The Bacteriophages of *Pseudomonas pyocyanea*

1. The Effect of Various Substances upon their Development

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SUMMARY: Bacteriophages were isolated from several strains of *Pseudomonas pyocyanea* and were assayed against an indicator strain. Counts of up to 10^{10} /ml. were obtained in both broth and a defined medium. The phages are stable at 60° but rapidly inactivated at 65°. They are unaffected by 50 % glycerol and are stable at pH 6.5–10.0 but not below 6.5.

About 500 compounds have been tested for their inhibitory action on the development of these bacteriophages. All the sulphonamides, amidines, pyrimidines, organo-metallic compounds, plant extracts, mould cultures and antibiotics tested were inactive at concentrations permitting the growth of the host. Proflavine exerted a viristatic effect while notatin and hydrogen peroxide were lethal in a defined medium but not in broth.

As part of an investigation into the chemotherapy of virus diseases, the bacteriophages of *Ps. pyocyanea* and, in particular, the effects of various substances on the bacteriolytic action of these phages have been studied. Whilst it is recognized that information obtained in such work may not be applicable to virus infections of animals, the direct investigation of the chemotherapy of these infections on a large scale presents such difficulties that preliminary investigations were made with bacteriophage as a test virus. Bacteriophages have also been used in this manner by Jones (1945), Klein, Kalter & Mudd (1945), Fitzgerald & Babbit (1946) and Fitzgerald & Lee (1946). It was felt that the use of a relatively resistant type of host, not nutritionally exacting, such as *Ps. pyocyanea* would offer decided advantages. This organism grows rapidly in synthetic media and active phage preparations can be obtained from such cultures. It is, therefore, a convenient host to use for more fundamental work on bacteriophage metabolism.

The lytic phenomena associated with *Ps. pyocyanea* were described by Hadley (1924). Fastier (1945) isolated one specific bacteriophage, and Bordet & Bordet (1946) studied the close relationship of the lytic agent to bacterial variation.

EXPERIMENTAL

Strains. Ten strains of *Ps. pyocyanea* were investigated. Six (nos. C1–C6) were originally isolated by Dr F. R. Selbie of the Bland Sutton Institute of Pathology from war wounds, and four (nos. C7–C10) were isolated in our own laboratory. Strains C1, C2, C3, C5 and C8 were characterized initially by marked pyocyanin production and a silvery sheen on the surface of confluent growth, typical of phage activity on this organism. These strains are referred to below as 'lysogenic', implying only that they can be subcultured without

loss of visible phage activity; presumably sensitive cells serving to maintain the phage are present. True lysogenesis, as defined by Delbrück (1946), does not entail lysis of the host. By rapidly repeated subculture in broth, however, phage-resistant strains were obtained. The lysogenic strains gradually lost their capacity to produce pyocyanin, unless it was maintained by passage through mice. At no time in two years' study did strains C4, C6 and C10 produce pyocyanin or show any evidence of phage action. This does not necessarily indicate that phage was not carried by these strains but rather that we found no bacteria susceptible to them. All ten strains produced fluorescein and retained this capacity throughout the investigation. Culture C9 was not studied in detail; its phage appeared to develop only after storage and even then its appearance was erratic.

Maintenance of strains. Cultures were grown at 37° on nutrient agar, in nutrient broth, or in a defined medium composed of lactic acid 2.4 g., NaCl 5.0 g., $(\text{NH}_4)_2\text{HPO}_4$ 1 g., KH_2PO_4 1 g., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g., and distilled water to 1 l., the pH being adjusted to 7.0 with NaOH and the medium sterilized for 15 min. in the autoclave at 15 lb. steam pressure. Stock cultures were subcultured at monthly intervals on agar and some were stored in the freeze-dried state. It was found advantageous to pass the lysogenic strains through mice occasionally, but passage through the yolk sac of 9-day chick embryos was a more satisfactory means of maintaining these strains and their associated phages, without the risk of contamination.

Cross-reactions of culture filtrates. In order to test the possibilities of cross infection by the phages of different strains, bacteria-free filtrates from 24 hr. broth cultures of each strain were 'spotted' on agar-plate cultures of all other strains not showing visible evidence of phage action (C4, C6, C10 and resistant variants of C1, C3, C5 and C8). The filtrates were designated by the number of the host preceded by the letter F, and individual phages isolated from the filtrate, by Pa, Pb. F1, the filtrate from C1, showed pronounced activity against C10 and C7, whilst F3, from C3, was moderately active against C10. No other cross reactions were observed, so that quantitative work was limited to the action of these filtrates on C10.

When F1 was grown on C10, a high titre filtrate was obtained; this gave rise to two distinct types of plaque (Pl. 1), a large one with a halo (Pa-type), and a smaller clear one (Pb-type). Four consecutive single plaque isolations were made from each of these two types. When grown on C10 in broth; Pa and Pb filtrates gave plaque counts up to 10^{10} /ml., but complete lysis of C10 never occurred. Pb filtrates gave only Pb-type plaques but Pa filtrates did not invariably give plaques of one type, although the Pa-type always greatly predominated. These phages were maintained as Seitz filtrates. It was not possible to produce a lysogenic strain of C10 by implanting F1 in it either *in vitro* or *in vivo* (mouse passage). This was however accomplished with F1 on C7, yielding the lysogenic strain C7X.

Titration of phages Pa and Pb against strain C10. The method of titration finally adopted was to mix 1 ml. of a series of a hundred-fold dilutions of the phage filtrate, or lysed culture, in Ringer's solution with 0.5 ml. 24 hr. broth

culture of C10, and add 2.0 ml. warm nutrient agar. After shaking, the tube was 'sloped' and examined after 18–24 hr. incubation at 37°. This method was much more rapid and convenient than the plate assay used by many workers. In titrations of F1 grown on C10, small numbers of C10 in the material did not matter, since excess of C10 was added in the course of the assay. Of the lysogenic strains, only C1, C3 and C7X possessed phages that could be titrated against C10.

Preparation of filtrates. Bacteria-free Seitz filtrates of 24–72 hr. broth cultures gave counts of up to 10^{10} /ml. for Pa and Pb phages. Seitz filtrates of cultures in defined media were, however, inactive. When Gradacol membranes of 500 μ porosity were used, the loss was still considerable, but counts of 10^6 /ml. were obtained. In the case of Pa, membranes of pore size 100 μ . allowed some phage to pass, but counts were diminished from 10^6 /ml. to less than 100/ml.

Properties of Pa bacteriophage

Heat stability. Type Pa filtrate (6×10^9 /ml.) in nutrient broth, in Ringer's solution and in lactate medium was stable between 50 and 60°, counts being only slightly diminished after 30 min. It was, however, inactivated within 10 min. at 65°. This heat stability was characteristic of all strains which could be assayed and was the basis of a convenient method of obtaining active phage from lysogenic strains, since the host cells were much less resistant; temperatures of 60° for 30 min. usually killed the host, provided the cell population was not very large.

Effect of glycerol. Glycerol was useful as a means of obtaining phage preparations from lysogenic strains, especially in lactate medium, because in a concentration of 50 % it killed the host cells of 24 hr. cultures of C7X and C1 within 7–10 days at 37° leaving the phage unaffected. At 0° the host cells were not killed within a month.

Effect of pH. Type Pa filtrate was kept for 24 hr. at 37° in phosphate and citric acid buffers (pH 2.2–8.0), and in boric acid KCl-NaOH buffers (pH 8.0–10.0). At pH 2.2–4.0 there was complete inactivation within 3 hr. and the count was very much diminished at pH 5.0 and 6.0. At pH 7.0–10.0 (in steps of 0.5 pH unit) there was a slight initial fall in every case, followed by a remarkable stability for at least 24 hr. The initial fall was quite common and occurred in all media, but after this the preparations could be kept for weeks at room temperature without any significant loss of activity, provided the pH was not below 6.5. Preparations of Pa phage in broth, lactate medium, and Ringer's solution at pH values about 7.0–7.5 were kept for several months at room temperature and maintained their titres.

Testing of drugs

In a preliminary test, the action of the drug was studied directly on agar plates and any substances found promising were tested by two quantitative methods.

Agar-plate test, carried out in the presence of multiplying host cells. 1 ml. amounts of a ten-fold series of drug dilutions were mixed with 9 ml. nutrient agar and plates poured. (On 'synthetic' lactic agar the phage action was very difficult to assess.) The plate was marked into four divisions. Three different lysogenic strains showing pronounced and visible phage activity were sown each on one-quarter of the plate. The fourth quarter of the plate was sown with C10 and a loopful of two active filtrates Pa and Pb were 'spotted' on it. After 24 hr. at 37° the lysogenic cultures were examined for silvery sheen and the C10 for lysis. The results were classified as follows.

Class A. Active: phage inhibited by less than 1/100, the drug concentration required to inactivate the host organism.

Class B. Slightly active: phage inhibited by 1/10–1/100, the drug concentration required to inactivate the host organism.

Class C. Inactive: no inhibition of phage at the highest concentration of the drug permitting growth of the host.

Titration of phage inhibitors in actively growing cultures. Filtrate F1 on Culture 10. A 24 hr. culture of C10 was diluted 1/10 in broth or lactate and 1 ml. F1 filtrate, containing 1000–10,000 phage particles/ml., was added to 9 ml. culture. After standing for 10 min. at room temperature a suitable series of concentrations of the test substance in 100 ml. bottles of the test medium were each sown with 1 ml. of the phage-host suspension, the maximum concentration permitting good growth of the host being determined beforehand. The test mixture was incubated at 37°. The phage content was determined at intervals up to 24 hr. by removing 1 ml. samples which were assayed unfiltered. This gave a figure for free phage plus phage in or on living host cells in the test bottles. Bacterial counts were made at the same time, and variation in phage counts were considered significant only if the host count were not itself influenced by the drug.

Lysogenic strains. 1 ml. of a 24 hr. culture of C1 or C7X was added to 100 ml. test medium and plaque counts of the filtered material assayed at intervals against C10. This count, therefore, determined only *free* phage passing the filter. Defined lactate media were filtered through Gradacol membranes of 500 m μ . porosity.

All tests were duplicated at different times and controls without drug were included in every experiment. After 24 hr. counts on the unfiltered material for both media were always between 10^7 and 10^{10} /ml., but values for the lysogenic culture controls were of the order of 10^6 /ml. for filtered material. In the broth titration the substances tested must affect the multiplying phage in the presence of its living host, but the actual assay was carried out at dilutions at which the drug could have no effect on the phage.

Titration of phage inhibitors. Contact test carried out in the absence of host cells. Compounds found to inhibit phage in growing cultures were tested by adding a series of dilutions of the drug to phage filtrates at 37°, in different media, and for varying periods. Subsequent counts indicated whether the drug were lethal.

RESULTS

Inhibition of phage multiplication

Agar plate test. About 500 substances were tested on agar plates against lysogenic strains; the great majority were inactive in the dilutions used (Table 1). The substances tested can be roughly classified as follows: sulphonamides and related compounds, amidines, pyrimidines and other heterocyclic compounds, organo-metallic compounds, antibiotics, plant extracts, mould cultures, and a miscellaneous group of substances thought for one reason or another to be worth while investigating.

Table 1. *Action of various substances against bacteriophages of lysogenic strains of Ps. pyocyanea in agar plate test*

Active	Inactive
Phenol	Fifty plant extracts
Agaric acid	Fifty mould cultures
Hexylresorcinol	Penicillin (100 u./ml.)
5-aminoacridine	Notatin*
2:7-diaminoacridine	Gramicidin
2-chloro-5-amino-7-methoxyacridine	Streptothricin
Proflavine	Streptomycin
	Patulin
	Organo-metallic compounds
	Phenanthridines
	Sulphonamides
	'Homosulphonamides'
	Formaldehyde

* In presence of 0.5% glucose.

Several dyes, such as eosin and brilliant green, showed class A activity against lysogenic strains but results were difficult to read, and the dyes were not investigated further in view of other work of greater promise. One mould appeared very active when the living mould was grown on the plate, but a culture filtrate was entirely inactive. This phenomenon has been noted by Jones & Schatz (1946) in the course of similar studies on staphylococcus and *Bacterium coli* phages. The acridines were the most active class of compound; 5-amino, 2:7-diamino, 2-chloro-5-amino-7-methoxyacridines and proflavine were all active both against lysogenic strains and in the spot tests. Since very active Pa and Pb filtrates were used, small degrees of inhibition were not detectable. Thus, phenol and all the other compounds showing class A activity were inactive by the spot test, but when Pa filtrate was tested in the presence in the assay plate of, for example, 5-amino-acridine, it was obvious that concentrations of 10^{-4} and 10^{-5} of the drug not only diminished the phage count, as compared with the control assay, but rendered the plaques very hazy and very difficult to see. It was partly because of this difficulty in assessing results by the agar plate test that the quantitative tests were developed to show whether compounds were inhibiting phage reproduction. The great majority of compounds could obviously be ruled out at once as inactive by the agar plate test.

The titration of drugs (Table 2) include some of the compounds classed as active in the agar plate test and of these, only proflavine was active. It is interesting to note that several acridines were found active against *Bact. coli*

Table 2. *Action of various substances on bacteriophage in growing cultures*

Substance	Medium	Strain	Approximate phage titre/ml.		
			At 0 hr.* (test and control)	At 24 hr.	
				Test	Control
Phenol					
10 ⁻⁴	Broth	F1 on C10	10° +	10 ⁻¹⁰ +	10 ⁻¹⁰ +
10 ⁻⁴	Lactate	C7X (lysogenic)	10 ⁻⁴ +	10 ⁻⁶ +	10 ⁻⁶ +
10 ⁻⁴	Broth	C1X (lysogenic)	10 ⁻³ +	10 ⁻⁶ +	10 ⁻⁴ ++
5-aminoacridine					
10 ⁻⁴	Broth	F1 on C10	10° +	10 ⁻⁷ ++	10 ⁻⁷ ++
10 ⁻⁴	Lactate	C7X (lysogenic)	10 ⁻⁴ +	10 ⁻⁶ +	10 ⁻⁶ +
10 ⁻⁴	Broth	C1X (lysogenic)	10 ⁻³ +	10 ⁻⁶ +	10 ⁻⁴ ++
Agaric acid					
10 ⁻⁴	Broth	F1 on C10	10° +	10 ⁻¹⁰ +	10 ⁻⁹ +
Hexylresorcinol					
10 ⁻⁴	Broth	F1 on C10	10° +	10 ⁻⁹ +	10 ⁻⁹ +
Proflavine					
10 ⁻⁴	Broth	F1 on C10	10 ⁻² +	10 ⁻² +	10 ⁻⁸ +
10 ⁻⁴	Lactate	F1 on C10	10 ⁻⁴ +	10 ⁻² +	10 ⁻⁸ +
5 × 10 ⁻⁴	Broth	C7X (lysogenic)	10 ⁻² +	10 ⁻⁶ ++	10 ⁻⁸ +
5 × 10 ⁻⁴	Broth	C1X (lysogenic)	10 ⁻² +	10 ⁻⁸ +	10 ⁻¹⁰ +
Patulin					
10 ⁻⁵	Lactate	F1 on C10	10 ⁻² ++	10 ⁻⁸ ++	10 ⁻⁸ ++
Notatin					
10 ⁻⁷	Lactate + 0.5 % glucose	F1 on C10	No growth of host		
10 ⁻⁸	Lactate + 0.5 % glucose	F1 on C10	10 ⁻² ++	10° Nil	10 ⁻⁸ ++
10 ⁻⁸	Lactate	F1 on C10	10 ⁻² ++	10 ⁻⁸ ++	10 ⁻⁸ ++
Streptomycin					
1 u./ml.	Lactate	F1 on C10	10° ∞	10 ⁻⁶ ∞	10 ⁻⁶ ∞
Hydrogen peroxide					
0.0002 % v/v	Lactate	F1 on C10	10° +	10° Nil	10 ⁻⁴ +
0.002 % v/v	Lactate	C1 (lysogenic)	10° ++	10 ⁻⁶ +	10 ⁻⁶ +
0.02 % v/v	Broth	F1 on C10	10° +	10 ⁻⁶ +	10 ⁻⁶ +
0.02 % v/v	Broth	C1 (lysogenic)	10° ++	10 ⁻⁶ +	10 ⁻⁶ +

Key to plaque counts: ∞ = more than 500; ++ = 100-500; + = less than 100.

* Calculated from amount of phage added.

phage by Fitzgerald & Babbit (1946) and Fitzgerald & Lee (1946). Although proflavine prevented the multiplication of F1 on culture C10, it had little, if any, effect, as judged by 24 hr. phage counts, on the lysogenic cultures C7X

and C1. Notatin, although inactive in the agar plate test (see below) was markedly active against F1 in 0.5 % glucose-lactate medium. It is known to be a glucose aerodehydrogenase, exerting its high *in vitro* antibacterial action by producing hydrogen peroxide in the presence of glucose and oxygen (Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick, 1945). Its inactivity in lactate medium without glucose suggested a similar mode of action against bacteriophage and results with hydrogen peroxide are in accord with this viewpoint.

Contact test. Contact tests were carried out with notatin and hydrogen peroxide in various media. A 10^{-6} concentration of a dried notatin preparation in lactate medium containing 0.5 % glucose, completely inactivated a Pa preparation with an initial content of 10^8 /ml., in 3 hr. at 37° and a 10^{-9} concentration inactivated it in 24 hr. Phage counts did not fall in lactate medium plus notatin, lactate medium plus 0.5 % glucose, broth plus notatin and glucose broth plus notatin. Similarly, hydrogen peroxide in a concentration of 0.02 % (v/v) inactivated Pa in lactate medium within 3 hr., but even 2 % (v/v) had no effect in broth. There is no explanation, at present, for this lack of action in broth.

As with proflavine, neither notatin nor hydrogen peroxide influenced the 24 hr. phage count of lysogenic strains. This could be accounted for to some extent by the inactivation of the hydrogen peroxide by catalase, which was produced by all strains of *Ps. pyocyanea*. In the case of F1 acting on culture C10, the hydrogen peroxide would inactivate the free phage before the production of catalase by the host had commenced. With lysogenic strains, if the phage initially within the host cell were unaffected by the hydrogen peroxide, when the latter had been inactivated by catalase, the bacteriophage could reproduce normally. For similar reasons, together with the fact that hydrogen peroxide has no action in broth, notatin would be inactive in the agar plate test even in the presence of glucose.

The author wishes to thank Dr W. J. Elford for his help and criticism, Sir Jack Drummond, F.R.S., and Mr C. E. Coulthard for their interest in this work, and Mr G. Inkley, B.Sc., and Miss S. Codd for technical assistance. Mr Johnson kindly provided the photograph.

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EXPLANATION OF PLATE

Ps. pyocyanea bacteriophage plaques ($\times 5$); showing the larger Pa-type with diffuse halo and the small, clear Pb-type.

(Received 11 November 1947)