Isolation and Preliminary Characterization of Three Bacteriophages which Adsorb Specifically to the Developing Daughter Cells of *Hyphomicrobium*

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**SUMMARY**

The isolation of three bacteriophages active on *Hyphomicrobium* is reported. These phages only infected *Hyphomicrobium* isolates; a variety of other Gram-negative bacteria were immune to infection with these phages. Each of these phages contained double-stranded DNA. Hyφ1a had a polyhedral head and a short, non-contractile tail. Hyφ22a was morphologically similar to Hyφ1a. They appear to be genetically distinct however, as they could be distinguished from one another by their host ranges, their densities in CsCl, and the mol. wt. of their major protein components. Hyφ32a had a polyhedral head and a long, flexible, non-contractile tail with fibres attached to its distal end. Each of these bacteriophages selectively adsorbed to daughter cells developing at the distal end of prosthecae on mother cells.

**INTRODUCTION**

Unlike the true bacteria, *Hyphomicrobium* species reproduce by budding and have a diphasic life cycle (Hirsch, 1974). The daughter cells, or swarmer cells, are motile and mature into non-motile cells. Each of these cells, in turn, produces a prostheca from which new daughter cells bud. Thus, those bacteria belonging to the genus *Hyphomicrobium* represent a model for studying cellular morphogenesis. Since the phages we have isolated for *Hyphomicrobium* adsorb exclusively to one of the stages in the development of the organism, they should be able to act as sensitive indicators of its morphological differentiation. The isolation and properties of one such bacteriophage have been reported (Gerencser & Voelz, 1971; Voelz et al. 1971; Kaplan et al. 1976).

Bacteriophage receptor sites, while highly specific, are generally scattered over the surface of a bacterium such that phage can adsorb to almost any point on the surface of the bacterium (Lindberg, 1973). Notable exceptions are the flagellotrophic phages (Meynell, 1961; Raimondo et al. 1968; Jollick & Wright, 1974; Lotz et al. 1977) and pilus-specific phages (Crawford & Gesteland, 1964; Caro & Schnös, 1966; Meynell & Lawn, 1968; Marvin & Hohn, 1969). A bacteriophage for *Hyphomicrobium* which adsorbs to developing daughter cells has been reported (Voelz et al. 1971).

Bacteriophages active on *Caulobacter*, another differentiating bacterial system, also show stage specific adsorption properties (Johnson et al. 1977; Lagenaaur et al. 1977). Most of these phages adsorb specifically to the swarmer cell stage. The RNA bacteriophage φCb5 adsorbs to pili which are only found at the flagellated poles of swarmer and predivisional cells (Schmidt, 1966). The DNA-containing bacteriophage φCbK also adsorbs to the
flagellated poles of swarmer and predivisional cells (Agabian-Keshishian & Shapiro, 1971). However, its receptor is not known; it does not adsorb to either flagella or pili (Kurn et al. 1974) but to some other structure which is synthesized co-ordinately with flagella and pili (Fukuda et al. 1976; Shapiro, 1976; Fukuda & Okada, 1977). Johnson et al. (1977) have suggested that stage-specific phages have a selective advantage over other phages as susceptible cells are continuously being produced as morphogenesis occurs. There are also bacteriophages active on Caulobacter which show more traditional adsorption patterns. Phages Cd1 (West et al. 1976) and Cr30 (Ely & Johnson, 1975) can adsorb to the entire surface of any of the cell types, swarmer, predivisional or mother cell. As such these last two phages are similar to the majority of phages for non-differentiating bacteria.

In an attempt to find bacteriophages specific for each of the stages in the cell growth cycle of Hyphomicrobium, we have begun isolating additional phages active on the bacterium. In this paper we report the isolation and preliminary characterization of three new bacteriophages specific for this genus. These phages specifically adsorb to daughter cells developing at the distal end of prosthecae.

**METHODS**

**Organisms and medium.** Various independent isolates of Hyphomicrobium were used in the present study. These isolates were obtained from different fresh water sources. Each isolate exhibited characteristics which distinguished it from the others. All of the other bacteria used for this study were obtained from stock cultures maintained at West Virginia University, Morgantown, U.S.A. Except where noted, medium MH-69 was used exclusively for growth of the organism and as a diluent (Gerencser & Voelz, 1975).

**Bacteriophage assays.** Bacteriophages were assayed for p.f.u. on log phase cultures of the appropriate strain of Hyphomicrobium using the agar overlayer method (Adams, 1959). Plaques were scored after incubation for 72 h at 30 °C.

**Bacteriophage purification.** For analysis, the phages were first purified. Cellular debris was removed by centrifugation at 10000 g for 30 min. The supernatant was treated with DNase (1 μg/ml) and RNase (1 μg/ml) at 37 °C for 1 h. The phage particles were then pelleted at 25000 rev/min for 3 h using a 30 rotor (Beckman Instruments). The phage pellet was resuspended in sterile MH-69. Particles at this stage were used for nucleic acid determinations. Further purification was achieved by sedimenting the particles through a 10% sucrose solution on to a CsCl cushion. The phage band was removed and re-run to equilibrium in CsCl. The band was collected and the CsCl removed by dialysis. Examination of the phage particles in the electron microscope revealed a pure preparation of phage but the majority of the particles (> 90%) had discharged their DNA and were empty. Particles purified through CsCl were used for gel electrophoresis.

**Host range studies.** The host range of the various phage isolates was determined by spotting 50 μl of a phage suspension (10^5 p.f.u./ml) on an agar overlayer containing the strain to be tested. The plates were incubated at 30 °C for 72 h and were examined daily for lysis.

**Density determinations.** The buoyant densities of the bacteriophages were determined by equilibrium centrifugation in CsCl. After centrifugation at 100000 g for 22 h, the bottom of the centrifuge tube was punctured and 0.2 ml fractions were collected. The density of every third fraction was determined from its refractive index. One ml of 0.01 M-tris-0.06 M-KCl buffer at pH 7.5 was added to each fraction and the absorbance at 280 nm was determined. The number of p.f.u. in each fraction was also determined.

**Nucleic acid determinations.** The type of nucleic acid contained in each phage was determined by staining with acridine orange followed by treatment with potassium tartrate (Bradley, 1966).
Hyphomicrobium bacteriophages

Gel electrophoresis. Polyacrylamide gel electrophoresis was performed according to Maizel (1971). A discontinuous SDS gel system using 13% acrylamide gels were employed. The gel buffer contained tris-HCl (pH 8.9) and 0.1% SDS. The electrode buffer was tris-glycine with 0.1% SDS. Twenty micrograms of purified virus protein were denatured by heating in a boiling water bath for 5 min in 1% SDS, 0.1% 2-mercaptoethanol and 10% glycerol. After cooling to room temperature, the samples were layered on to the gels. Twelve cm long cylindrical gels were run at 150 V for 5 h at a constant temperature of 20 °C. The gels were stained with Coomassie blue and then destained in 7% acetic acid. For mol. wt. estimations bovine serum albumin, ovalbumin and cytochrome c were run as markers alongside the virus samples.

Electron microscopy. All specimens were observed in an RCA EMU-3 G electron microscope at 100 kV. Samples were adsorbed on to carbon films and stained with 1% sodium phosphotungstate. The sizes of the phage particles were obtained by measuring ten micrographs of each isolate and averaging the results. For phage adsorption studies each bacteriophage was incubated with its host at an m.o.i. of about 50 p.f.u./c.f.u. for 15 min at 30 °C. The cells were then fixed in situ with 0.1% glutaraldehyde and processed as above for electron microscopy.

RESULTS

Isolation and propagation of bacteriophages

The bacteriophages were isolated from chloroform-treated sewage using established procedures (Schmidt & Stanier, 1965). After chloroform treatment the sewage was incubated for 48 h at room temperature and then centrifuged at 12000 g for 30 min to remove debris. Medium was prepared by dissolving MH-69 salts and methylamine in the sewage supernatant. Ten ml of a log phase culture of a strain of Hyphomicrobium were added to 100 ml of the medium. The culture was incubated on a shaker at 30 °C for 1 week. The cellular debris was removed by centrifugation and the supernatant fluid was passed through a membrane filter (0.45 μm) to remove cells. The filtrate was then assayed for p.f.u. on the same strain of Hyphomicrobium as that used in the enrichment procedure. Isolated plaques were picked and purified by serial passage. After three such passages, a phage stock was prepared from a single isolated plaque.

Phage was propagated by adding 10⁶ p.f.u. and 0.1 ml of a log phase culture (5 x 10⁷ cells) to an agar overlayer and incubating at 30 °C for 5 days. The overlayer was then scraped from the plate and was suspended in 5 ml of MH-69 broth. Two-tenths ml of chloroform was added and the mixture was shaken vigorously. After incubation at room temperature for 10 min, the agar debris was removed by centrifugation at 1200 g and the supernatant fluid containing the bacteriophage was filtered to remove any remaining cells. Titres ranging from 10¹⁰ to 10¹³ p.f.u./ml were routinely obtained in this manner for each of the bacteriophages.

Plaque morphology

Each of the bacteriophages gave distinct plaques when assayed on log phase cells of the strain on which it was isolated. Hyφ1a produced small clear plaques about 1 mm diam. Hyφ22a produced large clear plaques with diam. ranging from 3 to 5 mm. Hyφ32a produced clear plaques with a diam. of approx. 3 mm. If stationary phase cultures were used for assaying the phages smaller plaques were obtained.
Table 1. *Host range of bacteriophages active on Hyphomicrobium*  

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Hyφ1a</th>
<th>Hyφ22a</th>
<th>Hyφ32a</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> B</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> C600</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> K-12</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Ps. fluorescens</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Caulobacter vibrioides</em></td>
<td>CV 7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CV 14</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. bacteroides</em></td>
<td>CB 11a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CB 27</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Hyphomicrobium</em> isolate no. 1</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Hyphomicrobium</em> isolate no. 22</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Hyphomicrobium</em> isolate no. 30</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Hyphomicrobium</em> isolate no. 32</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* The tests with the *Hyphomicrobium* strains were performed using MH-69 medium. All other tests were performed using PYE medium (West et al. 1976).

† + = lysis; − = no lysis.

Fig. 1. Bacteriophage Hyφ1a. Note the icosahedral head and the short tail (a to c). A hexagonally shaped base plate is present at the distal end of the tail (a). Six pins are apparent on the base plate (a, c). All of the phage particles are adsorbed to the buds developing at the distal end of the prosthicae (d, e); the mother cells to the left do not have any particles adsorbed to them.
Hyphomicrobium *bacteriophages*

![Image](a) ![Image](b) ![Image](c) ![Image](d)

Fig. 2. Bacteriophage *Hyphi*22a. Note the icosahedral head and the short tail (*a* to *d*). Two cells are seen with phage particles adsorbed exclusively to the distal ends of the prosthecae (*e*). The inset shows a higher magnification of the bud of the cell on the left. Note the empty particles which have apparently injected their nucleic acid into the cell.

<table>
<thead>
<tr>
<th>Bacteriophage</th>
<th>Tail Length* (nm)</th>
<th>Tail Width (nm)</th>
<th>Head Apex to Side (nm)</th>
<th>Side to Side (nm)</th>
<th>Type†</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hyphi</em>1a</td>
<td>19</td>
<td>N.D.‡</td>
<td>67</td>
<td>60</td>
<td>C1</td>
</tr>
<tr>
<td><em>Hyphi</em>22a</td>
<td>13</td>
<td>N.D.‡</td>
<td>53</td>
<td>43</td>
<td>C1</td>
</tr>
<tr>
<td><em>Hyphi</em>32a</td>
<td>12†</td>
<td>6</td>
<td>71</td>
<td>62</td>
<td>B1</td>
</tr>
</tbody>
</table>

* The length of the tail was determined by measuring the distance between a phage particle and cellular debris to which it had adsorbed.
† Based on the Ackermann & Eisenstark (1974) scheme for bacteriophage classification.
‡ N.D. = not determined.

**Table 2. Size of bacteriophages active on Hyphomicrobium**

**Host range studies**

The results of the host range studies done with these bacteriophages are presented in Table 1. Several prosthecate and other Gram-negative bacteria were tested as potential hosts. Only strains of *Hyphomicrobium* were infected by these phages. *Hyphi*1a and *Hyphi*32a had similar host ranges.
Fig. 3. Bacteriophage Hyβ32a. Note the icosahedral head and the flexible, non-contractile tail (a to e). Fibres (arrows) can be seen extending from the distal end of the phage tail (a, b). Most of the phage particles are adsorbed to the bud (d). A few phage particles are seen alongside of the prostheca but they are not attached to it.
Hyphomicrobium bacteriophages

Bacteriophage morphology

Examination of the bacteriophages in the electron microscope revealed two distinct types of particles. Hy\(\phi\)1a and Hy\(\phi\)22a have icosahedral heads and very short tails. Hy\(\phi\)1a (Fig. 1) appears similar to coliphage T3 (Bradley, 1963) and is a Group C1 bacteriophage (Ackermann & Eisenstark, 1974). There appears to be a hexagonal plate with 6 spikes at the distal end of the tail. Hy\(\phi\)22a (Fig. 2) is similar in appearance to Salmonella phage P22 (Anderson, 1960) and is also a Group C1 bacteriophage. Hy\(\phi\)32a (Fig. 3) has an icosahedral head and a flexible, non-contractile tail. There appear to be some fibres at the distal end of the tail. This phage is morphologically similar to coliphage T5 (Bradley, 1963) and is a Group B1 bacteriophage. Table 2 summarizes the results of our morphological studies with these phages.

Bacteriophage adsorption sites

Adsorption of these bacteriophages to their host cells occurred at a specific stage in the morphogenic cycle of the cells. Each of the phages adsorbed to the distal end of the prostheca where a bud was developing (Fig. 1 to 3). These phages were never found adsorbed to cells in other stages of the morphogenic cycle nor were they found adsorbed to the mother cell from which a prostheca extended or to the prostheca itself. Fig. 1 (d, e) shows Hy\(\phi\)1a adsorbed to its host; all of the particles are seen clustered around the developing bud cell. Fig. 2 (e) shows Hy\(\phi\)22a adsorbed to its host. All of the particles are again seen clustered around the developing buds. The inset shows a higher magnification of the bud of the cell on the left; some empty particles which have apparently discharged their DNA following adsorption are clearly visible. Fig. 3 (d) shows the adsorption of Hy\(\phi\)32a to its host. Both full and empty particles are seen attached to the developing bud. A few particles are seen along the prostheca but they are not attached to the organelle.

Gel electrophoresis

Because Hy\(\phi\)1a and Hy\(\phi\)22a were so similar morphologically, we were concerned that they might be genetic variants of the same bacteriophage. In an attempt to determine whether they were in fact distinct phages, their major proteins were sized in polyacrylamide gels. Each of the phages gave two distinct bands which stained with Coomassie blue following SDS-gel electrophoresis. The results are shown in Table 3. It is likely from these data that the phages are distinct as their major proteins have different mol. wt.

Density of the bacteriophage particles

The buoyant density of each of the bacteriophage particles was determined by isopycnic gradient centrifugation in CsCl. The results of these experiments are shown in Table 4. In all of these determinations the absorbance peak at 280 nm occurred in the same fraction of the gradient as did the peak of plaque-forming activity. The densities of the phages were typical of DNA containing bacteriophage.

Bacteriophage nucleic acids

Determinations of the type of nucleic acid contained in each of the bacteriophages were done using acridine orange (Bradley, 1966). The results of these studies are presented in Table 4. Each of the bacteriophages apparently contained double-stranded DNA.
Table 3. Mol. wt. determinations of the major bacteriophage proteins*

<table>
<thead>
<tr>
<th>Designation of protein</th>
<th>Hyφ1a</th>
<th>Hyφ22a</th>
<th>Hyφ32a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rf</td>
<td>Approx. mol wt.</td>
<td>Rf</td>
</tr>
<tr>
<td>1</td>
<td>0.61</td>
<td>32000</td>
<td>0.50</td>
</tr>
<tr>
<td>2</td>
<td>0.86</td>
<td>16000</td>
<td>0.94</td>
</tr>
</tbody>
</table>

* Virus proteins were electrophoresed as described in the Methods. Rf is the distance the protein migrated/the distance the dye front migrated. Mol. wt. were obtained by plotting mol. wt. on a log scale versus Rf. As mol. wt. standards, bovine serum albumin (mol. wt. = 67000; Rf = 0.34), ovalbumin (mol. wt. = 43000; Rf = 0.49) and cytochrome c (mol. wt. = 11700; Rf = 0.97) were used. The numbers represent the averages of two separate determinations.

Table 4. Properties of bacteriophages active on Hyphomicrobium

<table>
<thead>
<tr>
<th>Bacteriophage</th>
<th>Density* (g/ml)</th>
<th>Nucleic acid type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyφ1a</td>
<td>1.505 ± 0.008</td>
<td>Green, Orange, 2-DNA</td>
</tr>
<tr>
<td>Hyφ22a</td>
<td>1.498 ± 0.009</td>
<td>Green, Orange, 2-DNA</td>
</tr>
<tr>
<td>Hyφ32a</td>
<td>1.528 ± 0.001</td>
<td>Green, Orange, 2-DNA</td>
</tr>
</tbody>
</table>

* Numbers represent the averages of three separate determinations.

DISCUSSION

Three new bacteriophages active on strains of *Hyphomicrobium*, a budding bacterium, have been isolated. These phages have morphologies similar to other known bacteriophages (Ackermann, 1973). They contain double-stranded DNA and are only active on strains of *Hyphomicrobium*. The receptor sites for these phage are not distributed randomly over their hosts but are either restricted to or only available at limited regions on the surface of the cell. As a result, these phages only adsorb to cells in a particular stage of morphological development.

All of the bacteriophages adsorb to the developing bud (daughter cell) at the end of a prostheca. A similar phenomenon has been reported with Hyφ30, the only other characterized phage active on this bacterium (Voelz et al. 1971). It is possible that the receptor sites for these phages are involved in the formation of new cell wall (membrane) material and hence are found only in the region of a developing daughter cell. The receptor site might be an intermediate in cell wall (membrane) formation or an enzyme complex involved in cell wall (membrane) synthesis. Alternatively, the receptor sites for these phages may be present over the entire surface of the cell but are only exposed in the area of a developing daughter cell. *Hyphomicrobium* is known to produce a holdfast material which is associated with the cell and not the prostheca (Hirsch & Rheinheimer, 1968; Staley, 1974). This material could mask receptor sites on the mother cell leaving only those at the distal end of prostheca available to the phage.

Despite the morphological similarity of phages Hyφ1a and Hyφ22a, it is apparent that they are distinct bacteriophages. They have different host ranges, their densities in CsCl are different and their major proteins are distinguishable by gel electrophoresis.

During studies of the host ranges of these phage, it was found that Hyφ1a and Hyφ32a produced plaques on isolates Hy1, Hy22 and Hy32; Hyφ22a plaqued only on isolate Hy22. The efficiencies of plating on Hyφ1a and Hyφ32a varied depending on which strain was used as an indicator and which strain was used to propagate the phage (D. B. Yelton, un-
Hyphomicrobium \textit{bacteriophages} published observations). The possibility of a restriction-modification system in \textit{Hyphomicrobium} is currently under investigation.

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REFERENCES


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