Isolation and Preliminary Characterization of Bacteriophages for *Bdellovibrio bacteriovorus*

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Received for publication 15 June 1972

Ten bacteriophages that attack and lyse saprophytic strains of *Bdellovibrio bacteriovorus* were isolated. Morphological, serological, and host-range studies revealed that there were four different bdellovibrio phages present among the isolates. One of the phages lysed a strain of *B. bacteriovorus* that requires the presence of a suitable bacterial host for growth. The phage attached to the bdellovibrio cells in the absence of the bacterial host cells; lysis occurred only in the presence of host cells. The 19 saprophytic bdellovibrio strains employed in the phage host-range studies were grouped on the basis of their susceptibility to phage lysis.

*Bdellovibrio bacteriovorus* was initially described as an ectoparasitic and bacteriolytic organism that attacks gram-negative bacteria (18). Subsequently, saprophytic strains were isolated (11, 13, 15, 17); some of these strains were capable of growth on artificial media and initially retained their parasitic abilities. A facultative strain designated UK12, which is capable of growing in host-free media or endoparasitically on *Escherichia coli* B/r, was reported (5). Studies of these parasitic, saprophytic, and facultative strains have considerably expanded our knowledge of the unique bacteria (14, 16). Nevertheless, the classification of *B. bacteriovorus* and the interrelationships of the various strains have not been clearly established.

Since bacteriophages for bdellovibrio could be used to classify the various strains of *B. bacteriovorus* and as probes for elucidating some aspects of the host-endsymbiont relationship, the availability of phages could prove to be useful. We have reported (6) the isolation of a bacteriophage which attacks and lyzes *B. bacteriovorus* strain UK12; this bdellovibrio can grow either in the presence or absence of host cells (5). We have isolated additional bdellovibrio phage which not only lysed saprophytic strains of *B. bacteriovorus*, but also were infective for obligately endosymbiotic strains of bdellovibrio in a system consisting of bdellovibrio, bacterial host, and phage (Mary Althauser and S. F. Conti, Bacteriol. Proc., p. 173, 1971; Abstr. Annu. Meeting Amer. Soc. Microbiol., p. 220, 1972). Varon and Levisohn (19) have recently also reported the isolation of bacteriophages which will attack a parasitic strain of *B. bacteriovorus*.

This paper describes the isolation and preliminary characterization of four different additional bacteriophages for *B. bacteriovorus*.

**MATERIALS AND METHODS**

**Media.** The liquid medium used throughout this investigation for growing the bacteria and diluting the phage suspensions was peptone-yeast extract (PYE) which contains 1% (w/v) peptone (Difco) and 0.3% (w/v) yeast extract (Difco). The pH was adjusted to 7.2 with 1 M NaOH. PYE agar plates for enumeration of phage and parasitic bdellovibrio were prepared by adding 1% (w/v) agar to PYE broth for the bottom layer and 0.7% agar in the overlay.

**Bacterial strains and cultural conditions.** The saprophytic *B. bacteriovorus* strains (100, 109, 110, 114, 118, 120, B, D, E, OX9-2, OX9-3, A3.12, 2484-2, 2484-3, Sa 109, SaD, Sm, Xty) were kindly supplied by R. J. Seidler and M. P. Starr. A facultative strain UK12 (5) and an additional saprophytic strain (UK11) were isolated from raw sewage. *Erwinia amylovora, Proteus mirabilis, Serratia marcescens, Spirillum serpens, Salmonella typhimurium*, and *E. coli* (ATCC 15144), furnished by D. Abram or M. Starr, were employed in the host-range determinations.

The saprophytic bdellovibrio strains were transferred every 12 hr using a 10% (v/v) inoculum. The members of the other genera were grown in a similar manner employing a 1% (v/v) inoculum for transfer. *B. bacteriovorus* 114 (ATCC 15362), a parasitic strain, was grown on *E. coli* (ATCC 15144) in PYE broth. For starter cultures of the parasitic bdellovibrio, 9 ml of a 12-hr PYE broth culture of *E. coli* (4 × 10⁹ cells/ml) in a 16 by 120 mm test tube was infected with 1 ml of an 18- to 24-hr *B. bacteriovorus* 114 culture (10⁹ cells/ml).

All liquid cultures were incubated at 30 C in a gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.).

**Enumeration of bacteria.** The saprophytic cells of
B. bacteriovorus and the bacterial host cells were enumerated by colony counts on PYE agar and the parasitic cells by plaque assay as previously described (5).

Isolation of bacteriophage. Two procedures were used to isolate bacteriophage. In the first isolation procedure 100 ml of raw sewage from the Lexington, Kentucky, sewage treatment plant were clarified by lowspeed centrifugation, and the supernatant fluid was passed through disposable filters of 0.45- and 0.2-μm pore size (Nalge Co., Rochester, N.Y.). The filtrate was centrifugated at 40,000 × g for 1 hr in a Beckman model L-2 ultracentrifuge. The pellet was resuspended in 10.0 ml of PYE broth, and 1 ml of the suspension was plated with 0.05 ml of a 12- to 14-hr culture of each of the following strains of B. bacteriovorus: 100, 109, 118, D, E, UK1, UK2, 2484-2, and Xty, using the double-layer plating procedure described below.

The second method used was a variation of the conventional enrichment technique described for isolating bacteriophage. A 2-liter flask containing 1 liter of raw sewage was inoculated with 20 ml of an overnight culture of each of the nine strains and incubated at 25°C for 72 hr. The flasks were occasionally shaken by hand during this period of time. After incubation, the suspensions were clarified and filtered as described above. A 10-ml amount of a 12- to 14-hr culture of each saprophytic bdellovibio added to a 1-liter flask containing 500 ml of filtrate from the first enrichment. A 5-ml amount of a 20% (w/v) yeast extract (Difco) solution was then added to the flask. After incubation at 25°C for 72 hr, the suspensions were clarified, filtered through a disposable filter of 0.45-μm pore size, and plated as described in the first enrichment procedure.

Plaques which appeared to be of different size or morphology, or both, were picked separately, and if subsequent platings showed these differences were consistent they were treated as different isolates. Plage were purified by at least three successive single plaque isolations. High-titered stocks of each of the phage were prepared by the plate lysis technique (1).

Enumeration of phage. For the plaque isolation experiments, petri dishes containing approximately 20 ml of PYE agar were overlaid with a mixture of 2 ml of PYE overlay agar, either 0.05 ml or 0.3 ml of an exponential-phase culture of the organism, and 1.0 ml of the material being tested for phage. The plating procedure was modified in the experiments to determine the host range of the phage and phage titers by mixing 0.1 ml of an appropriate dilution of the phage suspension with the overlay agar. All plates were incubated at 30°C for 24 to 48 hr. The organisms used in the plaque host-range studies included all the strains of bdellovibrio and the members of the other genera listed under bacterial strains.

Effect of phage MAC-3 in a three-membered system. The effect of one of the phages (MAC-3) on the B. bacteriovorus 114-E. coli system was tested by adding 4 × 10⁶ plaque-forming units (PFU)/ml to a 12-hr culture of E. coli (4 × 10⁹/ml) immediately after the addition of the bdellovibrio (10⁶ cells/ml). Samples were taken from the tube with the three-membered system at zero time and after 12 and 24 hr of incubation at 30°C and assayed for numbers of bdellovibrio, E. coli, and phage. Control tubes, lacking one or more components of the system, were treated similarly; experiments were repeated a number of times with equivalent and reproducible results.

To determine the adsorption kinetics of MAC-3 to B. bacteriovorus 114, 3 × 10⁶ PFU of bdellovibrio cells per ml were infected with MAC-3 at a multiplicity of infection (MOI) of 0.2. Samples (0.1 ml) were removed at various time intervals and diluted with 9.9 ml of cold PYE broth containing a few drops of CHCl₃. The mixtures were shaken on a Vortex shaker for 20 sec and titrated after 1 hr.

To test directly the effect of MAC-3 on the motility of the bdellovibrio cells, 2 × 10⁶ B. bacteriovorus 114 cells/ml were mixed with MAC-3 at ratios of phage to bdellovibrio of 30, 3, and 0.2 and incubated at 30°C. Control tubes contained bdellovibrio cells without phage. Samples were removed after 15, 30, and 60 min of incubation, and the motility of the cells was immediately checked by observation with a Zeiss Universal phase microscope.

To determine the effect of the phage on the ability of the bdellovibrio to attach to and penetrate the host cells, samples from the two-membered system described above and samples from the control tubes were added to 4 × 10⁶ E. coli cells. The attachment and penetration of the parasites within the host cell were observed by phase-contrast microscopy over a 2- to 3-hr period.

Serology. Antigen preparations were obtained by propagating each phage in PYE broth cultures of an appropriate saprophytic strain of B. bacteriovorus. The phage from the broth lysates were then purified by differential and sucrose density gradient centrifugation, followed by dialysis against phosphate-buffered saline as described by Bradley (2). The purity of each phage preparation was examined by direct electron microscopy, the amount of protein was determined by the procedure of Lowry et al. (7), and plaques were assayed. Six intravenous injections, each containing 30 to 50 μg of viral protein, were given to rabbits at 3- and 4-day intervals. Rabbits were bled from the heart 10 days after the last injection.

Each antiserum was tested first against the homologous phage and then against the remaining phages to detect cross-neutralization reactions. In both neutralization tests, heat-inactivated (56°C, 30 min) antiserum was diluted and mixed with an equal volume of a phage preparation containing 10⁵ PFU/ml. At 10-min intervals, 0.1-ml samples of the phage-serum mixture were withdrawn and added to 9.9 ml of diluent to stop the neutralizing action. From these tubes, further dilutions were made and samples were plated by the double layer procedure. Neutralization velocity constants (k min⁻¹) for each antiserum were determined as described by Adams (1). The percentage of phage neutralized was determined, and a velocity constant, k, was calculated at 99%ₐ neutralization by using the relationship k = 2.3 D(1 × log pₛ/p₀ ; where D is the dilution of antiserum used in the determination; tₛ, the time required to obtain 99% neutralization; pₛ, the plaque count before the addition of antiserum; and p₀, the plaque count after neutralization.
**Electron microscopy.** Phage specimens for negative staining were obtained by placing a drop of the purified phage preparation on a carbon-coated grid or by lightly touching the grid to an isolated phage plaque; specimens were stained with 0.2% uranyl acetate.

Thin sections were prepared by using previously described procedures (4); all specimens were examined in a Philips EM 200 electron microscope operating at 60 kv.

**RESULTS**

**Phage isolation and morphology.** Ten bacteriophages that attacked and lysed saprophytic bdellovibrios were isolated from raw sewage (Table 1). Two different morphological plaque types were observed on the plates of the phages initially designated as MAC-1 and MAC-4; each plaque was subsequently purified, and the second type was designated with a prime notation.

Microscopic examination of all ten phage isolates revealed four distinct morphological types. Seven of the phage isolates (MAC-1, 1', 2, 4, 4', 5, and 7) were of identical structure and correspond to Bradley's group E (3). All are tailless, have a regular hexagonal outline, and are approximately 25 nm in diameter (Fig. 1).

The remaining isolates were typical of Bradley's type A bacteriophage, possessing hexagonal heads, long tails, and contractile sheaths. MAC-3 (Fig. 2a) has an elongated hexagonal head 75 nm by 50 nm in diameter, and an overall length of 150 nm. Subunits of the tail are clearly visible (Fig. 2a), and its double base plate and four tail pins are best seen in Fig. 2b. The apparent swelling or collapsing of the head frequently seen when this phage attaches to its host is shown in Fig. 2c. MAC-6 has an overall length of 180 nm and a head diameter of 65 nm (Fig. 3a). The complex tail assembly, consisting of a single base plate and three or four tail pins with spherical structures on their distal end, is shown in Fig. 3b. HDC-2 has a head of 70 to 75 nm and is 150 to 160 nm long. A complex tail assembly as seen on MAC-6 was not seen on the intact or contracted HDC-2 particles (Fig. 4a, b).

Table 1 summarizes the results of cross-neutralization tests conducted to detect serological relationships among the various phage isolates. Antisera to phages MAC-3, MAC-6, HDC-2, and HDC-1 had neutralizing activity only against the homologous phage. The MAC-1 antiserum, however, neutralized the homologous phage, MAC-1, and also neutralized phages MAC-1', MAC-2, MAC-4, MAC-4', MAC-5, and MAC-7. Similar first-order inactivation kinetics were observed when each of the seven phages was exposed to MAC-1 antiserum, indicating that the seven spherical phage are closely related, if not identical.

**Host range of the phage isolates.** The host ranges of the phages among the saprophytic bdellovibrio strains are summarized in Table 2. The spherical phage MAC-1, like all of the other spherical phage isolates had a rather broad host range and lysed 12 of the 19 bdellovibrio strains tested. MAC-3, one of the tail-phages, had a more narrow host range lysing 7 of the 19 strains and was the only isolate to lyse strains 110 and 120. MAC-6, HDC-2, and HDC-1 had very specific host ranges. None of the phage isolates lysed strains UK1, A3.12, or any of the bacterial hosts used to propagate the bdellovibrio.

**Effect of MAC-3 on a host-dependent B. bacteriovorus.** The effect of MAC-3 on a parasitic strain of *B. bacteriovorus* 114 grown on *E. coli* is shown in Table 3. In the test system containing bdellovibrio and host cells, there was a 500-fold increase in bdellovibrio cells after 24 hr of incubation. In the presence of MAC-3 phage, the number of bdellovibrio cells decreased 100-fold in 24 hr, with an accompanying 1,000-fold increase in the phage titer. The MAC-3 phage did not multiply on either the parasitic bdellovibrio cells in the absence of host or on *E. coli*. In accordance with these observations, there was an increase in the number of viable *E. coli* cells remaining after incubation with the bdellovibrio in the presence of phage.

Since the frequency of mutation of parasitic to saprophytic cells is relatively high (11), during the course of the experimentation we assayed for saprophytic cells in the *B. bacteriovorus* strain 114 culture employed; none were detected.
FIG. 1. Negative stain preparation of the tailless, hexagonally shaped phage isolate MAC-1. Scale marker represents 200 nm and applies to Fig. 1 through 4.

FIG. 2. Negative stain preparations of phage MAC-3. The elongated head and subunits of the tail assembly are shown (a); the double base plate (arrows) and small tail pins are evident (b). The heads of MAC-3 are usually spherical or collapsed after attachment to its host (c).

FIG. 3. Negative stain preparations of phage MAC-6. The subunit structure of the uncontracted tail (a) and the complex structure of the tail pins on the contracted phage particles (arrows, b) are illustrated.

FIG. 4. Negative stain preparations of phage HDC-2. The phage has a hexagonal head and distinct tail subunits (a). The contracted phage particle (b) does not show any complex tail structures.

<table>
<thead>
<tr>
<th>Table 2. Host range of five B. bacteriovorus phages</th>
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<tr>
<td>Phage</td>
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<tr>
<td></td>
</tr>
<tr>
<td>MAC-1</td>
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<tr>
<td>MAC-3</td>
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<tr>
<td>MAC-6</td>
</tr>
<tr>
<td>HDC-2</td>
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<td>HDC-1</td>
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</table>

a The homologous host (saprophytic bdellovibrio strains used for initial phage isolation) of phage MAC-1, MAC-3, and MAC-6 are B. bacteriovorus strains Xty, 118, and E, respectively. The facultatively parasitic bdellovibrio strain UKi2 was used for initial isolation of phages HDC-1 and HDC-2.

b B. bacteriovorus strains UKi2 was used for initial isolation of phages HDC-1 and HDC-2.

c +, Lysis of cells; −, no lysis of cells.
Table 3. Effect of phage MAC-3 on the host-dependent Bdellovibrio bacteriovorus strain 114

<table>
<thead>
<tr>
<th>Test system</th>
<th>to</th>
<th>12 hr</th>
<th>24 hr</th>
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</thead>
<tbody>
<tr>
<td>1. Escherichia coli</td>
<td>4 X 10^9</td>
<td>3 X 10^9</td>
<td>3 X 10^9</td>
</tr>
<tr>
<td>B. bacteriovorus 114</td>
<td>1 X 10^7</td>
<td>7 X 10^7</td>
<td>1 X 10^7</td>
</tr>
<tr>
<td>MAC-3</td>
<td>4 X 10^7</td>
<td>6 X 10^9</td>
<td>3 X 10^7</td>
</tr>
<tr>
<td>2. E. coli</td>
<td>4 X 10^9</td>
<td>5 X 10^9</td>
<td>5 X 10^9</td>
</tr>
<tr>
<td>B. bacteriovorus 114</td>
<td>1 X 10^7</td>
<td>7 X 10^9</td>
<td>5 X 10^9</td>
</tr>
<tr>
<td>MAC-3</td>
<td>1 X 10^7</td>
<td>1 X 10^9</td>
<td>3 X 10^9</td>
</tr>
<tr>
<td>3. B. bacteriovorus 114</td>
<td>4 X 10^7</td>
<td>8 X 10^9</td>
<td>5 X 10^9</td>
</tr>
<tr>
<td>MAC-3</td>
<td>4 X 10^7</td>
<td>1 X 10^9</td>
<td>1 X 10^9</td>
</tr>
<tr>
<td>4. E. coli</td>
<td>4 X 10^9</td>
<td>4 X 10^9</td>
<td>4 X 10^9</td>
</tr>
<tr>
<td>MAC-3</td>
<td>4 X 10^7</td>
<td>1 X 10^9</td>
<td>1 X 10^9</td>
</tr>
<tr>
<td>5. MAC-3</td>
<td>4 X 10^7</td>
<td>1 X 10^9</td>
<td>1 X 10^9</td>
</tr>
<tr>
<td>6. B. bacteriovorus 114</td>
<td>1 X 10^7</td>
<td>1 X 10^9</td>
<td>5 X 10^9</td>
</tr>
<tr>
<td>7. E. coli</td>
<td>4 X 10^9</td>
<td>5 X 10^9</td>
<td>4 X 10^9</td>
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</table>

* The test systems, which contained bdellovibrio cells were assayed for the presence of saprophytic bdellovibrio cells at zero time and after 12 and 24 hr of incubation; none were detected. E. coli viable cell counts per milliliter were enumerated by plating on PYE agar, and parasitic cells by plaque assay as described in Materials and Methods; phage counts per milliliter were determined by the double-layer technique (1).

PYE broth was added to the test systems, which contained one or two members to maintain a volume of 10 ml. All cultures were incubated at 30°C on a gyratory shaker.

When the bdellovibrio cells and the phage were mixed in PYE broth at a ratio of 50 to 1, 99% of MAC-3 adsorbed to B. bacteriovorus 114 after 1.5 hr of incubation at 30°C (Fig. 5). Electron micrographs of negatively stained preparations from the three-membered system of phage, bdellovibrio, and E. coli (Fig. 6) revealed the attachment of a bdellovibrio cell to host after 1 hr of incubation of the bdellovibrio and MAC-3 in a ratio of 1 to 30. A section of a sample from the three-membered system of phage, bdellovibrio, and host which was incubated at 30°C for 4 hr shows MAC-3 particles inside a bdellovibrio cell which is within an E. coli cell (Fig. 7). MAC-3 did not affect either the motility of the bdellovibrio cells in the two-membered system or the attachment and penetration of the parasites into the host cells, even when ratios as high as 30 phage to 1 bdellovibrio cell were employed.

These data and observations support the conclusion that MAC-3 adsorbs to and multiplies on B. bacteriovorus strain 114 growing on E. coli.

**DISCUSSION**

Although ten bacteriophages were initially isolated for various strains of B. bacteriovorus, our results indicate that only four different phages were present among the initial isolates. The morphological and serological data and the host

**Fig. 5.** Adsorption of MAC-3 to B. bacteriovorus 114. Bdellovibrio cells (3 x 10^9 PFU/ml in PYE broth) were infected with MAC-3 at an MOI of 0.2. Samples of 0.1 ml were removed at various time intervals and diluted in 9.9 ml of cold PYE broth containing a few drops of CHCl₃. The mixtures were shaken in a Vortex mixer for 20 sec and assayed for phage after 1 hr.
Fig. 6. Negative stain preparation of a three-membered system. Contracted MAC-3 phage particles can be seen adhering to the bdellovibrio cell, which in turn is attached to an E. coli cell. Unattached, uncontracted phage are present (triple arrow).

Fig. 7. Thin-section preparation illustrating MAC-3 phage particles (P) within a bdellovibrio cell (Bd), which is located in the periplasmic space of an E. coli cell (E).
ranges of the phage support the conclusion that phages MAC-1, 1', 2, 4, 4', 5, and 7 are very similar, if not identical; MAC-1 has been selected as the representative of this group. The data also support the conclusion that MAC-3, MAC-6, and HDC-2 are separate and distinct bdellovibrio phage types. It thus appears that seven different phages now have been isolated which are infective for some strains of bdellovibrio: MAC-1, MAC-3, MAC-6, HDC-2, the previously described HDC-1 (6), and the VL-1 and VL-2 phages recently isolated by Varon and Levisohn (19). The apparent ease with which phages for *B. bacteriovorus* have been isolated indicates that utilization of conventional procedures, or minor modifications thereof, should result in the isolation of additional phages from natural habitats containing *B. bacteriovorus*.

The most extensive studies on the saprophic strains of bdellovibrio (9, 11, 12) indicate a very close similarity between all of the saprophic strains tested with the exception of strains A3.12 and the facultative *B. bacteriovorus* UKi2. Furthermore, there are no completely satisfactory methods available for differentiating between the 20 or so saprophic strains used in various laboratories, or for determining the number of truly distinct saprophic strains. Our data (Table 2) on the susceptibility of the various saprophic strains to the phage isolates illustrates that some of the strains can be differentiated from others. On the basis of phage susceptibility, the saprophic strains can be grouped as follows: Group I, strains 100, 109, 114, SaD3Sm, 118; Group II, strains D, O's9-2, 2484-2, 2484-3, Xty, Sa 109; Group III, strains 110 and 120; Group IV, strains B and E; Group V, strains UKi2; and Group VI, strains A3.12 and UKi1, which are strains not infected by any of the phage isolates. The availability of additional phages and appropriate antiserum, combined with other approaches, should allow differentiation between strains within a particular group, and perhaps establish more definitively whether a separate strain designation is warranted for all of the saprophic bdellovibrio isolates.

The recent report by Varon and Levisohn (19) and the data presented in this paper support our previous contention (Mary Althauser and S. F. Conti, Bacteriol. Proc., p. 173, 1971) that some phage which attack, lyse, and replicate on saprophic strains of bdellovibrio are also infective for parasitic strains, provided that a suitable host bacterium is present in the test system. The requirement for the presence of a host bacterium is presumably due to the inability of the parasitic bdellovibrio to grow and divide in the absence of host cells, although they are metabolically active (14).

The three-membered system (host, bdellovibrio, and phage) described by Varon and Levisohn (19) is similar to our system, but there are some significant differences. In their system, and under their conditions, host-free cultures of bdellovibrio which are unable to support phage growth can adsorb the phage particles and are subsequently killed. As a consequence, even a delay of a few minutes in the addition of the host cells to the bdellovibrio-phage system results in a diminished phage yield. In our system the incubation of the phage-bdellovibrio mixture for as long as 6 hr before addition of host cells did not have any noticeable effect on the phage yield. Varon and Levisohn also observed that the motility of the bdellovibrio cells ceased 10 to 20 min after addition of the phage; this did not occur in our system.

The observations that phage which can infect saprophic strains can also attack bdellovibrio which require suitable bacterial host cells for growth is not unexpected in view of the observation of Reiner and Shilo (8) that so called host-dependent or wild-type strains of *B. bacteriovorus* can elongate in the absence of host cells when incubated in cell-free microbial extracts. Subsequent work has resulted in the isolation of a factor which, when added to a parasitic strain of *B. bacteriovorus*, enables cells to grow and divide, i.e., cells go through at least one cycle of elongation and fragmentation (Shilo, personal communication). It would therefore appear that the major difference between saprophic and parasitic bdellovibrios is their ability to grow and divide in the absence of a “factor”; parasitic bdellovibrio can obtain the “factor” from suitable host bacteria whereas saprophic strains can either synthesize this factor or do not require it. Presumably, all so-called host-dependent bdellovibrio can be “transformed” into host-independent cells by addition of the “factor” to the medium under appropriate conditions. We are presently testing the infectivity of our five phage isolates against a number of parasitic bdellovibrio with the expectation that phage infective for saprophic strains will infect the closely related parasitic strains.

**ACKNOWLEDGMENT**

This research was supported by National Science Foundation grant GB 7972.

**LITERATURE CITED**

BACTERIOPHAGES FOR B. BACTERIOVORUS