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Interaction of *Bdellovibrio bacteriovorus* and Host Bacteria

I. Kinetic Studies of Attachment and Invasion of *Escherichia coli* B by *Bdellovibrio bacteriovorus*

MAZAL VARON AND M. SHILO

Department of Microbiological Chemistry, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

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Quantitative methods were developed for the study of the early stages in the interaction of *Bdellovibrio bacteriovorus* and host bacteria. Attachment measurements were based on the differential filtration of host and parasite. Invasion was measured by estimation of radioactively labeled *Bdellovibrio* cells remaining attached to the host cells after mechanical agitation. The kinetics of attachment and the final number of *Bdellovibrio* cells attached were dependent on the multiplicity of the parasite, the composition and *p*H of the medium, and the incubation temperature. Inhibitors of *Bdellovibrio* motility, including chelating agents, NaN_3, and low *p*H, all inhibited attachment, as did anaerobiosis. Ultraviolet-killed host cells retained their competence for attachment of *Bdellovibrio* cells, whereas heat-killed cells lost it. Invasion was selectively inhibited by inhibitors of protein synthesis, such as streptomycin, puromycin, and chloramphenicol. These antibiotics had no effect on attachment.

Bdellovibrio bacteriovorus, a unique group of parasitic bacteria first described by Stolp and Petzold (10), presents many interesting questions connected with the nature of its interaction with its hosts. These include the nature of host specificity, the existence and structure of host receptor sites, physical and enzymatic steps of the invasion process, parasite multiplication within the host cell, and the stages in the developmental cycle of the parasite outside the host.

Phase contrast and electron microscopic studies have shown that the actively motile *Bdellovibrio* cells attack and attach to host cells, damage them so that they are often transformed into spheroplasts, and cause their lysis (6, 7, 10, 11). Recent investigators (2, 5, 8; D. Abram and M. Shilo, Bacteriol. Proc., p. 41, 1967; J. C. C. Huang, J. Robinson, and R. G. E. Murray, Abstr. Ann. Meeting, Can. Soc. Microbiol, p. 45, 1966) have demonstrated intracellular growth and division of *B. bacteriovorus* within the host. At high multiplicity of *Bdellovibrio* cells, rapid lysis of the host cells similar to bacteriophage-induced "lysis from without" has been observed (Abram and Shilo, Bacteriol. Proc., p. 41, 1967).

In the present work, quantitative methods are given for the measurement of the early steps in the host-parasite interaction. The kinetics and requirements for attachment and invasion were studied with *B. bacteriovorus* 109 and with *Escherichia coli* as host.

MATERIALS AND METHODS

Bacterial strains and cultivation. B. bacteriovorus strain 109, described by Stolp and Starr (11), was used. A streptomycin-resistant mutant of *B. bacteriovorus* 109 was obtained after treatment with nitrosoguanidine by A. Oppenheim in our department.

The host used in these experiments was *E. coli* B-2262, from the stock collection of the Department of Bacteriology, University of California at Berkeley. We isolated a streptomycin-resistant mutant of this strain after ultraviolet irradiation.

B. bacteriovorus was grown in the dilute NB medium (DNB) described by Shilo and Bruff (7). The inoculum consisted of 5×10^8 to 10×10^8 plaque-forming units taken from a 24-hr culture and 4×10^9 to 6×10^9 host cells. Host and parasite were inoculated simultaneously into 30 ml of medium in a 100-ml Erlenmeyer flask and incubated at 30 C in a rotary New Brunswick PsychroTherm incubator shaker. Under these conditions, lysis of the host is almost complete after 16 to 20 hr, and the yield of *Bdellovibrio* cells is 10^9 to 2×10^9 plaque-forming units/ml (Fig. 1). The cells were harvested by centrifugation at 27,000 $\times$ *g* for 20 min in a Servall RC-2 centrifuge at 4 C. The sediment was suspended in DNB medium, and the suspension was successively filtered through cellulose

ester filters of an average pore size of 5, 3, and 1.2 μ in order to remove clumps and any remaining host bacteria. The cells were concentrated by centrifugation and washed with DNB medium. Finally, the *Bdellovibrio* concentration was adjusted by using a Klett-Summerson photoelectric colorimeter.

B. bacteriovorus was labeled by the addition of uniformly labeled ^{14}C-L-valine or ^{14}C-L-leucine (Radiochemical Centre, Amersham, England) at 0.33 μc per ml of growth medium. Under the growth conditions described, the incorporation of the label was approximately 10^8 counts per min for 3×10^7 plaque-forming units.

E. coli B was grown in Difco Nutrient Broth at 37 C on a New Brunswick rotary shaker and was harvested at the logarithmic phase of growth. The cells were centrifuged at 12,000 $\times$ *g* for 5 min, washed once, and suspended in DNB medium. Cell number was adjusted by using a Klett-Summerson colorimeter.

Plaque assay was carried out by the double-layer technique used for counting bacteriophage. A 0.2-ml sample of the appropriate *Bdellovibrio* dilution and 0.2 ml of the host suspension (containing 10^{10} cells per ml) were mixed in 2.5 ml of liquefied soft agar (DNB medium containing 0.6% Difco agar) kept in a water bath at 45 C. The mixture was immediately spread over the surface of DNB medium containing 1% agar in petri dishes. Plaques were counted after 4 to 5 days of incubation at 30 C.

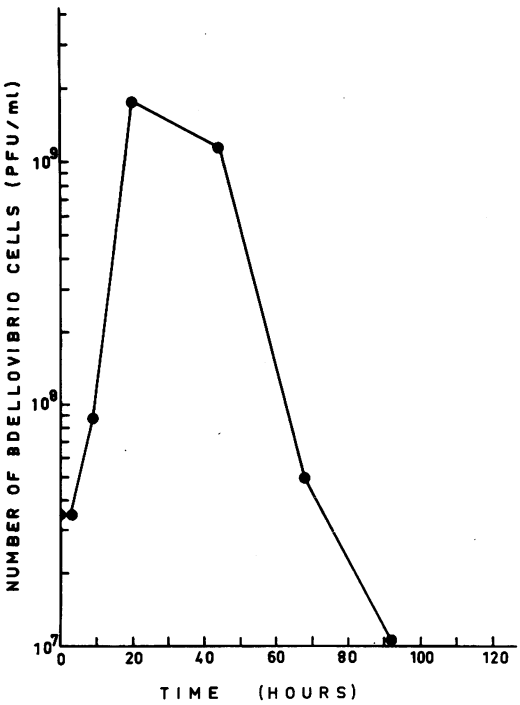

FIG. 1. *Growth curve of Bdellovibrio 109. B. bacteriovorus was grown with Escherichia coli B under standard conditions described. Portions of 0.5 ml were taken for enumeration of plaque-forming units.*

Chemicals. The following chemicals were used: *N* - methyl - *N'*-nitro - *N* - nitrosoguanidine (Aldrich Chemical Co. Inc., Milwaukee, Wis.); penicillin G (sodium salt) and streptomycin sulfate (Rafa Laboratories, Jerusalem); chloramphenicol B.P., (Abic Ltd., Ramat-Gan, Israel), puromycin (Nutritional Biochemical Corp., Cleveland, Ohio); iodoacetic, maleic, succinic, fumaric, and malonic acids, phenol, sodium azide, and the disodium salt of ethylenediaminetetraacetic acid (British Drug House, Poole, England), and tris(hydroxymethyl)aminomethane (Fluka, Switzerland).

Attachment experiments. Measurement of attachment of *B. bacteriovorus* to host cells was based on the differential filtration of host and parasite cells through filters of an average pore size of 1.2 μ. After washing, 97 to 99% of the host organisms were retained on the filter, whereas 90 to 95% of the free *Bdellovibrio* cells passed into the filtrate. To obtain reproducible results, careful attention was given to the growth stage of the cells, since this determined the cell size of both host and parasite and, thus, also the percentage of cells retained on the filter. One of the limitations of the method was the partial plugging of the filter by host bacteria at numbers higher than 3×10^8 to 4×10^8 cells.

Two different techniques for estimating attachment were possible: counting the radioactivity retained on the 1.2-μ filter (direct estimate), and counting the number of free *Bdellovibrio* cells as plaque-forming units in the filtrate (indirect estimate). Results of several experiments, in which the percentage of attached *Bdellovibrio* cells from the same sample were estimated in the two ways, showed a good correlation (within a limit of 6%). Therefore, we chose to employ the direct-estimate technique, which is simpler and faster, and makes frequent sampling possible.

Unless otherwise stated, 1 ml of the host suspension containing 3×10^9 cells was mixed with 1 ml of the parasite suspension containing 3×10^8 cells in a 50-ml Erlenmeyer flask. The mixture was shaken at 30 C; 0.2-ml portions were filtered through a 1.2-μ filter and washed with 100 ml of cold nutrient broth prefiltered through 1.2- and 0.8-μ filters. Nutrient broth was chosen for this purpose because it gave better results than several other washing solutions, including growth medium and NaCl solutions (0.015 M and 0.15 M). [These results on the washing efficiency of nutrient broth are similar to those obtained with f2 bacteriophage adsorption to filters (3).] After washing, the filter was removed to a scintillation vial and dried undeh a 250-w lamp. A 10-ml amount of scintillation fluid [containing 3 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene per liter of toluene] was added to each vial and the radioactivity was measured in a Tri-Carb scintillation counter (Packard Instruments Co. Inc., La Grange, Ill.). The duration of each count was 5 min.

A host-free control suspension of *Bdellovibrio* cells was prepared at the same dilution as the host-parasite suspension described above. Samples of this suspension were filtered as described through 1.2- and 0.22-μ filters, the last filter retaining all *Bdellovibrio* cells. The percentage of attached *Bdellovibrio* cells was calculated by subtracting the counts per minute

of host-free *Bdellovibrio* cells retained on the 1.2-μ filter from the counts per minute of *Bdellovibrio* cells plus host cells retained on the 1.2-μ filter, dividing the number obtained by the counts per minute of total *Bdellovibrio* cells (retained on the 0.22-μ filter), and multiplying times 100. (The "counts per minute" values used were the means of duplicate tests.) This calculation cannot exactly express the actual experimental conditions, since the numbers of free parasites retained on the 1.2-μ filter should decrease as attachment progresses. However, the size of the parasites was small enough to allow for no more than an initial 4 to 6% of the total free parasites to be retained on the filter; thus the calculated values are within the range of experimental error.

RESULTS

Attachment of B. bacteriovorus to E. coli at different relative concentrations. Figure 2 shows the effect of the parasite-host ratio on the kinetics of attachment of *B. bacteriovorus* to *E. coli* under conditions where (i) the number of *E. coli* cells was constant and the number of *Bdellovibrio* cells varied, and (ii) the number of *Bdellovibrio* cells was constant and the number of host cells varied. In both cases, the percentage of the parasite population attached increased progressively as the parasite-host ratio decreased. At a ratio of 0.1, the greatest part (up to 90%) of the parasite population was found to attach. At this ratio, there was a large excess of available competent host bacteria (Fig. 3). When *E. coli* cells were added to a parasite-host mixture after attachment saturation was attained, no further attachment of *Bdellovibrio* cells occurred. On the other hand, addition of fresh *B. bacteriovorus* cells to such a mixture resulted in an immediate increase in the number of *Bdellovibrio* cells attached.

In an experiment to determine the degree of attachment at high multiplicities, 5×10^9 *Bdellovibrio* cells were incubated with 5×10^8 host cells. After 20 min, a total of 1.6×10^9 *Bdellovibrio* cells, or 32% of the total population, had attached, a value that remained more or less constant until 40 min.

Effect of the age of B. bacteriovorus on its attachment to E. coli cells. Microscopic examination of free *Bdellovibrio* cells at different stages of the developmental cycle showed marked differences in their size. *Bdellovibrio* cells just liberated from the host cells were usually motile and small. Upon further incubation in the absence of the host, they lost motility and became larger. The mean length of *Bdellovibrio* cells, as measured in photomicrographs, was found to be 1.2 μ (range 1.0 to 1.4 μ) during the first 2 hr after lysis of the host, and 1.7 μ (range 1.1 to 2.3 μ) 8 to 10 hr later. Later, they gradually became small again and death ensued (Fig. 1). Another parameter associated with cell size, and which was also found to change with the age of the culture, was the filterability of the cells through filters of 1.2 μ average pore size. The percentage of *Bdellovibrio* cells retained on the filter ranged from 2 to 4 for the young and small cells to 11 to 12 for the older and larger ones. Figure 4 summarizes the majority of our attachment experiments with motile populations of *Bdellovibrio* cells taken at different stages before the onset of the decline phase in the growth curve. A correlation was found between attachment efficiency and filterability of *Bdellovibrio* cells; the small and more filterable cells were the most active.

Attachment of B. bacteriovorus to killed E. coli cells. E. coli cells killed by heating at 70 C for 15 min lost their capacity to bind *Bdellovibrio* cells. On the other hand, lethal ultraviolet irradia-

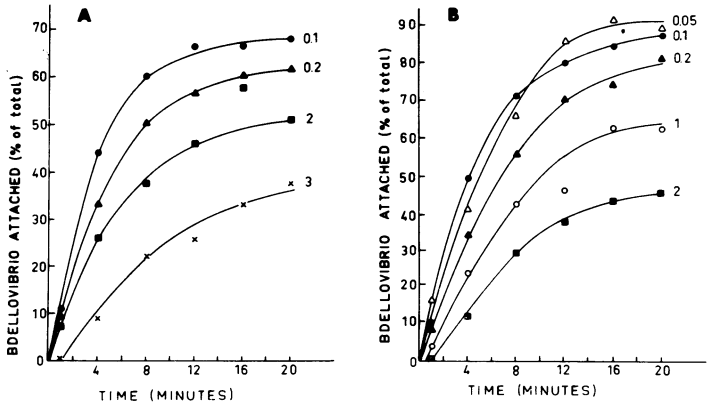

FIG. 2. *Effect of parasite-host ratio on the kinetics of attachment of Bdellovibrio bacteriovorus to Escherichia coli. (A) Concentration of host, 1.5 × 10⁸ cells per ml; concentration of the parasite varied to give the ratios indicated. (B) Concentration of Bdellovibrio cells, 1.5 × 10⁸ cells per ml, concentration of the host varied to give indicated ratio.*

tion of the host did not affect *Bdellovibrio* attachment (Fig. 5). The subsequent changes in the host cells and the first stages of the intracellular development of *Bdellovibrio* cells were also found

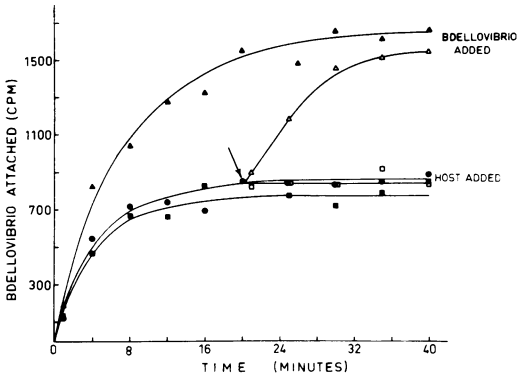

FIG. 3. *Effect of the interaction of host and parasite on the capacity of host cells to bind additional Bdellovibrio cells and on the ability of Bdellovibrio cells to attach to fresh Escherichia coli. The incubation mixtures contained, at zero time, 2 ml of each suspension. Arrow shows the addition of fresh host or Bdellovibrio cells at 20 min. The bacteria were added from concentrated (10×) suspension. Concentrations of bacteria (×10⁸/ml) are indicated by the following symbols: (●) 1.5 B. bacteriovorus and 15 E. coli at zero time; (▲) 3.0 B. bacteriovorus and 15 E. coli at zero time; (■) 1.5 B. bacteriovorus and 30 E. coli at zero time; (△) 1.5 B. bacteriovorus and 15 E. coli at zero time plus 1.5 B. bacteriovorus at 20 min; (□) 1.5 B. bacteriovorus and 15 E. coli at zero time plus 15 E. coli at 20 min.*

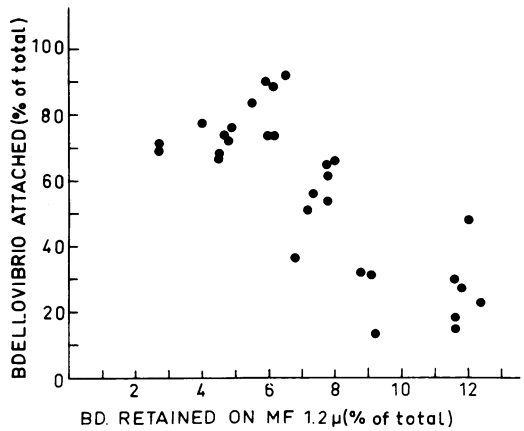

FIG. 4. *Correlation between filterability of Bdellovibrio bacteriovorus cells and their attachment efficiency. Filterability was measured as percentage of Bdellovibrio cells retained on a 1.2-μ filter out of the total population retained on a 0.22-μ filter. For these measurements, samples of 0.2 ml of Bdellovibrio suspension in DNB medium containing 1.5 × 10⁸ cells per ml were used. Attachment was determined after 20 min of incubation at standard conditions.*

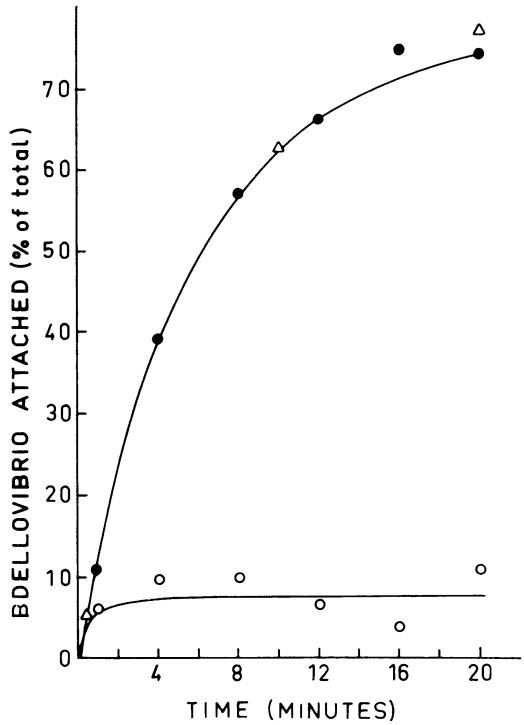

FIG. 5. *Attachment of Bdellovibrio bacteriovorus to killed Escherichia coli cells. Attachment of Bdellovibrio cells to host cells killed by heating at 70 C for 15 min (○), or irradiated by a Hanovia 1.25-w ultraviolet lamp with a dose sufficient to kill 99.9% of the population (△). Attachment to intact logarithmic phase E. coli cells (●).*

to be unaffected, as far as these could be observed under the phase-contrast microscope.

Attachment kinetics in various media. The attachment kinetics in several media are shown in Fig. 6. The highest rate and degree of attachment was achieved in DNB medium. In glass-distilled (2×) water, the attachment was about half that obtained in the DNB medium. Addition of buffers to distilled water diminished attachment; the inhibitory affect was related to the molar concentration of the buffer. The addition of buffer to DNB medium produced a less marked effect. Tris(hydroxymethyl)aminomethane and succinate dissolved in DNB medium at a concentration of 0.002 M exerted only slight inhibition, and these conditions were therefore chosen for testing the effect of *p*H on attachment. As shown in Fig. 7, no attachment occurred at *p*H 5.0 or below. To test whether this was a specific effect of succinic acid, the *p*H of the medium was lowered to 5.0 with acetic or hydrochloric acids. Microscopic examinations showed that under these conditions, also, there was no attachment. Under all the conditions tested, *Bdellovibrio* cells lost motility completely at *p*H 5.0.

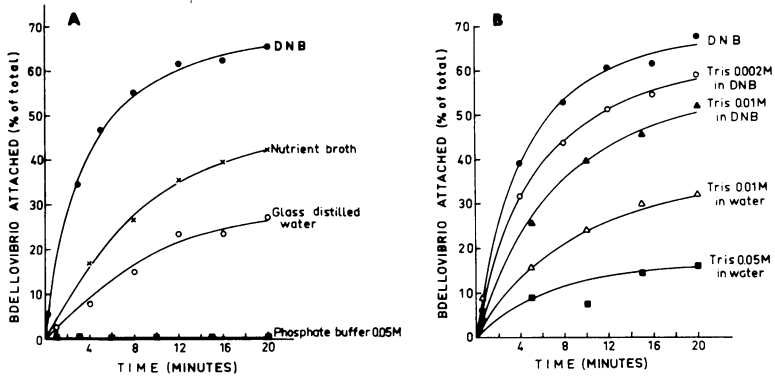

FIG. 6. *Kinetics of attachment of Bdellovibrio bacteriovorus in various media. Host and parasite cells were washed twice in 40 ml of the medium or buffer in which they were finally resuspended. The pH of all media was adjusted to 7.2.*

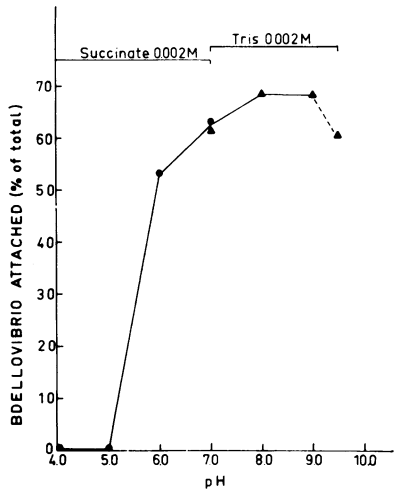

FIG. 7. *Effect of pH on the attachment of Bdellovibrio bacteriovorus. Host and parasite cells were washed twice in 40 ml of buffered medium and then resuspended in the respective medium. The pH above 9 was found to be unstable and decreased from 10 to 9.3 at the end of the experiment.*

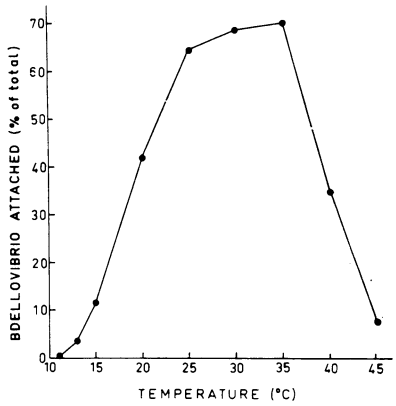

FIG. 8. *Attachment of Bdellovibrio bacteriovorus to Escherichia coli B at different temperatures. Host and parasite suspensions were incubated separately at the chosen temperature for 5 min before mixing. Percentage of attached cells was determined 20 min after mixing.*

Effect of incubation temperature on percentage of attachment. The effect of incubation temperature on attachment is shown in Fig. 8. The highest degree of attachment was achieved at 30 to 35 C. Attachment was slow at 20 C and negligible at temperatures lower than 15 C or at 45 C. It was established by plaque assay that incubation of *Bdellovibrio* suspension at 45 C for 10 min (a period usually sufficient for most cells to attach) did not significantly affect the viability of *B. bacteriovorus.*

Figure 9 shows the effect of shift in the incubation temperature of the parasite-host mixture from 20 C (conditions which allow only part of the *Bdellovibrio* cells to attach) to the optimal incubation temperature. After attachment had reached a plateau at 20 C, the mixture was transferred to 30 C. This temperature shift resulted in a new wave of attachment up to the level achieved in mixtures incubated at 30 C from the start.

Effect of aeration. Figure 10 shows that different aeration conditions have a marked effect on the attachment of *B. bacteriovorus to E. coli.* In the shaken mixture, the rate and level of attachment were noticeably higher than those obtained under nonshaken semianaerobic conditions.

Inhibitors. A high hydrogen-ion concentration prevents the attachment of *Bdellovibrio* cells and arrests their motility. Phase-contrast microscopy showed that a number of chemicals which prevented attachment had the same effect on the motility of *Bdellovibrio* cells. These included:

phenol (0.05%), NaCl (0.9%), and Na_2-ethylenediaminetetraacetic acid (1 μmole/ml); and Na-citrate, Na-maleate, Na-fumarate, and Na-malonate, each at concentrations of 25 μmoles/ml.

Table 1 shows the effect of metabolic inhibitors on the degree of attachment of *B. bacteriovorus*. Sodium azide at a concentration of 10 mM inhibited attachment almost completely. Sodium iodoacetate at similar concentrations showed only partial inhibition, and there was a significant attachment even in the presence of 20 mM sodium iodoacetate. Microscopic observations revealed that 10 mM sodium azide arrested the motility of

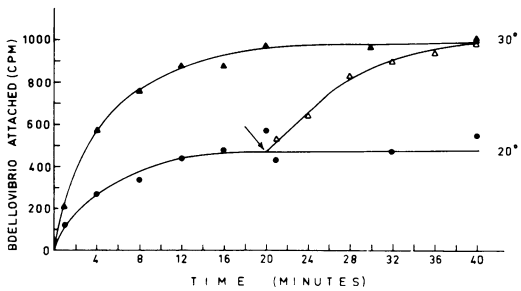

FIG. 9. *Effect of shift in the incubation temperature on the attachment of Bdellovibrio bacteriovorus to Escherichia coli. A mixture of host (2 ml) and parasite (2 ml) was incubated at 30 C (▲); 20 C (●); 20 C for the first 20 min and then transferred (arrow) to 30 C (△).*

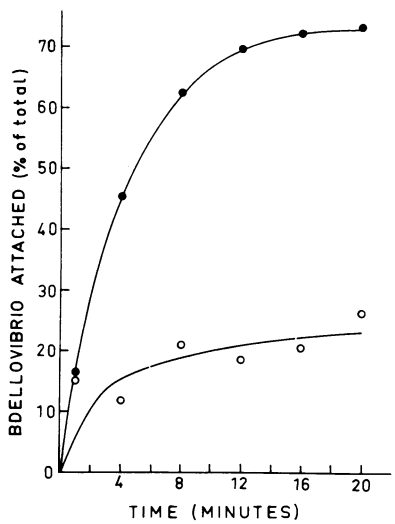

FIG. 10. *Effect of aeration on the attachment kinetics of Bdellovibrio bacteriovorus to Escherichia coli B. A 1.5 ml amount of the Bdellovibrio suspension and 1.5 ml of the host suspension were incubated at 30 C in a 50-ml Erlenmeyer flask shaken on a reciprocal shaker and in a stationary test tube of 5-mm diameter (○).*

TABLE 1. *Effect of metabolic inhibitors on the attachment of Bdellovibrio bacteriovorus to Escherichia coli B*

Inhibitor	Attachment of *B. bacteriovorus* (% of control) at different inhibitor concentrations[a]			
	0.5 mM	2.5 mM	10 mM	20 mM
NaN_3				
Experiment 1	101.0	90.1	7.2	
Experiment 2	97.2	84.8	0.0	
Na iodoacetate				
Experiment 1	84.3	78.7	69.3	
Experiment 2	81.3	70.7	51.0	29.3

[a] Attachment was determined 30 min after mixing host and parasite.

Bdellovibrio cells, whereas sodium iodoacetate, even at 20 mM, affected it only slightly.

Penetration of B. bacteriovorus into host cells. Mechanical agitation for 1 min in a Servall Omni-Mixer (micro-attachment) at 200 v, shortly after mixing host and parasite, caused the *Bdellovibrio* cells to detach. The detachment was complete in mixtures incubated for a few minutes; in mixtures incubated for longer periods, the effect of this treatment in causing detachment gradually decreased. From 20 to 30 min after the beginning of the reaction, agitation treatment no longer affected the attachment. Microscopic examinations at this stage showed that most *Bdellovibrio* cells were already inside the host cells (Fig. 11, b), whereas few were seen to be attached from outside, and fewer still were free. This simple technique enabled us to separate the early (attachment) step in the infection process from the following step, which is invasion of the host cells. Figure 12 shows that, whereas attachment starts immediately after mixing host and parasite, invasion begins after a lag and then increases rapidly. After 30 min, the *Bdellovibrio* cells penetrating host cells reach a value close to 90% of total attached cells in the controls (Table 2).

Role of protein synthesis in attachment and penetration of B. bacteriovorus. Table 2 shows the effect of several antibiotics on the early stages of *E. coli* B infection by *B. bacteriovorus* 109. The effect of streptomycin on attachment is shown in Fig. 13. None of the antibiotics tested had any effect on attachment. Invasion, however, was almost completely inhibited by streptomycin, chloramphenicol, or puromycin, all known to inhibit protein synthesis. Penicillin affected neither attachment nor invasion. To test whether the streptomycin-susceptible process takes place in the host or in the parasite, resistant strains

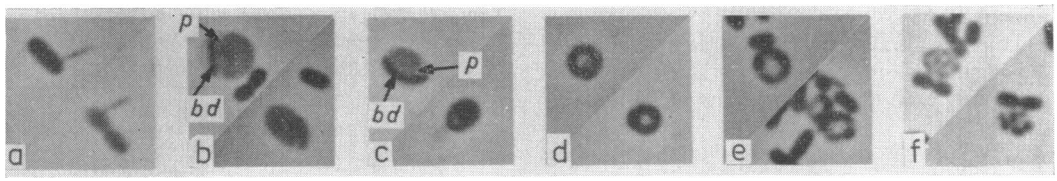

FIG. 11. *Attachment and intracellular life cycle of Bdellovibrio bacteriovorus 109 in host Escherichia coli B. Parasite (5 × 10⁸ cells) and host (1 × 10⁹ cells) were mixed in 2 ml of DNB medium and incubated at 30 C in a shaking bath. (a) B. bacteriovorus attached to E. coli immediately after mixing; (b) 30 min after mixing: parasite has entirely penetrated into host cell; note the exoparasitic position of Bdellovibrio (Bd) inside the cell wall, but outside the protoplast (P); (c) 1.5 hr: parasite has elongated; (d) 2.5 to 3 hr: parasite further elongates and assumes ring form; (e) 3.5 hr: parasite has begun fragmenting into daughter cells; (f) 4 hr: fragmentation completed, but daughter cells not yet released. (Phase-contrast microscope, 800×).*

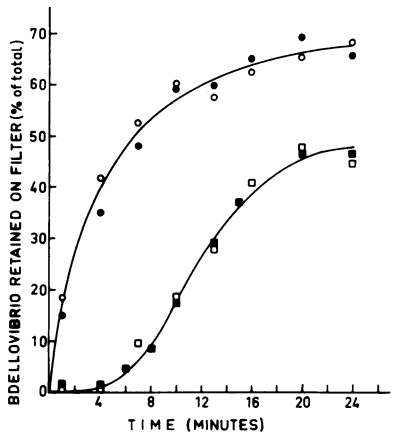

FIG. 12. *Kinetics of attachment and invasion of Bdellovibrio bacteriovorus. Escherichia coli B and Bdellovibrio suspensions were mixed and 0.2-ml samples were either taken into tubes with 5 ml of cold nutrient broth and filtered (circles), or were taken into tubes containing 1.8 ml of cold DNB medium, mechanically agitated, and then transferred into nutrient broth and filtered (squares). Open and closed symbols represent two different experiments.*

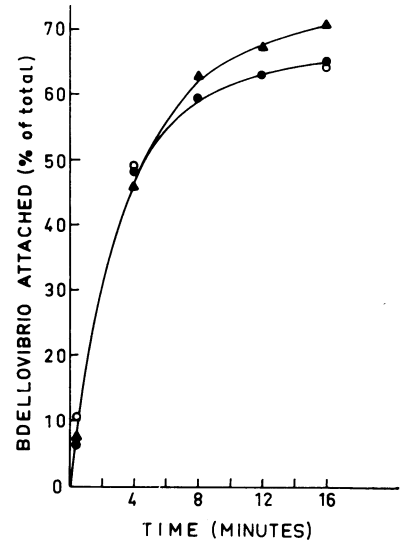

FIG. 13. *Kinetics of attachment of Bdellovibrio bacteriovorus to Escherichia coli B in the presence of streptomycin (50 μg/ml). Host and parasite susceptible (●), or resistant (○), to the antibiotic. Control without streptomycin (▲).*

TABLE 2. *Effect of penicillin and inhibitors of protein synthesis on penetration of Bdellovibrio bacteriovorus into host cells*[a]

Antibiotic	*Bdellovibrio* cells retained on filter (% of total)	
	After treatment	Without treatment
Streptomycin (50 μg/ml)	0.15	66.4
Chloramphenicol (50 μg/ml)	0.17	60.0
Puromycin (100 μg/ml)	4.15	59.0
Penicillin (100 units/ml)	62.4	67.0
None	62.5	66.2

[a] Suspensions of host and parasite were mixed; samples were removed after 30 min of incubation and were treated as described in Fig. 12.

were used. Table 3 shows that the inhibitory effect of streptomycin on invasion was expressed fully with either streptomycin-resistant or streptomycin-susceptible host cells, but it was abolished when a streptomycin-resistant *Bdellovibrio* strain was used.

DISCUSSION

The sequential steps in the attack of host bacteria by *B. bacteriovorus* have been described by a number of investigators on the basis of phase-contrast and electron microscopic studies (2, 5–8, 10, 11; D. Abram and M. Shilo, Bacteriol. Proc., p. 41, 1967). No method existed, however, for a quantitative description of the host-parasite interaction. We have developed a method for studying the kinetics of the early stages in this interaction. The method, based on the differential filtration

TABLE 3. *Penetration of Bdellovibrio bacteriovorus into Escherichia coli B in the presence of streptomycin*[a]

Parasite	Host	*Bdellovibrio* cells retained on filter after treatment (% of total)	
		Streptomycin (50 μg/ml)	Control
B. bacteriovorus 109	*E. coli* B	0.8	57.4
B. bacteriovorus 109	*E. coli* B str^r	8.0	64.8
B. bacteriovorus 109 str^r	*E. coli* B	42.6	56.0
B. bacteriovorus 109 str^r	*E. coli* B str^r	59.0	65.2

[a] The host and parasite cells were incubated together for 20 min; samples were removed for treatment in a homogenizer as described in Fig. 12.

of host and parasite through small pore-size filters, allows sampling at very short intervals and immediate separation of host and parasite, and is therefore suitable for kinetic studies. However, it is suitable for measuring irreversible attachment only; any reversible steps (which microscopically seem to occur) cannot be measured in this way.

The complete separation of *Bdellovibrio* cells from host bacteria by this method may be of use in the isolation and quantitation of *Bdellovibrio* populations from different natural environments. The method employed until now for isolating *B. bacteriovorus* from different ecosystems is based on a series of sequential filtrations (9, 10) from which only a small percentage of the original population is obtained.

The kinetics of attachment of *B. bacteriovorus* to *E. coli* invariably follows a saturation curve, the slope and saturation level of which vary according to experimental conditions. One of the most important factors determining both attachment rate and saturation level is the physiological state of the *Bdellovibrio* cells. In our experiments with actively motile *Bdellovibrio* cells, attachment of 60 to 90% of the total *Bdellovibrio* population was obtained after 20 min of incubation with the host under optimal conditions. The variations in percentage of attachment could be explained by the correlation found between the attachment efficiency in the different experiments and the size of the *Bdellovibrio* cells, which seems to be a function of the physiological age of the cells. The fact that *Bdellovibrio* cells grow only in two-membered cultures and that rapid death sets in soon after the host cells are exhausted are the main obstacles in obtaining standard *Bdellovibrio* populations. Thus, in different cultures, that part of the *Bdellovibrio* population which is incapable of attachment varies.

That portion of the population which is capable of attachment also seems to be heterogeneous, as was indicated by the temperature-shift experiment. Only part of the *Bdellovibrio* population capable of attachment at 30 C attaches to the host cells at 20 C, even upon prolonged incubation, and the plateau level reached within the first 20 min remains constant (Fig. 9). Low temperature effects on metabolic activities of host and parasite and on physical interaction between them may well affect the rate of the attachment, but would not explain the plateau observed at 20 C. It might be of interest to test whether such heterogeneity in the *Bdellovibrio* population underlies the limited attachment at other suboptimal conditions.

At the optimal conditions for attachment, the percentage of *Bdellovibrio* cells attached at the saturation level depends on the parasite-host ratio. The highest percentage is achieved when this ratio is 0.1. Higher ratios (2 to 3) yield significantly lower percentages of attachment, and saturation of the host is reached when every cell binds on the average up to one *Bdellovibrio* cell. Higher binding capacity is achieved only when the multiplicity is considerably higher. Thus, for example, at a multiplicity of 10 *Bdellovibrio* cells, every host cell binds on the average 3.2 *Bdellovibrio* cells. Microscopic examinations show that the host population is heterogeneous in its binding capacity. Part of the cells are observed to be attacked within seconds by tens of *Bdellovibrio* cells which entirely cover their surface and cause their rapid lysis ("lysis from without"). At the same time, there are other host cells which bind but few *Bdellovibrio* cells or even remain free for some time. It seems that only those cells which bind 1 to 2 *Bdellovibrio* cells allow for the intracellular life cycle which gives rise to a new generation of the parasite (Fig. 11).

Attachment is affected by the composition of the medium and the physicochemical conditions in which it takes place. Since significant attachment takes place in glass-distilled (2×) water, there may not be a requirement for cations or any other cofactors. However, as the attachment of *Bdellovibrio* cells to *E. coli* cells is inhibited by the addition of chelating agents such as ethylenediaminetetraacetate and citrate, the role of trace metals which may be present in the glass-distilled water cannot be excluded. J. Robinson and J. C. Huang (Bacteriol. Proc., p. 42, 1967) and Huang et al. (Abstr. Ann. Meeting, Can. Soc. Microbiol., p. 45, 1966) have mentioned the role of Ca^{++} and Mg^{++} in the establishment of the

host-parasite relationship and growth of *B. bacteriovorus* in two-membered cultures. Under such conditions, however, only the overall effect of the added cation can be measured, and it is impossible to relate the results to any one of the many steps involved in the interaction sequence of the parasite and its host.

Chelating agents, organic acids, phenol, azide, low *p*H, and sodium chloride all inhibit the motility of *Bdellovibrio* cells. *B. bacteriovorus* appears to be more sensitive to these agents than other bacteria, since both ethylenediaminetetraacetate and maleate at the concentration tested did not affect the motility of *Pseudomonas fluorescens* or *Salmonella enteritidis*. It has been shown (1) that only a 50 times higher concentration of ethylenediaminetetraacetate (5×10^{-2} M) inhibits motility of *E. coli* K-12, and lower concentrations promote motility. The high sensitivity of *B. bacteriovorus* motility to different agents may be connected with the unique structure of its flagellum, which has been shown in electron microscope studies to be sheathed along its entire length (D. Abram and M. Shilo, Bacteriol. Proc., p. 41, 1967; J. C. C. Huang, J. Robinson, and R. G. E. Murray, Abstr. Ann. Meeting, Can. Soc. Microbiol., p. 45, 1966; R. J. Seidler and M. P. Starr, Bacteriol. Proc., p. 42, 1967).

Stolp and Petzold (10) and Stolp and Starr (11) have stressed the importance of the active motility of *B. bacteriovorus* in its attack on the host. They suggested that the physical impact of the collision between the motile *Bdellovibrio* cell and its host plays an important role in the damage of the host-cell wall and may be a prerequisite for attachment. This view was given indirect corroboration by their observation that all nonparasitic, saprophytic strains of *B. bacteriovorus* isolated by them lacked motility. Our results, which show that many agents at similar concentrations inhibit both motility and attachment tend, therefore, to support this idea of Stolp and his colleagues.

Host viability is not a requirement for attachment of *Bdellovibrio* cells, as ultraviolet irradiation of host cells did not affect their competence to be attached. On the other hand, heat-killed cells bind *Bdellovibrio* cells very poorly or not at all. It is possible that heating alters the host-cell surface and thus prevents attachment. It might be of interest to study the effects of various other agents such as formaldehyde, *p*-chloromercuribenzoate, and many others which are known to affect the ability of *E. coli* cells to adsorb bacteriophages (4, 12). A second approach to the elucidation of the role of the host-cell wall in the attachment of *B. bacteriovorus* involves the use of bacterial mutants with defective cell wall synthesis. Experiments with such mutants are now under way in our laboratory.

The step following attachment is invasion. At this stage, mechanical agitation no longer detaches *Bdellovibrio* cells from the host, and microscopic observations reveal them inside the host cells. This step is completely blocked by inhibitors of protein synthesis. It is possible that one or more inducible enzymes have to be formed by the parasite for its invasion into the host cell. This presumed induction seems to depend upon the direct contact of the *Bdellovibrio* cell with its host or with inducers which, masked in the host cell wall, are exposed by the attachment.

Possible physical damage of the host-cell wall during the attachment process may also play a role in preparation for invasion.

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