JOURNAL OF BACTERIOLOGY, July 1977, p. 323-330
Vol. 131, No. 1

Printed in U.S.A.

Ultrastructural Changes During Encystment and Germination of *Bdellovibrio* sp.

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Received for publication 8 April 1977

Under proper conditions, *Bdellovibrio* sp. strain W cells develop into bdellocysts in appropriate prey bacteria. After attachment and penetration of the prey cell, the encysting bdellovibrio began to accumulate inclusion material and increase in size, and was surrounded by an outer layer of amorphous electron-dense material. The cytoplasm of the encysting cell appeared more electron dense, and the nuclear areas appeared more compact. During germination of bdellocysts, the outer wall was uniformly broken down, the inclusion material changed shape and affinity for the heavy metal stain, and the nuclear areas expanded. As the outer wall was dissolved, outgrowth began with the elongation of the germinant as it emerged from the prey ghost as an actively motile cell.

Bdellovibrio sp. strain W is an obligately predacious bacterium which, under proper conditions, will develop into a resting cell. We have designated these resting bodies bdellocysts based on their cystlike appearance (2, 8), reduced rate of respiration, and increased resistance to various physical and chemical stresses (19). Bdellocysts are capable of germination and outgrowth under suitable conditions. The resultant germinants are highly motile and predacious toward susceptible prey (19).

A brief account of ultrastructural changes occurring during encystment has been reported (8), but no study has been made of the ultrastructural events accompanying the germination process. This paper presents the ultrastructure of *Bdellovibrio* sp. strain W undergoing its complete morphogenetic cycle of encystment, germination, and outgrowth.

MATERIALS AND METHODS

Bacteria and cultural conditions. *Bdellovibrio* sp. strain W was obtained from G. Drews, University of Freiburg, Freiburg, Germany. The prey organism used was *Rhodospirillum rubrum* Hughes (obtained from G. Sojka, Indiana University, Bloomington, Ind.).

R. rubrum was grown in tryptic soy broth (Difco) and maintained on tryptic soy agar (Difco). Transfers to broth cultures were made from single colonies. For vegetative growth of the bdellovibrios, *R. rubrum* was grown to late exponential phase in tryptic soy broth, harvested by centrifugation at 5,000 × *g*, washed once by centrifugation in dilute nutrient broth (17) supplemented with 0.002 M $CaCl_2$ and 0.003 M $MgCl_2$, and suspended in dilute nutrient broth. The washed suspension of *R. rubrum* in dilute nutrient broth was inoculated with a 10% (vol/vol) inoculum of a *Bdellovibrio* sp. strain W lysate and incubated at 30°C in a shaker incubator at 150 rpm for 12 to 18 h until lysis of the prey was complete. *Bdellovibrio* sp. strain W was maintained as lysates at 4°C or as plaques formed when plated by the double-layer agar overlay method (4) onto peptone-yeast extract agar (1) supplemented with *R. rubrum*.

Encystment. For encystment of the bdellovibrios, *R. rubrum* was grown in tryptic soy broth to stationary phase, harvested by centrifugation at 5,000 × *g* and resuspended in 0.05 M potassium phosphate buffer, pH 7.5. Encystment conditions were as described previously (19). Cultures containing 2 × 10^9 cells per ml each of *R. rubrum* and *Bdellovibrio* sp. strain W in 0.05 M potassium phosphate buffer (pH 7.5) were incubated at 30°C in a shaker incubator at 150 rpm for 24 h. The resulting bdellocysts were purified by treatment with 0.02% Triton X-100 for 30 min at room temperature (22 to 25°C) to lyse vegetative bdellovibrios. This treatment resulted in no noticeable effect on either the morphology or viability of bdellocysts. Bdellocysts were harvested by centrifugation at 5,000 × *g*, washed five times by centrifugation with distilled water, and resuspended in distilled water. Bdellocyst suspensions were stored at 4°C until needed. Extended storage (up to 4 months) under these conditions had little, if any, effect on germinability or viability of bdellocysts.

Germination. Bdellocysts were germinated in peptone-yeast extract broth (pH 8.0) at 35°C.

Preparation of cells for electron microscopy. For negative staining, 1-ml samples of either encysting or germinating cultures were withdrawn at intervals, and aqueous glutaraldehyde (Polysciences) was added to a final concentration of 1%. Samples were placed on carbon-stabilized Formvar-coated 300-mesh copper grids (Ernest F. Fullam, Inc.) for 1 to 2 min, and excess liquid was removed with filter paper. Grids were allowed to air-dry and then were

negatively stained with 0.5% aqueous uranyl acetate (pH 4.0) for 1 min. The stain was absorbed to dryness with filter paper.

To prepare cells for thin sectioning, 10-ml samples were taken at intervals, prefixed by addition of 1 ml of aqueous 1% OsO_4, and harvested by centrifugation. Samples were then fixed according to a modification of the method of Kellenberger et al. in Veronal buffer (pH 6.1) containing 1% OsO_4 and 1% tryptone (10) for 2 h at room temperature. Samples were postfixed in 0.5% aqueous uranyl acetate for 2 h, dehydrated in a graded acetone series, embedded in a mixture of Epon 812 and 815 (3), and polymerized at 60°C for 22 h.

Thin sections were cut with an LKB Ultrotome (Stockholm, Sweden) with a diamond knife, mounted on 400-mesh copper grids and stained with lead citrate (13) for 30 s. All preparations for electron microscopy were observed through a Philips EM 200 electron microscope operated at 60 kV.

RESULTS

Encystment. Under the proper conditions, *Bdellovibrio* sp. strain W will attack and penetrate its prey, but instead of growing vegetatively, it will develop into a dormant cell, termed a bdellocyst (19). Ultrastructural changes that occurred during encystment are shown in Fig. 1. Samples for electron microscopy were taken at hourly intervals after mixing bdellovibrios and prey. By 2 h after mixing, the bdellovibrio had penetrated the prey cell wall and was positioned in the periplasmic space between the prey cell wall and cytoplasmic membrane (Fig. 1a). It was noted that the prey cell did not become swollen upon infection by the bdellovibrio; this was usually the case during bdellovibrio vegetative growth. At this stage, the bdellovibrio cell measured approximately 0.25 μm in diameter and 0.8 μm in length (comparable to the size of most vegetative bdellovibrios prior to infection).

Inclusion material was present at this early stage of encystment. These inclusion bodies were rarely observed in vegetatively growing bdellovibrios. After 3 to 4 h postinfection (Fig. 1b), the encysting bdellovibrio protoplast had increased in diameter, and a thin layer of electron-dense material had been deposited outside the bdellovibrio cell wall (Fig. 1c). This outer layer was approximately 5 to 10 nm thick. As encystment progressed, the outer layer increased in thickness, becoming 25 to 30 nm thick after 5 h (Fig. 1d). During encystment there was an invagination of the original bdellovibrio wall and of the adjacent cytoplasmic membrane on the concave side of the kidney-shaped bdellocyst (Fig. 1b and d). During the latter stages of encystment, the outer layer of the encysted bdellovibrio appeared to be intimately associated with the prey cell wall (Fig. 1d). Even after mild sonic treatment to remove the prey ghost from the mature bdellocysts, remnants of the prey wall remained attached to the outer bdellocyst layer.

The cytoplasm of the encysting cell began to increase in electron opacity 4 to 5 h after infection (Fig. 1d), and by 11 h the cytoplasm was considerably more electron dense, and the nuclear area was more compact than in vegetative cells (Fig. 2).

Germination. Ultrastructural changes occurring during germination were observed in both negatively stained preparations and thin sections. Samples were taken 0, 15, 30, 45, 60, 75, and 90 min after germination was initiated by suspending the bdellocysts in peptone-yeast extract broth. The first noticeable morphological change occurred after 15 to 30 min, when the outer bdellocyst layer began to expand and appeared fibrous and less dense (Fig. 3). During these initial stages of germination, the inclusion bodies assumed an irregular shape and possessed an increased affinity for the heavy metal stain. The germinating cell also appeared to have expanded somewhat, due to the disappearance of the cell wall invaginations present in mature bdellocysts. At this stage of germination, the cytoplasm was still electron dense, and the nuclear areas remained compact. At 45 to 60 min, the bdellocyst outer layer was further broken down, becoming less dense and appearing looser and more fibrous in structure (Fig. 4), and the prey cell wall became separated from the germinating cell (Fig. 4). The nuclear regions expanded, occupying a large portion of the cell, and the inclusion bodies assumed a highly irregular, multilobed appearance.

Subsequent to these germination events, outgrowth began with the cell increasing in length and producing an "S"-shaped cell, and finally emerging from the ghost of the prey (Fig. 5). After exposure to germination conditions for 90 min, most of the germinants gained motility and emerged from the prey ghost as highly motile vegetative bdellovibrios. Figure 6 shows a thin section of a fully germinated bdellocyst; essentially all of the bdellocyst outer layer material is gone. The ultrastructural appearance was similar to that of most free-living bdellovibrios, except that some inclusion material was still present.

Stages of germination and outgrowth as seen with negatively stained preparations corroborated the sequence of events deduced from thin sections (Fig. 7). A mature bdellocyst surrounded by its prey ghost is shown in Fig. 7a. As germination and outgrowth took place, the

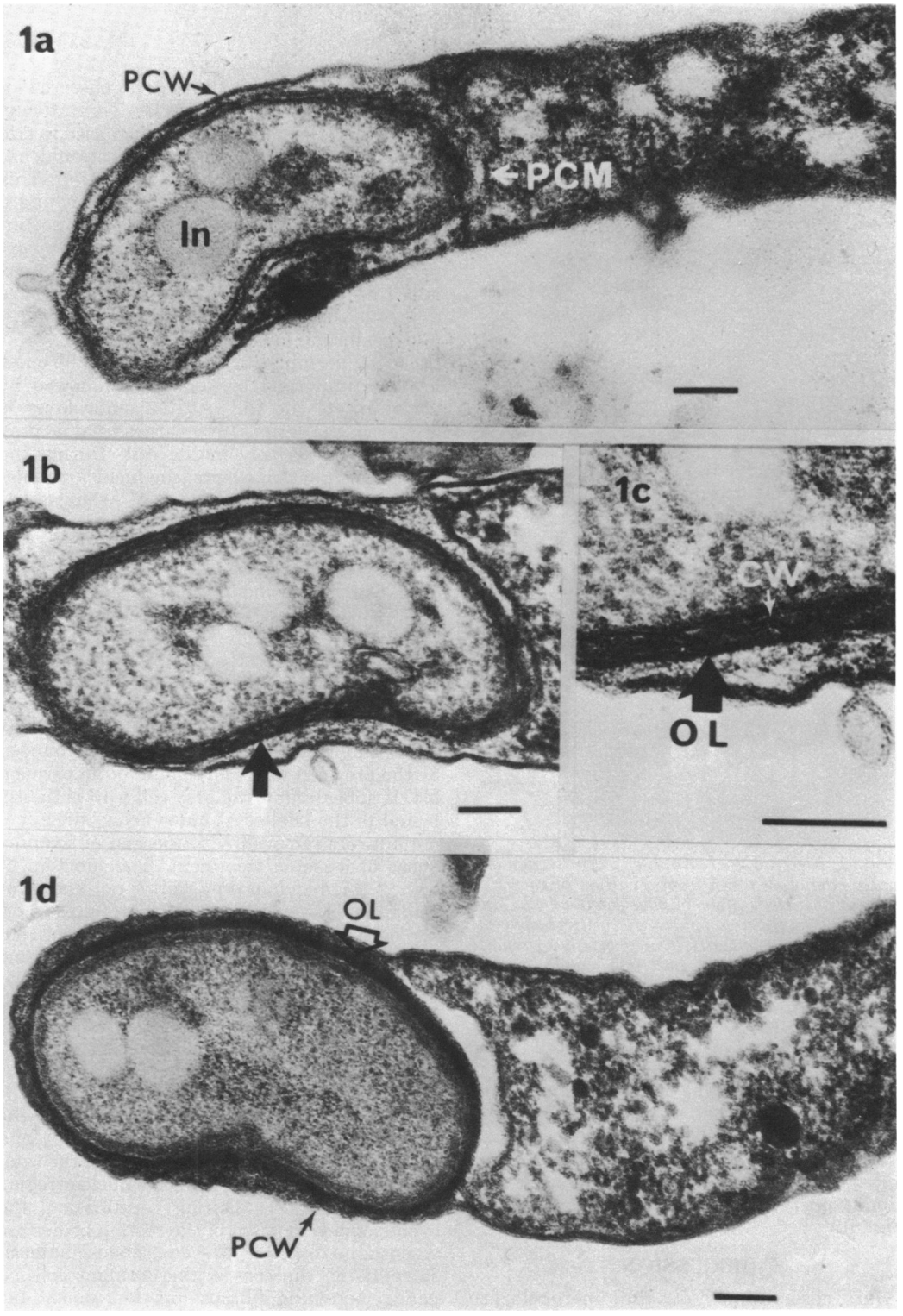


FIG. 1. *Electron micrographs of thin sections of Bdellovibrio sp. strain W during encystment in R. rubrum. (a) After penetration of the prey (2 h after mixing), the bdellovibrio is positioned in the periplasmic space between the prey cell wall (PCW) and prey cytoplasmic membrane (PCM). Inclusion bodies (In) are present in the bdellovibrio. (b) At 3 to 4 h after mixing bdellovibrios and prey, a thin layer of amorphous electron-dense material was laid down around the encysting cell (arrow). (c) Higher magnification of a portion of (b) showing the outer layer (OL), which lies just outside the bdellovibrio cell wall (CW). (d) At 5 to 6 h after mixing, the outer layer (OL) has increased in thickness and is intimately associated with the prey cell wall (PCW). Bar represents 0.1 μm.*

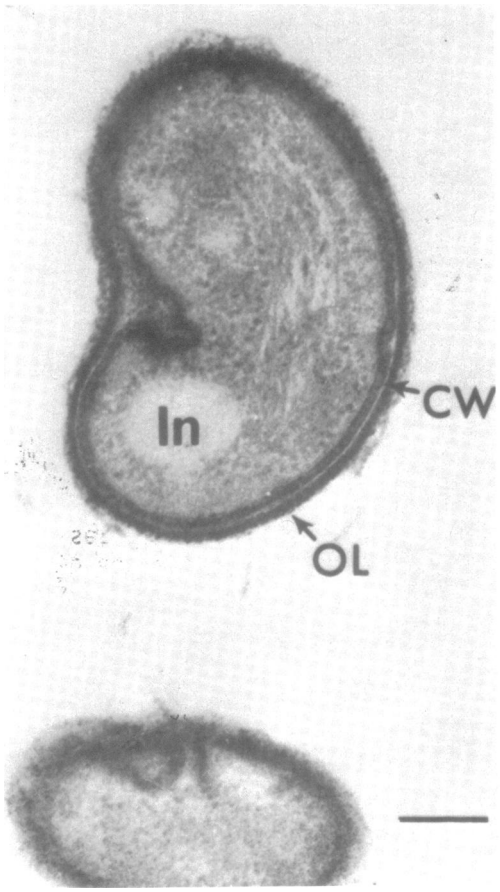


FIG. 2. *Electron micrograph of a thin section of a mature bdellocyst taken from a culture of encysting bdellovibrios 11 h after mixing bdellovibrios and prey. Prior to fixation for electron microscopy, the bdellocysts were sonically treated briefly to remove the prey ghost. The cell wall (CW) of the cyst is surrounded by a thick amorphous electron-dense outer layer (OL). Inclusions (In) are present in the bdellocyst. Bar represents 0.1 μm.*

cell elongated (Fig. 7b) inside the prey ghost and finally emerged as a freely motile vegetative bdellovibrio (Fig. 7c). This sequence has also been observed by phase-contrast microscopy (19). The outer layer from which the germinant emerged is the remains of the prey cell (Fig. 5).

DISCUSSION

Our observations of the morphological changes accompanying encystment of *Bdellovibrio* sp. strain W agree with those made by Hoeniger et al. (8). After penetration into the periplasmic space of its prey, the encysting *Bdellovibrio* accumulated inclusion material. This inclusion material was not observed in *Bdellovibrio* sp. strain W grown vegetatively. In addition, the encysting cell increased in size as an outer layer of amorphous electron-dense material was laid down outside the cell. This outer layer increased in thickness, reaching a maximum thickness of about 30 nm 5 h postinfection. During this time, the cytoplasm appeared more electron dense, the nuclear areas appeared more compact than in vegetative cells, and the inner wall and adjacent cytoplasmic membrane invaginated.

During germination of bdellocysts, the outer layer is uniformly broken down followed by outgrowth of the cell. As the outer layer is dissolved, the germinant emerges from its prey ghost as an actively motile cell. During the initial germination events, the inclusion material also appears to be changing. As suggested by Hoeniger et al. (8), these inclusion bodies may be storage granules, which are being mobilized for germination and outgrowth. However, neither the chemical composition nor the function(s) of these inclusions is known at this time.

Bdellocysts are structurally distinct from resting forms of other bacteria in that they are not surrounded by an impermeable barrier, such as a spore coat, in addition to the amorphous outer layer. Instead, they are surrounded by the prey cell wall and remain quite permeable. It appears that the prey cell wall is tightly bound to the bdellocyst outer layer, since it is not entirely removed with the rest of the prey ghost upon sonic treatment. The function, if any, of this residual prey wall is not known at this time. However, the sequence of gross morphological changes that occurrs during both encystment and germination parallels that found in most other spore- and cyst-forming bacteria. During development of the dormant cell, these morphological changes include: (i) the deposition of a thick layer of amorphous material just external to the dormant cell wall (e.g., the cortex of endospores [9, 12, 14, 18, 20] and the intine of *Azotobacter* cysts [5, 15, 21]); (ii) the appearance of inclusion material (5, 15); and (iii) the increase in electron density of the cytoplasm and decrease in size of the nuclear region (9, 14, 18, 20, 21). During germination, the events of development of the resting bodies are essentially reversed: the amorphous material surrounding the core of the dormant cell expands, becoming fibrous and less dense and appearing to be uniformly broken down; and the germinating cell swells with the concomitant expansion of the nuclear material (6, 7, 11, 14, 16, 18, 20, 21). Any storage material present also appears to be mobilized during germina-

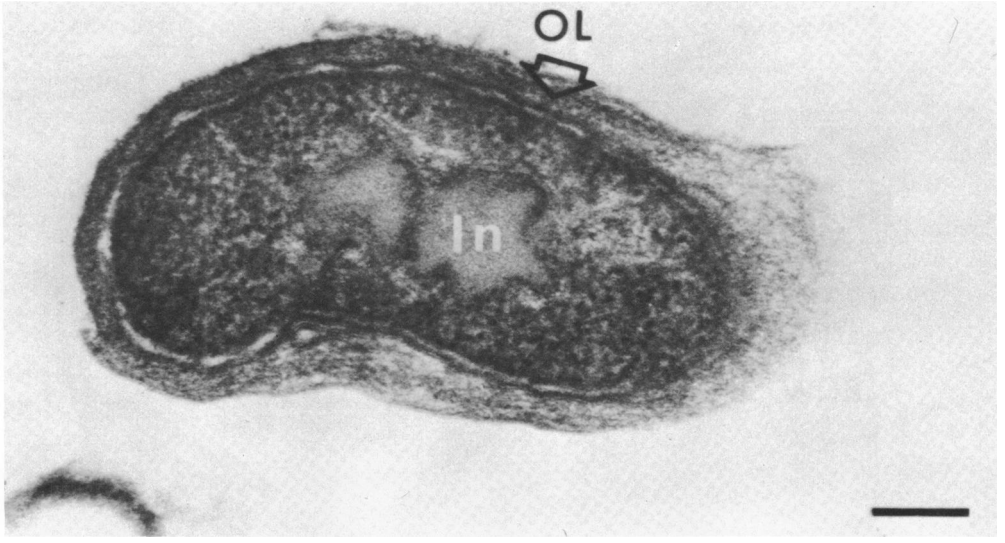


FIG. 3. *Section through a germinating bdellocyst exposed to germination conditions for 30 min. The outer layer (OL) has expanded and has become fibrous and less dense. Inclusion material (In) has become irregular in shape and has retained some of the lead citrate stain. Bar represents 0.1 μm.*

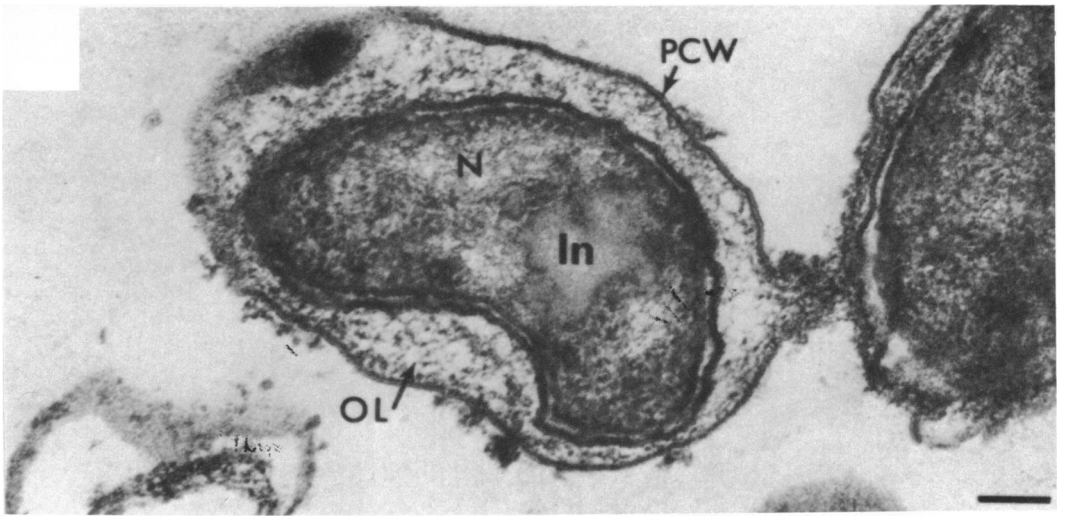
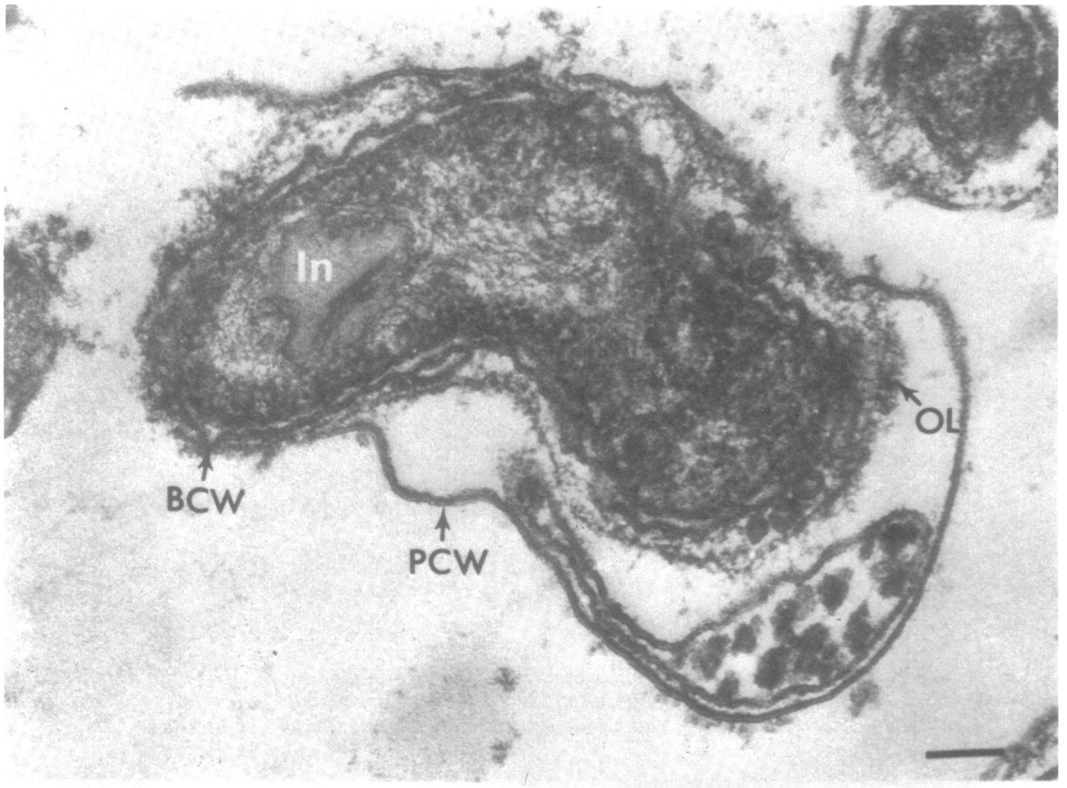


FIG. 4. *Thin section through germinating bdellocyst after exposure to germination conditions for 45 to 60 min. The outer layer (OL) has continued to break down and appears loose and fibrous in structure. The prey cell wall (PCW) is separated from the germinating cell. The cytoplasm is less dense, and the nuclear material (N) has expanded. Bar represents 0.1 μm.*



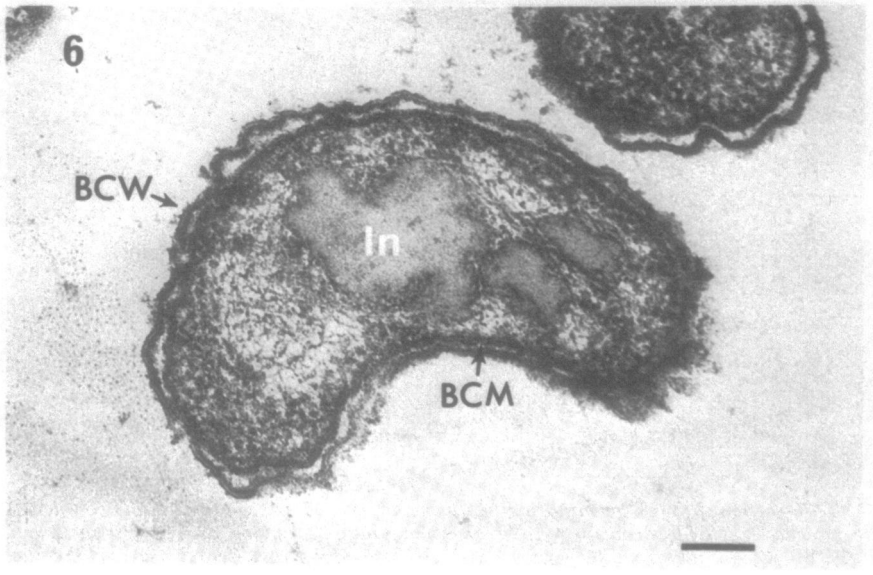


FIG. 5. *Thin section through a germinated bdellocyst during outgrowth and emergence from the prey ghost. The samples were taken from electron microscopy after bdellocysts had been exposed to germination conditions for 75 min. Small amounts of outer layer (OL) material still cling to the germinant. PCW, Prey cell wall; In, inclusion; BCW, bdellovibrio cell wall. Bar represents 0.1 μm.*

FIG. 6. *Section through a fully germinated bdellocyst. The samples were taken for electron microscopy after bdellocysts had been exposed to germination conditions for 90 min. BCW, Bdellovibrio cell wall; BCM, bdellovibrio cytoplasmic membrane; In, inclusion. Bar represents 0.1 μm.*

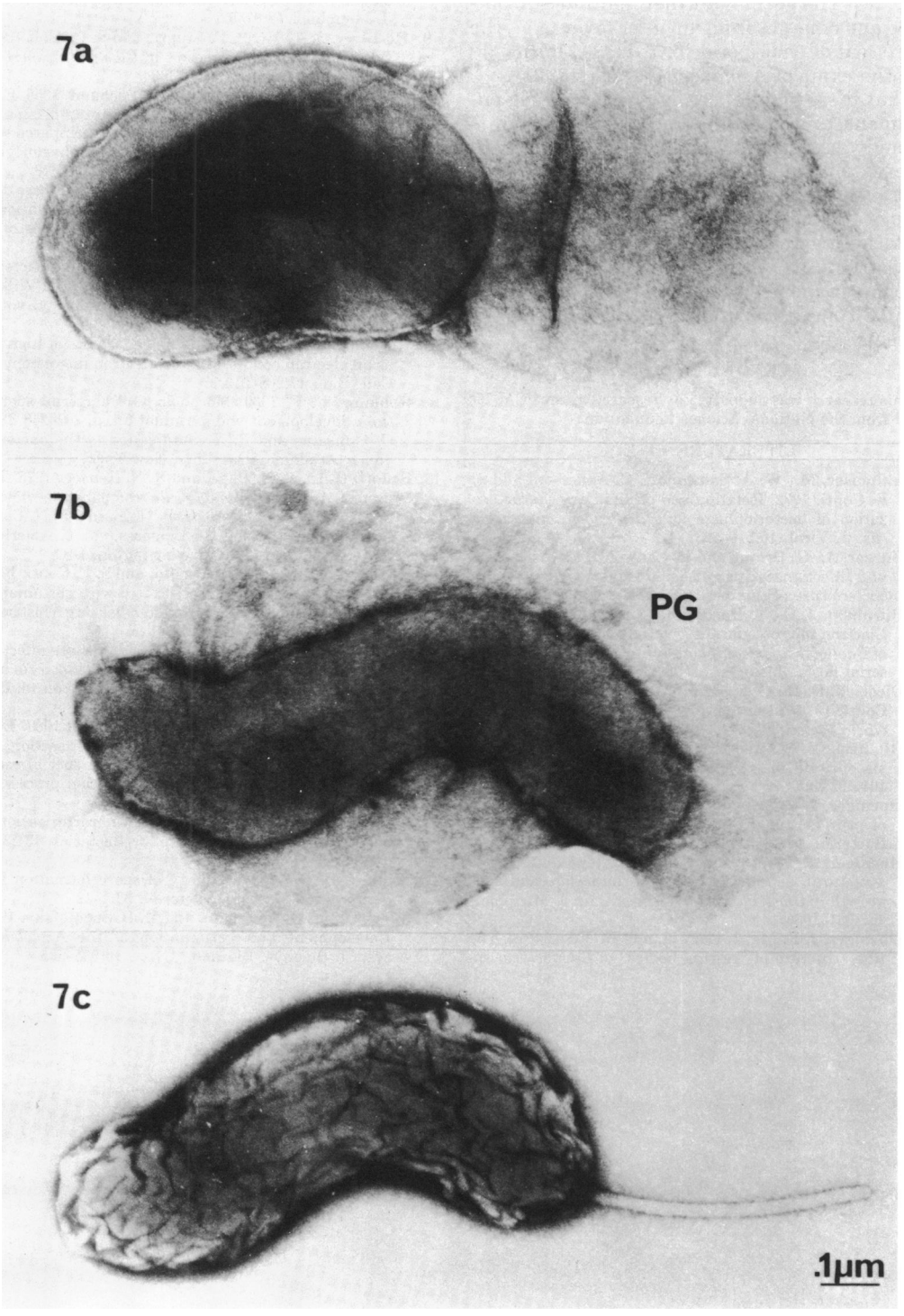


FIG. 7. *(a) Negatively stained mature bdellocyst surrounded by its prey ghost. (b) Negatively stained germinating bdellocyst after exposure to germination conditions for 60 min. PG, Prey ghost. (c) Negatively stained fully germinated bdellovibrio showing the typical convoluted surface and a single-sheathed polar flagellum. Bar represents 0.1 µm.*

tion (21). The germinant then continues to outgrow and emerges from an outer layer (e.g., the spore coat of endospores [6, 7, 11, 14, 16, 18, 20] and the exine of *Azotobacter* cysts [15, 21]).

That these general changes occur during encystment and germination of bdellocysts is strongly supported by the electron microscopic observations reported here. We do not propose that events during the formation and germination of "resting bodies" are analogous in all bacterial cryptobiotic systems, but simply that ultrastructural changes during bdellocyst development and germination do not differ significantly from those found in other, better known systems.

ACKNOWLEDGMENTS

This research was supported by research grant PCM 75-19781 from the National Science Foundation.

LITERATURE CITED

1. **Althauser, M., W. A. Samsonoff, C. Anderson, and S. F. Conti.** 1972. Isolation and preliminary characterization of bacteriophage for *Bdellovibrio bacteriovorus*. J. Virol. 10:516-523.
2. **Burger, A., G. Drews, and R. Ladwig.** 1968. Wirtskreis und Infectionscyclus eines neu isoliertes *Bdellovibrio bacteriovorus*-Stammes. Arch. Mikrobiol. 61:261-279.
3. **Burnham, J. C., T. Hashimoto, and S. F. Conti.** 1968. Electron microscopic observations on the penetration of *Bdellovibrio bacteriovorus* into gram-negative bacterial hosts. J. Bacteriol. 96:1366-1381.
4. **Diedrich, D. L., C. F. Denny, T. Hashimoto, and S. F. Conti.** 1970. Facultatively parasitic strain of *Bdellovibrio bacteriovorus*. J. Bacteriol. 101:989-996.
5. **Hitchens, V. M., and H. L. Sadoff.** 1970. Morphogenesis of cysts in *Azotobacter vinelandii*. J. Bacteriol. 104:492-498.
6. **Hoeniger, J. F. M., and C. L. Headley.** 1968. Cytology of spore germination in *Clostridium pectinovorum*. J. Bacteriol. 96:1835-1847.
7. **Hoeniger, J. F. M., and C. L. Headley.** 1969. Ultrastructural aspects of spore germination and outgrowth in *Clostridium sporogenes*. Can. J. Microbiol. 15:1061-1065.
8. **Hoeniger, J. F. M., R. Ladwig, and H. Moor.** 1972. The fine structure of "resting bodies" of *Bdellovibrio* sp. strain W developed in *Rhodospirillum rubrum*. Can. J. Microbiol. 18:87-92.
9. **Hoeniger, J. F. M., P. F. Stuart, and S. C. Holt.** 1968. Cytology of spore formation in *Clostridium perfringens*. J. Bacteriol. 96:1818-1834.
10. **Kellenberger, E., A. Ryter, and J. Sechaud.** 1958. Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. 4:671-678.
11. **Mayall, B. H., and C. F. Robinow.** 1957. Observations with the electron microscope on the organization of the cortex of resting and germinating spores of *B. megaterium*. J. Appl. Bacteriol. 20:333-341.
12. **Murrell, W. G.** 1967. The biochemistry of the bacterial endospore, p. 133-251. *In* A. H. Rose and J. F. Wilkinson (ed.), Advances in microbial physiology, vol. 1. Academic Press Inc., New York.
13. **Reynolds, F. S.** 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell. Biol. 17:208-212.
14. **Robinow, C. F.** 1960. Morphology of bacterial spores, their development and germination, p. 207-248. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria, vol. 1. Academic Press Inc., New York.
15. **Sadoff, H. L., W. J. Page, and R. N. Reusch.** 1975. The cyst of *Azotobacter vinelandii*: comparative view of morphogenesis, p. 52-60. *In* P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), Spores, vol. 6. American Society for Microbiology, Washington, D.C.
16. **Samsonoff, W. A., T. Hashimoto, and S. F. Conti.** 1970. Ultrastructural changes associated with germination and outgrowth of an appendage-bearing clostridial spore. J. Bacteriol. 101:1038-1045.
17. **Seidler, R. J., and M. P. Starr.** 1969. Factors affecting the intracellular parasitic growth of *Bdellovibrio bacteriovorus* developing within *Escherichia coli*. J. Bacteriol. 97:912-923.
18. **Takagi, A., T. Kawata, and S. Yamamoto.** 1960. Electron microscope studies on ultrathin sections of spores of the Clostridium group, with special reference to the sporulation and germination process. J. Bacteriol. 80:37-46.
19. **Tudor, J. J., and S. F. Conti.** 1977. Characterization of bdellocysts of *Bdellovibrio* sp. J. Bacteriol. 131:314-322.
20. **Walker, P. D.** 1970. Cytology of spore formation and germination. J. Appl. Bacteriol. 33:1-12.
21. **Wyss, O., M. G. Neumann, and M. D. Socolofsky.** 1961. Development and germination of the *Azotobacter* cyst. J. Biophys. Biochem. Cytol. 10:555-565.