Comparison of the Lipid-containing Bacteriophages PRD1, PR3, PR4, PR5 and L17

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SUMMARY

Five broad host range lipid-containing bacteriophages PRD1, PR3, PR4, PR5 and L17 isolated from different parts of the world were compared using biological and structural criteria. Virus morphology as well as genome sizes appeared to be identical. However, these viruses could be distinguished by restriction endonuclease mapping and by their structural protein patterns in SDS–gel electrophoresis. The viruses studied thus form a very close group of lipid-containing bacteriophages. We suggest PRD1 as a model organism for this group and that the group be called the PRD1 phage group.

INTRODUCTION

Two lipid-containing bacterial viruses PM2 and φ6 (for reviews, see Franklin, 1978; Mindich, 1978) have been studied in recent years as models for biological membranes. These investigations have been hampered by the lack of detailed knowledge on the molecular biology and genetics of the hosts, a marine pseudomonad and a plant pathogenic pseudomonad respectively. The plasmid-dependent broad host range bacteriophages PR4 (Stanisich, 1974; Bradley & Rutherford, 1975) and PR5 (Wong & Bryan, 1978) can replicate in Escherichia coli and Salmonella typhimurium. Since these phages contain lipids (Sands & Cadden, 1975; Wong & Bryan, 1978) obviously arranged as bilayer membranes (W. C. Earnshaw, personal communication) they are potential model systems for studying membrane biosynthesis and organization. Some other phages isolated from different parts of the world appear similar to PR4 and PR5. These are PRD1 (Olsen et al., 1974), PR3 (Bradley & Rutherford, 1975), L17 (N. Seeley, personal communication) and PR772 (Coetzee et al., 1979). In this study, certain aspects of the structure and biological characters of five of these phages are compared to clarify their relatedness, as well as to choose the most suitable member for further studies. The results show that the five phages are closely related. The obvious sixth member of this group, PR772 (Coetzee et al., 1979), was not available when this study was initiated.

METHODS

Phages. Bacteriophage PRD1 was kindly provided by Dr R. H. Olsen. It was originally isolated from Kalamazoo (Michigan, U.S.A.) sewage (Olsen et al., 1974). Bacteriophages PR3 and PR4 were isolated from sewage from different locations in Melbourne, Australia (Stanisich, 1974) and were kindly provided by Dr D. E. Bradley. Phage PR5 (Wong & Bryan, 1978) was isolated from sewage in Edmonton, Canada. Previously this phage was called P-RP4 (Shahrabadi et al., 1975). The phage was generously provided by Dr D. E. Bryan. Phage L17 (kindly provided by Dr N. Seeley) was isolated from the river Avon in

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England using a mixed indicator host comprising *Aeromonas hydrophila* (RP1) and *E. coli W3110* (RP1) (N. Seeley, personal communication). All the phages were purified by two successive single plaque isolations before analysis.

*Bacteria.* *S. typhimurium LT2* (RP1) originated from Dr R. H. Olsen's laboratory (Olsen et al., 1974). *Pseudomonas aeruginosa PAO 2605* (RP1), *E. coli CR34* (RP1) and *E. coli J53-1* (Sa) were from Dr D. E. Bradley's laboratory (Bradley & Rutherford, 1975).

**One-step growth curve and efficiency of plating.** *S. typhimurium LT2* (RP1) was grown to about $1 \times 10^9$ cells/ml in F-broth (15 g Bacto-peptone, 8 g Bacto-tryptone, 8 g NaCl and 1 g glucose per litre) at 37 °C. Phages were added to obtain an m.o.i. of about 5. After 5 min adsorption time the cells were centrifuged and resuspended in fresh broth to remove unadsorbed phages. The amount of unadsorbed phage was measured from the sample supernatants before lysis and found to be less than 1% of the input infectivity and thus was ignored.

For measurements of plating efficiency each phage was grown on *S. typhimurium LT2* (RP1), *P. aeruginosa PAO 2605* (RP1) and *E. coli J53-1* (Sa) using a method where the soft agar layer from a semi-confluently lysed TB plate (10 g Bacto-tryptone, 8 g NaCl and 15 g agar per litre) was transferred to 10 ml F-broth and grown with aeration for about 3 h. After this the debris was removed by low-speed centrifugation and the virus stock stored at 4 °C. With this method the cells were infected with high multiplicity. All these phage stocks were plaque assayed using the hosts *S. typhimurium LT2* (RP1), *P. aeruginosa PAO 2605* (RP1), *E. coli CR34* (RP1) and *E. coli J53-1* (Sa).

**Growth and purification of the phage.** The phages were grown on *S. typhimurium LT2* (RP1) or on *P. aeruginosa PAO 2605* (RP1) in F-broth containing 20 μg neomycin/ml at 37 °C. At a cell density of about $1 \times 10^9$, cells were infected with the phage using an m.o.i. between 5 and 10. After infection the phage were allowed to adsorb without shaking for 5 min and incubation was continued with aeration until lysis occurred. Cell debris was removed by low-speed centrifugation and the phage in the supernatant was concentrated by polyethylene glycol 6000 (PEG) precipitation as described by Yamamoto et al. (1970). The phage was purified by centrifugation in a linear 5 to 20% (w/w) sucrose gradient made in 10 mM-phosphate pH 7.2, 1 mM-Mg$^{2+}$ (phage buffer) using a Sorvall AH 627 rotor at 64 400 g (average) for 90 min at 4 °C. The virus material was transferred on to the top of a 37 to 45% (w/w) linear sucrose gradient and run to equilibrium in the same rotor. Running conditions were 76 600 g (average) for 20 h at 4 °C. The virus band was collected and diluted 1:4 with phage buffer and the phage pelleted in the AH 627 rotor at 76 600 g (average) for 2 h at 4 °C. The pellet was resuspended in the desired volume of phage buffer.

To measure the amount of top component produced by different phages, the infectivity of the (PEG) concentrated phages were measured and about $3 \times 10^{12}$ plaque-forming units (p.f.u.) of each phage were layered on to the top of a 5 to 20% (w/w) sucrose gradient made in phage buffer. The gradients were centrifuged in a Spincot SW50.1 rotor at 84 000 g (average) for 37 min at 4 °C. After the run the u.v. absorption at 280 nm was measured in a Uvicord S u.v. monitor and fractions were collected. The fractions were then assayed for infectivity, protein (Lowry et al., 1951) and in some cases, analysed by SDS–polyacrylamide gel electrophoresis. For buoyant density determinations, purified virus material was layered on to the top of a 37 to 45% (w/w) linear sucrose gradient and centrifuged in an SW50.1 rotor at 149 000 g (average) for 18 h at 4 °C. After the run the gradients were pumped through the u.v. monitor operating at A$_{280}$ and fractionated. The infectivity and refractive index of each fraction were measured.

**Characterization of the genomes.** The nucleic acid was isolated from the phages by phenol extraction in 1 × SSC (0·15 m-NaCl, 0·015 m-sodium citrate) after digestion with Pronase in the presence of 0·2% SDS essentially as described by Olsen et al. (1974). In later
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In experiments a better yield was obtained when the phages were incubated in 2% SDS at 50°C for 30 min prior to the phenol extraction. The water-phase was extracted three times with ether and the nucleic acid precipitated with ethanol, washed twice with 70% ethanol and finally resuspended in 10 mM-tris–HCl pH 7.4, 10 mM-NaCl. The A_{260}/A_{280} ratio of the preparations was 1.7 to 1.8.

Agarose gel electrophoresis was performed in horizontal gels (0.5 to 1.0%) in buffer E (Sharp et al., 1973). Molecular weights were calculated from mol. wt.\(^{3/2}\) versus mobility plots (Bearden, 1979) using the EcoRI, HindIII and EcoRI + HindIII fragments of phage λ DNA as standards (Szybalski & Szybalski, 1979). When DNA was cut with restriction enzymes giving a large number of fragments, the electrophoresis was performed in 3.5 to 10% acrylamide gradient gels as described by Jeppesen (1974). Restriction enzymes were purchased from New England Biolabs, Beverly, Mass., U.S.A. (AluI, BglII, HaeIII, Hhal, KpnI, MspI, PstI, XhoI and XmaI), from Boehringer-Mannheim (BamHI, HindIII, SalI and SmaI) and from Sigma (EcoRI). The digestions were done as recommended by the manufacturers. Pancreatic deoxyribonuclease (DNase I, EC 3.1.4.5, Sigma) and pancreatic ribonuclease (RNase, EC 3.1.4.22, Worthington) were used in 10 mM-tris–HCl pH 7.4, 100 mM-NaCl and 10 mM-MgCl\(_2\).

Structural proteins. The protein patterns of the purified viruses were studied using a discontinuous SDS–gel system which was essentially that described by Laemmli (1970), but with the following modifications. The separation gel had a pH of 8.9. The stacking gel (pH 7.8) contained 5% acrylamide, 62.5 mM-tris, 56 mM-sodium phosphate and 0.1% SDS. The electrode buffer (pH 8.6) contained 29 mM-tris, 220 mM-glycine and 0.1% SDS. The samples (pH 7.8) contained 1% SDS, 1% β-mercaptoethanol, 20 mM-tris, 8 mM-sodium phosphate and 9% glycerol. Bromophenol blue was used as the tracking dye. Proteins were solubilized by incubating for 5 min in a boiling water bath. The gels were stained with a solution containing 0.04% Coomassie Brilliant Blue, 25% isopropanol and 10% acetic acid and destained with 10% acetic acid.

Electron microscopy. Negative staining of the specimen was done on the grid with 1% uranyl acetate. Sectioned material was prepared as described previously (Bamford & Mindich, 1980). The micrographs were taken with a JEM-100B electron microscope.

RESULTS

One-step growth experiment

The one-step growth experiment of phages PRD1, PR3, PR4, PR5 and L17 using S. typhimurium LT2 (RP1) as the host is shown in Fig. 1. For all the phages the latent period was about 50 min and about 500 infective particles were released per cell except for phage PR3 which produced less than one tenth of the infective particles produced by the other viruses. In each experiment the cells lysed upon phage production, as indicated by the turbidity curve (dotted line in Fig. 1).

Efficiency of plating

Phages grown in S. typhimurium LT2 (RP1) (LT2 RP1), P. aeruginosa PAO 2605 (RP1) (PAO RP1) and E. coli 153-1 (Sa) (J53-1 Sa) were titred on all these hosts as well as on E. coli CR34 (RP1) (CR34 RP1). The results are shown in Table 1. The strain LT2 RP1 had the highest efficiency regardless of which host the phage was grown on. PAO RP1 was almost as good a host. Phage PR3 plated several orders of magnitude less efficiently when titred on CR34 RP1. The rest of the phages showed only slightly reduced efficiency when titred on this host. These results demonstrate that phage DNAs are not restricted when grown
in hosts from different genera. This was true regardless of the plasmid used, RP1 or Sa belonging to compatibility groups P and W respectively.

**Top component production**

Following rate zonal centrifugation all the viruses showed two u.v.-absorbing bands. The infectivity was associated with the faster sedimenting one while the slower band, the top component, obviously corresponded to the empty capsids previously described for PR4 (Lundström et al., 1979).

To measure the amount of the top component produced by each virus, a given amount of PEG-precipitated infectious phage was centrifuged in a 5 to 20% (w/w) linear sucrose gradient. The $A_{260}$ profile of the gradients, as well as the infectivity and protein content of the gradient fractions were determined (Fig. 2). Table 2 shows the results of two independent experiments where the amount of protein in the different fractions was compared. Phages

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**Table 1. Plating efficiency of phages grown on different hosts**

<table>
<thead>
<tr>
<th>Grown on</th>
<th>Plated on</th>
<th>PRD1</th>
<th>PR3</th>
<th>PR4</th>
<th>PR5</th>
<th>L17</th>
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<td>LT2 RP1</td>
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<td>$5.2 \times 10^{10}$</td>
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<tr>
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<td>$9.0 \times 10^{9}$</td>
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<td>$1.8 \times 10^{11}$</td>
<td>$2.5 \times 10^{10}$</td>
<td>$1.5 \times 10^{11}$</td>
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</table>

* Hosts used: S. typhimurium LT2 (RP1) referred to as LT2 RP1; P. aeruginosa PAO 2605 (RP1) referred to as PAO RP1; E. coli CR 34 (RP1) referred to as CR34 RP1; E. coli J 53-1 (Sa) referred to as J53-1 Sa.
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Fig. 2. The u.v. absorption profile of rate zonal centrifugation of about $3 \times 10^{12}$ p.f.u. of PRD1 (solid line) and PR3 (dotted line) bacteriophages. The linear 5 to 20% (w/w) sucrose gradient was centrifuged at 84,000 g for 37 min at 4°C in a Spinco SW50.1 rotor. The histogram shows the infectivity of the gradient fractions (open columns PRD1, shadowed columns PR3). Sedimentation is from left to right.

Fig. 3. Isopycnic centrifugation of PRD1 bacteriophage in a linear 37 to 45% (w/w) sucrose gradient, centrifuged at 149,000 g for 18 h at 4°C in the SW50.1 rotor. The histogram represents the infectivity of the gradient fractions.

Table 2. The distribution of protein* in infective virus and top component

<table>
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<th>Phage</th>
<th>Infective material</th>
<th>Top component</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1 2†</td>
<td>1 2</td>
</tr>
<tr>
<td>PRD1</td>
<td>66 72</td>
<td>34 28</td>
</tr>
<tr>
<td>PR3</td>
<td>27 35</td>
<td>73 65</td>
</tr>
<tr>
<td>PR4</td>
<td>73 80</td>
<td>27 20</td>
</tr>
<tr>
<td>PR5</td>
<td>73 78</td>
<td>27 22</td>
</tr>
<tr>
<td>L17</td>
<td>69 72</td>
<td>31 28</td>
</tr>
</tbody>
</table>

* Percentage of the total virus protein (Lowry et al., 1951) in the gradient.
† The columns (1, 2) give the results of two independent experiments.

PRD1, PR4, PR5 and L17 produce about three times more virus than top component, but for PR3 the virus to top component ratio was inverted. The infectivity of all the viruses was found solely in the faster sedimenting peak (Fig. 2).
Fig. 4. Comparison of the HaeIII restriction endonuclease patterns of the phage genomes. The isolated phage DNAs were digested to completion with fourfold over-digestion. The fragments were analysed in a 3.5 to 10% acrylamide gradient gel and stained with ethidium bromide. Lane 1, PR4; lane 2, PRD1; lane 3, PR3; lane 4, PR5; lane 5, L17; lane 6, plasmid pBR 322 HaeIII fragments as markers.

Electron microscopy of infected cells

Thin sectioning of infected cells showed identical intracellular particle morphology and location for all the phages (not shown). A virus diam. of about 65 nm was measured. Filled particles appeared more often in the cell periphery, whereas the empty ones were found mostly in the nucleic acid region. The size and location of intracellular particles are similar to those previously reported for PR4 (Lundström et al., 1979). Only PR3 differs from the others in sections. When the number of filled and empty particles in sectioned cells was counted 50 min or later post-infection, PR3-infected cells contained about 22% filled particles and about 78% empty ones. The rest of the viruses showed between 70 to 82% filled particles and 18 to 30% empty ones respectively. This figure is very close to the amount of top components found following rate zonal centrifugation (Table 2 and Fig. 2).

Buoyant density in sucrose

The infectious material and the top component were separated after rate zonal centrifugation and were centrifuged individually in an isopycnic sucrose gradient ranging from 37 to 45% (w/w). The viruses formed a single peak with a shoulder. In all cases the peak had a density between 1.204 to 1.211 g/ml and the shoulder 1.210 to 1.220 g/ml. The specific infectivity compared to the u.v. absorption in the peak and in the shoulder was the same. Fig. 3 shows the isopycnic gradient profile for phage PRD1. For other viruses the
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fig. 5. Polyacrylamide gel electrophoresis (14%) of purified bacteriophages grown on S. typhimurium LT2 (RP1) (a, c, e, g, i) and on P. aeruginosa FAO 2605 (RP1) (b, d, f, h, j). The gel was stained with Coomassie Brilliant Blue. (a, b) PRD1; (c, d) PR3; (e, f) PR4; (g, h) PR5; (i, j) L17. The numbers on the left refer to the PRD1 protein nomenclature (see Mindich & Bamford, 1981). The arrows on the right indicate the positions of the structural proteins of φ6 bacteriophage (for mol. wt. see Mindich, 1978).

figure is essentially the same. The shoulder height, however, varies between the different viruses. The top components of each virus formed a single peak between densities 1.201 to 1.205 g/ml (not shown). In the case of phage PR3, isopycnic centrifugation of the virus yielded material at the position of the top component, indicating that the PR3 particles are not as stable as those of the other viruses.

Virion morphology

Uranyl acetate staining of the virus particles and the corresponding top components made clear that the dimensions (60 to 65 nm diam.) and the morphology in all cases are similar, thus the distinction between these viruses cannot be made by electron microscopy (not shown).

Comparison of the genomes

The genomes of the five phages were isolated by phenol extraction. The extracted nucleic acids were subjected to agarose gel electrophoresis and for all the viruses a single distinct band was seen after staining with ethidium bromide. Digestion with DNase (100 μg/ml) prior to electrophoresis led to the loss of all stainable material, while RNase (100 μg/ml) had no effect (not shown). The mobilities of the single bands, presumably representing the DNA genomes, were the same for all the five phages in 0.5, 0.6 and 0.8% agarose gels. When the apparent mol. wt. for these bands was calculated using λ DNA fragments as markers, a value of 9.8 × 10^6 to 10.2 × 10^6 was obtained regardless of the host used to prepare phage stocks. This is slightly lower than the value (10.9 × 10^6) obtained by electron microscopy of PR4 DNA (Coetzee & Bekker, 1979).

To reveal possible dissimilarities between the genomes, isolated DNAs were digested with various restriction endonucleases followed by electrophoretic analysis. Strikingly, none of the
enzymes BamHI, BglII, EcoRI, HindIII, KpnI, PstI, SalI, SmaI, XhoI and XmnI, cut PRD1 or PR4 DNA. PR3, PR5 and L17 were not tested with these enzymes. A similar 'improbable' lack of sites has been described previously for bacteriophage φ26 DNA (Ito & Roberts, 1979). The DNA of all five phages contained many sites for enzymes HaeIII, HhaI, AluI and MspI. A comparison of the fragments formed, showed that the patterns were rather similar; each of the phages contained, however, fragments which distinguished it from the other viruses as shown for HaeIII digestion in Fig. 4. Several fragments with the same mobilities for all the viruses were seen; however, the differences in the restriction enzyme patterns show that none of the five phages are identical.

Structural proteins

No contaminating vesicles were seen in negatively stained virus preparations. In order to identify possible contaminating host proteins, viruses grown in different hosts (LT2 RP1 and PAO RP1) were analysed separately. The SDS–gel electrophoresis patterns of the virion proteins are shown in Fig. 5. The patterns resemble each other and there was no difference between the materials from different hosts. Thus, all the protein bands seen were virus-specific. The proteins of phage PRD1 are discussed in more detail elsewhere (Mindich & Bamford, 1981) and the numbering in Fig. 5 (P1, 2, 9, 18) refers to its proteins. The main differences in the patterns are in the minor bands below the major protein P2. The protein nomenclature proposed by Mindich & Bamford (1981) is valid only for PRD1 until the functional similarities of corresponding proteins in other phages are determined.

DISCUSSION

This work shows that the five viruses studied are closely related. The timing of the virus production is the same for all the viruses. Also the burst sizes are the same in all but PR3, which gave less than one tenth the virus yield per cell compared to the other viruses. One reason for low yield of infective particles in the case of PR3 might be in the inefficiency of the DNA packaging (Fig. 2, Table 2). Also, the infective particles are somewhat unstable in equilibrium centrifugation. In sectioned cells and following uranyl acetate staining of the purified viruses all the phages looked similar under the electron microscope.

The buoyant density profiles of all the viruses were similar. All had a shoulder slightly more dense than the main peak. The relative height of this shoulder, however, varied typically for each virus. The densities of the top components were essentially the same for all five phages.

The apparent mol. wt. of the genomes for the different viruses as determined by agarose gel electrophoresis appear to be the same. The calculated mol. wt. is about $10 \times 10^6$, which is close to that reported for PR4 using electron microscopic length determinations (Coetzee & Bekker, 1979). A preliminary restriction enzyme map for PRD1 suggested a slightly lower value for the genome mol. wt. (L. Mindich, personal communication). The size class of the genome fits well with the estimated genetic capacity (Mindich & Bamford, 1981).

It is striking that the genomes are resistant to all the restriction enzymes with six nucleotide recognition sequences which were tested. Since some bases are in recognition sequences of enzymes that both do cut and do not cut, it implies that the bases are not modified. It might be that certain sequences are selected against, due to the broad host range of these viruses. This in turn would also explain why no restriction modification system was detected when the phages were grown and plated on different hosts (Table 1).

The viruses included in this study were isolated in the United States, Australia, Canada and England by different workers, thus representing truly different virus isolates. The biological properties, genome sizes and morphology of these isolates were so similar, however, that no distinction could be done on the basis of these characters, except for phage PR3 which produces large amounts of empty particles. The distinction could be made, however, by
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restion enzyme mapping of the genomes and by the structural protein patterns. While these patterns generally resemble one another, there are sufficient differences within them to distinguish the different phages.

Wong & Bryan (1978) showed that phages PR3, PR4 and PR5 are serologically related so that PR5 is more closely related to PR4 than to PR3. We studied here the relationship of phages PRD1, PR3, PR4, PR5 and LI7. Phage PR772 from South Africa has a similar morphology and genome size (Coetzee & Bekker, 1979; Coetzee et al., 1979). The temperature denaturation map of PR772 DNA was very similar to that of PR4 (Coetzee & Bekker, 1979) allowing us to include this isolate in the phage group defined here. We use PRD1 as the model organism for this virus group and suggest that the group should be called the PRD1 phage group. The International Committee on Taxonomy of Viruses has given the name Tectiviridae for viruses resembling PRD1. Phages infecting Gram-positive hosts also belong to this phage group.

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