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Factors Affecting the Intracellular Parasitic Growth of *Bdellovibrio bacteriovorus* Developing Within *Escherichia coli*

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A procedure for one-step growth experiments on *Bdellovibrio bacteriovorus* growing parasitically in *Escherichia coli* B was developed. The resulting one-step growth curves showed that, under defined conditions at 30 C, each singly infected *E. coli* host cell, on the average, gave rise to 5.7 *Bdellovibrio* cells. This value was confirmed by single-burst experiments and by microscopic observations. In the temperature range of 25 to 38 C, the average burst size and the duration of the latent period were inversely proportional to the temperature. The effect of hydrogen ion concentration on the one-step growth kinetics in this system indicated a broad *p*H optimum, ranging from neutrality to slightly alkaline *p*H values. After *Bdellovibrio* cells and host cells were mixed, there was always a delay (the so-called "lag phase") before the parasite titer increased in terms of plaque-forming units. Phase-contrast microscopic observations indicated that this delay stems in part from the polyphasic nature of the *Bdellovibrio* life cycle. We propose the following five terms to make explicit the sequence of events in this life cycle: "attachment," "penetration," "elongation," "fragmentation," and "burst." Nutritional experiments revealed that *Bdellovibrio* obtains a major fraction of its cellular components from host-cell material. Infection of *E. coli* by *Bdellovibrio* without added Mg^{++} or Ca^{++} (0.003 M Mg^{++}, 0.002 M Ca^{++}) resulted in partial or total lysis of the host cell soon after infection. Protoplast integrity was necessary for the normal completion of the intracellular growth phase of *Bdellovibrio* in *E. coli*; normal development of the parasite took place only in the presence of Mg^{++} or Ca^{++}.

Most investigations of the nature of the parasitic attack by *Bdellovibrio bacteriovorus* upon host bacteria have been concerned mainly with morphological aspects (3, 5-7, 9, 11, 12). There have been only a few reports dealing with the intracellular growth of this unusual parasitic bacterium. Stolp and Starr (12) described, in a preliminary way, the kinetics of growth for one strain, and Starr and Baigent (9) showed that the time required for completion of the parasitic growth cycle was dependent upon the physiological state of the parasite at the time the cycle was initiated. Varon and Shilo (13) recently reported on attachment kinetics, and there has been a preliminary report on the effect of divalent cations on growth of *Bdellovibrio* (J. C. C. Huang, Bacteriol. Proc., p. 23, 1968).

During the 6 years since Stolp and Petzold's pioneering repc t (11) on *Bdellovibrio*, there have been no reports published of quantitative studies of the factors affecting the intracellular multiplication of *Bdellovibrio* and the yields of parasites from individually infected host cells. To gain a greater understanding of the growth of *Bdellovibrio* within its host, we initiated experiments leading to single cycles of growth. In the present communication, we will detail the methods involved in one-step growth experiments and provide a quantitative measurement of the multiplication of one strain of *Bdellovibrio* (*Bdellovibrio* 109)in individually infected host cells of *Escherichia coli* B. Phase-contrast photomicrographs are used to corroborate some of the results of the one-step growth curves.

MATERIALS AND METHODS

Cultures. The organisms studied were *E. coli* B (ICPB 2262, ATCC 15144) and *B. bacteriovorus* strain 109 (ICPB *Bd.* 109, ATCC 15143) of Stolp and Starr (12), hereafter referred to as *Bdellovibrio* 109.

Media. Enriched nutrient broth (NB) contained: Difco Nutrient Broth, 8 g; Difco Casamino Acids, 5 g; Difco yeast extract, 1 g; and distilled water, 1 liter (11).

Dilute nutrient broth (NB/10) was prepared from 1 volume of NB plus 9 volumes of distilled water; the *p*H was adjusted to 7.2 with 0.1 N NaOH. After autoclaving, the following salts were added from a filter-sterilized stock solution: $MgCl_2 \cdot 6H_2O$ (0.003 M Mg^{++}, final concentration) and $CaCl_2 \cdot 2H_2O$ (0.002 M Ca^{++}). NB/5 and NB/50 were prepared from 1 volume of NB plus 4 and 49 ml, respectively, of distilled water; Mg^{++} and Ca^{++} were added as in NB/10.

For NB/10 agar, the bottom layer was supplemented with 1% Difco agar, and the top layer contained 0.8% Difco agar.

Enumeration of plaques. A 0.5-ml amount of an overnight culture of *E. coli* B grown in NB was inoculated into 2.5 ml of NB/10 top agar along with an appropriate amount of the *Bdellovibrio* suspension (0.05 to 0.2 ml). After the top layer had been poured, plates were incubated inverted at 33 to 34 C for 2.5 to 3.5 days. To facilitate plaque counting, the plates were flooded with a nutrient solution (NB) containing 0.1% 2,3,5-triphenyl-tetrazolium chloride (TTC; Calbiochem, Los Angeles, Calif.). The plates were reincubated at 34 C for at least 4 to 5 hr while the dye was reduced by host cells. The TTC solution was decanted and the plaques were counted.

Preparation of host cells for one-step growth experiments. A 0.1-ml amount of an *E. coli* B culture in NB was inoculated into 50 ml of NB and grown overnight at 30 C until the stationary phase was reached. A 1-ml sample was removed and inoculated into 50 ml of prewarmed NB at 30 C. After about 1.5 hr of exponential growth, the culture, containing approximately 3×10^8 cells/ml, was chilled. A 30-ml portion of the culture was centrifuged at 15,000 to 20,000 $\times$ *g* for 10 min. The host cells were washed in 15 ml of NB/10 and resuspended in 3 ml of NB/10 at 30 C.

Preparation of Bdellovibrio 109 suspension for one-step growth experiments. The day before the experiment, 2 ml of a refrigerated, stationary-phase *E. coli* B culture in NB was inoculated into 25 ml of NB/10. After 0.5 ml of a refrigerated *Bdellovibrio* 109 lysate was added, the culture was shaken at 30 C for 16 to 17 hr. At this time, the culture was successively filtered through 5-, 3-, and 1.2-μm membrane filters (Millipore Corp., Bedford, Mass.) to remove host cells and debris, and then was centrifuged for 25 to 30 min at 30,000 $\times$ *g*. The *Bdellovibrio* 109 cells were resuspended at the appropriate concentration in NB/10 or in 0.001 M tris(hydroxymethyl)aminomethane (Tris) buffer at *p*H 7.2 (Sigma Chemical Co., St. Louis, Mo.).

Photomicroscopy. Phase-contrast observations and photomicrographs were made with a Zeiss photomicroscope equipped with a Zeiss VZ aplanatic condenser with a numerical aperture of 1.40 and a 100$\times$ Zeiss Neofluar objective lens with a numerical aperture of 1.30. Preparations were recorded on Adox KB-14 film and developed in Kodak DK-60a. Specimens were prepared from broth cultures and, at various times, mounted in a drop of molten NB/10 top agar and photographed immediately. Refer to the legends of Fig. 9–11 for other details.

Separation of free Bdellovibrio cells from host cells. Separation of the attached and free *Bdellovibrio* is accomplished by a differential filtration through a 1.2-μm membrane filter (13). Equal samples of an *E. coli* B suspension and an appropriately diluted *Bdellovibrio* 109 suspension [input multiplicity of infection (MOI), about 0.1] were mixed in prewarmed (30 C) NB/10 or Tris buffer (0.001 M, final concentration; *p*H 7.2). Immediately after mixing, a sample was removed and diluted 10^{-2} with chilled NB/10. A 1-ml amount of this dilution was filtered through a 1.2-μm membrane filter and washed with 99 ml of NB (prefiltered through a Millipore prefilter and a 0.65-μm Millipore filter). The titer of the filtrate was determined in terms of plaque-forming units (PFU) and represents the number of filterable, free *Bdellovibrio* cells in the attachment flask at time zero (T_0).

After host and *Bdellovibrio* cells had incubated for 30 min at 30 C, a portion was removed, chilled, and diluted 10^{-2} into cold NB/10. A 1.0-ml sample of the dilution was filtered and washed, as indicated above. The titer (T_{30}) of this filtrate was determined. From these values, the number of *Bdellovibrio* cells attached was calculated: $T_0 - T_{30}$ = number of *Bdellovibrio* cells attached to (and/or within) 1.5×10^9 host cells/ml after 30 min.

The T_{30} membrane filter, which contained infected and noninfected host cells (but no free *Bdellovibrio* cells), was inverted and transferred to another filter apparatus, and the cells were washed off by passing 10 ml of NB/10 through the filter. In some experiments, the filter was washed with additional NB/10 before the infected cells were removed. The filtrate was then inoculated into growth tubes (normally 0.4 ml into 19.6 ml of NB/10), which had been temperature-equilibrated. Samples were removed at intervals, and plated directly for determination of PFU.

RESULTS

Efficiency of separation of attached and free Bdellovibrio cells. Our modification of the previously published (13) differential filtration procedure increased the efficiency of separation of the unattached *Bdellovibrio* cells; before filtering a sample through a 1.2-μm filter, the host-parasite suspension was diluted 1:100 to prevent the plugging of the filter previously reported to occur (13). Table 1 illustrates the efficiency of filtration of a *Bdellovibrio* suspension by this method. In another experiment (Table 2), we tested the efficiency of filtration of a *Bdellovibrio* 109 suspension in the presence of host bacteria. *Bdellovibrio* 109 and *E. coli* B suspensions were prepared as described in Materials and Methods, and, to prevent attachment by the parasite, each was chilled in ice before mixing. The results shown in Tables 1 and 2 indicate an essentially complete recovery of the free *Bdellovibrio* cells. The presence of host cells (at the approximate concentration used in one-step growth experiments) did not affect the complete separation of unattached cells of the parasite from host cells.

TABLE 1. *Filtration of a suspension of Bdellovibrio cells through a 1.2-μm membrane filter*[a]

Titer of filtrate	Recovery
PFU/ml	%
282×10^6	98
272×10^6	94
290×10^6	100
278×10^6	96
276×10^6	96

[a] A 16-hr *Bdellovibrio* 109 culture was grown and filtered as described in Materials and Methods section. The resulting suspension was diluted 1:100 in NB/10 and the titer in plaque-forming units (PFU) was determined directly to be 289×10^6 PFU/ml. To determine the filtrate titer, 1-ml portions of the 1:100 dilution were filtered through a 1.2-μm membrane filter and washed with 99 ml of prefiltered NB; the titer was then determined. The filtrate titer represents the averaged results of five independent determinations.

TABLE 2. *Efficiency of separation of cells of Bdellovibrio from cells of E. coli B*[a]

Titer of filtrate	Recovery
PFU/ml	%
113×10^6	95
123×10^6	103
118×10^6	99

[a] A chilled *Bdellovibrio* 109 suspension containing approximately 2.4×10^8 PFU was mixed with an equal volume containing 3×10^9 *E. coli* B cells prepared from a washed and chilled exponentially growing culture. The culture was treated as described in Table 1. The direct titer was 119×10^6 PFU/ml.

Growth curve of Bdellovibrio 109 growing in E. coli B. Figure 1 illustrates the nonsynchronous growth kinetics of *Bdellovibrio* 109 at 30 C in NB/10 with *E. coli* B as the host. The "lag period" of the parasite under these conditions was about 4 hr. (We shall clarify the meaning of this "lag period" in the Discussion section.) During the exponential growth phase of this *Bdellovibrio* strain, the average generation time was about 1.3 hr. Cultures of the parasites were normally taken from the early stationary phase (16 to 17 hr of incubation) for experiments involving single growth cycles.

Plaque formation and relative Bdellovibrio concentration. Figure 2 shows the relationship between the relative concentration of *Bdellovibrio* cells and the number of PFU plotted on a log-log scale (1). The results indicate a linear relationship between the two parameters, at least in the range of 12 to approximately 500 plaques per plate.

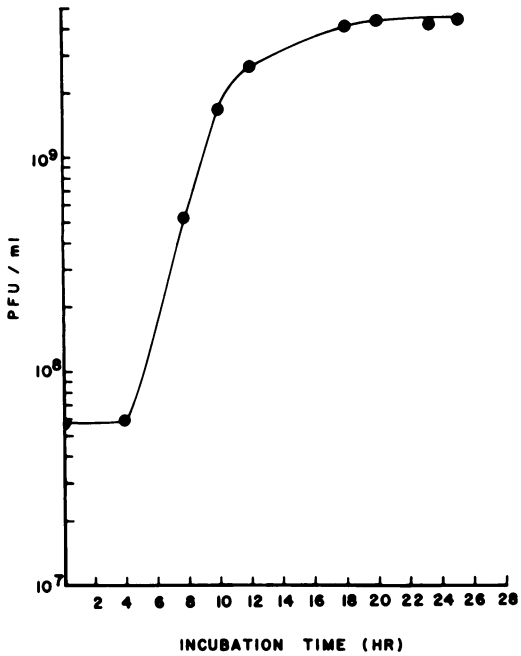

FIG. 1. *Growth curve of Bdellovibrio bacteriovorus in its host in terms of plaque-forming units (PFU). A 2-ml amount of an overnight NB culture of E. coli B and 0.5 ml of a Bdellovibrio 109 lysate were inoculated into 25 ml of NB/10 and incubated at 30 C in a New Brunswick shaker. Samples were withdrawn and plated periodically for PFU on NB/10 double-layer plates.*

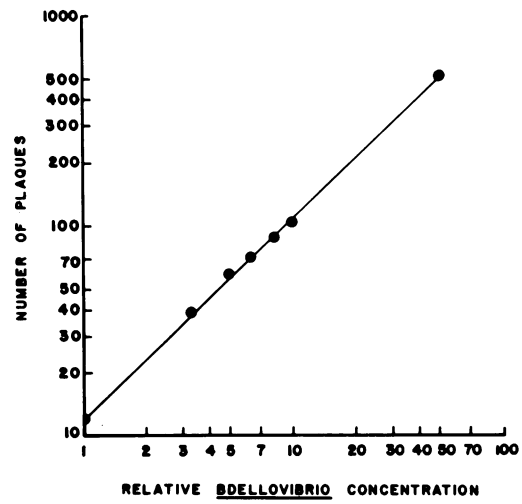

FIG. 2. *Relationship between plaque-forming units (PFU) and relative Bdellovibrio concentration. A sample was removed from a 17-hr NB/10 lysate, and 0.1-ml amounts of appropriate dilutions were plated onto NB/10 agar plates. Each point represents an average of the counts from six plates. The slope of the log-log plot is approximately unity (i.e., the number of plaques/relative Bdellovibrio concentration = 1).*

The fact that the slope of the line is approximately unity would indicate that under these conditions of enumeration every viable *Bdellovibrio* cell forms one plaque.

One-step growth experiments. Figure 3 shows a mean one-step growth curve, averaging the results of four one-step growth curves carried out at 30 C under the standard conditions given in Materials and Methods. At 30 C, the termination of the latent period for a 17-hr *Bdellovibrio* lysate was about 3 hr, following a 30-min period of attachment. The results of these experiments are expressed in terms of the "average burst size," which is the quotient of the titer (in PFU) at the end of the rise period and the average titer (in PFU) over the lag period. These experiments indicated that each infected *E. coli* B cell, on the average, gives rise to 5.7 *Bdellovibrio* cells.

Even though attachment proceeded for only 30 min, the rise spanned about a 2-hr period. Since there was a maintenance of infective centers after the rise period, a second burst cycle was prevented by our experimental procedure. Finally, the fact that the counts during the latent period remained essentially constant indicates that the plating efficiency remained stable during this time.

Physiological state of the parasite and the one-step growth curve. In an attempt to explore some of the variables affecting the average burst size and the duration of the latent period, several one-step growth cycles were carried out with parasites taken from different stages of growth (cf. Fig. 1). The results with parasites taken from three stages during nonsynchronous growth are shown in Fig. 4.

The major effect is seen in the actively growing 10-hr exponential culture of *Bdellovibrio*. The latent period in this case lasted for about 2.5 hr after the attachment period. When the *Bdellovibrio* inoculum was taken from the stationary phase (after 14- or 17-hr incubation), the latent period was about 3 hr. There was a small difference (less than 10%) in the average burst size between the later stationary-phase inoculum and the log-phase inoculum.

Effect of temperature on latent period and burst size. Using the normal one-step growth cycle protocol, we inoculated infected cells into growth tubes of NB/10 prewarmed to various temperatures (25, 30, 35, 38, and 42 C). The effects of temperature on the average burst size and duration of the latent period are illustrated in Fig. 5. The results indicate that as the temperature was lowered the latent period became longer and the average burst size increased. At 42 C, there was no burst; indeed, after about 2 hr of incubation, there was an exponential disappearance of infective centers.

To determine the overall effect of temperature on multiplication of *Bdellovibrio*, one must take into account both variables affected by temperature, i.e., the duration of the latent period and the average burst size. To take both of these two variables into account, we used a relationship

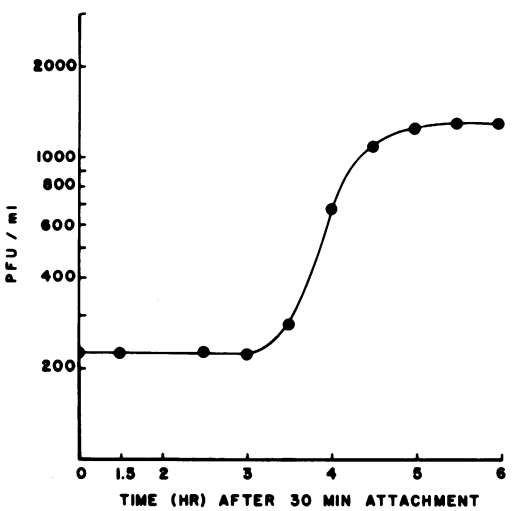

FIG. 3. *Single-step growth curve of Bdellovibrio bacteriovorus 109 growing in E. coli B in NB/10 broth at 30 C. The latent period ended about 3 hr after the termination of the 30-min attachment period. The average burst size in four experiments was 5.7.*

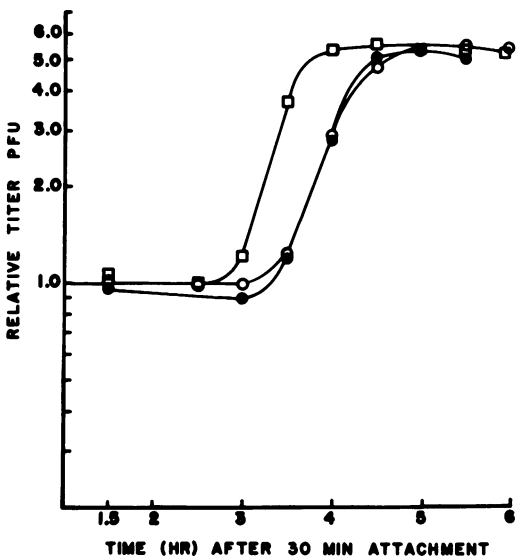

FIG. 4. *Effect of the physiological state of Bdellovibrio 109 growing on E. coli B on the latent period and burst size. Cultures were grown as described in Fig. 1. Samples were removed after 10, 14, and 17 hr of nonsynchronous growth; □, 10 hr; ●, 14 hr; ○, 17 hr.*

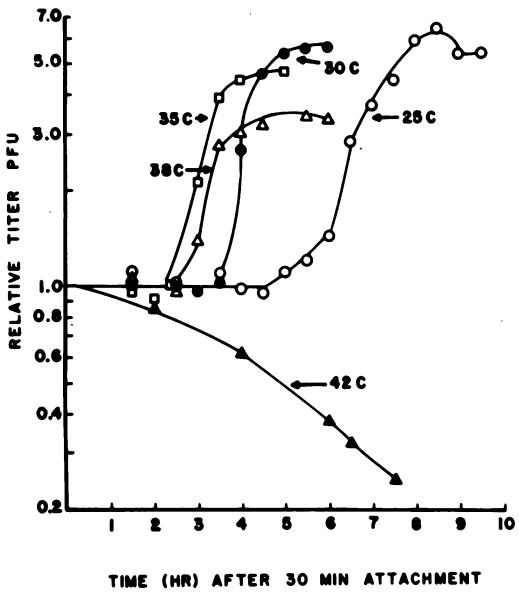

FIG. 5. *Effect of temperature on the latent period and average burst size of Bdellovibrio 109 growing on E. coli B. After the 30-min attachment period at 30 C, samples were treated as described in Materials and Methods and were inoculated into prewarmed NB/10. The counts were normalized to unity at time zero, and were plotted in terms of the relative titer increase. Symbols: ○, 25 C; ●, 30 C; □, 35 C; △, 38 C; ▲, 42 C.*

which we term the "burst coefficient." The burst coefficient equals the quotient of the average burst size and the latent period in hours. The temperature corresponding to the highest burst coefficient would represent the optimal temperature for growth of the parasite in this system. For *Bdellovibrio* 109, the burst coefficient was highest in the range of 30 to 35 C (Table 3).

Effect of pH on the latent period and burst size. After the attachment period at 30 C in NB/10, samples of infected cells were transferred into NB/10 at *p*H increments of approximately 0.5 from *p*H 6.0 to 8.5. The medium was made up in 0.001 M Tris buffer, and the *p*H was adjusted with 0.1 N NaOH or 0.1 N HCl. The results of one-step growth curves at four *p*H values are shown in Fig. 6. At *p*H 6.2, the latent period was extended from the normal 3 hr to about 4.5 hr after the attachment period. The average burst coefficients at *p*H 6.2 and 6.6 were 0.7 and 1.2, respectively. In the *p*H range of 7.0 to 8.5, the burst coefficients ranged from 1.3 to 1.5.

The effect of hydrogen ion concentration on *Bdellovibrio* multiplication might also be expressed as "percentages of the final burst size" at a particular time after the bursts commenced. The results of one such calculation are shown in Table 4. Here, the relative titer is calculated in terms of percentages of the final burst size at that particular *p*H at 3.5 hr after T_{30}. There was an increase in the percentages of the final burst size with increasing *p*H up to *p*H 8.5. These results demonstrate that the optimal *p*H for growth of *Bdellovibrio* 109 is in the range of *p*H 7.5 to 8.1.

Effect of nutrient concentration on the average

TABLE 3. *Summary of the effect of temperature on the duration of the latent period and the burst size in Bdellovibrio growing on E. coli B*[a]

Temp	Latent period	Avg burst	Burst coefficient
C			
25	5	6.4	1.3
30	3.5	5.7	1.6
35	3	4.7	1.6
38	3	3.4	1.1

[a] The latent period listed here includes the 30-min attachment period. Burst coefficient = average burst size/latent period (in hours). Each value for the burst size represents the average of at least two experiments.

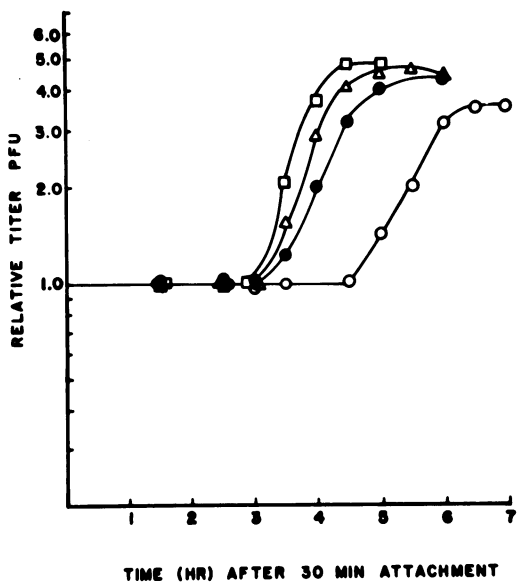

FIG. 6. *Effect of pH on the latent period and average burst size in Bdellovibrio 109 growing on E. coli B. Following the standard single-step protocol, 0.4 ml of infected cells was transferred into 19.6 ml of NB/10 in 0.001 M Tris buffer at the pH values indicated. The counts were normalized to unity at time zero. Symbols: ○, pH 6.2; ●, pH 6.6; △, pH 7.0; □, pH 8.1.*

TABLE 4. *Effect of pH on burst size in Bdellovibrio growing on E. coli B*[a]

Percentage of final burst at 3.5 hr from T_{30}	*p*H
0	6.2
27	6.6
34	7.0
43	7.5
43	8.1
40	8.5

[a] Cultures were prepared as described in the text. The *p*H values listed were measured at the beginning and end of the one-step growth experiment. The counts of PFU at zero time (T_{30}) were adjusted to 1, and the titers relative to this adjustment were determined during the course of the experiment (Fig. 6). The percentage of the final burst size was calculated by dividing the relative titer at 3.5 hr after T_{30} by the final relative titer after the end of the rise period.

TABLE 5. *Effect of suspending medium on average burst size in Bdellovibrio growing on E. coli B*[a]

Suspending medium	Avg burst size	
	Attachment in NB/10	Attachment in Tris
NB/5	6.4 ± 0.3	5.6 ± 0.3
NB/10	6.3 ± 0.5	5.4 ± 0.1
NB/50	5.5 ± 0.3	5.2 ± 0.2
Tris, 0.001 M	4.8 ± 0.4	5.1 ± 0.4

[a] All media were supplemented with Tris buffer, 0.001 M final concentration, *p*H 7.2. Attachment took place either in NB/10 or in 0.001 M Tris buffer (*p*H 7.2) for 30 min. Average burst size was determined from samples taken during the latent period (0 to 1.5 hr) and after the end of the rise period (5 to 7 hr); the values reported represent the means of three replicate determinations.

burst size. To determine the degree of dependence of *Bdellovibrio* on the presence of nutrients other than host cells, experiments to determine the average burst size were carried out in various concentrations of NB and in 0.001 M Tris buffer (*p*H 7.2). In the first experiments, attachment took place in NB/10. The *Bdellovibrio*-infected host cells were then washed with an additional 10 ml of NB/10. Samples of the suspension (0.4 ml) were inoculated into 19.6 ml of various concentrations of NB or Tris buffer. The results are shown in Table 5.

The data indicate a correlation between the nutrient concentration and the average burst size. A significant burst was obtained from the infected cells suspended in Tris buffer (approximately 75% of the NB/10 control).

In a second nutritional experiment, in which attachment took place in Tris buffer alone (0.001 M, *p*H 7.2), a suspension of washed host cells was used. The *Bdellovibrio* lysate was prepared in the normal manner, washed once in Tris buffer with added Mg^{++} (0.003 M) and Ca^{++} (0.002 M), and diluted approximately 60-fold in buffer before being mixed with the hosts. The percentage of attachment of the *Bdellovibrio* cells in this experiment was about the same as that observed in other experiments with cells suspended in NB/10, i.e., about 70%. Following the NB wash for the T_{20} sample, the infected cells were washed from the filter with 10 ml of NB/10 plus the added cations. The average bursts are shown in Table 5. In this case, there was less than a 10% drop in the average burst size between the control in NB/10 and the trial in Tris buffer.

Single-burst experiments. To determine the yield of parasites from individually infected cells, it became necessary to do single-burst experiments. A one-step growth curve accompanied the single-burst experiments. In this manner, it was possible to compare average burst sizes, determined from a statistical consideration of the single-burst data (Table 6), with those obtained from the one-step growth curve (Fig. 7). The average burst size from the one-step experiment was about 5.7. This compares with the average value of 5 obtained from the single-burst experiments.

TABLE 6. *Distribution of plaques in a single-burst experiment for Bdellovibrio growing on E. coli B*[a]

Bursts				
0	0	6	0	4
0	5	8	4	13
8	14	0	0	0
4	9	0	0	3
0	0	6	6	6

[a] The sample was removed from a single-step experiment growth tube at 2.5 hr after T_{30}, and diluted 1:100; 0.2 ml was pipetted into each of 25 tubes and was incubated at 30 C for 3 hr. Host cells and melted agar were added, and the entire contents of each tube were poured onto an NB/10 plate. The fraction of tubes (P_0) not receiving any infected cells can be determined by Poisson's law and calculated as shown by Stent (10). From Poisson's law, $P_r = (n^r/r!)e^{-n}$, where P_r = fraction of tubes with r infected cells, r = number of infected cells/tube, and n = number of infected cells/tube. $P_0 = e^{n-} = 11/25$; $n = 0.82$. Total number of infected cells: $0.82 \times 25 = 20$. Total number of plaques: 96. Average burst: 96/20 = 5.

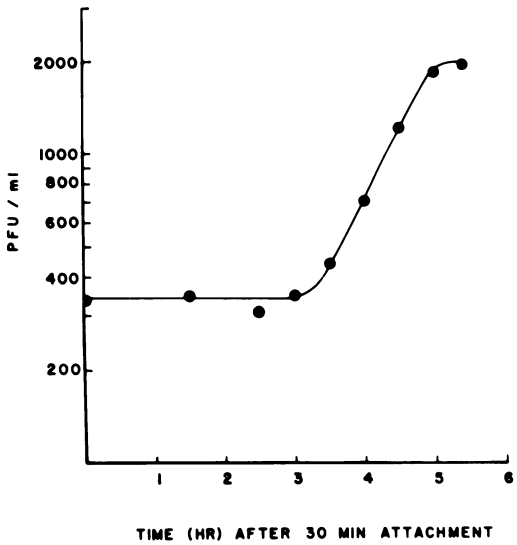

FIG. 7. *Single-step growth curve at 30 C of Bdellovibrio 109 growing in E. coli B. A sample was removed 2.5 hr after the attachment period for the single-burst experiment. The burst was complete at 5.5 hr, at which time samples were plated from the single-burst tubes. The average burst size in the one-step growth experiments was 5.7.*

Based on an average of 0.8 infected cells per tube, the number of tubes expected to contain one infected cell is about 37% or nine tubes (Table 6). An inspection of nine tubes with the lowest number of PFU indicates that the single burst from individually infected *E. coli* B cells varied from three to six *Bdellovibrio* cells.

Effect of divalent cations on Bdellovibrio-host interactions. Experiments involving attachment (T_0 versus T_{30}) indicated that the nonaddition of magnesium and calcium ions to the system during the attachment period resulted in a reduction in attachments by the parasite. In NB/10, attachment was about 50 to 60% that of the control with the added cations; in 0.001 M Tris buffer, the comparable value was only 20 to 30% without the added cations.

A more striking effect was observed when the divalent cations were omitted from the growth tube during a one-step growth experiment in NB/10. Under these conditions, the latent period was greatly extended and the average burst size was only about one-half that of the cultures containing added Ca^{++} (0.002 M) and Mg^{++} (0.003 M). Addition of either Ca^{++} or Mg^{++} alone resulted in a normal one-step growth curve (Fig. 8).

The culture in NB/10 with added Ca^{++} and Mg^{++} showed an average burst size of about 4.3. This is some 15 to 20% less than the burst size normally observed under these conditions. The probable cause of this reduction can be attributed to the experimental design which involved washing of the cells (in this experiment only) with NB/10 without added cations to prevent carry over of Ca^{++} and Mg^{++} from the attachment flask. The results clearly show, however, that addition of either Mg^{++} or Ca^{++} alone can produce the same average burst size as in the control culture (4.1 for Mg^{++} and 4.2 for Ca^{++}). The culture without added cations had an average burst of only about 2.

In an attempt to determine the function of cations in the growth of *Bdellovibrio*, phase-contrast microscopic observations were made with infected hosts in the presence and absence of Mg^{++} and Ca^{++}. Periodic microscopic observations permitted us to detect the role played by these cations and to follow the intracellular growth of the *Bdellovibrio* parasite.

Figures 9–11 are photomicrographs of *Bdellovibrio* 109 and *E. coli* B taken at various times after the two cultures were mixed. Regardless of the suspending medium, the infected host cells were always more transparent when the cations were omitted from the culture (compare Fig. 9a and b with 10a and b and 11a and b). In the

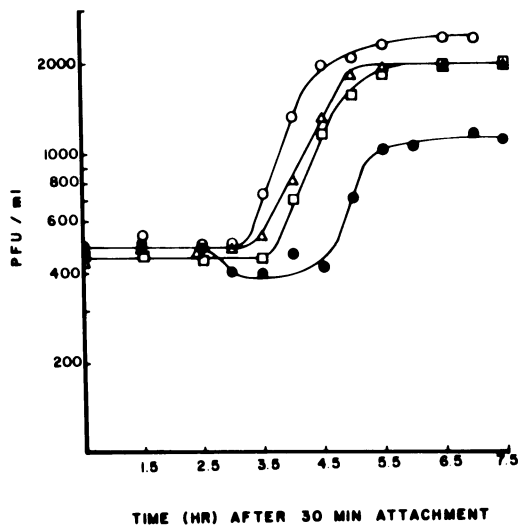

FIG. 8. *Effect of the addition of calcium or magnesium ions on the average burst size for Bdellovibrio 109 growing in E. coli B. Following the attachment period and dilution, the infected cells were rinsed on the filter with 10 ml of NB/10 without added cations. This process resulted in a lysing of a fraction of the infected cells. Symbols:* ○, *both calcium and magnesium ions (0.002 M Ca^{++}, 0.003 M Mg^{++}) added to growth tubes;* △, *only Mg^{++} added;* □, *only Ca^{++} added;* ●, *no added cations.*

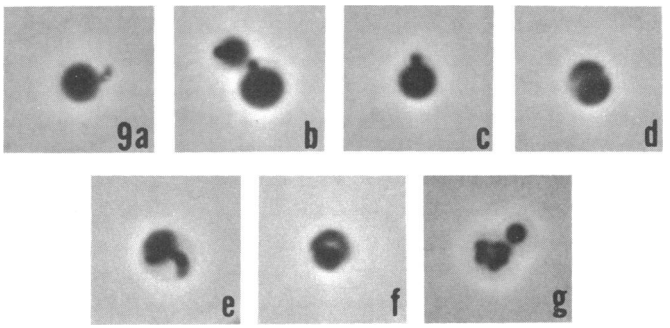

FIG. 9. *Life cycle of Bdellovibrio bacteriovorus 109 infecting E. coli B. Cultures were prepared as described in Materials and Methods, but were not diluted or filtered; NB/10 with added Ca^{++} and Mg^{++} (0.002 M Ca^{++}, 0.003 M Mg^{++}). To determine the corresponding time in the one-step growth curves, subtract 30 min to account for the attachment period. (a) After 20 min; (b and c) 1 hr; (d) 3 hr; (e) 3.5 hr; (f and g) 4 hr.* × *2,400.*

absence of the cations, the *Bdellovibrio*-infected host cells appeared more transparent under phase microscopy and lysed prematurely. Lysis of the host cells by *Bdellovibrio* 109 without addition of these cations resulted in a two- to five-fold increase in the absorbance at 260 and 280 nm of the culture supernatant liquids over that of control cultures with cations. Since infected host cells do not appear to lyse (in the same premature manner) in the presence of Mg^{++} and Ca^{++}, and because lysis (when it does occur) clearly results from deterioration of the membrane, this finding suggests that the cations play a role in stabilization of the host plasma membrane after *Bdellovibrio* infection.

Observations of intracellular growth of Bdellovibrio with and without added cations. Under ordinary conditions (NB/10 plus cations, 30 C), the *E. coli* host cell formed a spheroplast within several minutes after attachment by the *Bdellovibrio* cell (Fig. 9a). The host cell remained dark-appearing under phase-contrast illumination through the entire developmental cycle of the parasite (Fig. 9a–f). In 20 to 40 min after attachment, the parasite was almost completely within the host cell (Fig. 10b). Once inside the host, the *Bdellovibrio* cell appeared to occupy a position between the host cell wall and the host protoplast (Fig. 9c). The small vibrio-shaped parasite became thicker and elongated into a C- or spiral-shaped cell (Fig. 9c–e). Within about 4 hr after the *Bdellovibrio* and host cells were mixed at 30 C, most of the parasites had completed their intracellular development and the progeny soon left the remnants of their host. Figure 9f illustrates an infected host cell in which about five *Bdellovibrio* progeny have been produced. The completion of the cycle of development of *Bdellovibrio*, as observed by phase-contrast microscopy, corresponds to the termination of the latent period in the one-step growth curves.

There were at least two consequences of infection in NB/10 without added cations. In some cases, infection and penetration by the *Bdellovibrio* cell resulted in a clearing of the host cell. Some of these infected cells did not form a spheroplast but, to varying degrees, merely swelled slightly and more or less retained their rod shape (Fig. 10a and b). Under such conditions, the parasite was sometimes weakly motile and moved throughout the ghosted host cell. In a few instances, the parasite was observed to leave its host; the fate of the parasite after this "aborted infection" is unknown.

Another alternative in the infection process in the NB/10 medium without added cations was the formation of a spherically shaped host cell which, to various degrees, retained its cytoplasmic contents. Examples can be seen in the photomicrographs of infected hosts (Fig. 10c and d). In Fig. 10d, the *Bdellovibrio* is readily observed between the retracted host protoplast and cell wall.

The single-burst data obtained in NB/10 without added cations have shown that at least some of the *Bdellovibrio* cells complete their cycle of growth. Figures 10e–g illustrate several stages in the growth cycle of *Bdellovibrio* at a time when bursts were beginning to occur in the one-step growth curves. In the transparent hosts illustrated, the intracellular growth phases of the parasite are readily observed. The vibrio-shaped parasite has elongated into a spiral- or C-shaped cell similar to those observed in NB/10 with added cations. After the curved parasite cell nearly fills the circumference of the host spheroplast, the parasite divides (Fig. 10f and g). In the three cases illustrated, four to six parasites have been produced

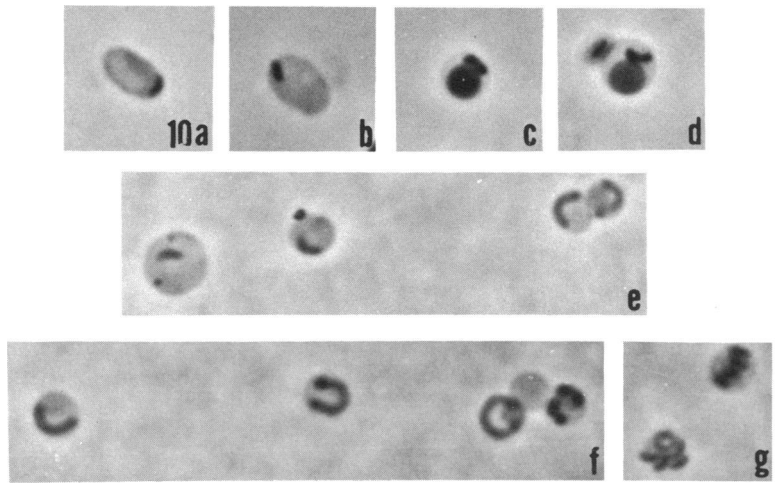

FIG. 10. *Conditions as in Fig. 9, except no cations were added to the medium. (a and b) After 20 to 40 min; (c) 3 hr; (d) 3.5 hr; (e, f, and g) 5.5 hr.* × *2,400.*

per host cell. Thus, even in the absence of added cations, at least some of the *Bdellovibrio* cells complete their life cycle within lysed host cells in NB/10. When parasites were mixed with host cells in 0.001 M Tris buffer (*p*H 7.2) without added cations, the parasites quickly caused a ghosting of the host after attachment. The lysing of the host was usually accompanied by blebs or extrusions of cell material (Fig. 11a and b).

Figure 11c illustrates an infected host cell in Tris buffer plus cations in which a *Bdellovibrio* has completed its cycle of growth. There appear to have been four or five *Bdellovibrio* cells produced within this host cell.

DISCUSSION

Because of the intracellular nature of *Bdellovibrio* growth (3, 5, 9), we thought it would be feasible to develop one-step growth curve assays (akin to those used with bacteriophage) and thus to evaluate some of the parameters affecting the yield from single cycles of parasitic growth of *Bdellovibrio*. The events occurring during the intracellular growth of the parasites could be followed by phase-contrast microscopy. This combination of techniques has indeed been rewarding in clarifying some aspects of the parasite's growth within its host cell.

The technique for the one-step growth curves is similar to that employed in bacteriophage systems, first developed in 1939 (1). The method involves mixing a parasite population with an actively growing host suspension at a low MOI (about 0.1) so that each infected host contains only one parasite. After a short attachment period, the culture is diluted and filtered through a membrane filter to remove all free *Bdellovibrio* cells. The mixture of infected and uninfected host cells is diluted 500-fold upon inoculation into growth tubes. The perfection of the one-step growth assay has permitted us to investigate a variety of processes involved in the intracellular growth and the yield of these parasites.

By infecting an actively growing host cell suspension at a low MOI, it was possible consistently to obtain at least 70% attachment in a 30-min period at 30 C. Our results on the percentage of attachment are in agreement with those reported by Varon and Shilo (13).

The average burst size was computed by dividing the average titer (in PFU) over the "lag period" into the final yield of parasites (in PFU) at the end of the rise period; by "lag period," we mean that initial phase of the overall growth cycle where there is no apparent rise in PFU. An accurate estimation of the average burst size is dependent upon obtaining a supension of infected host cells which contain very few, if any, of the free parasites. If the base-line counts (i.e., those over the "lag period") also included free *Bdellovibrio* cells, the average burst size would have been

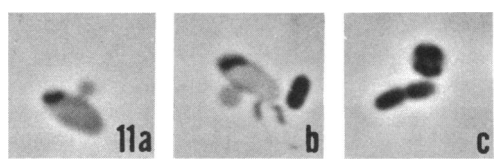

FIG. 11. *Conditions as in Fig. 9, except cells were suspended in 0.001 M Tris buffer, pH 7.2. (a and b). After 3.5 hr, no cations added; (c) after 4 hr, with calcium and magnesium ions added.* × *2,400.*

lowered by the percentage of free *Bdellovibrio* cells in the suspension. The results shown in Table 2 indicate that our procedure results in an essentially complete recovery of the unattached *Bdellovibrio* cells from a mixed population of host and parasite cells.

The physiological state of the parasite plays a role in the kinetics of its life cycle. When the parasites are taken from the stationary phase of growth in a nonsynchronous culture, the time of burst is delayed in comparison with that observed with actively growing parasites (Fig. 4). A more drastic effect on the time required for completion of the life-cycle has been reported for parasites which have been deprived of hosts for several days (9). It is not presently known whether the delay in the burst is distributed evenly throughout the various phases of the *Bdellovibrio* life cycle (including attachment) or whether there is a delay only in the initiation of intracellular growth.

One of the major factors affecting the one-step growth curves is temperature. Our studies indicated an inverse relationship between the average burst size and the temperature of incubation. The same relationship is true for the length of the latent period. The prolongation of the latent period would be expected from a consideration of the effect of lower temperatures on the overall metabolism. There was, however, no *a priori* reason to expect such a drastic change in the average burst size (nearly twice as large at 25 C as at 38 C). Ellis and Delbrück showed that temperature did not similarly affect the burst size of an *E. coli* phage (1). The optimal temperature for multiplication of *Bdellovibrio* 109 in *E. coli* B is about 30 to 35 C (Table 3). Under the conditions tested, 42 C was above the maximal temperature for growth.

The only major effect of *p*H on the one-step growth curves in the range tested occurred at *p*H 6.2. At this *p*H, the burst occurred 1.5 hr after those at *p*H 6.6, 7.0, 7.5, 8.1, or 8.5. At *p*H 6.2, there was also a 20% reduction in the average burst size from those cultures at *p*H 7 or above. In the *p*H range of 6.5 to 8, there was a step-wise increase in the relative *Bdellovibrio* titer with increasing *p*H after 3.5 hr of incubation in the growth tubes (Table 4). The burst coefficients indicate a broad *p*H optimum, ranging from neutrality to slightly alkaline *p*H values (7 to 8.5).

The optimal temperature and *p*H for multiplication of *Bdellovibrio* in one-step growth curves are the same as those found to be optimal for attachment (13). Although the attachment and multiplication processes appear to be continuous events, the former takes place in the external milieu, whereas the latter occurs in the intracellular microhabitat of the host. This requirement for similar environmental conditions may be indicative of the degree of adaptation and the intimate association of events involved in the life cycle of this unusual parasite.

When samples of *Bdellovibrio* and host suspensions are mixed in a broth culture, there is always a "lag period" of variable length (3.5 to 5 or 6 hr) preceding the exponential growth phase (Fig. 1; references 12 and 13). Even when the *Bdellovibrio* inoculum is taken from a culture exhibiting an exponential increase in PFU (Fig. 4, 10-hr sample), a "lag phase" of 2.5 hr still occurs before there is an increase in PFU titer.

Our phase-contrast photomicrographs (Fig. 9 and 10) indicate that during what is termed the "lag period" with respect to PFU, the parasite is increasing in size and proceeding to multiply within its host cell. Indeed, there is already a four- to sixfold increase in the number of intracellular *Bdellovibrio* cells just as our one-step assay in terms of PFU begins to show an increase in titer (Fig. 9g, 10f, and 10g). Thus, as long as all infected cells are removed from the suspension at the time of mixing host and *Bdellovibrio* cells, there will always be such a "lag period" before there is any increase in PFU.

We believe that the single term, "lag period," is an inappropriate designation for the complex of events prior to the burst of *Bdellovibrio*-infected hosts. The following five terms are proposed to make explicit the sequence of the major events of the polyphasic *Bdellovibrio* life cycle: "attachment," "penetration," "elongation," "fragmentation," and "burst."

Within several minutes after attachment by *Bdellovibrio*, the *E. coli* host cell forms a spheroplast (Fig. 9a). Once this spheroplast is formed, we have never observed the parasite detaching from its host, as is occasionally observed moments after an abortive collision by the parasite with a host cell.

Within 10 to 15 min after the cultures are mixed, the penetration phase becomes microscopically apparent. This phase proceeds for at least 1 to 1.5 hr at 30 C (Fig. 9b and c), at which time the entire parasite has completely entered its host (Fig. 9d). The parasite lodges between the host wall and protoplast (Fig. 9d–f and 10c–d). We have never observed the parasite entering the host protoplast. This is in agreement with a recent preliminary report (J. C. Burnham, T. Hashimoto, and S. F. Conti, Bacteriol. Proc., p. 22, 1968).

The phase of elongation begins when the parasite has entered its host. This phase is analogous to the lag phase in the growth of ordinary bac-

teria. During this period, the small, vibrio-shaped parasite increases in thickness and elongates into a C- or spiral-shaped cell without undergoing cell division (Fig. 9e–f and 10e–f). At 30 C in NB/10, this phase proceeds for 2 to 3 hr.

The phase of elongation terminates with the fragmentation of the enlarged cell into daughter cells (Fig. 9g and 10f–g). This entire series of events, in which there is no increase in the infective PFU titer, can be termed the latent period (10). The formation of the C- or spiral-shaped cell, which has been reported by others (5, 9), and its subsequent cleavage into daughter cells, is a different kind of life cycle that, so far as we know, is unique to this group of bacteria. The *Bdellovibrio* life cycle thus involves a dimorphism alternating between a small vibrio-shaped cell and a much larger C- or spiral-shaped unicellular unit which subsequently divides into small units and gives rise again to the vibrio.

Stolp and Starr (12) and Robinson and Huang (Bacteriol. Proc., p. 42, 1967) noted that *Bdellovibrio* could lyse a host suspension in Tris buffer; however, details of the extent of multiplication of the parasite under these conditions were not published. In our experiments, the quantitative effect on the burst size brought about by the presence of nutrients was noted when attachment proceeded in NB/10 (Table 5). There was a 15% decrease in Tris buffer, compared with the NB/10 control. However, when attachment proceeded in Tris buffer, there was a smaller difference in the subsequent effect of nutrients on the burst size (Table 5). In this case, there is only about a 6% decrease in the burst size in Tris compared with NB/10. These results indicate that the composition of the medium during attachment plays some role in the subsequent burst size, since there was a much smaller differential in the burst size between Tris and NB/10 when attachment had proceeded in Tris. Since a significant burst occurs in Tris buffer alone, the *Bdellovibrio* must obtain nearly all of its major cellular components from host-cell material. The presence of small amounts of essential cofactors carried over into the growth tubes cannot be ruled out in these experiments.

The one-step growth curves of *Bdellovibrio* represent only a determination of the average yield of parasites produced from a population of infected host cells. Single-burst experiments, which were initiated to determine the burst size from individual infected host cells, have indicated that there is relatively little variation in the burst size among host cells infected with a single parasite. Those tubes containing a single infected host cell showed a variation in burst size from three to six parasites. Since binary fission of the *E. coli* host produces two daughter cells, each one-half the size of the mother cell (4), it is reasonable to expect such a twofold difference in the average burst size. We must emphasize, however, that we do not know whether the host cell is the sole determinant of the burst size in *Bdellovibrio*. Since the nutritional experiments indicate that *Bdellovibrio* must obtain a major fraction of its cell material from its host, it is not unreasonable to expect a proportionality between the burst size and the amount of host protoplasm. Moreover, the mechanisms regulating the size of the intracellular *Bdellovibrio* spiral and its subsequent effect on the burst size are still unknown.

Single-burst experiments also provide another approach to the calculation of average burst sizes. For the experiment illustrated (Fig. 7, Table 6), there is only a 10% difference between the two independent measurements of the average burst size.

Huang, Robinson, and Murray (Abstr. Ann. Meeting, Can. Soc. Microbiol., p. 45, 1966) were the first to mention that a successful *Bdellovibrio*-host relationship is established only in the presence of added Ca^{++} or Mg^{++} ions. Recently, Huang (Bacteriol. Proc., p. 23, 1968) presented a preliminary report to the effect that the growth of nonsynchronous cultures of *Bdellovibrio* is, within limits, proportional to the concentration of divalent cations. Since the late addition of cations to a host-parasite culture resulted in immediate growth of the parasite, he concluded that the cations supported growth of the parasite without affecting attachment. Huang mentioned also that the cations do not appear to maintain the integrity of the spheroplasted, infected *Spirillum serpens* host.

Our experiments have indicated that only about one-half of the number of parasites are capable of attachment in NB/10 without added Ca^{++} or Mg^{++} ions. The number is reduced to between one-fifth and one-third of the control number when cations are not added to the Tris buffer. Attachment by only a fraction of the number of *Bdellovibrio* cells in the control may indicate some type of heterogeneity in the parasite population, as mentioned by Varon and Shilo in their temperature-shift experiments pertaining to attachment (13). Similar experiments involving a shift to media containing Ca^{++} and Mg^{++} may be helpful in revealing such heterogeneities in the *Bdellovibrio* population with respect to the cation requirement.

After attachment, when infected hosts are inoculated into growth tubes of NB/10 without added cations, the latent period is extended 1.5 hr beyond the control. The average burst is only about one-half of that of the control which contains Mg^{++} or Ca^{++} or both ions (Fig. 8). Phase-

contrast microscopic observations have revealed a plausible explanation for the decrease in burst size. In NB/10 broth without added Ca^{++} or Mg^{++}, *Bdellovibrio* causes premature lysis of the host cell (Fig. 10a, b, and e). This does not happen, however, in all infected cells.

Some host cells retain at least a portion of their cytoplasmic contents (Fig. 10c and d). When the host does lyse rapidly at an early stage in the infection process, it does not form a spheroplast (Fig. 10a and b) as is characteristic of the normal events of infection in the presence of cations (Fig. 9a–e). When a normal spheroplast is not formed, the host and *Bdellovibrio* cells become more transparent as time progresses; presumably the parasite becomes nonviable within the ghosted host. This may account for the 20% drop in PFU during the latent period (Fig. 8). Furthermore, after 5 hr into the one-step growth curve (when the majority of the bursts have occurred), some *Bdellovibrio* cells are still at an early stage in the infection process and have barely begun to elongate within their host cell (Fig. 10e). Such parasites cannot complete their development within the following 0.5 hr, at which time the burst has terminated (Fig. 8). Microscopic observations have indicated, however, that at least a fraction of the *Bdellovibrio* cells complete their life cycle (Fig. 10f and g) under these conditions, thus resulting in the burst observed in the one-step growth curve.

When similar observations are carried out in Tris buffer, the infection causes a rapid lysis of the host cell. The host retains its rod shape and shows extrusions at one site on its surface (Fig. 11a and b). This extrustion probably corresponds to the site of penetration by the *Bdellovibrio*. In 4 to 5 hr, the host and *Bdellovibrio* cells become quite transparent, and it becomes increasingly difficult to find *Bdellovibrio* and ghosted host cells in the culture. The results of a one-step growth curve under these conditions showed a one-log drop in the *Bdellovibrio* PFU titer after 1.5 hr of incubation. When such a system is stabilized with Ca^{++} and Mg^{++} ions, a burst does occur (Fig. 11c).

The stabilization of *E. coli* spheroplasts by Ca^{++} or Mg^{++} ions is not a new observation. Lederberg and St. Clair (2) showed that these cations were required to stabilize lysozyme-induced spheroplasts, and that stabilization also needed the presence of 0.3 M sucrose. Weibull (14) believed that these cations may be essential for stabilizing lipid residues of the plasma membrane.

The preliminary report by Burnham, Hashimoto, and Conti (Bacteriol. Proc., p. 22, 1968) mentioned that the host cell membrane is osmotically protected during the penetration by *Bdellovibrio*. We conclude from our photomicrographs that this osmotic protection occurs only in the presence of added Ca^{++} and Mg^{++} ions. Also, the maintenance of the protoplast integrity is necessary for the normal completion of the intracellular growth phase of *Bdellovibrio* in *E. coli*.

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