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ISOLATION AND PRELIMINARY CHARACTERISTICS OF THREE BACTERIOPHAGES ASSOCIATED WITH A LYSOGENIC STRAIN OF *PSEUDOMONAS AERUGINOSA*[1, 2]

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ABSTRACT

FEARY, THOMAS W. (Tulane University School of Medicine, New Orleans, La.), EARL FISHER, JR., AND THELMA N. FISHER. Isolation and preliminary characteristics of three bacteriophages associated with a lysogenic strain of *Pseudomonas aeruginosa*. J. Bacteriol. **87**:196–208. 1964.—Three bacteriophages designated 7v, 7m, and 7s were isolated from a lysogenic strain of *Pseudomonas aeruginosa* designated Ps-7. The three viruses were found to be completely unrelated on the basis of plaque morphology, host range, serology, ultraviolet induction, sensitivity to heat, and particle morphology as revealed by electron microscopy. In addition, it was shown that the three phages were incapable of plaque formation on bacteria other than various strains of *P. aeruginosa*. Of the three phages, only phage 7v was capable of plaque formation on strain Ps-7. The growth of phage 7v on strain Ps-7 exhibited properties which suggest that this virus arises as the result of mutation in a temperate phage for which strain Ps-7 is lysogenic. Phages 7m and 7s are incapable of plaque formation on strain Ps-7, but are adsorbed at characteristic rates to cell suspensions of strain Ps-7. The relationship between phage 7m and strain Ps-7 was shown to meet the classical criteria for lysogeny. Because phage 7s contains ribonucleic acid as its nucleic acid component, it was concluded that its production by strain Ps-7 and the demonstration of immunity of strain Ps-7 to infection by phage 7s were not sufficient evidence to define the nature of the relationship between phage 7s and *P. aeruginosa* strain Ps-7. It was observed that under certain conditions the infectious titer of phage 7s preparations are markedly reduced in the presence of ribonuclease.

One of the properties conferred on lysogenic bacteria by the presence of prophage is immunity to an infection by mature phage released into the culture medium as a consequence of spontaneous lysis by a small fraction of the bacterial population (Jacob and Wollman, 1959). McCloy (1951) reported an apparent exception, since she isolated a *Bacillus* phage, Wα, which could be propagated on and cause the lysis of the lysogenic organism from which it was isolated. Subsequent investigations regarding the relationship between phage Wα and the lysogenic *B. cereus* strain W (McCloy, 1958) showed that bacterial strain W was lysogenic for a temperate phage, Wβ, which, as a result of mutation, gives rise to a variant, Wα, which can form plaques on strain W itself and on other bacterial strains lysogenic with phage Wβ. Recently, it was reported (Meynell 1962) that *B. cereus* strain W produces at least five antigenically related bacteriophages of which only one, phage Wα, is able to overcome the immunity conferred by the Wβ prophage.

The present investigation was undertaken in an attempt to characterize the relationship between a bacteriophage designated 7v and the lysogenic *Pseudomonas aeruginosa* strain Ps-7 from which phage 7v was isolated. Like phage Wα, phage 7v can be propagated on and will cause the lysis of broth cultures of *P. aeruginosa* strain Ps-7; however, unlike the *B. cereus*-phage Wα system, attempts to isolate phages related to phage 7v have not been successful. To date, two additional bacteriophages, designated 7m and 7s, have been isolated which are not related to each other or to phage 7v. Recently, it was shown (Feary, Fisher, and Fisher, 1963*a*) that phage 7s contains ribonucleic acid (RNA) as its nucleic acid com-

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ponent. The host ranges of phages 7v and 7m on 95 strains of *P. aeruginosa*, and the failure to detect nonlysogenic bacterial hosts for any of the three viruses, have also been reported (Feary et al., 1963*b*).

The present paper relates data concerning the isolation of phages 7v, 7m, and 7s and some of the properties by which these viruses can be shown to be unrelated. Features of the multiplication of phage 7v on *P. aeruginosa* strain Ps-7 are described, and evidence is presented which suggests that all three viruses are permanently associated with strain Ps-7.

MATERIALS AND METHODS

Media. A medium, designated NBYE, used for the growth of various strains of *P. aeruginosa*, consisted of nutrient broth (Difco) supplemented with 0.5% yeast extract (BBL) and 0.5% NaCl. For liquid cultures, 1% glucose was added to this basal preparation, while plating medium contained 1.5% agar (Difco) but no glucose. In several experiments, the M-9, 1% glucose synthetic medium of Watson (1950) was used. Overnight growth of *Pseudomonas* species other than *P. aeruginosa* was obtained in NBYE broth supplemented with 0.08% NH_4NO_3; Trypticase Soy Broth (BBL) served as a medium when overnight growth of bacteria belonging to genera other than *Pseudomonas* was required.

Bacterial strains. The origin of *P. aeruginosa* strains 1, Ps-7, and Ps-105, and their maintenance as stock cultures have been reported (Feary et al., 1963*b*). Strains of *Pseudomonas* species other than *P. aeruginosa* were kindly sent to us by Pingui V. Lui, Department of Microbiology, University of Louisville School of Medicine. Bacterial strains of the genera *Escherichia*, *Salmonella*, *Shigella*, *Proteus*, and *Klebsiella*, used for a survey of bacteriophage host ranges, were obtained from the stock culture collection, Department of Microbiology, Tulane University Medical School.

Bacteriophages. The three bacteriophages designated 7v, 7m, and 7s are all associated with *P. aeruginosa* strain Ps-7. The procedure for preparation of stock phage lysates by confluent lysis techniques was presented earlier (Feary et al., 1963*b*). Plaque-forming titers of all bacteriophage samples were estimated by the soft-agar layer method described by Adams (1950, 1959).

Preparation of phage antisera. Antisera against phages 7v, 7m, and 7s were prepared in the following manner. Rabbits, in groups of three for each phage, were injected intravenously with 0.5 ml of phage lysates ranging in titer from 5×10^{11} to 2×10^{12} plaque-forming units (PFU) per ml at 2-day intervals for a total of nine injections each. At 1 week after the last injection, all rabbits were bled from the ear vein, and the sera so obtained were heated for 30 min at 56 C and tested for the ability to neutralize homologous phage. To examine for neutralizing activity, antisera at various dilutions in NBYE broth were prewarmed to 37 C; homologous phage was then added to give a final concentration of 10^7 to 2×10^7 PFU per ml in NBYE broth controls without antiserum. The phage-antiserum mixtures were allowed to react for 15 min at 37 C; then duplicate 0.1-ml samples of suitable dilutions were plated on the appropriate bacterial host to assay for surviving plaque formers. If the test showed that an antiserum dilution of 1:100 or better would neutralize 90% of the plaque-forming ability of the homologous phage in 15 min, 50 ml of blood were taken from each rabbit by cardiac puncture. The sera from each group of rabbits were pooled, heated 30 min at 56 C, and then dispensed in sterile screw-capped tubes and stored in the frozen state. The *K* value of each pooled antiserum preparation was determined against its homologous phage by methods described by Adams (1959), and each was tested for its ability to neutralize heterologous phage by the method outlined above.

Phage sensitivity to heat at 60 C. To test the heat sensitivity of phages 7v, 7m, and 7s, phage suspensions at concentrations of 10^9 to 2×10^9 PFU per ml were diluted 1:100 in NBYE broth prewarmed to 60 C in a constant-temperature water bath. At given intervals, samples were diluted 1:10,000 in NBYE broth held at room temperature. Duplicate 0.1-ml samples of this final dilution were plated to assay surviving plaque formers on the appropriate bacterial host.

Growth of phage 7v on bacterial strain Ps-7. The growth of phage 7v on *P. aeruginosa* Ps-7 was followed with bacterial cells in the logarithmic phase of growth. Logarithmic growth was initiated by making 1:500 dilutions of overnight NBYE broth cultures of strain Ps-7 in fresh NBYE broth prewarmed to 37 C. Bacterial growth was estimated by turbidity measurements at 660 mμ in a Coleman Universal spectrophotometer; it was then compared with plate count values of viable cell numbers obtained by

plating duplicate 0.1-ml culture samples on NBYE agar. All cultures were aerated, except where indicated, before and after the addition of phage. The growth of phage 7v was followed by changes in the turbidity of infected cultures and by assays for PFU. The latent period and burst size of phage 7v were estimated by the one-step growth technique of Ellis and Delbruck (1939).

Phage adsorption to cell suspensions of bacterial strain Ps-7. The adsorption rates of phages 7v, 7m, and 7s to cell suspensions of bacterial strain Ps-7 were calculated from data obtained from assaying adsorption mixtures for unadsorbed phage. Procedures described by Adams (1959) for the determination of the velocity constant, K, were followed. For these experiments, bacteria were grown to a concentration of 2×10^8 viable cells per ml in NBYE broth at 37 C with aeration. At zero time, phage was added to give a multiplicity of 0.05; aeration was continued throughout each experiment. To assay for unadsorbed phage, samples of the adsorption mixtures were diluted 1:100 in NBYE broth and centrifuged for 5 min at 6,000 $\times$ g. The supernatant fluids obtained were again diluted by a factor of 100, and duplicate 0.1-ml samples of this final dilution were plated on the appropriate bacterial host for estimation of plaque formers.

The adsorption of phage 7v to cells of strain Ps-7 was also estimated by assaying adsorption mixtures for infected bacteria. To determine the number of infected bacteria, samples of the adsorption mixture were diluted 1:100 in anti-phage 7v antiserum at a concentration sufficient to inactivate 99% of the unadsorbed phage in 5 min at 37 C. Duplicate 0.1-ml samples of a 1:100 dilution of the adsorption-antiserum mixture were plated for estimation of the number of infected bacteria. All experimental manipulations were completed by the end of 20 min after the addition of phage to the bacterial suspension.

Bacteriophage host range determinations. The methods used to test the ability of phages 7v, 7m, and 7s to form plaques on bacterial strains other than *P. aeruginosa* were those previously reported (Feary et al., 1963*b*) with the exception that each non-*Pseudomonas* strain was tested against suspensions of each phage at concentrations of 10^8, 10^6, 10^4, and 10^2 PFU per ml. As controls, each phage suspension was plated on *P. aeruginosa* strains 1, Ps-7, and Ps-105.

Ultraviolet irradiation of bacterial strain Ps-7. Exponentially growing cultures of strain Ps-7 in NBYE broth were transferred in 10-ml samples to flat petri dishes and subjected to ultraviolet irradiation from a 4-w General Electric ultraviolet lamp placed 30 cm above the petri dish. Irradiated cultures were diluted 1:5 in fresh NBYE broth prewarmed to 37 C in aeration tubes. Duplicate 0.1-ml samples of suitable dilutions of the lysates obtained in this manner were plated on *P. aeruginosa* strains 1, Ps-7, and Ps-105 for the estimation of phages 7v, 7m, and 7s.

Effect of ribonuclease on phage 7s. Partially purified preparations of phage 7s obtained by treatment of crude 7s lysates with deoxyribonuclease and ribonuclease, followed by differential centrifugation, contain more ribonucleic acid than can be accounted for on the basis of PFU present in such preparations, considering the small size of the virus (Feary et al., 1963*a*). To test the hypothesis that estimates of infectious titers might be low owing to clumping or packing of virus in the presence of nonviral RNA, various dilutions of a phage 7s preparation containing 5×10^{13} PFU per ml were made in 0.005 M phosphate buffer (pH 7.2) containing 20 μg of ribonuclease (Sigma Chemical Co., St. Louis, Mo.) per ml, and in phosphate buffer containing 20 μg of ribonuclease per ml with 0.005 M ethylenediaminetetraacetic acid (EDTA). As a control, identical dilutions were made in phosphate buffer alone. Samples were withdrawn from each mixture before and after a 30-min incubation interval at 37 C to assay for plaque formers.

Electron microscopy. To prepare for electron microscopy, crude phage 7v and 7m lysates were centrifuged for 30 min at 40,000 $\times$ g, and crude phage 7s lysates were centrifuged for 120 min at 70,000 $\times$ g. The pellets obtained were covered with 5 ml of 1% ammonium acetate and left at 4 C without agitation for 24 hr. The softened pellets were then resuspended by gentle swirling and clarified by centrifugation for 30 min at 6,000 $\times$ g. Dilutions of each phage suspension in 1% ammonium acetate were mixed with equal volumes of 2% aqueous phosphotungstic acid brought to pH 7.0 with potassium hydroxide (Brenner and Horne, 1959). Drops of each phage suspension were then transferred to Formvar-covered electron microscope specimen grids by the spreading technique described by Bradley and Kay (1960). Specimens were examined in a Siemens Elmiskop I electron microscope at in-

strumental magnifications of 20,000, 40,000, and 80,000.

RESULTS

Isolation of phages 7v, 7m, and 7s. Phage 7v was originally isolated from clear plaques which infrequently appear spontaneously in areas of confluent growth when bacterial strain Ps-7 is streaked on solid media. The features of the growth of phage 7v on strain Ps-7 will be described below; however, it is pertinent to state here that phage 7v has not been isolated from overnight NBYE cultures, nor has the frequency of the spontaneous appearance of 7v plaques been increased by incubating NBYE broth cultures of strain Ps-7 at 37 C for as long as 10 days. In addition, spontaneous phage 7v plaques have not been observed at any time over an 18-month interval as a result of plating cells of strain Ps-7 by the soft-agar layer method over prepoured NBYE agar plates to obtain bacterial lawns for phage plaque count assays. Phage 7m was isolated from plaques which formed when supernatant fluids from centrifuged, overnight NBYE broth cultures of strain Ps-7 were mixed and plated with cell suspensions of bacterial strain Ps-105; similarly, phage 7s was isolated from plaques which appeared when similarly treated NBYE broth cultures of strain Ps-7 were plated with cell suspensions of bacterial strain 1. It was found that overnight NBYE broth cultures of strain Ps-7, grown at 37 C with aeration, contain phage 7m in concentrations ranging from 10^6 to 10^7 PFU per ml when plated on strain Ps-105 and phage 7s in concentrations ranging from less than 10 to 10^3 PFU per ml when mixed and plated with cell suspensions of strain 1.

The plaque titers of crude lysates of the three bacteriophages on *P. aeruginosa* strains 1, Ps-7, and Ps-105 are shown in Table 1. Within this group of three bacterial strains, Ps-7 serves as a specific host for phage 7v, while strain 1 serves as a specific host for phage 7s. Phages 7v and 7m can be distinguished from each other by plaque morphology when mixtures of the two phages are plated on strain Ps-105; lysates of phage 7m prepared on strain Ps-105 contain no phage 7v plaques.

The plaque morphology of phages 7v, 7m, and 7s, when plated on their respective bacterial host, is depicted in Fig. 1. Phage 7v forms clear plaques, 2 to 4 mm in diameter, surrounded by a slightly turbid band with irregular edges when plated on strain Ps-7. When plated on bacterial strain Ps-105, phage 7m forms plaques with turbid centers, 1 to 2 mm in diameter, surrounded by a band of clear lysis; the whole is surrounded by a halo. Clear plaque mutants of phage 7m occur at a frequency of 10^{-4} to 10^{-5}. Phage 7s forms plaques which are clear to slightly turbid and range in size from 3 to 6 mm when plated with its host, *P. aeruginosa* strain 1.

TABLE 1. *Plaque titer* of crude lysates of phages 7v, 7m, and 7s on three strains of Pseudomonas aeruginosa*

Phage	Strain		
	Ps-7	Ps-105	1
7v	9×10^{10}	7×10^9	0
7m	0	1.5×10^{12}	0
7s	0	0	4.3×10^{11}

* Expressed as PFU per ml.

Serology. The pooled antiserum prepared against phage 7v was found to have a K value of 48; when the same group of rabbits used to obtain this antiserum were reimmunized 4 months after their first series of inoculations, an antiphage 7v antiserum with a K value of 4,600 was obtained. The pooled antiserum prepared against phage 7m was found to have a K value of 80 after a single series of inoculations with phage 7m lysate. While the inactivation of phages 7v and 7m by their respective antisera could be described as an exponential decrease of plaque-forming ability with time, the inactivation of phage 7s by its antiserum appeared to occur at different rates with respect to time. This observation is illustrated in Fig. 2, which shows the inactivation of phage 7s at different antiphage 7s serum dilutions. The results of several experiments suggest that this antiserum has a K value of 350 during the earlier stage of the reaction (0 to 5 min) and a K value of 200 during the later stages of the reaction (5 to 20 min).

The results obtained when the three antisera, each diluted 1:50 in NBYE broth, were reacted against homologous and heterologous phages at reaction mixture concentrations of 2×10^7 to 3×10^7 PFU per ml are presented in Table 2. No serological relationships could be demonstrated between any two of the three viruses as a result of using antiserum dilutions lower than 1:50, or as a result of increasing the reaction time from 15 to 30 min.

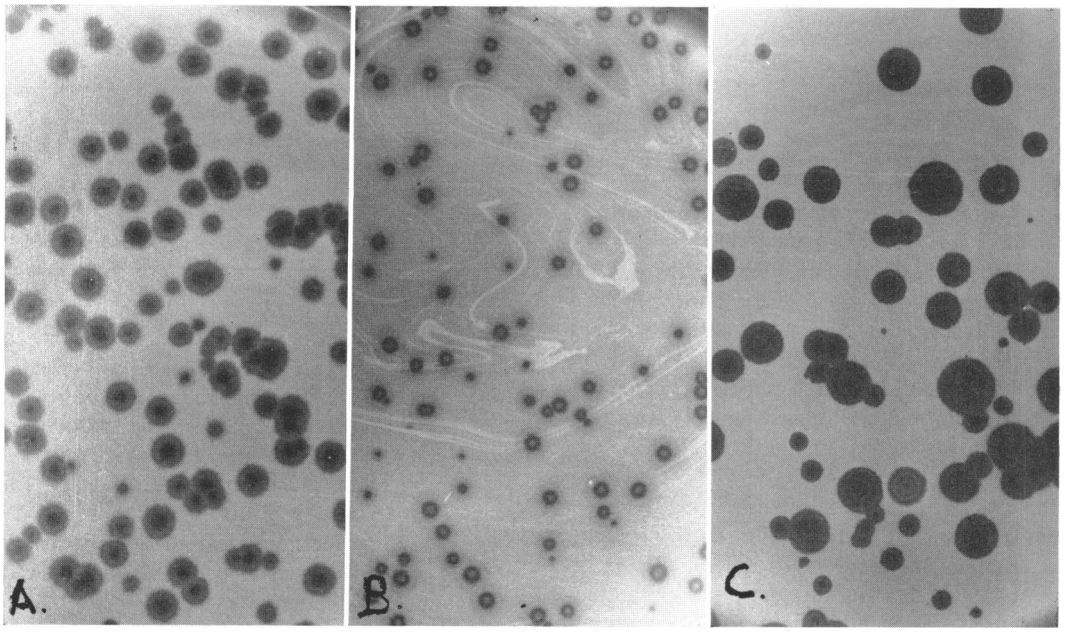

FIG. 1. *Plaque morphology of bacteriophages isolated from Pseudomonas aeruginosa strain Ps-7. (A) Phage 7v plated on strain Ps-7. (B) Phage 7m plated on strain Ps-105. (C) Phage 7s plated on strain 1.*

Phage sensitivity to heat at 60 C. Dickinson (1948) observed that a group of *P. aeruginosa* bacteriophages under investigation in her laboratory were resistant to inactivation for 30 min at 60 C, but were rapidly inactivated in 10 min at 65 C. An extension of this observation has been the development of a routine method for the isolation of *P. aeruginosa* bacteriophages which involves heating supernatant fluids obtained from centrifuged broth cultures of lysogenic strains of *P. aeruginosa* at 58 to 60 C for as long as 2 hr to kill residual bacterial cells (Gould and McLeod, 1960; Postic and Finland, 1961; Graber et al., 1962). Because phages 7v, 7m, and 7s are all associated with a single lysogenic strain of *P. aeruginosa*, it was of interest to determine their stability at 60 C. Figure 3 illustrates that these three viruses vary greatly in their response to heat at this temperature. While phage 7m appears to be relatively stable, over 90% of the plaque-forming ability of phage 7s was inactivated at the end of the 60-min interval tested, and 99% of phage 7v plaque formers were inactivated within 20 min.

Ultraviolet irradiation. Exposure of exponentially growing NBYE broth cultures of strain Ps-7 to ultraviolet light for a period of 2.5 min resulted in lysis, which began 90 min after ultraviolet exposure and was completed within 150 min. When appropriate dilutions of such lysates were plated on bacterial host strains 1, Ps-7, and Ps-105, only plaques formed by phage 7m on strain Ps-105 were observed. In an additional attempt to detect ultraviolet induction of phage production, colonies of bacterial strain Ps-7 on NBYE agar plates were exposed to ultraviolet light and then overlaid with melted soft agar containing cells of either strain 1 or strain Ps-105. When these plates were examined after overnight incubation at 37 C, only the plates that had been seeded with cells of strain Ps-105 showed areas of lysis around the embedded Ps-7 colonies.

Electron microscopy. The most striking differences among phages 7v, 7m, and 7s were found to be differences in particle morphology as exhibited in an electron microscope. Typical electron micrographs of each bacteriophage are shown in Fig. 4. It can be seen that phage 7v possesses a contractile tail sheath and base plate; however, no tail fibers have been observed in association with this virus. The tail of phage 7m is often curled and shows a barbed structure at the terminal end. No tail structures have been observed in association with phage 7s. The dimensions of the three viruses, as determined

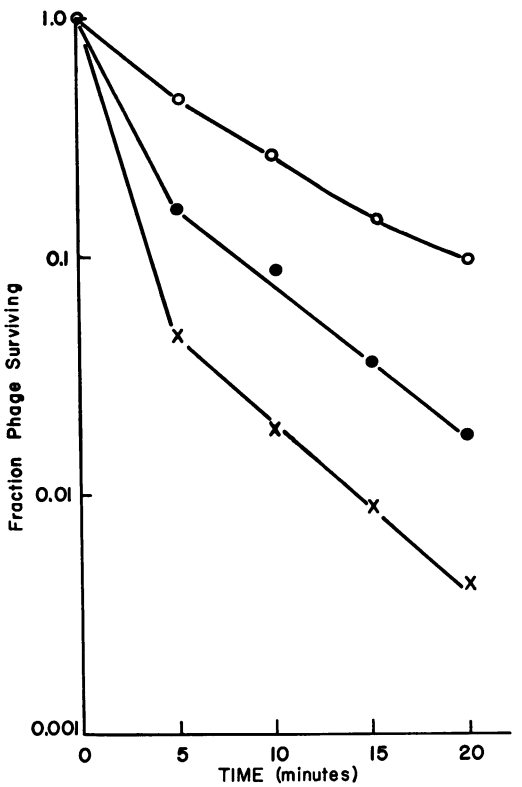

FIG. 2. *Antiphage 7s antiserum inactivation of phage 7s. Symbols:* ○ = *antiserum diluted 1:2,500* ● = *antiserum diluted 1:1,000;* × = *antiserum diluted 1:250.*

from the instrumental magnifications of the electron microscope, are given in Table 3.

The examination of crude phage 7v lysates, which had been prepared by the propagation of phage 7v on *P. aeruginosa* strain Ps-7, by electron microscopy revealed a heavy contamination with phage 7m. In several preparations, an occasional tailed phage particle was observed which did not resemble either phage 7v or phage 7m with respect to particle morphology; whether these particles represent morphological mutants of either phage 7v or phage 7m, or whether they are completely different and unrelated phages, is not known. Attempts to find hosts for phages other than phages 7v, 7m, and 7s among 95 different strains of *P. aeruginosa* were unsuccessful (Feary et al., 1963*b*). Phage 7s was not observed as a contaminant in phage 7v lysates.

Phage host ranges. It was found that all three phages were incapable of plaque formation on bacteria other than various strains of *P. aeruginosa*. The bacteria tested for their susceptibility to phages 7v, 7m, and 7s and the number of strains of each genus and species tested were as

TABLE 2. *Reaction of antiphage antisera against homologous and heterologous phage for 15 min at 37 C*

Antisera diluted 1:50 in NBYE broth	Average plaque count per 0.1 ml of antiserum-phage mixture diluted 1:10,000 in NBYE broth		
	Phage 7v	Phage 7m	Phage 7s
Antiphage 7v (K = 4,600)	0	328	261
Antiphage 7m (K = 80)	396	0	248
Antiphage 7s (K = 200)	352	336	0
NBYE broth (no antiserum)	370	340	252

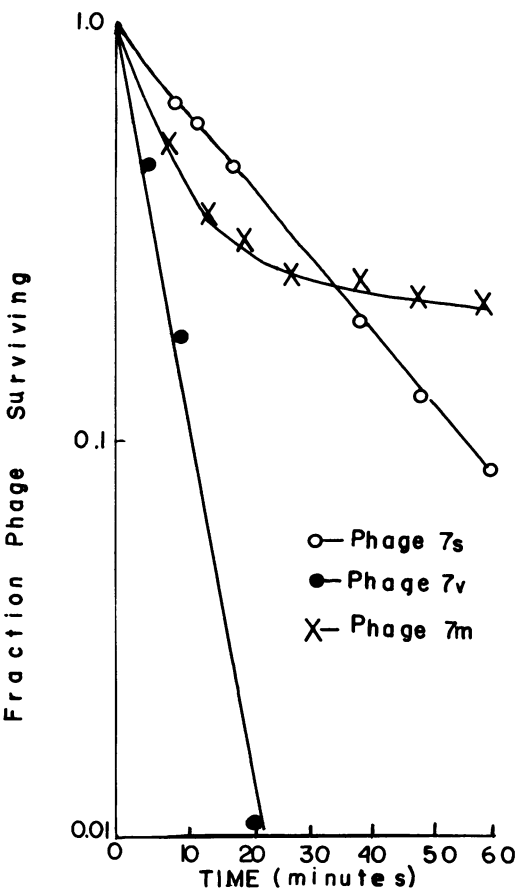

FIG. 3. *Inactivation of phages 7v, 7m, and 7s at 60 C.*

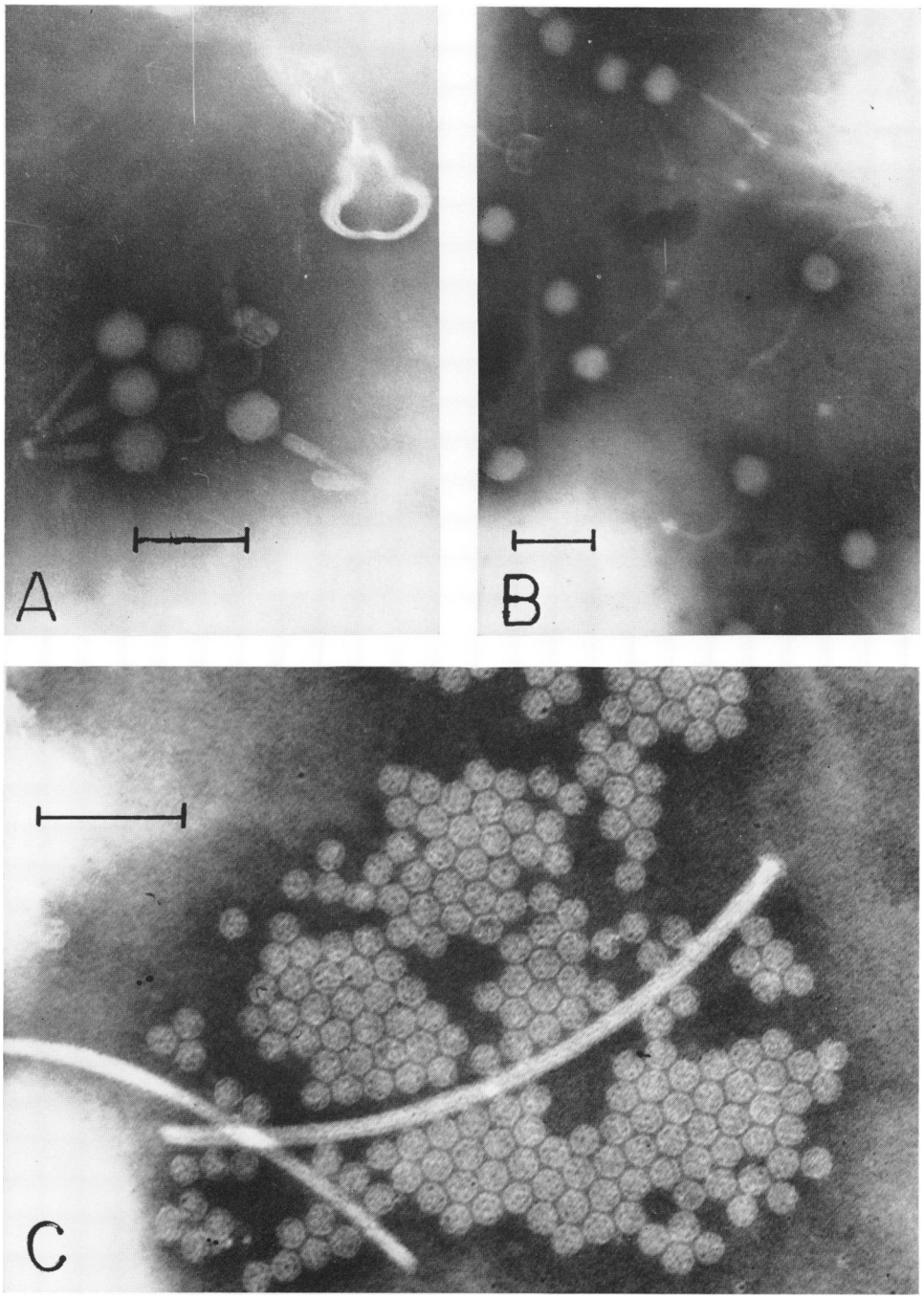

FIG. 4. *Electron micrographs of crude phage lysates negatively stained by phosphotungstic acid. The line in each photograph represents a distance of 0.1 μ. (A) Phage 7v. (B) Phage 7 m. (C) Phage 7s.*

follows: *P. fluorescens*, 10; *Proteus vulgaris*, 10; *Escherichia coli*, 23; *Shigella sonnei*, 12; *Salmonella typhimurium*, 9; *S. typhosa*, 14; and *Klebsiella pneumoniae*, 4.

Growth of phage 7v on bacterial strain Ps-7. When logarithmic cultures of strain Ps-7 in NBYE broth were infected with phage 7v at multiplicities less than 1, lysis, as measured by changes in turbidity, was seldom complete and occurred very slowly. On the other hand, when the multiplicity of infection was greater than 1, lysis of infected cultures was always complete and occurred within 4 to 6 hr after addition of phage. The length of time required for the complete lysis of multiply infected, aerated cultures varied to some extent according to the initial bacterial concentration at the time of phage addition. This observation is summarized in Fig. 5 where samples of a multiply infected culture were diluted in fresh NBYE broth prewarmed to 37 C after a 15-min interval to allow for phage adsorption. These data also show that the turbidity of multiply infected Ps-7 cultures increases up to 2 hr after the addition of phage. Figure 6 illustrates the results obtained when identical Ps-7 cultures, multiply infected with phage 7v, were aerated for varying lengths of time after phage had been added. As might be expected, no lysis occurred in the absence of aeration; however, a degree of lysis, dependent upon the length of time the infected culture was aerated after the addition of phage, could be initiated by an abrupt cessation of aeration. The degree of lysis obtained in this manner occurred within 10 min after aeration was discontinued and reached a level which remained constant for up to 2 hr. No further lysis occurred when aeration was again resumed 15 to 30 min after being discontinued.

The increase in phage 7v plaque formers, which accompanied turbidity changes in multiply infected cultures, is presented in Fig. 7. It can be seen that phage multiplication began within 30 min after infection and continued at a constant rate for approximately 120 min, or until lysis began, as evidenced by a drop in turbidity. Under these conditions, highly viscous 7v lysates were obtained with plaque titers of approximately 5×10^{11} PFU per ml in a synthetic medium such as M-9, or as high as 5×10^{12} PFU per ml in a complete medium such as NBYE broth.

The results of a one-step growth experiment with phage 7v propagated on strain Ps-7 are shown in Fig. 9. Under conditions of single infection, phage 7v was found to have a latent period of 23 min and a burst size of 230.

TABLE 3. *Dimensions of phages 7v, 7m, and 7s*

Phage	Head	Tail
	mμ	mμ
7v	55 × 65	20 × 90
7m	45 × 50	15 × 125
7s	25 × 25	—

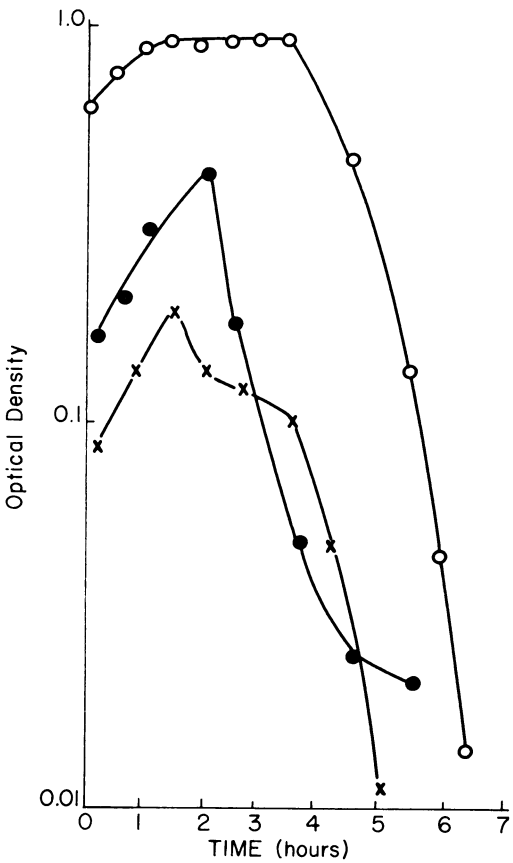

FIG. 5. *Effect of dilution on lysis of phage 7v-infected Pseudomonas aeruginosa strain Ps-7. A culture of strain Ps-7 in NBYE broth at a concentration of 5×10^8 viable cells per ml was infected with phage 7v to give a multiplicity of 10. After 15 min for adsorption the mixture was diluted into fresh NBYE broth prewarmed to 37 C, and turbidity measurements were obtained until lysis was complete in all tubes. Symbols: ○ = undiluted; ● = diluted 1:5; × = diluted 1:10.*

Adsorption of phage 7v to strain Ps-7. The adsorption of phage 7v to cells of strain Ps-7 suspended in NBYE broth is depicted in Fig. 8. At

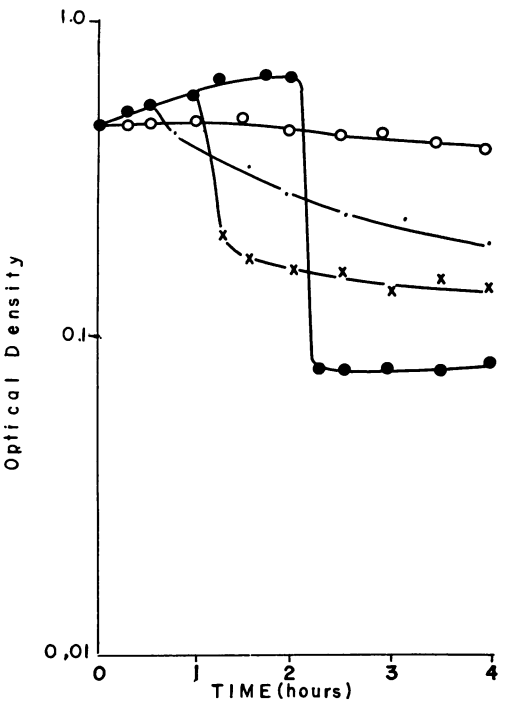

FIG. 6. *Effect of aeration on lysis of cells multiply infected with phage 7v. A culture of Pseudomonas aeruginosa strain Ps-7 at a concentration of 5 × 10⁸ viable cells per ml was infected with sufficient phage 7v to give a multiplicity of 10. Samples of the mixture were immediately transferred to separate aeration tubes, and optical density measurements were obtained under varying conditions of aeration. Symbols:* ● = *aeration discontinued 120 min after addition of phage;* × = *aeration discontinued 60 min after addition of phage;* • = *aeration discontinued 30 min after addition of phage;* ○ = *aeration discontinued immediately after addition of phage.*

the end of 4 min, less than 20% of the initial phage added at zero time remained unadsorbed when adsorption was estimated by assays for free phage. At any time between 2 and 9 min after the addition of phage, the sum of the phage fraction measured as free phage and the fraction measured as infected bacteria was approximately 60% of the initial phage concentration added at zero time. Subsequently, it was found that supernatant fluids obtained from centrifuged NBYE broth cultures of strain Ps-7 do not contain materials which inhibit the plaque-forming ability of phage 7v. Since the multiplicity of infection in all the adsorption experiments was 0.05, it is unlikely that a significant number of bacterial cells to which phage had adsorbed received more than 1 phage particle. Thus, it appears that a significant number of Ps-7 cells to which a single phage 7v particle has adsorbed are incapable of forming infectious centers.

When the adsorption rate of phage 7v to cells of strain Ps-7 in NBYE broth at 37 C with constant aeration was measured by assaying adsorption mixtures at appropriate intervals for unadsorbed phage, it was found that the velocity constant, K, had a value of 2.5×10^{-9} ml/min.

Adsorption of phages 7m and 7s to strain Ps-7. Phage 7m is incapable of forming plaques when mixed and plated with cells of strain Ps-7; however, the frequency of spontaneous phage 7m production by strain Ps-7 is such that viable Ps-7 cells mixed and plated with cells of strain Ps-105, the host for phage 7m, form turbid plaques which cannot be distinguished from plaques formed by phage 7m. Consequently, phage 7m-strain Ps-7

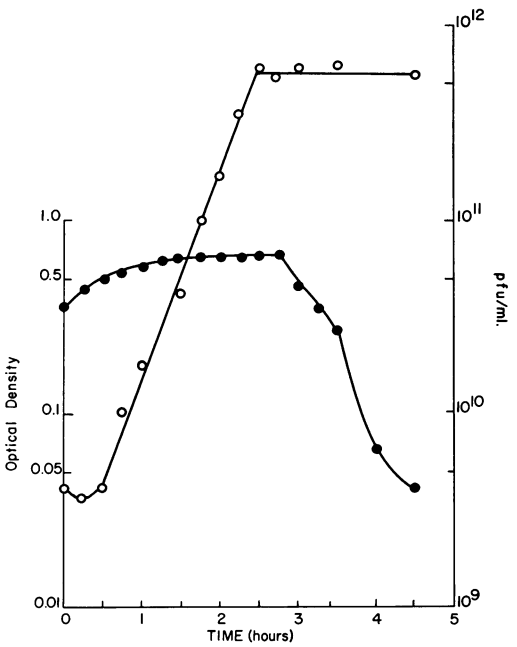

FIG. 7. *Multiplication of phage 7v on strain Ps-7 at 37 C in M-9. A culture of Pseudomonas aeruginosa strain Ps-7 was grown in M-9 medium with 1% glucose to 5 × 10⁸ viable cells per ml and infected with phage 7v to give a multiplicity of 10. At appropriate intervals, samples were withdrawn for plaque count assays and optical density changes were noted until lysis was completed. Symbols:* ● = *changes in optical density;* ○ = *changes in plaque assays.*

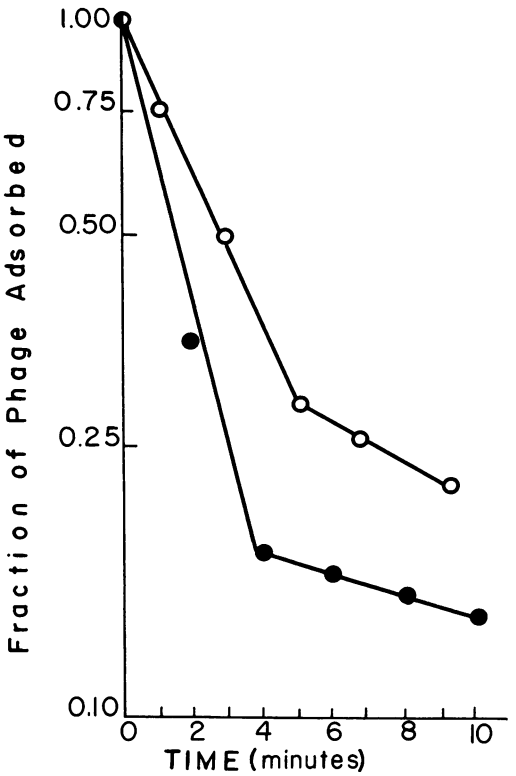

FIG. 8. *Adsorption of phage 7v to strain Ps-7. The initial cell concentration was 2 × 10⁸ viable cells per ml, and phage 7v was added to give a multiplicity of 0.05. At appropriate intervals, samples of the adsorption mixture were withdrawn and assayed for either free phage or infected bacteria. Symbols:* ● = *free phage;* ○ = *infected bacteria.*

adsorption mixtures were diluted by a factor of 100 to stop adsorption, and were centrifuged for 10 min at 8,000 × *g* to sediment Ps-7 cells. Under conditions identical to those described above for phage 7v adsorption experiments, the adsorption of phage 7m to cells of strain Ps-7 was found to have a velocity constant of 1.8 × 10^{-9} ml/min.

Like phage 7m, phage 7s is incapable of plaque formation on bacterial strain Ps-7. Viable Ps-7 cells do not initiate plaque formation when they are mixed and plated by the soft-agar layer method with an excess of cells of the host strain for phage 7s, *P. aeruginosa* strain 1. Thus, the adsorption of phage 7s to strain Ps-7 was studied by plating duplicate 0.1-ml samples of a 1:10,000 dilution of phage 7s–strain Ps-7 adsorption mixtures directly on strain 1 at appropriate intervals. Under the same conditions described for studying the adsorption of phages 7m and 7v to strain Ps-7, the adsorption of phage 7s to strain Ps-7 was found to have a velocity constant of 4.1 × 10^{-8} ml/min. When the ratio of phage 7s to cells of strain Ps-7 was increased from 0.05 to 1.8, the reduction of unadsorbed phage with time was barely discernible; under these conditions, the adsorption of phage 7s to strain Ps-7 was not influenced by the inclusion of $CaCl_2$ to a final concentration of 0.01 M in the adsorption mixture.

Effect of ribonuclease on phage 7s. The effect of ribonuclease on the plaque titer of a partially purified phage 7s preparation under differing conditions of dilution and temperature is shown in Table 4. It can be seen that 99% of the plaque titer of such a preparation is lost when diluted 1:100 in phosphate buffer containing 20 μg of ribonuclease per ml and incubated for 30 min at 37 C. At the same dilution, a 30-min incubation at 25 C resulted in a 90% reduction of plaque titer, while a 1:9 dilution incubated at 37 C resulted in a loss of only 13%. Independent of dilution, the presence of EDTA at a concentration of 0.005 M reduced the loss in plaque titer observed in the presence of ribonuclease alone.

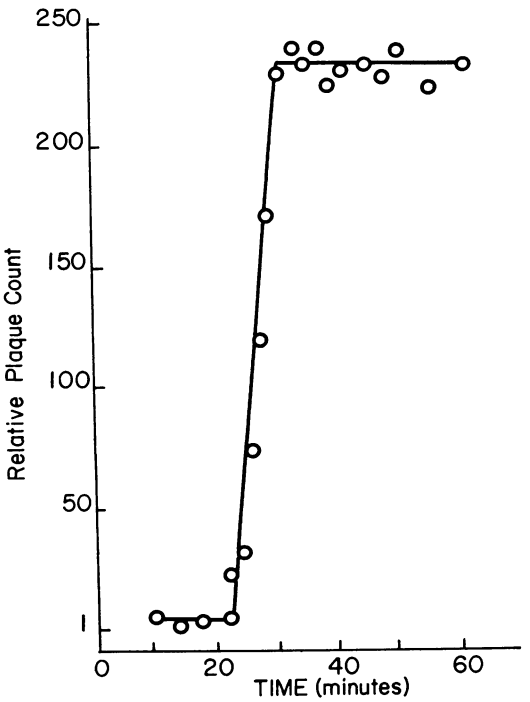

FIG. 9. *One-step growth curve at 37 C with phage 7v on Pseudomonas aeruginosa strain Ps-7 in NBYE broth.*

TABLE 4. *Changes in plaque titer of a partially purified phage 7s preparation after a 30-min exposure to ribonuclease or ribonuclease and EDTA**

Diluent	Dilution		
	1:9 at 37 C	1:100 at 37 C	1:100 at 25 C
Phosphate buffer (0.005 M, pH 7.2)	5.2×10^{13}	4.9×10^{13}	5.1×10^{13}
Phosphate buffer plus ribonuclease (20 μg/ml)	4.4×10^{13}	0.05×10^{13}	0.4×10^{13}
Phosphate buffer plus ribonuclease (20 μg/ml) plus 0.005 M EDTA	4.7×10^{13}	1.7×10^{13}	5.0×10^{13}

* Each figure is an average value in PFU per ml obtained from three experiments.

DISCUSSION

The study reported in this communication was undertaken initially to inquire into the relationship between phage 7v and *P. aeruginosa* strain Ps-7. As a result of this investigation, two additional phages were isolated from cultures of strain Ps-7 which have been shown to be unrelated to each other or to phage 7v. Since the phages concerned have properties of individual interest, each will be discussed separately.

Phage 7v. Our attention was originally directed towards the presence of phage 7v by the observation that occasionally, and unpredictably, clear plaques appear spontaneously in areas of confluent growth when strain Ps-7 is streaked on solid media. During this investigation, numerous unsuccessful attempts were made to isolate phage 7v as a contaminant in the laboratory; thus, the infrequent appearance of clear plaques from which phage 7v can be isolated appears to be a stable characteristic of *P. aeruginosa* strain Ps-7. The failure to isolate phage 7v from overnight broth cultures of strain Ps-7, or to increase the frequency of spontaneously appearing 7v plaques by prolonged incubation of broth cultures of Ps-7, does not support the hypothesis that *Pseudomonas*-7 is a carrier strain (Lwoff, 1953) for phage 7v. Alternatively, the infrequent appearance of spontaneous phage 7v plaques suggested the hypothesis that phage 7v probably arises as the result of a rare mutation, or a series of mutations, occurring in a temperate phage in a manner analogous to that reported for *Bacillus* phage Wα (McCloy, 1951, 1958). Although attempts to demonstrate the presence of a temperate phage related to phage 7v have not as yet met with success, it is highly probable that this is a result of the unavailability of sensitive indicator strains. This conclusion is supported by the observation that temperate *P. aeruginosa* bacteriophages tend to exhibit extremely limited host ranges (Feary et al., 1963*b*).

Phage 7v shows some similarity to *P. aeruginosa* phage E79 described by Holloway, Egan, and Monk (1960). These two viruses are not antigenically related (Feary, *unpublished data*); however, like phage E79, phage 7v exhibits a relatively wide host range and is capable of causing the complete lysis of broth cultures of sensitive strains. Unlike phage E79, which brings about a marked reduction of bacterial growth when used to infect an exponentially growing culture (Holloway et al., 1960), the infection of sensitive strains with phage 7v, under conditions which insure infection of the majority of cells in the culture, does not appreciably affect the rate of increase in the turbidity of exponentially growing cultures. This observation suggests that phage 7v is indeed a virulent mutant of a temperate phage (Lwoff, 1953; Jacob and Wollman, 1953); however, the finding that abortive infection is a prominent feature following adsorption of phage 7v to host cells of strain Ps-7 raises questions regarding the validity of the criterion for this system. In relation to the above, it would be of interest to learn the events which bring about the lysis inhibition observed in broth cultures of strain Ps-7 multiply infected with phage 7v. In particular, it would be well to know whether the lysis inhibition observed is a consequence of superinfection by phage 7v particles, as has been shown to be the case for the lysis inhibition of phage T_2 (Doermann, 1948), or whether it is a result of superinfection by temperate phages such as phage 7m which are present in Ps-7 cultures in relatively high concentrations. In view of the demonstration (Fry and Gros, 1959) that significant metabolic disturbances in bacterial cells are brought about during the establishment of lysogeny, it could be postulated that the abortive infection as well as the lysis inhibition observed in this system is a consequence of super-

infection by temperate phages. Unfortunately, the results obtained by the infection of a multilysogenic host system cannot be entirely attributed to the interaction of a single virus with the host cell; our attempts to isolate nonlysogenic, sensitive strains of *P. aeruginosa* have not been successful (Feary et al., 1963*b*).

Phage 7m. This bacteriophage is found in constant association with strain Ps-7 and is present in overnight broth cultures in characteristic concentrations. Its production can be induced by exposure of exponentially growing cultures of Ps-7 to ultraviolet light, and strains sensitive to phage 7m are lysogenized. In addition, strain Ps-7 has been shown to be immune to infection by phage 7m. Thus, it is concluded that the relationship between *P. aeruginosa* strain Ps-7 and phage 7m meets the classical criteria for lysogeny (Lwoff, 1953; Jacob and Wollman, 1959).

Phage 7s. Throughout this investigation, we have consistently been able to demonstrate the presence of phage 7s in broth cultures of *P. aeruginosa* strain Ps-7. In a careful check for possible contamination, it was found that phage 7s does not share properties in common with any of the bacteriophages under investigation in our laboratory. Shortly after the preparation of large quantities of lysates of phage 7s (Feary et al., 1963*a*), laboratory contamination was detected; however, it was found that this could be eliminated by diligent application of disinfectant to floors, benches, and shelves in the work areas, and could be prevented by strict application of sterile techniques. Our original observation was confirmed when phage 7s was reisolated from a broth culture of strain Ps-7 that had been started from a lyophilized preparation of this organism stored for 2 years prior to the original isolation of phage 7s.

On the basis of data presently available, the nature of phage 7s is somewhat of an enigma. Although it can be demonstrated in association with a lysogenic bacterial strain and its adsorption to this strain suggests some sort of an immunity mechanism, because it is a RNA virus, its mode of association with the constituents of the host cell may differ markedly from that observed in the case of temperate deoxyribonucleic acid (DNA) viruses. Information regarding the nature of the relationship between phage 7s and *P. aeruginosa* strain Ps-7 could be obtained by application of the sequence complimentarity test devised by Hall and Speigelman (1961) and recently used (Doi and Speigelman, 1962) to show that no homology exists between the RNA of phage MS2 and the DNA of its host strain *E. coli* Hfr strain K-16. In addition, genetic recombination (conjugation) has been described for *P. aeruginosa* (Holloway, 1955; Holloway and Jennings, 1958; Holloway and Fargie, 1960). Genetic experiments, employing strains of *P. aeruginosa* in which a permanent relationship with phage 7s has been established, should shed additional light on the mode of association of this virus with the constituents of its host strain.

The sensitivity of phage 7s to ribonuclease was unexpected and warrants further study. Among the many possibilities, there are at least three reasonable suppositions which may help to explain this observation. First, partially purified preparations of phage 7s may contain significant levels of proteolytic enzymes which, under the proper conditions, break down the protein coat of the virus, allowing ribonuclease in turn to inactivate the viral nucleic acid. This hypothesis would account for the partial protection observed in the presence of EDTA in cases where metals serve as cofactors for proteolytic enzymes. A second tentative explanation for the observed effect of ribonuclease is that perhaps incomplete virus protein coats form which carry a full complement of RNA. Support for this hypothesis is gained by the observation (Feary et al., 1963*a*) that partially purified phage 7s preparations contain particles of two different sizes. The heavier particles (with a sedimentation coefficient of 88s) do not dissociate in the presence of EDTA, while the lighter particles (with a sedimentation coefficient of 53s) are completely dissociated in the presence of EDTA. Support is lent to the proposition that the smaller particles represent incomplete virus by the finding that, although there is a significant loss in total RNA after the treatment of such preparations with ribonuclease and EDTA, there is no significant change in the base ratios of the RNA that resists such treatment. This second tentative hypothesis does not account for the partial protection observed in the presence of EDTA. A third explanation for the observed sensitivity of phage 7s in the presence of ribonuclease might be that at high dilutions in a dilute buffer system, dissociation of the phage protein coat occurs to a degree sufficient to allow inactivation of infectious viral RNA by the enzymes. Again, this provisional hypothesis does not account for the partial protection obtained in the presence of EDTA.

The sensitivity of phage 7s to ribonuclease offers additional proof that this virus contains RNA as its nucleic acid component. This observation may also account for the low concentration of phage 7s found in broth cultures of *P. aeruginosa* strain Ps-7.

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