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Freeze-Fracture Study of the Filamentous, Segmented Microorganism Attached to the Murine Small Bowel

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A freeze-fracture study has provided new information about the filamentous, segmented microorganism known to live in the murine small bowel. The intracellular bodies produced by this microbe appear to arise by a modified sporogenesis so that they are enclosed in an envelopment membrane at least prior to release by the filament mother cell. At least some of the intracellular bodies divide while still within the mother cell, suggesting a reproductive role for these structures. The host epithelial membrane remains intact at the site of attachment, but does appear to have a reduced concentration of intramembrane particles. Changes in the host cytoplasm adjacent to the attachment site are documented and interpreted to be a sol-gel transformation which may stabilize the attachment socket.

There is considerable interest in the filamentous, segmented microorganism that inhabits the murine gastrointestinal tract (4-6, 9, 12, 14, 15). Although there are no confirmed reports that this organism can be cultured axenically, the published data have contributed to our understanding of the possible life cycle and ultrastructure of the microbe as well as the nature of its impact on the host epithelial membrane. The microbe is particularly remarkable in its ability to induce the formation of an "attachment receptacle" in the epithelial cell to which it is anchored. Previous findings (4, 5, 9) indicate that microvilli in the vicinity of the attachment site are destroyed and that the host does not produce a significant inflammatory response to the attached microbe. As yet, however, little is known about the structural details of these attachment sites or the mechanisms by which they are formed and maintained.

The freeze-fracture technique is particularly well suited to the study of the attachment site, since the fracture face characteristically reveals membrane surfaces that are not visible by any other method. This paper presents new information regarding the ultrastructure of the filamentous organism and of the attachment site. Also, we suggest an hypothesis for the mechanism by which the attachment site is formed and stabilized.

MATERIALS AND METHODS

Animals. Long Evans hooded rats (5-week-old males) were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass. The rats were housed individually in wire cages and given Lab Blox ration (Allied Mills, Chicago, Ill.) and acidified water (0.001 N HCl) ad libitum.

Sample collection. All animals were killed at 2 to 5 months of age by chloroform inhalation. The gut was exposed, and a 4- to 5-cm length of distal ileum was tied off using either surgical suture or 10-pound-test fishing line. Glutaraldehyde (2%) in Millonig buffer (11) without glucose (pH 7.2) was prewarmed to 37°C and injected into the ileal loop, which then was removed and slit open. The ileal contents were brushed gently aside, and the tissue was immersed in fixative. Fixation time was either 1 to 2 h at room temperature or overnight at 4°C. After fixation, the tissue was washed gently in fresh Millonig buffer and stored at 4°C in the same buffer supplemented with 0.1% sodium azide.

Freeze-fracture. Small pieces of tissue were immersed at room temperature for 1 to 2 h in a solution of 20% glycerol in Millonig buffer and then divided into tissue blocks no larger than 1 mm per side. These blocks were mounted either on copper disks or on gold specimen mounts (Balzers Co.) and frozen in Freon 12 cooled with liquid nitrogen. Tissue blocks usually were mounted with the villi oriented upward. Tissue blocks were freeze-fractured and replicated at −100°C in a Balzers (model BAF 300) freeze-etch machine. The fractured tissue was thawed into 20% glycerol-Millonig buffer, and the replica was removed and cleaned by overnight treatment with 5% sodium hypochlorite (Clorox bleach). The cleaned replicas were mounted on uncoated 200-mesh copper grids and examined with either a Jeol 100 C or a Siemens 102 electron microscope.

RESULTS

Figures 1 and 2 show the typical appearance of a filament mother cell containing intracellular bodies as seen in a freeze-fracture preparation. Although this organism has been described as "gram-variable" (5), the wall has the typical

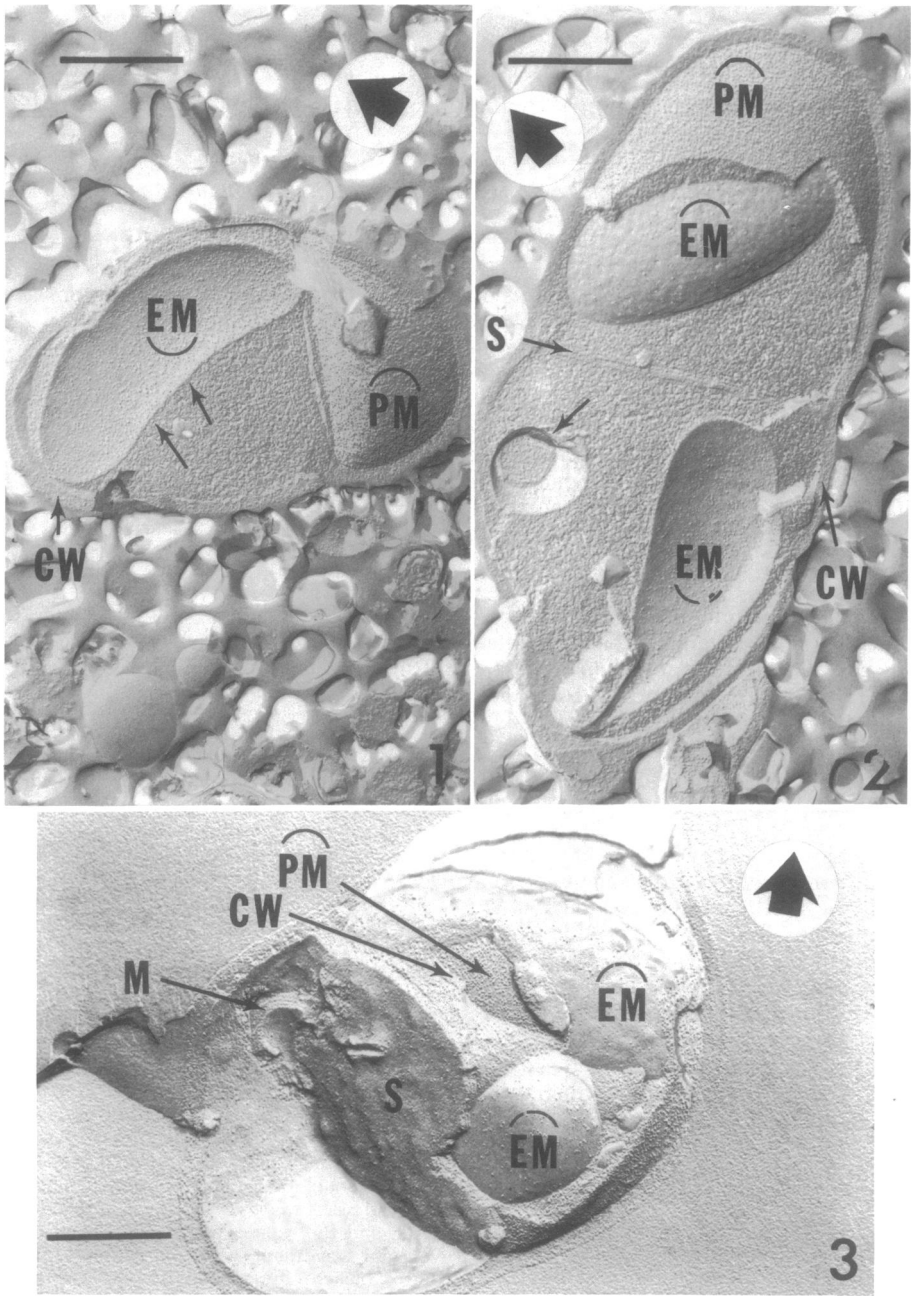

FIG. 1. *Freeze-fracture preparation of the segmented microbe in the ileal lumen. The fracture is at an oblique angle so that only a small portion of the filament and one of the filament cell plasma membranes (PM) are visible. The fractured ICB membrane surface (EM) is not a plasma membrane "B" face, as might be expected, but is the "A" face of an envelopment membrane. Note that there is no cell wall visible exterior to this membrane (arrows), as would be expected if this were a fracture face of the plasma membrane. Since the curvature of the envelopment membrane is reversed with respect to the plasma membrane, the "A" face and "B" face are concave and convex, respectively. In this figure as well as in all subsequent figures, the bar represents 0.5 μm. Symbols: PM and EM, surfaces of the plasma and envelopment membranes, respectively; ⌣ and ⌢, respectively, concave or convex surface; CW, cell wall. Shadow direction is indicated by the thick encircled arrows.*

appearance of that of a gram-positive bacterium. In Fig. 1, a concave fracture face of an intracellular body (ICB) can be seen inside one of the filament mother cells. Although this structure resembles the "B" face of a conventional cytoplasmic membrane, there is no indication of a wall external to the membrane (arrows). Since it is likely that the ICBs arise via a modified sporogenesis (4, 7), the fracture face seen here probably represents the envelopment membrane rather than the plasma membrane of the ICB. Also, since the curvature of the envelopment membrane is reversed from that of a normal cytoplasmic membrane, the surface seen here would be an "A" face.

In Fig. 2 a pair of adjacent filament mother cells have been cross-fractured. An envelopment membrane "A" face (EM) is exposed in the lower mother cell along with the fractured tip (arrow) of a second ICB in the same cell. The cytoplasm of the upper mother cell is partially fractured away to reveal the convex "B" face of an envelopment membrane. The face of this membrane is very sparsely populated with intramembrane particles. Even the envelopment membrane "A" faces in Fig. 1 and 2 are more sparsely populated than is the conventional-appearing "A" face of the mother cell plasma membrane (PM).

Figure 3 shows a filament that has been fractured at an oblique angle so that the fracture plane follows the surface of the cross wall between two adjacent mother cells. The cytoplasm is fractured away entirely in one cell, revealing a laminar, mesosome-like structure resembling that described by Chase and Erlandsen (4). The neighboring mother cell contains a pair of ICBs, one of which is fractured so as to reveal the plasma membrane "A" face as well as the envelopment membrane "B" face. The ICB cell wall is visible between the plasma and envelopment membranes. The fragment of plasma membrane "A" face appears comparable in structure to the mother cell plasma membrane "A" face (Fig. 2).

As previously reported (4), the mother cell frequently contains two ICBs (Fig. 2 to 6), although we have not, to date, found any more than two. Chase and Erlandsen suggest that the ICBs potentially can divide while inside the filament mother cell. This finding is supported by Fig. 2 to 6, which suggest strongly that the ICBs shown have divided or are in the process of dividing. In Fig. 6 the ICBs appear to have just finished division. The filamentous microbe produces endospores (4, 6), some of which are seen in Fig. 7 to 9. Figure 7 shows a longitudinal fracture of three consecutive cells, each of which contains a single endospore. The "B" face of the core protoplast membrane is visible in two of the fractured endospores, and the cortex and spore coat are seen in cross-fracture. The third spore has fractured in such a way as to reveal the outer surface of the cortex (2). The endospores are seen in better detail in Fig. 8 and 9. The plasma membrane, cortex, and spore coat are visible in both micrographs, and the endospore itself is ovoid in shape as reported previously (4). We never observed an example of the highly sculptured spore coat characteristic of some bacterial endospores (2), although we cannot say whether such structures might appear later in the development of these endospores.

Figure 10 shows a near-longitudinal fracture of a filament embedded in an epithelial cell. The general appearance of the attachment site is similar to that seen in thin sections (4, 5, 9). A small amount of tight junction visible at one edge of the micrograph indicates that the filament is attached near the boundary between two epithelial cells. In this as well as other micrographs of attachment sites, the epithelial cell cytoplasm immediately adjacent to the attachment site has undergone a change in appearance similar to that seen via electron and optical phase-contrast microscopy (4, 5).

As reported previously (4, 5, 9), the microvilli in the vicinity of the attachment site have degenerated. The intact microvilli shown in Fig. 10 are well preserved and normal in appearance, with the core elements clearly visible. Normally, a single core extends from the terminal web into each microvillus; however, a group of at least four cores extends into what appears to be a single microvillar tip immediately adjacent to the attachment site, suggesting that the micro-

FIG. 2. *Both the "A" (concave) and "B" (convex) faces of a pair of ICB envelopment membranes (EM) are visible within two adjacent mother cells. The "B" (convex) face contains very few intramembrane particles, and the "A" (concave) face contains relatively few particles compared to the normal mother cell plasma membrane "A" (convex) face (PM). The septum (S) between the two mother cells is clearly visible, and the cell wall appears to be of the gram-positive type. Mother cells frequently contain two ICBs; the unlabeled arrow indicates the fractured tip of a second ICB in the lower mother cell.*

FIG. 3. *Oblique fracture of a filament in which the fracture plane has followed the surface of a transverse septum (S). A mesosome-like body (M) can be seen in one corner of the hollowed-out mother cell, and two ICBs are visible in the adjacent mother cell. The cross-fractured cell wall (CW) of one of the ICBs is visible, as well as a portion of the plasma membrane "A" face. Both ICBs are enclosed within an envelopment membrane, and the ICB cell wall is seen to lie between the plasma and envelopment membranes.*

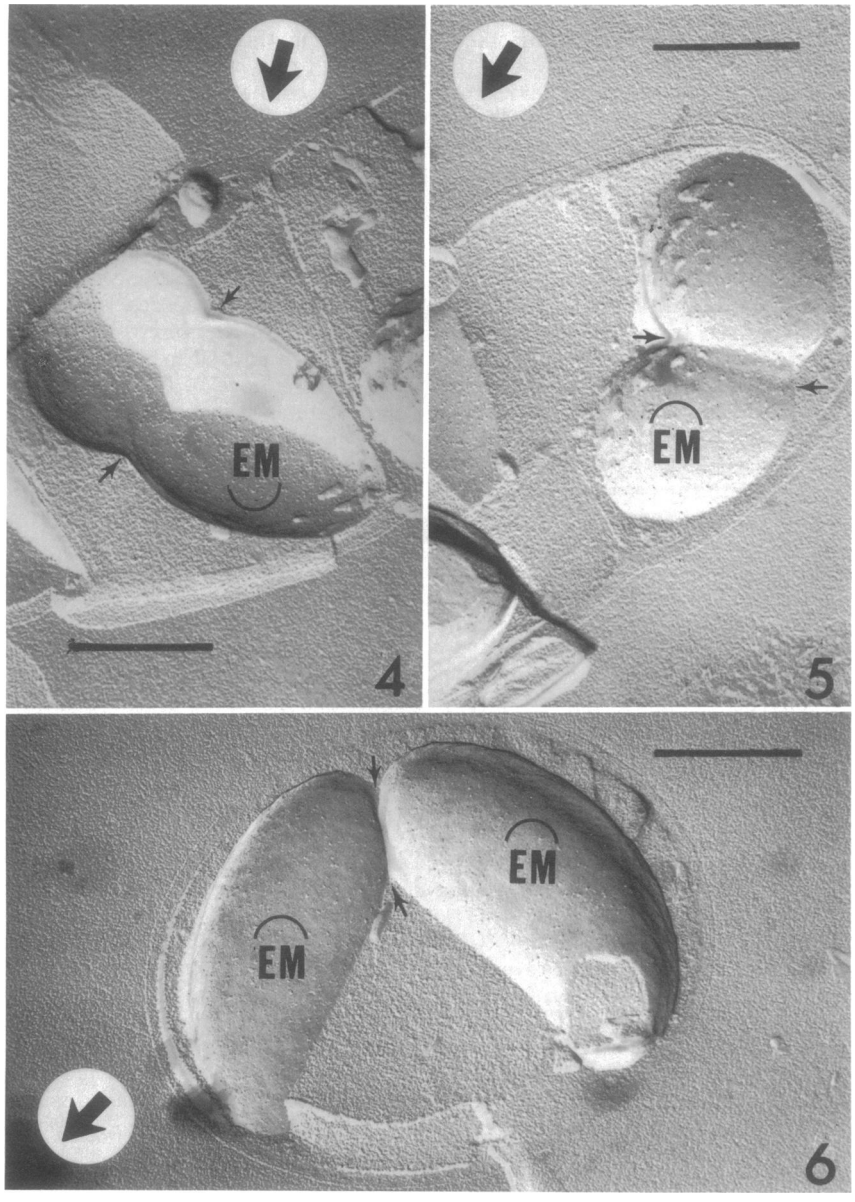

FIG. 4–6. *The ICBs shown here appear to be in the process of dividing, and in Fig. 5 the division is nearly complete. Arrows indicate the point of constriction. The ICBs remain enclosed within the envelopment membrane (EM) at least through the completion of binary fission (Fig. 6).*

villi are destroyed by a process of fusion. The segmented microbe has made an extensive indentation in the epithelial membrane even though only a small portion of the microbe tip actually is in close contact with the epithelial cell. Many attachment sites, however, are quite shallow (4, 5).

The fracture shown in Fig. 11 has cleaved a shallow attachment site so as to reveal a fragment of epithelial membrane (arrow) in close contact with the microbial tip. We interpret this convex fracture face to be the inner surface of the outer leaf of the epithelial membrane ("B" face), as it is very unlikely that the fracture plane would pass between the bacterial wall and the epithelial membrane (3). This membrane

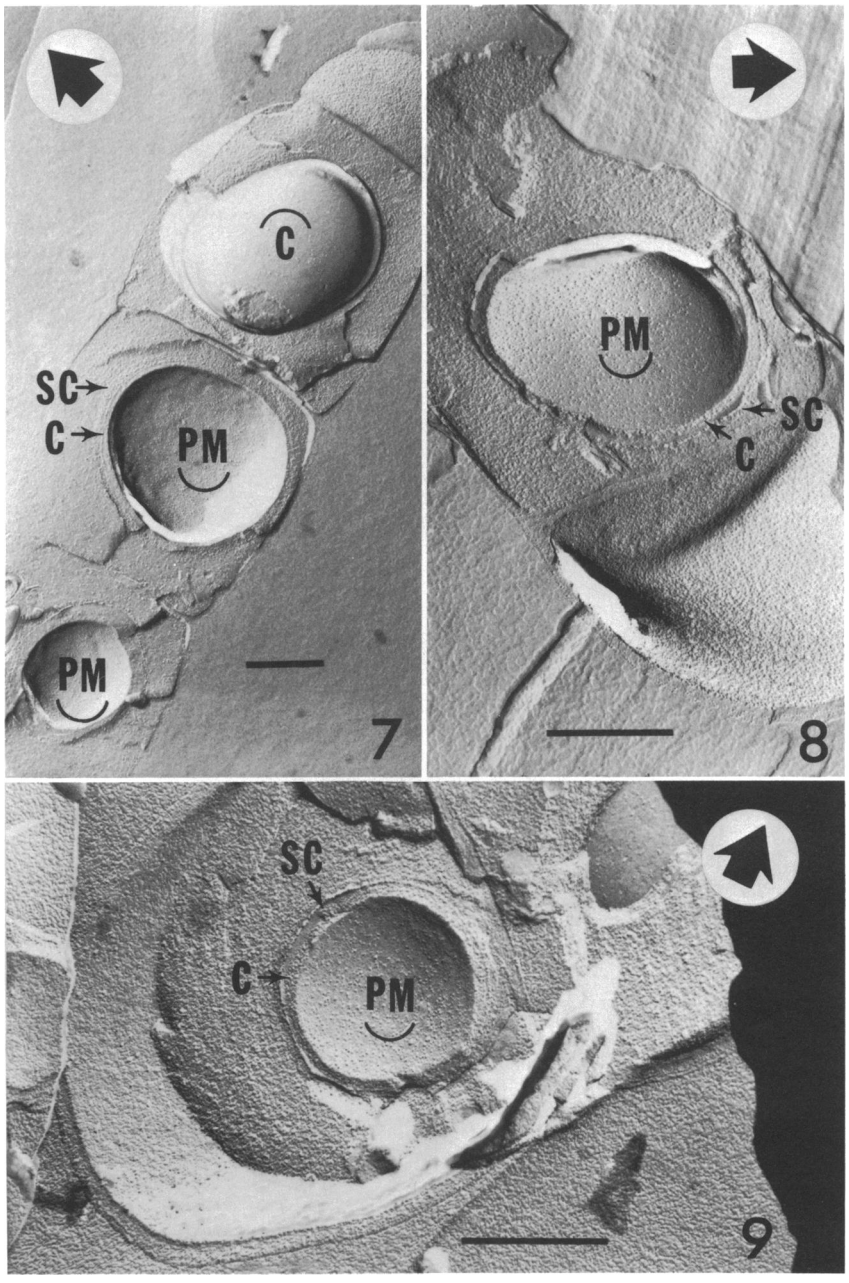

FIG. 7. *Longitudinal fracture of a filament showing a series of endospores. The plasma membrane of two of the spores is revealed and is normal in appearance; the third spore has fractured along the surface of the cortex (C). The cortex and spore coat (SC) of the other two spores are visible in cross-fracture.*

FIG. 8–9. *Each micrograph shows a concave fracture of an endospore; both the cortex (C) and spore coat (SC) can be seen in cross-fracture.*

fracture face appears essentially to be devoid of the particles normally associated with this surface.

Another shallow attachment site is seen in Fig. 12; in this micrograph, most of the bacterial cell is fractured away, revealing the "B" face of the bacterial cytoplasmic membrane and the "A" face of the epithelial membrane. Again, the

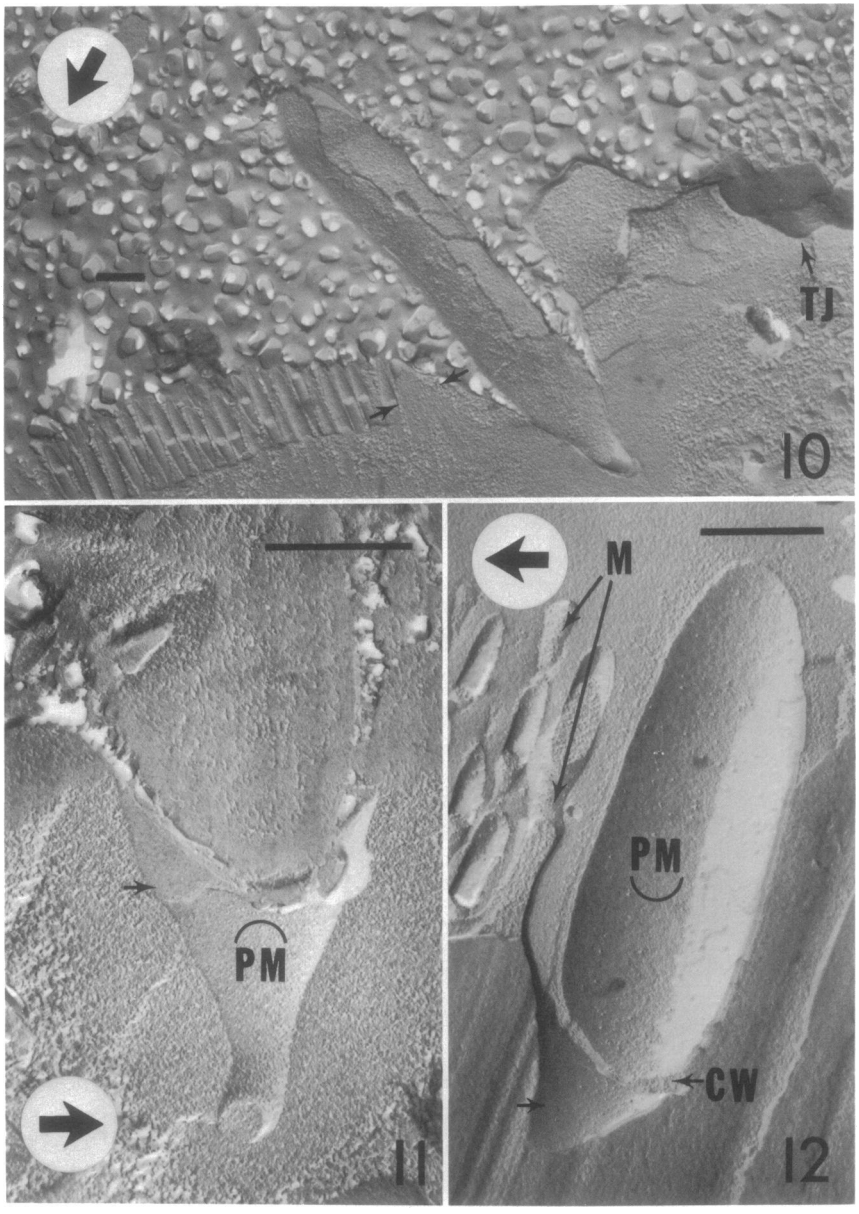

FIG. 10. *Longitudinal fracture of a filament and attachment site. The microvilli have been destroyed in the vicinity of the attachment site, apparently by a process of fusion (arrows). A small portion of tight junction (TJ) indicates that this filament lies near the junction of two epithelial cells. Note the change in texture of the cytoplasmic matrix in the immediate vicinity of the attachment site.*

FIG. 11–12. *Attachment sites in which part of the epithelial cell plasma membrane is visible (arrows). The membrane fracture face in each case appears to be relatively smooth, with fewer than normal numbers of intramembrane particles present; this suggests that the host plasma membrane at the attachment site may have experienced a phase change. Note that since the plasma membrane has been invaginated by the microbe, the convex plasma membrane face in Fig. 11 is a "B" face, while the corresponding concave surface in Fig. 12 is an "A" face.*

epithelial membrane is nearly devoid of particles, although the plasma membrane "A" face normally is well populated with such particles as seen on the microvillar "A" face (M) visible in the same micrograph.

A deep attachment site is shown in Fig. 13. In

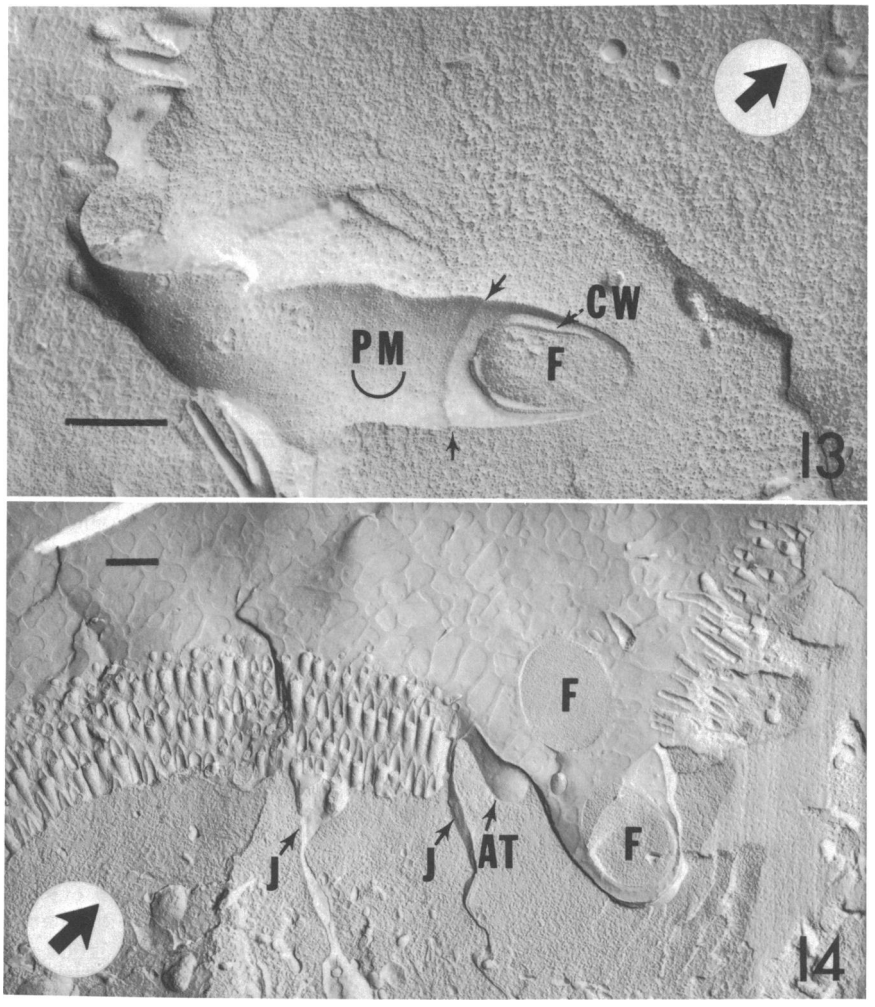

FIG. 13. *Attachment site in which the filament (F) has been nearly fractured away and only the tip remains in a cross-fracture view. The area of the host plasma membrane in intimate contact with the filament is not visible, but a pronounced ridge is visible (arrows) where the plasma membrane presumably was flexed by the filament. The invaginated concave plasma membrane surface actually is a "B" face.*

FIG. 14. *Multiple attachment site in which two filaments (F) are visible in cross-fracture and evidence of a third filament can be deduced from an attachment site (AT). The two cell junctions (J) indicate that the attachment site lies at the boundary of three cells.*

this micrograph, all but the tip of the microbe has been fractured, exposing a large expanse of epithelial membrane ("A" face). The membrane surface at the extreme tip of the attachment site is not visible, but a ridge (arrows) is exposed. The location of this ridge suggests that it may be the boundary where bacterial and mammalian surfaces come into close contact. In contrast to the epithelial membranes in Fig. 11 and 12, the membrane surface shown here is heavily populated with intramembrane particles. It is not known whether this is true also for the epithelial membrane in the area of close contact with the bacterial cell.

Occasionally, we found multiple attachment sites, as seen in Fig. 14. The multiple site seen here lies near the junction of three epithelial cells, as determined by the proximity both of lateral membranes and tight junction. Two cross-fractured filaments are visible, and there is an indirect indication of a third filament attached to the epithelium below the plane of view.

DISCUSSION

The growing body of information regarding the filamentous, segmented microbe found in the murine ileum indicates that this organism

almost certainly is a natural component of the gut flora which evolved with its animal host. Several types of microbes are known to have mechanisms for attachment to the gut wall (15). However, with the possible exception of an unusual colonic organism (16), none have been found to modify the epithelial membrane as extensively as the segmented microbe. The available evidence supports the hypothesis that the ICBs are reproductive elements which permit the microbe constantly to reimplant itself on the epithelial surface, at least within the same animal host (4).

It is possible that the endospores observed by us and by others (4, 6) transmit the segmented organism from one host to the next; however, it is not clear whether the ICBs might serve the same function, since it is not known if these structures can remain viable outside the host.

As suggested by our findings, and in agreement with those of Chase and Erlandsen (4), the ICBs appear to arise by a modification of sporogenesis (7) and then to undergo at least one binary fission. Our results indicate that the ICBs remain enclosed in the envelopment membrane at least through the completion of the binary fission, although we do not know whether they remain so enclosed by the time of their release from the mother cell. The selective advantage of division is obvious. If the segmented organism depends upon the ICBs to reimplant itself in the constantly exfoliating epithelium, then its reimplantation frequency should be in proportion to the number of ICBs produced.

Previous findings (4, 5) indicate that the epithelial cell cytoplasm adjacent to the attachment site becomes more osmophilic, as viewed by thin-section transmission electron microscopy, and more phase dark, as viewed by phase-contrast microscopy, than cytoplasm remote from the site. Our results confirm these observations. In our freeze-fracture preparations, epithelial cell cytoplasm adjacent to the attachment site differed strikingly in texture from cytoplasm remote from the site. The phase darkening seen by optical microscopy suggests that this cytoplasm has a higher than normal refractive index, as might be expected had it changed from a sol to a gel. That a sol-gel transition has occurred at the attachment site is also suggested by the difference in texture between cytoplasm adjacent to the site and cytoplasm remote from the site, as seen in our freeze-fracture micrographs.

The means by which the attachment site is formed and then stabilized remains unknown. It is not likely to be understood until it becomes possible to culture the filamentous microbe and monoassociate it with germ-free animals. We speculate that attachment site formation might be initiated if the attachment segment is able to induce a phagocytic-like event in the epithelial cell with which it is in contact. Presumably, this attachment segment has the ability to halt the progress of the "phagocytosis" at some point so that a stable attachment pocket is formed. The domain of modified host-cell cytoplasm associated with the attachment site suggests to us as well as to others (4) that stabilization of the attachment site might be mediated by a sol-to-gel conversion in the host-cell cytoplasm.

Early studies of cytoplasmic sol-gel transformations are based upon the model of amoeboid movement (8, 10). These workers suggested that such a transformation could be mediated by the appearance/disappearance of long filamentous molecular structures in the cytoplasmic matrix and that ATP caused the gelled cytoplasm to liquify presumably by relaxing the molecular structure of the cytoplasmic matrix. More recent findings (1) indicate that the structure of microtubules and microfilaments is affected by nucleoside triphosphates. It may be, then, that the segmented microbe somehow influences the state of the actin-like microfibrils known to be present in the apical cytoplasm of intestinal epithelial cells (13), possibly by influencing the adenylate charge in the immediate vicinity of the attachment site. If this is true, then it is conceivable that such a mechanism might explain how the attachment site forms as a phagocytic event and then stabilizes by a subsequent gelation of the epithelial cell cytoplasm in the immediate vicinity of the attachment site. It would be of considerable interest to determine whether either colchicine or cytochalasin B blocks attachment site formation by the filamentous organism. Unfortunately, experiments of this sort must await a practical in vitro model which permits axenic culture of the filamentous organism in a tissue culture system.

Previous observations (4, 5, 9) suggest that the host cytoplasmic membrane essentially remains intact at the site of contact with the attachment segment of the filamentous microbe, so that the microbe remains topologically outside the epithelial cell. In addition, the host membrane at the site of contact appears to have an unusually low concentration of intramembrane particles, suggesting a possible transition from liquid to solid state.

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