OBSERVATIONS ON BACTERIOPHAGE TYPING OF
PSEUDOMONAS AERUGINOSA

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Recent reports indicate that there have been increasing numbers of serious infections due to *Pseudomonas aeruginosa* during the past few years (1–3). This has also been true at this hospital where the number of cases of bacteremic infection with this organism and of deaths in such cases has been steadily increasing since the introduction of potent antibacterial agents (4). *Ps. aeruginosa* has become one of the most important infectious agents, rivaling the staphylococcus as a cause of septicemia and death in patients with burns (5–7); it is being found frequently in infected surgical wounds (8), and outbreaks of serious and fatal infections with this organism have been reported in nurseries (9). It causes notoriously intractable urinary tract infections (10, 11), and it is being recognized as a major cause of serious and fatal superinfections in patients under intensive treatment with multiple or broad spectrum antibiotics (12–14). Most recently it has proved to be a major cause of bacteremia and death in patients under treatment for staphylococcal infections with methicillin (dimethoxyphenyl penicillin) (15–17). Most of the pseudomonas infections seem to arise in hospitals (12, 18, 19) and some studies have implicated inhalation of humid air (20–22) and contaminated water (23) as sources of isolated outbreaks in nurseries, but the sources of most of the sporadic infections within hospitals remain obscure.

The use of bacteriophage typing for the characterization of strains within certain genera of bacteria is now well known and has had many applications (24). This method, applied to strains of *Staphylococcus aureus* has proved particularly useful in recent years for the identification of sources of endemic and epidemic spread of infections with this organism, particularly in nurseries

and surgical operating rooms (25–27). However, although phages of *Ps. aeruginosa* have been recognized and studied in some detail in various laboratories, and they have been used to some extent for the classification of strains of this species and in attempts to distinguish strains from various sources, they have not been widely used for "typing" strains or in the epidemiology of pseudomonas infections in hospitals. For these reasons it seemed appropriate to explore the possible uses of pseudomonas phages for such purposes.

The present paper deals with the isolation of pseudomonas phages from clinical sources, some observations on their properties, and a preliminary exploration of the application of these and some other available phages to the typing of strains of *Ps. aeruginosa* from clinical infections. Soon after this study got under way, Gould and McLeod (28) reported in detail on their study, based on bacteriophage and serological techniques, of pseudomonas infections in hospitals. Their report, which contains the pertinent references to previous work bearing directly on this subject, has proved most valuable in the conduct of the present study.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. Seven phages and their propagating strains (PS) were obtained from the American Type Culture Collection ATCC (29). The phages had been designated 12055-B1, -B2, and -B3; and 12175-B1, -B2, -B3, and -B4; the propagating strain of *Ps. aeruginosa* for the first three phages was ATCC-12055,[1] and for the other four it was ATCC-12175. All other strains of *Ps. aeruginosa* were isolated in the bacteriology laboratory of the Boston City Hospital and all other phages were developed from these strains in the course of this study.

The following criteria were used for identification of *Ps. aeruginosa*: motile, gram-negative rods; typical colonial morphology on 10 per cent sheep's blood agar and on eosin methylene blue agar; for most, but not all,

[1] This is host C10 of Dickinson and Codd (30, 31).

strains a diffusible green, or, in a few, a reddish-brown pigment on trypticase soybean agar; pellicle formation in liquid media; growth at 30 to 42° C; glucose the only one of the commonly-used sugars fermented; and characteristic odor. In a previous study of pseudomonas strains reported from this laboratory (32), the formation of a green or brown color on Gessard's glycerol agar was found to be the most reliable test for the production of pyocyanin; by that criterion 103 of 110 strains were classified as *Ps. aeruginosa* and 7 were considered to be atypical strains of pseudomonas, since they failed to produce the pigment. No such differentiation was attempted in the present study.

In all, 161 strains of *Ps. aeruginosa* were isolated and used in this study. Most of them were obtained from cultures of urine or infected wounds, some from blood, peritoneal exudate or cerebrospinal fluid, and occasional ones from specimens of feces that were negative for enteric pathogens and in which the predominating coliform bacilli had first been suppressed by growth in liquid tetrathionate medium.

Isolation, propagation and characterization of Ps. aeruginosa bacteriophages. The procedures were similar in many respects to those used by Gould and McLeod (28). The media used routinely were: trypticase soy broth [2] (TSB), 1.5 per cent trypticase soy agar [2] (TSA), and a semisolid medium consisting of equal parts TSB and TSA.

Each of the phages obtained from ATCC was propagated as follows: 1 ml of a 5-hour TSB culture of the propagating strain was flooded on a TSA plate and the excess fluid removed. The plate was placed in an incubator at 37° C with the lid tilted for 25 minutes to permit drying, after which 1 ml of concentrated phage suspension containing 10^6 to 10^8 phage particles was poured over the surface and permitted to dry again in the same manner after removing the excess fluid. The plate was covered and incubated overnight (16 to 18 hours) at 31° C. The surface of the plate then appeared to be largely free of the bacterial lawn and had only isolated colonies, which usually represented phage-resistant or lysogenic mutants. The "bare" plate was refrigerated at −20° to −30° C for 24 to 72 hours and then thawed at room temperature, yielding varying amounts of clear fluid containing concentrated phage, which was then harvested as the clear supernatant after centrifugation at 3,000 rpm for 30 to 60 minutes. The phage suspension was heated at 58° to 60° C for 2 hours to kill any residual bacterial cells. This procedure did not affect the titer of phage appreciably, thus confirming the observations of Dickinson (33).

The titer of phage in the suspension was estimated by plaque counting (24) and also roughly by delivering single drops of 10-fold dilutions of the suspension with a Pasteur pipet on a dried lawn of the propagating strain. The plaque-counting method also permitted visualization of plaque morphology. The routine test dilution (RTD) for that phage suspension was then selected. This differed from that employed in phage typing of staphylo-

cocci (34) in that 10 or, more often, 100 times the highest dilution giving confluent lysis was used as the RTD for the present study in order to permit visualization of various types of lysis produced by the same phage on different strains; this also served to reduce to some extent the variations in the degrees of susceptibility of different strains of *Ps. aeruginosa* to any given phage. The RTD for most of the phages was a 10^{-2} dilution of the phage suspension, but it ranged from 10^{-1} to 10^{-4} (equivalent to about 10^4 to 10^8 or more phage particles per ml by the plaque-counting method).

In the isolation of new phages, advantage was taken of the spontaneous lysis observed in some cultures of *Ps. aeruginosa* on solid media as evidenced by the moth-eaten appearance of individual colonies, probably representing an unstable lysogenic state of that culture (temperate phage).[3] An isolated moth-eaten colony together with its base agar was cut out and put into TSB, which was then refrigerated for 48 or 72 hours at 4° C and centrifuged. The supernatant was removed, heated, and used as a phage suspension. A lawn of each strain of *Ps. aeruginosa* to be tested was then prepared on the surface of an agar plate and individual drops of the various phage suspensions were placed on this lawn. The susceptibility of the bacterial strain to these phages was evidenced by the development of clear plaques. Bacterial strains showing confluent lysis with any phage suspension were then chosen as indicator and propagating strain.[4] When a suitable host or indicator strain was not found in this manner, a bacterial lawn was flooded with the phage suspension and isolated clear plaques were then looked for as representing virulent phage. Phage mutants which had a wider host-range than their parent phages were sometimes derived in this manner. Each isolated plaque was then picked and placed in a growing 4-hour broth culture of the bacterial strain which had yielded the plaque. The mixture was incubated for only 6 hours, at which time the maximal yield of phage was obtained. The suspension of the lysed areas was then refrigerated and subsequently subjected to centrifugation, heating, titration, and propagation on agar plates as already described for the known phages.

The "phage typing" of strains of pseudomonas was carried out essentially as with staphylococci, using a lawn of a 5-hour TSB culture on "checkerboard" plates on which a drop of RTD (as defined above) of each of the phages was placed. The plates were read after overnight incubation. Clear, confluent lysis was read as +++, 20 to 30 clear plaques as ++, less than 20 clear plaques as +, and no lysis as −. Another type of reproducible lysis, best described as "turbid plaque" formation was also re-

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[2] Obtained from Baltimore Biological Laboratories.

[3] The "metallic spots" sometimes noted in pseudomonas cultures are morphologically different and probably do not represent a phage phenomenon (35).

[4] An "indicator" bacterial strain is one that is lysed by a particular phage but not by some others; a "propagating" strain is one that is maintained and used to prepare a stock of a particular phage (and sometimes more than one) to which it is susceptible.

corded and designated by the addition of "T" after the phage designation. The type or pattern of a pseudomonas strain was then represented by the designation of the phages which produced confluent, that is, +++ or +++ T lysis.

The visualization and counting of plaques was done with double-layered TSA plates. The base layer contained 10 ml TSA (1.5 per cent), and the surface layer consisted of 2 ml of semisolid agar (0.75 per cent TSA) containing 0.1 ml of the desired dilution of phage suspension and 0.1 ml of a 5-hour TSB culture of the propagating strain. The soft agar hardened promptly after being delivered over the previously hardened base layer. The number and morphology of the plaques were recorded after overnight incubation.

Bacterial mutants resistant to a particular phage were developed from a susceptible bacterial strain by flooding a suspension of phage on a lawn of the culture and allowing it to grow overnight. Individual phage-resistant colonies were picked into TSB and grown overnight at 37° C. The resulting culture was washed in TSB, diluted, and plated so as to produce individual colonies. A culture from a single colony was retyped and then used as an indicator strain in the plaque visualization and counting procedure for the phage in question in order to ascertain the nature and degree of the resistance of the mutant strain to that phage.

RESULTS

Development of the set of typing phages. Three criteria were used in selecting phages for inclusion in typing set: *1*) host range, as determined by exposure of the 161 isolates from this hospital

and the 2 host strains obtained from ATCC; *2*) development of resistance to individual phages and tests of cross resistance to other phages; and *3*) visualization of plaque morphology. When two or more preparations of phage suspensions derived from different sources behaved in identical manner with respect to these three criteria, only one of them was chosen for inclusion in the set together with the propagating strain that gave the best yield of that phage. The 13 distinct phages thus chosen are listed in Table I, which also lists the relevant data concerning their sources as well as those of their respective propagating strains. The phages were divided into four groups, based on similarities with respect to one or more of the three criteria. The lytic patterns produced by these phages on all of the propagating strains are shown in Table II.

Phage typing of clinical isolates of Ps. aeruginosa. The set of 13 phages thus selected was used in the "phage typing" of 161 isolates of *Ps. aeruginosa* obtained from various clinical sources throughout the hospital. The largest number, 49, were from infected urines; 25 from infected wounds; 22 from sputum or tracheal aspirates; 14 from cultures of feces which, as already noted, had been found negative for enteric pathogens and from which the predominant coliform organisms had been eliminated by cultivation in tetrathionate

TABLE I

The set of Pseudomonas aeruginosa typing phages

Group	Type	Phage Origin*	No. (PS)	Propagating strain Strain of origin	Source	Yield, plaques per ml	Plaque morphology† Size	Edges¶
A	1	Phage ATCC-12055B₁	1	BCH-12	Sputum	10^8	Small	Irreg. [5]
	2	Phage ATCC-12055B₁M	2	BCH-47a	Urine	10^8	Very large	Sharp.
	3	BCH-165a (ear)	3	ATCC-12175m	Mutant‡	10^{10}	Very large	Irreg. [1]
	4	ATCC-12175B₁	4–5	ATCC-12175		10^{10}	Large	Sharp
B	5	ATCC-12175B₃	4–5	ATCC-12175		10^{10}	Large	Sharp
	6	BCH-15 (wound)	6	BCH-29	Urine	10^8	Medium	Sharp
	7	BCH-79 (urine)	7	BCH-129 brown	Urine	10^7	Variable	
C	8	BCH-5 (blood)	8	BCH-30	Urine	10^8	Medium	Sharp
	9	BCH-94 (urine)	9	BCH-88	Feces	10^{10}	Variable	
	10	BCH-94§ (urine)	10	BCH-137	Urine	10^{10}	Medium	Irreg. [2]
	11	BCH-137 (urine)	11	BCH-127	Sputum	10^9	Medium	Irreg. [4]
D	12	ATCC-12055B₁	12–13	ATCC-12055		10^9	Medium	Irreg.
	13	ATCC-12055B₂M‖	12–13	ATCC-12055		10^9	Medium	Irreg.

* Source of phage or original designation of the lysogenic donor strain of *Ps. aeruginosa*, the source of which is given in parentheses.
† All plaques were clear.
¶ See Figure [ ].
‡ Mutant bacterial strain selected for resistance to phage 12175B₃ (which is here designated as phage 5).
§ Mutant phage isolated from the same lysogenic strain of *Ps. aeruginosa* that yielded phage 9.
‖ This mutant phage produced clear confluent lysis.

TABLE II

Pattern of lysis of propagating strains (PS) by the set of typing phages

| No. (PS) | Phages | | | | | | | | | | | | | Phage pattern* |
| | A | | | | B | | | C | | | | D | | |
	1	2	3	4	5	6	7	8	9	10	11	12	13	
1	+++	+	±	±	−	−	−	−	−	−	−	−	−	A(1)
2	+	+++	−	−	+	−	−	−	−	−	−	−	−	A(2)
3	+++T†	+++T	+++	+++	−	−	−	−	−	−	−	−	−	A(3/4)
4–5	+++T	+++T	+++	+++	+++	+++	−	−	−	−	−	−	−	A/B(3/4/5/6)
6	+++T	+	++	++	+	+++	−	−	−	−	−	−	−	B(6)
7	−	−	−	−	−	−	+++	−	−	−	−	−	−	B(7)
8	−	−	−	−	+++	+++	−	+++	−	−	−	−	−	B/C(5/6/8)
9	−	−	−	−	−	−	−	+++	+++	+++	−	−	−	C(8/9/10)
10	−	−	−	−	−	−	−	−	+	+++	−	−	−	C(10)
11	−	+++T	−	−	−	−	−	−	−	−	+++	−	−	C(11)
12–13	+	−	−	+	−	−	−	−	−	−	−	+++	+++	D(12/13)

* Pattern based only on confluent (+++), complete (clear) lysis.
† T = turbid or hazy lysis, not listed in the phage pattern.

broth; 10 from otitic exudate; 6 from blood cultures; and the rest from other or unknown sources. The latter included 20 strains that had been isolated 18 or more months previously for different studies and had been kept frozen, whereas all of the others were recent isolates made after this study began.

The strains were then classified according to the patterns of lysis with RTD of the different phages, separating those on which the lysis was confluent and clear from those yielding only turbid lysis. In every instance in which confluent lysis, clear or turbid, was produced with RTD (as here defined) of any given phage, the same type of lysis was reproduced on subcultures of the same strain with multiples of RTD. Both the clear and turbid lyses were reproduced on different occasions at least once, and the latter was reproduced numerous times with two strains. Moreover, the same pattern of both kinds of lysis was reproduced whenever multiple isolates of *Ps. aeruginosa* were obtained from the same or different sources in the same patient. A third type of lysis was observed with phage 7 when it was applied undiluted on certain strains. This was an irregular pattern of lysis with fuzzy edges which was also reproducible with subcultures of the same strains.

The results of the phage typing of the 161 isolates are summarized in Table III, which also lists the sources of the strains of each phage pattern. From part A of this table it is seen that 78 strains (48.4 per cent) could be typed on the basis of the patterns of clear and confluent lysis, and that 19 phage patterns of such lysis were discerned among the 78 strains. Included among the 78 were 2 to

4 isolates obtained from the same or different sources in each of seven patients. In each of these patients all of the isolates produced the identical phage pattern; in three this was B(5/6); in three others it was B(5/7); and the isolates from the seventh patient all typed at B(7), each producing the same type of reddish-brown pigment. The proportion of strains from each source (i.e., from urines, wounds, and so forth) that could be typed on the basis of clear lysis did not differ markedly from that for the total of all strains except that a somewhat smaller proportion of those of miscellaneous or unknown sources could be typed in this manner. Of the 29 strains in the latter category, only 9 (31 per cent) produced patterns of clear lysis.

Part B of Table III lists the number of isolates from different sources that failed to produce confluent clear lysis but which yielded reproducible patterns of uniformly turbid lysis with RTD of some of these typing phages. There were 50 such strains and it is interesting to note that all of them reacted only with RTD of the four phages classified here as group A. Also listed as of the last phage pattern in part B of Table III are 16 strains that yielded irregular plaques of turbid lysis with fuzzy edges only with the undiluted suspension of phage 7. A total of 65 strains (40.4 per cent) could be typed in this manner, and they fell into nine patterns, including B(7T). Multiple isolates of *Ps. aeruginosa* were obtained at different times from the same or different sources in four patients; here again all isolates from any one patient yielded the same phage pattern.

In all therefore, 143, or 88.8 per cent, of the 161

TABLE III

"Phage patterns" and sources of 161 clinical isolates of Ps. aeruginosa

Phage pattern	Infected urine	Infected wounds	Sputum or tracheal exudate	Feces[a]	Otitic exudate	Blood culture	Deep abscess	Others, or unknown source	All sources
A. Patterns of confluent clear lysis with RTD									
A(1)			1						1
A(1/2)		1							1
A(2)	2								2
A(2/3/4)	1								1
B(5)	1			1					2
B(5/6)	3	6	2		3	1	1	5	21[b]
B(5/7)	6	1	6			2		3	18[c]
B(6)	2	1	3		1				7
B(7)	3[d]	1							4
B/C(5/8)				1					1
B/C(5/6/8)	1								1
B/C(7/8)		1							1
C(8)		3	1	1				1	6
C(9)	1	1							2
C(10)	1								1
C(11)			1						1
C(8/9/10)	2			4					6
C(8/9/10/11)				1					1
C(9/10)			1						1
Total	23	16	14	8	4	3	1	9	78
B. Patterns of confluent but turbid or hazy lysis with RTD									
A(1T)	2	2						1	5
A(1T/2T)	3	3	4	4	3		2[e]	1[f]	20[g]
A(1T/2T/3T)	1								1
A(1T/2T/4T)			1					4	5
A(1T/4T)	5	1						1	7
A(1T/2T/3T/4T)		1			1			4	6
A(2T)	1	1							2
A(4T)				1		1		1	3
B(7T)[h]	8	1	2			1	1	3	16[i]
Total	20	9	7	5	4	2	3	15	65
C. Nontypable (NT) with RTD or concentrated phages									
NT	6		1	1	2	1	2	5	18[j]
All strains	49	25	22	14	10	6	6	29	161

[a] All fecal strains were from specimens negative for enteric pathogens.

[b] Four separate isolates (3 from leg ulcer and 1 from urine) from one patient; 3 separate isolates (2 ear and 1 throat culture) from a second patient and 2 (both from wound) from a third.

[c] Two separate isolates from each of 3 patients (both from urine in one, both from sputum in the second and 1 from urine and the other from a blood culture in the third).

[d] All from separate urines obtained at different times from the same patient and each produced reddish-brown pigment.

[e] One from osteomyelitis, 1 from empyema.

[f] From cerebrospinal fluid.

[g] Two strains isolated from separate stools of same patient.

[h] Irregular plaques of turbid or hazy lysis produced only with undiluted phage.

[i] Four separate isolates from one patient (1 each from urine, nephrostomy drainage, blood culture and cerebrospinal fluid); 2 isolates from different urine specimens in a second patient and 2 isolates (one from sputum and the other from a burn wound) in a third.

[j] Two of these were separate isolates from an abdominal abscess in one patient and 2 others were from otitic exudate in a second patient.

TABLE IV

Susceptibility of parent and phage-resistant mutant strains of Ps. aeruginosa to the set of typing phages

Strain of Ps. aeruginosa*	Phages													Phage pattern†
	A				B			C				D		
	1	2	3	4	5	6	7	8	9	10	11	12	13	
BCH-63 Parent	+++T	+++T	–	–	+++	–	+++	–	–	–	–	–	–	B(5/7)
Mutant‡	+++T	+++T	–	–	+++	–	–	–	–	–	–	–	–	B(5)
BCH-31 Parent	–	–	–	–	–	–	–	+++	+++	+++	–	–	–	C(8/9/10)
Mutant§	–	–	–	–	–	–	–	–	+++	+++	–	–	–	(C9/10)

* Another such pair of strains, shown in Table II, consists of PS4–5 (parent) and PS3 (mutant resistant to phages 5 and 6).
† Confluent, clear lysis.
‡ Resistant to phage 7.
§ Resistant to phage 8.

strains could be classified with these phages. The remaining 18 strains (11.2 per cent) failed to produce any lysis with any of the 13 phages of the typing set and were therefore classified as non-typable (NT), as shown in part C of Table III.

Not shown in Table III is the distribution of the patients from whom the strains were obtained among the different hospital services and wards. Actually, the strains of each of the phage patterns were obtained from many wards and there was no concentration of these patients in any given part of the hospital. Three was the largest number of patients with strains of the same phage pattern on any one ward; they were among the 15 patients from whom strains of B(5/6) were isolated. There were four wards in which two patients yielded strains of the same phage pattern, a different phage pattern being represented in each ward.[5]

Development of phage-resistant mutants from phage-susceptible bacterial strains. In the course of this study, isolated colonies of bacterial growth were sometimes observed within an area of otherwise clear and confluent lysis produced on the lawn of a strain of *Ps. aeruginosa* on which one of the phage suspensions had been placed. Some of these colonies were subcultured, washed free of residual phage and studied for their susceptibility to the phages of the typing set. The results thus obtained with two mutant bacterial strains, resistant to the respective phages by which they were recognized, with their respective parent strains are shown in Table IV. A third pair of such strains was included among the propagating strains of the typing set of phages, namely, the

[5] These pairs of patients were, respectively, from among 7 patients with type B(6); 15 with B(5/7); 4 with C(8/9/10); and 19 with phage pattern A(1T/2T).

parent strain PS4–5 and the derived mutant PS3 isolated from the area of lysis of PS4–5 produced with phage 5.

From Table IV it is seen that the mutant bacterial strain derived from BCH-63 after it had been acted on by phage 7 gave a phage pattern identical with that of the parent strain except for its failure to be lysed by that phage. Likewise, the phage pattern of the mutant derived from BCH-31 when that strain reacted with phage 8 was identical with its parent except for its resistance to phage 8. From Table II it is seen that PS3 which was derived from a colony within the area of lysis produced on PS4–5 by phage 5 was partially resistant to phage 5 and completely resistant to phage 6, to which PS4–5 was fully sensitive.

Observations on the color produced by some strains of Ps. aeruginosa and their phage susceptibility. Phage 7 of the present typing set is characterized by a rather wide host range. Among the 161 clinical isolates, it produced clear and confluent lysis in RDT of 23 strains, and irregular and atypical lysis of 16 others when applied as the undiluted suspension, each type of lysis being reproducible in every instance. The action of phage 7 on the propagating strains for the typing set of phages, however, was highly specific, as shown in Table II. Only its own PS (BCH-129), which regularly produced a diffusible reddish-brown pigment on TSA, was fully susceptible to phage 7. The development of this phage is of interest.

Early in the present study it was noted that strains yielding a diffusible brownish-red or brown-to-purple color [probably due to pyorubrin (36)] were not susceptible to any of the phages originally obtained from ATCC. Only a phage derived

from the lysogenic yield of a clinical strain of
Ps. aeruginosa, BCH-79, when propagated on an-
other strain, BCH-63, produced a few plaques of
lysis on three available strains that produced deep
brown pigment, *viz.,* BCH-90, -98 and -129.
These three isolates had all been obtained from
cultures of separate urine specimens of one pa-
tient with chronic pyelonephritis. The plaques
produced on BCH-129 were then propagated first
in a growing broth culture of that bacterial strain,
the heated supernatant was further propagated on
TSA plates seeded with BCH-129 which was then
selected as PS7, and the resulting phage was in-
cluded in the typing set as phage 7.

The sequence of events and probable mecha-
nisms involved were somewhat as follows. The
original phage yield from the brown, lysogenic
strain BCH-79, when propagated on the green
strain BCH-63, contained a phage mutant with a
wider host range which included the brown iso-
lates BCH-90, -98 and -129. After propagation
on the brown strain BCH-129, now PS7, the
phage (now labeled phage 7) retained its wide
host range irrespective of whether it was propa-
gated on the green BCH-63 or the brown PS7.
Furthermore, the concentrated suspension of phage
7 could lyse several strains of pseudomonas not
susceptible to other phages and some of these
strains yielded a diffusible purplish color on TSA,
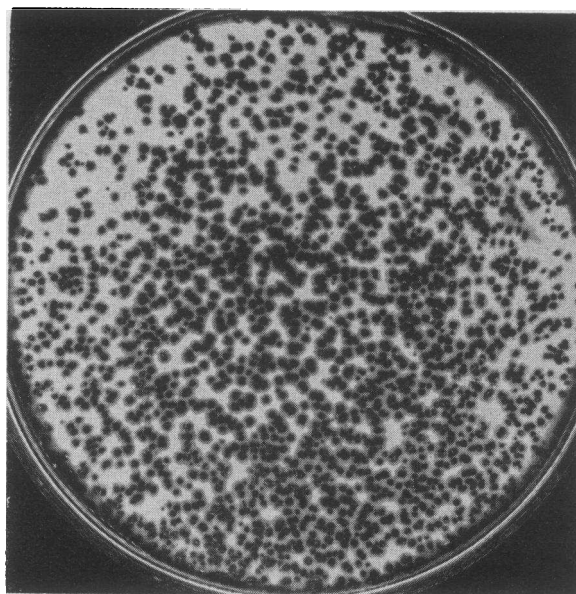especially if the growth was allowed to stand for

Fig. 2. Dilution 10⁻⁷ of phage 10 on PS10. The
plaques are clear, of medium size, and sharply defined
but of irregular outline.

1 or 2 days exposed to daylight. Three such
strains were BCH-19, -20 and -27. However,
although the concentrated phage 7 produced ir-
regular lysis, and individual plaques of varying
size could be demonstrated by the plaque-visuali-
zation method, repeated attempts to propagate
phage from these plaques in growing cultures of
strains BCH-19, -20 and -27 failed. It therefore
seems highly probable that the irregular lysis was
the result of a direct lethal effect of the large
numbers of phage particles on the bacterial strains.

The loss of color production frequently ob-
served in some strains of *Ps. aeruginosa* (32, 37)
makes the estimation of the yield of pigment an
unstable genetic marker for these organisms. The
ability of phage 7 to lyse both green and brown
(red) strains is therefore of some interest.

Observations on plaque morphology. The final
separation of phage clones was carried out by the
plaque-counting technique which also permitted
visualization of the morphology of the individual
plaques. Figures 1 and 2 illustrate the appearance
of the plaques of two of these phages.

The turbid⁶ plaque was of particular interest
and the subject of more detailed study because it

Fig. 1. Dilution 10⁻⁸ of phage 3 on PS3. The plaques
are clear, very large and have irregular edges.

⁶ The term is used here to describe the centeral lytic
area of the plaque and does not refer to the peripheral zones
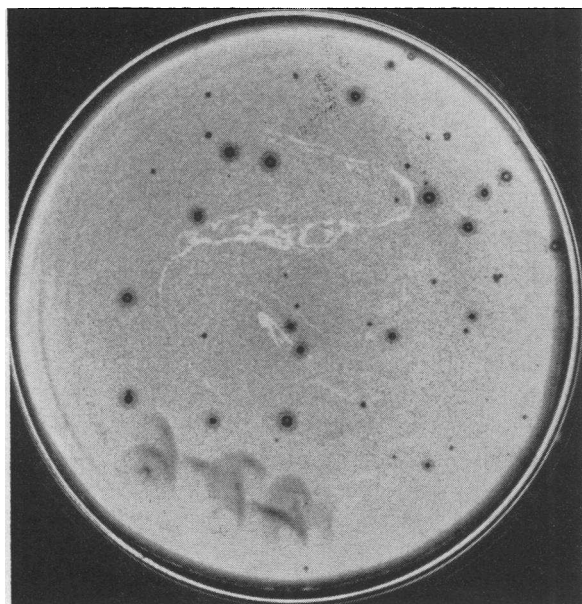of partial lysis, such as those shown in Figures 4 and 5.

FIG. 3. DILUTION 10⁻⁷ OF PHAGE 11 AS ORIGINALLY ISO-
LATED FROM THE PHAGE YIELD OF BCH-127 PROPAGATED
ON THAT STRAIN AS AN INDICATOR. Note the three types
of plaques: clear center with irregular edges; turbid cen-
ter within a clear area and irregular outer borders; very
small clear plaques. A fourth type, small and diffusely
turbid, was also present but not seen in the photograph.

was frequently employed, under special conditions,
in the typing of strains of pseudomonas, as al-
ready noted. Several possibilities were consid-
ered to explain this variety of plaque morphology:
1) mixed host bacterial culture; 2) lysogenic
variants or phage-resistant mutants selected out
of the bacterial culture during exposure to a spe-
cific phage; 3) a mixture of phages; 4) develop-
ment of host-range mutants of the phage; and 5)
temperate phage with lysogenization of part of
the host culture exposed to the phage and lysis
of the rest of the culture by virulent phage.

An example of an attempt to analyze the mecha-
nism of turbid plaque formation may be cited from
the experience with the early development of typ-
ing phage 11. The initial phage suspension from
which this phage was derived produced a mixture
of clear and turbid plaques on the indicator strain
that was used, as shown in Figure 3. When one
of the clear plaques was picked and propagated on
the same host culture, it gave rise to clear plaques
exclusively, as shown in Figure 4.

To rule out the possibility that a mixed cul-
ture accounted for the turbid plaque, PS11 was

grown in TSB and then 10 indivdual colonies were
picked from TSA pour plates of end-dilution of
that culture. Each colony was grown separately
in broth and then tested with the original phage
suspension. Both clear and turbid plaques were
produced on the cultures from each of the 10
colonies in a manner similar to that depicted in
Figure 3. However, when purified phage sus-
pensions were prepared by propagating single
clear plaques on each of these single colony cul-
tures, only clear plaques resulted, and these were
always reproducible. Likewise, phage suspensions
derived from single turbid plaques prepared in the
same manner always yielded turbid plaques when
propagated on each of the single colony cultures.
This seems to indicate that the differences in
plaque morphology were not the result of a mixed
host culture.

When a drop of RTD of a suspension of phage
prepared from one of the turbid plaques was used
on a lawn of PS11, it produced confluent turbid
lysis. A turbid section of agar was cut out of
such a plate, inoculated into TSB, and a number
of individual colony cultures prepared, as above.
Each of the resulting cultures was then tested
separately for susceptibility to the clear plaque-
forming phage and to the phage prepared from
the turbid plaque. All were found to be resistant
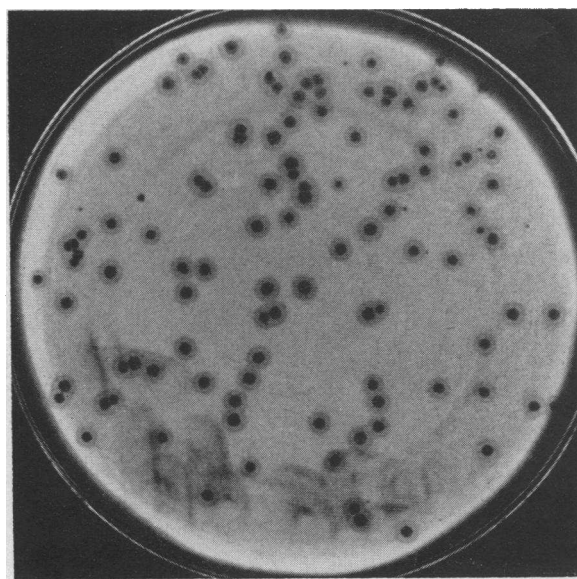to both varieties of phage. On the other hand,

FIG. 4. DILUTION 10⁻⁶ OF THE CLEAR PLAQUE VARIANT SE-
LECTED AS PHAGE 11 WHEN GROWN WITH PS11.

when the clear plaques were cultured in TSB, they yielded no growth, indicating the presence of virulent phage exclusively, which lysed all of the bacterial cells within the clear plaque.

This phenomenon of hazy plaque formation was noted by Dickinson (31) with her host strain C-10 of *Ps. pyocyanea* which served as PS for phages ATCC-12055-B_1, -B_2 and -B_3. She concluded that the clear plaque-forming phage was a virulent mutant of a predominantly temperate phage yield of a lysogenic strain of pseudomonas. The temperate phage, on the other hand, produced hazy plaques capable of establishing a lysogenic state in the host (indicator) strain.

Turbid plaques could also be produced artificially with a single virulent phage suspension screened against a background of a mixture of phage-sensitive and phage-resistant bacteria. Thus, when phage 7, which is lytic for *Ps. aeruginosa* strain BCH-63 was used to infect a bacterial lawn consisting of a mixture of BCH-63 and a phage 7-resistant mutant of that strain (Table IV), confluent turbid plaques resulted. Normally, phage 7 produced only clear plaques on the susceptible indicator strain, BCH-63, and had no effect on the phage 7-resistant mutant of that strain.
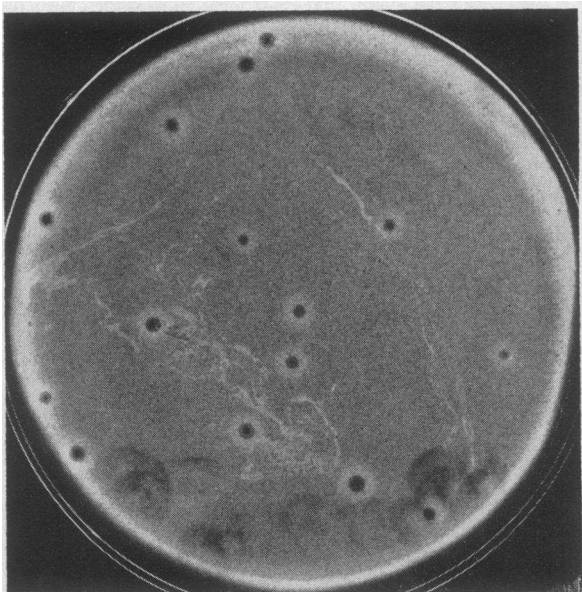
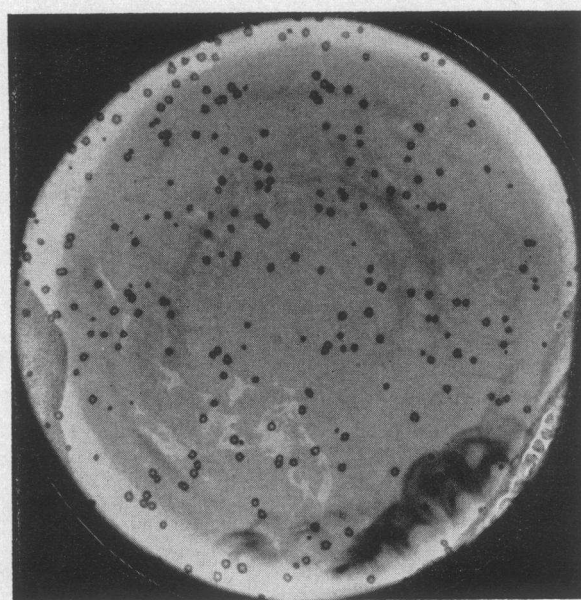In the present study some of the typing phages

Fig. 6. Dilution 10^{-6} of phage 1 on indicator strain BCH-138. Note the turbid centers of the plaques and compare them with the clear plaques produced by this phage on PS1 (Fig. 5).

produced confluent clear plaques on their own propagating strains and on very few other strains, but they produced almost exclusively turbid plaques on all other susceptible bacterial host cultures. This was the case with phage 1 which produced clear lysis on PS1, as shown in Figure 5, and on only one other clinical strain of *Ps. aeruginosa,* whereas it produced only turbid plaques on a large number of hospital strains of pseudomonas (Table III) as illustrated in Figure 6. This is probably the result of the different degrees of virulence of phage 1 for the different bacterial strains. Phages 2, 3 and 4 also behaved in the same manner as phage 1, as can be seen from parts A and B of Table III. The frequent and reproducible finding of turbid plaque formation in the typing of clinical isolates of *Ps. aeruginosa* by the set of typing phages, when used in concentrations greater than the minimum that produced confluent lysis on the PS, was the reason for selecting RTD in the manner used in this study.

In the isolation of new phages, when a lysogenic bacterial strain serving as a phage donor was screened alone on semisolid agar, no plaques were visualized. This was to be expected, since only a certain proportion of the bacterial cells of the moth-eaten colony would lyse and yield phage to

Fig. 5. Dilution 10^{-6} of phage 1 on PS1. Note the clear centers of the plaques and their irregular edges.

which the remaining cells were presumably resistant. If the moth-eaten colony were incubated in broth, the phage-resistant bacteria would multiply without an increase in the yield of phage. For that reason, refrigeration at below freezing temperatures, followed by thawing of the suspension of the unstable lysogenic phage donor colony was used rather than incubation, as recommended by Gould and McLeod (28).

DISCUSSION

The present study should be considered only as an exploration of the possibility of utilizing bacteriophage for the classification of human pathogenic strains of *Ps. aeruginosa* and for the possible use of phage typing in the epidemiology of serious infections caused by this species. From that point of view the study seems to have been successful. It has been possible, with relative ease, to apply some of the basic phage technology and the rather limited previous data available on the application of bacteriophage to clinical strains of this organism, to establish a set of typing phages and to apply them in a manner somewhat similar to, but not identical with that used in phage typing of *Staphylococcus aureus*. Thus far the clinical virulence of the strains does not seem to be correlated with the phage types.

Most of the phages in the typing set were developed in the course of this study and only four of them were used directly from other sources or adapted from previously known phages. Those developed here have not yet been compared with those developed and used in other laboratories (28, 38–41). Moreover, it is recognized that phages of pseudomonas, as well demonstrated in this study, may be rather difficult to maintain in "pure" lines because of the frequency of lysogenization and the unstable lysogenic state of the bacterial hosts. However, the possibility of "purifying" the phages by the methods applied here seems feasible so that it is reasonable to look forward to a system of phage typing not much less satisfactory than that now available for *Staphylococcus aureus*.

Several favorable and promising aspects were brought out in this study. Among them is the fact that once the possibility of a variant or mixture of phage or culture was recognized, it proved feasible in most instances to select and purify the phage and its propagating strain so that they were again useful for typing purposes. Even more important is the fact that typing of any given clinical strain was reproducible with the same cultures and with subsequent subcultures. Moreover, in every instance in which multiple isolates of *Ps. aeruginosa* were made at the same time from different sources or at different times from the same or different sources in the same patient, all of them produced a phage pattern essentially identical with the typing set. This suggests that the organisms retain their specific pattern of phage susceptibility in any given patient, a property which is essential if typing is to be useful in clinical and epidemiological work. Finally, it is most encouraging that nearly 90 per cent of 161 strains isolated from clinical materials in this hospital over a period of several months could be classified with the typing set that was developed.

It remains to be determined to what extent the typing set can be maintained in reasonably stable form over long periods. It will also be necessary to determine how widely these phages can be applied in the identification of strains from other sources within this hospital and from patients and other sources in other hospitals or outside of hospitals. The stability of the individual phages and the susceptibility of bacterial strains under various types of storage and for prolonged periods must also be studied.

SUMMARY AND CONCLUSIONS

The method used in the isolation, purification and characterization of bacteriophages for *Pseudomonas aeruginosa* has been presented. A set of 13 distinct phages was separated on the basis of host range, plaque morphology, mutation and cross resistance. These phages were employed in the classification of 161 strains of *Ps. aeruginosa* isolated from clinical sources in this hospital. It was possible to classify or type 88.8 per cent of these bacterial strains on the basis of the patterns of lysis produced by this set of typing phages. Some observations are presented on the relation of phage susceptibility to the reddish-brown color of some strains of *Ps. aeruginosa*. Studies on the significance of turbid plaque formation and its use in typing are also described.

The reproducibility of the phage patterns with any given strain and the similarity of the phage

pattern of different isolates from the same patient suggest that phage typing of *Ps. aeruginosa* may prove useful for epidemiological purposes.

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