Presence of Lipids in Mycobacteriophage 13

By MOHAN L. GOPE AND K. P. GOPINATHAN*

Microbiology and Cell Biology Laboratory, Indian Institute of Science,
Bangalore 560012, India

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SUMMARY

The presence of lipids has been demonstrated in mycobacteriophage 13. The total lipid was composed of 69% phospholipids and 31% neutral lipids. More than two-thirds of phospholipids present in the phage were synthesized in the host prior to infection. The fatty acid composition of the phage differed markedly from that of its host, both in chain length and the degree of saturation. The phage lipid was mostly composed of saturated fatty acids of which more than 50% were short chain fatty acids. Changes in growth temperatures reflected variations in fatty acid composition, characteristic of the phage, and which were distinctly different from those of the host. Electron microscopic observations revealed that the phage has a membranous bilayer structure. The presence of lipids may facilitate the phage–host interaction especially in lipid-rich organisms like mycobacteria.

INTRODUCTION

The sensitivity of bacteriophages to chloroform or other organic solvents is indicative of the presence of lipids in them. Most of the mycobacteriophages are highly sensitive to solvents such as chloroform, methanol and ether. Bowman et al. (1973) have reported the sensitivity of mycobacteriophages Leo, D29, R1 and DS6A. Furthermore, Sellers & Tokunaga (1970) have reported that phages D4, D32, D29 and D28 are sensitive to butanol, chloroform, ether, methanol and benzene, in that order. However, Schafer et al. (1977) failed to find any evidence for the presence of lipids in their preparation of D29. On the other hand, firm evidence for the presence of lipids in phage R1 has been provided recently (Soloff et al., 1978).

Structural damage or the loss of infectivity to a phage caused by organic solvents cannot be taken as definite evidence for the presence of lipids because inactivation or damage can also result from the denaturation of proteins, as suggested for phage pf 1 (Amako & Yasunaka, 1977). Even when lipids are directly demonstrated the purity of phage preparation is often suspect and their presence is attributed to contamination with host lipids. In this communication, we present definite evidence for the presence of lipids in mycobacteriophage 13. These lipids appear to be essential for maintaining the structural integrity and infectivity of the phage.

METHODS

Bacteria and phage. The host strain was Mycobacterium smegmatis SN2. The phage used was a clear-plaque mutant of the transducing mycobacteriophage 13. The growth of host and phage were carried out as described previously (Karnik & Gopinathan, 1980; Nagaraja & Gopinathan, 1980). For the preparation of large quantities of phage, 1 to 2 l batches of host cells, grown in synthetic medium (Karnik & Gopinathan, 1980) to early exponential phase,
were infected with phage I3 at an m.o.i. of 3 in the presence of 1 mM-CaCl₂. Incubation was continued for 14 h and the clarified lysates were treated with 10% polyethylene glycol 6000 (PEG) and 0.5 M-NaCl. The samples were left for 24 h at 4 °C and centrifuged at 10 000 g in the cold for 10 min. The pellet was suspended in 15 ml 10 x SSC (1.5 M-NaCl, 0.15 M-sodium citrate) containing 10 mM-tris-HCl pH 7.2. The phage was purified by centrifugation through a series of different gradients. First, the phage was sedimented through 15% (w/v) sucrose in 10 mM-tris-HCl pH 7.2 on to a cushion of 60% (w/v) sucrose. This band was collected and centrifuged through a linear gradient of 15 to 60% (w/v) sucrose (see Fig. 1 for details). The peak fractions containing infective particles were pooled, dialysed and finally purified through a CsCl step gradient consisting of seven steps ranging in density from 1.3 to 1.6 g/ml. Centrifugation was carried out at 40000 rev/min for 6 h at 16 °C in a Beckman SW50 rotor. The narrow sharp band of the phage was collected and dialysed against SSC containing 10 mM-tris-HCl pH 7.2. During purification, no significant inactivation of phage I3 was observed. ³²P-labelled phage was prepared as described previously (Karnik & Gopinathan, 1980). Phosphates were completely omitted from the growth medium and, instead, 0.5 μCi/ml ³²P (carrier-free, BARC, Bombay, India) was added. The labelled phage was purified on sucrose and CsCl gradients.

Organic solvent treatment of the phage. The phage lysates (1 × 10⁹ p.f.u./ml) were treated with organic solvents such as chloroform, ether, methanol, butanol and benzene independently or in combination (1:1) at a concentration of 33% (v/v) at 37 °C for 45 min. After treatment, the samples were centrifuged at 4 °C to obtain phase separation, and the organic phase was discarded. The samples were spread in Petri plates to evaporate the remaining traces of solvents. Whenever phase separation was not possible, the whole mixture was placed in Petri plates and the solvent allowed to evaporate inside a sterile hood (Sellers & Tokunaga, 1970).

Lipid extraction and estimation. The phage preparations purified in CsCl were used for lipid extraction according to the method of Bligh & Dyer (1959). A part of the lipid fraction was spotted on thin-layer chromatographic (t.l.c.) plates for the separation of phospholipids and neutral lipids. The remaining portion was used for the estimation of total lipids. Fatty acids were determined as the copper soaps by the method of Duncome (1963). Triglycerides were determined after digesting them to free fatty acids with 2% alcoholic KOH for 45 min at 65 °C and neutralization with 1 M-HCl. Control and standard triglycerides were given the same treatment. The fatty acids obtained after triglyceride digestion were estimated according to the method of Duncome (1963). Lipid phosphorus was estimated by a modified method of Bartlett (1959) as described by Marinetti (1962).

Total lipid, DNA and protein estimation. Total lipid was estimated according to the method of Bragdon (1951), DNA was estimated according to Burton (1968) and protein was estimated by the method of Lowry et al. (1951).

Phospholipid synthesis. The observation that phage I3 has a very high phospholipid content led us to investigate the extent to which various phospholipids that become a part of phage particles are synthesized in the host cell prior to, or after, infection. For this, ³²P-labelled phage stocks were prepared according to the following labelling protocols. (i) The host cells were grown in the presence of 0.5 μCi [³²P]phosphate/ml for 14 h, centrifuged and resuspended in fresh medium containing non-radioactive phosphates. The cells were infected with phage I3 and incubation was continued for 14 h. (ii) Host cells were grown in the presence of non-radioactive phosphate for 14 h. These cells were centrifuged and resuspended in fresh medium containing [³²P]phosphate. The cells were immediately infected with phage and the lysates were prepared after 14 h. As a control, phage grown in the presence of [³²P] throughout were used.

Influence of growth temperature on the fatty acid composition of phage lipids. The host
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and phage were grown under different temperature regimes in the following way: (i) 40 °C for both host growth and phage growth after infection; (ii) 37 °C for both host growth and phage growth after infection; (iii) 18 °C for both host growth and phage growth after infection; (iv) 40 °C for host growth and 18 °C for phage growth after infection. Since temperature affected the growth rates of the host and the phage, appropriate compensations for time for phage infection and duration of phage growth were incorporated. The phage was purified and lipid was extracted as described earlier. The lipid was hydrolysed with 0.5 M-KOH in 50% alcohol at 60 °C for 2 h. The free fatty acids were re-extracted with ether. The fatty acid methyl esters were prepared by dropwise addition of diazomethane in ether (prepared by addition of 3 M-KOH to 2,2-dimethylnitrosourea in the cold and trapping the liberated diazomethane in ether) until a persistent yellow colour was obtained. The sample was concentrated prior to loading for gas–liquid chromatographic analysis. Similarly, the host cells were washed extensively in tris–HCl buffer and processed for extraction of lipids, methyl ester formation of fatty acids and gas–liquid chromatography in the same manner.

Separation of fatty acid methyl esters. The methyl esters were separated by using a gas–liquid chromatograph equipped with a flame ionization detector and recorder. The chromatography was carried out on a 6 foot glass column packed with 6% PEG adipate and nitrogen as the carrier gas [15 lb/in² (103.5 kPa) at 200 °C]. The peaks were identified by comparison to known standards and from the retention time. The percentage fatty acid composition was calculated by triangulation.

Electron microscopy. Phage samples for electron microscopy were prepared according to Bradley & Rutherford (1975) with some modifications. Bacteriophage particles were mounted by floating Formvar-coated grids on the surface of a suspension of purified phage or a crude lysate for 10 to 12 h. Grids were then washed and stained with 1% neutral sodium phosphotungstate or 1% alcoholic uranyl acetate. Alternatively, the phage suspension was mixed with 2% neutral sodium phosphotungstate or 2% alcoholic uranyl acetate and dried on to Formvar-coated grids.

RESULTS

Purification profile of mycobacteriophage 13

The phage preparation was extensively purified by linear sucrose gradient and CsCl density gradient centrifugations. The traces of contaminating materials arising from the host were removed at these steps (Fig. 1). While the phage viability was affected after the CsCl purification step, the addition of 2 × SSC into the dialysis buffer significantly improved the recovery.

Effect of solvent treatment

The phage lysates showed a loss in titre after treatment with various organic solvents (Table 1). Maximum inactivation of 6 log₁₀ orders was observed with butanol, followed by 3 log₁₀ inactivation with methanol. Less than 1 log₁₀ inactivation was observed with chloroform, ether and benzene. The solvents, when used in combination, were more effective in inactivating the phage than each of them independently. The effect on phage viability of varying concentrations of chloroform ranging from 10 to 60% (v/v) was studied. The chloroform effect was fairly constant over the entire range of concentrations tested, resulting in the inactivation of 50% of the particles in 45 min at 37 °C. The chloroform effect was dependent on the temperature of treatment (Fig. 2a). An increase in temperature from 37 °C to 40 °C resulted in a significant increase in its action. Furthermore, the inactivating action of chloroform could be enhanced by increasing the time of treatment with the solvent (Fig. 2b).
Fig. 1. Purification of phage 13 by sucrose gradient centrifugation. The phage band collected from the interphase of 15 and 60% sucrose was dialysed against 2× SSC and loaded on to a linear gradient of 15 to 60% (w/v) sucrose (in 10 mM-tris–HCl pH 7-2). Centrifugations were carried out for 3 h at 22000 rev/min in an SW25 rotor. Dropwise fractions were collected and the infectivity and radioactivity were determined in samples after appropriate dilutions. The inset shows the appearance of bands as seen in the centrifuge tube. The phage particles corresponded to the bands A and a. Fractions 13 to 18 (corresponding to A) were pooled, and centrifuged through the CsCl gradient, which gave a sharp band. If the trailing portions from the peaks of sucrose gradient centrifugation were not eliminated, the second band (corresponding to a) was seen in CsCl centrifugation step also. ●, 32P; ○, infective phage.

Table 1. Effect of organic solvents on phage viability

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Phage titre (p.f.u./ml)</th>
<th>Solvent</th>
<th>Phage titre (p.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no solvent)</td>
<td>1.5 × 10⁹</td>
<td>Benzene</td>
<td>8.0 × 10⁸</td>
</tr>
<tr>
<td>Butanol</td>
<td>1.3 × 10⁴</td>
<td>Chloroform + methanol</td>
<td>1.5 × 10⁵</td>
</tr>
<tr>
<td>Methanol</td>
<td>1.4 × 10⁶</td>
<td>Ether + methanol</td>
<td>1.5 × 10⁴</td>
</tr>
<tr>
<td>Chloroform</td>
<td>6.5 × 10⁸</td>
<td>Chloroform + ether</td>
<td>3.0 × 10⁸</td>
</tr>
<tr>
<td>Ether</td>
<td>6.3 × 10⁸</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The solvent concentration used was 33% (v/v), either as a single solvent or a mixture (16.5% each) of two solvents. The treatment was at 37 °C for 45 min.

Macromolecular composition of 13

Since the phage preparations showed sensitivity to the solvents, suggesting the presence of lipids, quantitative estimation of lipids in the purified virions was carried out. The DNA and protein contents of the phage were also estimated. The phage contained 42% DNA, 43% protein and 15% lipid.

Characterization of phage lipids

The phospholipid content was very high compared to that of neutral lipids: 69% phospholipid and 31% neutral lipid formed the total lipids. The neutral lipids consisted of...
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Fig. 2. Influence of time and temperature of chloroform treatment. (a) Treatment with 33\% (v/v) chloroform for 45 min at various temperatures. (b) Phage lysates were treated with 33\% (v/v) chloroform at 37 °C for the time intervals shown.

Table 2. Incorporation of $^{32}P$ into phage phospholipids*

<table>
<thead>
<tr>
<th>Labelling time</th>
<th>Percent of control</th>
<th>Percentage distribution of phage phospholipids synthesized†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before infection (−14 h to 0 h)</td>
<td>70</td>
<td>PE 30 PG 61 UI 9</td>
</tr>
<tr>
<td>After infection (0 h to 14 h)</td>
<td>30</td>
<td>PE 20 PG 63 UI 17</td>
</tr>
</tbody>
</table>

*Phage were purified through a sucrose linear gradient followed by a CsCl step gradient (see text for details).

†PE, Phosphatidylethanolamine; PG, phosphatidylglycerol; UI, unidentified.

only two species, triglycerides and free fatty acids. Free fatty acids were found to contribute 15\% and triglycerides 16\% of the total lipid.

On chromatography, phage phospholipid was separated into three distinct spots. Two spots were identified as PE (phosphatidylethanolamine) and PG (phosphatidylglycerol), and the third was unidentified.

Phospholipid synthesis

The phage particles produced by the labelling protocols described earlier were purified and then analysed for the distribution of $^{32}P$ in the phage phospholipids. These results are presented in Table 2. It is evident from the results that more than two-thirds of the phospholipids were synthesized before infection. The percentage of the different phage phospholipids synthesized during these intervals are also shown in the table. After infection, the percentage of PG remained the same whereas the percentage of PE decreased with a concomitant increase in the percentage of unidentified phospholipid.

Effect of growth temperature on fatty acid composition of phage

In order to see how a change of growth temperature would affect the fatty acid composition of the phage, the phage was propagated at different temperatures. The results are shown in Table 3. At all temperatures, the fatty acid composition of the phage differed markedly from that of its host in both chain length and the degree of saturation. The phage lipid was mostly composed of saturated fatty acids, of which more than 50\% were short chain
Fig. 3. Electron micrographs of phage 13. (a) Negatively stained with phosphotungstic acid. (b) Staining with alcoholic uranyl acetate of phage dialysed after sucrose gradient run. Inset: same before dialysis. (c) Stored phage particles stained with alcoholic uranyl acetate (arrows show membranous structures). (d) Phage particles, after chloroform treatment, stained with alcoholic uranyl acetate. Changed ultrastructural features can be seen.

Table 3. Effect of temperature on fatty acid composition of phage 13 and its host*

<table>
<thead>
<tr>
<th>Temperature of host/phage (°C)</th>
<th>Short chain FA (%)</th>
<th>Long chain FA (%)</th>
<th>Total of (1) and (2)</th>
<th>Unsaturated FA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18/18</td>
<td>60</td>
<td>30</td>
<td>90</td>
<td>9.6</td>
</tr>
<tr>
<td>37/37</td>
<td>46</td>
<td>41</td>
<td>87</td>
<td>13</td>
</tr>
<tr>
<td>40/40</td>
<td>37</td>
<td>46</td>
<td>83</td>
<td>17</td>
</tr>
<tr>
<td>40/18</td>
<td>55</td>
<td>36</td>
<td>91</td>
<td>9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fatty acid composition of host lipids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short chain FA (%)</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>11</td>
</tr>
<tr>
<td>14</td>
</tr>
</tbody>
</table>

*The details of the experiment are given in Methods. The size classes of fatty acids were defined as: short chain, C 8, 10 and 12; long chain, C 14, 16 and 18; unsaturated fatty acids, C 16:1, 18:1 and 18:2. Phage unsaturated fatty acids consisted of only 16:1 and 18:1.

fatty acids. As the temperature of growth was lowered, there was an increase in the short chain fatty acids in the phage whereas in the host the content of short chain fatty acids decreased. On the other hand, the percentage of unsaturated fatty acids increased in the host concomitantly with decreasing temperature of growth. The difference in fatty acid composi-
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In this last instance where the phage was propagated at 18 °C in a host which was grown at 40 °C. In this case the fatty acid composition reflected very nearly the composition of the phage grown at 18 °C throughout.

Phage ultrastructure

Images of particles negatively stained with sodium phosphotungstate showed that the stain penetrated to the particle interior leaving an unstained band (Fig. 3a) which may correspond to a lipid bilayer. The particles, when dialysed, stored in the cold for about 3 weeks and then positively stained with uranyl acetate, showed a ‘double track’ pattern at their edge similar to the unit membrane structure (Fig. 3c). Fresh phage preparations stained with uranyl acetate (prior to ghosting) are shown in Fig. 3(b). Chloroform treatment of the phage resulted in their aggregation and an alteration in ultrastructure (Fig. 3d).

Discussion

The presence of lipid has been reported in all the mycobacteriophages examined so far. This conclusion, however, is mainly based on the inactivation of phage by treatment with organic solvents. Even when lipids are demonstrated in phage preparations, they could be attributed to contamination with host material. Thus the presence of lipid seen in the case of phage D29 (Jones et al., 1970) has been challenged recently by Schafer et al. (1977). When extensively purified preparations of phage D29 were used, the presence of lipids could not be demonstrated. Furthermore, there were no structural changes in the phage particles after inactivation by treatment with chloroform and an electron paramagnetic resonance spectrum characteristic of the lipids was totally absent.

Since the samples of phage I3 used in the present study for the demonstration of lipids have been purified extensively by two consecutive centrifugations through sucrose gradients followed by banding on CsCl, the chance of non-specific contamination due to host lipids can be ruled out. This was further confirmed by electron microscopy. Moreover, the lipid composition of the phage and host were different when they were propagated at different temperatures.

The lipids of the phage are evidently picked up from the host during phage assembly but in a specific manner. In fact, two-thirds of the phospholipid content of the phage was from the lipids of the host synthesized prior to infection. The remaining third of the phospholipid, that was synthesized after phage infection, showed a decrease in the PE fraction and concomitant abundance of the unidentified component. In the case of lipid-containing phage PR4 it has been shown that two-thirds of the phospholipids are synthesized after infection (Sands, 1976).

Phage I3 resembles mycobacteriophage R1 in its total lipid content (14%; Soloff et al., 1978). However, it differs significantly from the latter with respect to the phospholipid content. Phage R1 contains 1% of total lipids as phospholipid whereas phage I3 contains 69% of the total as phospholipids. The major phospholipids are PG and PE which form the bulk of phospholipids in other lipid-containing bacteriophages such as PM2, φ6 and PR4 (Braunstein & Franklin, 1971; Sands, 1973, 1976).

The phospholipid content also correlated with a higher susceptibility of phage I3 to the killing action of polar solvents. By comparison with other mycobacteriophages (Sellers & Tokunaga, 1970), phage I3 is relatively insensitive to chloroform. This might be due to the fact that the lipids are deeply embedded as complexes with protein or are inside a protein layer and hence not readily accessible to solvents. There are other known instances where viruses containing lipids are only partially inactivated by solvents. Thus, iridescent virus type 2, which contains 9% lipid (Kelly & Vance, 1973), is not sensitive to ether (Day & Mercer, 1964), and phage φ6 is only partially inactivated in 25% ether (Vidaver et al., 1973).
The lipids of mycobacteriophage 13 appear to be present as bilayer membrane-like structures under the electron microscope just as with PM2, PR3 and PR4 (Harrison et al., 1971; Bradley & Rutherford, 1975). These structures were clearly seen and quite pronounced in stored preparations of phage 13 which had become partially ghosted. Considering the lipid-rich nature of the host organism the presence of lipids as a structural component of mycobacteriophages may certainly be a desirable trait to facilitate host-phage interactions. Hydrophobic interactions may be necessary for successful infection when the phage adsorbs to the host cell surface. Phage 13 capsid membrane is mostly composed of saturated and short chain fatty acids and its membrane fluidity is perhaps modulated to a limited extent by changing the content of short chain fatty acids. It is known that some degree of compensation can be achieved by changing the chain length of fatty acids.

REFERENCES


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