JOURNAL OF BACTERIOLOGY, May, 1966
Vol. 91, No. 5


# Parasitic Interaction of *Bdellovibrio bacteriovorus* with Other Bacteria

MORTIMER P. STARR AND NANCY L. BAIGENT
*Department of Bacteriology, University of California, Davis, California*

Received for publication 17 January 1966

ABSTRACT

STARR, MORTIMER P. (University of California, Davis), AND NANCY L. BAIGENT. Parasitic interaction of *Bdellovibrio bacteriovorus* with other bacteria. J. Bacteriol. **91**:2006–2017. 1966.—The interactions of the predatory parasite, *Bdellovibrio bacteriovorus*, with *Erwinia amylovora*, *Pseudomonas tabaci*, and *P. phaseolicola* were examined by means of phase-contrast and electron microscopy. Attachment of the bdellovibrio to the host cell is apparently initially reversible; detachment occurs infrequently in the later stages. Formation of a pore in the host cell wall is followed by disorganization of the host nucleus and of the murein layer of the host cell wall. Short host cells become totally spheroplasted; the longer rods of *Pseudomonas* usually are partially spheroplasted. The parasite completely invades the host cell, and the cell contents of the host are digested. Bdellovibrios living as parasites inside the host increase considerably in size in comparison with those which have been living away from the host for a time. When the host protoplast is entirely lysed, the parasites leave the disintegrating "ghosted" cell envelope, and are ready to reinitiate the parasitic cycle. The time taken for a mature *Bdellovibrio* cell to complete the parasitic cycle may vary depending on the length of time the parasite has been away from its hosts.

*Bdellovibrio bacteriovorus* (12, 13) is an unusual bacterium which is predatory and parasitic upon other bacteria. The initial predatory phase involves the seeming "choice" of a susceptible host, followed by a violent contact between one or more of the highly motile *Bdellovibrio* cells and the much more massive host cell. Shortly thereafter, all or part of the host cell becomes a spherical body, presumably a spheroplast. The ultimate results of this predatory and parasitic interaction are a complete disintegration of the host bacterium and an increase in the number of *Bdellovibrio* cells.

It is the purpose of the present communication to record our observations, made with phase-contrast and electron microscopy, which bear on the parasitic interaction of *Bdellovibrio bacteriovorus* with three representative host species, *Erwinia amylovora*, *Pseudomonas phaseolicola*, and *P. tabaci*—all phytopathogenic bacteria. Ultrastructure, per se, of *Bdellovibrio* will be the subject of a separate report, as will our other studies on this unusual microbe.

## MATERIALS AND METHODS

*Media.* Tris, yeast extract, and peptone (TYP) broth contained Difco yeast extract, 3.0 g; Difco peptone, 0.6 g; 0.5 M Tris buffer at *p*H 7.5 [tris(hydroxymethyl)aminomethane, Sigma Chemical Co., St. Louis, Mo.], 100 ml; and distilled water, 900 ml. Double-layered agar plates were made by use of TYP broth with the addition of 1.5% agar (Difco) for the bottom layer, and 0.7% non-nutrient water agar for the top layer. Yeast extract, glucose, and calcium carbonate (YDC) agar contained Difco yeast extract, 1.0%; glucose, 2.0%; finely divided, USP precipitated calcium carbonate, 2.0%; and Difco agar, 1.5%.

*Bacteria.* All bacterial cultures were obtained from the International Collection of Phytopathogenic Bacteria (ICPB) maintained in this department. The three strains of *B. bacteriovorus* are subcultures of the isolates described by Stolp and Starr (13). Strains 100 (ATCC 15356) and 233 (ATCC 15372) have the same host activity spectrum, including a wide range of enterobacteria and pseudomonads, all gram-negative; they differ in being independent isolates which have been propagated and maintained continuously in stock culture on different hosts; i.e., *E. amylovora* ICPB-EA137 and *P. tabaci* ICPB-PT1, respectively. Strain 321 (ATCC 15142) attacks a limited range of *Pseudomonas* spp., including its propagating strain, *P. phaseolicola* ICPB-PM142. In the interest of brevity, few details will be presented here concerning our study of strain 321 and its pseudomonad hosts; the results are similar to those reported herein for strain 233 acting on pseudomonads.

Stock cultures of the host bacteria were routinely kept on YDC agar slants, and those of *Bdellovibrio* in TYP broth. Periodically, the *Bdellovibrio* lysate was filtered through a 0.45-$\mu$ Millipore filter to remove nonlysed bacteria and cellular debris, and 18-hr-old shaker-grown cells in TYP broth of the appropriate host bacterium were added to the filtrate containing the *Bdellovibrio* cells.

*Phase-contrast microscopy.* Phase-contrast microscopic observations and photomicrographs were made with a Zeiss Photomicroscope, with the use of a Zeiss VZ aplanatic condenser (na 1.40) and a 100× Zeiss Neofluar objective lens (na 1.30). The film, Adox KB14, was developed in Kodak DK-60a developer. Continuous observation of microcultures, recorded intermittently by photomicrography, is a very important step (*see* 11) in establishing the precise sequence of events during the interaction (Fig. 1). The microcultures were prepared by mixing approximately 3 volumes of an interacting host-plus-parasite preparation with one volume of molten 2% water agar and quickly pressing out a drop of the mixture to a very thin film between cover slip and slide; care was taken to trap air bubbles in the semisolid agar preparation. The cover slip was sealed to the slide with Vaspar. The phase-contrast photomicrographs presented herein are all at the same magnification, × 2,750.

*Preparation of bacteria for electron microscopy.* The first samples were prepared by flooding, with TYP broth, plates in which the bdellovibrio and its host bacterium had been incubated together for 3 to 4 days on double-layer TYP agar. After standing for 15 min, the supernatant fluid was poured off and was found by phase-contrast microscopy to contain cells of bdellovibrio and host in various stages of interaction. Some preparations were examined after staining with 1.0% phosphotungstic acid (*p*H 7.2). More usually, samples were fixed with osmic acid, dehydrated according to the method of Ryter and Kellenberger (9), and then embedded in epoxy resins. A moderate degree of plasmolysis can be observed in the electron micrographs. The flagella, missing from the preparations described herein, are known to be attached to the cells after fixation, centrifugation, and embedding in agar; detachments occur during the dehydration or the epoxy embedding. Attempted corrections of these defects are in progress, and any solutions will be reported elsewhere.

Later in the study, as the time-sequence of the interaction of the bdellovibrios with the host cells became important, samples were prepared by the following procedure. The stock culture of the bdellovibrio was diluted in a decimal series. Each dilution was plated in replicate with the appropriate host bacterium. After 4 days, the replicate plates of that dilution which had shown confluent lysis on the 3rd day were flooded with a minimal volume of TYP broth and allowed to remain undisturbed for 20 min. The supernatant fluid, which contained numerous *Bdellovibrio* cells, was then poured into a sterile flask. Bdellovibrios taken from such plates are, in general, at the peak of their activity, having lysed all the host cells but not yet approaching starvation. New 18- to 24-hr-old shaker-grown bacterial host cells in TYP broth were then added to the *Bdellovibrio* cells in the flask. The total addition of new host cells was made within a period of 5 min, but the number of cells added was controlled by microscopic examination of the cell suspension after each limited addition of host cells. The addition of host cells was halted when microscopic examination showed that the great majority of the added cells had one or more bdellovibrios attached to them, and that there were still a few bdellovibrios moving freely in the medium. Attachment of these very active *Bdellovibrio* cells occurs rapidly and with a certain amount of uniformity. Less active *Bdellovibrio* cells attach irregularly over a much greater period of time.

To secure a sequence of stages in the interaction, the fixative was applied to the interacting bacteria after various periods of time. The parasite-plus-host suspension was distributed in 10-ml amounts in sterile petri dishes. Fixation was started at the end of the appropriate time period with a 30-min prefix with osmic acid vapors. Fixation and dehydration then proceeded according to the Ryter and Kellenberger (9) method, before embedding in epoxy resins. Sections (approximately 60 m$\mu$) were cut on a Porter-Blum ultramicrotome fitted with a diamond knife; they were mounted on 200- or 400-mesh copper grids, and stained with saturated aqueous uranyl acetate or lead citrate. The specimens were then examined in a RCA EMU-3 electron microscope. For ease of comparison of stages in the host-parasite interaction, all electron micrographs are presented at the same magnification, × 60,000.

## RESULTS

Attachment of the bdellovibrio to the host cell is, apparently, initially a surface contact which is at first reversible. Individual parasites, after attacking one host cell, may become detached from that cell and go on to attack another. The prior entrance of a bdellovibrio into a host cell does not preclude further attacks and penetrations of that host cell by other bdellovibrios. The attachment to the host cell is not passive, and the term "attack" is indeed appropriate. The violent movement at the moment of contact appears to result primarily from the velocity with which the tiny *Bdellovibrio* cell strikes the relatively massive host cell; possibly, there is some component of the movement which stems from an attempt of the motile host cell to "escape." There is frequently observed a rotatory movement of the bdellovibrio relative to its point of attachment to the host cell, suggestive of an arm-in-socket type of action.

The continuous observation of microcultures by phase-contrast microscopy reveals additional features of the interaction. Figure 1, and particularly Fig. 1A, demonstrates that the bdellovibrios actually enter the host cell. Further, the interaction is seen to result in the complete or partial spheroplasting of the host cell. The foregoing

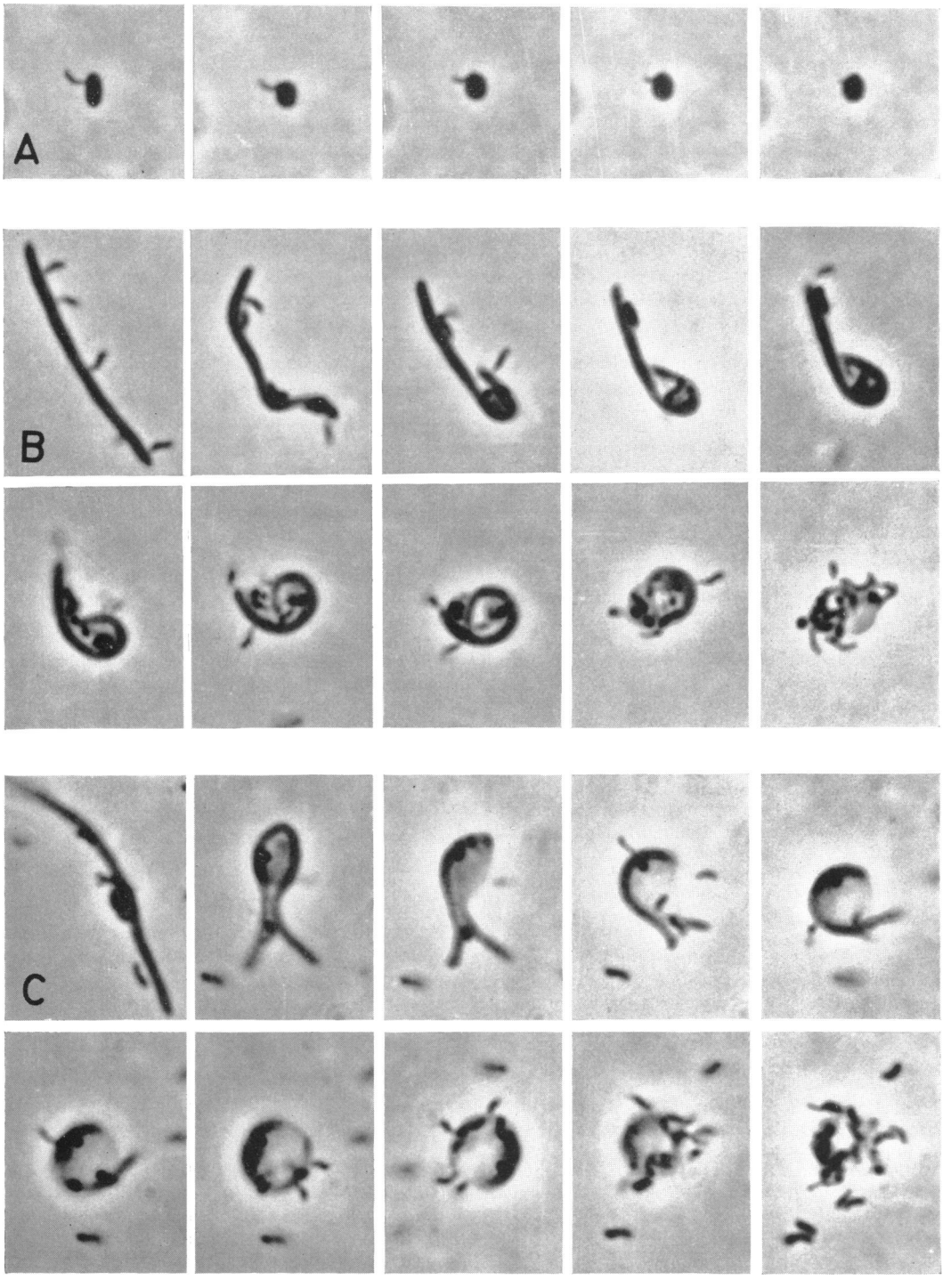


FIG. 1. *Time sequence, recorded by phase-contrast photomicrography, of the interactions (A) between Bdellovibrio bacteriovorus strain 100 and Erwinia amylovora ICPB-EA137; and (B and C) between B. bacteriovorus strain 233 and Pseudomonas tabaci ICPB-PT1.* × *2,750. A (top row): after 5, 20, 25, 25.5, and 30 min. B (second and third rows): after 15, 25, 26, 33, 48, 69, 75, 80, 150, and 275 min. C (fourth and bottom rows): after 25, 35, 39, 42, 48, 48.5, 69, 80, 150, and 275 min.*

observations are confirmed, and further details of the interaction are revealed, by the electron micrographs of thin sections (Fig. 2 to 7).

In these and other micrographs, the *Bdellovibrio* cell wall is shown to consist of two electron-dense layers separated by one of lesser density; inside this rather thin cell wall is a double-track plasma membrane. The cell walls of our strain of *E. amylovora* show three electron-dense areas interspersed by two layers of lesser density, exterior to a double-track plasma membrane (Fig. 2A). In *P. tabaci* and *P. phaseolicola*, there are certainly two such electron-dense areas (Fig. 5A); in higher-magnification electron micrographs which are not presented here, there are indications of an interrupted, poorly discernible, interior third layer (*see* 3). Electron micrographs (Fig. 2B, 5B) of the interacting cells, after irreversible attachment, show that the *Bdellovibrio* cell wall in the attachment area is easily distinguishable as a separate double-track layer in simple contour with the multilayered cell wall of the host.

After the initial surface contact, the next step in penetration is the formation of a pore (Fig. 3A, 5C) in the host cell wall. No differentiation or organization of the cell contents of the bdellovibrios has yet been observed in the electron micrographs which might explain why the bdellovibrios attach to the host cell by the nonflagellated end of the parasite (compare references 12 and 13 and our consistent observations with negatively-stained phosphotungstic acid preparations). There may be significance in the location of mesosomes in some *Bdellovibrio* cells near the attachment area. Cytoplasmic extrusions extending from the plasma membrane to the cell wall of the bdellovibrios have been observed in plasmolyzed cells. This may be an artifact resulting from the preparative procedures, but it is also possible that these are the "tramlines" along which enzymatic or enzyme-control substances are transmitted to result in production of the initial pore in the host cell wall.

Many micrographs (e.g., Fig. 3) show a definite constriction of the *Bdellovibrio* cell as it passes through the host cell wall, presumably resulting from the relatively small pore and the rigidity of the host cell wall. After the wall is penetrated and as the cell becomes spheroplasted, this constriction relaxes, allowing a deeper penetration by the bdellovibrio.

The large volume of deoxyribonucleic acid (DNA)—large, that is, relative to cell size—appears to remain centrally located. Some micrographs (e.g., Fig. 3A, 5) might be interpreted as showing extrusion or leakage of bdellovibrio DNA into the host cell upon penetration. Other electron micrographs show the entire *Bdellovibrio* cell completely within the host cell with no evidence of DNA extrusion. While not dismissing the possibility that the DNA of the bdellovibrio might play a fairly direct role in lysis of the host cell contents, it must also be recognized that the process of preparing the samples for electron microscopy and thin-sectioning could have produced these effects.

An early effect of attack by *Bdellovibrio* is the complete disorganization of the host cell contents (Fig. 3, 5C). First, the relatively discrete nucleus becomes dispersed and disappears; then, the ribosomes and cytoplasm become granular and unevenly distributed. Enzymatic activity on the host cell wall is evident; the smooth contour of the inner wall layer [presumably the layer consisting of the mucopeptide, murein (7, 14)] becomes irregular and interrupted, finally disappearing to leave the double-track wall typical of the spheroplast. The spheroplast increases considerably in size; with the apparent relaxing of the spheroplast wall, the *Bdellovibrio* cell forces its way completely into the host, progressively digesting the cell contents. It is not known whether all of these effects are caused directly by enzymes of the bdellovibrio, or whether the bdellovibrios upset the control mechanisms of the host cell involving a process which is autolytic rather than heterolytic (*see* 14).

The spheroplasts formed by *Erwinia* (Fig. 1A) vary in size, but eventually may be several times the size of the original host cells. Total spheroplasts are not formed as readily with our strains of *Pseudomonas*; the typical double-track spheroplast wall results from attack by bdellovibrio, but the increase in diameter may be restricted to one particular area of the rod. Approximately 30 min after the parasite attaches to and invades the host, a rapid hairpin bending of the host cell occurs, centering around the swollen portion of the rod (Fig. 1B, 1C). Nonspheroplasted parts of the cell were observed gradually to wrap around the swollen portion. The shorter pseudomonads become spheroplasted in a manner similar to the ballooning of the *Erwinia* cell.

A most striking feature in the invasion of the host cell is the considerable increase in size of the individual *Bdellovibrio* cell during its parasitic stage. The fully nourished parasite may reach three or four times the cell volume of the original attacking parasite (compare Fig. 2B and Fig. 4), particularly if the entering bdellovibrio had been away from its host for some time. Stolp and Starr (13) had reported that the size of *Bdellovibrio* is about 0.25 by 1.0 $\mu$. However, it would be diffi-

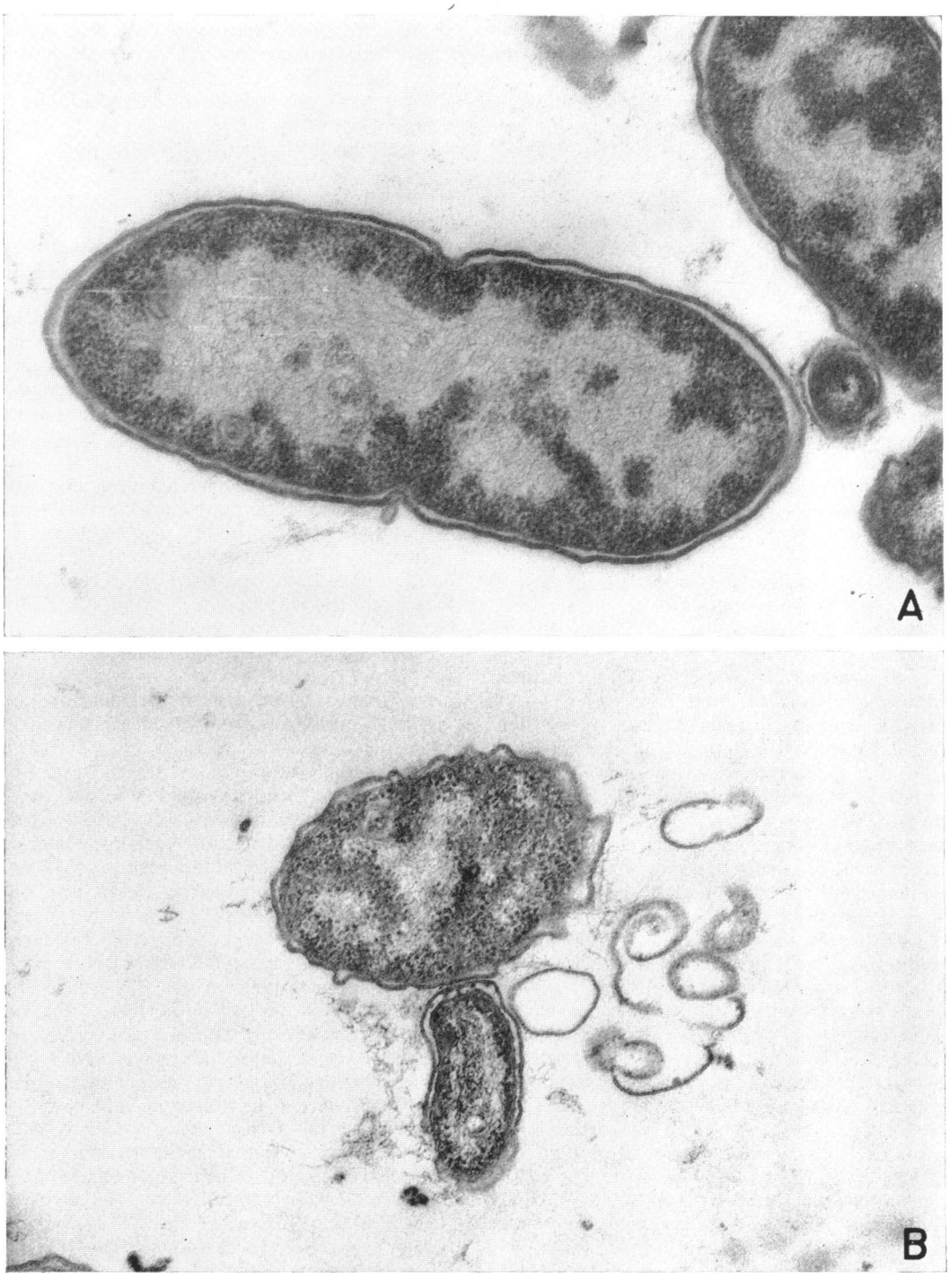


FIG. 2. *Sequences in the interaction between Bdellovibrio bacteriovorus strain 100 and Erwinia amylovora ICPB-EA137.* × *60,000. (A) Nonparasitized host bacterium; (B) initial contact of Bdellovibrio with Erwinia.*

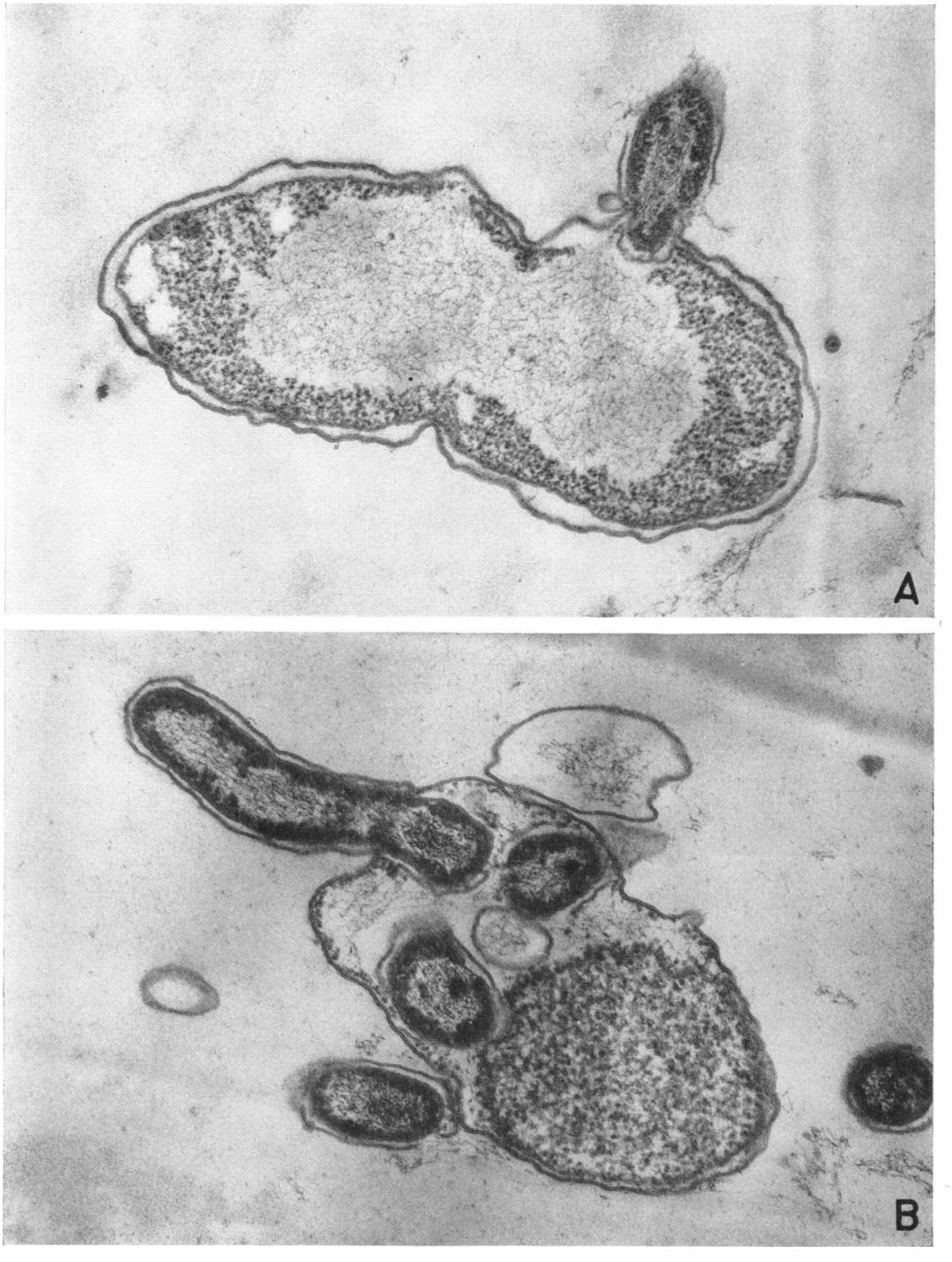


FIG. 3. *Continuation of the sequence of Fig. 2. (A) Entrance of Bdellovibrio through pore; (B) invasion of host cell.*

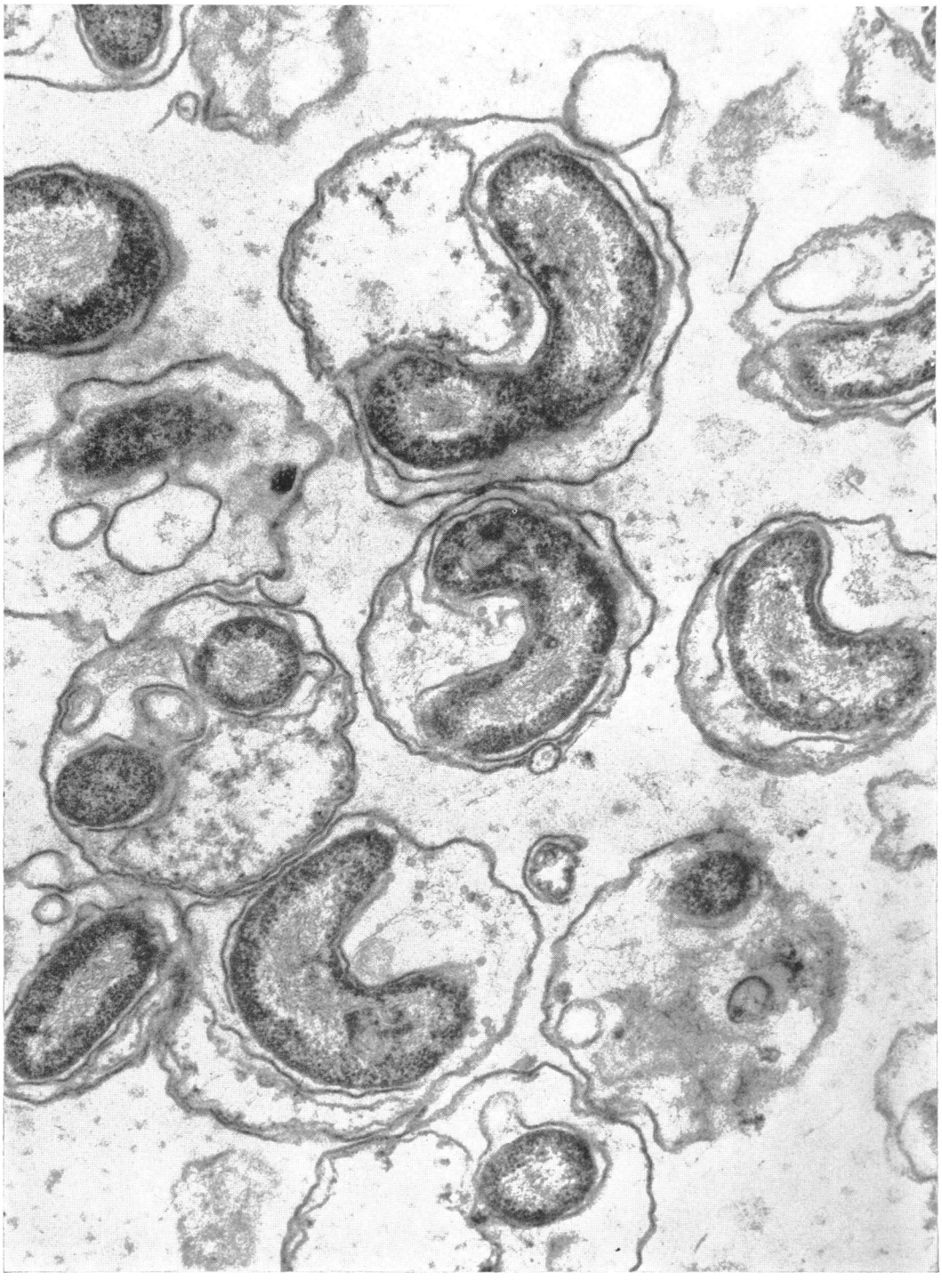

FIG. 4. *Continuation of the sequence of Fig. 2 and 3. Spheroplasts and developing bdellovibrios.*

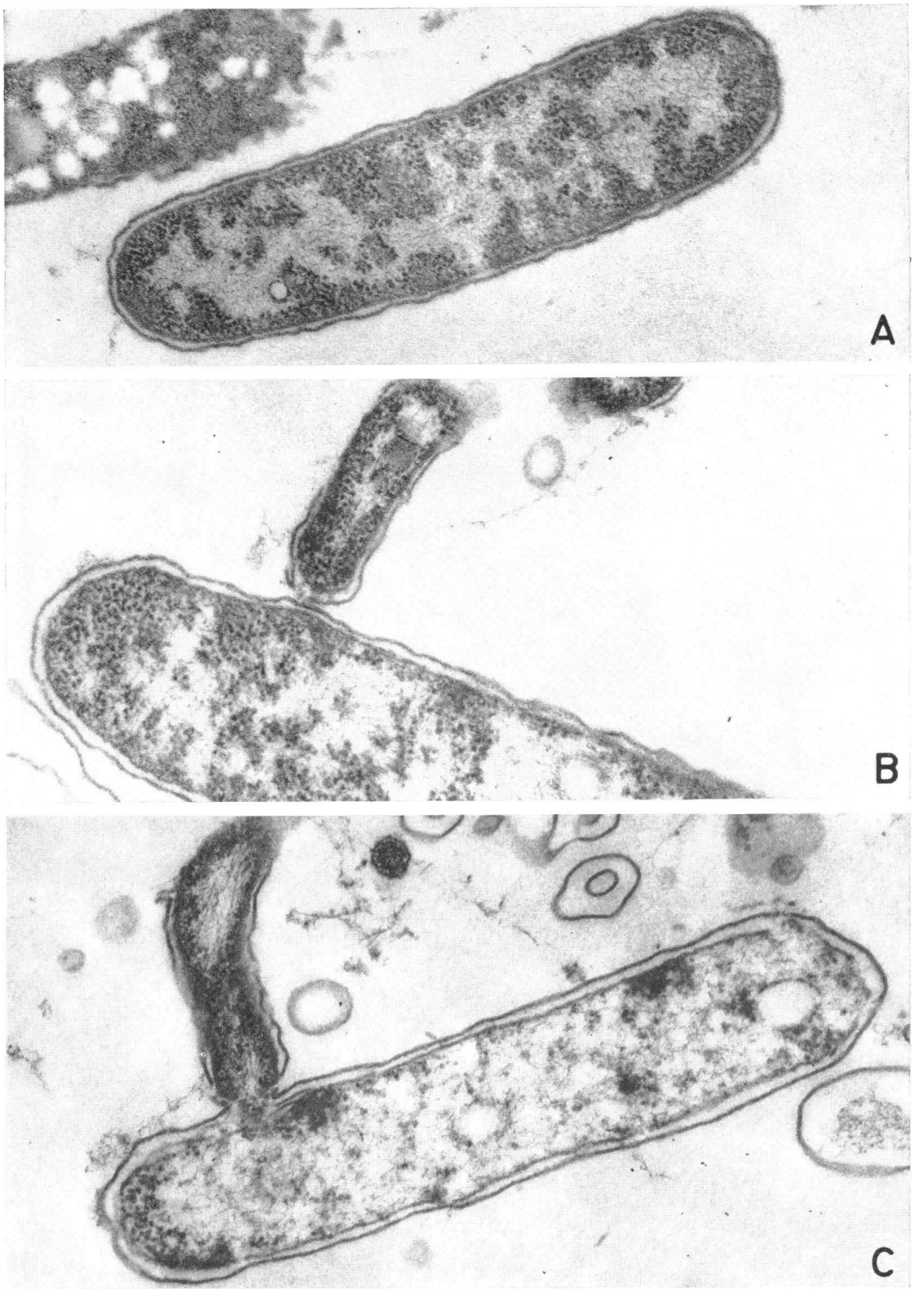


FIG. 5. *Sequences in the interaction between Bdellovibrio bacteriovorus strain 233 and Pseudomonas tabaci ICPB-PT1.* × *60,000. (A) Nonparasitized host bacterium; (B) initial contact; (C) entrance of Bdellovibrio through pore.*

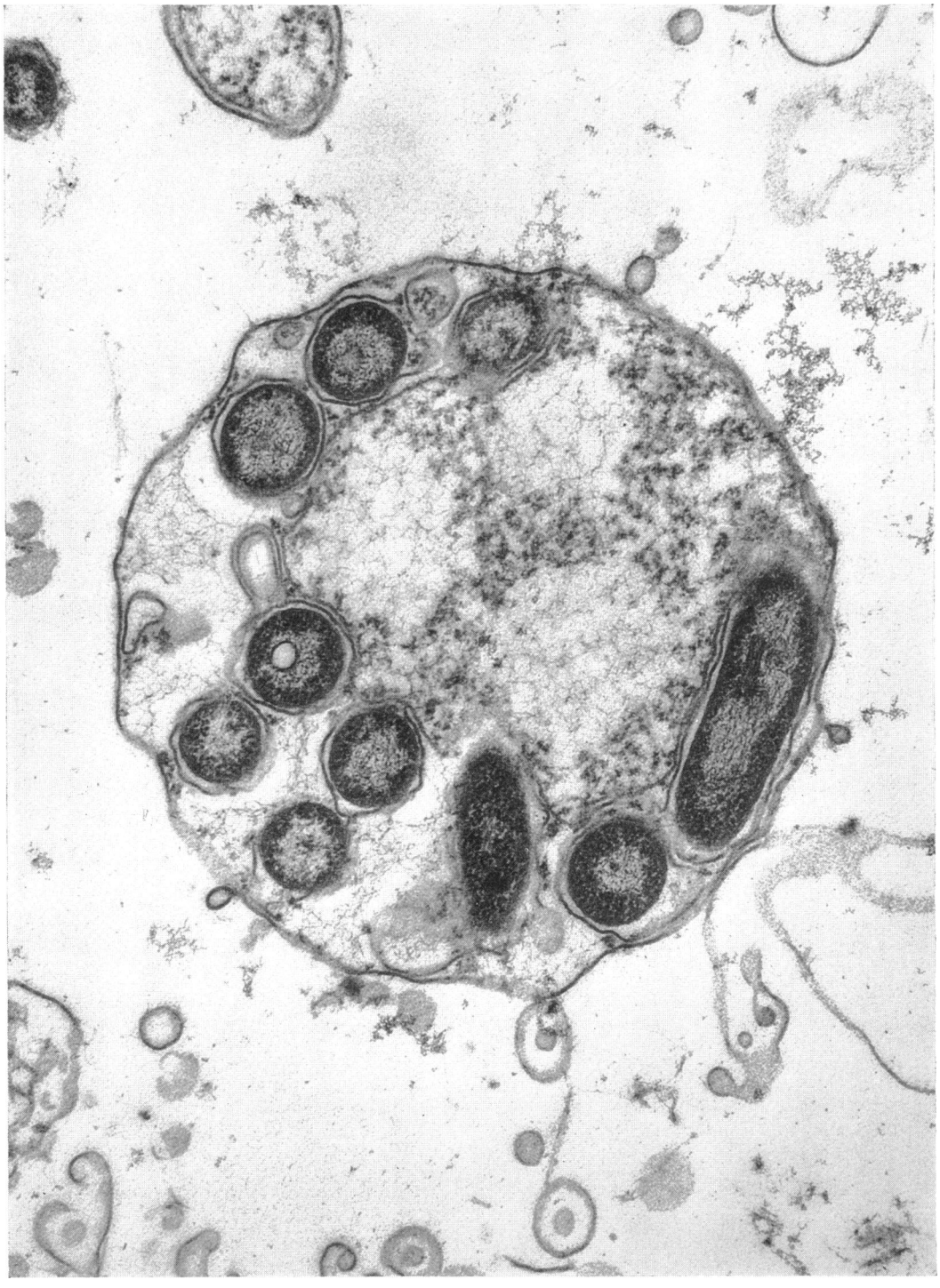

FIG. 6. *Continuation of the sequence of Fig. 5. Complete invasion of the host cell.*

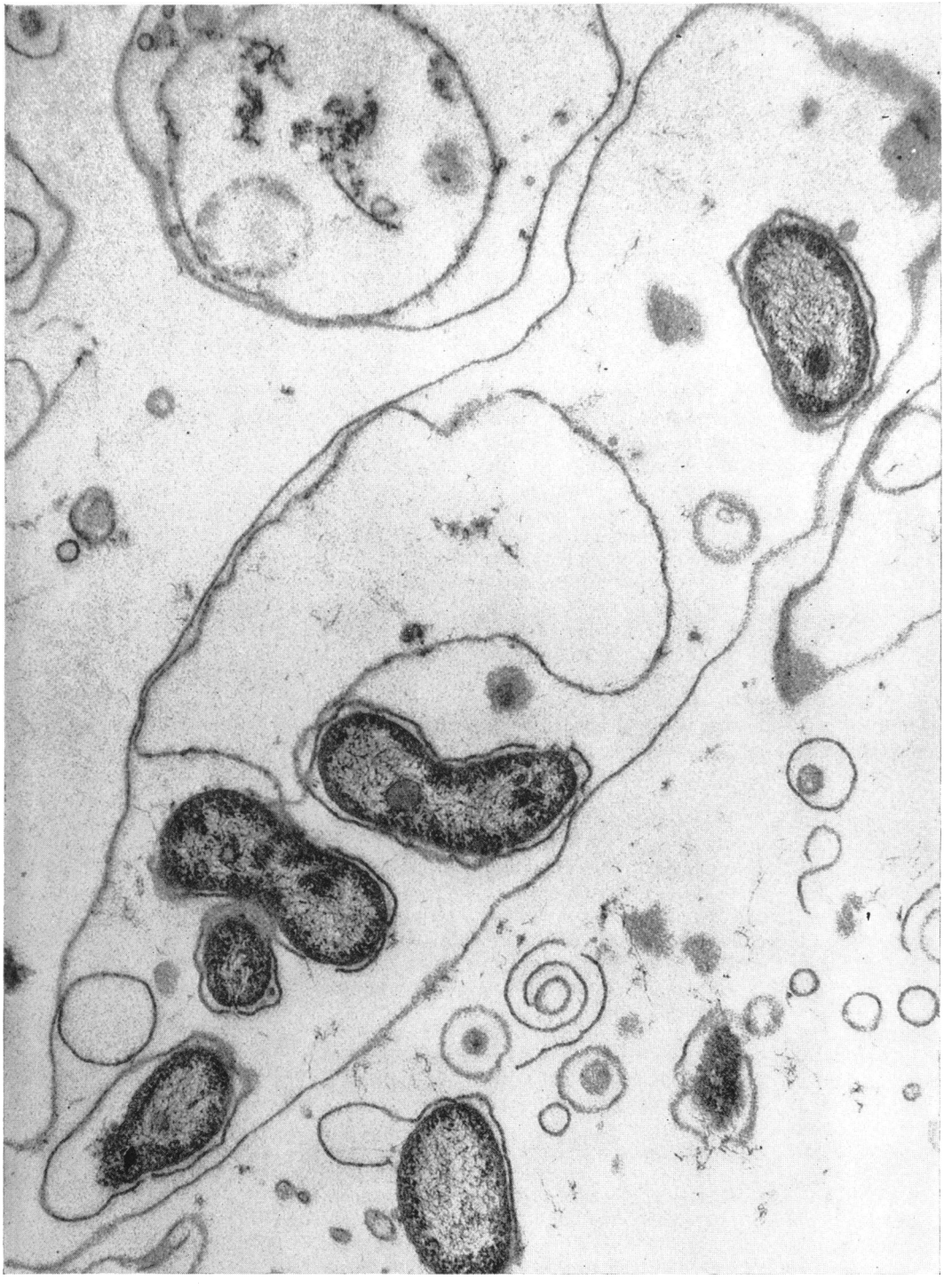

FIG. 7. *Continuation of the sequence of Fig. 5 and 6. Ghosted spheroplast and fully developed bdellovibrios.*

cult to establish a reliable and meaningful size range for any particular strain of *Bdellovibrio*, since the dimensions will depend upon the stage in the life cycle at which the cells are measured.

The *Bdellovibrio* cells remain inside the spheroplast usually until the entire cell contents are digested (Fig. 7). Absorption of the cell contents appears to be a rather localized process, since electron micrographs show the cell contents being digested away piecemeal, with the *Bdellovibrio* cells always being found adjacent to the area being digested (Fig. 3B, 6). A current paper (10) suggests some possible mechanisms for the lysis of the host organisms by the bdellovibrios.

Bdellovibrios which have digested the entire contents of the spheroplast now leave the empty "ghosted" cell envelope and reinitiate the parasitic cycle. They can attach immediately to suitable host cells or may attach to cells that already are parasitized (Fig. 1B, 1C). Exactly how the mature *Bdellovibrio* cells leave the ghost is not understood; in many cases, the host cell is so disintegrated that departure would require no more than swimming away from the debris. Phase-contrast and electron microscopic observations have shown that some of the bdellovibrios develop into corkscrew-like spirals (chains of vibrios?) within the spheroplast; others retain their vibrio shape but are considerably increased in size (Fig. 4). There is variation in the stage at which detachment occurs of individual vibrios from the corkscrew-like spirals. Indications of the formation of cell walls between individual vibrios in the spiral within the spheroplast have been seen in some micrographs. Because of the problem of focusing inherent in the vibrio and spirillum shapes, thin sections obtained to date have not been particularly revealing on this point and we are turning to stereoscopic techniques. By phase microscopy, vibrios have been seen apparently to extrude from the spheroplast wall and move vigorously for several seconds in a rotary action, before swimming away very actively to attack other host cells. On other occasions spirals have been seen protruding passively from spheroplasts before being released in some way into the medium. Examination of these spirals by negatively stained phosphotungstic acid electron microscope preparations have shown them to be strings of vibrios.

The time taken for a mature *Bdellovibrio* cell to complete the parasitic cycle may vary, depending upon the activity of the parasite—which, in turn, reflects the length of time the parasite has been away from its host. Parasites which have been free-living for several days may take more than 24 hr to complete the cycle. Cells of *B. bacteriovorus* strain 100, taken immediately after release from the spheroplast and allowed to interact with fresh *Erwinia* host cells, completed the cycle within 6 hr; fresh cells of *Bdellovibrio* 321 completed the cycle in 5 hr. At 1 hr after mixing the cells, most of the parasites were almost entirely or completely inside the host cells.

## DISCUSSION

The seeming lack of a device (holdfast?) which could explain the physical attachment in the early stages of the irreversible contact of the bdellovibrio with the host cell, in conjunction with the arm-in-socket movement of the bdellovibrio upon the host cell, suggests strong surface bonding between the two cells. The latter could also perhaps explain in part the exceeding rapidity with which the bdellovibrios attach to the host cells. Stolp and Starr (14) suggested a possible mechanical drilling of the pore in the host cell, and our electron micrographs neither support nor deny this possibility. Thin sections have been examined in which the attached bdellovibrio had been torn from the host cell, presumably by centrifugation in the preparative process. Part of the bdellovibrio cell wall is frequently left attached to the host cell, suggesting a strong bonding rather than a mechanical invagination.

Certain aspects of the parasitism of the bdellovibrios within the host cell are not understood. How, or whether, the spheroplast wall blocks off the entry pore is not known, except that the spheroplast wall is somewhat elastic. The means whereby the bdellovibrios escape from the ghosted spheroplasts is uncertain. Possibly the pressure exerted by the developing spiral of cells ruptures the wall at a weakened area. If flagella are developed within the spheroplast, an impetus may be given by movement of the unusually stout flagellum which is known from other work (13) to be produced by the mature parasite.

The partial spheroplasting brought about by the action of the bdellovibrios upon the elongate rods of pseudomonads (Fig. 1B, 1C) is strikingly reminiscent of the result of exposing gram-negative bacteria to penicillin. Here, rod-shaped cells develop an osmotically sensitive swelling or extrusion, either centrally or near the end, which enlarges to form a sphere which increases in size (compare reference 6, Fig. 2 of reference 5, and Fig. 6 of reference 8).

*B. bacteriovorus* is an endoparasite, rather than an ectoparasite as previously reported (10, 13), capable of entering and killing the host bacterium by disorganization and digestion of the cell contents, with a resultant increase in parasite population. In the use of the term endoparasite, we

refer solely to the fact that the *Bdellovibrio* cell completely enters the host cell. However, the parasite may not initially breach the plasma membrane (Fig. 4 and 6).

The resemblance, in their "outward manifestations" (13), between *Bdellovibrio* activity and bacteriophage action appears to depend largely on the trait that each agent causes a lysis of its bacterial host. *Bdellovibrio* is undoubtedly cellular and procaryotic; adsorption to the bacterial host cell is by direct cell-to-cell contact; the entire *Bdellovibrio* cell enters the host cell (compare phage DNA penetration) and remains a discrete body during digestion of the cell contents; direct microscopic observations have shown that each *Bdellovibrio* cell gives rise to one or few parasites which are greatly increased in size relative to that of the invading parasite, and which appear to be produced by cell division from the parent cell.

Much closer parallels might be drawn between the *Bdellovibrio* system and several other bacteria which penetrate plant and animal cellular habitats for the necessities of life; for example, the various intracellular arthropod symbionts (2) and the rickettsiae. Perhaps more pertinently, the interaction of *Bdellovibrio* with its host might be contrasted with the infection by rhizobia of legume roots (4). The motile nodule bacteria are attracted to the root hairs, somewhat specifically and probably chemotactically; they excrete enzymes which dissolve the walls of adjacent cells in the root hair and thus penetrate into the plant cells; the rhizobia also increase enormously in volume while living as intracellular bacteroids. A similar comparison might be made concerning the endoparasites of *Alnus* and other nonleguminous angiosperms (1).

## ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant GM-12643 from the National Institute of General Medical Sciences.

We are indebted to J. Pangborn of the Davis Campus Electron Microscope Laboratory for helpful advice and cooperation concerning electron microscopy, and to J. R. Woods for assistance with the microtomy.

## LITERATURE CITED

1. BOND, G. 1963. The root nodules of non-leguminous angiosperms. Symp. Soc. Gen. Microbiol. **13**:72–91.
2. BROOKS, M. A. 1963. Symbiosis and aposymbiosis in arthropods. Symp. Soc. Gen. Microbiol. **13**:200–231.
3. BUCKMIRE, F. L. A., AND R. A. MACLEOD. 1965. Nutrition and metabolism of marine bacteria. XIV. On the mechanism of lysis of a marine bacterium. Can. J. Microbiol. **11**:677–691.
4. DIXON, R. O. D. 1964. The structure of infection threads, bacteria and bacteroids in pea and clover root nodules. Arch. Mikrobiol. **48**:166–178.
5. HAHN, F. E., AND J. CIAK. 1957. Penicillin-induced lysis of *Escherichia coli*. Science **125**: 119–120.
6. MARTIN, H. H. 1963. Bacterial protoplasts—a review. J. Theoret. Biol. **5**:1–34.
7. MURRAY, R. G. E., P. STEED, AND H. E. ELSON. 1965. The location of the mucopeptide in sections of the cell wall of *Escherichia coli* and other Gram-negative bacteria. Can. J. Microbiol. **11**:547–560.
8. MCQUILLEN, K. 1960. Bacterial protoplasts, p. 249–359. *In* I. C. Gunsalus and R. Y. Stanier [ed.], The bacteria, vol. 1. Academic Press, Inc., New York.
9. RYTER, A., AND E. KELLENBERGER. 1958. Étude au microscope électronique de plasmas contenant de l'acide déoxyribonucléique. Z. Naturforsch. **13b**:597–605.
10. SHILO, M., AND B. BRUFF. 1965. Lysis of Gram-negative bacteria by host-independent ectoparasitic *Bdellovibrio bacteriovorus* isolates. J. Gen. Microbiol. **40**:317–328.
11. STARR, M. P., AND D. A. KUHN. 1962. On the origin of V-forms in *Arthrobacter atrocyaneus*. Arch. Mikrobiol. **42**:289–298.
12. STOLP, H., AND H. PETZOLD. 1962. Untersuchungen über einen obligat parasitischen Mikroorganismus mit lytischer Activität für *Pseudomonas*-Bakterien. Phytopathol. Z. **45**: 364–390.
13. STOLP, H., AND M. P. STARR. 1963. *Bdellovibrio bacteriovorus* gen. et sp. n., a predatory, ectoparasitic, and bacteriolytic microorganism. Antonie van Leeuwenhoek J. Microbiol. Serol. **29**:217–248.
14. STOLP, H., AND M. P. STARR. 1965. Bacteriolysis. Ann. Rev. Microbiol. **19**:79–104.