Lipid-containing Bacteriophage PR4: Structure and Life Cycle

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SUMMARY

The structure of the purified lipid-containing phage PR4 was studied electron microscopically using thin sectioning, negative staining and freeze-fracturing techniques. The lipid layer was located inside a rigid capsid and had a bilayer structure. The innermost dense core was probably composed solely of DNA since no major structural proteins were missing in empty phage particles lacking DNA. During infection, phages were attached to the cell wall of the host. They apparently inject their DNA through the cell envelope. The lipid layer of the phage might play an active role in the injection process. The maturation of the phage capsid takes place within the nuclear region of the cell, whereas intact phages with DNA were always seen in the cell periphery.

INTRODUCTION

Bacteriophage PR4 (Stanisich, 1974; Bradley & Rutherford, 1975; Sands & Cadden, 1975) is one of the few lipid-containing phages isolated so far. Phage PR3 was isolated along with PR4 (Stanisich, 1974) and was shown to be serologically related to phage PR4 (Bradley & Rutherford, 1975) and to phage PR5 (Wong & Bryan, 1978). Electron microscopic studies of phage PR3 and PR4 using a negative staining technique (Bradley, 1974; Bradley & Rutherford, 1975) indicate that a chloroform sensitive layer may be surrounded by a capsid, as is the case with the well characterized lipid phage PM2 (Franklin, 1974), but contrary to the other well-known lipid phage φ6, where the lipid layer forms an envelope around the nucleocapsid (Vidaver et al. 1973; Bamford et al. 1976).

PR4 can infect a variety of Gram-negative bacteria including Escherichia coli and Salmonella typhimurium. The sensitivity of the host depends on an appropriate drug-resistance plasmid of either P, N or W compatibility groups (Stanisich, 1974; Bradley & Rutherford, 1975; Bradley, 1976). The nature of the phage receptors, probably coded by the plasmid genes, for this and for a related phage PRD1 (Olsen et al. 1974) still remains somewhat controversial (Olsen et al. 1974, 1977; Bradley, 1974, 1976; Bradley & Cohen, 1977).

The present communication describes the structure and composition of the purified phage PR4, as well as the infection and maturation process of the phage using electron microscopic techniques.

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Growth and purification of the phage. Bacteriophage PR4 and its hosts Pseudomonas aeruginosa K/1PO₄ (RP1), Escherichia coli CR 34 (RP1), E. coli J53-1 (R, ) and E. coli J53 (RN3) were kindly provided by Dr D. E. Bradley, Memorial University of Newfoundland, St John's, Newfoundland, Canada. The phage was first purified by three successive single plaque isolations on phage agar (Clowes & Hayes, 1968). Phage broth (Clowes & Hayes, 1968) was used for bacterial growth and phage production. Phage lysates were produced by infecting logarithmically growing cells with PR4 at a multiplicity of infection (m.o.i.) of 20 to 30. After lysis, cellular debris was removed by low speed centrifugation. The supernatant routinely contained about 2 × 10¹⁰ p.f.u./ml with E. coli CR 34 (RP1) as the host. The phage in the supernatant was concentrated with 10% (w/v) polyethylene glycol (PEG) by the method of Yamamoto et al. (1970). The PEG-treated phage was allowed to stand at 4 °C overnight and was pelleted by centrifugation at 8000 g for 15 min. The pellet was resuspended in 1/200 of the original lysate vol. in 10 mM-phosphate buffer, pH 7.2, containing 1 mM-MgSO₄ or in TGN medium (Sands, 1976). The phage was purified by centrifugation in a 5 ml 10 to 40% (w/v) linear sucrose gradient in phosphate buffer or in TGN medium at 84,000 g for 50 min using a Spinco SW 50.1 rotor. Fractions of about 100 µl were collected from the bottom. Assays for protein and DNA were performed according to Lowry et al. (1951) and Burton (1956) respectively. The phage material was diluted 1:2 in phosphate buffer and pelleted at 150,000 g for 60 min. The phage pellets were resuspended in phosphate buffer and the two centrifugation steps repeated.

Electron microscopy. The purified phage suspensions were negatively stained on the grid with 1% potassium phoshotungstate, pH 6.5. For freeze-fracturing the purified phage suspension was frozen in liquid Freon 22 cooled by liquid nitrogen. The fracturing was performed at −120 °C in a Balzers freeze-etching apparatus (Liechtenstein). Samples for thin sectioning were pre-fixed in two steps with ice cold 3% glutaraldehyde in 0.1 M-phosphate buffer, pH 7.2, for 45 min and post-fixed with 1% osmium tetroxide for 2 h at room temperature in the same buffer. After dehydration in a rising ethanol series the samples were embedded in Epon 812. Thin sections were prepared with an LKB Ultramicrotome 1 and were stained with uranyl acetate (Hayat, 1972) and lead citrate (Reynolds, 1963). All micrographs were taken with a JEM-100B electron microscope operating at 80 kV.

Sodium dodecyl sulphate (SDS) gel electrophoresis. Discontinuous SDS-gel electrophoresis method was that described by Laemmli (1970), except that the stacking gel contained 5% (w/v) acrylamide, 0.062 M-tris, 0.056 M-sodium phosphate, pH 7.8, 0.1% SDS, and the samples contained the final concentrations of 0.02 M-tris, 0.008 M-sodium phosphate, pH 7.8, 1% SDS, 1% β-mercaptoethanol, 10% glycerol and phenol red as a dye. The separating gel was 1 mm thick 14% (w/v) acrylamide slab gel. The electrophoresis was carried out at 100 V until the samples were in the gel and after that 140 V until the dye was in the lower end of the gel. The gels were stained with Coomassie brilliant blue according to Fairbanks et al. (1971).

RESULTS
Purification and structural proteins of PR4

When PR4 was purified after PEG concentration in a 10 to 40% sucrose gradient, two separate bands were always obtained. Both bands contained protein, but infectivity and DNA were mainly associated with the fatter sedimenting band A (Fig. 1). The results were identical in phosphate buffer, as well as in the TGN medium used by Sands (1976). After pelleting, when these bands were separately re-centrifuged in a similar gradient, both
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Fig. 1. PEG-concentrated phage purified in a 10 to 40% sucrose gradient using Spinco SW 50.1 rotor at 84,000 g for 50 min. After fractionation the gradients were assayed for protein concentration (●---●), DNA concentration (○---○) and infectivity (▲---▲) as described in the Methods.

Fig. 2. Coomassie brilliant blue stained discontinuous SDS polyacrylamide gel (14%) showing proteins from the infectious band A (slot 1) and the uninfectious band B (slot 2). Arrows at the left indicate the positions of the mol.wt. standards: β-galactosidase (mol.wt. 130,000); bovine serum albumin (mol.wt. 68,000); γ-globulin, heavy chain (mol.wt. 50,000); ovalbumin (mol.wt. 43,000); creatine kinase (mol.wt. 40,000); γ-globulin, light chain (mol.wt. 23,000); trypsinogen (mol.wt. 24,000) and lysozyme (mol.wt. 14,300).

Formed only a single protein peak at the original position. The faster sedimenting band A contained about $1 \times 10^{12}$ p.f.u./mg protein and band B about $5 \times 10^7$ p.f.u./mg protein. Thus band A contained the infective virus and band B defective particles. The DNA content of the material in band B was less than one-tenth of the band A material. The protein composition of the separately pelleted material from band A and B was analysed using 14% discontinuous SDS-polyacrylamide gel electrophoresis. No differences in the major proteins were detected between A and B band material (Fig. 2) after Coomassie brilliant blue staining. However, some minor changes may exist for the bands migrating in the region of the 23K and 14.3K standards. Using this gel system at least fifteen proteins with mol.wt. ranging from 12,000 to 68,000 were found. The major structural protein had a mol.wt. of 39,000.
Morphology of the purified PR4

The purified virus was examined electron microscopically using thin sectioning, negative staining and freeze-fracturing techniques. Fig. 3(a) shows sectioned material from the infective band A. With this technique the overall diam. of the virus was 60 to 70 nm and that of the electron dense core, probably formed by nucleic acid, was 35 to 45 nm. Between the core and the outer layer of the virus lies an electron translucent area. Negative staining of this material (Fig. 3b, c) revealed particles of 55 to 65 nm diam. in good agreement with
previous reports (Bradley & Rutherford, 1975). Some intact particles seemed to have a tail 120 to 130 nm in length (Fig. 3 c). The frequency of tail appearance varied from one preparation to another perhaps indicating a fragile structure of the tail. The same phenomenon was also described by Bradley & Rutherford (1975) in unpurified phage preparations. The phage head appeared somewhat elongated perpendicular to the tail-like structure. This shape is already apparent in Fig. 3 (a).

A section of the material from the non-infective band B containing a low amount of DNA is seen in Fig. 3 (d). This fraction contained particles of the size of intact phages, but mostly lacking the electron dense cores. The most interesting feature of these particles was the bilayer structure seen just inside the capsid (arrowheads in Fig. 3 d). This layer had the appearance and the thickness (7 to 8 nm) of a unit membrane (Salton, 1964). The bilayer usually followed the capsid wall closely, but in some particles the bilayer was folded inside (arrowhead a in Fig. 3 d). Negative staining of the band B material also showed empty particles with a double wall layer (Fig. 3 e). Short (30 to 50 nm) hollow tail structures were seen connected to some particles. This tail structure penetrated the outer capsid and was apparently associated with the inner (lipid) layer of the phage (arrowhead in Fig. 3 e). The empty particles were similar to the chloroform-treated, altered PR3 particles (Bradley & Rutherford, 1975).

The freeze-fracturing image of the infective purified phage PR4 is seen in Fig. 4 (a) to (d). The fracturing took place either over the phage and revealed the fractured surface (FF) of the capsids or through an internal fracture plane exposing both the concave (FF) and convex (FF) fracture faces of the phage. The fractured surfaces of the phage are pointed out by arrowheads and a hexagonal outline of the capsid is seen (Fig. 4 a). The surface of the phage seemed to be smooth without any particles and the size of the head was about 70 nm. The FF and FF of the phage in Fig. 4 (b) to (d) demonstrate the presence of a fracture plane inside the phage. The FF appears smooth (Fig. 4 b, c) while the FF contains particles 8 to 10 nm in diam. (Fig. 4 b, d).

Infection

Samples for thin sectioning were taken 4, 8, 15, 45, 60, 80 and 120 min post-infection (p.i.). The bacteria were infected with m.o.i. of about 20 using the material from the purified infective band. Similar results were obtained with all the hosts used in this study. The micrographs shown, however, were all taken from infections of Escherichia coli CR 34
Fig. 5. Thin sections of *Escherichia coli* CR 34 (RP1) infected with PR4. (a) 4 min p.i., (b) 8 min p.i., (c) and (d) 15 min p.i.

(RP1). At 4 min p.i. (Fig. 5a) phages were seen attached to the cell wall. Most of the particles appeared intact and the capsids were located at about 10 nm from the cell wall (arrowheads a in Fig. 5a). The first particles with contracted inner parts were seen at this stage of infection (arrowhead b in Fig. 5a). This preparation also contained some empty capsids both free and in contact with cells (not shown). Particles with contracted inner parts were commonly observed in preparations fixed 8 min p.i. (Fig. 5b). The capsids of these particles were in close contact with the cell wall, and the bilayer structure seen in empty non-infective particles (Fig. 3d) was no longer distinguishable. Instead a heavily stained area appeared inside these particles. The relative number of empty particles in contact with cells increased during infection. At 4 min p.i. about 20% of the particles were empty and at 8 min p.i. about 50%. At 15 min p.i. most attached phage particles were empty although some intact particles
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Fig. 6. Maturation of PR4 with Escherichia coli CR 34 (RP1) as the host. (a) 60 min p.i. (b) A higher magnification of (a). (c) 120 min p.i.
and ones with contracted inner parts were also found (Fig. 5 c, d). Clearly single layered empty capsids (arrowhead a in Fig. 5 d) as well as capsids with a thick wall (arrowhead b in Fig. 5 d) were seen in contact with cells.

**Maturation**

At 45 min p.i. intracellular empty particles were observed in the nuclear region of the cells. The first mature particles also appeared in the cell periphery at this stage of infection (not shown). At 60 min p.i. the empty particles were clearly visible almost entirely in the nuclear area (Fig. 6 a) and the number of mature particles in the cell periphery was increased. The empty capsids can clearly be seen in the larger magnification of a 60 min p.i. fixed cell (Fig. 6 b). As far as can be concluded from the empty intracellular particles, the thick wall indicates that they contain the bilayer shown in Fig. 3 (d). The mature particles in the cell periphery were seldom in contact with the bacterial cytoplasmic membrane. The maximal number of mature particles counted in about 60 nm thick sections of the cells was from 20 to 40. The first lysed cells were also seen at this time. At 80 min p.i. the intracellular particles described above appeared although most of the cells had lysed (not shown). Phages being liberated often appeared inside single membrane vesicles as shown in the preparation fixed at 120 min p.i. (Fig. 6 c).

One step growth experiments with *Escherichia coli* CR 34 (RP1) gave a burst size of about 100 when the results were corrected for free phages (Adams, 1959) and a latent period of 80 to 90 min as previously reported (Sands, 1976).

**DISCUSSION**

The major difference between material obtained after purification into band A (infectious phage) or band B (empty capsids) appeared to be the lack of DNA in the slower sedimenting band B material. This indicates that the electron dense core (Fig. 3 a) probably consists of the nucleic acid of the virus. None of the major structural proteins was missing in the empty particles. The protein composition obtained in SDS gels differs somewhat from that published previously (Cadden & Sands, 1977), when only six proteins were observed. This could be at least partly due to the discontinuous gel system we used having a greater resolving power. Protein 2 (Cadden & Sands, 1977), the major protein, appears to be identical to our 39,000 mol.wt. protein and is probably the capsid protein of the virus. Even if the minor bands in the gels are included, the total mol.wt. of the proteins is within the coding capacity of the virus.

The electron translucent layer in sectioned phages (Fig. 3 a) and the bilayer structure seen in empty capsids (Fig. 3 d) suggests that the virus has an outer (protein) capsid in close contact with an inner lipid bilayer. This is in agreement with previous results (Bradley & Rutherford, 1975) where the inner layer was found to be chloroform-sensitive. The phage DNA seems to lie inside the lipid membrane in close contact with it. The fracturing plane inside the virus (Fig. 4 a) corresponds well to the location of the bilayer structure seen in Fig. 3 (d). The smooth appearance of the convex (FF) and the rough appearance of the concave (FF) fracture faces are structurally similar to those found in the envelope of bacteriophage φ6 (Bamford & Lounatmaa, 1978).

A 120 to 130 nm long tail was seen in some apparently intact phages (Fig. 3 c). When the infective process was studied, the phage capsids were very close to the cell wall and were at no time 120 to 130 nm away from it. In band B material some particles also had a short hollow tail (Fig. 3 e) which was in contact with the lipid layer of the phage. The different appearance of the tails in the intact and empty particles may reflect changes in the tail structure taking place during injection. The changes of the frequency of tail appearance
from one preparation to another is apparently not due to the purification process, because the same phenomenon was described with unpurified phage (Bradley & Rutherford, 1975) and because no noteworthy loss of infectivity was detected during purification. We cannot, however, completely rule out the possibility that the tailed phages in Fig. 3(c) are contaminants, although this phage stock was plaque purified also in our laboratory and the phage was propagated in Escherichia coli CR 34 (RP1).

The burst size determined from the one-step growth curve was about 100. The maximum value of 20 to 40 mature phage particles counted in many cell sections taken late in infection is not in contradiction with a burst size of about one hundred.

Intact or apparently intact particles were in contact with cells early in infection (Fig. 5a). However, the capsids of these particles were about 10 nm away from the cell wall and this may reflect a reversible step in the beginning of infection. Later, closer contacts between the phage capsids and the cell wall were seen (Fig. 5b). During the infection process phages with contracted inner parts appear. The frequency of these particles was highest at 8 min p.i. and they contained a heavily staining area while no bilayer structure was observed. Using this osmium tetroxide fixation technique the lipids are known to stain very heavily (Hayat, 1970) and this would support the idea that the strongly stained area inside the virion contains lipids. From these results it would appear that the phage recognizes the cell wall receptor and DNA injection follows. The lipid layer would be continuously in contact with the DNA and also at least partly pulled out by it. Because the membrane of PR4 obviously lies inside the protein capsid, a membrane fusion such as one taking place with bacteriophage φ6 (Bamford et al. 1976) cannot occur. However, the lipids of this phage may still interact with the cell envelope possibly by means of the tail, which seems to be in contact with the phage lipid layer. The observation that certain unsaturated fatty acids inhibited the virus infection (Reinhardt et al. 1978) supports the idea that the virus lipids play an important role in infection. The first reversible step of infection was not affected (Reinhardt et al. 1978), but the fatty acids might prevent the formation of particles with contracted inner parts.

During maturation two kinds of particles were seen. At 45 min p.i. empty particles appeared in the nuclear region of the cell. Later dark intact-looking phages accumulated in the cell periphery. The empty particles had a rather thick wall structure, which could indicate that they already contained the lipid layer. The maturation of PR4 differs from that of φ6, where nucleic acid containing capsids appear about halfway through the life cycle and the membrane is formed around this nucleocapsid during the latter half of the life cycle (Bamford et al. 1976). Cell lysis was first seen at 60 min p.i. In spite of careful attempts to find bursting cells we could not observe any at either 80 or 120 min p.i. Instead, large single membrane vesicles containing intact phages were commonly seen.

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REFERENCES


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