The Effect of Temperature on the Ecology of Aquatic Bacteriophages

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(Accepted 2 August 1979)

SUMMARY

Three physiological types of coliphage were recognized on the basis of the effect of temperature on their e.o.p. High temperature (HT) phages plated at or above 25 °C, low temperature (LT) phages at or below 30 °C and mid-temperature (MT) phages in the range 15 to 42 °C. Only LT phages were found for Aeromonas hydrophila, an indigenous water organism. The maximum and minimum plating temperatures were stable properties of the virus and were not influenced by the growth temperature of the host. Temperature was found to affect the adsorption of two phages and to affect multiplication, but not adsorption, of another two. The distribution of the three types of phage correlated closely with the temperature of the environment from which they were isolated. The ecological implication of these results is discussed.

INTRODUCTION

The occurrence and distribution of bacteriophages in aquatic environments, and particularly coliphages in sewage, have been investigated by many workers (Dhillon et al. 1970; Dhillon & Dhillon, 1974; Reali et al. 1975; Fannin et al. 1977; Furuse et al. 1978). However, relatively little is known about the factors which influence the ecology of bacteriophages. Current knowledge of the physiology of bacteriophage infection (Stent, 1963) suggests that important factors would be concentration of host cells, divalent cation concentration, pH and temperature. To date there have been no detailed investigations of the effects of any of these parameters on the activities of bacteriophages in the natural environment. With certain bacterial hosts additional factors may have a role in bacteriophage ecology. Thus Zachary (1974) found that the distribution of bacteriophages infecting the marine bacterium Vibrio natriegens was salinity dependent. In addition, laboratory studies showed that V. natriegens bacteriophages require sodium ions for replication, thus explaining their distribution (Zachary, 1976).

We have investigated the effects of temperature on the multiplication of phages isolated from different environments and report here the identification of three physiological types of coliphage. The significance of our results in terms of the ecology of phages is discussed.

METHODS

Bacteria and bacteriophages. These are described in Table 1 with the exception of those bacteriophages isolated in this study.

Media and solutions. These have been described previously (Seeley & Primrose, 1979).

Isolation of bacteriophages from river water. Twenty to 40 l samples of river water were concentrated to approx. 10 ml using a portable phage concentrater (Seeley et al. 1979).

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Table 1. Properties of bacteria and bacteriophages used in this study

<table>
<thead>
<tr>
<th>Organism</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
</tr>
<tr>
<td>W3110</td>
<td>Female K12 strain</td>
</tr>
<tr>
<td>ED391</td>
<td>Derived from W3110 by introduction of F'</td>
</tr>
<tr>
<td>HfrH</td>
<td>Male K12 strain</td>
</tr>
<tr>
<td>C</td>
<td></td>
</tr>
<tr>
<td><em>Aeromonas hydrophila</em></td>
<td></td>
</tr>
<tr>
<td>TW1</td>
<td>Fish pathogen supplied by Thames Water Authority</td>
</tr>
<tr>
<td>Bacteriophage MS2</td>
<td>Male specific. Assayed on <em>E. coli</em> HfrH or ED391</td>
</tr>
<tr>
<td>Bacteriophage φX174</td>
<td>Single-stranded DNA phage. Assayed on <em>E. coli</em> C</td>
</tr>
</tbody>
</table>

Filter-sterilized concentrates were plated in duplicate and incubated at two temperatures, one near the optimum for host cell growth (30 or 37 °C) and the other at either 15 or 20 °C, depending on the temperature of the river water at the time of sampling.

Isolation of bacteriophages from sewage. Samples (10 ml) of raw sewage were filtered through 47 mm diam. 0.22 μm membrane filters which had been flushed with 2 ml nutrient broth to minimize phage adsorption to the filter. The filtered sewage was plaque assayed without further treatment.

Isolation of bacteriophages from faeces. Samples of freshly voided human, sheep, horse and cattle faeces were added to weighed screw-capped bottles and suspended in nutrient broth at a concentration of 0.25 g/ml wet weight; 1.5 ml of the faecal suspensions were centrifuged at 14000 g for 3 min and the supernatant fluid assayed without further treatment.

Purification of bacteriophages. Different bacteriophages were selected on the basis of plating host or plaque morphology and purified by at least two single plaque isolations. Lysates were prepared by flooding each of 20 confl uently lysed phage assay plates with 10 ml phage buffer. The plates were stored overnight at 4 °C and the buffer collected. Phages were concentrated by precipitation with polyethylene glycol (Yamamoto et al. 1970) and isopycnic centrifugation in CsCl. Opalescent bands of bacteriophage were collected from CsCl gradients, dialysed overnight at 4 °C against 11 ammonium bicarbonate (0.05 M, pH 7.6) and stored at 4 °C.

Determination of efficiency of plating (e.o.p.) of purified phages. Phage preparations were diluted and plated in duplicate at eight different temperatures. Plates were transferred to incubators as soon as the top agar solidified. Plates incubated at 45, 42, 37, 30 and 25 °C were read after 18 h, those at 20 and 15 °C after 48 h and those at 10 °C after 2 weeks. The temperature at which the highest number of plaques formed was taken as the standard for calculating the e.o.p.

Rapid determination of the effect of temperature on plaque formation. To avoid the lengthy procedure described above for determining the e.o.p. at different temperatures the following rapid method was developed. Using a sterile Pasteur pipette, plaques were removed at random from primary isolation plates and transferred to 0.4 ml phage buffer contained in the wells of a phage typing block. After allowing the phage to diffuse from the agar plugs for 10 min, 10⁻² and 10⁻⁴ dilutions of each phage suspension were made in two identical phage typing blocks. A 25-pin multi-point inoculator was used to spot each phage suspension on freshly prepared soft-agar lawns of host cells. This procedure was repeated with the 10⁻² and 10⁻⁴ dilutions after sterilization of the inoculator pins with alcohol and flaming. This method gave results which agreed closely with those obtained by the more lengthy determination of e.o.p. at different temperatures.

Adsorption experiments. The method used was based on that of Adams (1959). Host cultures were grown to approx. 2 × 10⁸ cells/ml in nutrient broth at the temperature at which adsorption was to be measured. The exponentially growing cells were harvested by centri-
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fugation at 5000 g for 5 min and washed twice in ice-cold phage buffer. The final cell pellet was resuspended in 0.5 ml phage buffer and added drop-wise to cold nutrient broth containing 10 mM-KCN until an absorption at 550 nm, corresponding to a cell count of $2 \times 10^8$ colony forming units/ml, was obtained. The cell suspension was equilibrated to the required temperature in a circulating water bath and a 20 μl sample removed for viable cell count. Phage was added to an m.o.i. of 0.1 and the suspension mixed and aerated by bubbling with filtered air. At intervals, 20 μl samples were removed, diluted in 2 ml ice-cold phage buffer containing 0.1 ml chloroform and vigorously agitated for 10 s. Chloroformed samples were stored on ice until all further samples had been taken. They were then diluted and plated in duplicate. A control tube containing broth, KCN and phage, but no cells, was always included to check for reductions in phage titres. Assay plates were incubated at the optimum temperature as determined in previous experiments. Adsorption velocity constant values ($k$) were calculated as described by Adams (1959).

**One-step growth experiments.** Phage were adsorbed to cells in the presence of KCN as described above until there was 90% adsorption. The suspension was then diluted $10^{-4}$ and $10^{-5}$ into pre-equilibrated broth and samples assayed at intervals thereafter for p.f.u.

**RESULTS**

**Preliminary observations**

A 20 l phage concentrate (Seeley et al. 1979) from a fish farm fed with water from Draycote drinking water reservoir contained 100 p.f.u. active against *Aeromonas hydrophila* at 25 °C and 1300 p.f.u. active against *Escherichia coli* HfrH at 37 °C. The possible origins of these phages were questioned by determining the effects of temperature on their ability to form plaques. One of the coliphages failed to plaque below 25 °C. By contrast, all the *A. hydrophila* phages which we have isolated from this and other freshwater sources have high e.o.p., relative to their optimum temperature, in the range 10 to 25 °C (Fig. 1). Indeed, with some of the *A. hydrophila* phages there is virtually no variation in e.o.p. across this temperature range. Since the temperatures of most rivers and reservoirs in central England seldom rise above 20 °C (Fig. 2) it seems likely that the coliphage would not have been capable of replication in the reservoir or fish pond, whereas the *A. hydrophila* phages were probably better suited to replication in these environments.

**Effect of temperature on the e.o.p. of common laboratory bacteriophages**

The failure of the coliphage from the fish pond to plaque at low temperatures prompted us to test the effect of temperature on the e.o.p. of a number of bacteriophages from our culture collection. The results are shown in Fig. 3. All the phages tested had a low e.o.p. at 20 °C and most failed to plaque at this temperature. We call such phages high temperature (HT) phages.

**Isolation of coliphages which plaque at low temperatures**

To determine whether phages adapted to the low temperatures of the natural environment exist, a concentrate was prepared from the river Avon (Leicestershire) near its source. This location was chosen so that the water sampled was free of gross faecal pollution, a likely source of HT phages (see later). When the concentrate was plated at 20 and 37 °C on four strains of *E. coli* the plaque count was 20- to 200-fold higher at 20 °C. Representative plaque types were selected from the 20 °C plates and re-tested at 20 and 37 °C. Over 75% of the phages failed to plaque at 37 °C. We have termed such phages 'low-temperature (LT) phages'. The e.o.p. at different temperatures was examined for 7 LT phages and results for a representative phage (E2) are given in Fig. 4. All the LT phages tested had an optimum
Fig. 1. Effects of temperature on the efficiency of plating of phages isolated from Draycote fish farm: (a) coliphage H2; and *Aeromonas hydrophila* phages, (b) ah1, (c) ah2 and (d) ah3.

Fig. 2. Mean (---) and maximum (△—△) weekly temperatures of the River Derwent from 11th December, 1977 to 26th November, 1978. (Data courtesy of Severn Trent Water Authority.)

The temperature for plating between 20 and 30 °C and the e.o.p. dropped sharply to 0 between 33 and 37 °C.

**Effect of growth temperature of the phage on subsequent e.o.p. at different temperatures**

It is possible that the initial temperature of plating of the phages could influence the subsequent e.o.p. versus temperature profiles. Consequently, a HT phage was plaque purified three times at its lowest plaquing temperature (25 °C) and its highest plaquing temperature (45 °C) and an e.o.p. versus temperature profile then determined in each case. The profiles obtained were almost identical to those obtained with the original phage lysate.
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Fig. 3. Effects of incubation temperature on the efficiency of plating of laboratory coliphages: \(\phi X174\) (\(\triangle\)); \(\phi 80\) (\(\square\)); and MS2 (\(\bullet\)).

Fig. 4. Effects of incubation temperature on the efficiency of plating of typical low, mid and high temperature coliphages: E2 (\(\square\)); CSH2 (\(\bullet\)); and HSW2 (\(\triangle\)).

Table 2. Temperature profiles of bacteriophages from different habitats

<table>
<thead>
<tr>
<th>Source of phage</th>
<th>No. tested</th>
<th>15-30 °C (LT type)</th>
<th>25-42 °C (HT type)</th>
<th>30-45 °C (HT type)</th>
<th>15-45 °C (MT type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human faeces</td>
<td>50</td>
<td>0</td>
<td>54</td>
<td>0</td>
<td>46</td>
</tr>
<tr>
<td>Raw sewage</td>
<td>49</td>
<td>7</td>
<td>0</td>
<td>9</td>
<td>84</td>
</tr>
<tr>
<td>Cow faeces</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>72</td>
<td>28</td>
</tr>
<tr>
<td>River Swift site 1</td>
<td>19</td>
<td>53</td>
<td>0</td>
<td>5</td>
<td>42</td>
</tr>
<tr>
<td>River Swift site 2</td>
<td>25</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>67</td>
</tr>
<tr>
<td>River Swift site 3</td>
<td>18</td>
<td>16</td>
<td>0</td>
<td>8</td>
<td>78</td>
</tr>
<tr>
<td>River Swift site 4</td>
<td>24</td>
<td>33</td>
<td>0</td>
<td>4</td>
<td>63</td>
</tr>
<tr>
<td>River Swift site 5</td>
<td>24</td>
<td>42</td>
<td>0</td>
<td>8</td>
<td>50</td>
</tr>
</tbody>
</table>

Attempts were also made to isolate HT mutants of two LT coliphages. High-titre lysates \((6 \times 10^9 \text{ p.f.u./ml} \text{ and } 1.6 \times 10^{11} \text{ p.f.u./ml})\) were assayed at 37 °C but no plaques were observed.

Effect of growth temperature of the host on the e.o.p. versus temperature profile

To eliminate the possibility that the temperature at which the host was grown had any influence on the results obtained so far, \(E. coli\) was grown at 15 and 37 °C and then used to determine the e.o.p. versus temperature profiles of two phages. \(E. coli\) C was used for the profile of \(\phi X174\) and \(E. coli\) HfrH for that of a phage isolated from water. With both phage–host combinations identical e.o.p. profiles were obtained with hosts grown at 15 and 37 °C.

Origins of HT and LT phages

The most obvious source of HT phages is the gut of warm-blooded animals. Consequently we isolated phages from horse, sheep, cow and human faeces. Since there is a possibility that the faeces of laboratory workers may contain phages used in the laboratory, human
faeces were collected from hospital patients. All phage isolations were done at 30 °C, a temperature which permits growth of all LT and HT phages isolated by us.

E.o.p. versus temperature profiles were determined for two phages from horse faeces, three from cow faeces and two from sheep faeces. Five of these phages were typical HT phages and the remaining two, one each from cow and sheep faeces, showed a new profile in that they plated over a range of temperatures between those of LT and HT phages. We call such phages mid temperature (MT) phages. Fig. 4 shows e.o.p. profiles of representative MT (CSH2) and HT (HSW2) phages compared with an LT phage (E2). The effect of temperature on the plating of a further 57 phages, seven from cow faeces and 50 from human faeces was examined by means of spot tests (see Methods). The results are shown in Table 2. Human faeces contained typical MT phages and also phages which behaved similarly to the HT type but plaqued at the slightly lower temperature range of 25 to 42 °C.

A possible source of LT phages is the faeces of cold-blooded animals, e.g. fish. However, we failed to isolate any coliphages from the contents of fish guts, either by direct plating or after enrichment in liquid culture.
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**Distribution of LT, HT and MT phages in a water course**

Concentrates were prepared from water taken at five locations on the river Swift in Leicestershire and Warwickshire (Fig. 5) and the proportion of each type of phage determined by the rapid spot test method (Table 2). It should be noted that the proportion of LT phages is greatest near the source of the river, decreases with increasing pollution from sewage treatment plants and increases again as the river self-purifies.

**Physiological properties of HT and LT phages**

There are at least two possible reasons for the inability of a bacteriophage to plaque at normal environmental temperatures: failure to adsorb to the host cell or failure to multiply. The adsorption velocity constants ($k$) at 15 °C for two HT and two LT coliphages are shown in Table 3. E2, a typical LT phage completely failed to adsorb to *E. coli* at 37 °C whereas the $k$ value of HSW2, a typical HT phage, was 25-fold lower at 15 than 37 °C. Both these results adequately account for the effects of temperature on e.o.p. With the other two phages the differences in $k$ values at the two temperatures were not enough to identify them as HT or LT phages. Their failure to form plaques at their restrictive temperature (15 or 37 °C) was probably due to an inability to multiply.

Because the latent period increases with decreasing temperature it is difficult to study the effect of low temperature on the multiplication of an HT phage. An apparent failure to multiply could in fact be due to an extremely long latent period. Consequently we have studied only the effects of increased temperature on the multiplication of LT phages. The results obtained with *Aeromonas* phage ah1, a typical LT phage (Fig. 1), are shown in Fig. 6. Note that although there was a significant decrease in the latent period as the temperature was raised from 10 to 30 °C, the burst size was relatively constant. When the temperature was raised to 35 °C the adsorption of the phage was unaffected but the burst size was reduced to less than 2 p.f.u. cell. Similar results were obtained with *Aeromonas* phage ah2 (data not shown).
DISCUSSION

Our data on the effect of temperature on the e.o.p. of coliphages indicate the existence of three physiological types of coliphage, termed by us LT, HT and MT. The fact that LT phages have remained undiscovered until now is simply a reflection on the methods used to isolate coliphages. Usually these involve enrichment or direct plating at 37 °C of faecal samples or raw sewage. Only MT and HT phages would be selected at this temperature. Indeed most of the bacteriophages in our culture collection are typical HT phages and all were obtained by plating concentrates (Primrose & Day, 1977) at 37 °C. We found that all three classes of phage plate at 30 °C and this is now the incubation temperature of our choice for coliphage isolation.

Our results suggest that the LT and HT phage types are phenotypically stable, as low temperature mutants of an HT phage, and high temperature mutants of two LT phages were not isolated. The ready isolation of mutants of \( \phi X174 \) which replicate normally at 43 °C, a temperature at which wild type \( \phi X174 \) plates poorly (Primrose et al. 1971; Haworth et al. 1975), suggests that high temperature mutants of some LT phages might be produced spontaneously. It is possible, however, that \( \phi X174 \) is unusually susceptible to such mutations as the genome codes for only nine genes and a mutation in one of them is sufficient to affect the maximum and minimum plating temperatures (Segal & Dowell, 1974; Haworth et al. 1975). With more complex phages the maximum and minimum temperatures for growth will be influenced by more than a single protein, resulting in the stable phenotype which we observed.

Reduced adsorption was shown to be the reason for the failure of one HT phage to plaque at 15 °C and one LT phage to plaque at 37 °C. The two other phages which we tested adsorbed efficiently at 15 and 37 °C (Table 3) and thus the block at restrictive temperatures must be in the injection step and/or subsequent multiplication. Our experiments did not distinguish between those two events but Fig. 6 shows that some step subsequent to adsorption was defective at 35 °C with an \textit{Aeromonas} phage. Surprisingly, temperatures up to 30 °C had relatively little effect on the burst size of the two \textit{Aeromonas} phages and a similar observation was made with a coliphage by Ellis & Delbrück (1939). Thus the reduced e.o.p. seen at permissive temperatures other than the optimum cannot be explained by variation in burst size. A more likely explanation is that, as with phage \( \lambda \) and \( \varphi 60b \) (Mackay & Bode, 1976; McConnell et al. 1979), temperature affects the proportion of irreversibly adsorbed phages which inject their DNA.

Can laboratory observations on the effects of temperature on the e.o.p. of phages be used to explain their distribution in the environment? The data presented in Table 2 and Fig. 5 suggest that they can. Thus faeces of warm-blooded animals contained only HT and MT phages. Although the bulk of coliforms in domestic sewage come from human faeces the temperature of sewage is close to that of the environment and would select MT faecal phages. Growth of host cells in sewage would be enhanced by the high nutrient concentrations, and any LT phages present could also replicate. The temperature of natural waters free of gross faecal pollution will also favour the multiplication of LT and MT phages and the number of HT phages would be expected to be low. In such environments the ratio of LT to MT phages will clearly be influenced by many factors but pollution with domestic or agricultural waste would increase the number of MT phages as observed in samples from the River Swift.

A number of workers (Kott et al. 1974; Berry & Noton, 1976) have suggested that coliphages be used as indicators of faecal pollution of water. Our results show that coliphages can be found in waters free of gross faecal pollution and that these coliphages, as well as some from human and animal faeces, could replicate in water.
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During the course of this work N. D. S. was in receipt of a studentship from the Natural Environment Research Council.

REFERENCES


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