

# Three-Membered Parasitic System: a Bacteriophage, *Bdellovibrio bacteriovorus*, and *Escherichia coli*

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Two bacteriophages for *Bdellovibrio bacteriovorus* were isolated. One of the phages (VL-1) was isolated on a host-independent *Bdellovibrio* strain, and the other (VL-2) was isolated on a host-dependent strain. Both phages grew on host-dependent as well as on host-independent *Bdellovibrio* strains. The development of the phages in host-dependent bdellovibrios occurred only when the phage-infected bdellovibrios parasitized cells of other bacteria. In the absence of other bacteria, the phages adsorbed to the bdellovibrios and killed them and in the process lost their own plaque-forming ability.

*Bdellovibrio bacteriovorus* is a bacterium which grows as a parasite of other bacteria. The bdellovibrio cells penetrate through the cell wall of the host bacteria into the periplasm, where they increase in size and finally divide into a number of progeny cells (6, 9, 14). In the absence of a suitable host, the bdellovibrios are unable to divide, although they are not metabolically inactive (11). The factor(s) contributed by the host that allows *Bdellovibrio* to complete its life cycle is not known. Host-independent mutants have been isolated from several *Bdellovibrio* strains (4, 10, 12). These mutants grow well in a host-free medium but often lose the ability to parasitize other bacteria when transferred repeatedly in the absence of a host. The frequency of mutation from host dependence to host independence is relatively high (10), indicating that this might be a single mutational event. The nature of the mutation is, however, not known.

Bacteriophages whose development and maturation depend on the metabolic activity of their host cells could be a useful tool in the study of the nature of *Bdellovibrio* parasitism. We have, therefore, looked for bacteriophages for host-dependent, parasitic *Bdellovibrio*. Recently, a phage that attacks the host-independent mutant of *B. bacteriovorus* UKi2 was described (5).

During the present study, we isolated several bacteriophages for *Bdellovibrio* (bdellophages) which grew both on host-independent as well as on host-dependent *Bdellovibrio* strains. The properties of two of these bdellophages and their behavior in a two-membered system (phage + *Bdellovibrio*) and a three-membered system (phage + *Bdellovibrio* + *Escherichia coli*) are described.

## MATERIALS AND METHODS

**Culture media and chemicals.** Media ENB (= NBA), PY, and YP were described by Stolp and Starr (15). DNB medium and TM buffer were described by Varon and Shilo (18). For solid media, 1% Difco agar was added. Streptomycin sulfate was obtained from Rafa, Jerusalem, Israel. Membrane filters were obtained from Sartorius-Membranfilter GMBH, Göttingen, West Germany. Polyethylene glycol (molecular weight 35,000 to 40,000) was purchased from Serva, Heidelberg, West Germany.

**Bacterial strains and culture conditions.** *E. coli* B was obtained from S. P. Champe. *B. bacteriovorus* 109 (host-dependent) and a host-independent (HI) mutant of *B. bacteriovorus* A3.12 were obtained from M. Shilo. HI streptomycin-resistant (Str<sup>r</sup>) mutants of *B. bacteriovorus* 109, XTY, and 3258 were obtained from M. P. Starr. The original strain (109 Davis) from which *B. bacteriovorus* 109 HI Str<sup>r</sup> was derived is not identical to the host-dependent *B. bacteriovorus* 109 (109 Jerusalem) obtained from M. Shilo (S. C. Rittenberg, *personal communication*). However, in our experiments, there was no difference between the two host-dependent strains of *B. bacteriovorus* 109 with respect to bdellophages VL-1 and VL-2.

We isolated a Str<sup>r</sup> mutant of *E. coli* B from a colony that appeared on a streptomycin-containing (100 µg/ml) ENB plate and a Str<sup>r</sup> *B. bacteriovorus* 109 from a plaque that appeared on a lawn of *E. coli* B Str<sup>r</sup> growing on a DNB plate containing streptomycin (100 µg/ml). Mutants of *B. bacteriovorus* 109 HI resistant to VL-1 or VL-2 were obtained by spreading approximately 10<sup>8</sup> bdellovibrios and 10<sup>6</sup> phage plaque-forming units (PFU) on ENB plates and picking colonies that appeared after 1 week of incubation at 32 C. Mutants of *B. bacteriovorus* 109 (the wild-type, host-dependent strain) resistant to VL-1 or VL-2 were obtained by spreading approximately 10<sup>7</sup> bdellovibrios, 5 × 10<sup>9</sup> *E. coli* cells, and

$10^6$  phage PFU and DNB plates and picking plaques which appeared after 5 to 6 days of incubation at 32 C.

*E. coli* strains and host-independent *Bdellovibrio* strains were grown in ENB medium with or without streptomycin (50  $\mu$ g/ml). Host-dependent *Bdellovibrio* strains were grown with *E. coli* on DNB medium as previously described (16).

**Enumeration of bacteria and bdellophages.** *E. coli* and host-independent *Bdellovibrio* strains were enumerated by colony counts on ENB plates. Host-dependent *Bdellovibrio* strains were enumerated by a plaque assay as described previously (17). Bdellophages were enumerated by a plaque assay on ENB plates with approximately  $10^8$  HI bdellovibriosis per plate with the double-layer technique (1).

**Isolation of bdellophage VL-1.** Thirty-five milliliters of sewage water was mixed with 5 ml of 10 times concentrated YP medium, 10 ml of a fresh *B. bacteriovorus* 109 HI culture, and 2.5 mg of streptomycin. After 5 days of shaking at 32 C, the culture was centrifuged at  $12,000 \times g$  for 5 min. The supernatant fluid was filtered through Sartorius membrane filters of 0.45  $\mu$ m pore size. Samples of 0.1 ml of the filtrate were plated on streptomycin-containing YP plates with *B. bacteriovorus* 109 HI as indicator. Several plaques appeared after 2 to 3 days of incubation at 32 C. One of the plaques which appeared after 2 days was transferred three times on YP plates to obtain a clone and designated VL-1.

**Preparation of phage stocks.** An exponentially growing *B. bacteriovorus* 109 HI culture was infected with a fresh lysate at a multiplicity of infection (MOI) of 0.01 to 0.05 phage per bacterium and incu-

bated overnight at 32 C on a New Brunswick gyratory shaker. Lysis was completed with chloroform, and the debris was removed by centrifugation for 10 min at  $12,000 \times g$ . The phages were concentrated by dialysis against polyethylene glycol to 10 to 20% of the original volume. The concentrated suspension was then subjected to two cycles of low-speed (10,000 rev/min, 10 min) and high-speed (45,000 rev/min, 2 hr) centrifugation in a Beckman ultracentrifuge (rotor SW-50), the final step being the removal of debris at low speed. The stock suspensions were made in ENB containing streptomycin (50  $\mu$ g/ml) and kept in the refrigerator over a drop of chloroform or at -20 C with 20% glycerol. The half-life time of such a suspension was approximately 35 days.

## RESULTS

**Properties of bdellophage VL-1.** Electron micrographs of negatively stained concentrated VL-1 lysate revealed a phage with a hexagonal head 60 nm in diameter and a contractile tail which at the extended state reaches a length of 80 nm (Fig. 1). It can thus be classified in Bradley's group A (3). Fluorescent staining by the method of Bradley (2) and sensitivity of the nucleic acid to treatment with deoxyribonuclease I (20  $\mu$ g/ml for 2 hr at 37 C), but not to pancreatic ribonuclease (20  $\mu$ g/ml for 2 hr at 37 C), indicate that this phage contains double-stranded deoxyribonucleic acid.

**Effect of VL-1 on HI *Bdellovibrio*.** Bdellophage

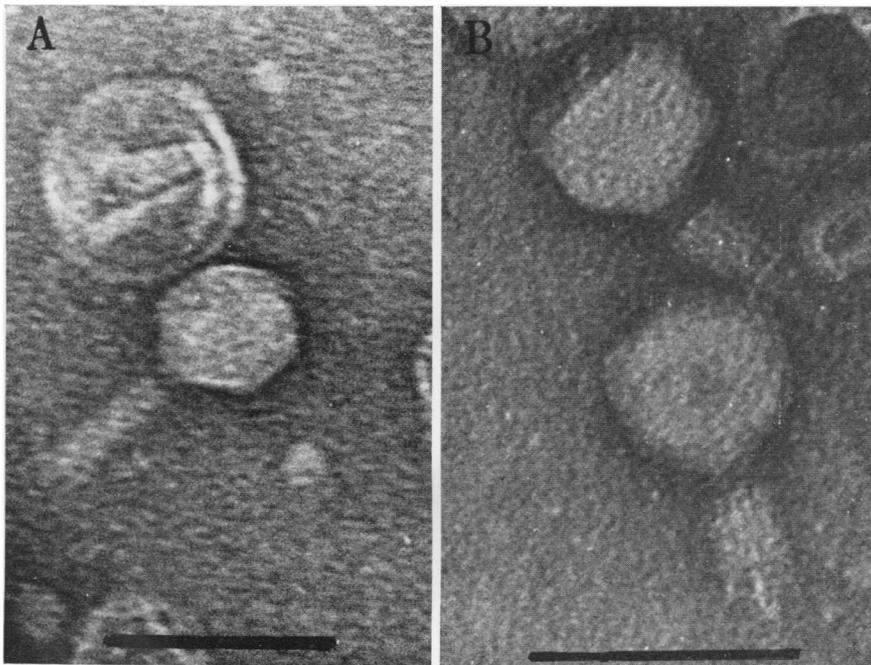


FIG. 1. Electron micrographs of bacteriophage VL-1 negatively stained by 0.5% uranyl acetate. Magnification bars represent 100 nm. (A) Phages with extended tail. (B) Phages with contracted tail.

TABLE 1. Effect of VL-1 on host-dependent wild-type and phage-resistant *Bdellovibrio bacteriovorus* 109

Members of the system <sup>a</sup>	Titer per ml after 22 hr at 32 C					
	<i>Bdellovibrio</i> 109			<i>Bdellovibrio</i> 109 VL-1 <sup>r</sup>		
	<i>E. coli</i>	<i>Bdellovibrio</i>	VL-1	<i>E. coli</i>	<i>Bdellovibrio</i>	VL-1
<i>E. coli</i> .....	2 × 10 <sup>9</sup>			4 × 10 <sup>9</sup>		
<i>Bdellovibrio</i> .....		7 × 10 <sup>7</sup>			5 × 10 <sup>7</sup>	
VL-1.....			2 × 10 <sup>7</sup>			1 × 10 <sup>7</sup>
<i>E. coli</i> + <i>Bdellovibrio</i> ....	1 × 10 <sup>9</sup>	1 × 10 <sup>10</sup>		1 × 10 <sup>9</sup>	3 × 10 <sup>9</sup>	
<i>Bdellovibrio</i> + VL-1.....		2 × 10 <sup>7</sup>	2 × 10 <sup>5</sup>		5 × 10 <sup>7</sup>	3 × 10 <sup>6</sup>
<i>E. coli</i> + VL-1.....	1 × 10 <sup>9</sup>		4 × 10 <sup>6</sup>	3 × 10 <sup>9</sup>		1 × 10 <sup>7</sup>
<i>E. coli</i> + <i>Bdellovibrio</i> + VL-1.....	2 × 10 <sup>9</sup>	1 × 10 <sup>6</sup>	1 × 10 <sup>8</sup>	1 × 10 <sup>1</sup>	3 × 10 <sup>9</sup>	4 × 10 <sup>7</sup>

<sup>a</sup> Starting concentrations in DNB medium were: *E. coli* B, 3.5 × 10<sup>9</sup> cells/ml; *Bdellovibrio* 109 (wild type or phage-resistant), 6.0 × 10<sup>7</sup> PFU/ml; bdellophage, 1.3 × 10<sup>7</sup> PFU/ml.

VL-1 formed plaques on lawns of HI mutants of *B. bacteriovorus* 109, XTY, and 3258. The plaques were visible after 1 day of incubation at 32 C, but, since the growth of the indicator was slow, contrast at this time was very poor. The plaques were better seen after 2 days and they continued to grow for several days. VL-1 did not form plaques on a HI mutant of *B. bacteriovorus* A3.12.

Liquid cultures of *B. bacteriovorus* 109 HI growing in ENB medium and infected with VL-1 at an MOI 0.01 to 0.05 lysed within 16 to 24 hr, yielding 10<sup>9</sup> to 10<sup>10</sup> PFU/ml.

**Effect of VL-1 on host-dependent *Bdellovibrio*.** The effect of VL-1 on host-dependent *Bdellovibrio* was examined in a three-membered system containing *E. coli*, *Bdellovibrio*, and phage and in a two-membered system containing *Bdellovibrio* and phage. The mixtures were incubated for 22 hr, and the final number of each member was determined (Table 1). In a mixture of *E. coli* and *Bdellovibrio*, there was a large decrease in the number of *E. coli* cells and a large increase in the number of bdellovibrios compared to the respective single organism controls. When, however, bdellophages were added to the mixture, the *E. coli* cells were "saved" and the titer of *Bdellovibrio* dropped. The phage titer in the three-membered system increased although it did not reach the titer observed in an HI *Bdellovibrio* lysate. In the two-membered *Bdellovibrio* 109 phage system, there was no multiplication of the phage; on the contrary, the phage titer dropped to about 1% of the single-membered phage control, and at the same time there was a small but significant decrease in the *Bdellovibrio* titer.

In a three-membered system in which the wild-type *Bdellovibrio* was replaced by a phage-resistant mutant (Table 1), *E. coli* cells were killed and the bdellovibrios multiplied regardless of

whether the phage was present. In a two-membered system of *Bdellovibrio* and phage, the decrease in the titers of both phage and *Bdellovibrio* was much smaller compared to the decreases observed with the wild type.

To test directly the effect of the phage on the parasitic properties of the bdellovibrios, a mixture was prepared containing 2.5 × 10<sup>9</sup> phage PFU/ml and 0.5 × 10<sup>9</sup> *Bdellovibrio* PFU/ml. The mixture was divided into several portions and incubated at 32 C. At intervals, *E. coli* was added to a final concentration of 0.7 × 10<sup>9</sup> cells/ml, and, after further incubation for 30 and 60 min, samples were withdrawn for microscopic observation. The bdellovibrios lost their motility as well as their ability to attach within 10 to 20 min after the addition of phage. In a control experiment in which the bdellovibrios were pre-incubated in the absence of phage, they attached to the *E. coli* cells and penetrated into them at the usual rate (17).

**Adsorption of VL-1 to free and intracellular host-dependent *B. bacteriovorus* 109.** As seen from Table 1, phage VL-1 did not multiply in *Bdellovibrio* cells, unless *E. coli* was added. Moreover, the phage titer was reduced by about 99% in the two-membered system. This decrease could be explained if the phages were irreversibly adsorbed to the nongrowing bdellovibrios or inactivated because of adsorption. We measured, therefore, the adsorption kinetics of VL-1 to host-dependent *B. bacteriovorus* 109 in the absence of *E. coli*. The experiment showed (Fig. 2) that the bdellophages did adsorb to the bdellovibrios. A separate experiment showed that the adsorption rate of phage VL-1 to the host-dependent *Bdellovibrio* was similar to the rate of adsorption to the HI mutant. Adsorption of VL-1 to *B. bacteriovorus* 109 VL-1<sup>r</sup> was much slower (Fig. 2).

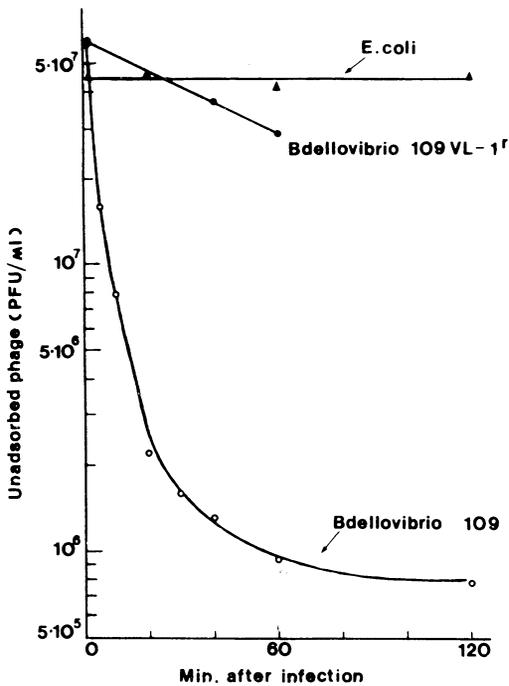


FIG. 2. Adsorption of VL-1 to *Bdellovibrio bacteriovorus* 109. *Bdellovibrio* 109 ( $6.2 \times 10^8$  PFU/ml in DNB medium) was infected with VL-1 at an MOI of 0.1. At intervals, samples of 0.1 ml were withdrawn and diluted into 9.9 ml of ice-cold,  $\text{CHCl}_3$ -containing DNB medium, shaken for 30 sec on a Vortex mixer, and titrated after 1 hr. The figure also shows adsorption of VL-1 to *Bdellovibrio* 109 VL-1<sup>r</sup> ( $7.0 \times 10^8$  PFU/ml, MOI of 0.1) and to *E. coli* B ( $3.7 \times 10^{10}$  cells/ml, MOI of 0.001).

To examine whether bdellophages can adsorb to *Bdellovibrio* cells after they have penetrated into *E. coli* cells, we infected *E. coli* with *Bdellovibrio* under conditions in which the majority of the bdellovibrios penetrate into the *E. coli* cells within 20 min (17). At different intervals after mixing host and parasite, we added bdellophages at a multiplicity of 0.1 phage per bdellovibrio. After additional incubation for 60 min, the number of free bdellophages was determined. As shown in Fig. 3, the fraction of bdellophages able to adsorb decreased as the number of extracellular bdellovibrios decreased. If we compare the curve in Fig. 3 to the known kinetics of *Bdellovibrio* penetration (17), it seems that the decrease in phage receptor sites parallels the decrease in the number of extracellular bdellovibrios. In other words, it seems that once inside the *E. coli* cells the bdellovibrios are no longer accessible to the phages.

#### Growth of VL-1 in a three-membered system.

As indicated in the experiment depicted in Fig. 3,

infection of bdellovibrios by phage was not efficient when *E. coli* cells were added earlier. On the other hand, addition of *E. coli* after infection of bdellovibrios by phage resulted in a poor infectivity of the parasites as observed microscopically. Poor infectivity is likely to reduce the chances of both *Bdellovibrio* and phage to grow. This was confirmed by the following experiment. *B. bacteriovorus* 109 ( $2.6 \times 10^8$  PFU/ml) was infected with VL-1 at an MOI of 0.13. Ten and 20 min after phage infection, a 50-fold excess of *E. coli* cells was added to two different portions of the two-membered system. After 1 hr at

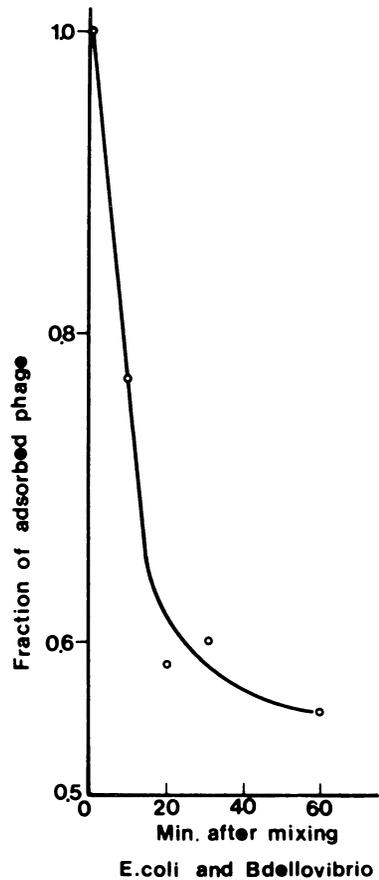


FIG. 3. Adsorption of VL-1 to intracellular *Bdellovibrio bacteriovorus* 109. *E. coli* was infected with *Bdellovibrio* at an MOI of 0.05 in DNB medium, and the mixture was divided into 0.9-ml portions in 50-ml Erlenmeyer flasks. At different times, 0.1 ml of the phage suspension was added to one of the flasks. The final concentration of bdellovibrios was  $1.1 \times 10^9$  PFU/ml and the MOI was 0.07 phages per bdellovibrio. The number of unadsorbed phages was determined after 60 min of incubation, and the fraction of adsorbed phages was calculated from the total input number. The number of unadsorbed phages at zero time was determined from an experiment in which *E. coli* was omitted.

32 C, the number of adsorbed phages was  $3.1 \times 10^7$  PFU/ml. At this time, the three-membered cultures were diluted 1:1,000 to prevent further adsorption and incubated for an additional 5 hr. The phage titer obtained was  $1.2 \times 10^9$  PFU/ml when *E. coli* was added at 10 min, but only  $3.1 \times 10^8$  PFU/ml when *E. coli* was added at 20 min, after phage infection.

In Fig. 4, a one-step growth experiment in a three-membered system is shown. *B. bacteriovorus* 109 was infected with VL-1; 10 min later *E. coli* was added. The general pattern is similar to the growth of other bacteriophages, but the latent period was not constant and it varied in different experiments from 2 to 4 hr. The duration of the latent period is thus similar in time and

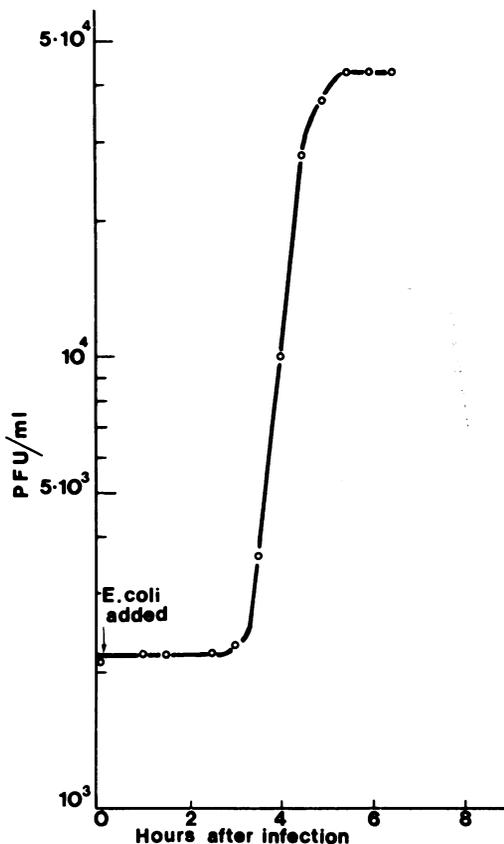


FIG. 4. One-step growth of VL-1 in a three-membered system. *Bdellovibrio* 109 ( $8.0 \times 10^8$  PFU) was infected with VL-1 ( $2.4 \times 10^7$  PFU) in DNB medium and incubated at 32 C. *E. coli* ( $1.6 \times 10^{10}$  cells) was added 10 min later. The final volume was 1 ml. Sixty minutes after infection, the mixture was diluted  $10^4$ -fold in DNB medium and the diluted suspension was incubated further. Samples of 0.1 ml were withdrawn at intervals for phage titration.

variation to the latent period in the *Bdellovibrio* life cycle (18).

**Isolation of bdellophage for host-dependent *Bdellovibrio*.** The results presented in Table 1 suggest a way for enrichment and isolation of bdellophages for host-dependent *Bdellovibrio* strains. Such bdellophages were indeed obtained with the following procedure. A 50-ml amount of sewage water was inoculated with 1 ml of fresh culture of *B. bacteriovorus* 109 and 1 ml of an overnight culture of *E. coli* B and incubated for 3 days at 32 C. One milliliter of a filtrate of this enrichment culture was inoculated into 50 ml of TM buffer containing approximately  $2 \times 10^{11}$  *E. coli* cells and  $2 \times 10^9$  bdellovibrios. The buffer itself does not support growth of bacteria, but the bdellovibrios grow well inside the *E. coli* cells (18). Thus, the only bacteriophages that would grow under these conditions would be bdellophages. The culture was incubated for 24 hr at 32 C and examined microscopically. It contained *E. coli* cells only and no bdellovibrios could be detected. The bacteria were removed by centrifugation (10,000  $\times$  g, 10 min), and the supernatant fluid was titrated by the end-point dilution technique with *B. bacteriovorus* 109 and *E. coli* in TM buffer. The last dilution which prevented *Bdellovibrio* growth was  $10^{-6}$ . Several transfers were made from the highest dilution, and the phage thus obtained was designated VL-2.

**Effect of VL-2 on *B. bacteriovorus* 109.** Similarly to bdellophage VL-1, VL-2 immobilized the host-dependent bdellovibrios at an MOI of 3 within 10 to 20 min after infection. At this time, the bdellovibrios aggregated to form clumps. The clumps enlarged until they contained dozens of cells.

Table 2 shows the effect of phage VL-2 on the

TABLE 2. Effect of VL-2 on host-dependent *Bdellovibrio bacteriovorus* 109

Members of the system <sup>a</sup>	Titer per ml after 22 hr at 32 C		
	<i>E. coli</i>	<i>Bdellovibrio</i>	VL-2
<i>E. coli</i> .....	$2 \times 10^9$		
<i>Bdellovibrio</i> .....		$5 \times 10^7$	
VL-2 .....			$1 \times 10^7$
<i>E. coli</i> + <i>Bdellovibrio</i> .....	$5 \times 10^4$	$6 \times 10^9$	
<i>Bdellovibrio</i> + VL-2 .....		$2 \times 10^7$	$4 \times 10^5$
<i>E. coli</i> + VL-2 .....	$1 \times 10^9$		$1 \times 10^7$
<i>E. coli</i> + <i>Bdellovibrio</i> + VL-2 .....	$2 \times 10^9$	$4 \times 10^5$	$2 \times 10^8$

<sup>a</sup> Starting concentrations in DNB medium were: *E. coli* B,  $3.8 \times 10^9$  cells/ml; *Bdellovibrio* 109,  $1.2 \times 10^8$  PFU/ml; and VL-2,  $1.2 \times 10^7$  PFU/ml.

ability of *B. bacteriovorus* 109 to kill *E. coli*. Like VL-1, phage VL-2 also prevented *Bdellovibrio* growth and "saved" the *E. coli*. The phage titer increased only in a three-membered system and dropped significantly in the presence of *Bdellovibrio* alone.

**Effect of VL-2 on HI *Bdellovibrio* strains.** Bdellophage VL-2 grew on *B. bacteriovorus* 109 HI and yielded a similar titer as VL-1. The plaque morphology was, however, different, VL-2 plaques being smaller and more heterogeneous in size. Their appearance was slower and they could be counted easily only after 3 to 4 days of incubation.

VL-2 seemed to adsorb to different receptor sites than VL-1 on both the HI mutant and the host-dependent wild type, since mutants resistant to one phage but not to the other were obtained.

Like VL-1, VL-2 lysed the HI mutants of *B. bacteriovorus* XTY and 3258 but not the HI mutant of *B. bacteriovorus* A3.12.

## DISCUSSION

Since their discovery in 1970 by Hashimoto et al. (5), several bdellophages have been isolated (Althausen and Conti, *Bacteriol. Proc.*, p. 173, 1971; Varon and Levisohn, *unpublished data*). They seem to be abundant in sewage and to include different phage types. The two phages described here do not distinguish between the wild-type, parasitic *Bdellovibrio* and its HI mutant. They grow well on both bacteria. However, in the case of the parasitic *Bdellovibrio*, the phage is propagated only when infection takes place in the presence of a host for the *Bdellovibrio*.

It is now known that, although they require host bacteria for their multiplication, the host-dependent bdellovibrios are able to perform a number of functions in the absence of host cells; they can, for example, carry out a significant amount of endogeneous respiration (8), produce energy (13), move rapidly (15), incorporate amino acids into acid-insoluble material, and regenerate artificially sheared flagella (M. Varon, Ph.D. thesis, The Hebrew University, Jerusalem, 1969). Our results show that the functions supplied by host-dependent bdellovibrio, in the absence of host, are not sufficient to support bacteriophage growth, at least not under the conditions employed in this study.

Upon infection of a host bacterium, the *Bdellovibrio* cell undergoes a differentiation process from a rapidly motile, nongrowing cell to an aflagellated cell with increased biosynthetic activities, which grows and divides into a number of progeny cells (11). Triggering a similar differentiation process in vitro might be possible, and

it has already been attempted by Reiner and Shilo, who examined the response of bdellovibrio suspensions to externally added bacterial extracts (7). A possible shift in the host-dependent bdellovibrio metabolism in response to an examined factor might allow phage development, even though the bdellovibrio itself need not divide. A relatively large burst of the phage would then afford a sensitive means to follow the effects of various factors on bdellovibrio differentiation.

Under the conditions employed in this study, host-free cultures of *Bdellovibrio* which are unable to support phage growth can adsorb the phage particles and are subsequently killed. Such adsorption can be suicidal for phages because they become inactivated by the adsorption or trapped through irreversible binding to the cells which they kill (Table 1). As shown, even a few minutes delay in the addition of *E. coli* resulted in a diminished phage yield. How then do these phages survive in nature, where a suitable host for the bdellovibrio may not be immediately present? It is possible that, under different conditions from the ones utilized in this study, the adsorption of phages to free bdellovibrios is reversible and becomes irreversible only after the bdellovibrio is firmly attached to its host. Alternatively, were the bdellovibrios in nature able to grow in the absence of host cells [as they do under certain laboratory conditions (7)], the phages might have been able to reproduce. Moreover, it may be that *Bdellovibrio* in nature exists mostly in the HI form, in which case there would be no need for secondary host bacteria for the development of bdellophages. At present no HI *Bdellovibrio* strains are known to exist in nature; however, with the techniques currently employed for *Bdellovibrio* isolation, such strains probably would not appear. It is also possible that bdellophages multiply in nature in a similar way to our isolated three-membered system; yet the frequency of successful successive events (phage adsorbing to bdellovibrio and infected bdellovibrio attaching to another host bacterium soon afterwards, or vice versa) is sufficient for the survival of the phage species.

There is at present no indication as to the mechanism of release of the newly formed bdellophages from their host bacteria. Assuming that bdellophages are reproduced in the intracellularly growing bdellovibrios, they, unlike other bacteriophages, have two barriers to cross at the end of their growth cycle: the envelope of their immediate host as well as that of the secondary bacterial host. Whether this is accomplished by phage enzymes, *Bdellovibrio* enzymes, or in any other way is a matter for further investigation.

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