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Structural Properties and Features of Parasitic *Bdellovibrio bacteriovorus*

DINAH ABRAM AND BRIDGETT K. DAVIS

Department of Microbiology, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15213

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The structure of five parasitic strains of *Bdellovibrio bacteriovorus* was studied by electron microscope after negative staining and in shadow-case and etched freeze-fractured preparations. Special attention was paid to the cell wall and the flagellar sheath which is continuous with the wall or part of it. These structural components reveal distinct features which are induced by certain staining substances; they are exceedingly susceptible to disruption by physical treatments, and in old cells often appear impaired. In freeze-fractured cells the wall shows characteristic fracturing tendencies not known in other microorganisms. These structural properties and features are distinct to *Bdellovibrio* wall and flagellar sheath, the structural integrity of which is a fundamental requirement for the infectivity and survival of this organism. The anterior end of *Bdellovibrio* is differentiated: 6 to 12 ring-like structures (9 to 12 nm, outer diameter) are built into its wall and several fibers (7 to 10 nm wide, up to 1.5 μm long) emerge from it. Intracellular structures, which are revealed as compact oval bodies bulging from the cell border and have internal laminated organization, are characteristic of *Bdellovibrio* after negative staining with certain compounds. These findings on the structure of parasitic *Bdellovibrio* substantiate previous observations indicating the uniqueness of this organism and add criteria for the identification of this genus.

The recognition of the new genus and new species *Bdellovibrio bacteriovorus* was originally based on its predatory and parasitic nature (35). The uniqueness of this organism has been further supported by biochemical studies of parasitic strains and their host-independent derivatives (27, 31). Aspects of the morphology, pleomorphism, and the fine structure of *Bdellovibrio* have been described from observations by phase-contrast and electron microscopy (5, 6, 13, 20, 24, 25, 30, 32 to 35). However, when parasitic activity and morphology are used as criteria for identification, they can lead to an approximate description of bdellovibrio-like organisms (12). In fact, it is not certain at this time whether *Bdellovibrio* represents a single species or if this genus includes a heterogeneous group of organisms. Moreover, the base compositions of the deoxyribonucleic acid of parasitic and host-independent organisms also indicate the possible existence of at least two different genera among the organisms currently classified as *Bdellovibrio* (28).

The object of this study was to further elucidate the structure of *Bdellovibrio*. Several electron microscope procedures previously not used in structural investigations with this organism were exploited. These procedures revealed features distinct to parasitic *Bdellovibrio* and permitted an insight into the structural properties of the cell wall and the flagellar sheath, which is continuous with the wall or part of it.

This paper presents electron micrographs which are pertinent to the specialized features of *Bdellovibrio* with emphasis on the structural properties of the cell wall. These observations may have a bearing on the parasitic nature of *Bdellovibrio*, and provide additional criteria for the identification of organisms belonging to this genus. A preliminary report of a part of this study was presented earlier (Abram and Shilo, Bacteriol. Proc., p. 41, 1967).

MATERIALS AND METHODS

Organisms. Five strains of *B. bacteriovorus* were employed: 109 and D (obtained from M. Shilo, Hebrew University Medical School, Jerusalem, Israel), and ATCC 15143, ATCC 15362, and ATCC 15360 (obtained from the American Type Culture Collection). *Escherichia coli* B (obtained from M. Shilo) was used as host organism to maintain stock cultures of *Bdellovibrio* 109 and D. *E. coli* ATCC 15144 and *Proteus mirabilis* ATCC 15363 were used as host organisms to maintain stock cultures of *Bdellovibrio* 15143 and 15362, and *Bdellovibrio* 15360, respectively. *Enterobacter aerogenes* ATCC 15361, *Spirillum serpens* NCIB 9510 (obtained from the National Col-

lection of Industrial Bacteria in Torry Research Station, Aberdeen, England) and *Proteus vulgaris* (Purdue strain) were also used as hosts. All five *Bdellovibrio* strains were grown in two-membered cultures with any one of the above organisms as host at one time or another during this study.

Media. TY medium consisted of 1% trypticase (BBL), 0.2% yeast extract (Difco); in the solid form it was supplemented with 1.4% agar (Difco). TY/5 and TY/10 broth consisted of 0.2% trypticase and 0.04% yeast extract, and 0.1% trypticase and 0.02% yeast extract, respectively, supplemented with 1.25×10^{-3} M $Ca(NO_3)_2$. TY/5 agar consisted of TY/5 broth supplemented with 1% or 0.55% agar; these were used as bottom and top layers for plaque assays. STY/5 agar consisted of TY/5 broth supplemented with 6.5×10^{-5} M $MnSO_4$, 6.5×10^{-5} M $FeSO_4$, and 1% agar. The *p*H of all the TY media was 6.8 after autoclaving. NB, NB/5 and NB/10 broth were prepared by the method of Seidler et al. (26), and DNB broth was prepared as described by Varon and Shilo (36).

Culturing conditions. *Bdellovibrio* stock cultures were maintained on petri plates containing STY/5 agar (40 to 50 ml) inoculated in the center with host-parasite mixtures. The plates were kept at 24 to 26 C in a moist atmosphere. Viable parasite and host cells from the periphery of the inoculated area, where growth continued slowly, served to start new stock cultures (every 4 to 6 weeks), and two-membered broth cultures. Routinely, two-membered cultures were established by inoculating: (i) 5 ml of host cells (5×10^9 to 10^{10} cells/ml), grown overnight at 37 C on TY slants and washed off the agar with TY/5 broth, and (ii) 1 ml containing 10^9 to 3×10^9 *Bdellovibrio* cells, from a two-membered culture in TY/5 broth after 16 to 24 hr of incubation, into 65 ml of TY/5 broth in 250-ml flasks. After 14 to 16 hr of incubation at 30 C with shaking, almost all host cells (more than 99.99%) are lysed and the lysates contain 10^9 to 3×10^9 *Bdellovibrio* cells per ml. To study cells of lysates in the other diluted media (TY/10, NB/5, NB/10, and DNB), the same procedure was followed, except that the host cells were washed off the slants with the medium to be used, and *Bdellovibrio* inoculum was obtained from lysates of cultures which had been previously transferred in the same medium at least twice. *Bdellovibrio* 109 or D were grown with *E. coli* B also on the enriched media (TY and NB). The cultures were established as above but had to be incubated for 28 to 34 hr before almost all host cells lysed, and the lysates contained 5×10^9 to 10^{10} *Bdellovibrio* cells per ml. Parasite-host ratio in the inoculum of the two-membered cultures never exceeded 1:5.

Enumeration of Bdellovibrio. Viable cells were enumerated by plaque counting. An 0.6-ml amount of *E. coli* B grown overnight in TY broth at 30 C was added to 7.5 ml of top layer TY/5 agar at 50 C and thoroughly mixed; 0.3 ml of an appropriate dilution of *Bdellovibrio* in TY/10 or TY/5 broth was added and well mixed. One-milliliter samples were spread on 12 to 15 ml of TY/5 bottom-layer agar in 9-cm petri dishes. Plaques (15 to 80 per plate) were counted after incubation at 30 C for 2 days.

Preparation of cells for electron microscopy. Lysates of *Bdellovibrio* were used within 4 hr after most host cells had lysed. To examine "old" *Bdellovibrio*, the above lysates were left at room temperature (20 to 24 C) for 2 to 8 days, or two-membered cultures were incubated for 2 to 4 days. Lysates were often but not always filtered through a double layer of Whatman no. 41 paper to remove some of the host cells debris. This step did not affect *Bdellovibrio* in any way. Lysates and filtrates obtained from them were centrifuged at $3,500 \times g$ for 5 to 10 min at room temperature, yielding loosely packed sediments which were gently suspended in TY/10 broth or in distilled water. These suspensions (ca. 10^{10} cells/ml) were kept at room temperature and were used for electron microscopy within 4 hr, with or without further treatment. Cells suspended in TY/10 broth were disrupted by a freeze-thaw cycle or by sonic oscillation. (i) Suspensions (0.5- to 3.0-ml samples) were rapidly frozen (20 to 40 sec) in liquid nitrogen. Samples were withdrawn for electron microscopy while the material was thawing (3 to 6 min) at room temperature and for 2 hr after the completion of the freeze-thaw cycle. (ii) Suspensions (2- to 4-ml samples) were treated by sonic oscillation for 20 to 30 sec, by using an ultrasonic generator, Sonifier model S110 (Branson Instruments Inc., Stamford, Conn.), set for 3 to 4 amp output and operating at 3/8 of its maximal power. Samples for electron microscopy were prepared within 2 hr. Suspensions of host organisms (10^9 to 10^{10} cells/ml), obtained by washing off overnight growth on TY slants with TY broth, were treated as described above for *Bdellovibrio*. The surviving cells in the treated suspensions were estimated by plaque or colony counts.

Aqueous solutions of the acids and salts listed in Table 1 were used at 0.5% (w/v) concentration (before *p*H adjustment) for negative staining. Uranyl acetate (UA) was also used at 0.3% (w/v) concentration. Preparations for electron microscopy were obtained as previously described (1). Whenever necessary, suspensions were diluted directly on the support grids with distilled water to obtain specimens for shadow casting, or with the desired staining solution to obtain a negatively stained preparation.

To examine the effect of the stains on cells in suspensions, *Bdellovibrio* (5×10^9 to 10^{10} cells/ml) were suspended in solutions of lithium tungstate (LiT) and UA at final concentrations of 0.9 to 3% and 0.9 to 1.9%, respectively. After 15 to 30 min at room temperature, the suspensions were centrifuged at $3,500 \times g$ for 10 min. The sediments from the LiT suspensions were washed with TY/10 or distilled water to dilute the stain 50- to 100-fold, and those from the UA suspensions were washed thoroughly with distilled water to dilute the stain 1,000-fold. The cells were recovered by centrifugation at $3,500 \times g$ for 10 min, resuspended in TY/10 broth or water, and examined after negative staining with LiT and potassium phosphotungstate (KPT) at *p*H 7.0. As controls, the cells recovered from the above suspension were examined without further staining and after shadow-casting, and native cells were examined in unstained preparations to determine their inherent density and in preparations stained with 0.001% UA.

For freeze-etching studies, *Bdellovibrio* lysates in

TABLE 1. *Negative stains*

Compound[a]	*p*H adjusted with	Final *p*H
Phosphotungstic acid	KOH (1.0 and 0.1 N)	4.0, 5.0, 6.0, 6.5,. 68, 7.2, 7.5, and 8.1
Borotungstic acid	KOH (1.0 and 0.1 N)	6.8–7.2
Silicotungstic acid	KOH (1.0 and 0.1 N)	6.8–7.2
Sodium tungstate		7.1
Lithium tungstate		7.2
Ammonium tungstate meta		6.2
Ammonium molybdate	NH_4OH (1.5 N)	6.8–7.0
Uranyl acetate/EDTA[b]	NaOH (1 N)	6.6–6.8
Uranyl acetate		3.8–4.2

[a] All chemicals were obtained from K & K Laboratories, Inc.

[b] Prepared by mixing equal volumes of 1% aqueous solutions of disodium ethylenediamine-tetraacetate (EDTA) and uranyl acetate to give a final concentration of 0.5%.

TY/5 broth and suspensions of *E. coli* in the exponential phase of growth were centrifuged at 3,000 to 4,000 × *g* for 5 to 10 min at room temperature. After all the supernatant fluid was withdrawn, the loose sedimented cell paste was placed on a commercial 3-mm gold-nickel alloy specimen carrier to form a droplet, 2 mm in diameter. This droplet was rapidly frozen in Freon 12 and kept in liquid nitrogen (1 to 4 hr) until it was transferred to a Balzers model 360 M apparatus to obtain replicas of etched freeze-fractured material. We followed the Balzers operating instructions in bulletin no. A11-3992e (May 1969) which are essentially the method described previously by Moor and Mühlethaler (16). Etching time was 1 min at −100 C; the replicas were picked up on collodion-coated grids and dried before examination in an electron microscope. The Balzers apparatus (without the electrode screen) was also used to shadow-cast preparations with platinum-carbon.

The specimens were examined in an AEI 6B electron microscope at accelerating voltages of 60 and 80 kv, and were recorded at instrumental magnifications of ×7,500 to ×60,000. Micrographs illustrating shadow-cast material were printed as negatives from contact intermediates.

RESULTS

Cell wall. Surface projections continuous with the cell wall or part of it are revealed on intact *Bdellovibrio* cells after negative staining with all the substances listed in Table 1 except UA (Fig. 1, 2, 5, 6, 23, 24, 37–40). The staining substances fall into two groups: (i) LiT, KPT at *p*H 6.8 to 8.1, ammonium molybdate (AmMo), sodium tungstate (NaT), and UA/ethylenediaminetetraacetate (EDTA); and (ii) borotungstic acid (KBT), silicotungstic acid (KST), and KPT at *p*H 4.0 to 6.5, and ammonium tungstate meta (AmT-m). Cells stained with compounds of the first group (Fig. 1, 5, 6, 23, 24, 37–40) exhibit intricate surface projections which are larger and longer than those of cells stained with compounds of the second group (Fig. 2). The features of the projections are characteristic of each staining compound and the *p*H of the staining solution. In preparations stained with LiT, NaT, and KPT at *p*H 6.8 to 8.0 (Fig. 5, 37, and 38), these projections are delineated from within by the electron-dense salts which have penetrated them and appear as vesicular and tubular structures.

Fragments of material usually surround cells negatively stained with all the compounds used except UA (Fig. 6). Their shape, size, texture, and density are related to the staining substance and are similar to those of the surface projections; these fragments appear as vesicular and tubular structures when penetrated by the same salts which also penetrate the surface projections.

Bdellovibrio cells usually do not reveal material extending from their surface in shadow-cast preparations (Fig. 22) except in old lysates. As the lysates age, the preparation of cells surrounded by envelope extensions in shadow-cast preparations and revealing dispersed envelopes after negative staining with KPT at *p*H 7.0 (similar to Fig. 29 and 30) increases. The number of noninfective *Bdellovibrio* cells also increases in old lysates, i.e., only 50% cells are infective in lysates after 36 hr of incubation, and less than 10% cells are infective in lysates after 3 days at room temperature. In no case are envelope extensions revealed in shadow-cast preparations as abundant and as elaborate as the surface projections of negatively stained intact cells.

Cells stained with UA have smooth surfaces with folds and appear collapsed (Fig. 4). Cells recovered from UA suspensions are more electron-dense than untreated cells both in unstained preparations and after staining with 0.001% UA; they exhibit smooth envelopes after negative staining with KPT at *p*H 7.0 and LiT (Fig. 3). These results show that UA reacts with wall material and stains it positively. The staining reaction with UA is irreversible and consequently the wall material no longer interacts with LiT which induces surface projections on native cells. On the other hand, cells recovered from LiT suspensions, after negative staining with LiT or KPT at *p*H 7.0, appear similar to native cells.

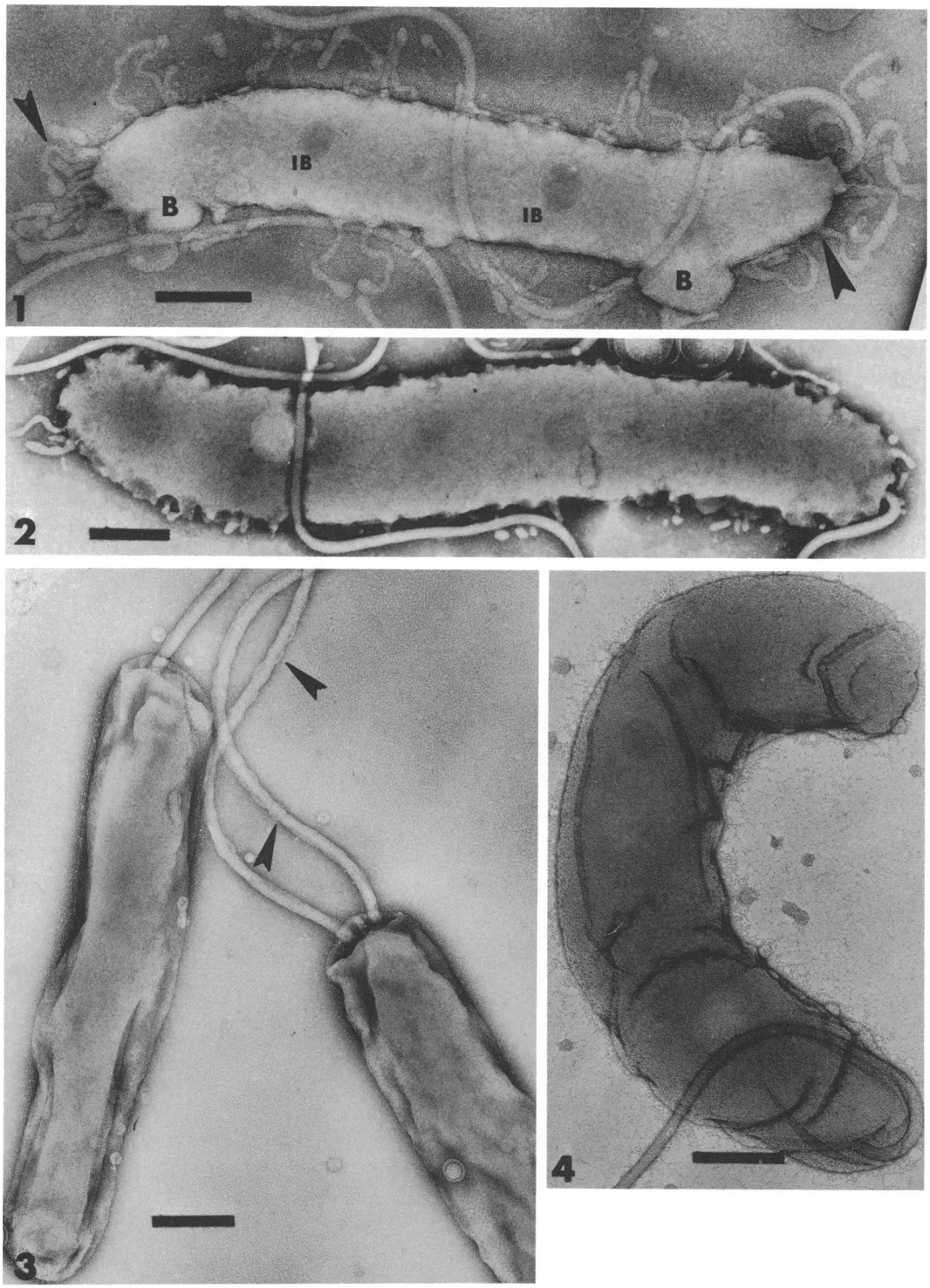


FIG. 1. *Long surface projections distributed around the entire cell are more abundant at both poles (arrows). Note the structures bulging from the cell border (B) which do not show internal details, and the electron-dense inclusion bodies (IB). Negatively stained with UA/EDTA. ×76,000. (The markers on these and subsequent figures, unless otherwise stated, indicate 0.2 μm. Whenever not specified, the micrographs are of Bdellovibrio bacteriovorus 109.)*

FIG. 2. *Multiple short surface projections on a cell stained with KBT. ×60,000.*

FIG. 3. *Cells recovered from a suspension in UA and then stained with LiT show smooth surfaces and slightly disrupted flagellar sheaths (arrows). Note the cell which bears two flagella. ×64,000.*

FIG. 4. *A vibrio-shaped cell stained with UA appears collapsed and shows smooth surface with folds. ×70,000.*

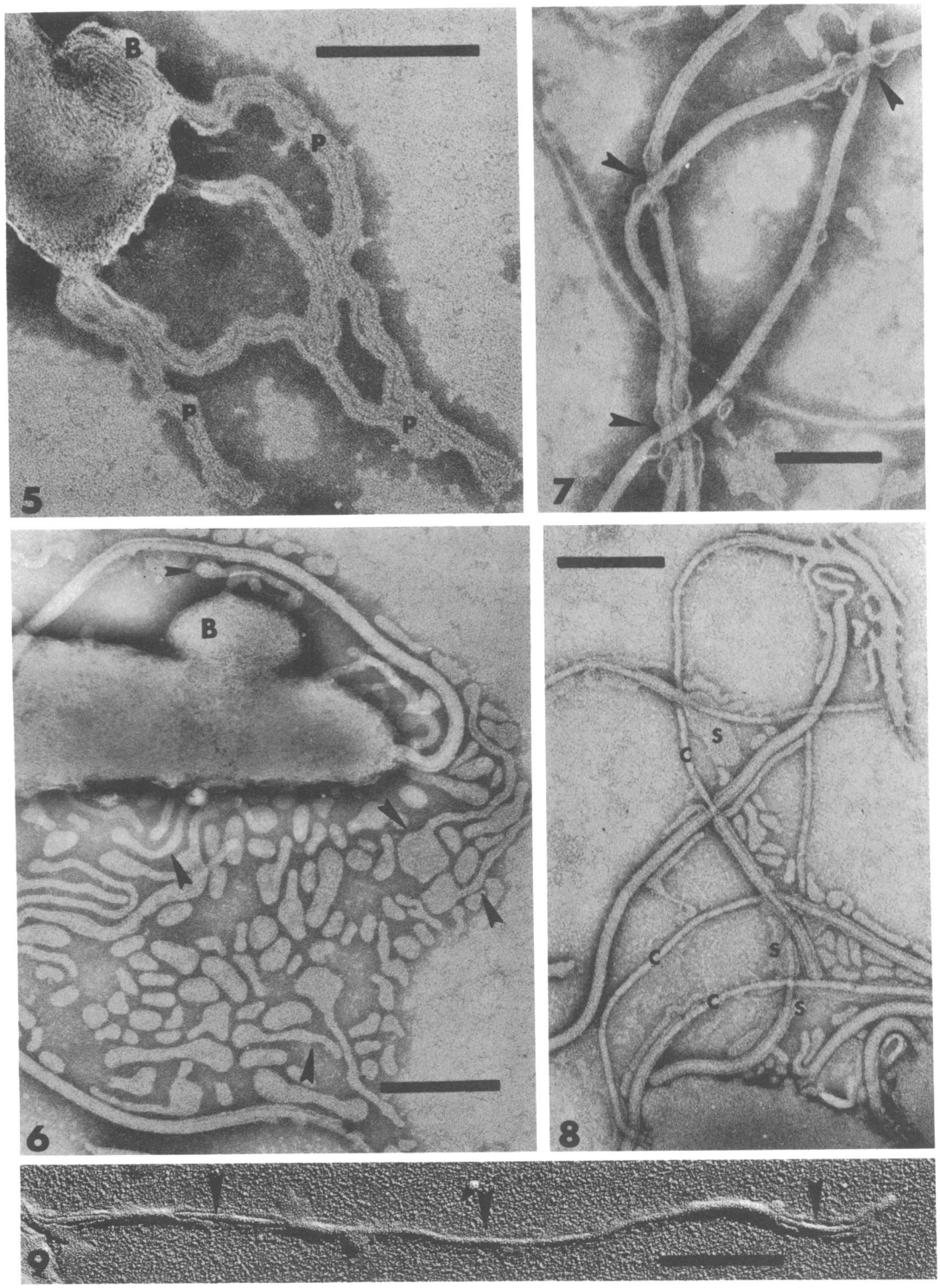


FIG. 5. *Tubular projections (P) at an anterior end of a cell are delineated by the penetrated electron-dense salt. Note the bulging body which shows laminated internal structure (B). Negatively stained with LiT.* ×*130,000.*

FIG. 6. *Fragmented material surrounding cells stained negatively with KPT at pH 7.0. Some fragments appear as tubules (arrows), and the body bulging from the cell border (B) does not reveal internal details.* ×*95,000.*

FIG. 7. *Flagellar sheaths are expanded and separated from the core filaments mostly at the crossing sites where the stain accumulates (arrows). Negatively stained with KPT at pH 8.1.* ×*83,000.*

FIG. 8. *Flagella exhibiting intact core filaments (C) and sheath material (S) disrupted to various degrees in a preparation of Bdellovibrio bacteriovorus D. Negatively stained with KPT at pH 4.0.* ×*65,000.*

FIG. 9. *Flagella revealing the core filament (arrows) and disrupted sheath material. Shadow cast.* ×*40,000.*

Their density is similar to that of untreated cells in unstained preparations, and they do not exhibit material extending from their envelope in shadow-cast preparations. Thus, LiT induces the surface projections only when its concentration is increased during the drying of the specimen.

Bdellovibrio cells are more susceptible than host cells to sonic oscillation or to a freeze-thaw cycle. For instance, 5 to 15% of *Bdellovibrio* cells, compared with 70 to 80% of *E. coli* cells, survive a freeze-thaw cycle. Also, the proportion of cells with damaged wall in these treated suspensions is higher for *Bdellovibrio* than for the host organisms (Fig. 25 to 30). The features of the damaged wall revealed in negatively stained preparations are related to the staining compound: the wall appears expanded and flattened after staining with KPT at *p*H 4.0 to 6.0 (Fig. 27), and flattened and fragmented after staining with KBT and AmT-m (Fig. 28). In these cases the damaged wall which is relatively transparent surrounds an elongated, nearly intact protoplast. The wall appears dispersed to a great extent and surrounds either nearly intact or disorganized protoplasts after staining with KPT at *p*H 6.8 to 8.1 (Fig. 29 and 30), LiT, AmMo, NaT, and UA/EDTA. On the other hand, after staining with UA the damaged wall appears smooth and continuous (Fig. 25 and 26), but the cells can easily be distinguished from native cells which in similar preparations appear characteristically collapsed (Fig. 4). Uranyl acetate never penetrates the wall to reveal the protoplast. When the above appearances of damaged walls in negatively stained preparations are considered, the staining substances, except for UA, fall into the same two groups previously mentioned in discussing the surface projections induced on native cells. The compounds which induce the larger projections are the same ones which greatly disperse damaged cell walls. These results show that the staining substances interact with and affect the integrity of wall material.

Small rounded forms of *Bdellovibrio*, 0.4 to 0.6 μm in diameter, are always present in old lysates and their number increases as the lysates age (Fig. 10 to 13). They are more numerous in old lysates in the enriched media (TY and NB) than in the corresponding diluted ones, i.e., 10 to 20% and 30 to 50% of the cells of 4-day-old lysates in TY/5 and TY broth, respectively, are rounded forms. The cell wall of all the rounded forms, after negative staining, reveals features similar to those of intact vibrio-shaped *Bdellovibrio*, indicating that the components which are responsible for these features are present in both the curved and the rounded forms.

Bdellovibrio cells bear one and occasionally two or three sheathed polar flagella (Fig. 3) with uniform outer diameter of 21 to 25 nm which encloses a core filament, 11 to 15 nm in diameter (5, 6, 27; Abram and Shilo, Bacteriol. Proc. p. 41, 1967). The flagellar sheath, which is continuous with the cell wall or part of it, often is affected by the staining substances (except UA) at sites where flagella cross one another and at the flagellar distal ends. At the crossing sites the electron-dense salt accumulates, penetrates the sheath, causes its expansion, and delineates the flagellar core filament (Fig. 7). The distal ends of the flagella show strands of material extending from the sheath (Fig. 15). Similar strands have never been identified in shadow-cast preparations and after staining with UA. In the latter case the distal ends of the flagella are usually rounded, slightly wider than the rest of the sheathed filament, and occasionally appear twisted and hollow (Fig. 14). Such ends probably represent only sheath material. In preparations stained with KPT at *p*H 4.0, a high proportion of flagella reveals disrupted sheath material along the entire filament (Fig. 8), but this is probably due to the *p*H of this stain. Flagella with defective sheaths (Fig. 9) are often present in shadow-cast preparation. Therefore, the disrupted sheath material observed in negatively stained preparations cannot be solely a result of interaction with the staining substances. In fact, sheathless flagella or flagella with defective sheaths are present on most rounded forms (Fig. 10 and 11) and on cells with damaged walls (in old lysates, in suspensions after a freeze-thaw cycle and treatment with sonic oscillation) as well as on many cells with intact walls. After a freeze-thaw cycle, the flagellar sheath has a characteristic appearance in preparations stained with UA: it is expanded and portions of it appear to have been pulled along the core filament towards the cell (Fig. 25 and 26). These observations indicate that the flagellar sheath material is more susceptible to disruption than the cell wall.

The number of infective *Bdellovibrio* in lysates is proportional to the nutrients in the medium. The composition of the medium affects mostly the growth of the host cells, the final density of which determines the ultimate yield of *Bdellovibrio* in the lysate. However, *Bdellovibrio* populations of lysates from the enriched media (TY and NB) are not only denser than those of lysates from the diluted ones, but are also characteristically morphologically heterogeneous (Fig. 23, 24). They contain 5 to 20% long forms (6 to 20 μm), which are straight or spiral with varying degrees of coiling. Some of these reveal constrictions and appear ready to separate into

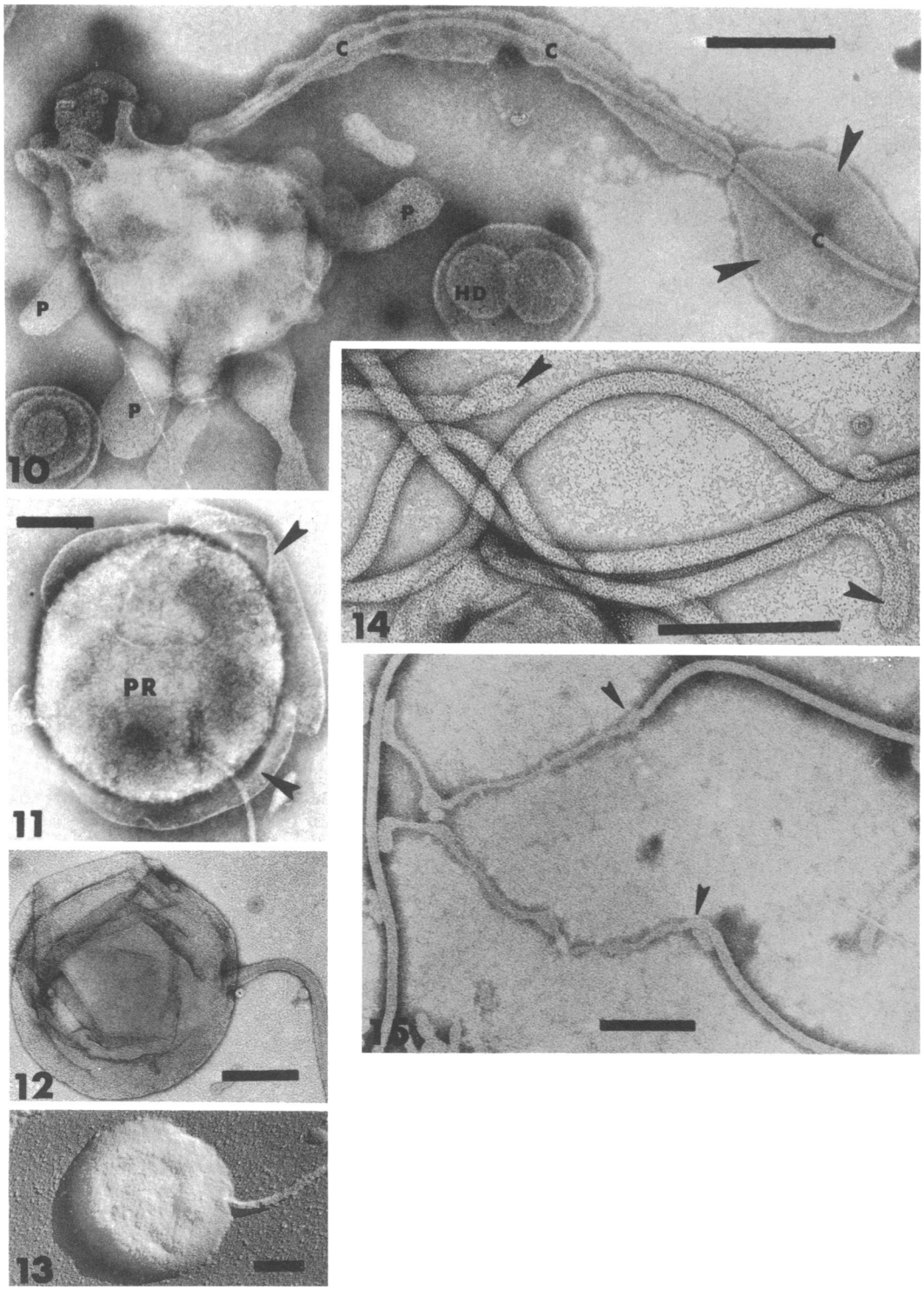


FIG. 10. *Rounded cell stained with LiT shows surface projections (P). The disrupted flagellar sheath is revealed as a flattened membrane. Note the flagellar core filament (arrows) and the host cell wall debris (HD).* ×*103,000.*

FIG. 11. *Spherical cell from a suspension after a freeze-thaw cycle stained with KPT at pH 5.0. The rounded protoplast is separated from the damaged cell envelope which appears as delicate transparent membranous material. A sheathless flagellum emerges from the protoplast.* ×*60,000.*

FIG. 12. *Rounded cell stained with UA appears collpased and shows smooth surface with folds.* ×*60,000.*

FIG. 13. *Spherical cell in shadow-cast preparation.* ×*40,000.*

FIG. 14. *Distal ends (arrows) of intact sheathed flagella stained with UA appear bent and twisted. They are slightly wider than the filaments and extend beyond the part of the flagellum which encloses the core filament.* ×*143,000.*

individual cells. Generally the spirals are wider (0.3 to 0.5 μm) than the curved cells of average size (1 to 2 μm long and 0.2 to 0.3 μm wide). Many of the long forms bear a flagellum and show translational and rotational motion, but they move slower than the jerking vibrio-shaped cells. After negative staining the walls of all the morphologically different forms found in lysates in enriched media reveal features similar to those of the vibrio-shaped cells of lysates in the diluted media.

Freeze-etching. Replicas of etched freeze-fractured surfaces of intact *Bdellovibrio* and *E. coli* are shown for comparison in Fig. 16 to 21. The outermost surfaces of both organisms appear either flat or moderately wavy (Fig. 16, 17, 19–21). However, *Bdellovibrio* more frequently reveal undulated outer surfaces which often appear irregularly layered (Fig. 16). In *E. coli*, when portions of the wall break off, the fractured boundaries usually are clean and in profile reveal a double-layered structure (Fig. 20, 21). The fractured wall of *Bdellovibrio* usually shows two smooth surfaces which appear to belong to two layers of a cleaved wall (Fig. 17). The boundaries of the inner wall layer (adjacent to the cytoplasmic membrane) are serrated and at a distance from the relatively straight fractured edges of the outermost layer. Clean fractured wall boundaries, similar to the ones of *E. coli*, are rare in *Bdellovibrio*. In *E. coli* two surfaces of a cleaved wall occasionally are observed (Fig. 21): the outermost smooth wall layer reveals relatively straight fractured edges, whereas the inner layer always has irregular boundaries. The latter, unlike the smooth inner wall layer in *Bdellovibrio*, shows particles (3 to 5 nm) and fine fibers scattered on its outer surface, and fibrous connections with the cytoplasmic membrane. The outer (convex) surface of the cytoplasmic membrane of *Bdellovibrio* is covered with scattered particles, 6 to 10 nm in diameter (Fig. 17 to 19), and also often shows patches of flattened material with smooth outer surfaces which appear to be fragments of the inner layer of the cleaved wall (Fig. 18). The outer surface of the cytoplasmic membrane of *E. coli* usually is completely covered by particles, 6 to 8 nm in diameter (Fig. 20 and 21), but on rare instances shows smooth areas devoid of particles (*not shown*) similar to those previously reported (3, 9). However, unlike the scattered particles of *Bdellovibrio*, those of *E. coli* are always densely packed. In *Bdellovibrio* several "islands" of the wall break off occasionally and reveal fractures irregularly guided along the inner wall layer and the outer surface of the cytoplasmic membrane (Fig. 19). Fractures similar to the latter have never been observed in *E. coli*.

Structures associated with the anterior end of the cell. Fibers which vary in length (up to 1.5 μm) emerge from the anterior end of a small proportion of cells (Fig. 33 to 36). Their diameter (8 to 10 nm) varies from one preparation to another and even on the same fiber (Fig. 34), suggesting partial disorganization. They appear either straight (Fig. 36) or irregularly curved (Fig. 35) and often show angular bends (Fig. 33 and 34). In stained preparations their proximal ends are often located very close to the cells but are not connected to them (Fig. 33, 34), giving the appearance that they had been pulled away from the cell during the drying of the specimens or had been affected by the stain which accumulates at the cell's border (Fig. 33 and 34). The higher proportion of cells bearing these fibers in shadow-cast preparations than in negatively stained ones suggests a disruptive action of the staining substances. Generally cells bear two to three fibers, but occasionally as many as six have been observed. These structures have never been seen on the rounded forms of *Bdellovibrio* and are seldom present on cells of old lysates and cells with damaged envelopes.

Structures (6 to 12) which appear as electron-dense circles are revealed at the anterior ends of intact *Bdellovibrio* in preparations stained with UA, AmT-m, and KPT at *p*H 5 to 6 (*not shown*). They are easily revealed as electron-transparent rings (outer diameter 9 to 12 nm) on cells with damaged envelopes (Fig. 28 to 32) in preparations stained negatively with all the substances, except UA/EDTA, but frequently are partially obscured after staining with UA (Fig. 25). Thus, they can serve to identify the anterior ends of deflagellated cells. Ring-like structures scattered on expanded envelope material at the cell anterior end (Fig. 28 and 29) appear to be sites of structural differentiation built into the cell wall. However, they are often observed in clusters (Fig. 30 and 31), usually at the anterior ends of cells with greatly dispersed cell wall, suggesting association with the protoplast also. These structures have never been observed on the rounded forms.

Intracellular structures. Compact bodies, usually oval (150 to 300 nm long and 70 to 120 nm wide), bulging from the cell border but located within the cell wall of intact *Bdellovibrio* are always observed after staining with LiT, AmMo, UA/EDTA, or KPT at *p*H above 6.5, and NaT (Fig. 1, 5, 6, 23, 24, 37–41). Generally two (occasionally one or more than two) such structures

FIG. 15. *Strands of sheath material extending from the proximal ends of intact sheathed flagella (arrows). Negatively stained with KPT at pH 7.0. ×75,000.*

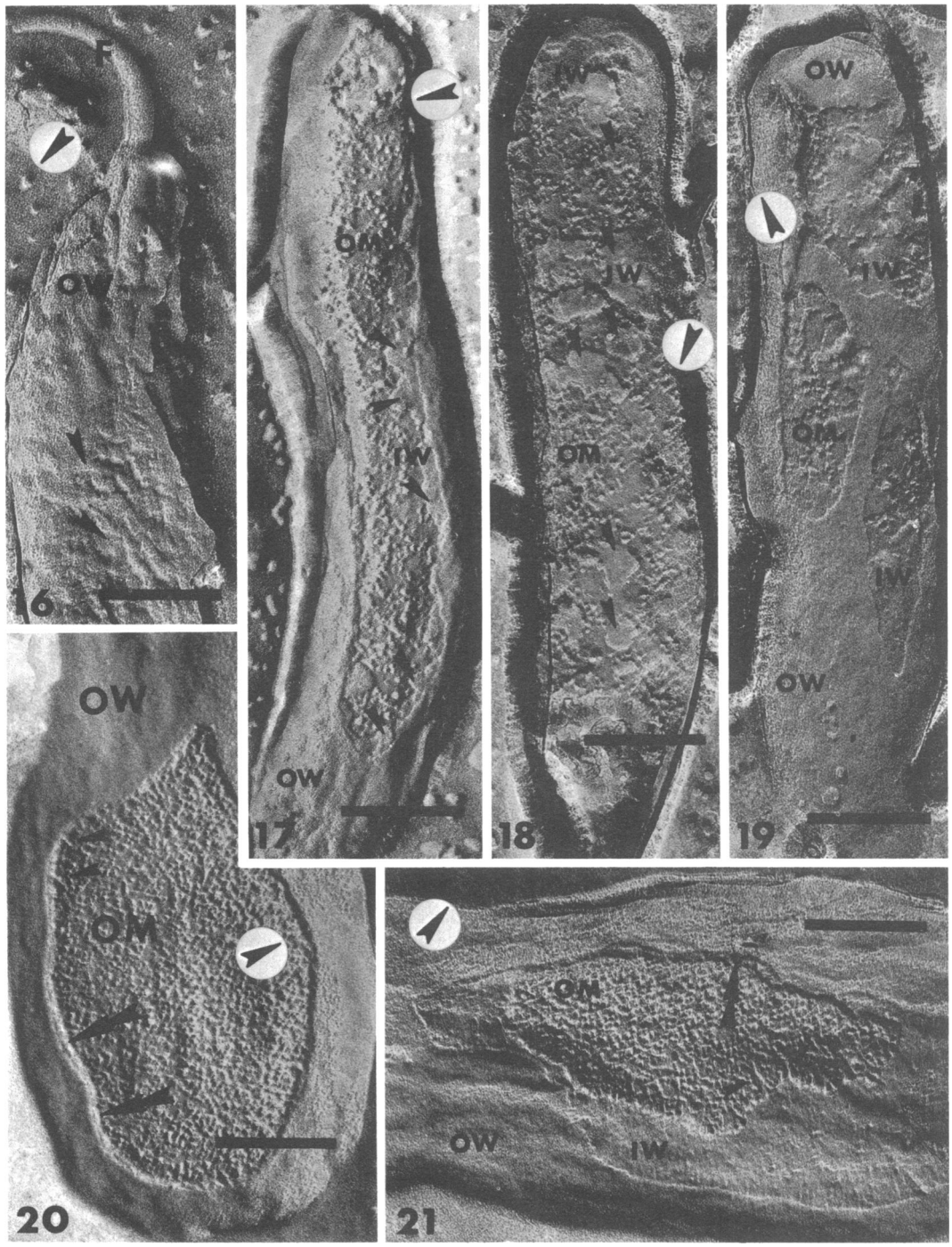


FIG. 16–21. *Shadowed replicas of etched freeze-fractured cells of Bdellovibrio bacteriovorus strain 109 (Fig. 16 to 18), strain D (Fig. 19), and E. coli B (Fig. 20 and 21). The circled arrows indicate the direction of the cast metal.* ×*90,000.*

FIG. 16. *Fracture guided along the outer surface of the cell wall (OW) and the flagellum (F). The surface of the flagellum is smooth and that of the cell is undulated; the fractured surface towards the center of the cell (arrows) appears irregularly layered.*

FIG. 17. *Fracture guided along the outermost smooth and slightly undulated cell surface (OW), the outer smooth surface of an inner cell wall layer (IW) and the cytoplasmic membrane outer (convex) surface (OM). The fractured boundaries of the inner layer of the cleaved wall are serrated (arrows), whereas those of the outermost layer are relatively straight. The particles on the cytoplasmic membrane outer surface are irregularly scattered.*

FIG. 18. *Fracture revealing the cytoplasmic membrane outer surface (OM) covered with irregularly scattered particles and patches of smooth material (IW and arrows), which appear to be fragments of an inner wall layer adhering to the cytoplasmic membrane.*

are present in a vibrio-shaped cell of average length (1 to 2 μm). They usually are located close to both ends of the cell (Fig. 1 and 6), but have been observed in the middle or at the very end of cells. Long straight and spiral forms of *Bdellovibrio* show several such structures (Fig. 23 and 24). These bodies have not been observed in intact cells after negative staining with KPT at *p*H 4.3 to 6.5, KBT, KST, AmT-m, and UA, or in shadow-cast preparations, in cells with damaged wall and in the rounded forms. They also could not be identified in replicas of etched freeze-fractured cells either on the surface of the cytoplasmic membrane or within the cytoplasm. Internal details of these bodies are revealed as regular laminated structures which appear as "fingerprint" patterns in preparations stained with LiT (Fig. 5, 37, and 38) and KPT at *p*H 7.5, whereas, in preparations stained with KPT at *p*H 8.1 (Fig. 39 to 41), they are revealed as both laminated and vesiculated structures. The internal details are not visible after staining with UA/EDTA, AmMo, NaT, and KPT at *p*H 6.5 to 7.0 (Fig. 1 and 6), either owing to poor contrast or to inability of these salts to penetrate. The bulging bodies occasionally are adjacent to a confined electron-dense space appearing empty and located in the protoplast (Fig. 38). Fingerprint patterns which bulge only slightly from the cell border are occasionally observed within a confined space similar to the one seen in Fig. 38.

Electron-dense inclusion bodies are revealed in the cells after negative staining with various compounds (Fig. 1), and in unstained (*not shown*) and shadow-cast preparations (Fig. 22).

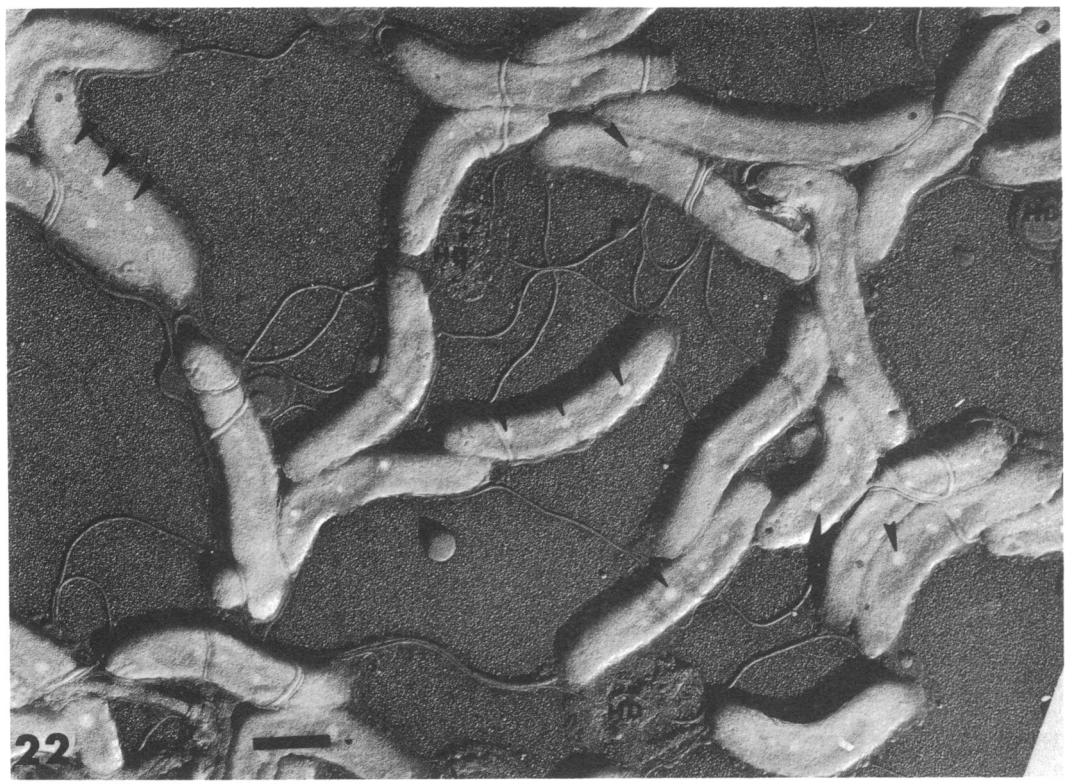
Although the illustrations in this paper are mostly of *Bdellovibrio* 109, all the five strains employed in this study, irrespective of the host organism and the growth medium, showed similar features of the cell wall and the flagellar sheath, polar ring-like structures and fibers, intracellular laminated structures, and inclusion bodies.

DISCUSSION

The most likely surface topography of *Bdellovibrio* cells is that revealed in freeze-fractured and shadow-cast material and after staining with UA. In the latter case the wall material is stained positively and appears to have been fixed. However, the most striking structural feature of *Bdellovibrio*, not known in other organisms, is the intricate surface projections induced by certain substances used for negative staining. These structures easily detach from the cells, fragment, and fuse to form vesicles and tubules which often surround the cells. The surface projections which are continuous with the cell wall appear to result from interaction between wall components and the staining substances and represent altered wall material. They probably reflect a chemical composition distinct to *Bdellovibrio* wall. Since projections are apparent only on cells with intact wall, their formation may also be related to the intracellular osmotic pressure affected by the staining substance. Other gram-negative organisms, after negative staining with the same substances which induce surface projections in *Bdellovibrio*, reveal their protoplasts retracted from the cell walls, indicating that plasmolysis has occurred (Davis and Abram, Bacteriol. Proc., p. 52, 1970), whereas plasmolyzed *Bdellovibrio* have never been observed. This may indicate that the association between the wall and the cytoplasmic membrane in *Bdellovibrio* is tighter than that in the organisms which reveal plasmolysis, and is mostly preserved after negative staining. The appearances of the *Bdellovibrio* surface in etched freeze-fractured cells also indicate the distinct organization of its cell wall. The fracturing tendencies and fragmentation of the wall material can result from loose bonds between cell wall components or from a firm association between an inner wall layer and the cytoplasmic membrane, or both. The second possibility is supported by the absence of apparent plasmolysis in negatively stained cells. A similar fragmentation of the inner layer of cleaved wall of other organisms is not known. In *E. coli*, occasionally cells frozen from sucrose suspensions show patches of material on the outer surface of the cytoplasmic membrane. These, however, represent fragments of the entire wall material (3). The outer surface of the cytoplasmic membrane of freeze-fractured *Bdellovibrio* cells is covered with scattered particles, whereas in *E. coli* and other organisms similar particles are densely packed (3, 9, 19, 21, 22). The exact function of these particles is not known, but they are believed to be indicative of active membranes (10, 15, 17, 39) since inactive ones such as myelin layers are

FIG. 19. *Smooth outer surface of a cell (OW) shows several fractured "islands" guided irregularly along the inner wall layer (IW) and the cytoplasmic membrane outer surface (OM).*

FIG. 20 and 21. *Portions of E. coli cells showing a fracture guided along the outermost cell surface (OW), the outer surface of an inner wall layer (IW), and the cytoplasmic membrane outer surface (OM) which is covered with densely distributed particles. The clean fractured boundaries reveal the cell wall as a double layered structure (double arrows). The fractured inner wall layer shows fibrous connections (arrows) with the cytoplasmic membrane and its outer surface is covered with particles and fine fibers.*



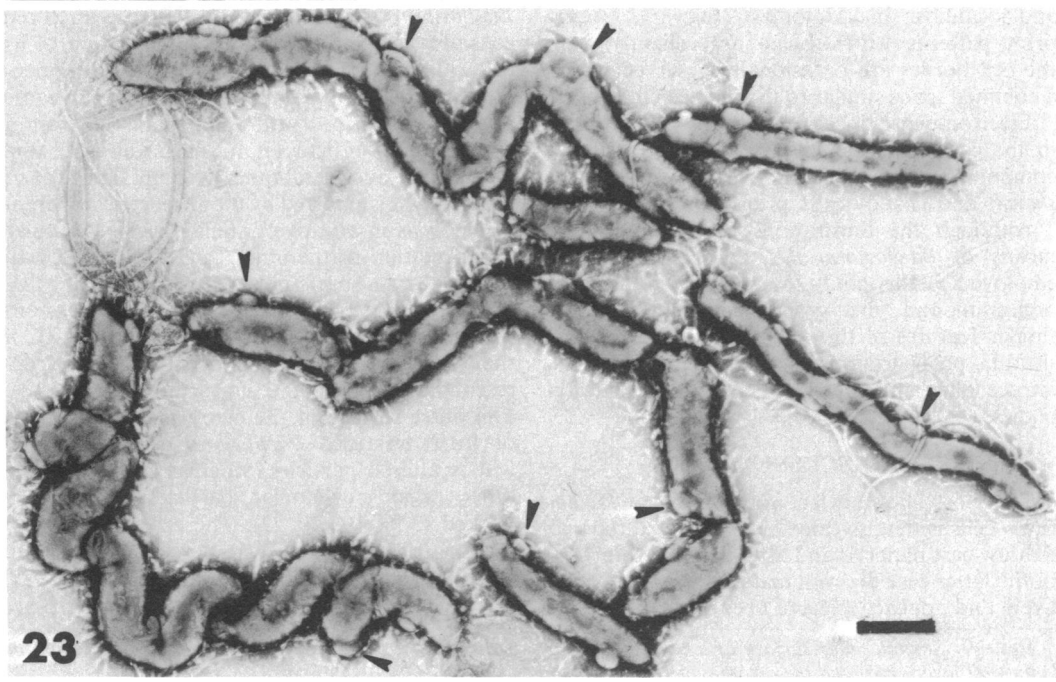


FIG. 22. *Vibrio-shaped cells of lysate in TY/5 broth 12 hr after almost all host cells had lysed show relatively smooth surfaces. Note the relaxed flagella, the electron-dense inclusion bodies (arrows), and the host cell debris (HD). Shadow cast. ×21,000. Marker indicates 0.5 μm.*

FIG. 23. *Morphologically heterogeneous cells of lysates in enriched medium (TY). All cell forms show surface projections and intracellular bodies which bulge from their borders (arrows). Negatively stained with LiT. ×21,000. Marker indicates 0.5 μm.*

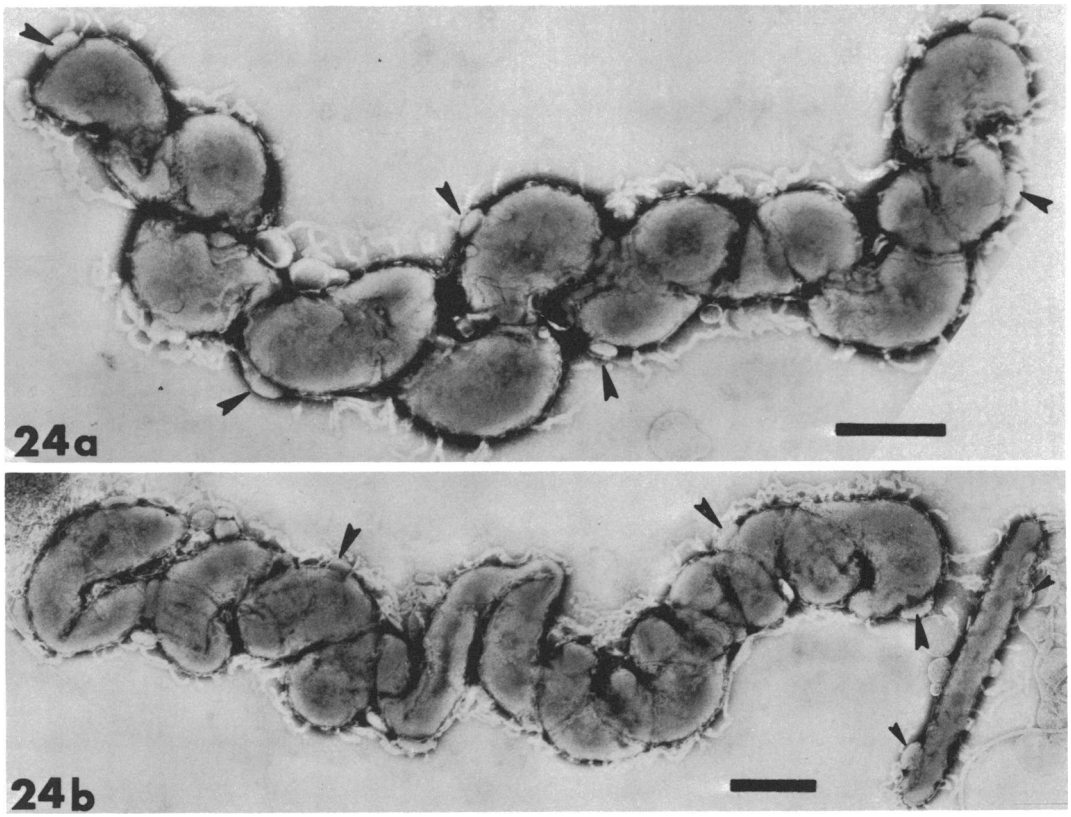


FIG. 24. *Same as Fig. 23. Long spiral forms with septa (Fig. 24a) are wider than the cells of average size. Fig. 24a, × 30,000; Fig. 24b, ×23,000. Marker indicates 0.5 μm.*

devoid of them (4). In *Bdellovibrio* the scattered arrangement of the particles on the cytoplasmic membrane may reflect a distinct function. However, if the wall is tightly associated with the membrane some of the particles could have been pulled away during the fracturing process.

Bdellovibrio cells are highly susceptible to physical treatment (sonic oscillation and freeze-thaw cycle) which results in only a small proportion of surviving cells. After negative staining most of the treated cells show damaged envelopes which often surround nearly intact elongated protoplasts. Thus, the cell wall, or part of it, clearly is the cell component most affected by the treatment.

Structural properties of *Bdellovibrio* cell wall have not been apparent in thin sections after chemical fixation. In these preparations convoluted and serrated cell wall has the nonspecific appearance of a unit membrane (5, 6, 13, 24, 32), and an inner wall layer, corresponding to the mucopeptide layer of the cell wall in other gram-negative organisms (7, 18), has not been observed. The exaggerated convolutions and serrations at the anterior ends of the cells (both in thin sections and negatively stained preparations) were interpreted by Burnham et al. (6) to be caused by the methods employed for preparing specimens for electron microscopy at the cell region which is more susceptible to distortion. Our observations indicate that the entire surface of *Bdellovibrio* is susceptible to disorganization. In lysates, a few hours after most host cells had lysed, some *Bdellovibrio* cells reveal defective envelopes and the proportion of such cells increases as the lysates age. Material extending from the wall seen in shadow-cast preparations appears to represent damaged cell wall rather than a drying artifact, as suggested by Burger et al. (5). The properties of newly synthesized wall cannot account for the frequent disorganized appearances of the cell poles in cells of old lysates. It is more likely that at the poles wider surface areas are exposed to the adverse conditions. In stained preparations, when one or both cell poles are markedly affected by a staining substance and bear elaborate surface projections, the other parts of the cell are also distorted but to a lesser degree, and the true structural features of the cells' surfaces probably are not revealed. Therefore, it is possible that the chemical fixation procedure, which is known to be

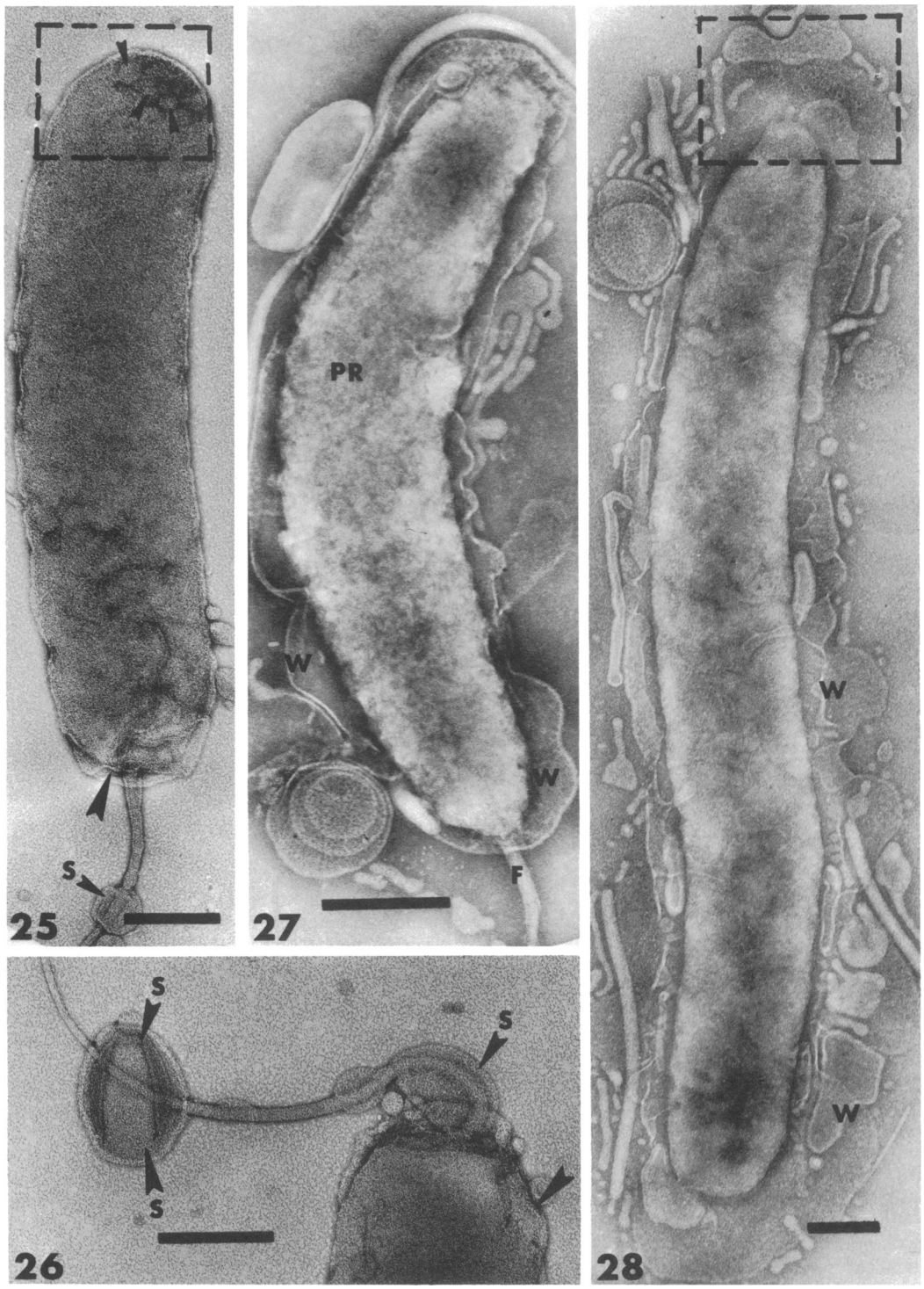


FIG. 25 to 32. *Samples withdrawn during the thawing of frozen suspensions.*

FIG. 25 and 26. *Cells stained with UA have intact wall and exhibit only slight folds (arrows). The disrupted flagellar sheath material appears expanded and pulled towards the cells (S). The anterior end of the cell in Fig. 25 shows partially obscured ring-like structures and sites which appear differentiated (boxed). Fig. 25, ×75,000; Fig. 26, × 91,000.*

FIG. 27. *Transparent, slightly damaged and expanded wall (W) surrounds an intact elongated protoplast (PR). Negatively stained with KPT at pH 4.0. ×100,000.*

FIG. 28. *The elongated intact protoplast is surrounded by disrupted wall which appears as flattened and fragmented membranous material (W). The anterior end of this deflagellated cell can be recognized by the ring-like structures scattered in the expanded envelope material (boxed). Negatively stained with AmT-m. × 54,000.*

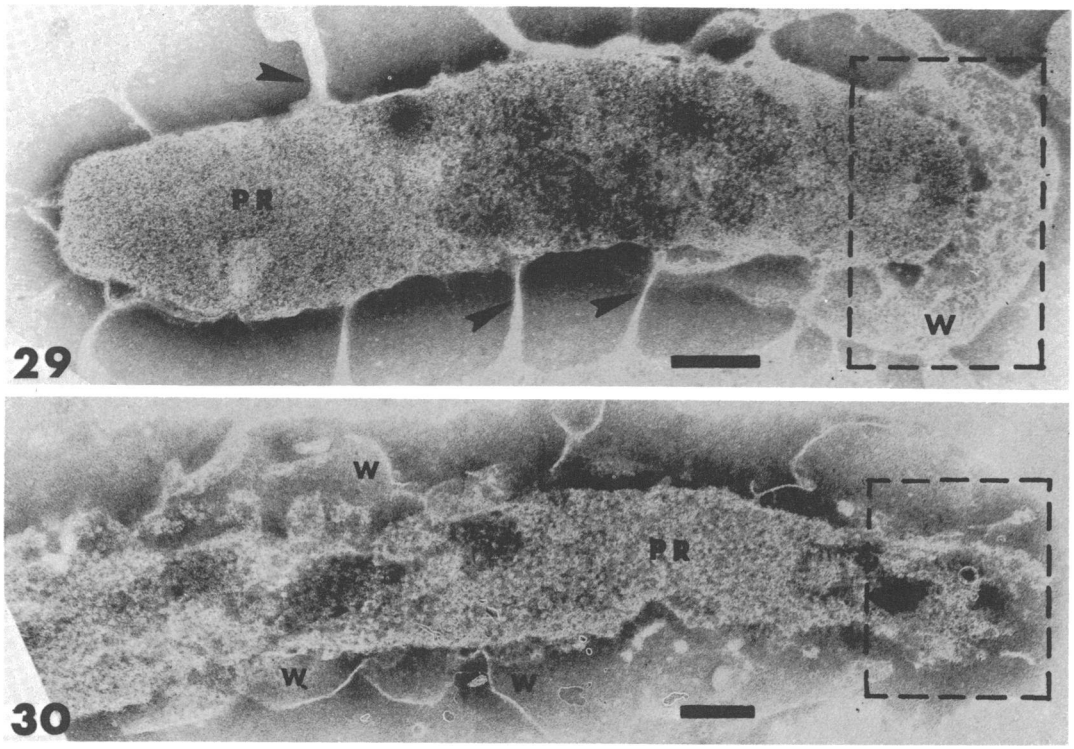


FIG. 29. *At the anterior end the expanded cell envelope (W) appears as a flattened cap (boxed); at the outer parts of the cell, the envelope is disorganized to a greater extent and appears as electron-transparent strands (arrows). The elongated protoplast (PR) is nearly intact. Negative staining with KPT at pH 7.0. ×60,000.*

FIG. 30. *The disorganized cell wall appears as delicate flattened membranous material (W). The elongated protoplast (PR) is disrupted at the anterior end of the cell (boxed) where the wall material can hardly be distinguished. Negatively stained with KPT at pH 7.0. ×50,000.*

satisfactory for the host organism (*E. coli*) but which produces distorted images of the anterior ends of *Bdellovibrio* (6), is not suitable for revealing the true structural features of the latter or the real structural nature of host-parasite interaction.

The decrease in infective *Bdellovibrio* cells (in suspensions after a freeze-thaw cycle or sonic oscillation, and in old lysates) always coincides with the appearance of cells showing disrupted flagellar sheaths, which appear more frequently disorganized than the cell wall. It has been shown that the flagellar sheath is continuous with the cell wall (5, 6, 27; Abram and Shilo, Bacteriol. Proc., p. 41, 1967), but it is not known whether both are built of identical material. The higher susceptibility of the sheath to disruption may result from its specific composition, or from its large exposed surface area. The structural integrity of the sheath may be essential for the function of the flagellum. Since motility appears to be a prerequisite for the attachment of the parasites to host cells (32, 35, 36), nonfunctioning flagella with defective sheaths may account, at least in part, for the loss of infectivity of *Bdellovibrio* in the above suspensions. It has been shown that flagellated protoplasts of *B. megaterium* are not motile (38), suggesting that the intact cell wall may be essential for motility. In *Bdellovibrio*, when the more exposed flagellar sheath appears damaged, the cell envelope may be damaged to a lesser degree, and thus, both probably have an effect on the function of the flagellum. It is our opinion that the survival of *Bdellovibrio* under laboratory conditions is greatly related to the structural stability of the wall and the flagellar sheath.

The growth conditions of two-membered cultures in the enriched media (TY and NB) appear to support both intracellular and extracellular development of *Bdellovibrio*. Lysates of these cultures consist of populations which characteristically are morphologically heterogeneous and contain long cells, many of which appear too large to have emerged as such from the host cells. These forms probably develop, partially or completely, extracellularly. In the enriched media complete lysis of host cells occurs 16 to 28 hr after they have reached the stationary growth phase. In these media a relatively high proportion of the

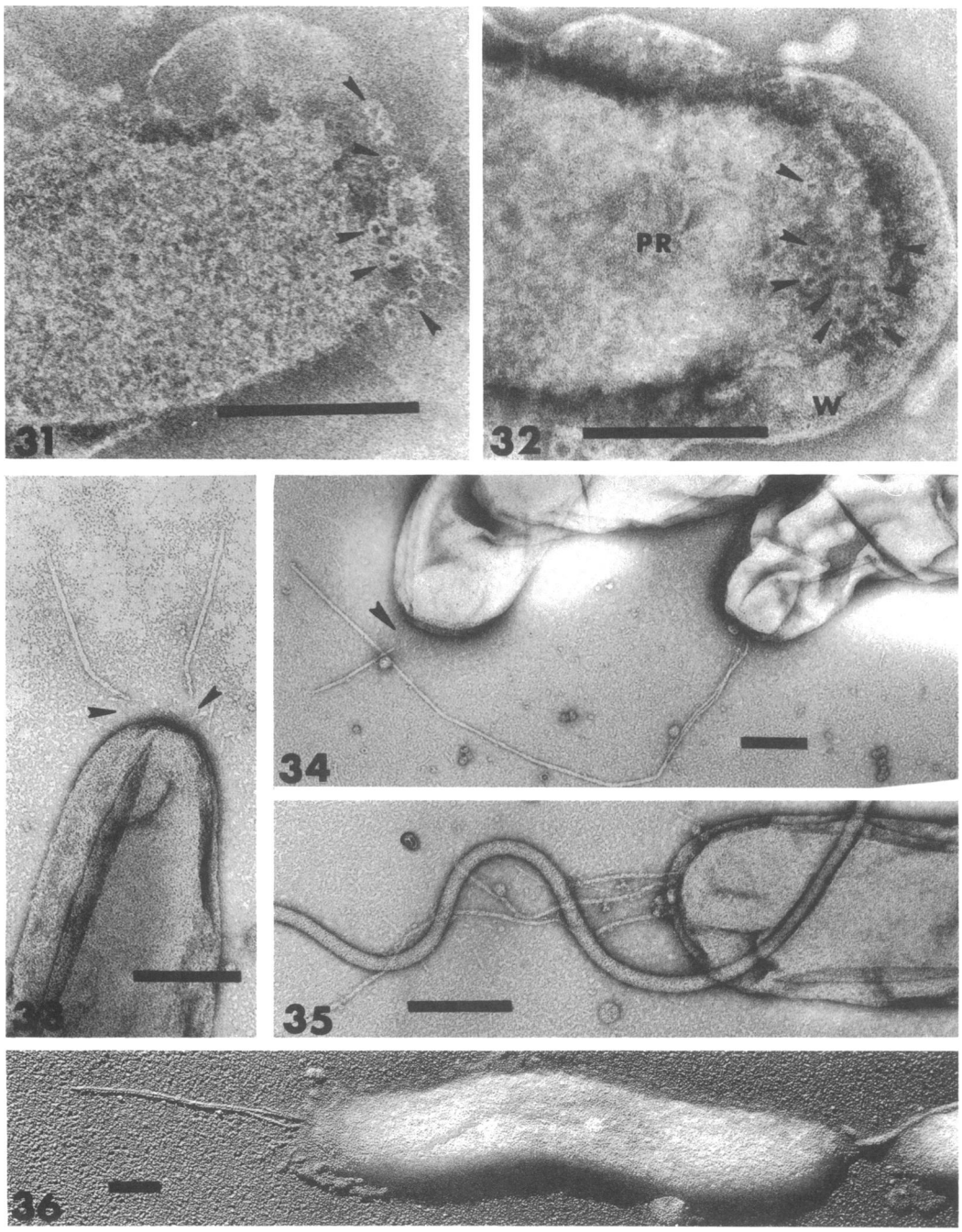


FIG. 31. *Clusters of ring-like structures (arrows) are associated with the nearly intact anterior end of the protoplast. Negatively stained with KPT at pH 7.0. ×147,000.*

FIG. 32. *Ring-like structures (arrows) seen in the cell with a nearly intact transparent cell wall which surrounds the protoplast (PR). Negatively stained with KPT at pH 4.0. ×135,000.*

FIG. 33 to 36. *Anterior fibers in preparations negatively stained with UA and in a shadow-cast preparation (Fig. 36) exhibit variations in size and shape, and show angular bends (Fig. 33 and 34). Note the gaps between the proximal ends of the fibers and the cells (arrows, Fig. 33 and 34). Fig. 33, ×80,000; Fig. 34, ×52,000; Fig. 35, ×76,000; Fig. 36, ×38,000.*

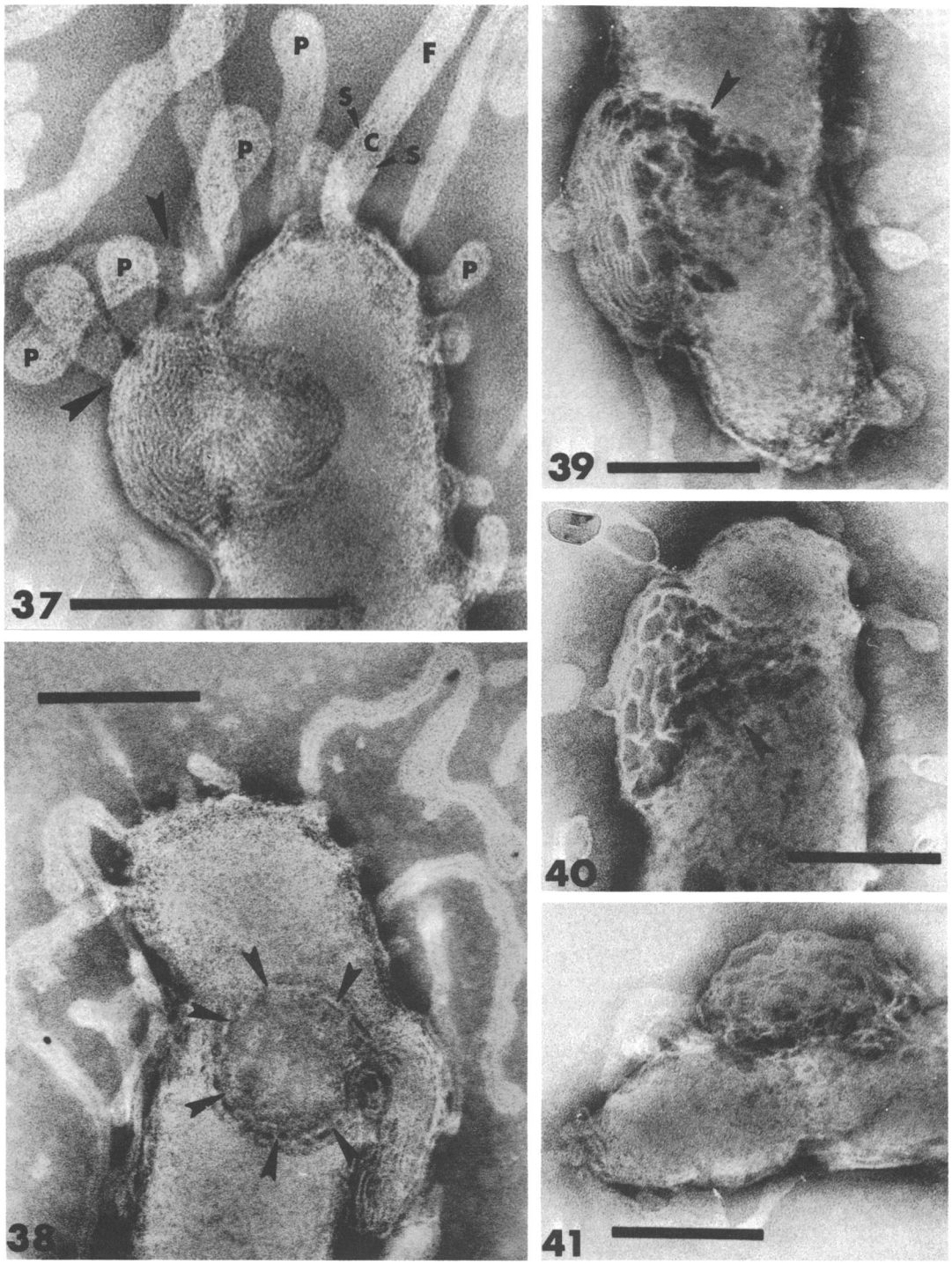


FIG. 37 to 41. *Portions of intact cells stained negatively with LiT (Fig. 37 and 38) and KPT at pH 8.1 (Fig. 39 to 41), showing oval intracellular structures bulging from the borders of the cells.*

FIG. 37. *Bulging body close to the flagellated end of the cell shows a laminated fingerprint pattern. The surface projections (P) extend also from the border of the bulging body (arrows). Note the flagellum (F) which reveals the core filament (C) separated from the sheath (S). ×210,000.*

FIG. 38. *Bulging body located close to the cell anterior pole has internal laminated structure and is adjacent to an electron dense confined space (arrows) from which it appears to have been extruded. The tubular surface projections are delineated by the stain. ×125,000.*

FIG. 39 to 41. *Bulging bodies in cells stained with KPT at pH 8.1 vary in their internal organization; they exhibit both laminated and vesiculated structures (Fig. 39), loose lamellae irregularly spread apart (Fig. 41), and irregular vesicles (Fig. 40). Often they are adjacent to areas within the protoplast into which the stain had penetrated (arrows). Fig. 39 to 41, ×118,000.*

host cells is lysed from without, probably releasing growth substances which support the extracellular development of some *Bdellovibrio*. This development, however, follows a course similar to that occurring intracellularly, and to those of the facultatively parasitic *B. bacteriovorus* UKi2 grown in the absence of host cells (8) and of parasitic strains grown on microbial extracts (20). Namely, it occurs via long forms which fragment to new progeny. It appears that the facultative tendencies of *Bdellovibrio* which have been suggested from several other observations (5, 20, 37) are expressed also in two-membered cultures. However, the same distinct structural features of the wall are observed both in the vibrio-shaped cells which have been released from host cells and in the extracellularly growing long forms. The same features are also common to the wall of the degenerated nonviable rounded forms of *Bdellovibrio* which appear to have the essential outer plastic parts of the cell wall.

Our observations do not support the suggestion that parasitic *Bdellovibrio* possess, at their anterior ends, a suction disc (35), an infection cushion (24), or a distinct structural "hold fast" (29). Rounded anterior ends such as observed in *Bdellovibrio* A3.12 (29) are not a common feature of other parasitic strains, and, therefore, it is doubtful that they represent a real functioning structural entity. However, *Bdellovibrio* anterior pole is differentiated; it shows 6 to 12 distinct ring-like structures which are built into the cell wall and also appear to be associated with the protoplast, and 2 to 3 fibers emerging from it are observed on a small proportion of the cells. It is likely that the ring-like structures represent differentiated sites of association between the fibers and the cell wall. The functions of these specialized structures are not known, but since they are present at the attachment pole of the cell they may be related to *Bdellovibrio* parasitic activity. Although the fibers appear to be fragile and detachable, it is possible that several such structures can support a firm connection between parasite and host cells. If the fibers are formed after attachment to the host cell and if they then secure a firm connection between host and parasite, there would be only a small chance to observe these structures on free *Bdellovibrio*. Another possibility is that enzymatic activity is associated with the fibers which, upon attachment, are injected through the cell wall and affect the host periplasmic matrix or cytoplasmic membrane.

Intracellular structures which appear as compact bodies characteristically bulging from the borders of intact *Bdellovibrio* are seen after negative staining with any one of the stains which also induce the larger surface projections. These bodies can be observed only in intact cells with wall modified by the stains; they appear to have been extruded from the protoplast due to an osmotic effect on the cells by the staining substances. Further penetration by LiT or KPT at *p*H 7.5 reveals in these bodies internal regular laminated structures which appear as fingerprint patterns. However, further penetration by KPT at *p*H 8.1 appears to cause the disruption of the internal organization in some of these bodies which reveal loosely packed irregular vesicles. These bodies probably are not mesosomes, since *Bdellovibrio* 15143, which shows more than one such body per cell, has only a single anterior mesosome (6). Similar structures which appear to have been extruded from the protopast (a few per cell, depending on the organism) and exhibit fingerprint patterns also have been observed in several gram-negative organisms (*E. coli*, *P. vulgaris*, *Pseudomonas putida*, *S. serpens*, and others) stained negatively with LiT, AmMo, and occasionally KPT at *p*H 7.5 (Davis and Abram, Bacteriol. Proc., p. 52, 1970). In these organisms, unlike *Bdellovibrio* which never appears plasmolyzed, these structures are located in the periplasmic space. Fingerprint patterns have also been observed in negatively stained preparations of lecithin, cholesterol, and phosphatidylethanolamine alone or in mixtures with lipopolysaccharides (2, 14, 23). One of these studies (23) noted, similar to our observations, that the organized pattern depends on the staining substance; it was apparent when LiT and AmMo were used for staining, but when KPT was used, appreciable disruption of the basic lamellar structure occurred. The appearance of organized helical assemblies of lecithin, cholesterol, and saponin also is affected by the composition, *p*H, and ionic constitution of the staining solution (11). The nature of the laminated bodies in *Bdellovibrio* and the other gram-negative organisms is not yet understood. It is assumed that they represent compartmentalized sites of organized chemical differentiation within the protoplast, which may be confined by infoldings of the cytoplasmic membrane. Perhaps they contain phospholipids or related compounds which accumulate when in excess, or before being integrated into the cytoplasmic membrane or cell wall, of which they are common components.

The observations reported are relevant to an understanding of the nature of *Bdellovibrio* and its parasitic activities. It is hoped that further examination of other known parasitic strains, various types of host-independent mutants, and bdellovibrio-like organisms will lead to a better understanding of the evolution of the parasitic mode of life of *Bdellovibrio*.

ACKNOWLEDGMENTS

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