Bdellovibrio bacteriovorus gen. et sp. n., a predatory, ectoparasitic, and bacteriolytic microorganism

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ABSTRACT

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Bdellovibrio bacteriovorus, gen. et sp. n., a predatory and ectoparasitic microorganism with lytic activity against susceptible bacteria, is described, as are techniques for isolation and cultivation. These unusual bacteria cause reactions that are similar in their outward manifestations to bacteriophage-induced lysis. Upon plating a mixture of host bacteria and parasites, confluent lysis or single plaque formation occurs, just as in titration experiments with bacteriophage. However, the parasite plaques develop more slowly than phage plaques. Lysis of host bacteria in liquid culture is accompanied by a decrease in optical density; actually, a population of infected host bacteria is replaced by a population of the tiny parasite.

Individual cells of the presently known strains of *Bdellovibrio bacteriovorus* are typically about 0.3 μ in width and, thus, are considerably narrower than ordinary bacteria. Therefore, they can pass Millipore filters of 0.45 μ pore size diameter. Their shape is often vibrio-like. They possess one unusually thick polar flagellum of about 50 mμ diameter, and they show a distinctive type of motility.

The interaction between *Bdellovibrio* and the attacked host bacterium can be followed in the phase-contrast microscope; it is characterized by a physical attack of the highly motile parasite, attachment to the bacterial cell surface, and lysis of the host cell.

It has not yet been possible to cultivate *Bdellovibrio* in its parasitic form on any artificial substrate. All parasitic strains require living host cells for their propagation. However, saprophytic mutants can be selected from a population of the parasite. These saprophytic derivatives are unable to lyse living bacteria as does the wild-type parasite. On the basis of morphological and physiological properties, a sapro-

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phyte strain which has been examined in some detail shows no close relationship to any of the already known categories of bacteria.

A study of the kinetics of growth of *Bdellovibrio* in mixed culture with a susceptible host has disclosed that the number of parasites produced is not proportional to the number of host bacteria killed during the same period. After the majority of the host cells has been destroyed, there is still a considerable increase in parasites, indicating that they grow at the expense of material released from the lysed bacteria. Under the conditions of this trial, the generation time is about 100 minutes.

All presently known isolates of *Bdellovibrio* possess lytic activity only against gram-negative bacteria. The individual strains, however, show certain differences in their host activity spectra; some have a restricted host range, while others are able to attack a broad spectrum of host bacteria.

INTRODUCTION

In 1962, in the course of experiments designed for the isolation of bacteriophages from soil, the first strain was found of a new group of bacteria which are parasitic upon other bacteria. This particular strain, which has been described by Stolp and Petzold (1962), is characterized by a lytic activity limited to members of the Pseudomonadales. Using a special isolation technique, we have recently succeeded in isolating a number of additional strains from California soil and sewage samples (Stolp and Starr, 1963). These new isolates and the current studies broaden our concept of this group. Although the same general type of parasitic microorganism is involved, some of the isolates differ from the original strain in their host activity spectra. In the presence of susceptible bacteria, the parasites cause reactions which are very similar in their outward manifestations to bacteriophage-induced lysis. Upon plating a mixture of host bacteria and parasites in a layer of semisolid agar, confluent lysis or plaque formation occurs just as in titration experiments with bacteriophage. In liquid culture, under suitable conditions, infection of susceptible host bacteria with the parasite results in lysis, which is accompanied by a decrease in optical density. The interaction between parasite and host cell is characterized by physical attack of the highly motile parasite, and attachment to the bacterial cells. This action is often succeeded by an intermediate spherical stage and finally by disintegration of the infected host cell. A population of the host bacteria is replaced by a population of the parasites. From the nature of the attack, the organism must be regarded as a predatory and bacteriolytic ectoparasite.

The experiments so far completed indicate that this hitherto unknown group of microorganisms exists in many natural habitats, and probably re-

presents an integral component of the microbial flora. Later in this report we propose formally the designation *Bdellovibrio* for this group; for convenience in the presentation, we anticipate our formal taxonomic recommendation and refer to these tiny, ectoparasitic bacteria by that name.

MATERIAL AND METHODS

Culture media

YDC agar: 1% Difco yeast extract, 2% glucose, 2% finely divided calcium carbonate, and 1.5% agar. Stock cultures of the various host bacteria are kept on YDC agar slants.

NBA: 1,000 ml distilled water, 8 g Difco dehydrated nutrient broth, 5 g Difco Casamino acids, 1 g Difco yeast extract, pH 6.8.

PY: 1,000 ml distilled water, 10 g Difco peptone, 3 g Difco yeast extract, pH 6.8.

YP: 1,000 ml distilled water, 3 g Difco yeast extract, 0.6 g Difco peptone, pH 7.2.

Agar concentrations for double-layer technique: The bottom layer is made with 1.9% agar, and the top layer with 0.6% agar, usually in YP solution; other compositions are mentioned in the text.

Organisms

Table 1 lists the bacterial cultures used as indicator host strains for the isolation of parasites. Most of these originate from the International Collection of Phytopathogenic Bacteria (ICPB) maintained in this department.

Isolation of Bdellovibrio

The following procedure has proved to be effective for the isolation of *Bdellovibrio*. A soil sample (500 g) is suspended in tap water (500 ml) and shaken vigorously for one hr. The suspension is centrifuged for 5 min at 2,000 rpm, and the relatively dirty supernatant, which contains all types of microbes extracted from the soil, is submitted to a series of differential filtrations. The step-wise filtration through Millipore filters of different porosities is started with filters of 3 μ average pore size diameter, and continued with filters of 1.2, 0.8, 0.65, and 0.45 μ pore size. Portions of the last two or three fractions, which have a diminishing content of ordinary bacteria, are then mixed with a suspension of the prospective host strain, which is used as an indicator bacterium. These mixtures are plated in the same way as is done in phage isolation experiments, using the double-layer technique.

TABLE 1

Cultures used in this investigation[1])

Bacterial strain No.	Species and strain	*Bdellovibrio* strain isolated
100	*Erwinia amylovora* EA137	*Bd.* 100
101	*Erwinia carotovora* EC153	*Bd.* 101
102	*Corynebacterium insidiosum* CI13A	—
103	*Corynebacterium poinsettiae* CP2	—
104	*Agrobacterium tumefaciens* TT10	—
105	*Bacillus polymyxa* 2120	—
106	*Bacillus megaterium* 2363	—
107	*Bacillus subtilis* 2035	—
108	*Agrobacterium radiobacter* TR1	—
109	*Escherichia coli*-B 2262	*Bd.* 109
110	*Aerobacter aerogenes* 2001	*Bd.* 110
111	*Pseudomonas polycolor* PP2	—
112	*Acetobacter aceti* 2108	—
113	*Staphylococcus aureus* 2092	—
114	*Proteus mirabilis*	*Bd.* 114
115	*Caulobacter* sp. CB15	—
116	*Pseudomonas aeruginosa* 2019	—
117	*Arthrobacter atrocyaneus* 2396	—
118	*Serratia marcescens* 2031	*Bd.* 118
119	*Sarcina lutea* 2040	—
120	*Bacterium stewartii* SS12	*Bd.* 120
121	*Streptococcus lactis* 2194	—
122	*Lactobacillus casei* 2193	—
123	*Aeromonas* sp. 2326	—
124	*Rhizobium leguminosarum* 2067	—
125	*Corynebacterium michiganense* CM9	—
126	*Protaminobacter rubrum* 2144	—
127	*Pseudomonas solanacearum* PS138	*Bd.* 127
128	*Aerobacter cloacae* 2112	*Bd.* 128
129	*Pseudomonas caryophylli* PC102	—
130	*Rhodospirillum rubrum* S1	—
321	*Pseudomonas phaseolicola* ATCC 11355	*Bd.* 321
233	*Pseudomonas tabaci* NRRL B877	*Bd.* 233
A3.12	*Pseudomonas fluorescens* ATCC 12633	*Bd.* A3.12

[1]) The cultures listed are maintained in lyophilized condition at the International Collection of Phytopathogenic Bacteria, Department of Bacteriology, University of California, Davis, California, U.S.A.

If the added fraction of the soil filtrate contains parasitic organisms of the type under discussion, lytic spots appear after 2 to 4 days which – in their initial stages – are externally identical to phage plaques. The isolation of

Bdellovibrio from sewage or other material can be undertaken in a fashion similar to that described for soil.

The parasite plaques which were first observed appeared accidentally during a phage isolation experiment, in a lawn of *Pseudomonas phaseolicola* growing on a medium of high nutrient content (NBA). It has since been learned that growth and activity of the parasite are much better in a medium which does not support maximum growth of the host; therefore, a medium of relatively low nutrient content (YP) is now used for isolation. An adequate supply of substrate for the parasite is assured by inoculating a concentrated cell-suspension into the top layer (approximately 10^{10} bacteria per plate). One-half ml portions of the bacterial suspension and of the filtrate which is to be checked for *Bdellovibrio* are placed upon the solidified bottom layer, and then mixed with about 4 ml of the molten semisolid agar medium of the same nutrient composition (YP).

After overnight growth, the plates are checked for phage plaques. Although the bacteriophage content of native soil or sewage samples is usually extremely low, phage plaques occur occasionally and must be marked at this point in order to avoid confusion with the parasite plaques which develop somewhat more slowly. The phage enrichment technique, which is based on a selective phage multiplication by massive inoculation of the indicator bacteria into soil samples, has proved to be of no advantage in the isolation of *Bdellovibrio*. On the contrary, in many instances where single phage particles present in some unenriched natural material will not interfere with the isolation of parasites, the phage multiplication upon enrichment results in so high a phage titer that it is impossible to detect the parasites in a dilution series because of their relatively low numbers. In contrast to bacteriophages which produce plaques during the exponential growth phase of their host cells, *Bdellovibrio* normally requires at least 2 days in order to develop visible plaques in isolation experiments. The single parasitic cells embedded in the lawn of growing bacteria have to overcome their own lag phase before they can start multiplying. In addition, they grow slowly with a generation time of about 100 min (cf. Fig. 8). Therefore, the development of a continuous lawn by the rapidly growing host bacteria will necessarily be accomplished before plaque formation by the parasite becomes visible. These conditions do not exclude the prospect that single host cells might be lysed by metabolically active parasites in a few minutes after infection.

Plaques in isolation plates, which are suspected to be caused by *Bdellovibrio*, are cut out from the top layer, suspended in YP solution, and plated in a lawn of the corresponding host bacteria, using a dilution series in order to get single plaque formation. If lytic spots develop after about 2 days of

incubation, one of the plaques may be checked microscopically for the presence of parasites.

The procedure for obtaining pure cultures of *Bdellovibrio* is the same as used for purification of bacteriophage. Parasites from a single plaque are suspended in sterile YP solution, which is then passed through a 0.45 μ Millipore filter, diluted, and plated for plaque formation with an excess of the homologous host. After 3 successive single plaque isolations, the strain is regarded as pure in the sense of representing the descendents of a single cell, that is, a clone.

Maintenance of cultures

Stock cultures of the different strains of *Bdellovibrio* are kept in flasks (125 ml) containing the host bacteria in the top layer of YP. They are transferred by depositing a drop of suspension of lysed material containing parasites upon the semisolid top layer, which had previously been inoculated with the host bacterium originally used for the isolation of the corresponding *Bdellovibrio* strain. Transfers of the stock cultures are made every 4 weeks[1]). The viability of the parasites is heavily influenced by the composition of the substrate used for the propagation of the host-parasite system. As already mentioned in connection with the medium used for primary isolations, the growth of *Bdellovibrio* is favored in a substrate that limits the development of the host bacteria. The viability is influenced in the same direction. In a lawn of luxuriantly growing host (for example, in NBA), there is a considerable reduction in the viability of the parasites, although they are able to grow and lyse. It becomes difficult to make successful transfers from such cultures when they are more than 2 weeks old. For this reason, the stock cultures are kept in YP medium.

RESULTS

Bdellovibrio strains isolated

In addition to the original strain *Bd.* 321, 11 separate strains of *Bdellovibrio* have now been isolated using individual soil samples for the different indicator bacteria (Table 1). The other bacteria listed in Table 1 have not yet elicited *Bdellovibrio* parasites which are active upon them.

Up to this point, the isolation of *Bdellovibrio* has been possible only with

[1]) Thanks to Mrs. Gladys Cosens, all *Bdellovibrio* strains have been preserved by lyophilization of lysates in skim milk.

gram-negative bacteria as propagating hosts. These findings do not, of course, disprove the existence of similar parasites attacking gram-positive bacteria. The number of isolation trials is still far too small to allow a conclusive judgment on this matter. However, if such parasites exist, they are certainly much less common than the existent strains, whose lytic activity is restricted to the gram-negative bacteria. Alternatively, such hypothetical organisms may require a modified isolation technique. On the other hand, there are gram-negative bacteria (for example, *Agrobacterium*, *Caulobacter*, *Rhizobium*) which have shown neither suitability for the isolation of *Bdellovibrio*, nor susceptibility to any strain presently available (see Tables 1 and 2). Here again, the isolation experiments thus far performed cannot decide the question as to whether *Bdellovibrio* strains exist with lytic action against these latter bacteria.

The number of parasite plaques observed in isolation plates is normally low, usually less than 20 from 0.5 ml of the filtrate of a soil suspension. In one particular case, using *Aerobacter cloacae*, more than 100 plaques appeared from 0.5 ml of the 0.45 μ Millipore filtrate. Considering that not all the parasitic individuals go into the suspension, and that a certain part is held back during the filtration process, the actual content in soil samples might be considerably higher than determined by this isolation method. In samples of sewage checked for *Bdellovibrio* active against *Escherichia coli*, the number of parasites was about 2 to 10 times as high as in the soil samples examined. Surprisingly, the number of phage particles (without previous enrichment) was smaller than the number of *Bdellovibrio*. These lytic parasites, therefore, may play an important role in the destruction of bacteria in polluted waters and sewage.

Light microscopic observations on the parasitic action of Bdellovibrio

Although the individual cells of *Bdellovibrio* are considerably narrower than most of the ordinary bacteria, they can be seen quite well under the phase-contrast microscope (Fig. 1). The interactions between the two participants can be studied in a mixture of host cells and parasites from a lysing culture, or from an actively developing plaque. The highly motile parasites move several times as fast as a typical pseudomonad. These tiny organisms actually attack single host cells, and attach momentarily to the cell surface. This process of attachment is reversible; occasionally, one observes an individual parasite which, after being attached to a host cell, leaves that cell after a short time and attacks another one. Motile host cells usually stop moving a few seconds after being attacked by a parasite; with *Rhodospirillum rubrum* and *Bd.* 100, motility regularly stops about 5 seconds after attachment of *Bdellovibrio*. The time required for lysis seems to be influenced by the physiological condi-

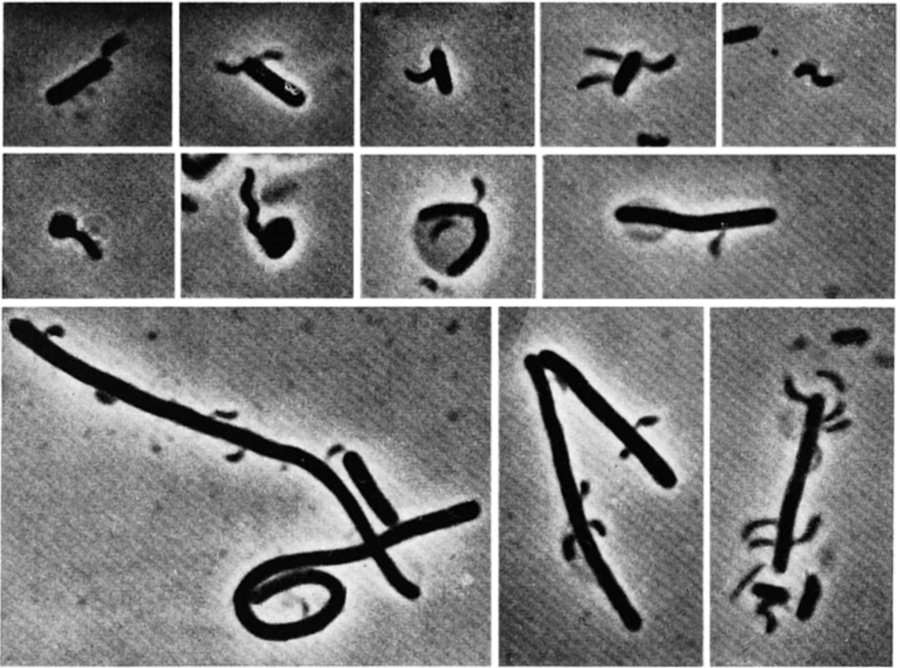

Fig. 1. *Bdellovibrio bacteriovorus*, strain *Bd.* A3.12, in mixed culture with *Pseudomonas fluorescens*, strain A3.12, demonstrating an assortment of typical interactions between parasites and host bacteria. Zeiss Photomicroscope; 100 × Neofluar Ph 3, phasecontrast oil immersion objective, na 1.32; phase-contrast condenser V Z na 1.40. Final magnification 2,060 ×.

tions of both host and parasite. When a suspension of parasites from a lysed culture is centrifuged in order to separate them from the nutrients available in the lysate, the cells are especially aggressive. After being mixed with young host cells, they attach to the bacteria within a few seconds. If many parasites and few host cells are mixed, up to 10 or more parasitic individuals have been observed sticking to the surface of a single bacterium. Under such conditions, lysis of the attacked host cell can occur within a few minutes. The parasite requires a good supply of oxygen for its motility; on a microscope slide this condition is usually found in the vicinity of an air bubble. When mixed with *Bdellovibrio* strains, non-susceptible host cells, for example *Bacillus megaterium*, are completely ignored and no attachments occur.

Because of their small size, details of the morphology of *Bdellovibrio* cannot be observed by phase microscopy, except that individual isolates exhibit differences in size and shape. Some are vibrio-like; others are more straight.

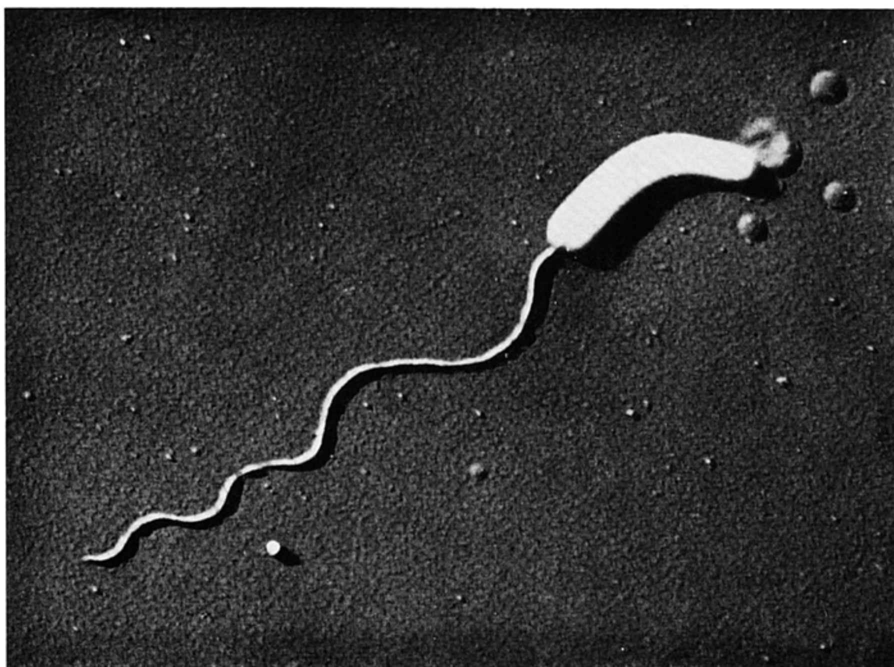

Fig. 2. *Bdellovibrio bacteriovorus*, strain *Bd.* 100. Electron micrograph, 14,000 ×.

Cell division occurs by binary fission, and chains of *Bdellovibrio* cells often have the superficial appearance of a spirillum. The type of motility is characteristic; the cells usually do not move in a straight line and with a constant speed, but seem to jump from place to place. When they hit a bacterium, they do so with considerable force. As a result of the collision, the attacked bacterium is often shoved by the parasite for a distance equivalent to several cell-lengths. Possibly this impact is necessary for successful attachment.

Gram stains of *Bdellovibrio* have shown that it is gram-negative. Like most bacteria it stains with methylene blue and gentian violet.

Electron microscopic observations of Bdellovibrio

Fig. 2 shows a cell of *Bdellovibrio*, strain *Bd.* 100. The organism is clearly vibrio-shaped, and possesses one long polar flagellum of about 55 mμ diameter. The length of this particular cell is about 2.0 μ, and its breadth is about 0.35 μ. *Bdellovibrio* attaches to the host cell by means of the end opposite to the flagellum. This orientation is demonstrated in Fig. 3, which depicts a cell of *Erwinia amylovora*, strain 100, early in the process of being attacked by *Bdello-*

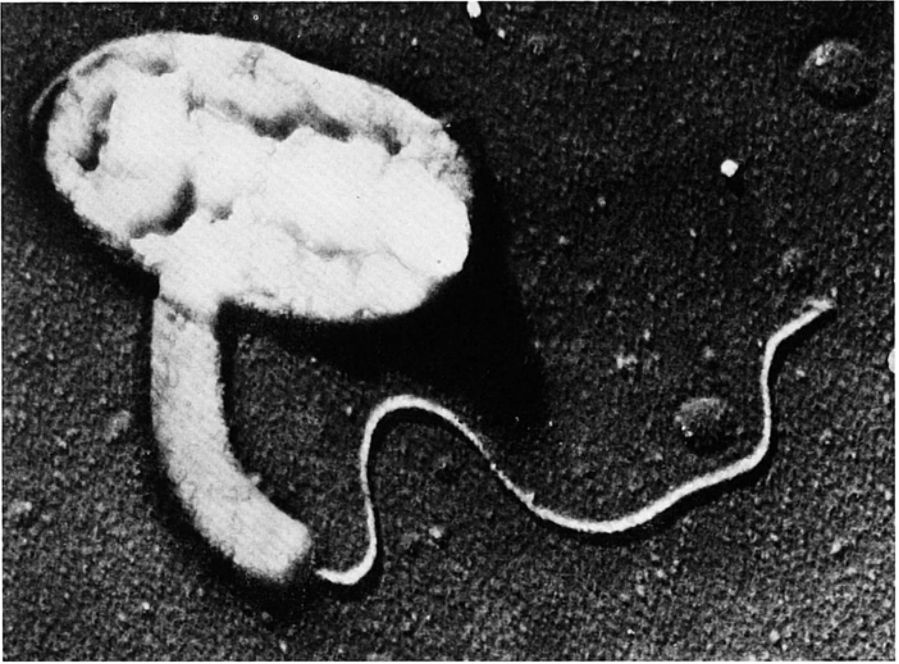

Fig. 3. *Bdellovibrio bacteriovorus*, strain *Bd.* 100, attached to a host cell of *Erwinia amylovora*, strain 100. Electron micrograph. 26,200 ×

vibrio, strain *Bd.* 100. As in the electron micrographs of *Bd.* 321 previously presented (Stolp and Petzold, 1962), a modification of the aflagellated end of the cell will be noted. The parasite seems to cling to the cell surface like a leech attaches to the skin. The fine structure of the flagellum has not been studied as yet; the electron micrographs do not show any differentiation, and it is reasonable to expect that the flagellum – despite its unusual thickness – is similar to that of other bacteria. The thin sections of *Bdellovibrio* which are currently being prepared may shed some light upon this and other cytological features.

Demonstration of the lytic action of Bdellovibrio

When plated with an excess of susceptible host bacteria, all *Bdellovibrio* strains are capable of producing single plaques or confluent lysis. Fig. 4 shows plaque formation after 2 days of incubation (*Bd.* A3.12 on its homologous host *P. fluorescens* A3.12). Fig. 5 shows almost confluent lysis, and Fig. 6 demonstrates the lytic effect produced by dropping a concentrated parasite

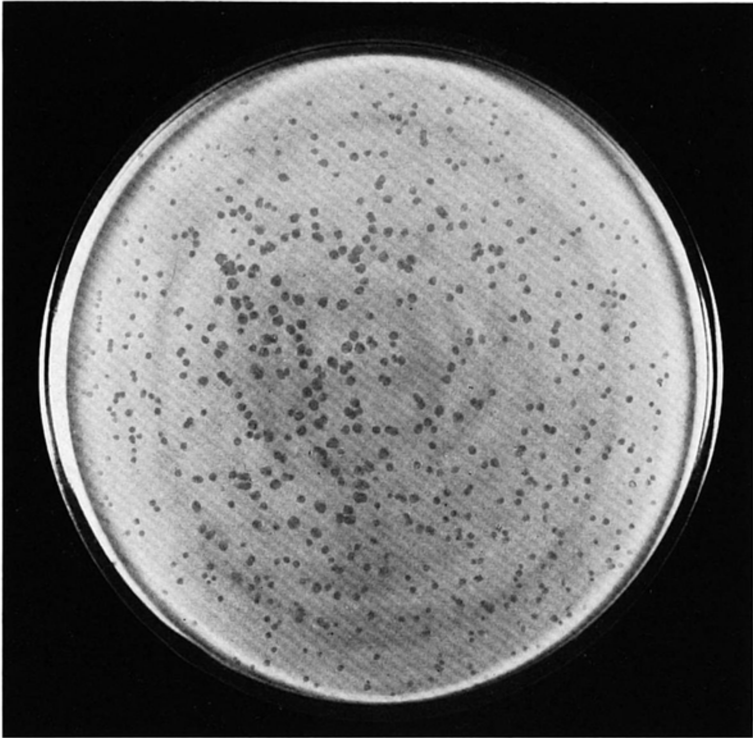

Fig. 4. Plaque formation by *Bdellovibrio bacteriovorus*, strain *Bd.* A3.12, on its homologous host, *Pseudomonas fluorescens*, strain A3.12.

suspension on the inoculated top-layer (Figs. 5 and 6 with *Bd.* 109 and *E. coli* B). Fig. 7 demonstrates the action of *Bd.* 109 against *E. coli* B, where a mixture of host bacteria and parasites has been streaked on a nutrient agar plate. At those sites where host bacteria and parasites have been inoculated in low numbers, single colonies of the host bacterium develop and, by their extension, overgrow the spots where single parasites are located. Here, the development of lytic areas is initiated by the action of the parasite. The lytic spots actually represent colonies of the parasite which develop on the bacterial substrate. Because of their inability to grow without the host, parasitic cells that do not get contact with host bacteria do not give rise to colony formation. Where *E. coli* cells and parasites have been inoculated in great numbers and close to each other, the confluent bacterial growth is replaced by confluent lysis.

The demonstration of the lytic activity in liquid culture is influenced considerably by the composition of the host medium. As in plaque formation, reduced bacterial growth favors the parasite development. The type of metab-

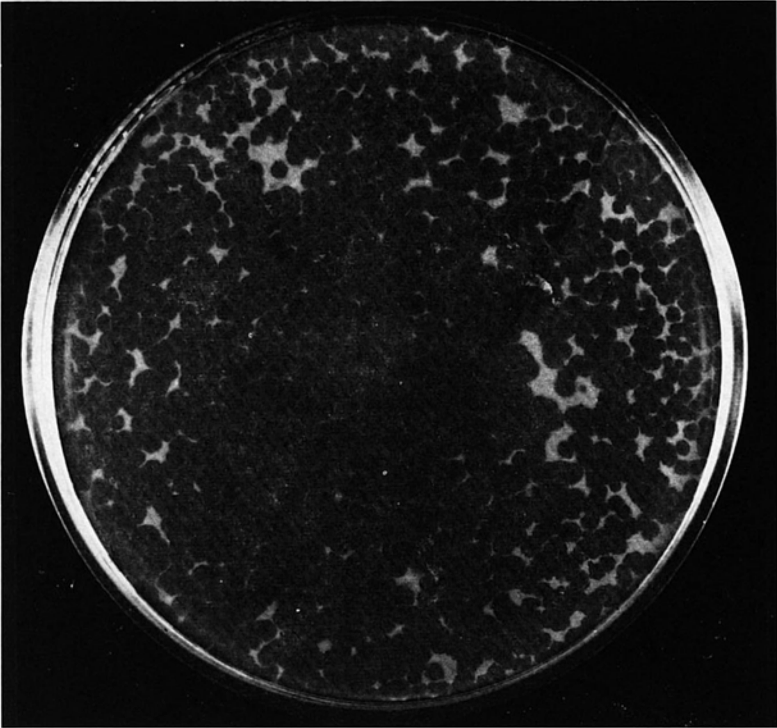

Fig. 5. Almost confluent lysis of *Escherichia coli* B by *Bdellovibrio bacteriovorus*, strain *Bd.* 109.

olism seems also to be involved; under the same nutritional conditions, lysis is complete in some systems, but not in others. In nutrient broths, such as NBA diluted 1 : 10 or YP diluted 1 : 4, good lysis could generally be achieved. Lysis of living cells is possible in tris-buffer (0.05 M; pH 7.2) without any nutrients, if relatively large amounts of inoculum (i.e., parasite) are used. In the systems *Erwinia amylovora* plus *Bd.* 100, *Pseudomonas phaseolicola* plus *Bd.* 321, and *Pseudomonas fluorescens* plus *Bd.* A3.12, complete lysis was obtained in the tris-buffer alone. A comparative study of the other systems, using non-nutrient buffer solution, has not yet been done.

It can easily be demonstrated that the lytic action caused by *Bdellovibrio* has nothing to do with bacteriophage. The plaque-forming capacity of a lysate which contains parasites is lost after centrifugation at high speed (20 min at 15,000 rpm) and passage through a filter with less than 0.1 μ pore size diameter. Naturally, this procedure would allow bacteriophages to pass into the filtrate. In contrast to bacteriophage, plaque formation by *Bdellovibrio* on a strepto-

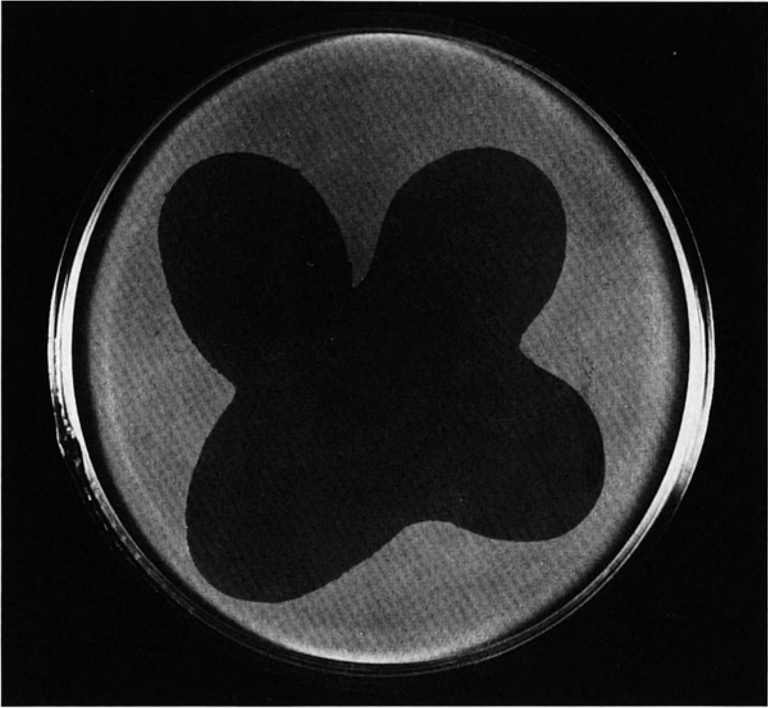

Fig. 6. Lytic action of *Bdellovibrio bacteriovorus*, strain *Bd.* 109, on *Escherichia coli* B, provoked by drops of a suspension which contained the parasite.

mycin-resistant (Sm^r) host is inhibited in the presence of streptomycin, although the same host is susceptible in the absence of streptomycin. All wild-type isolates of *Bdellovibrio* have been found to be sensitive to streptomycin, but it has been possible to select Sm^r mutants of *Bdellovibrio*. Furthermore, the kinetics of plaque-formation are different in bacteriophage and *Bdellovibrio*. Bacteriophage multiplication is essentially connected with active growth of the host; hence the size of phage plaques is determined at the time the bacteria have entered the stationary phase. Contrary to these conditions, plaques produced by *Bdellovibrio* continue to increase in size for about 1 week, because the parasites also attack and lyse living host cells that are no longer multiplying. However, the most cogent reason for rejecting the idea of phage involvement is the fact that one can actually see these tiny, parasitic organisms in the act of attacking and lysing the host bacteria.

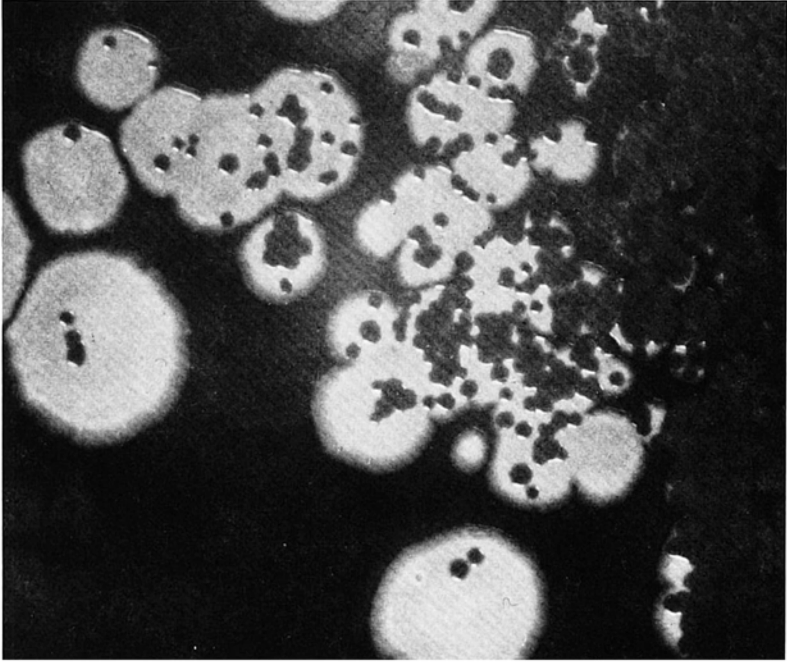

Fig. 7. Lytic action of *Bdellovibrio bacteriovorus*, strain *Bd.* 109, on *Escherichia coli* B, after streaking a mixture of parasites and host bacteria on nutrient agar.

Kinetics of growth of Bdellovibrio

A few cells of *Bdellovibrio* are able to bring about lysis of a concentrated bacterial cell suspension. For example, 18 parasite cells per ml effected complete lysis of a culture containing 4×10^{10} host cells per ml (*E. amylovora* 100 Sm^r plus *Bd.* 100, in 50 ml YP solution). In the same system, the kinetics of the host cell and the parasite were studied by following the development of both partners during the process of lysis (Fig. 8). The number of viable host cells was determined by plating dilutions on streptomycin agar (500 μg/ml), which killed the streptomycin-sensitive parasite. The number of parasites was determined by plaque counts of dilutions plated in a lawn of *E. amylovora* 100 Sm^r in the absence of streptomycin. The initial concentration of host was 16×10^8 per ml, and of parasite 16×10^5 per ml. The culture was incubated at 28 C on the shaker. After lysis, the reaction medium contained only 18 viable cells from 0.5 ml of *E. amylovora* 100 Sm^r. These 18 surviving cells were propagated individually, and all proved to be susceptible to *Bd.* 100. This result confirms the previous observation that the development of *Bdellovibrio*-resistant mutants does not seem to occur.

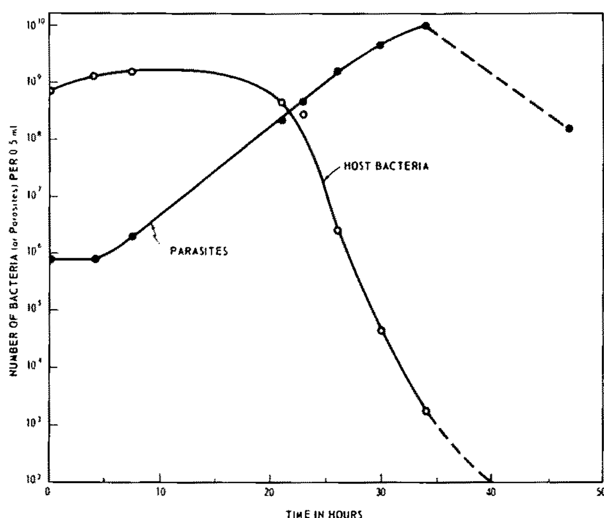

Fig. 8. Development of *Bdellovibrio bacteriovorus*, strain *Bd.* 100, on *Erwinia amylovora*, strain 100 Sm^r.

After lysis of the majority of the host cells in liquid culture, *Bdellovibrio* continues to multiply. In contrast to bacteriophage development, there is no proportionality between number of host cells killed and number of parasites produced during equal time periods of the parasite development. During the time of intensive lysis, the ratio of "parasites produced per hour" to "host cells killed per hour" was as follows:

Time of culture development	A Bacteria killed per hr per 0.5 ml	B Parasites produced per hr per 0.5 ml	B : A
21 — 23 hr	72×10^6	87×10^6	1.2
23 — 26 hr	120×10^6	250×10^6	2.1
26 — 30 hr	0.6×10^6	375×10^6	625.0

After 26 hr, the killing rate decreased considerably, because practically no living host-cells remained to be killed; nevertheless, there was a tremendous increase in numbers of parasites. Clearly, they develop at the expense of nutrients they have made available by their own lytic activity.

Activity spectra of Bdellovibrio strains

An extensive study of the host range of the first *Bdellovibrio* strain that had been isolated (Bd. 321) had shown that this organism lyses exclusively fluores-

cent pseudomonads and xanthomonads (Stolp and Petzold, 1962). Many saprophytic strains of the *Pseudomonas fluorescens* type, and the majority of more than 100 phytopathogenic *Pseudomonas* strains belonging to a great number of "species", proved to be susceptible to *Bd.* 321. It was striking to note that representatives of certain phytopathogenic *Pseudomonas* "species" – such as *P. solanacearum* or *P. caryophylli* – which, on the basis of comparative phage susceptibility and other criteria, apparently are not closely related to the fluorescent pseudomonads (Stolp, 1961), likewise are not attacked by *Bdellovibrio* strain 321.

The host activity of the recently isolated strains has been investigated in a comparative study using the same set of bacteria (numbers 100 to 130) which had been used for the isolation experiments, and which represent members of several different systematic groups. In addition, two streptomycin-resistant host strains (100 Sm^r and A3.12 Sm^r) were included.

A given bacterial culture is regarded as susceptible for a given strain of *Bdellovibrio* if the parasite is able to induce plaque formation or confluent lysis when plated in a dilution series with an excess of host cells. These tests were performed with YP medium using the double-layer technique. The host bacteria were 16 to 24 hr old, and each plate was inoculated with 10^{10} cells. The parasite dilutions in these experiments were prepared by the following procedure: a single plaque (2 to 4 days old) was cut out of a plate inoculated with the homologous host strain; the material was mechanically homogenized in 5 ml YP solution, centrifuged (about 30 sec at 2,000 rpm) for removal of agar pieces, and filtered through 0.45 μ Millipore filters. The dilutions were prepared in YP solution. In addition to the dilution test leading to single plaque formation, one plate of each series was inoculated by dropping the most concentrated parasite solution onto the bacterial lawn. The initial parasite concentration, representing the parasites of one single plaque, was usually between 10^7 and 10^8 plaque-forming units per ml.

The method described may have some limitations in connection with the metabolism of the host bacteria and the accumulation of products possibly inhibitory for the parasite. If such a situation should arise with one or the other bacterial strain, plaque formation might be inhibited or reduced to micro-plaques that are not recognizable. Because of the massive inoculation of the host bacteria and the reduced growth on the poor YP medium, this possible limitation probably does not play a major role. This assumption is supported by the observation that plaque formation by single parasites parallels the confluent lysis which results in the drop test. If single plaque formation were reduced to a nonvisible size, one should expect that a great number of micro-plaques in the drop test would cause a visible reaction. The best

way to avoid any possible interaction by growth and metabolism of the bacteria, would be to test with living bacteria in the absence of any nutrients. Here, on the other hand, it may be possible that the development of *Bdellovibrio* is suppressed for other reasons. The parasite would be blocked in its activity if it required nutrients from the medium in addition to the bacterial host cells or the products of their lysis. The same would be true if the development of *Bdellovibrio* depended on some metabolic product of the host bacterium. As these points have not yet been studied in detail, we have avoided the two possible extremes for the performance of the activity test; that is, maximum growth of the host by optimal supply of nutrients, or no growth of the host by withdrawal of nutrients. Instead, we chose – for the sake of convenience – a middle course, using conditions of restricted bacterial growth. Since the different parasite strains give good lysis and plaque formation with their homologous host bacteria under the conditions employed, we regard this test as adequate for the evaluation of host specificity.

In some host-parasite systems (*Bd.* 100, *Bd.* 321, and *Bd.* A3.12 plus their homologous hosts), plaque formation could be demonstrated in tris-buffer agar (pH 7.5; 0.05 M) without any nutrients. But there are obviously differences from strain to strain; *Bdellovibrio* strain 128, for instance, when acting on *Aerobacter cloacae* (128), requires conditions that allow bacterial growth. With non-proliferating host bacteria, plaque-formation with this *Bdellovibrio* strain could not be achieved.

Table 2 summarizes the activity spectra of the different isolates of *Bdellovibrio*, as based upon single plaque formation under the described experimental conditions. It can be noted that the 12 independently isolated *Bdellovibrio* strains are restricted in their activity to gram-negative bacteria. On the basis of the host ranges they can be differentiated into 5 groups:

1) *Bd.* 128, active only against its homologous host,

2) *Bd.* 321, activity restricted to fluorescent pseudomonads and xanthomonads,

3) *Bd.* A3.12, active against *Pseudomonas* "species", including *P. solanacearum* and *P. caryophylli*,

4) *Bd.* 100, 109, 110, 118, 120, 127, 233, active against some enterobacteria and pseudomonads, but not against *Proteus*,

5) *Bd.* 101 and 114; the same as type 4, but in addition active against *Proteus*.

In order to find out whether the results obtained by the plaque-test coincide with lysis of living host bacteria in liquid culture, the activity of *Bd.* A3.12 was examined comparatively by both methods, using as host strains all those cultures that have been shown to be susceptible to any strain of parasite.

TABLE 2

Host activity spectra of *Bdellovibrio* strains

Bacterial host	*Bdellovibrio*: 100	101	109	110	114	118	120	127	128	321	233	A3.12
100	+	+	+	+	+	+	+	+	—	—	+	—
101	+	+	+	+	+	+	+	+	—	—	+	—
102	—	—	—	—	—	—	—	—	—	—	—	—
103	—	—	—	—	—	—	—	—	—	—	—	—
104	—	—	—	—	—	—	—	—	—	—	—	—
105	—	—	—	—	—	—	—	—	—	—	—	—
106	—	—	—	—	—	—	—	—	—	—	—	—
107	—	—	—	—	—	—	—	—	—	—	—	—
108	—	—	—	—	—	—	—	—	—	—	—	—
109	+	+	+	+	+	+	+	+	—	—	+	—
110	+	+	+	+	+	+	+	+	—	—	+	—
111	—	—	—	—	—	—	—	—	—	—	—	—
112	—	—	—	—	—	—	—	—	—	—	—	—
113	—	—	—	—	—	—	—	—	—	—	—	—
114	—	+	—	—	+	—	—	—	—	—	—	—
115	—	—	—	—	—	—	—	—	—	—	—	—
116	—	—	—	—	—	—	—	—	—	—	—	—
117	—	—	—	—	—	—	—	—	—	—	—	—
118	+	+	+	+	+	+	+	+	—	—	+	—
119	—	—	—	—	—	—	—	—	—	—	—	—
120	+	+	+	+	+	+	+	+	—	—	+	—
121	—	—	—	—	—	—	—	—	—	—	—	—
122	—	—	—	—	—	—	—	—	—	—	—	—
123	+	+	+	+	+	+	+	+	—	—	+	—
124	—	—	—	—	—	—	—	—	—	—	—	—
125	—	—	—	—	—	—	—	—	—	—	—	—
126	—	—	—	—	—	—	—	—	—	—	—	—
127	+	+	+	+	+	+	+	+	—	—	+	+
128	—	—	—	—	—	—	—	—	+	—	—	—
129	—	+	—	—	+	—	—	—	—	—	—	+
130	+	+	+	+	+	+	+	+	—	—	+	—
321	+	+	+	+	+	+	+	+	—	+	+	+
233	+	+	+	+	+	+	+	+	—	+	+	+
A3.12	+	+	+	+	+	+	+	+	—	+	+	+
100 Sm^r	+	+	+	+	+	+	+	+	—	—	+	—
A3.12 Sm^r	+	+	+	+	+	+	+	+	—	+	+	+

The results shown in Table 3 suggest that susceptible strains show lytic reactions with both methods. Since plaque tests are easier to perform, we prefer this method for the determination of host specificity.

TABLE 3

Comparison of the activity of *Bdellovibrio* A3.12 as measured by plaque formation on bacterial lawns, and by lysis in liquid cultures

Host bacterium	Plaque-forming ability	Lysis in liquid culture[1]) (Klett units after 24 hr)
100	—	250[2])
101	—	260
109	—	280
110	—	280
114	—	260
118	—	220
120	—	200
123	—	170
127	+	100
128	—	230
129	+	105
130	—	260
321	+	130
233	+	130
A3.12	+	95

[1]) Lysis by *Bd.* A3.12 in liquid culture has been determined under the following conditions:
a) Substrate: YP, diluted 1 : 4 with tris-buffer (0.05 M; pH 7.5), 50 ml in 250 ml flasks.
b) *Bd.* A3.12: 5 ml of a filtered suspension from a lysate of *P. fluorescens* A3.12 (parasite concentration about 10^8/ml) inoculated into each flask.
c) Host bacteria: Young bacteria (from an overnight culture on YDC) suspended in tris-buffer and added to the solution which contains the parasite, adjusting the turbidity to 200 Klett units (blue filter).
d) Culture conditions: The infected cultures were shaken at 28 C.
e) Determination of lytic activity: After 24 hours, the optical density was determined.
[2]) The increase of turbidity in the non-lysed cultures is due to slight growth of the bacteria in the diluted YP medium.

Experiments with heat-killed host cells

It was noted, in connection with the *Bdellovibrio* strain which was first isolated, that a suspension of parasites develops confluent lysis or single plaques only with living host cells. On the basis of this observation, and on account of the failure to cultivate the parasite on artificial substrates, the organism had been characterized, with certain reservations, as an obligate parasite (Stolp and Petzold, 1962). The present study of the kinetics of growth, has revealed that *Bdellovibrio* – through its parasitic and lytic activity – actually prepares the substrate which allows growth and multiplication. After lysis

of the majority of susceptible host cells, there is still a considerable increase in number of parasites, indicating that the lysate they create by their own activity contains all the nutrients required for their multiplication. This fact makes it probable that a detailed study of the nutritional requirements will lead, sooner or later, to the formulation of an artificial substrate on which *Bdellovibrio* can be cultivated. Up to now, however, we have not been successful in growing *Bdellovibrio* in its parasitic form on a bacteria-free medium of known composition. The selection of saprophytic mutants from a wild-type population of *Bdellovibrio* is another matter, which will be discussed in the next section.

Some of the recently isolated *Bdellovibrio* strains, and the original strain *Bd.* 321, have been examined in a comparative study of their ability to cıeate lytic reactions in a lawn of heat-killed host cells (15 min at 100 C). A suspension of parasites, prepared from two-day old plaques, was diluted 1 : 10^3 in YP solution, and titrated after filtration (0.45 μ) in parallel experiments on living and on dead cells. The living bacteria were inoculated as usual (about 10^{10} cells per plate), the heat-killed bacteria were added at a concentration which causes turbidity equal to that obtained with living bacteria at the end-point of their development. The results were as follows:

Host	Parasite	Plaque formation on Living Cells	Dead Cells
321	*Bd.* 321	+	—
A3.12	*Bd.* A3.12	+	—
100	*Bd.* 100	+	—
109	*Bd.* 109	+	—

The most concentrated test solutions of each dilution series contained approximately 10^5 parasites peı ml (1 : 10^3 dilution of the initial parasite suspension of 10^8 cells per ml). Drops of this suspension caused complete lysis in the lawn of living cells, but had no effect on dead cells.

Drops of an undiluted lysate from a liquid culture after complete lysis (A3.12 lysed by *Bd.* A3.12; parasite concentration 10^{10} per ml) caused lytic spots on living and on dead cells. However, after dilution of this lysate, plaque formation could be observed only on living cells. This experiment discloses that the parasites are able to lyse heat-killed cells only when their growth is permitted by the carryover of essential nutrient substances present in the lysate.

Since the chemical and physico-chemical conditions in lawns of living and heat-killed host cells are different, two factors were investigated which might have been responsible for the failure of plaque formation with heat

treated cells; namely, pH and redox-potential. Under the conditions chosen for these exploratory experiments, the final pH value in the lawn of living bacteria was pH 8.4. When the substrate used for the inoculation of heat-killed cells was adjusted to pH 8.4 prior to inoculation, the parasites remained inactive as in the case of a lawn at pH 7.2. Several substances known to decrease the redox-potential were added to the lawn prepared from dead cells; namely, sodium ascorbinate, sodium thioglycolate, cysteine, and glutathione – all at concentrations of 0.05%. The addition of these compounds to living host cells had no inhibitory effect on *Bdellovibrio*, but their addition to dead host cells did not permit the parasite to start plaque formation. Subject to the limitations of these exploratory trials, one could conclude that changes of pH and redox-potential as they occur in a lawn of living bacteria are probably not the factors that decide the failure of parasite development in a lawn of heat-killed cells. It becomes more likely, therefore, that the lytic reaction observed in a lawn of dead cells, after application of a concentrated lysate, is connected with the carryover of essential material. This assumption was confirmed moreover, by examination of the influence of a lysate freed from parasites upon development of plaques in lawns of heat-killed host cells. A suspension of parasites (*Bd.* A3.12) was diluted in tris-buffer and equal amounts of a dilution series were tested for plaque-forming ability on living and on heat-killed cells in YP agar. The top layer containing dead bacteria was supplemented either with 0.5 ml of a sterile lysate (A3.12 lysed by *Bd.* A3.12 in liquid culture, separated from parasites by filtration); or with 0.5 ml of a definitely sterile filtrate of a culture of A3.12 (after 24 hours development in NBA solution). In addition plaque-formation was checked in a mixture of living and heat-killed cells. Results were as follows:

Top layer containing:	Plaque development:
living bacteria	+
living plus heat-killed bacteria	+
heat-killed bacteria	
15 min at 80 C	—
15 min at 100 C	—
heat-killed bacteria (15 min at 100 C)	
+ 0.5 ml lysate	+
+ 0.5 ml culture filtrate	—
+ 0.5 ml medium NBA	—

These results support the supposition that plaque-development on heat-killed cells depends upon some material produced by lysis of living host cells. The nature of this material has not yet been determined.

In liquid culture, with living or heat-killed cells, the activity of *Bdellovibrio* was the same as described in the plating experiments. The results obtained with *Erwinia amylovora* (100 Sm^r) and *Bd.* 100 in YP solution (25 ml in 125 ml flask) are summarized herewith:

Development of *Bd.* 100 on:	Lysis after 2 days	Viable count after 2 days	
		Bacteria	Parasites
living cells	+	3×10^2/ml	3×10^9/ml
heat-killed cells (15 min at 100 C)	—	0	6×10^2/ml
heat-killed cells (15 min at 121 C)	—	0	1.6×10^3/ml
control in plain YP solution	—	0	1×10^2/ml

The initial cell concentration was 2×10^9 bacteria per ml (the same for living and dead cells) and 1×10^4 parasites per ml. These figures reveal that a relatively small inoculum of *Bd.* 100 (10^4 cells per ml) brings about lysis only in the presence of living host cells; this is accompanied by an increase in parasite titer. On the other hand, *Bd.* 100 does not grow with heat-killed cells, nor in plain YP solution. The number of viable parasites determined by plaque test after 2 days actually decreases considerably. We shall discuss in the following section the finding that massive inoculation of a concentrated parasite suspension in a medium free of living host cells permits selection of parasite derivatives (variants or mutants) which grow saprophytically, but differ from the wild type in respect to lytic activity and other characters.

Selection of saprophytic derivatives from a wild-type population of Bdellovibrio

When studying the first isolated *Bdellovibrio* strain (*Bd.* 321), it was found that individuals which were able to grow on PY medium could be selected from a population of the wild-type parasite (Stolp and Petzold, 1962). By inoculation of 5×10^8 parasite cells (concentration determined by plaque test), only a single developing colony on the average was obtained. The saprophytic strains produced a yellowish pigment. With respect to their morphology, they were less homogeneous than the parental wild type. Some strains consisted mainly of spherical cells, in others, vibrio- or spirilli-form individuals prevailed. In contrast to the parasitic form which possesses only one flagellum, various kinds of flagellation were observed in cultures of the saprophytes; the unusual thickness (50 mμ) was the same as in the parasite, however, the number

of polarly inserted flagella ranged from one to three in the saprophyte in contrast to the single flagellum of the parasite. A series of electron micrographs disclosed these morphological differences. In terms of plaque-forming ability, the lytic action of the saprophyte derivatives was drastically changed, as compared with the parental wild type. The situation was described as follows: "Die isolierten Stämme besassen im Aktivitätstest auf dem Wirtsbakterium (321) entweder gar keine Aktivität, z.B. Stamm Sp. 6, oder sie bildeten lytische Plaques mit zentraler Kolonieentwicklung des Saprophyten (z. B. Stamm Sp. 32; Abb. 8). Nach mehreren Passagen auf künstlichem Substrat ging die Eigenschaft, Bakterien zu lysieren, meist ganz verloren, und mit dem Verlust der lytischen Aktivität war in der Regel der Verlust der Beweglichkeit gekoppelt". (Stolp and Petzold, 1962, p. 380). According to these observations, the different isolates were not identical with respect to their lytic properties but they all shared the tendency to lose lytic activity and motility after successive transfers on a bacteria-free substrate. That these saprophytically growing descendents of the parasitic wild-type isolate were not contaminants, was proven by morphological resemblance including the unusually thick flagellum, and by the fact that parasitic strains with properties of the wild type can be reselected when massively inoculated into a culture of living host cells.

The isolation of saprophytic mutants from a parasitic culture of *Bdellovibrio* can be achieved principally by two different methods; namely, inoculation of a concentrated parasite suspension (at least 10^9 cells per ml) into a top layer of PY medium, or inoculation of a corresponding parasite suspension into a liquid nutrient medium. The first method has the advantage of giving individual colonies which represent development into clones of cells with saprophytic growth ability. Using the second method, a mixture of different clones will be obtained if the inoculum contains saprophytic cells originating from independent mutational events. A more important disadvantage of the second method is the high content of parasites from the initial inoculum.

With several of the recently isolated *Bdellovibrio* strains, saprophytic mutants have been selected by using the first method. Their general features are similar to those described for saprophytic mutants of *Bd.* 321. One striking property is a strong proteolytic activity which allows good colony growth and causes lysis on heat-killed bacteria, as demonstrated in Fig. 9. The same result is obtained with casein as substrate. Another property of significance that is shared by all parasitic and saprophytic strains is a negative reaction for catalase. A detailed and comparative study of a series of saprophytic derivatives of different *Bdellovibrio* strains is in progress; the results will be published separately. We feel that our investigation of these saprophytic mutants will contribute to a clarification of the taxonomic position of *Bdellovibrio*.

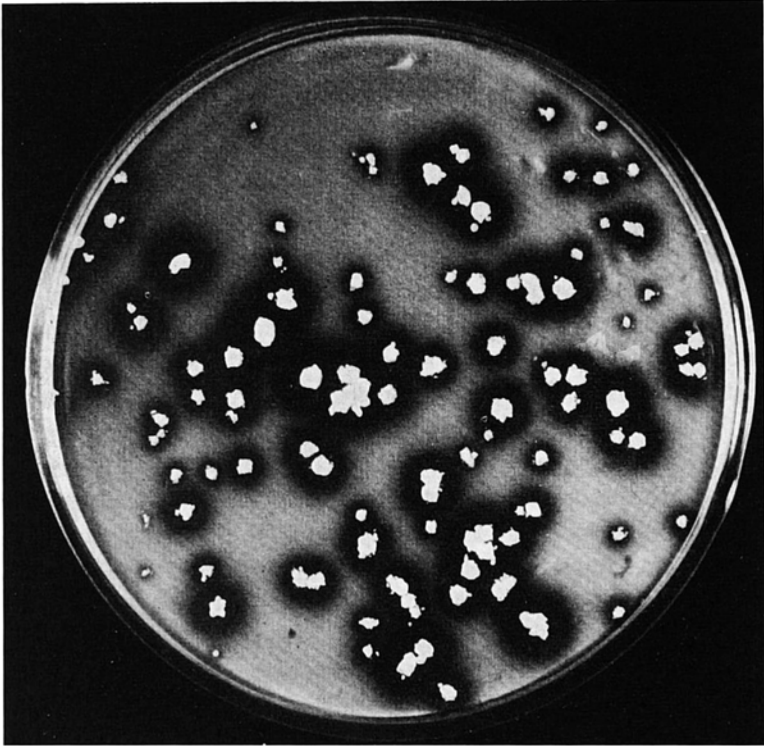

Fig. 9. Lysis of heat-killed cells of *Pseudomonas fluorescens*, strain A3.12, by a saprophytic derivative (strain T II) of *Bdellovibrio bacteriovorus*, strain *Bd.* A3.12.

One saprophytic culture [1]) of *Bd.* A3.12 which had developed in broth (NBA) after inoculation of a concentrated parasite-suspension (the second method described) has been analyzed for lytic ability of its individuals. We found that the population consisted of a mixture of parasitic and saprophytic individuals. In this culture, only 1 out of 10^6 cells possessed plaque-forming ability. When a single colony developing in PY medium was isolated, the new population contained only 1 out of 10^8 cells with plaque-forming ability. Such a broth culture of *Bd.* A3.12, therefore, is no longer the wild-type parasite, but rather a mixed population in which the saprophytic type has a selective advantage.

We derived a second broth culture of *Bd.* A3.12 using the second isolation technique; it showed exactly the same characteristics. After serial transfers, the number of individuals with plaque-forming ability decreased to approxi-

[1]) We are obliged to Dr. M. Shilo for this particular derivative of our original *Bd.* A3.12 isolate.

mately 1 in 10^8 cells. Although the population shift in broth cultures has not yet been studied thoroughly, we feel that mutation and selection are the working principles. This suggestion is supported by the observation that the number of saprophytic colonies developing after mass inoculation of the wild type into PY medium is at the level of mutation rates (1 in 10^8) generally found in bacteria for other properties.

Some physiological and biochemical reactions were studied of one saprophytic strain (Sp. 19), selected from *Bd.* 321. The results are summarized in Table 4.

TABLE 4

Characteristics of a saprophytic strain (Sp. 19) derived from *Bdellovibrio* 321

Characteristic	Saprophyte strain 19
1) Effect of temperature (on complex medium)	No growth at 35 C. Optimum around 22 C.
2) pH range for growth	6.0 to 8.5.
3) Nitrate test (complex medium plus 1% KNO_3)	Accumulates both nitrite and ammonia.
4) Gelatin liquefaction (12% gelatin in complex medium)	Strongly positive.
5) Catalase production	Negative.
6) Dehydrogenase test (0.1% triphenyl tetrazolium chloride)	Positive.
7) Utilization of carbon sources	
a) Synthetic medium plus carbon source[1])	No growth[2]).
b) Complex medium[3]) plus 0.5% carbon source	

Carbon source	Growth[4])	Carbon source	Growth[4])
Pyruvate	+	Fructose	—
Succinate	+	Glucose	+
Malate	+	Maltose	+
Fumarate	+	Lactose	+
Lactate	+	Sucrose	+
Citrate	—	Sorbitol	+
Mannitol	—	Dextrin	+

8) Utilization of nitrogen sources in synthetic medium[1]) plus glucose

Nitrate	No growth[2]).	Urea	No growth[2]).
Asparagine	No growth[2]).		

Characteristic	Saprophyte strain 19
9) Gram stain	Gram negative.
10) Oxygen requirement	Aerobic.
11) Pigmentation	Yellow[5])

[1]) Synthetic medium: 0.1% $NH_4H_2PO_4$, 0.1% KCl, 0.02% $MgSO_4 \cdot 7H_2O$, 1.2% Difco agar, pH 7.2. Carbon sources as in 7b.

[2]) "No growth" in 7a and 8 probably reflects lack of an essential growth factor.

[3]) Complex medium: 1.0% Difco peptone, 0.3% Difco yeast extract, 1.5% Difco agar, pH 7.2.

[4]) Since growth is possible on the complex medium alone, the indicated positive growth reaction does not mean that the carbon source is utilized. In no instance was a pH shift observed. As far as glucose is concerned, the strain Sp. 19 is non-oxidative and non-fermentative.

[5]) The yellow pigment produced by Sp. 19 has been identified as a xanthophyll; we are indebted to Dr. W. L. Stephens for this determination. Some saprophytic mutants that have been isolated from *Bd.* A3.12 are colorless.

On the basis of its morphological and physiological properties, it was impossible to identify this saprophyte as a representative of any one of the already known categories of bacteria.

Since it is possible to select individuals with bacteriolytic properties from a saprophytic strain of *Bdellovibrio*, attempts have been made to select such parasites from saprophytic bacteria of known systematic position. In preparations of *Bd.* 321, a few cells had been found in electron micrographs that showed some similarity to *Caulobacter*, and it had been suggested cautiously (Stolp and Petzold, 1962) that the two groups of organisms might be related. In order to verify this assumption, more than 50 *Caulobacter* strains[1]) were examined for their ability to give rise to parasitic forms when handled in the same way as the saprophytic cultures derived from *Bdellovibrio*. For this purpose concentrated suspensions of the different *Caulobacter* strains (about 10^{10} cells) were inoculated into lawns of prospective host bacteria, using the strains 100 to 130 as shown in Table 1. In none of these experiments, could plaque formation be observed. This result we regard as sufficient evidence that *Caulobacter* is not a saprophytic form of *Bdellovibrio*. It is more likely that the stalked cells found in preparations of *Bdellovibrio* (strain *Bd.* 321) might have been true caulobacters as contaminants.

Vibrio is a second group of bacteria which, because of morphological similarity, might be suspected to be related to *Bdellovibrio*. Here again, all attempts to isolate parasitic derivatives from a set of *Vibrio* cultures[2]) were unsuccessful. On the contrary, 2 of these cultures (*V. percolans* and *V. neocistes*) proved to be susceptible for *Bd.* 100. All the saprophytic strains selected from *Bdellovibrio*, however, are resistant against the parasite strains available. Hence, it is unlikely that members of the genus *Vibrio* represent saprophytes from which parasitic mutants of the *Bdellovibrio* type can be selected. The same is true for *Rhodospirillum rubrum*, which is lysed by several of the *Bdellovibrio* strains.

DISCUSSION

A diversity of organisms, including microbes, can attack plants and animals parasitically. *Bdellovibrio* represents the first parasitic organism that attacks bacteria in a recognizably predatory fashion. By the nature of its action, it has the characteristics of an ectoparasite. The organism physically attacks

[1]) We are grateful to Dr. J. L. Stove (Stove and Stanier, 1962) for her generosity in making these *Caulobacter* cultures available to us.

[2]) We are indebted to Dr. W. A. Clark for the following 10 cultures of *Vibrio* spp. from the American Type Culture Collection: *Vibrio cuneatus* 6972, *V. percolans* 8461, *V. algosus* 14390, *V. ponticus* 14391, *V. haloplanktis* 14393, *V. marinovulgaris* 14394, *V. marinofulvus* 14395, *V. marinagilis* 14398, *V. neocistes* 14636, and *V. cyclosites* 14635.

susceptible bacteria, attaches to their soma, and causes death by cell disorganization and lysis.

The systematic position of the parasite described in this report is still in doubt. It is clearly bacterial; that is, procaryotic. *Bdellovibrio* has a single polar flagellum – albeit an unusually thick one – which suggests placement in the order Pseudomonadales. It is not photosynthetic, and hence is to be referred to the suborder Pseudomonadineae. Because of its curved, vibrio-like shape, it can be placed in the family Spirillaceae. From one standpoint or another, it might be considered as a member of the genus *Vibrio*. However, we feel that the unusual predatory and ectoparasitic character is a sufficiently distinctive and unusual feature to warrant establishment of a separate genus. We, therefore, propose placement of this type of bacterial parasite in a new genus, *Bdellovibrio*[1]). At the present point, we will refer all of the foregoing individual isolates, notwithstanding the differences in host range and the minor variations in morphology, to a single species for which we propose the name *Bdellovibrio bacteriovorus*. We adopt this conservative approach in view of the present lack of information on stability of host range and other properties.

By its activities, *Bdellovibrio* in its wild-type form makes available the nutrients required for its growth and development. The fact that saprophytically growing derivatives can be selected from a population of *Bdellovibrio*, opens perspectives for a comparative study with already known bacteria. Our current systematic study of several saprophytic strains might disclose some natural relationships to already described bacteria. Logically, it should be expected that saprophytes of this type might have been isolated and described under other names.

The possibility of reisolating – from a population of the saprophytic type – parasitic derivatives with lytic activity against living bacteria has led to the speculation that parasites can be selected from already known saprophytic bacterial species. For *Caulobacter* and *Vibrio*, which were suspected to be possibly related to *Bdellovibrio*, the results indicate that a close relationship is not likely. This conclusion is supported by the observation that *Vibrio* species are susceptible to attack by *Bdellovibrio*, although the saprophytic mutants of *Bdellovibrio* have proved to be resistant against all available parasite strains. For the same reason, the susceptibility of *Rhodospirillum rubrum* to *Bdellovibrio*

[1]) We thank Dr. R. E. Buchanan for nomenclatural advice. The generic name "*Bdellovibrio*" describes the organism in respect to shape and behavior. It has the morphology of a vibrio and attaches to the bacterial host cell in a fashion similar to the action of a leech. "*Bdello*" is derived from the Greek word for leech. Strain *Bd.* 100 is designated as the nomenclatural type culture, and has been deposited in the American Type Culture Collection (ATCC), Washington, D.C., and in the International Collection of Phytopathogenic Bacteria (ICPB), Davis, California, together with its original host, *Erwinia amylovora* ICPB-EA137.

makes unlikely a close relationship to the photosynthetic spirilliform bacteria.

Most of the exciting questions connected with the mode of action of *Bdellovibrio* are unsolved. The morphological basis is a physical attack and attachment, but we are still far from understanding the lytic process in mechanistic terms. At least two steps are involved: a direct action on the cell wall; and a lysis of the bacterial protoplast resulting in liberation of substances which are metabolizable by the parasite. There is presently no indication that the breakdown of the cell wall is connected with the action of an exoenzyme secreted by *Bdellovibrio*. A lysate of susceptible host cells, after separation from the parasites, has no detrimental effect on living bacteria. The individual contact with the parasite is an essential precursor of the destruction of the host cell. A lysate of a host culture containing high concentrations of the parasite, however, possesses strong digestive activity against heat-treated bacteria. This property is shared by the saprophytic derivatives (Fig. 9) and is similar to that shown by myxobacteria filtrates (Norén, 1953, 1955, 1960; Salton, 1953, 1955). Once the living cell has been destroyed by the primary reaction on the cell wall, the parasite can digest the bacterial proteins.

The experimental results have shown that single cells of the wild-type *Bdellovibrio* have the ability to produce plaques (colonies) in a lawn only of living host cells; however, saprophytically growing strains can be selected. These strains usually have lost their lytic activity against living bacteria, or they lose this capacity after successive transfers on artificial substrate. They possess strong proteolytic activity, and are capable of digesting heat-killed host cells, but they are unable to lyse living cells. The population changes have not yet been studied thoroughly, but obviously they result from mutation and selection. Theoretically, from wild-type parasitism to complete saprophytism, one might imagine a series of intermediate forms. Let us assume that the wild-type *Bdellovibrio*, which alone can be regarded as the parasite, requires a growth factor present only in living host cells (r^+), that it is capable of attachment (a^+), that it has the capability of cell wall breakdown (w^+), that it possesses proteolytic activity (p^+), and that it is motile and hence can collide with force against the host bacterium (m^+). The "genetic" recipe for the wild-type parasite would then be I. $r^+a^+w^+p^+m^+$.

Since the most extreme saprophytic condition, as exemplified by Sp. 19, is still proteolytic, we assume that the proteolytic activity remains stable, but that the other factors may vary independently; hence, the following saprophyte types are possible:

II. $r^+a^+w^-p^+m^+$	V. $r^-a^+w^+p^+m^+$	VII. $r^-a^-w^+p^+m^\pm$
III. $r^+a^-w^+p^+m^\pm$	VI. $r^-a^+w^-p^+m^+$	VIII. $r^-a^-w^-p^+m^\pm$
IV. $r^+a^-w^-p^+m^\pm$		

The reasoning behind these analyses, might be summarized in the following way. Cells that are capable of active attachment (a^+) must be motile (m^+); cells that have lost this capacity (a^-) can be motile but need not be so ($m^\pm$). If attachment capacity is lost (a^-), the breakdown of the cell wall is not possible because there is no demonstrable secretion of an exogenous enzyme; if the capability of breaking down the cell wall of living cells is lost (w^-), the attachment to the host cell may occur, but there would be no lytic effect. The same would be true with cells that do not attach (a^-), but have retained their capability for cell wall breakdown (w^+). The loss of either the capacity of attachment (a), or the capacity to break the cell wall (w), or both, would render impossible a parasitic reaction on living cells. Therefore, types II to VIII (with exception of type V) cannot act on living cells.

When cultivated on living host bacteria, only types I and V should be able to propagate. Type V (r^-), that should show lytic activity (in terms of plaque-forming ability) against both living and dead cells, has not yet been found. On artificial substrates, the population shifts toward type VIII which does not possess those factors associated with an attack on living cells (a^-, w^-), and which is independent of the growth factor (r^-).

Before one can make conclusions concerning the parasitic or saprophytic nature of a culture in the foregoing terms, it is necessary to analyze the individual activities comparatively. When the substrate offered to *Bdellovibrio* consists of living bacteria, the wild-type with its parasitic and lytic activity has a selective advantage. Under these conditions, the population of *Bdellovibrio* statistically represents a "parasite". It is at the same time a potential saprophyte, as has been shown by the selection of saprophytic derivatives. A saprophytically growing population of *Bdellovibrio* statistically represents a "saprophyte" but, at the same time, it is a potential parasite because individuals with wild-type characteristics can usually be reisolated. The question of parasite versus saprophyte, therefore, can be related only to individual organisms. Whether a given culture gives the appearance of one or the other form is a matter of statistics and population genetics. By definition, we regard a culture of *Bdellovibrio* as a parasitic culture if the majority of the individuals of a population have the ability to lyse living host cells by physical attack and attachment. This property is expressed by the ability of individual cells to produce plaques (colonies) in a lawn of susceptible host cells. Massive inoculation of saprophytically growing *Bdellovibrio* into a liquid culture of living host cells may result in lysis due to selective development of a few wild-type organisms (back-mutants). In any case, it is difficult to judge on the parasitic or saprophytic nature of a culture unless an analysis is undertaken of the individuals representing that population. For this reason, one has to check

cultures of *Bdellovibrio* in parallel for plaque formation on living cells, and for colony production on dead (heat-killed) cells or on bacteria-free medium. A population which, at the same time and with quantitative equivalence, is able to form colonies on an artificial (bacteria-free) substrate, and to form plaques on living bacteria, has not yet been found. Possibly, the two conditions exclude one another.

The occurrence of cells with saprophytic growth ability in a population of the wild-type parasite, and the existence of individuals with parasitic properties in a population of the saprophytic *Bdellovibrio*, very probably originate from mutational events. If all individuals of a population would have the capacity to adapt to changes in environmental conditions by alteration of their metabolic activity, one would have to expect that inoculation of one type into a substrate favorable to the other would initiate the development of many cells through adaptation. The experimental data, however, are not compatible with this theory. From a population of parasites, kept on living host cells as substrate, only a few saprophytic derivatives can be selected. In *Bd.* 321, they occur at a frequency of about 1 in 5×10^8 cells. The change from saprophytic to parasitic growth ability is in the same order. These events correspond to the usual mutational rates; hence, we consider the derivatives under discussion as mutant strains.

The very existence of *Bdellovibrio* raises many intriguing problems for the biologist. Up to this point, our work has emphasized a general view of this new kind of microorganism. Detailed studies of the many interesting vistas are now required. We find that parasites of the *Bdellovibrio* type can easily be isolated from soil or sewage. The few isolation experiments which have been carried out, naturally, cannot give more than an outline of the distribution, frequency of occurrence, and ecological significance of these organisms. The presently known isolates show some differences with respect to morphology and host range, but they represent one group of unique microorganisms able to attack and lyse living bacteria. Without doubt, many more strains with differences in host range and other properties exist and await isolation. In our isolation experiments, we have not yet found any *Bdellovibrio* plaques on lawns of gram-positive bacteria, but this does not definitely mean that they do not exist, or that nature has exempted the gram-positive bacteria from such parasitic attack. Because of the fundamental differences in structure and composition of the cell walls of the gram-positive and gram-negative bacteria, a parasite that attacks and lyses gram-positive bacilli must be expected to have some properties different from the presently known *Bdellovibrio*.

In this connection, we wish to point out that various writers (Houwink, 1951, 1955; Hund and Kandler, 1956; Masudo, 1957) have presented evidence

that *Caulobacter* cells can attach to cells of *Bacillus* spp. They concluded from their studies that the invested cells undergo lysis as a result of the "parasitic" action of *Caulobacter*. In the present report, we have recorded our observations that caulobacters are unable to cause lytic reactions in lawns of bacteria belonging to different systematic groups including bacilli and, for this reason, have rejected the idea of a relationship between *Bdellovibrio* and *Caulobacter*. We are convinced that the attachment of *Caulobacter* to other bacteria has nothing to do with "parasitism", and that caulobacters are unable to provoke the lysis of invested bacteria. This conviction is strongly supported by the critical and much more extensive investigation of Stove (1963). She confirmed that bacterial cells – not only bacilli, but also gram-negative organisms such as *Azotobacter vinelandii* – are suitable substrata for attachment of *Caulobacter*. However, neither lysis, nor enhanced growth of the caulobacters, nor cytotoxic effects could be found in relation to such attachments. Indeed, Stove concluded categorically that "the results of these studies on the alleged parasitism of other bacteria by *Caulobacter*, supposedly made possible by the ability of caulobacters to attach to the surface of many types of bacteria, have been uniformly negative."

We have omitted reference to other lytic phenomena in bacteria, caused by antibiotics or lytic enzymes excreted by antagonistic microorganisms, because of their basic difference from the predatory and parasitic nature of *Bdellovibrio*. Its mode of action is a fascinating subject. What is the nature of host specificity? How do the cells of *Bdellovibrio* attach to the bacterial surface? What is the biochemical situation with respect to cell wall disintegration and lysis? Is the breakdown of the cell wall caused exclusively by enzymatic activity of the parasite, or is it referable to an induction of autolytic processes in the attacked host? These and many other questions need to be investigated before the mechanism of lysis and the nature of the fatal parasitism can be understood. Related to these problems are the nutritional requirements of *Bdellovibrio*; ultimately, the "purpose" of the parasitic action of *Bdellovibrio* is a "search" for food.

The fact that this type of plaque-former has escaped detection for so long, in spite of numerous phage isolation trials, can be explained on the basis of the methods used. Phage workers, when isolating bacteriophages from natural habitat, usually do (and should do!) everything necessary to avoid bacterial contamination of the phage-containing filtrate (chloroform treatment, high-speed centrifugation, filtration through filters of very small pore size diameter, etc.). Besides, by use of phage enrichment techniques, high phage concentrations are produced so that *Bdellovibrio* – even if it were present in the original filtrate – would have been diluted out in the series leading to single phage

plaques. Last but not least, the development of *Bdellovibrio* in isolation experiments leads to visible plaque formation not before 2 to 4 days. Since bacteriophages usually form plaques within 24 hours, negative phage isolation plates are discarded long before *Bdellovibrio* plaques could develop. All these points have conspired to prevent the earlier detection of *Bdellovibrio* as a "contaminant" in phage isolations. On the other hand, the organism has escaped discovery by microbial ecologists because it does not grow in culture media, because it is quite small and – most importantly – because there was no a priori reason to expect the existence of this unusual creature!

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