The Behaviour of a Temperate Phage of *Pseudomonas aeruginosa* compared with that of a Serologically related, Virulent Mutant

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**SUMMARY:** A temperate phage of *Pseudomonas aeruginosa* (*P. pyocyanea*), on addition to a non-lysogenic indicator strain of the organism, produced a lysogenic culture with a high spontaneous rate of free phage production. A virulent mutant, serologically related to the temperate phage, was obtained from this lysogenic culture; this mutant did not give rise to lysogenically infected bacteria. Both virulent and temperate phages, acting on an indicator strain, had latent periods of 90 min., and the multiplication of each was inhibited by a phenanthridine added immediately after adsorption. This indicated that the initial stage of infection is the same for both types of phage. The production of free phage (i.e. the prophage to phage change) by the lysogenic culture derived from the temperate phage was not inhibited by the drug.

Filtrates of lysogenic strain C7X of *Pseudomonas aeruginosa* yielded two types of plaques when assayed against an indicator strain (Dickinson & Codd, 1952). One type was clearly visible after 18 hr. incubation of the assay plate while the other gave hazy plaques, only visible after 48 hr. In view of Boyd's (1951) work on lytic and symbiotic phages of *Salmonella typhimurium*, the results of further investigations into the case of *P. aeruginosa* are of interest. Lwoff & Gutman (1950) and Lwoff, Siminovitch & Kjeldgaard (1950), using *Bacillus megaterium*, considered that whether or not phage was reduced to prophage in a given bacterium was primarily a function of host resistance. Lieb (1953) analysed the factors involved in the establishment of lysogenesis in a strain of *Escherichia coli* and suggested that the cell response was due to physiological conditions. Lwoff (1958) has recently reviewed the subject of lysogeny. The present work compares the behaviour of a temperate phage with a virulent mutant.

**Definitions.** In this paper the terms proposed by Jacob, Lwoff, Siminovitch & Wollman (1953) are used, in particular:

- **Temperate phage**, meaning a phage capable of producing a lysogenic system with a given host.
- **Virulent phage**, meaning a phage incapable of producing a lysogenic system with a given host.

Cultures are denoted by C and a culture, e.g. C10, made resistant to a given phage, P1, is denoted by C10/c. The *total* phage count of a culture means the number of infectious centres, including free phage. The *free* phage count is that obtained when all bacteria have been removed.
L. Dickinson

METHODS

Strains. Iridescent strains C1 and C7X and the non-iridescent indicator strain C10 (Dickinson & Codd, 1952) were employed. The phages produced by these strains are noted in Table 1. Phages Pa and Pb, originally isolated from C1 (Dickinson, 1948), had been passaged on C10 at intervals, using a phage:bacteria ratio of about 1:1000. These phages were quite distinct serologically and were used only for typing strains.

Phage preparations. Except where otherwise stated, phage preparations were obtained by filtration through Ford Sterimats. Several recent batches of Sterimats adsorbed nearly all the phage; through the courtesy of Mr D. McLean Wyllie of Messrs T. B. Ford Ltd., samples of the raw materials of the mats were examined. As a result of this work Messrs Ford supplied a new grade of mat, SB/B, which was satisfactory for phage filtrations.

Titration of phages. Phage dilutions (1 ml.) in Ringer's solution were added to 1 ml. indicator and 4 ml. nutrient agar and the mixture was poured on to nutrient agar plates. Assay plates were read after 18 hr. incubation at 37°.

Determination of latent periods. The method of Dickinson & Codd (1952) was used.

Cross-resistance tests. Cultures were spread on nutrient agar plates and dried in the incubator. Undiluted filtrates from the cultures under test were spotted on this; when required, resistant organisms were recovered from the spotted area.

Serological tests. Phage antisera were prepared in rabbits inoculated intravenously with 1 ml. high titre filtrates once weekly for 6 weeks. All sera were inactivated by heating at 56° for 20 min. and then kept at -20°.

For preliminary neutralization tests equal volumes of sera dilutions were mixed with dilutions of the phage preparation, and the mixture incubated at 37° for 30 min. in a water-bath.

For determining the rates of neutralization of phages, 25 ml. of the phage preparation and 0.5 ml. antiserum were incubated at 37° and samples removed for phage assay at the intervals 3, 15, 45, 75 min., 3, 5 hr. Controls, using normal serum, showed no loss in titre at 5 hr.

EXPERIMENTAL

Derivation of temperate (Pc) and virulent (Pv) phages

The derivation of the virulent and temperate phages appears in Table 1. There were essentially three types of phage plaques in this work: (1) uniformly hazy; (2) those with a solid centre surrounded by a zone of complete lysis; (3) uniformly clear. A hazy plaque from a 48 hr. assay plate of a filtrate of C7X on C10 was passaged twice on C10, using single plaque isolations. The resulting culture, C10/h, was lysogenic and of twenty colonies tested all produced phage, giving a mixture of hazy plaques with about 1% of the zoned type; after passage on C10 even the hazy plaques were visible at 24 hr. but they never showed a lytic zone. These hazy plaques were often extremely difficult to count.
Phages of P. aeruginosa

Unlike C7X, C10/h was not iridescent but, like C7X, it was resistant to both typing phages Pa and Pb. It was difficult to prove whether all the zoned plaques present were the result of phage mutation or whether different phages were involved. All hazy plaques were neutralized by antiserum to Pb but some of the zoned plaques were neutralized by antiserum to Pa, whereas others were neutralized by antiserum to Pb. One zoned plaque, which gave progeny neutralized by antiserum to Pb, was selected for further passage on C10.

Table 1. Derivation of phages of P. aeruginosa

<table>
<thead>
<tr>
<th>Source</th>
<th>Ref. no.</th>
<th>Phages isolated after C10 passage</th>
<th>Plaque description</th>
<th>Serological type of phage (Pa or Pb)</th>
<th>Lyso-</th>
<th>Resistant to</th>
</tr>
</thead>
<tbody>
<tr>
<td>War-wound</td>
<td>C1*</td>
<td>Pa</td>
<td>Zoned</td>
<td>Pa</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pb</td>
<td>Zoned</td>
<td>Pb</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lab. isolate</td>
<td>C7</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C7+C1 filtrate (mouse passage)</td>
<td>C7X*</td>
<td>P18</td>
<td>Zoned</td>
<td>Pa</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ph</td>
<td>Hazy</td>
<td>Pb</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lab. isolate</td>
<td>C10</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C10+Ph</td>
<td>C10/h</td>
<td>Ph</td>
<td>Hazy</td>
<td>Pb</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Single colony of C10/h</td>
<td>C10/z</td>
<td>Pz</td>
<td>Zoned</td>
<td>Pb</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pe (rare)</td>
<td>Clear</td>
<td>Pb</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C10/c</td>
<td>Pe</td>
<td>Clear</td>
<td>Pb</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* = iridescent culture; ? = not studied in detail.

Three consecutive single-plaque isolations of this zoned plaque from a filtrate of C10/h were made, the resulting culture being termed C10/z. For two passages, only zoned type plaques were observed. After the third passage the titre of the filtrate was $37 \times 10^8$/ml. but at the 1/10^6 dilution there were five absolutely clear plaques. One of these was picked off with the C10, incubated and filtered (free phage titre, $10^9$/ml.); all the plaques were of the same clear type and this phage was termed Pc in contrast to Pz (zoned type plaque) and Ph (hazy type plaque).

Relationship between phages Pb, Ph, Pz and Pc

Preliminary neutralization tests, using a 30 min. contact period at 37°, indicated a serological relationship between Pb, Ph, Pz and Pc. Antiserum to Pa had no effect on any of these phages, less than ten phage particles being neutralized by 1 ml. serum even after 3 hr.; 1 ml. antisera to either Pb or Pc neutralized more than 10,000 phage particles of all four phages, but antiserum to Ph was much less active against Pb and Pc than against Pz and Ph.

The rates of neutralization by two sera against the four phages are shown graphically in Fig. 1. Antiserum to Pc produced a very rapid decrease in titre; as soon as readings were practicable (about 3 min.) this was apparent and at
15 min. all counts were zero. Antiserum to Pb gave a similar result, all counts being zero at 20 min.

Antiserum to Ph gave less steep slopes against all four phages, but particularly was this evident against Pc and Pb. The Pb curve levelled out after 3 hr. but the initial slope was almost exactly equal to that of Pc; this slow rate of inactivation explains the apparent lack of action on Pb and Pc by this antiserum in the 80 min. test. The slopes of the Pz and Ph curves are steeper than those against Pb and Pc but the rate of inactivation is less than that produced by antiserum to Pc. The two antisera prepared against Pz were not very potent. A 1/25 dilution was inactive against even the homologous antigen but a 1/10 dilution gave slopes similar to those produced by antiserum to Ph.

It appeared that Pz and Ph were identical except for the appearance of plaques formed. On certain batches of medium, distinguished by the fact that on them C10 produced a dirty blue-green pigment instead of the normal yellowish green, Pz preparations gave a mixture of zoned and hazy plaques, whereas the same preparation gave only zoned plaques on the normal agar. The only difference traced to the medium was the method of digestion of the meat; the normal batches were trypic or weak papain digests, the abnormal were concentrated papain digests.

Cross-resistance tests showed no difference between any of these phages. Cultures made resistant to any one were resistant to all, but still sensitive to Pa.

Comparison of culture of C10 treated with phages Pz and Pc

Three well-isolated plaques of each type of phage, Pz and Pc, were picked off into broth (plus accompanying C10) and the cultures, C10 + Pz and
Phages of P. aeruginosa

C10 + Pc, examined after incubation. The following observations were made:
(1) the total phage count of the unfiltered culture, and the free phage count of
the filtrate were found and all plaques observed; (2) the cultures were
plated out for individual colonies, followed by subculture into broth (for phage
production) and to agar (for resistance tests); (3) cultures derived from single
colonies of C10/z and C10/c were grown in the presence of compound B
(2:7-bis-(-2-dihydroglyoxalineyl)-9-phenylphenanthridine trihydrochloride).
This compound was known to prevent the reproduction of phage Pb on C10,
when added within a few minutes of infection, but to have little effect on the
total phage titres of lysogenic cultures (Dickinson & Codd, 1952; Mills, 1953).
The cultures were diluted 1/104 in broth containing 1 mg. compound B/ml.
Three passages were made in this medium and the cultures were then titrated
for total phage and tested for their ability to produce phage in the absence of
the drug.

Total and free phage counts were about 10^8–10^9/ml. for both C10 + Pz and
C10 + Pc cultures. Examination of the assay plates, involving about 10,000
plaques, revealed only Pc type from C10 + Pc cultures and filtrates. There was
only one clear plaque amongst the thousands of the Pz plaques examined from
C10 + Pz. All broth cultures derived from Pc or Pz plaques were resistant to
Pz, Pc and Pb phages but not to Pa.

The thirty single colony cultures tested from C10 + Pz were resistant to Pz,
Pc and Pb phages but sensitive to Pa; all these thirty broth cultures produced
phage. It was difficult to obtain colonies from C10 + Pc cultures (containing
only few unlysed organisms) except where free phage was carried over. On
second subculture, however, phage-resistant but not phage-producing colonies
were obtained. Compound B had little effect on the phage titres of cultures of
C10/z; both treated and untreated cultures gave high titres even after three
passages. C10/c cultures did not produce phage.

These results showed that the serologically related phages Pz and Pc
established different relations with C10. Pz set up a lysogenic system; with
Pc no lysogenic organisms were obtained. Pl. 1, figs. 1 and 2, show the plaques
produced by Pz and Pc.

Properties of temperate phage Pz and virulent phage Pc: stability. Preparations
of Pz and Pc in broth or Ringer’s solution maintained their titres when held
at 55° for 30 min., 37° for 4 days and at 18 and 4° for 6 months. Dilutions in
0.1M-phosphate citrate buffers of pH’s 5.0, 7.0 and 9.0 were unaffected in
titres after 24 hr. at 37°. As mentioned above, Pz preparations on some media
gave mixtures of hazy and zoned plaques.

Latent period. There was an increase in phage titres at 90 min. for both Pz
and Pc when acting on C10. Each tube contained about 10–50 phage particles
and 1000 host cells; the average phage titres rose from 14 to 71 in the case of
Pz and from 48 to 118 for Pc. When a culture of C10/z was diluted so that each
tube had both a host count and total phage count of about 10/tube there was
no regular time for a rise to occur in all tubes. Taking readings up to 5½ hr.,
one tube showed an increase in titre (forty plaques) at 3½ hr., but six others
remained at the initial level up to 5½ hr. With an average initial level of one
bacterium/tube none of the ten tubes showed an increase of phage titre within $5/4$ hr. Four of these presumably contained no bacteria; after $5/4$ hr. incubation half of the contents of each tube was plated out for bacterial colonies and the other half assayed for phage, but even on prolonged incubation (48 hr.) of the respective assay plates there was neither colony nor plaque. The above results indicated that only a proportion of C 10/z cells produced free phage (certainly less than 1 in 10, probably more than 1 in 100).

Amount of free phage in cultures of C 10/z and C 10/c. A broth culture of C 10/z (colony count $10^8$/ml.) had a total phage count of $1.27 \times 10^8$/ml.; after heating at $55^\circ$ for 30 min. to kill the bacteria, the free phage count was $4.7 \times 10^7$/ml. Bacteria-free filtrates and cultures treated with $50\%$ (v/v) glycerol for 13 days at room temperature also had high free phage titres, never less than one-tenth of the culture titres. Similar results were obtained for lactate medium except that filtrates (Sterimats and Gradocol membranes) gave negative results; this is usual for the phages of P. aeruginosa in lactate medium of Ringer's solution, since marked adsorption occurs.

When phage Pc was grown on C 10 in broth or lactate medium the resulting cultures contained up to $10^{10}$ total phage particles/ml. and there was no decrease in titre when the bacteria were killed. Broth filtrates, but not lactate medium filtrates, also had very high phage counts.

Effect of varying the phage : host ratios for phages Pz and Pc. To see whether the infection ratio of the phages affected the culture response, each phage was serially diluted and the tenfold dilutions added to serial dilutions of C 10 in broth. After incubation the cultures were spotted on to C 10. All Pc tubes yielded clear lytic spots and all Pz tubes gave centred spots. Ten colonies from each culture containing the phage : host ratios $10^8:10^8$, $10^8:10$, $10:10^8$ and $10:10$ were examined for phage production and resistance to Pc and Pz. All forty colonies from C 10/z tubes were phage-producing and resistant. From C 10/c tubes, mixtures of resistant-non-phage producing organisms and unchanged C 10 were obtained; out of 100 resistant colonies none produced phage. Thus, multiple infection by Pc did not establish lysogenesis.

Growth of mixtures of phages Pz and Pc on C 10. As expected, the result of adding the mixed phages, in Pz : Pc proportions of $10^6:10^6$, $10^6:10^2$, $10^2:10^6$ and $10^2:10^2$, was the rapid elimination of Pz; even where Pz was present in 10,000-fold excess it was eliminated within four subcultures.

Action of compound B on growth of phages Pz and Pc

One ml. of a 24 hr. broth culture of C 10 and 1 ml. of the phage filtrate, diluted to contain about $10^8$ phage particles/ml., were added to 100 ml. broth. After a 5 min. adsorption period, 1 ml. of the mixture was added to fivefold dilutions of compound B in broth. After incubation for 18 hr. the tubes were tested for the presence of phage by spotting on to C 10. Results, expressed as:

$$\frac{\text{amount drug (mg./ml.) inhibiting phage growth}}{\text{amount drug (mg./ml.) inhibiting host growth}}$$

were the same for Pz and Pc, i.e. $0.016/0.4$. Total phage counts for both
Phages of P. aeruginosa

Pz and Pc controls were $10^6$ to $10^8$/ml., while total phage counts for the treated cultures remained at the initial level. Even when cultures were incubated for 7 days the phage did not increase in the treated cultures; however, such treated cultures still produced phage to high titre when subcultured into drug-free medium. The activity of the compound against Pz acting on C10 was in contrast to its lack of action on the phage production in established lysogenic cultures of C10/z.

Before seeking an explanation for this behaviour, evidence that the effect of the phenanthridine was not due to action on free phage or on the adsorption of phage on to host was needed. Both Dickinson & Codd (1952) and Mills (1953) showed that the action of this compound on Pb phage could not be accounted for by the slight viricidal action at $37^\circ$. Mills also found that when adsorption was carried out in the presence of the drug and the phage (Pb) host (C10) suspension, then diluted 1/1000 before distribution into the latent-period test tubes (i.e. to below the effective concentration of the drug), then a burst at the normal time still occurred. When adsorption was carried out in the control medium, the drug then prevented phage growth if added within 3 min. of the dilution of the adsorption mixture.

These facts were confirmed for phages Pz and Pc in latent-period experiments. The phenanthridine prevented phage growth when added immediately after the dilution of the adsorption mixture, but had no effect when the drug addition was made at 15 min.

**Total and free phage titres of phage cultures C10+Pz, C10+Pc and C10/z with and without compound B.** In all the following experiments compound B was used at 0.4 mg./ml. in nutrient broth. Total phage was assayed in the usual way and free phage was estimated after heating the cultures (diluted 1/100) at 55° for 30 min. The dilution was necessary because at 55° compound B had a marked viricidal action at 0.4 mg./ml. on both Pz and Pc; the bacteria in the diluted culture were all killed after 30 min. It should be noted that in all control cultures the free phage counts are almost as high as the total phage counts (Table 2). In all cases of C10+Pz and C10/z the total phage counts were probably low since not every lysogenically infected bacterium will produce a plaque; the error cannot be very great since both the free phage titres and the host colony counts were of the order of 10s/ml. Since there were only resistant (non-lysogenic) bacteria in 24 hr. cultures of C10+Pc, the total and free phage titres were the same.

Table 2 shows that compound B suppressed the rise in titres of Pz and Pc when acting on C10; incidentally, it confirmed the fact that there was no contact action. The important point is that it had no effect on the free phage titre of C10/z, i.e. it did not prevent the development of prophage to phage in this culture.

**DISCUSSION**

C10/z, produced by the action of the temperate phage Pz on C10, is a typical lysogenic culture. As high phage titres are obtained in lactate medium it does not appear that any external amino acids are essential for phage maturation,
L. Dickinson

unlike the temperate phage of *Escherichia coli* (Gots & Hunt, 1953). However, the finding of Gots & Hunt referred to an inducible system, whereas C10/z has a high spontaneous rate of phage production.

Although phage Pc arose as a mutant of phage Pz it is not responsible for the lysis of the cells in C10/z culture, for if it were one would expect to find frequently the clear plaques of phage Pc. In fact, in the latent period experiments using ten cells of a C10/z culture, all the plaques which were produced at 8½ hr. were zoned. Only a proportion of the cells (between 1:10 and 1:100) produced phage spontaneously, as in other lysogenic systems. Phage Pc is a typical virulent phage and no lysogenically infected organisms have been obtained from it, only resistant non-phage producing bacteria. It is soon eliminated when in competition with Pz for the same host, because it cannot attack either the lysogenically infected organisms, produced by the action of Pz, or the C10/c organisms.

Table 2. *Total and free phage counts in cultures derived from temperate phage, Pz, and virulent phage, Pc, grown on C10 in the presence and absence of compound B. These are compared to the lysogenic culture C10/z*

<table>
<thead>
<tr>
<th>Counts (particles/ml.)</th>
<th>C10 + Pc</th>
<th>C10 + Pz</th>
<th>C10/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial total phage</td>
<td>$1 \times 10^6$</td>
<td>$1 \times 10^5$</td>
<td>$1 \times 10^4$</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hr. total phage</td>
<td>$5 \times 10^8$</td>
<td>$1.3 \times 10^8$</td>
<td>$1.3 \times 10^8$</td>
</tr>
<tr>
<td>24 hr. free phage</td>
<td>$6 \times 10^8$</td>
<td>$8 \times 10^8$</td>
<td>$6.3 \times 10^7$</td>
</tr>
<tr>
<td>Compound B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hr. total phage</td>
<td>$3.6 \times 10^8$</td>
<td>$2.8 \times 10^8$</td>
<td>$5.8 \times 10^7$</td>
</tr>
<tr>
<td>24 hr. free phage</td>
<td>$1.3 \times 10^8$</td>
<td>$9 \times 10^7$</td>
<td>$3.6 \times 10^7$</td>
</tr>
</tbody>
</table>

The inhibition of the action of Pz and of Pc on C10 by the phenanthridinium compound, provided that the drug is given very soon after adsorption, indicates that after adsorption the initial stages in the action of temperate and virulent phages are the same. Since the free phage titre of C10/z cultures is still high in drug-treated cultures, the change from prophage to phage cannot be affected by the phenanthridine. This suggests that the step affected may be the reduction of phage to prophage.

The relationship between the various phages Pb, Ph, Pz and Pc suggests that Pc is a virulent mutant of the original Pb, being derived as shown:

\[ \text{Pb} \rightarrow \text{Ph} \rightarrow \text{Pz} \rightarrow \text{Pc}. \]

Phages Ph and Pz are very similar except in plaque formation; Pz was derived from Ph and on certain media Pz showed many hazy plaques. Pz is not, however, identical with Pb since antiserum to Ph neutralizes Pb and Pz at different rates.

This paper is No. 3 in a series entitled 'Bacteriophages of *Pseudomonas pyocyanea*'. (*P. aeruginosa*). The author wishes to thank Mr C. E. Coulthard for his continued interest in this work, Miss M. A. Wheater for technical assistance and Mr. G. Whiting for the photograph.
L. Dickinson

Unlike the temperate phage of Escherichia coli (Gots & Hunt, 1953). However, the finding of Gots & Hunt referred to an inducible system, whereas ClO/z has a high spontaneous rate of phage production. Although phage Pc arose as a mutant of phage Pz it is not responsible for the lysis of the cells in ClO/z culture, for if it were one would expect to find frequently the clear plaques of phage Pc. In fact, in the latent period experiments using ten cells of a ClO/z culture, all the plaques which were produced at 3\& hr. were zoned. Only a proportion of the cells (between 1:10 and 1:100) produced phage spontaneously, as in other lysogenic systems. Phage Pc is a typical virulent phage and no lysogenically infected organisms have been obtained from it, only resistant non-phage producing bacteria. It is soon eliminated when in competition with Pz for the same host, because it cannot attack either the lysogenically infected organisms, produced by the action of Pz, or the ClO/c organisms.

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<table>
<thead>
<tr>
<th>Counts (particles/ml.)</th>
<th>ClO+Pc</th>
<th>ClO+Pz</th>
<th>ClO/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial total phage</td>
<td>1\times10^6</td>
<td>1\times10^5</td>
<td>1\times10^4</td>
</tr>
<tr>
<td>Control 24 hr. total phage</td>
<td>5\times10^8</td>
<td>1.3\times10^9</td>
<td>1.3\times10^8</td>
</tr>
<tr>
<td>24 hr. free phage</td>
<td>6\times10^8</td>
<td>8\times10^8</td>
<td>6-8\times10^7</td>
</tr>
<tr>
<td>Compound B 24 hr. total phage</td>
<td>3.6\times10^6</td>
<td>2.8\times10^6</td>
<td>5.8\times10^7</td>
</tr>
<tr>
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</tr>
</tbody>
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L. Dickinson—Phages of *P. aeruginosa*. 
REFERENCES


EXPLANATION OF PLATE

Fig. 1. Plaques produced by the temperate phage, Pz, of P. aeruginosa (x 1).

Fig. 2. Plaques produced by the virulent phage, Pc, of P. aeruginosa (x 1).

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