The Structure and Infective Process of a *Pseudomonas aeruginosa* Bacteriophage Containing Ribonucleic Acid

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SUMMARY

The electron microscope shows that there are a number of different morphological types of bacteriophages which grow on *Pseudomonas aeruginosa*. Some are conventional ones with contractile or non-contractile tails, but the most interesting is a tail-less phage containing RNA. The structure of both conventional and RNA phages is described. It is shown that the RNA phage probably infects the cell via polar pili. Intracellular multiplication and lysis by the RNA phage is followed in thin sections of infected cells. In the early stages, the nuclear region is much reduced and dense granular areas appear. These subsequently differentiate into crystal-line aggregates of virus particles; at the same time a large bulge, identical to that found associated with spheroplast formation, appears. The crystals continue to increase in size until the spheroplast ruptures and lysis occurs.

INTRODUCTION

The specific bacteriolytic agents of the genus *Pseudomonas* consist of several morphological types of bacteriophages and a number of bacteriocins. The natural habitats of *Pseudomonas aeruginosa* include the intestinal canal, water, sewage, pus and the human skin. The habitat of its bacteriophages will of course be the same, and it has been found that large numbers can be obtained from sewage. In the present paper, isolates from this source are illustrated and compared with phages of other species in the genus. *P. aeruginosa* phages are represented by four basic morphological types (Bradley, 1965*a*); those with contractile tails, those with long non-contractile tails, tail-less phages with small capsomeres and containing ribonucleic acid (RNA), and a rod-shaped or filamentous type (Takeya & Amako, 1966).

Pseudomonas aeruginosa, unlike other members of the genus, is mildly pathogenic to man and animals and has been well studied; its bacteriophages are therefore of special interest. In addition, a fertility factor, known as FP, has been demonstrated for it (Holloway & Jennings, 1958; Holloway & Fargie, 1960); it is thus of some importance to determine whether or not its RNA phages infect via male-specific pili as appears to be the case with those of *Escherichia coli* (Brinton, 1965; Edgell & Ginoza, 1965). With *E. coli* the pili are coded for by the F factor (Brinton, Gemski & Carnahan, 1964) and the RNA phages adsorb to them (Crawford & Gesteland, 1964; Bradley, 1964).

Since RNA phages have only been found for three genera of bacteria, *Escherichia*, *Pseudomonas* and *Caulobacter* (Schmidt & Stanier, 1965), and intracellular

multiplication has only been studied for the first of these, it is important to follow the infective process in the other two. It is by no means certain that the same cytological changes in infected cells will be observed in all three cases. For this reason a detailed electron-microscopic study has been made of the whole infective process of a *Pseudomonas* RNA phage from adsorption to lysis.

METHODS

Culturing media and methods. Oxoid nutrient broth was used for both plate and broth cultures, the former containing 2% (w/v) agar. Phages were grown by confluent lysing bacteria in double agar layer plates (Adams, 1959), the soft agar layer containing 1% (w/v) agar + 1% (w/v) peptone.

Isolation and purification of bacteriophages. Untreated sewage influent was plated by the double agar layer method with *Pseudomonas aeruginosa* strain 1. Each ml. of sewage contained between 100 and several thousands of phage particles so that discrete plaques were produced with 0.1 ml. to 0.01 ml. of sewage per plate. After incubation at 37° overnight, plaques of differing morphology were picked and suspended in 1.0 ml. broth. Subsequent purification was achieved by several cycles of plaque-picking. High-titre phage preparations were obtained from successive single plaque isolations by the confluent lysis of bacteria growing on double agar layer plates using 0.25 ml. plaque eluate per plate. The plates were extracted with 0.1 M-ammonium acetate solution (neutral) for electron microscopy and purified by alternate high- and low-speed centrifugation.

Sources of host bacteria and phages. *Pseudomonas aeruginosa* strain 1 and the RNA phage 7S were kindly supplied by Dr T. Feary. Strain c 10 was obtained from Dr L. Dickinson together with phage Pc. Other pseudomonas species were provided by Dr E. Billing. The pyocin indicator, *P. aeruginosa* strain 1050 was provided by Dr Y. Hamon (for addresses see Acknowledgements). Other phages were obtained from various sewage samples and labelled as follows. The prefixes PP, PB and PL denote the source locations Pangbourne (Berks), Basingstoke (Hants) and Linlithgow (West Lothian) respectively. The isolate PP 7, an RNA phage from Pangbourne, is the most important described here.

The isolation of phage-resistant bacteria. It was found that the RNA phages 7S and PP 7 easily contaminated the laboratory in a short time. While care was taken to only work with one at a time, the growth of other phages on the host *Pseudomonas aeruginosa* strain 1 was facilitated by the isolation of a mutant resistant to the RNA phages. The spot-test method was used: a loopful of RNA phage suspension was placed on the surface of a double agar layer plate of host bacterium, and resistant clones obtained from the resulting plaque. One of these was designated *P. aeruginosa* strain 1/7; though it was obtained with phage PP 7 it was also resistant to phage 7S (see Table 1).

Lytic activity tests. The various phages isolated on *Pseudomonas aeruginosa* strain 1 were tested against three pseudomonas species and other strains of *P. aeruginosa* for lytic activity using the spot-test as described above. In addition, the activity of phages on double agar layer plates of host bacterium (*P. aeruginosa* strain 1) containing 1 mg./ml. of ribonuclease (RNase) in the top layer of agar was tested.

Acridine-orange staining. The type of nucleic acid in phage PP 7 was identified as single-stranded RNA (1-RNA) using a modified fluorescent staining procedure (Bradley, 1965*b*, 1966) based on that of Mayor & Hill (1961). A suspension of the phage was obtained by extracting confluent lysed soft agar plates with phosphate buffered saline (g./l.: Na_2HPO_4 , 1.27; KH_2PO_4 , 0.41; NaCl , 7.36; pH 7.2). This was partly purified by cycles of alternate high- and low-speed centrifugation and also treated with deoxyribonuclease (DNase) and RNase at concentrations of about 10 γ /ml. and 100 γ /ml. respectively for 1 hr at 37°. Droplets of phage suspension were dried on microscope slides, fixed in Carnoy's fluid for 5 min. and rinsed in absolute alcohol. After drying in a stream of warm air they were stained in 0.01 % acridine orange in modified McIlvaine's buffer (0.1 M-citric acid, 6.0 ml.; 0.15 M- Na_2HPO_4 , 4.0 ml.; pH 3.8) for 5 min. They were treated with 0.15 M- Na_2HPO_4 (for 15 min. and finally viewed under 2570 Å ultraviolet light. A post-stain treatment was carried out by immersing the slides in solutions of molybdic or tartaric acids as described elsewhere (Bradley, 1966), the colours under u.v. also being noted.

Lysis of broth culture by phage PP 7. Thin sections of RNA phage-infected cells were obtained from a culture of *Pseudomonas aeruginosa* strain 1 as follows. A log-phase broth culture was diluted so that 250 ml. contained about 1.5×10^8 bacteria/ml., 50 ml. of this was removed to serve as a control culture and the remaining 200 ml. were infected with 15 ml. of a phage suspension containing 1×10^{11} plaque-forming units (p.f.u.) per ml. This gave a multiplicity of infection of about 50:1, a sufficiently high value to ensure the nearly simultaneous infection of all the host cells. Approximately 15 ml. samples were removed for fixation, embedding and sectioning at 15, 35, 50 and 75 min. after infection. In addition, bacterial concentration was estimated by optical absorption at intervals for both cultures so that growth curves could be obtained.

Fixation, embedding and sectioning of bacteria. About 15 ml. of a culture of bacteria previously infected with phage PP 7 as above was centrifuged at 7000 *g* for 3 min. and the pellet resuspended in 5 ml. of 6.25 % (w/v) glutaraldehyde in Sørensen's buffer at pH 7.2 (Sabatini, Bensch & Barnett, 1963). After 1½ hr at room temperature the bacteria were washed twice by centrifugation in buffer. The pellet was resuspended in 1 % (w/v) osmic acid in buffer (Sørensen's, pH 7.2) and post fixed at room temperature for 1½ hr. It was found that, if required, the bacteria could be stored overnight at this stage at 2° in buffer; no ill effects were observed in the final sections. The bacteria were again centrifuged into pellets at 7000 *g*; these were sufficiently hard to be passed through dehydration, etc., using a wire loop without breaking.

The pellets were then dehydrated for 20 min. in each concentration of 30 %, 50 %, 75 % and 100 % acetone; the first three solutions contained 1 % (w/v) uranyl acetate for staining. Two further treatments for ½ hr and 1 hr in 100 % acetone were then carried out, the acetone having been dehydrated overnight with anhydrous calcium chloride. The pellets were next transferred to a 50 + 50 (v/v) mixture of acetone + Vestopal in a shallow dish. After about 2 hr at room temperature, a stream of warm air was passed over the dish from a hair drier for another 2 hr to ensure that all the acetone had evaporated. The pellets were next transferred to 100 % Vestopal containing 1 % (v/v) each of initiator and accelerator, where they remained overnight at room temperature. The following morning they were transferred to fresh

Vestopal mixture for 7 hr. The pellets were finally broken into two or three pieces and transferred to gelatine capsules containing Vestopal mixture; these were baked with the lids off at 60° until hard (18–36 hr). Sections were cut on a Huxley ultra-microtome with a diamond knife; they were mounted on grids unsupported, and then stained for 2–5 min. in lead citrate (Reynolds, 1963) before examination in the electron microscope.

Negative staining for electron microscopy. Phage suspensions prepared by extracting confluent lysed plates with neutral 0.1 M-ammonium acetate were mixed with an equal volume of neutral potassium phosphotungstate (2 %, w/v) solution. A carbon-coated grid was touched on to the surface of the mixture and dried under a lamp after removing excess liquid by touching the edge with a filter paper.

Preparation and use of anti-7s serum. Anti-serum for phage 7s was prepared in order to determine the serological relationship between phages 7s and PP 7. A rabbit was inoculated intravenously with about 5×10^{11} p.f.u. phage 7s in 0.5 ml. phosphate buffered saline three times over a period of 1 month. About 1 week after the last inoculation 10 ml. of blood was removed by superficial venesection of the ear. The serum was prepared in the normal way and complement removed by heating at 56° for $\frac{1}{2}$ hr. About 5×10^5 p.f.u. of phage were then added to 10 ml. broth containing 0.1 % (v/v) anti-serum. The mixture was incubated with shaking at 37° and the phage particles counted at intervals over 1 hr. A graph was then plotted of percentage survivors (on a log scale) against time, for the two phages in question.

RESULTS

Phages with contractile tails

Plate 1, figs. 1, 4 illustrate the octahedral shape of the head of phage BP 1, which is 750 Å in size. The tail, which is 1500 Å long, has a fine subunit structure, the organization of which is not clear. The lower portions of the tails of phage PP 1 in Pl. 1, figs. 2, 3 appear to be thickened, and in Pl. 1, fig. 4 this can be seen to be due to fibres folded up against the sheath for the last 600 Å of its length. Plate 1, fig. 5 shows a PP 1 virion without a head but with a contracted tail sheath. The top of the tail has a double disc arrangement similar to that found in coliphage ZG3A (Bradley, 1963), a *Pseudomonas syringae* phage (Matthews & Bradley, 1964), and a bacillus killer particle (Bradley, 1965*c*). In Pl. 1, fig. 6, two PB 1 virions can be seen adsorbed to a piece of debris. Their sheaths are contracted and the tail fibres are just visible, no longer folded back. A change in subunit packing has taken place in the sheath on contraction since the fine structure of Pl. 1, fig. 4 has been replaced by parallel longitudinal striations. This sort of change has been observed in other phages of similar morphology (Bradley, 1963).

Phages with non-contractile tails

This morphological group is divided into two: phages with regular heads and phages with elongated heads. Two of the former type are shown here, differing only in the structure of the tail tip. Phage Pc (Pl. 1, figs. 9, 10) has a distinct knob on the end of the tail. This can be seen to have a hole in it in the case of an empty virion (Pl. 1, fig. 9), presumably where the tail core passes through. With a full virion, the knob, as well as the tail, is solid, doubtless filled with a strand of nucleic acid.

This phage has been described in detail elsewhere (Bradley & Kay, 1960) but is included here for comparison with the rather similar phage PP 4. Again the head has a regular hexagonal outline and is probably octahedral (Pl. 1, fig. 7). The tail, however, is quite different, the tip resembling a cross (Pl. 1, fig. 8). It appears to be identical to the *Pseudomonas syringae* phage PS 4 (Matthews & Bradley, 1964). A complete virion is shown in Pl. 2, fig. 11. The diameter of the head is 600 Å and the tail is 1950 Å long. Phage Pc has a head of similar dimensions but the tail is rather shorter (1650 Å).

The phage with a non-contractile tail and an elongated head (isolate PB 2) is shown in Pl. 2, fig. 12. The head is 1000 Å long and 700 Å wide with an appearance very similar to that of the T-even coliphages. The tail is similar to that of phage PP 4 though shorter (1750 Å); it exhibits a helical structure (Pl. 2, fig. 12, top left particle and inset) and like PP 4 has a crossed tip. In many respects it is similar to the coliphage ZG3A (Bradley, 1963).

The RNA phages

Because of the importance of the RNA *Pseudomonas aeruginosa* phages both the structure and the infective process of one of these (PP 7) have been studied in detail.

Structure. In Pl. 2, fig. 13, virions of the isolate PP 7 are illustrated in a micrograph which is close to focus and so has rather low contrast. They have the characteristic transparent appearance of other RNA phages specific to *Escherichia coli* and caulobacter species with a regular hexagonal outline. Apart from this, no other details can be seen. In Pl. 3, fig. 16, 17 the electron microscope was slightly under-focused to increase contrast, and some fine detail can be discerned. In Pl. 3, fig. 16, a number of particles show a dark spot on their surface suggesting a pore (two examples are arrowed *P*). One virion also has a number of distinct white spots on it (arrowed *C*), probably representing capsomeres. These are clearer along an edge of a virion in Pl. 3, fig. 17 (arrowed *C*); here they have a centre-to-centre spacing of 30 Å. An empty virion can be seen on the right in this figure. The size of phage PP 7 is about 250 Å.

Adsorption. Plate 3, fig. 14 shows a cell of *Pseudomonas aeruginosa* heavily infected with phage PP 7. It can be seen that the virions are lying in dense aggregates extending from one pole of the bacterium, and also that one or two of them have adhered to the cell surface. A close examination at higher magnification (Pl. 3, fig. 15) reveals that the aggregates are interlaced with very fine strands, which in fact arise from the bacterium. Plate 2, fig. 13 and Pl. 3, fig. 17 show that there is undoubted adsorption to the surface of these filaments, and that they cause the aggregates. Little structure can be seen in the filaments themselves; a portion to the right of the arrow in Pl. 3, fig. 17 appears to be hollow but there is no helical structure visible. The diameter of the strands is about 45 Å. The micrographs thus show two kinds of adsorption: the majority of phages adhere to the filaments and a very small minority to the cell surface. The cell illustrated looks rather transparent for a healthy bacterium and has probably undergone cytological changes associated with phage infection.

Intracellular multiplication. A broth culture infected at a multiplicity of infection of about 50:1 was used to prepare the sections illustrated. As stated in Methods above, cell concentration estimates were taken of this culture so that suitable

sampling times could be determined. These estimates, together with those of the control culture, were plotted against time and the curves obtained are shown in Fig. 1. The sampling positions on the curve are marked C, D, E, F (A and B being control samples of an uninfected culture); these letters correspond to those on the blocks from which the sections were cut (see explanation of plates).

The electron micrographs of sections taken from the blocks designated C, D, E and F on the curve in Fig. 1 follow the morphological changes during the intracellular multiplication of the RNA phage. In Pl. 4, fig. 18 an uninfected cell of *Pseudomonas aeruginosa* strain 1 is seen to have well dispersed nuclear material (nucleoplasm), the DNA fibres being barely visible in this micrograph. There are also numerous dark particles which may be ribosomes. In sample C, only 15 min. after infection, definite changes can be seen (Pl. 4, figs. 19, 20). Dense areas (marked P) appear at the poles of the cell, and also at the centre. This has the effect of reducing the area occupied by the nucleoplasm most markedly (see Pl. 4, fig. 20). The latter micrograph also shows a mesosome (arrowed M; Fitz-James, 1960; Salton & Chapman, 1962; Vanderwinkel & Murray, 1962). The appearance of sections of cells from block C was noticeably uniform but cells from the remaining blocks showed differences between individual bacteria as would be expected if each had reached a different stage in infection.

Plate 5, fig. 21, which was obtained from block F, shows the appearance of an infected cell at a later stage in the infective cycle than that in block C (see Fig. 1). The shape of the cell, which is typical of a spheroplast, will be discussed below; it is pointed out, however, that it appeared (from light-microscope observations) about $\frac{1}{2}$ hr after infection and increased in frequency thereafter up to lysis. In Pl. 5, fig. 21 the dense patches of undifferentiated viral RNA and protein can be seen at the periphery of the bulge, and in the ends of the two undistorted lengths of cell, often called 'rabbit's ears', and also where these protrusions join. A patch of material can be seen to possess organization in Pl. 5, fig. 22. The plane of this section lies lengthwise through one of the 'ears' and the bulge. The periodicity visible in the phage material is small (about 80 Å) compared with the size of the intact virion. The cell wall shows indications of a break at the top of the bulge.

Many cells in block D had crystalline inclusions of virus similar to those found in *Escherichia coli* by Schwartz & Zinder (1963) though generally larger. In Pl. 5, fig. 23 the cell is beginning to lyse and the phage particles are just breaking away from the right of the crystal. In Pl. 5, fig. 24 a higher magnification shows two different orientations in a crystal, perhaps analogous to twins in metals. In Pl. 6, fig. 25 the cell has not lysed so that the bulge is intact. Two 'ears' can be seen, one tapering away from the plane of the section and one completely filled with a crystal of virions. There are some 30 rows of them, each containing about 40 phage particles making a total of 1200 in this section. In Pl. 7, fig. 26 the earliest stage of crystal formation can be seen; a few spheres are beginning to line up against the cell wall.

Lysis. The moment of lysis is shown in Pl. 7, figs. 27, 28. It is emphasized that the plane of the section is passing through only one 'ear' in Pl. 7, fig. 27; a section through the other 'ear' would probably look similar. A small crystal is present in the 'ear', and a number of virions have passed from a second, now disorganized crystal into the broken bulge. Together with cytoplasmic debris and nucleic acid they are passing through the massive rupture in the cell wall. Many sections have

shown that this is typical and that lysis is achieved by the breakdown of the wall of the spheroplast. In Pl. 7, fig. 28 the same stage is shown in transverse section through the bulge. There are two disorganized crystals, presumably one from each 'ear'. It can be seen here and in other sections, that the spheroplast wall is apparently complete with its various layers and that lysis is caused by fracture rather than general disintegration.

Lytic activity of Pseudomonas aeruginosa phages

Table 1 shows the results of lytic activity tests described above. It can be seen that the RNA phages lyse only *Pseudomonas aeruginosa* strain 1, but other phages have a wider range of activity. In addition lytic activity by the RNA phages on their host is inhibited by the presence of RNase in the top layer of agar in the double layer plates used for the test. This suggests that both phages contain 1-RNA.

Table 1. *Lytic activity of Pseudomonas aeruginosa phages*

Host	Bacteriophages							
	Contractile		Non-contractile				RNA	
	PP 1	PB 1	PP 4	Pc	PB 2	PL 2	7S	PP 7
<i>P. aeruginosa</i> , 1	+	+	+	+	+	+	+	+
<i>P. aeruginosa</i> , 1 with RNase	+	+	+	+	+	+	—	—
<i>P. aeruginosa</i> , 1/7	+	+	+	—	+	—	—	—
<i>P. aeruginosa</i> , c10	+	+	—	+	+	+	—	—
<i>P. aeruginosa</i> , 1050	—	—	—	—	—	—	—	—
<i>P. syringae</i> , (NCPPB 1072)	—	+	—	—	(+)	+	—	—
<i>P. fluorescens</i> , (NCTC 10038)	—	—	—	—	—	—	—	—
<i>P. phaseolicola</i> , (NCPPB 52)	—	+	(+)	—	+	+	—	—

NOTE. A + signifies strong phage activity (a clear area), (+) weak activity (a veiled area), and — no activity.

Table 2. *Acridine-orange staining of phages of 7S and PP7*

Phage	Colours			
	Nucleic acid	After Na ₂ HPO ₄	After molybdic acid	After tartaric acid
T 4	2-DNA	Bright green	Bright green	Bright red
WTV RNA	2-RNA	Bright green	Stayed green but faded out	Pale red
ZJ/2	1-DNA	Bright red	Pale green	Pale green
ZIK/1	1-RNA	Bright red	Pale red	Pale red
7 S	1-RNA	Bright red	Pale red	Bright red
PP 7	?	Bright red	Pale red	Bright red

NOTE. WTV stands for wound tumour virus. Coliphages ZJ/2 and ZIK/1 have been described by Bradley (1964).

Acridine-orange staining

The object of this test was to confirm the presence of 1-RNA in phage PP 7. The results are given in Table 2 with other representative types of nucleic acid for comparison. It can be seen that the colours agree with other phages known to contain 1-RNA.

Inactivation of phage PP 7 by anti-7S serum

The graph in Fig. 2 shows that there is a definite reaction between anti-7S serum and phage PP 7. The rate of inactivation is not, however, as great as with the homologous phage. There is therefore a definite serological relationship between phage 7S and phage PP 7, but they are not identical.

Fig. 1

Fig. 2

Fig. 1. Rate of bacterial growth in non-infected (x—x) and PP 7-infected (O—O) broth cultures of *Pseudomonas aeruginosa* strain 1.

Fig. 2. Rate of inactivation by anti-7S serum (dilution 1000:1) of phages 7S (x—x) and PP 7 (O—O).

DISCUSSION

Tailed phages

The tailed phages of *Pseudomonas aeruginosa* are similar in appearance to those associated with other genera of bacteria, the form with contractile tail and octahedral head being particularly common especially amongst coliphages. The electron

micrographs shown here have, however, provided a useful observation. The coliphage E1 (one of the common forms mentioned), which is almost identical with phages PP 1 and PB 1, has four tail fibres (Bradley, 1963). These can usually be seen splayed out at the tip of the sheath. Other contractile types seemed to lack these fibres, but it is shown here that they are in fact folded up against the sheath. On adsorption they appear to splay out (Pl. 1, fig. 6). It would seem that they play a role in adsorption as is the case with the T-even coliphage tail fibres (Kellenberger *et al.* 1965); with T4 they form a network round the sheath and are attached by a reversible bond to the collar under conditions when adsorption is not possible; the bond breaks under favourable circumstances and the fibres are released and assist attachment in some way (see also Bradley, 1963). It seems highly probable from observations shown here that the tail fibres of other contractile phages have a similar function. The non-contractile types have only one unique feature: their tail tip. This cross-like form is common amongst phages specific to other *pseudomonas* species. The non-contractile phages with regular hexagonal heads seem to be octahedral.

Comparatively few *Pseudomonas aeruginosa* phages have been described in the literature but three examples are given by Slayter, Holloway & Hall (1964). Phage B3 is similar to phage PP 4 and phage E79 to PP 1 and PB 1 save for slight dimensional differences. The third phage they described (F 116) had an octahedral head with an unstable non-contractile tail; this form has not been found in any sewage samples to far. In addition to these isolates, Takeya & Amako (1966) have described a filamentous type similar in many respects to those specific for *Escherichia coli*. These authors give no indication as to how or where this phage was isolated.

RNA phages

Nucleic acid. The size (about 250 Å) and appearance of all 1-RNA phages so far isolated has been generally similar and they are readily recognizable in the electron microscope as small semi-transparent spheres. Nevertheless, once such a phage has been isolated, it is important to show that it does, in fact, contain 1-RNA. Apart from the morphology of the virion, the ability of RNase to inhibit plaque formation (see Table 1) is valuable evidence in favour of the nucleic acid being 1-RNA. In spite of this apparent certainty, it is obviously desirable to have a further confirmatory test, and acridine-orange staining (Mayor & Hill, 1961; Bradley, 1965*b*; Bradley, 1966) provides this. The results given here: morphology, the effect of RNase, and acridine-orange staining, confirm without doubt that PP 7 contains 1-RNA.

Structure. As has been stated, micrographs of the RNA phages all look similar regardless of host genus. There is, however, one exception, namely 7S. Feary, Fisher & Fisher (1964) show a micrograph illustrating a high degree of organization within the capsid. The present author has not found such organization in 7S, PP 7 or indeed any other RNA phage. If the micrographs of Feary *et al.* (1964) are compared to those shown here, it becomes immediately obvious that the virions in the former are better preserved. This would mean that capsomere organization is not disturbed. Lack of preservation will probably be a function of the negative staining material, phosphotungstic acid being known to vary in efficiency according to source. Thus it may be worth while fixing RNA phages for negative staining, a practice not normally

used. In the present case, simple formaldehyde fixation was attempted but with no effect.

The micrographs of Feary *et al.* (1964) appear to show the structure produced by the faces of the capsid rather than individual capsomeres. This appearance is in agreement with an icosahedral form, which has been observed for the RNA coliphage ZIK/1 (Bradley, 1964). The latter had 92 capsomeres with a centre-to-centre spacing of about 45 Å; PP 7 has smaller ones spaced at 30 Å. They are more difficult to see, but being smaller will provide a sharper outline to the faces of the capsid, which perhaps accounts for the appearance of the pictures of Feary *et al.* (1964). A 30 Å spacing in an edge length of 180 Å on an icosahedron would make the total number of capsomeres 252 (6 per side). Since it is difficult to be accurate about the edge length, 5 capsomeres per side, making a total of 162, is another possibility. It is certainly evident from these observations that the RNA coliphages are not structurally identical with the pseudomonas ones. One feature which does seem to be shared is the pore. This is a point of asymmetry in the capsid, formed perhaps by a missing capsomere. In Pl. 3, fig. 17 the pore does not seem to be associated with adsorption to pili since some can be seen on virions attached to the filament.

Adsorption. There is little doubt that adsorption takes place to fine polar pili. Whether or not this is associated with infection is another question. In the case of coliphages all the evidence is in favour of this (Brinton, 1965; Edgell & Ginosa, 1965) and by analogy one can justifiably assume that the same is likely to be the case with pseudomonas phages. The appearance of the electron micrographs of Crawford & Gesteland (1964), Bradley (1964) and others is remarkably similar to those shown here. In a previous observation (Bradley, 1965*a*) no virions could be seen attached to the *Pseudomonas aeruginosa* cell wall. In that case the bacterium was not yet morphologically changed by the phage infective process and appeared typically dense in the electron micrograph. Here (Pl. 3, fig. 14), the bacterium is apparently damaged, a factor which might produce non-infective adsorption to the cell wall. Another explanation is that some pili, with virions attached, have folded back across the bacterium.

The pili themselves are of special interest. Similar ones have been demonstrated in *Escherichia coli* to be unique and to be associated with and coded for by the F (fertility) factor (Brinton *et al.* 1964; Brinton, 1965). They are thus called F-pili (Brinton, 1965; Valentine & Strand, 1965). Now an infectious fertility factor (FP) has been demonstrated for *Pseudomonas aeruginosa* (Holloway & Jennings, 1958; Holloway & Fargie, 1960): the pili observed here (Pl. 3, figs. 15–18) are probably the same, being adsorbed to by RNA phages. In fact it is the phage particles which have revealed their presence. Negative staining of uninfected cells does not reveal the pili; perhaps they stick to the cell surface or wind round the polar flagellum. The RNA phages thus have the effect of delineating them as is the case with *E. coli* F-pili (Brinton, 1965). The *P. aeruginosa* pili are rather thinner than those of *E. coli*: about 45 Å as opposed to 70 Å. They appear to be hollow in places and so could act as a tube for the transfer of infective RNA from the phages, in which case they should be called FP-pili. They also resemble the filamentous bacteriophages of *E. coli*. These viruses are so similar in appearance to *E. coli* F-pili that one cannot but wonder whether the latter evolved in some way into an infective viral form. The

recently discovered filamentous *P. aeruginosa* phage (Takeya & Amako, 1966) is thicker than the pili illustrated here.

Intracellular multiplication. While the fundamentals of intracellular multiplication appear to be similar to those encountered in *Escherichia coli* (Schwartz & Zinder, 1963), the formation of a spheroplast is quite unique.

The early stages of the infective cycle are marked by the appearance in characteristic positions in the cell of dark masses of material which subsequently crystallize into virions. These crystals seem rather bigger than those illustrated for *Escherichia coli*, particularly in Pl. 6, fig. 25. The number of particles present in this single slice (1200) reflects the huge burst size of the phage. If one assumes that the thickness of the section includes one layer of phages in a perpendicular plane (this would be about 300 Å) then the total number in the crystal would be about 30×1200 , assuming a circular cross-section. A burst size of 36,000 is, if anything, rather bigger than that usually quoted for RNA coliphages.

Lysis. The apparent maintenance of the integrity of the cell wall until lysis strongly suggests that the rupture of the spheroplast is brought about by osmosis, the way in which normal spheroplasts lyse. This process is quite different from that associated with *Escherichia coli* with any phage: here the cessation of cell-wall synthesis together with the release of lysozyme causes lysis (Cota-Robles, 1964).

The rapid growth of resistant bacteria after the lysis of a broth culture (Fig. 1) shows that a fairly large proportion of the original bacterial population is resistant to infection; indeed, it is extremely easy to obtain resistant clones. The important question is whether or not resistance is due to the absence of pili. This can only be found out when a method is devised for detecting pili on uninfected cells.

The taxonomy of Pseudomonas aeruginosa phages

The morphological groups fit in with the scheme of classification proposed by Bradley (1965*a*), there being examples of contractile phages, phages with long non-contractile tails, tail-less phages with small capsomeres containing RNA, and a filamentous form containing 1-DNA (Takeya & Amako, 1966). It is interesting to note from Table 1 that the lytic activity of phages isolated on *Pseudomonas aeruginosa* extends to other species of the genus *Pseudomonas* save in the case of the RNA types.

Serological characteristics give a very fine distinction between phages. Thus the fact that there is a definite serological relationship between the two RNA phages 7 s and PP 7 indicates that they have only small chemical differences in the capsid. Similar relationships have been found for RNA coliphages (Bishop & Bradley, 1965; Scott, 1965).

CONCLUSION

The observations described here have revealed a number of fundamental features about *Pseudomonas aeruginosa* and its phages. Perhaps the most important is the presence of what are probably FP-pili. It is clear that much more should be learnt about these appendages to see if they perform the same functions as those of *Escherichia coli*, namely to act as a tube for the passage of DNA and the fertility factor in the conjugation process (Hayes, personal communication).

The second major feature is the association of spheroplast formation with lysis.

A study of lysis by other *Pseudomonas aeruginosa* phages, to see if the same process takes place, would be of particular interest. *Pseudomonas aeruginosa* is a sufficiently common and important organism to warrant more detailed study, and it is hoped that the present work will stimulate this.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Phage PB 1, $\times 165,000$.
- Figs. 2, 3. Phage PP 1, $\times 165,000$.
- Fig. 4. Phage PB 1, $\times 275,000$.
- Fig. 5. Headless phage PP 1, $\times 275,000$.
- Fig. 6. Phage PB 1 adsorbed to debris, $\times 275,000$.
- Fig. 7. Head of phage PP 4, $\times 275,000$.
- Fig. 8. Tail of phage PP 4, $\times 275,000$.
- Fig. 9. Empty virion of phage Pc, $\times 165,000$.
- Fig. 10. Intact virion of phage Pc, $\times 275,000$.

PLATE 2

- Fig. 11. Phage PP 4 intact, $\times 275,000$.
- Fig. 12. Phage PB 2 (inset tail of phage PL 2), $\times 165,000$.
- Fig. 13. Phage PP 7 particles adsorbed to pilus; near focus electron micrograph, $\times 275,000$.

PLATE 3

- Fig. 14. Phage PP 7 adsorbed to host cell, $\times 37,500$.
- Fig. 15. Detail of the same, $\times 75,000$.
- Figs. 16, 17. Phage PP 7 underfocused; *P* are pores and *C* capsomeres, $\times 275,000$.

PLATE 4

Fig. 18. Section of an uninfected cell of *Pseudomonas aeruginosa*, strain 1, $\times 37,500$.

Figs. 19, 20. Sections of PP 7-infected cells from sample C (Fig. 1), $\times 37,500$.

PLATE 5

Fig. 21. Section of PP 7-infected cell of *P. aeruginosa*, strain 1 from sample F (Fig. 1), $\times 12,500$

Figs. 22, 23. Sections of infected cells from sample D (Fig. 1), $\times 50,000$.

Fig. 24. Crystal from sample D, $\times 83,000$.

PLATE 6

Fig. 25. Sectioned PP 7-infected cell of *P. aeruginosa* strain 1 from sample F, $\times 37,500$.

PLATE 7

Fig. 26. Section of part of PP 7-infected cell from sample C (Fig. 1). $\times 83,000$.

Fig. 27. Section of lysing cell from sample E, $\times 50,000$.

Fig. 28. Section of lysing cell from sample D, $\times 37,500$

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