Development of Bdellophage VL-1 in Parasitic and Saprophytic Bdellovibrios

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Ultrastructure was correlated with growth kinetics of bdellophage VL-1 infecting host-dependent ("parasitic") *Bdellovibrio bacteriovorus* 109J in its *Escherichia coli* B host (the three-membered system), as well as in the host-independent ("saprophytic") derivative of the *Bdellovibrio*. Electron microscope observations showed the arrested growth of the phage-infected bdellovibrios, polar localization of the phage progeny, and stages in their release. Present evidence indicates that bdellophage DNA is derived from both the *Bdellovibrio* and its host cell.

A unique parasitic system has recently been described consisting of a bacteriophage (bdellophage) developing inside a bacterium (*Bdellovibrio*) which is itself a parasite developing in the periplasm of still another bacterium (1, 17). Bdellophage development in parasitic bdellovibrios can only proceed in the presence of a suitable host for the *Bdellovibrio*, although host-independent *Bdellovibrio* mutants can support normal phage growth in an ordinary medium (17). In this investigation we have compared the development of bdellophage VL-1 in both parasitic and saprophytic bdellovibrios, and attempted to follow the various stages in the establishment, development, and final breakdown of the three-membered system.

MATERIALS AND METHODS

Culture media and chemicals. Media ENB (= NBA) and YP were described by Stolp and Starr (14), DNB medium was described by Shilo and Bruff (12) and TM buffer by Varon and Shilo (18). Difco agar (1%) was added for solid media. Polyethylene glycol (molecular weight 35,000 to 40,000) was purchased from Serva, Heidelberg, West Germany. [*Methyl-³H*]thymidine was obtained from the Nuclear Research Center, Beersheba, Israel. Millipore membrane filters used were of type HA, 0.45 μ m average pore size.

Bacterial strains and culture conditions. *Escherichia coli* B, its streptomycin-resistant mutant (Str^r), and its host-dependent (HD) *Bdellovibrio bacteriovorus* 109J Str^r were grown as described by Varon and Shilo (18). A host-independent (HI) mutant of this bdellovibrio strain was isolated from a two-membered culture with *E. coli* B by selection on streptomycin-containing ENB plates. Stocks of bdellophage VL-1

were prepared as described by Varon and Levisohn (17).

E. coli strains and HI *Bdellovibrio* were grown in ENB medium with or without streptomycin (50 μ g/ml).

The HD *Bdellovibrio* titer was determined by plaque assay with *E. coli* B Str^r as a host (18); phage titer was determined by plaque assay with HI *Bdellovibrio* as host (17).

Measurement of phage DNA injection. Phage DNA was labeled by addition of adenosine (250 μ g/ml) and [*methyl-³H*]thymidine (5 μ Ci/ml, specific activity 26 Ci/mmol) to the medium. *Bdellovibrio* (3×10^8 cells) was infected with the labeled phages at an input multiplicity of 1 in DNB medium. Samples of 0.2 ml were withdrawn into 1.8 ml of ice-cold DNB medium. The dilute samples were immediately filtered on 0.45- μ m membrane filters to determine adsorption, or after 2-min treatment in a Servall Omnimix (microattachment) to determine the amount of DNA injected into the cells (7). Each filter was washed with 100 ml of cold nutrient broth to remove nonadsorbed phages. The filters were dried and the radioactivity remaining on them was determined in a liquid scintillation counter (Packard) with a toluene-based scintillation fluid.

Electron microscopy. Cells for ultrathin sectioning were fixed by the standard Ryter-Kellenberger procedure (9) and embedded in Spurr's low viscosity medium (13). Sections were cut with a diamond knife on an LKB III Ultratome and mounted on uncoated 300-mesh grids. Serial sections were mounted on carbon coated-collodion 100-mesh grids. The sections were stained with lead citrate and uranyl acetate. For negative staining, cells and phages were stained with 1% aqueous ammonium molybdate (pH 7.0) and 1% aqueous uranyl acetate (pH 5.0). Specimens were viewed in a Philips EM300 operating at either 60 kV or 80 kV with a 50- μ m aperture. Micrographs were

recorded on Kodak Electron Image Plates.

General design of growth experiments. Either HD or HI bdellovibrios, at concentrations of 1.5×10^9 PFU/ml, were infected in various media, as noted, with bdellophage VL-1 at a multiplicity of infection of 1 to 2, and were incubated in a shaking water bath at 32 C. Thirty minutes after phage infection, the mixture was diluted 1:100 in the same medium to prevent further adsorption.

For the three-membered system *E. coli* was added to the bdellovibrio suspension 10 min before phage infection to a final concentration of 2.5×10^8 cells/ml.

For total phage count the cells were further diluted 1:100 and disrupted by a 30-s treatment in a Braun disintegrator with 0.1-mm glass beads. Samples of 80 ml were taken for electron microscopy preparation.

RESULTS

Bdellophage development in a three-membered system (phage, Bdellovibrio and *E. coli*). Figure 1 shows a one-step curve of bdellophage VL-1 in a three-membered system with *Bdellovibrio* 109J and *E. coli* B incubated in DNB medium at 32 C. The eclipse period lasted for 60 min and the latent period ended 45 min later. The average burst size measured under these conditions was 15.

FIG. 1. One-step growth curve of phage VL-1 in a three-membered system with *Bdellovibrio* and *E. coli* in DNB medium. Symbols: infective centers, O; total phage count after premature lysis by mechanical disintegration, ●.

Electron microscope examination of samples taken at various times during the growth cycle shows the following: attachment (Fig. 2) and entry (Fig. 3) of phage-infected bdellovibrios into the *E. coli* are similar to that observed in the two-membered system with uninfected bdellovibrio and *E. coli* host (Fig. 4, 5). The bdellovibrio constricts as it enters through the narrow opening ("penetration pore") into the periplasmic space. In the three-membered system the adsorbed phages are scraped off the bdellovibrio as it enters its host (see Fig. 3) and remain clustered at the penetration pore (Fig. 6). In the periplasmic space of the *E. coli* cell, the noninfected bdellovibrio elongates into a horseshoe shape (Fig. 7) and finally fragments into several progeny (Fig. 8), whereas the host protoplast dissolves. Phage-infected bdellovibrios, on the other hand, do not elongate (Fig. 9) and the *E. coli* protoplast remains cytologically intact.

No further unusual morphological features are evident throughout the eclipse period in either the bdellovibrio or the *E. coli*. At the end of the eclipse period, the first phage heads become visible in the bdellovibrio cytoplasm. Phage progeny are localized at the poles of the bdellovibrio cell apparently in the cytoplasm rather than in the nucleoplasm (Fig. 10). This polar localization of the developing phages is best demonstrated in serial sections at this stage (Fig. 11). At about 3 h, corresponding to the end of the rise period, the wavy outer wall layer of the infected bdellovibrio ruptures (Fig. 12), whereas its plasma membrane remains intact. The resulting cell wall fragments vesicularize and may be seen in the periplasmic space. After bdellovibrio wall dissolution, the bdellovibrio membrane ruptures and phage particles (Fig. 13) can be observed within the remnants of the bdellovibrio cell.

At an advanced stage of lysis of the bdellovibrio cell, free phages are observed within the periplasmic space (Fig. 14). A study of serial sections at this stage determined that the phages were indeed free and not connected to the disintegrating bdellovibrio. The *E. coli* spheroplast eventually collapses and lyses, releasing the phages which are immediately adsorbed to other noninfected bdellovibrios or to bacterial cell debris.

Since *Bdellovibrio* 109J can grow normally in resting *E. coli* cells (18), we examined the development of phage VL-1 in the three-membered system suspended in TM buffer. Microscopically there were no differences between this system and that incubated in the DNB

FIG. 2. Attachment of phage-infected HD *Bdellovibrio* to host. *Bdellophage* VL-1 was added to the incubation system 10 min after mixing *E. coli* and *Bdellovibrio*. Empty-headed phages (P) are seen attached by their tails to the *bdellovibrio* (B), attached to *E. coli* (E). Negatively stained with 1% aqueous uranyl acetate. Magnification $\times 47,196$. Scale represents $0.5 \mu\text{m}$.

FIG. 3. Phage-infected HD *Bdellovibrio* at a late stage of penetration. *Bdellophages* are seen attached only to the *bdellovibrio* surface remaining outside the host; *E. coli* cell wall (EW), *E. coli* protoplast (EP). Negatively stained with 1% aqueous ammonium molybdate. Magnification $\times 47,196$. Scale represents $0.5 \mu\text{m}$.

FIG. 4. Thin sections of uninfected HD *Bdellovibrio* interaction with *E. coli*. Early stage of *bdellovibrio* penetration into its host. The outer wall membrane of the *E. coli* (EW) has been ruptured at the point of entry of the *bdellovibrio*. Magnification $\times 46,170$. Scale represents $0.5 \mu\text{m}$.

FIG. 5. Further penetration showing constriction of the *bdellovibrio* entering cell B; in the adjacent cell, A, a *bdellovibrio* is seen in the periplasmic space, between the cell wall (EW) and the plasma membrane (EPM) of the *E. coli*. Note smoothness of *E. coli* wall compared to its wavy appearance at early stages of infection (Fig. 4).

FIG. 6. Phage-infected *Bdellovibrio* after entry into the *E. coli* host. Empty headed phages are seen at the penetration pore (PP) where they cluster after being scraped off the *bdellovibrio* during its entry. Negatively stained with 1% aqueous ammonium molybdate. Magnification $\times 57,680$. Scale represents $0.5 \mu\text{m}$.

FIG. 7. Thin section of horseshoe-shaped *bdellovibrio* at almost maximum elongation before segmentation into progeny cells, 120 min after infection. Magnification $\times 46,170$. Scale represents $0.5 \mu\text{m}$.

FIG. 8. Thin section of host cell containing progeny *bdellovibrio* with newly synthesized flagella (F) immediately prior to lysis, 150 min after infection. Magnification $\times 46,170$. Scale represents $0.5 \mu\text{m}$.

medium. Repeated one-step experiments showed, however, that the average burst size in buffer is smaller by a factor of 2 to 4.

Bdellophage development in HI *Bdellovibrio*. Figure 15 shows the growth of bdellophage VL-1 on HI *Bdellovibrio* in YP medium. The eclipse and latent periods are similar to that observed in the three-membered system (see Fig. 1). The average burst size is also similar but the rise is slower, and the plateau is reached only after 3.5 h. Similar results were obtained in ENB medium.

Electron microscope examination of thin sections (Fig. 16) 2 h after infection shows mature phage particles in a polar arrangement similar to that in phage-infected intraperiplasmic bdellovibrios (see Fig. 10, 11). An hour later, the cell wall begins to rupture with the eventual release of phage progeny.

Interaction of bdellophage with HD bdellovibrios in the absence of *E. coli*. Bdellophage

VL-1 adsorbs to but does not multiply within HD bdellovibrios in the absence of a suitable host (17). Using radioactively labeled phages, we found that phage DNA is injected into free HD bdellovibrios (Fig. 17). Injection is a rapid process, lagging behind the adsorption by only a few minutes. The presence of apparently empty phage heads attached to the free bdellovibrios observed in the electron microscope also suggests that the phage DNA was injected.

No further development of the phage could be demonstrated in thin sections of infected HD bdellovibrios in the absence of *E. coli*. Morphologically, they resembled thin sections of uninfected bdellovibrios incubated under the same conditions.

The possibility that limited bdellophage reproduction took place, but the progeny were not released from the cells was ruled out by plaque counts of mechanically disintegrated, infected bdellovibrios. No increase in phage titer was

FIG. 10. Thin section of phage-infected bdellovibrio in the periplasmic space showing polar concentrations of bdellophages. *Bdellovibrio* cell wall (BW), and plasma membrane (BPM), ribosomes (BR) and nucleoplasm (BN) are clearly distinguished. The infected bdellovibrio is in the periplasmic space, is bordered by the double track of the modified *E. coli* cell wall (EW). Magnification $\times 57,680$. Scale represents $0.5 \mu\text{m}$.

FIG. 9. Thin section of phage-infected bdellovibrio in the periplasmic space. An electron transparent space (S) is seen in the nucleoplasm of the bdellovibrio. Ribosomes (RE) are seen in *E. coli* protoplast bounded by the plasma membrane (EPM). All are contained within *E. coli* cell wall (EW). Magnification $\times 57,680$. Scale represents $0.5 \mu\text{m}$.

FIG. 11. Consecutive serial sections of the three-membered system 150 min after phage infection. As the plane of longitudinal section traverses the bdellovibrio, phage concentrations are seen in Fig. 11a and b, first at the lower right of the cell and later in Fig. 11f at the upper left. Magnification $\times 50,000$. Scale represents $0.5 \mu\text{m}$.

FIG. 12. Thin section of infected bdellovibrio showing rupture of bdellovibrio cell wall at 180 min after phage infection. Bdellovibrio cell seen in cross section. The outer wall membrane (BW) ruptures and appears to form vesicles (V) which are seen in the periplasmic space. The bdellovibrio plasma membrane (BPM) remains intact. Magnification $\times 57,680$. Scale represents $0.5 \mu\text{m}$.

FIG. 13. Thin section of lysed bdellovibrio devoid of cell contents and with bdellophage progeny localized by remnant bdellovibrio plasma membrane (BPM). The *E. coli* plasma membrane remains intact. Magnification $\times 57,680$. Scale represents $0.5 \mu\text{m}$.

FIG. 14. Free phage progeny within the periplasmic space of the collapsing bdelloplast. Ribosomes (R) and membranous vesicles (V) are also seen. Magnification $\times 50,000$. Scale represents $0.5 \mu\text{m}$.

FIG. 15. One-step growth curve of VL-1 on *Bdellovibrio* 109HI in YP medium. Symbols: Infectious centers, O; total phage count after premature lysis by mechanical disintegration, ●.

observed in samples taken 1, 3, and 5 h after adsorption.

Nonspecific degenerative symptoms in bdellovibrios. By the time mature phage particles appear in the three-membered system and in infected HI bdellovibrios, electron transparent spaces bordered by a double membrane become evident within the nucleoplasm of the bdellovibrios (Fig. 18, see also Fig. 9, 11d, and 16). Similar spaces appear in the nucleoplasm of HD bdellovibrios whether infected by phage or incubated in the absence of phage in YP medium. This phenomenon seems unrelated to the phage maturation, but may reflect a degenerative process occurring in bdellovibrios under unfavorable conditions. Similar structures have been observed by Hoeniger et al. (4) in *Bdellovibrio* W during cyst formation where they were considered to be storage granules.

DISCUSSION

Development of the bdellophage in host-dependent bdellovibrios depends upon success-

FIG. 16. HI bdellovibrio 120 min after infection by bdellophage VL-1 showing polar localisation of the developing phages and prominent electron transparent spaces (S). Magnification $\times 50,000$. Scale represents $0.5 \mu\text{m}$.

FIG. 17. Phage adsorption and DNA injection into free parasitic bdellovibrios; total radioactivity adsorbed to the cells O; radioactivity remaining adsorbed after omnimix treatment, ●.

ful parasitization of the *E. coli*. Earlier experiments showed that bdellovibrios infected by phage VL-1 lost their motility and ability to attach to *E. coli* within a few minutes after infection (17). This raises the question of how the three-membered system is established. Our investigation shows that phages adsorb to already attached bdellovibrios, and probably inject their nucleic acid before penetration is completed. The phage infection has no effect on the normal penetration process of the bdellovibrio, which enters the host periplasm while the empty phage coats are scraped off and left in a cluster at the penetration pore. Whole phages do not seem to penetrate the *E. coli* envelope.

Since phage VL-1 infection arrests the motility of free *Bdellovibrio* 109J (17), the fact that penetration occurs suggests that this process does not depend upon the active motility of the bdellovibrios. This notion gains further support from our observation that noninfected bdellovibrios lose their flagella soon after attachment to the host (M. Kessel, unpublished data).

The transformation of the *E. coli* from a rod to sphere ("bdelloplast") in the three-membered system appears no different than normal

bdelloplast formation in the two-membered system. In both cases the bdelloplast is limited by a single clearly defined smooth unit membrane which is the layer usually associated with the lipopolysaccharide of the cell wall (3).

The noninfected intraperiplasmic bdellovibrio induces breakdown of host DNA which is reutilized by the growing bdellovibrio (8). Other host components are possibly also utilized by the bdellovibrio since the parasite develops in nonnutrient buffer (18). Phage-infected bdellovibrios, in contrast, do not elongate in the periplasmic space and at the end of the phage growth cycle lyse, whereas the *E. coli* protoplast appears intact.

The nuclear region of the phage-infected bdellovibrios appears normal without any cytologically detectable DNA degradation (see Fig. 9, 10), similar to observations reported for other bacteriophages (15, 16). This is in contrast to T-4 phage, for example, which is known to cause rapid breakdown of host DNA, followed by the appearance of pools of phage DNA at the periphery of the cell (6).

No empty phage heads were detected before the appearance of the mature phages. As pointed out by Sechaud and Kellenberger (10), the standard Ryter-Kellenberger fixation, used

FIG. 18. High magnification of the electron transparent spaces (S) associated with bdellovibrio nucleoplasm (BN). Arrows point to double track appearance of the membrane surrounding the transparent space. Magnification $\times 100,000$. Scale represents $0.1 \mu\text{m}$.

in this investigation, may not preserve fragile incomplete viral particles due to the slow action of osmiumtetroxide fixation.

The polar arrangement of the developing phage progeny suggests a specific localization, similar to the situation found in cyanophages (11). This phenomenon is rare among DNA bacteriophages, where newly formed phages are usually randomly dispersed in the host cell (2).

The question arises as to the source of phage DNA, which could be derived from incorporation of DNA precursors from the medium and de novo biosynthesis, breakdown, and reutilization of *Bdellovibrio* DNA or of *E. coli* DNA, or a combination of either two or all these possibilities. The experiments showing that phage VL-1 can grow normally in a three-membered system incubated in a nonnutrient buffer solution exclude the possibility that the medium is the sole source for phage DNA. However, the difference between average burst size in medium compared to buffer suggests that nutrients in the medium do contribute to phage development. Cytological evidence suggests that most of the *Bdellovibrio* DNA remains intact until lysis. Nevertheless, degradation of a small percentage of the *Bdellovibrio* DNA and reincorporation into phage DNA could take place and remain undetected in the micrographs. The possibility that phage utilizes *E. coli* DNA would require that at least some of the capacity of *Bdellovibrio* to break down this DNA is not impaired by phage infection. Our preliminary experiments with radiolabeled bacteria suggest that both the *Bdellovibrio* and *E. coli* contribute precursors for the phage DNA. Further experiments are required to estimate the relative contribution of each and the possible contribution of the external medium.

The question of the mechanism of release of newly formed bdellophages has been raised (17). The cytological evidence shows that, at the end of the rise period, the *Bdellovibrio* cell envelope is disrupted and the mature phages are released either into the medium (in the case of HI *Bdellovibrios*) or into the periplasmic space of the *E. coli* (in the three-membered system). Although this initial process is similar to release of bacteriophages from their hosts, the release of the bdellophages from the "bdelloplast" in the three-membered system involves a further step. Assuming that in the normal two-membered system the bdelloplast is ruptured through enzymatic activity of the *Bdellovibrio*, as suggested by Huang and Starr (5), does the infected *Bdellovibrio* indeed carry out this late function despite cessation of its normal

development? An alternative possibility is that having lysed the *Bdellovibrio* cell wall, phage enzyme(s) continue their activity on the *E. coli* envelope. Present evidence does not allow us to choose between these alternatives.

The number of phage progeny in a single cell of HD or HI *Bdellovibrio* as seen in the electron microscope is approximately 40 to 50. This number is two to three times higher than the average burst size obtained by plaque counts. The possibility of incomplete release from the bdelloplast was ruled out by microscope observation and the technique of complete disintegration of cells used for plaque counts. However, the frequent observation that newly released phages immediately re-adsorb to cell debris might explain a significant decrease in phage titer. Another possible explanation for the low average burst size is that a certain percentage of phage-bacterium interactions are abortive.

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