 in 9·8) as in platings from specimens in the home series (1 in 22·1).

TABLE VII

Frequency with which more than one pyocine type of Pseudomonas pyocyanea was found in specimens from patients at home and patients in hospital

Nature of specimen	Number of specimens from patients				Frequency ratio * in	
	at home		in hospital		home cases	hospital cases
	with more than one type	Total	with more than one type	Total		
Faeces	10	158	1	18	1/15·8	1/18
Urine	2 †	77	10	76	1/38·5	1/7·6
Others ‡	1	53	12	131	1/53	1/10·9
Total	13	288	23	225	1/22·1	1/9·8

* Total number of specimens in category divided by the number of these specimens from which strains of more than one pyocine type were isolated.

† These two patients had recently returned home from hospital.

‡ Swabs from ears, throats, burns and bedsores; blood cultures, cerebrospinal fluid, and sputum.

DISCUSSION

The continuation of our studies on the pyocine typing of strains of *Pseudomonas pyocyanea* has confirmed the validity of the method for epidemiological studies. Our technique has been used successfully by workers in Britain (Brumfitt, Percival and Leigh, 1967; Tinne *et al.*, 1967) and abroad (Fierer, Taylor and Gezon, 1967), and a new pyocine type, type 37, has been added after strains with a distinctive pattern of inhibition (table I) had been isolated from patients in several centres on different occasions during the last 2 yr.

The sensitivity and usefulness of pyocine typing are enhanced by the fact that it is now possible to recognise eight subtypes within type 1, which is the type most commonly encountered in our series. Workers in other countries tell us that pyocine type-1 strains are also the most prevalent strains in their areas. The critical nature of the conditions under which the pyocine-producing strains should be incubated in typing tests has been emphasised previously (Gillies and Govan, 1966) and these conditions have been found equally important in the subtyping of pyocine type-1 strains. In the experiment shown in figs. 1-3 the producer strain of *Ps. pyocyanea* (pyocine type 1, subtype b; i.e., type 1b) was identical on all three plates, but the plates were incubated at different temperatures, namely 32°, 35° and 37°C, for 14 hr during pyocine production. The

clear-cut inhibition of all indicator strains except strain A when the producer strain was incubated at 32°C (fig. 1) contrasts strongly with the lack of inhibition of any of the indicator strains when incubation of the producer strain was at 37°C (fig. 3). When incubation of the producer strain was at 35°C (fig. 2) areas of central growth occurred in the inhibition zones of indicator strains C, D and E. This latter phenomenon was noted by Wahba (1963), who attempted to eliminate it by adding a triad of chemicals to his medium. However, we have shown that by adhering to the lower temperature for incubation of the producer strain and by restricting the period of incubation to not more than 18 hr it is possible to obtain clear-cut patterns of inhibition of the indicator strains in the subtyping of pyocine type-1 strains. Among others, Dixon (personal communication) and Phillips *et al.* (1968) have found our method of subdividing strains of pyocine type 1 to be reliable and epidemiologically valuable.

It has already been noted that not all replicate isolates of *Ps. pyocyanea* from any one site in a patient may belong to the same pyocine type or subtype as the strain originally isolated from that site (tables III and V). Two possible reasons for this lack of constancy of pyocine type or subtype are (1) a variability of pyocine production in a single strain and (2) the infection of a patient with two or more strains originally different in type. While collecting the information summarised in tables III and V we noted that strains of more than one pyocine type were isolated from the same lesion more frequently in patients being treated in hospital than in patients being treated at home. It seems unlikely that the patient's environment *per se* would dictate instability of pyocine production. On the other hand, patients in the hospital environment are more liable to become infected with different strains of *Ps. pyocyanea* than are patients being treated at home. Another significant finding in our prospective survey (table VII) is that the frequency with which more than one pyocine type is found in the same lesion varies between the different categories of specimens more greatly in the home series than in the hospital series. The only similarity between the two series is in the results for faecal specimens. These specimens were from patients suffering from gastro-intestinal upset and most of them yielded bacteria of one or other species belonging to the family Enterobacteriaceae; the isolation of *Ps. pyocyanea* from them was therefore considered to be incidental.

Although in table VII two patients have been allocated to the home series as cases from whose urine more than one pyocine type of *Ps. pyocyanea* had been isolated, it must be stated that both patients had recently returned from hospital, one after prostatectomy and the other, a woman, after investigation of genito-urinary disease. Thus the great frequency with which more than one pyocine type of *Ps. pyocyanea* was found in the hospital series, coupled with the fact that there was relatively little difference in such frequency among the various types of specimens in that series, reflects the great risk of patients becoming infected with new strains of *Ps. pyocyanea* when they are treated in the hospital environment. If it were postulated that inconstancy of pyocine type was due to instability of pyocine production *in vivo* it would be very difficult to explain the contrasting findings in the two series of patients.

SUMMARY

Further experience of the typing of strains of *Pseudomonas pyocyanea* (*Ps. aeruginosa*) by their production of pyocines has confirmed the value of pyocine typing for epidemiological studies.

The introduction of five new indicator strains has made it possible to distinguish eight subtypes of the commonly occurring pyocine type 1. The subtyping of the type-1 strains shows the same dependence on use of the correct temperature and duration of incubation of the producer strain as does the main typing procedure.

In studies of the reliability of the typing and subtyping methods for epidemiological purposes it was noted that not all strains of *Ps. pyocyanea* isolated from the same site in the same patient were of the same pyocine type or subtype. When several colonies of *Ps. pyocyanea* from individual diagnostic plates were typed it was found that more than one pyocine type or subtype was more frequently present in the same lesion in patients in hospital than in patients being treated at home. This finding suggests that the occurrence of more than one pyocine type or subtype of *Ps. pyocyanea* in a patient is generally due to his having been separately infected with strains of more than one type, rather than to instability of pyocine production in a single infecting strain.

The number of colleagues who have supplied us with strains of *Pseudomonas pyocyanea* is too great for us to thank them individually, but we wish particularly to thank Dr J. H. Bowie and other colleagues in the Bacteriology Department, Royal Infirmary, Edinburgh. Mr D. O. Brown has given us unstinted technical help. These investigations were made possible by Public Health Service Grants AI-04833-02 and -03 from the National Institute of Allergy and Infectious Diseases and by a grant from the Scottish Hospitals Endowments Research Trust.

REFERENCES

- BRUMFITT, W., PERCIVAL, A., AND LEIGH, D. A. 1967. *Lancet*, **1**, 1289.
- CRUICKSHANK, R. 1965. Medical microbiology, 11th ed., *Edinburgh and London*, p. 740.
- DARRELL, J. H., AND WAHBA, A. H. . . 1964. *J. Clin. Path.*, **17**, 236.
- FIERER, J., TAYLOR, P. M., AND GEZON, H. M. 1967. *New Engl. J. Med.*, **276**, 991.
- GILLIES, R. R., AND GOVAN, J. R. W. . 1966. *J. Path. Bact.*, **91**, 339.
- PHILLIPS, I., LOBO, A. Z., FERNANDES, R., AND GUNDARA, N. S. 1968. *Lancet*, **1**, 11.
- TINNE, J. E., GORDON, A. M., BAIN, W. H., AND MACKEY, W. A. 1967. *Br. Med. J.*, **4**, 313.
- WAHBA, A. H. 1963. *J. Hyg., Camb.*, **61**, 431.