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Evidence for a Complex Life Cycle and Endospore Formation in the Attached, Filamentous, Segmented Bacterium from Murine Ileum

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Light and electron microscope observations showed that the filamentous, segmented bacterium commonly found attached to the ileal epithelium of rats and mice undergoes a complex life cycle. Filaments comprising up to 90 segments were attached to the microvillous border of absorptive epithelial cells by a specialized terminal holdfast segment. Starting at the free end of the filament and progressing toward the attached end, undifferentiated segments were converted into reproductive or mother segments. Within each mother cell two new holdfast segments developed. As the holdfasts matured, their mother cells degenerated and released them into the intervillar space where they attached, grew, and divided to produce new segmented filaments. Alternately, in some filaments, newly formed but not yet released holdfasts were converted into endospores, which were released in the same manner as holdfasts, presumably to spread the bacterial colony to other members of the rodent population.

Many systems have been described in recent years in which bacterial attachment to cellular substrates is important in microbial pathology or ecology (6). One such system has been studied primarily by electron microscopy and involves an unusual filamentous, segmented microbe that attaches, without apparent pathological consequences, to the ileal mucosa of mice (3, 8, 9) and rats (3, 4, 11). A similar microbe has also been observed by light microscopy in the ileum of the fowl (7).

The filamentous microbes from murine ileum are easily recognized in the transmission electron microscope by the distinctive structure of their filaments and attachment sites. Morphologically, the filaments and attachment sites are identical in rats and mice (3). Although the filamentous microbe was first identified at *Streptobacillus moniliformis* (8, 9), more recent observations suggest that it belongs in the *Arthromitaceae* (3).

The filamentous microbe is indigenous to the murine ileum and colonizes young mice within 20 to 25 days after birth (3). However, little is known about the manner in which colonies of attached filaments are maintained on the transient intestinal epithelium or how filaments are transmitted to newborn individuals. The present communication addresses these questions by presenting morphological evidence for a complex life cycle and endospore formation in the filamentous microbe. In addition, new observations on the ultrastructure and distribution of filaments are presented.

MATERIALS AND METHODS

Animals. Ten male and female rats, ranging in age from six weeks to one year, from several animal suppliers were examined in this study. A single male Sprague-Dawley rat provided the tissue for all but one of the transmission electron micrographs shown here.

Transmission electron microscopy. Tissues were collected from animals killed by ether fumes or intraabdominal injection of Nembutol. Ileum was taken from within 2 cm of the ileocecal junction, fixed for 1 h in buffered 1.5% glutaraldehyde, washed briefly in buffer, and postfixed for 1 h in buffered 2% osmium tetroxide. In all cases the buffer was 0.1 M phosphate, pH 7.4. The specimen shown in Fig. 5b was treated differently from all others in that it was immersed overnight in 0.5% aqueous uranyl acetate after a brief wash in distilled water. All tissues were dehydrated in a graded alcohol series followed by propylene oxide and embedded in Epon 812. Silver-grey sections were cut with diamond knives (Dupont), picked up on Parlodion- and carbon-coated grids, stained in saturated aqueous uranyl acetate followed by lead citrate, and examined at 80 kV in a Philips EM 200 electron microscope equipped with a 40-μm aperture.

Scanning electron microscopy. Small samples of intestine were fixed for 1 h in a mixture of 1.25% glutaraldehyde and 2% osmium tetroxide in 0.1 M cacodylate-HCl buffer at pH 7.4, dehydrated in ethanol, transferred to isoamyl acetate, and critical point dried according to Anderson (1). These speci-

mens were mounted on copper conduction tape (3M) attached to stubs with silver paint and coated first with carbon and then with a 10- to 30-nm layer of gold-palladium alloy, using a tilting omnirotary stage in a vacuum evaporator. Tissues thus prepared were examined in a Kent Cambridge Stereoscan S-4 scanning electron microscope at accelerating voltages of 5 to 20 kV and recorded on Polaroid 55 P/N film.

Light microscopy. For sectioned material, tissues were collected, processed, and embedded as for transmission electron microscopy. One-micrometer sections were cut, transferred to glass slides, and stained with toluidine blue. For light microscope observations of unfixed tissues, 1-cm-long segments of small intestine were taken at 7-cm intervals throughout the length of the small intestine, beginning within 2 cm of the ileocecal junction. These segments were opened and a scalpel blade, held perpendicular to the flattened sheet of intestine, was lightly scraped across the mucosa. The material collected on the scapel blade was smeared on a slide, covered with a cover slip, and examined with regular and differential interference phase optics on a Zeiss WL light microscope. Photomicrographs were made on Polaroid 55 P/N film.

RESULTS

Distribution of filaments in the intestine. Observations on fresh mucosal scrapings with the light microscope and on fixed specimens from different levels of the small intestine with the scanning electron microscope indicated that the frequency of attached microorganisms was greatest in the terminal ileum and gradually decreased toward the duodenum. The number of attached filaments dropped abruptly to zero at the ileocecal junction (Fig. 1), and the large intestine was devoid of these organisms. Within the ileum, filaments were most numerous on the distal half of the villi, although filaments in all stages of development were found from the tips of the villi to the floor of the intervillar space (Fig. 2). In agreement with Hampton and Rosario (9), we saw no filaments in crypts. In the otherwise normal appearing ileum of one rat, small numbers of filamentous microorganisms, as well as other morphologically distinct bacterial types, were present in capillaries of the lamina propria.

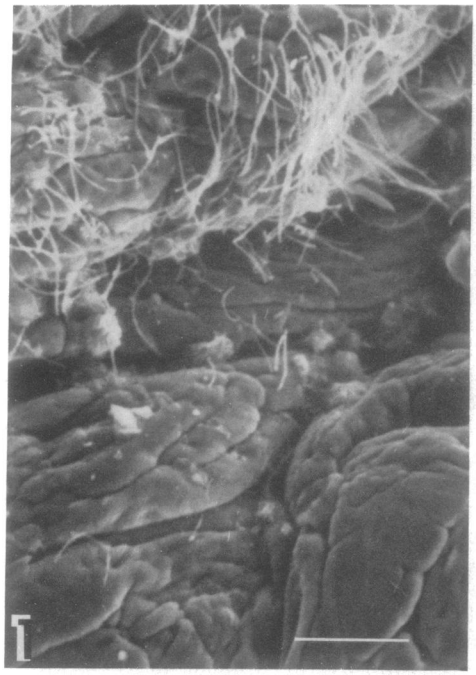
Morphology of filaments and segments. Both internal and external morphology of filaments varied with the stage of development. Filaments ranged from 0.7 to 1.8 μm in diameter and from 2 to about 80 μm in length. Younger filaments were narrow with straight sides and smoothly rounded, free ends (A, Fig. 3). Older filaments were wider and were often beaded in appearance (B, Fig. 3). Older beaded filaments of maximum length, like all younger filaments, had smoothly rounded free ends, but short beaded filaments had rough, irregular, free ends (C, Fig. 3).

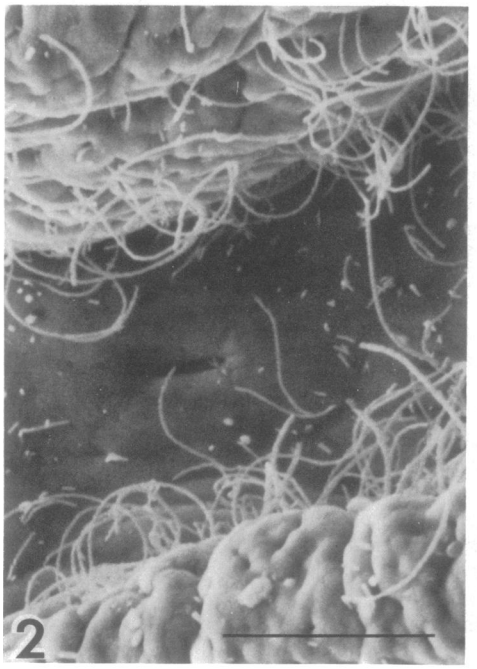
With the exception of several filaments that contained phagelike particles, all filaments or portions of filaments over approximately 8 μm long were segmented (Fig. 4). Segment length, width, shape, and content varied from filament to filament and often within single filaments. Though diverse in morphology, segments could be divided into three broad categories: undifferentiated segments, reproductive segments, and holdfast segments.

Undifferentiated segments (B, Fig. 4) were cylinders 1 to 3.5 μm long and 0.8 to 1.0 μm wide. They contained large numbers of ribosomes and a nucleoid represented by scattered electron-lucent areas. Mesosome-like structures, which were lamellar, tubular, or homogeneous, depending on plane of sectioning and perhaps also metabolic state or stage of development, were seen in some segments (Fig. 5a). The cell wall averaged about 40 nm in thickness and usually contained four layers designated, from outside inward, outer dense, outer lucent, inner dense, and inner lucent layers (Fig. 5a). A fifth layer was sometimes seen as a thin dense line at the outer margin of the outer lucent layer. The significance of the sporadic expression of this layer was not determined. Its presence was not related to the developmental stage of the filament.

The inner dense layer was the only visible component of the cell wall that extended into the septal wall (Fig. 5a). Immediately inside the inner dense layer or septal wall was a lucent space (arrow, Fig. 5a), which occasionally contained a faint dense line. This structure was seen more clearly in tissue treated with aqueous uranyl acetate after osmium postfixation (arrow, Fig. 5b). Because of its position and because it followed minute variations in the cytoplasmic profile with great fidelity, we assume that this dense line represents the outer lamella of the trilaminar cell membrane.

Individual holdfast segments were found within filaments (Fig. 6), free in the intervillar space (Fig. 7), and attached to absorbtive epithelial cells (Fig. 8). Individual holdfasts from these three locations shared several distinctive features, including a teardrop shape. The pointed end contained a finely textured matrix from which ribosomes were excluded. The nucleoid was a compact, rounded, lucent area centered in the dense mass of ribosomes that crowded the remainder of the cell. The cell wall was 22 nm thick and contained one dense and one lucent layer. The presence of individual holdfasts in these three sites and the obvious relationship between individual and anchoring





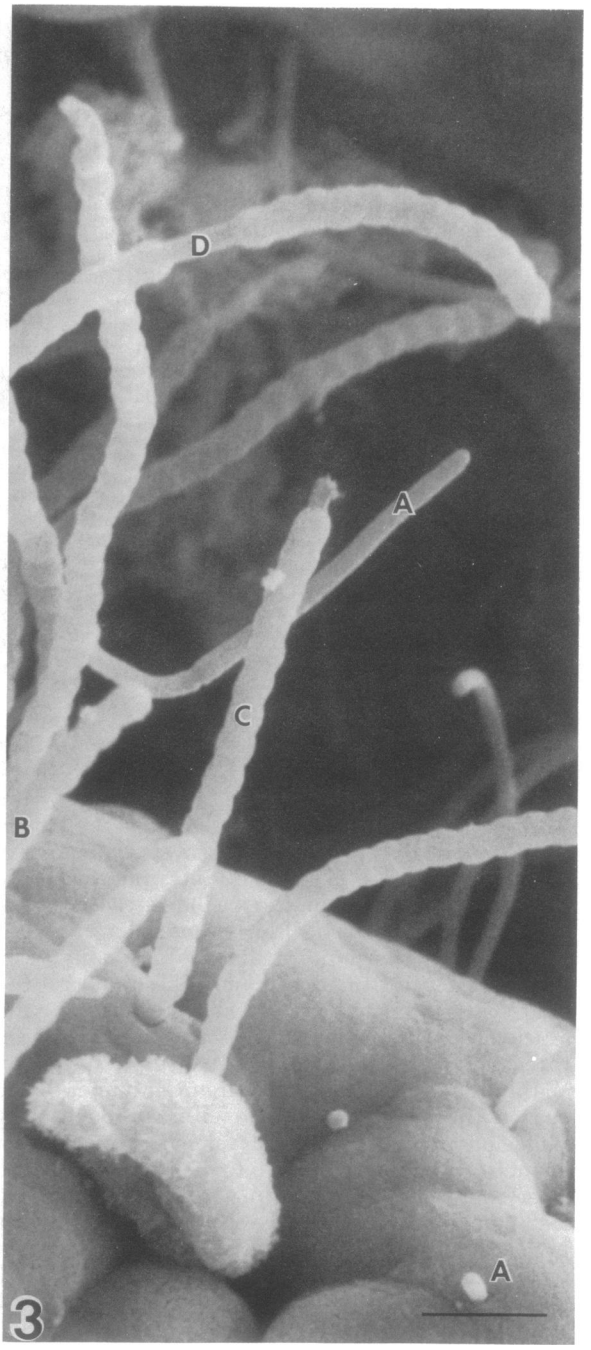


FIG. 1. *Scanning electron micrograph showing the junction between ileum and cecum. The ileal mucosa at the top of the figure is covered with filamentous microorganisms, whereas the cecal mucosa at the bottom is bare. Scale = 50 μm.*

FIG. 2. *Scanning electron micrograph showing portions of two villi and the mouth of a crypt centered in the floor of the intervillar space. Filaments of all sizes are present on the intervillar floor and the sides and tips of the villi. Scale = 50 μm.*

FIG. 3. *Scanning electron micrograph showing filaments of various ages at higher magnification. Young slender filaments with smoothly rounded ends are seen at A. Most of the filaments in the foreground are older, broader, beaded filaments composed of barrel-shaped segments. The smoothly rounded end of a long beaded filament (at least 40 more segments were present out of the frame of this figure) is indicated at B. A short beaded filament with a rough irregular end is shown at C. A short region of narrow unexpanded segments interrupts a long sequence of barrel-shaped segments at D. The large irregular mass at the lower left is extruded mucous. Scale = 5 μm.*

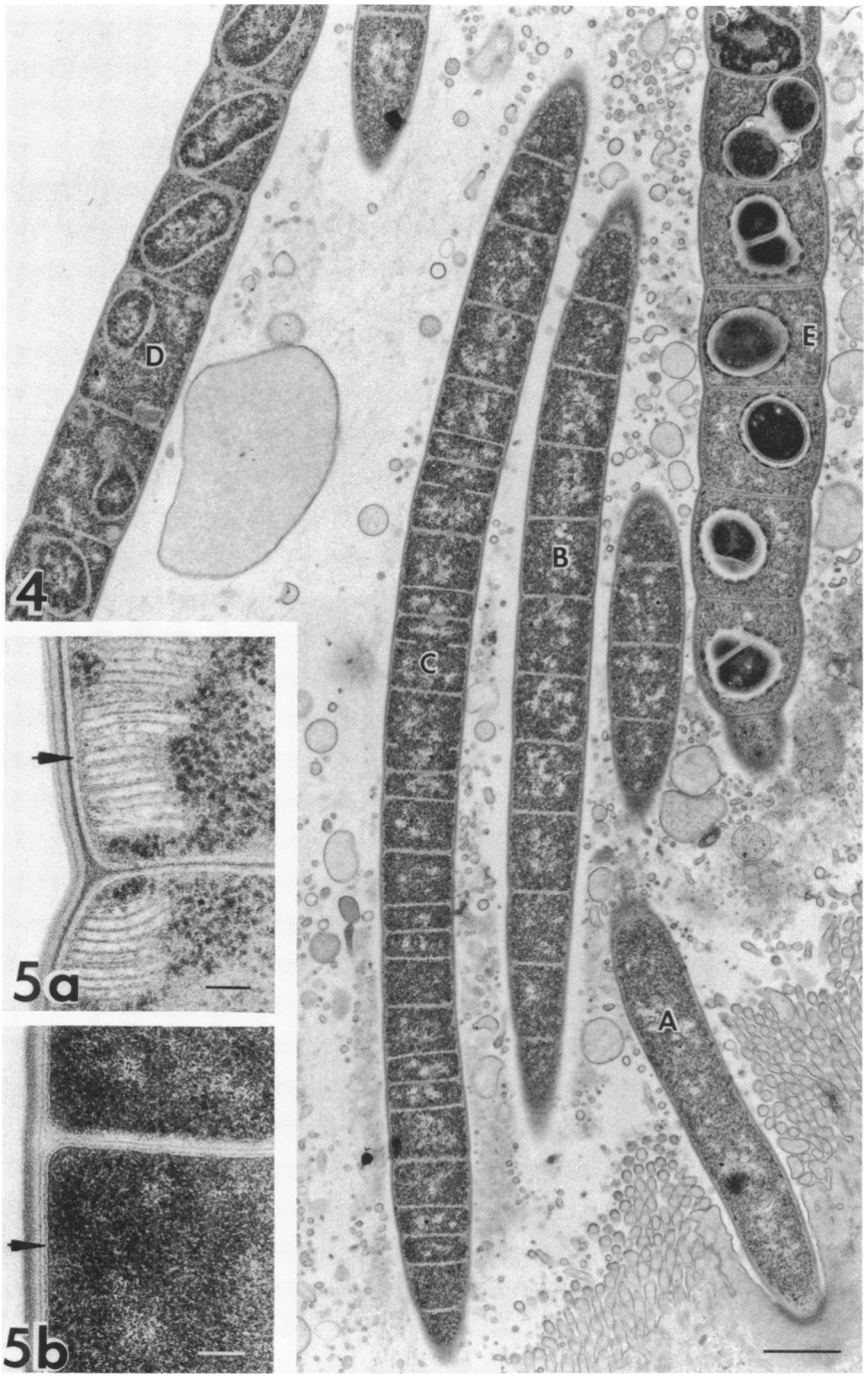


FIG. 4. *Thin section through portions of several filaments in various stages of development. An anchoring holdfast segment is present at A. At B is a filament composed of secondary segments, and at C is a filament containing short tertiary segments, which were destined to become intrasegmental inclusion bodies like those in the filaments at D and E. The inclusions in filament E are developing endospores. Scale = 1.0 μm.*

FIG. 5. *Portions of two pairs of contiguous segments showing lamellar mesosome-like organelles (a) and the structure of the cell wall and transverse septum (a and b). Arrows indicate the position of the bacterial cell membrane. In (b), which was postfixed with uranyl acetate, the tip of the arrow rests on the outer lamina of the trilaminar unit membrane. In (a), which was not postfixed in uranyl acetate, the arrow rests in the same relative position, but the unit membrane was not preserved. Scale = 100 nm.*

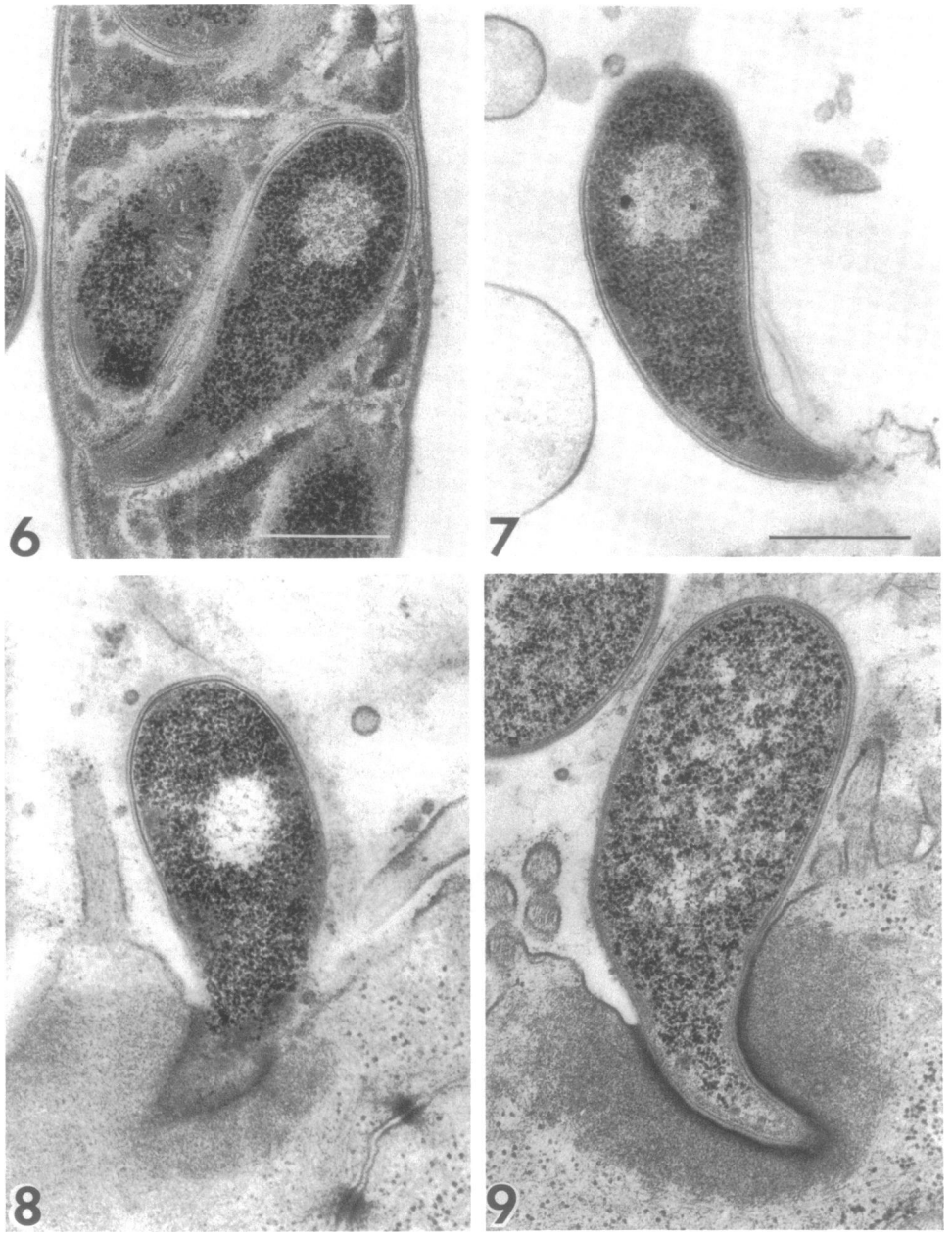


FIG. 6. *Individual holdfast segments, recognizable by their pointed ribosome-free ends, compact nucleoids, and two-layered cell walls, in a section of filament in which transverse septa have disappeared. Scale = 500 nm.*

FIG. 7. *Individual holdfast segment free in the intervillar space. Scale = 500 nm.*

FIG. 8. *Individual holdfast segment attached to an absorptive epithelial cell.*

FIG. 9. *Early stage in filament growth in which the cell wall structure is like that of an individual holdfast but the nucleoid is dispersed like that of undifferentiated segments.*

holdfasts suggest the existence of a life cycle in which filaments produce and release holdfasts that attach and generate new filaments.

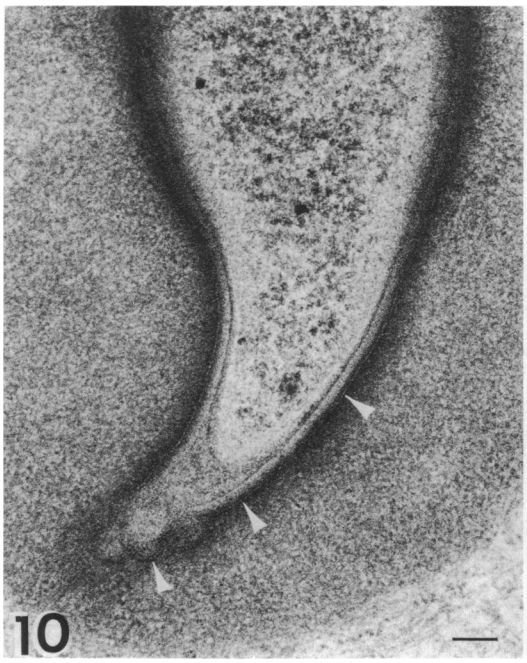
Anchoring holdfasts attached filaments to the microvillous border of absorbtive epithelial cells (Fig. 12). The morphology of the microfilament-reinforced attachment sites has been described (3, 4, 11), and only a few points relating

to holdfast morphology need be added here. The conical, attached end of anchoring holdfasts was sometimes studded with rounded protrusions (Fig. 10) whose significance is undetermined. The free cell wall was similar in morphology and dimensions to that of undifferentiated and reproductive segments, but the attached cell wall, like the cell wall of individual holdfasts, was thinner (20 nm) and contained only two, rather than four, layers (Fig. 11). In other respects anchoring holdfasts were similar to undifferentiated segments.

Previous workers tentatively expressed the belief that the plasma membrane of the host cell remains intact at the attachment site but failed to present convincing data (3, 9). Figure 11 shows the juncture between free and contact surfaces in a section perpendicular to the plane of the membrane. The limiting unit membrane of the host cell can be traced on both sides of the holdfast from free cell surface to a point deep in the contact area. Since both outer and inner dense lamina of the unit membrane of the host cell merge with surrounding dense structures after contact with the bacterial cell wall, the most easily traced entity is the lucent inner lamina, which is seen as a thin bright line in Fig. 10 and 11. In Fig. 10 the host cell plasma membrane is visible on the innermost tip of the holdfast in regions where it passes perpendicular to the plane of sectioning (arrows). These micrographs do not prove membrane continuity over the whole contact surface at a single attachment site. Nevertheless, in combination with our failure to observe membrane discontinuities in any of a large number of sections of attachment sites made in various planes relative to the holdfast, they strongly support the conclusion that the holdfast does not breach the host cell plasma membrane.

Filament growth. Figure 9 shows an early stage in growth in which the filament was only slightly larger than an individual holdfast segment. The cell wall was still two layered, as in individual holdfasts, but the nucleoid had become dispersed like that of undifferentiated cells. The addition of new material to free (i.e., unattached) cell wall must occur after the increase in filament length begins, but before the start of segmentation, since an unsegmented 5-μm-long filament we observed possessed the four-layered cell wall typical of older filaments.

Filaments did not grow to their final length before becoming segmented; rather, they were



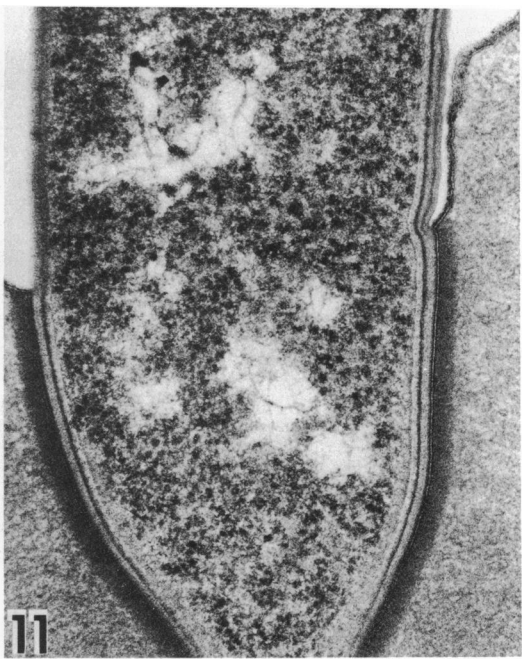


FIG. 10. *Rounded protrusions and host cell plasma membrane (indicated by arrows) are shown at the conical end of an attached holdfast. Scale = 100 nm.*

FIG. 11. *Attachment site in which unattached host cell plasma membrane at the upper right can be traced well beyond the point of contact with bacterial cell wall. A change in the thickness and number of layers in the cell wall and in the density of the cytoplasm underlying the epithelial cell membrane are evident at the junction between free and attached surfaces. Scale = 100 nm.*

divided by transverse septum formation (Fig. 13) into "primary" undifferentiated segments of 2 to 3.4 μm in length as they elongated (Fig. 12 and 13). Although the length of primary segments varied as much as 1.4 μm among different filaments, it varied no more than 0.3 μm in single filaments. On reaching some final filament length in the range of 50 to 80 μm, a new wave of transverse septum formation began at the free end of the filament and gradually progressed towards the attached end, dividing primary segments into pairs of "secondary" undifferentiated segments (B, Fig. 4) whose length ranged from 1 to 1.7 μm in different filaments. This sequence of events was indicated by the following observations. Short, therefore presumably young, filaments were always made up of primary undifferentiated segments. In long filaments containing both primary and secondary undifferentiated segments, the secondary segments were located at the free end of the filament. Primary undifferentiated segments were often seen being divided into equal halves by incomplete transverse septa. These dividing segments were located between groups of primary and secondary segments. Holdfast formation always began in segments 1 to 1.5 μm long, indicating that secondary segments were more mature than primary segments.

Holdfast formation. The inclusions of reproductive segments, when arranged in the order of increasing complexity, suggested a sequence of stages in the formation of new holdfasts or endospores.

Holdfast formation was initiated by a third round of cell divisions which, by the development of septa on either side of alternate existing cross walls, partitioned each secondary segment into two compartments of unequal length (C, Fig. 4 and Fig. 14 and 19). Through growth and rearrangement of the new septa (Fig. 14 and 15) the short compartments were transformed into spherical bodies enclosed within mother cells derived from the original secondary segments. The spherical inclusions were bounded by structures that developed from the septum and, therefore, comprised a portion of the cell wall sandwiched between two membranes, which, for reasons already discussed, are not shown in Fig. 15. Up to this point the process was morphologically very similar to forespore formation in gram-positive spore-forming rods (5). However, in the filamentous organism the spherical inclusions never devel-

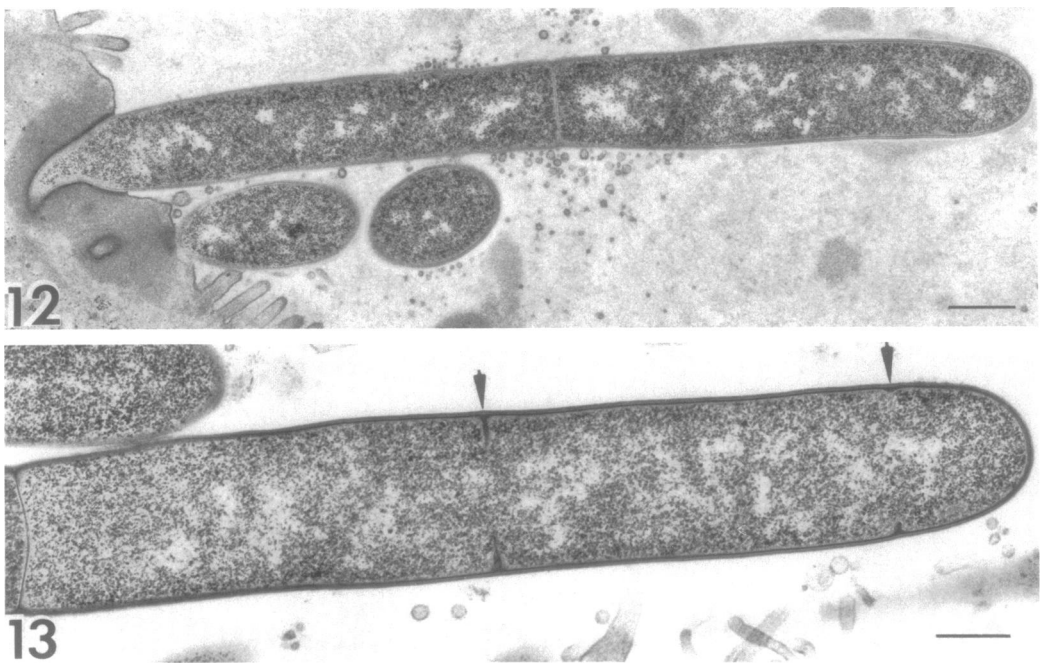


FIG. 12. *Short filament consisting of one undifferentiated primary segment and one anchoring holdfast segment. The full extent of the curved conical tip of the holdfast and of the underlying modified host cell cytoplasm are shown. Scale = 500 nm.*

FIG. 13. *Free end of a growing filament showing one complete and two developing (arrows) transverse septa. Scale = 500 nm.*

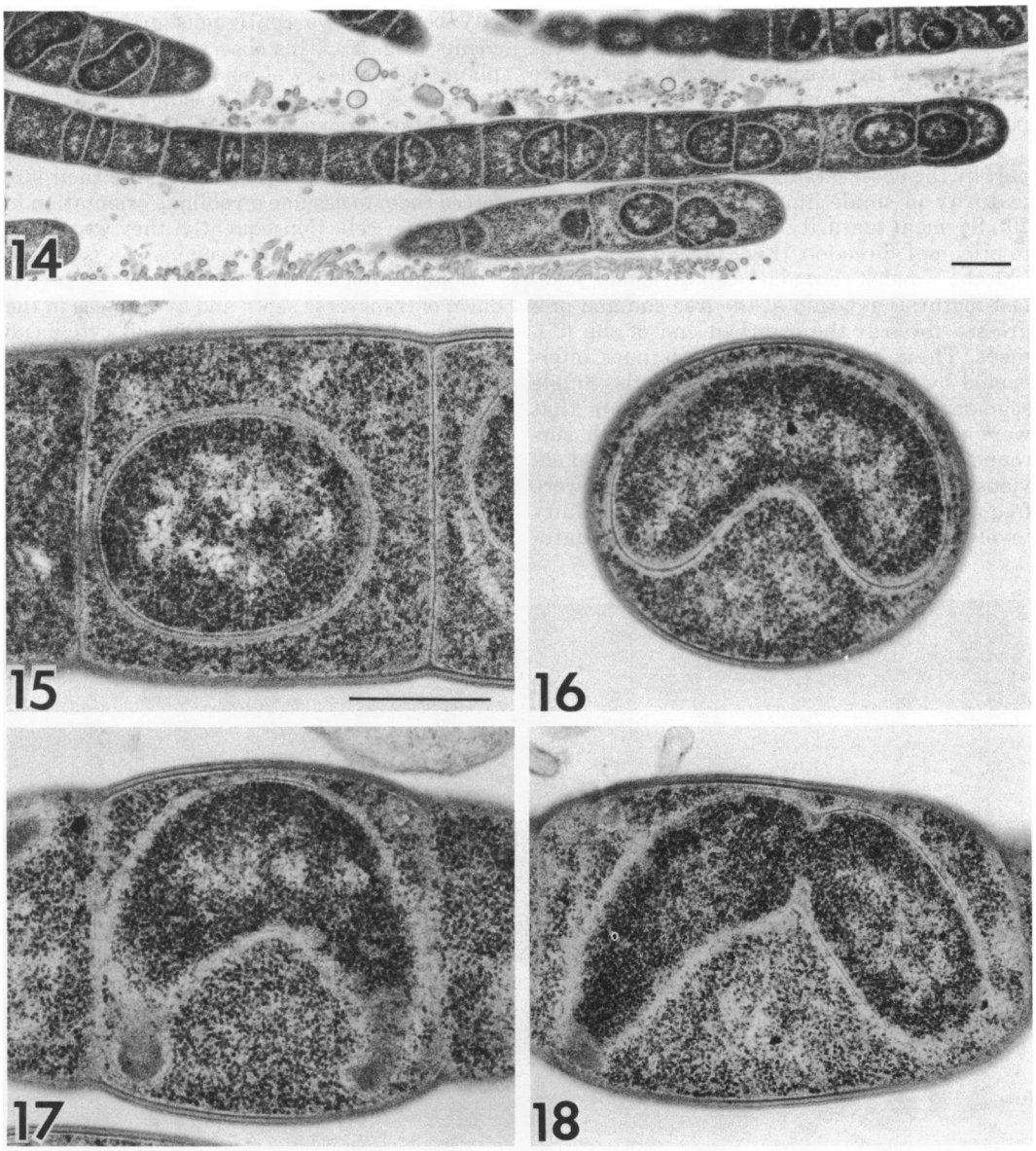


FIG. 14. *Free end of a filament the segments of which contained the earliest stages of holdfast formation, beginning with tertiary segments on the left and grading through stages destined to produce spherical forespore-like inclusions on the right. Scale = 10 μm.*

FIG. 15. *Spherical forespore-like inclusion in a mother cell. Scale = 500 nm.*

FIG. 16. *C-shaped inclusion body.*

FIG. 17. *C-shaped inclusion with pointed ribosome-free ends.*

FIG. 18. *C-shaped inclusion with pointed ends in the process of dividing into two presumptive holdfasts. Note that the nucleoid is still diffuse rather than compact as in mature individual holdfasts.*

oped directly into spores. Instead they were always converted into pairs of holdfasts, which were either released directly from the filament as individual holdfasts or converted into spores.

Holdfast differentiation continued with the conversion of the spherical forespore-like inclusion into a "c"-shaped inclusion with rounded ends (Fig. 16). The ends of this structure then become pointed and ribosome free (Fig. 17), and the resulting inclusion developed into two pre-

sumptive holdfasts by constricting in two at the arch of the "c" (Fig. 18). Final maturation of the holdfast also involved the transition at a late stage from the diffuse (Fig. 16–18) to the compact (Fig. 6–8) nucleoid.

The foregoing sequence was confirmed in part by the observation of gradients of segment maturity in single filaments (Fig. 14, 19, and 20). Segment maturity decreased in the distal-to-proximal direction (Fig. 14 and 20). Thus, like the wave of secondary cell divisions, holdfast maturation began at the free end and progressed towards the attached end of the filament. These gradients were sometimes interrupted by discontinuities of two kinds. Single segments or small groups of segments that were either well out of synchrony with surrounding segments (D, Fig. 3) or contained obviously abnormal inclusions (E, Fig. 4) were frequently encountered. Another discontinuity involved an abrupt change in segment maturity between two contiguous groups of segments (Fig. 20). This observation no doubt explains the tendency of holdfasts to mature and be released in groups. The stages of holdfast maturation shown in Fig. 16 through 18 were not seen in their entirety in sections cut along the long axis of the filament because their size forced them to assume an oblique orientation in the mother cell. Consequently, they were fully revealed only in oblique sections.

Holdfast release was signaled by the breakdown of transverse septa and by changes in the texture and density of mother cell cytoplasm that suggested autolysis (Fig. 6 and 21) and was completed with the disintegration of the lateral cell wall. The rough irregular ends of short beaded filaments described earlier (C, Fig. 3) may represent scars remaining after the release of groups of mature holdfasts.

Spore formation. In some filaments newly formed holdfasts, rather than being released

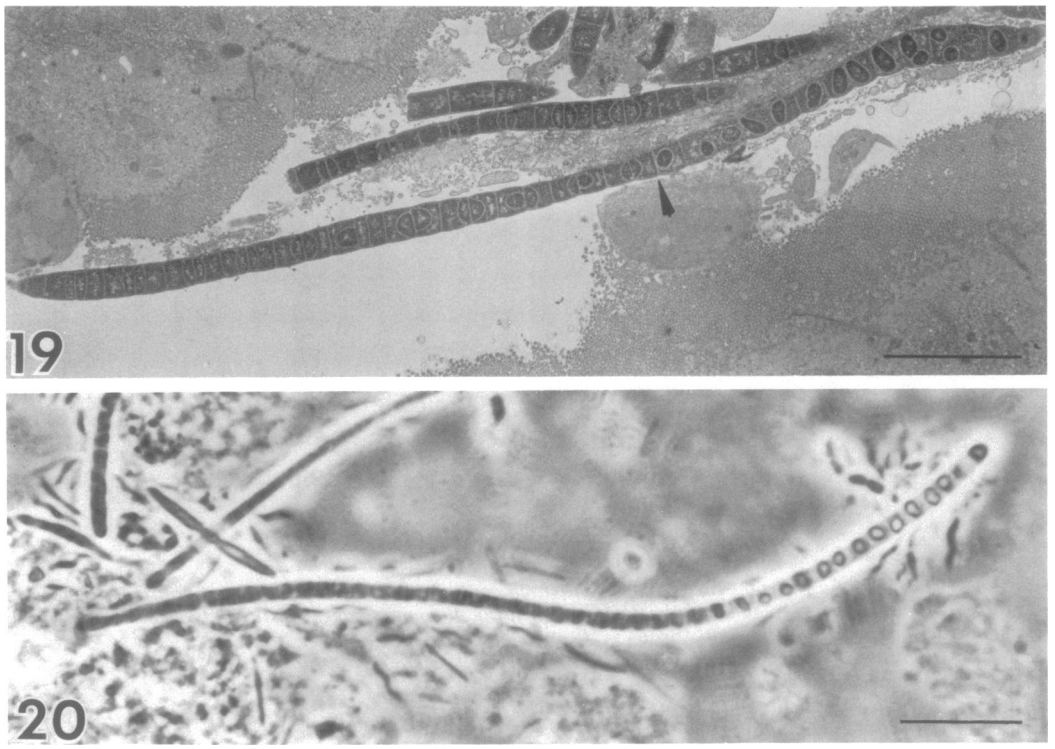


FIG. 19. *Long chain of reproductive segments which show various stages in holdfast formation ranging from tertiary segments on the left to dividing c-shaped inclusions on the right. There is an abrupt transition in the stage of segment maturity at the arrow. To the right are advanced c-shaped inclusions; to the left are stages preceding the spherical inclusion. Scale = 5 μm.*

FIG. 20. *Light micrograph (phase) of a complete filament. The attachment site is seen on the left as a darker area around the end of the filament. The smoothly rounded free end is at the right. The refractile bodies in the free terminal segments are developing spores. More proximal segments contain developing holdfasts. Scale = 10 μm.*

from the filament, were incorporated into spores. Like holdfasts, spores developed synchronously in terminal groups of contiguous segments (Fig. 20). One spore developed in each segment and contained two holdfasts. The manner of deposition of spore walls in the filamentous microorganism was similar to endospore formation in *Clostridium* and *Bacillus* (reviewed in reference 5).

Spore formation was indicated by an increase in holdfast density relative to mother cell density and by the appearance of what we assumed to be cortex and spore coat material. The latter developed around holdfasts as irregularly spaced, dense, wafer-like plaques (Fig. 22) whose margins were surrounded by single layers of ribosome-sized dense granules (Fig. 23). The electron-lucent cortex was found within the incipient spore coat and immediately external to the inner dense layer (Fig. 22), the only layer of cell wall material around the holdfasts at this stage. As maturation progressed, the two holdfasts in each mother cell were drawn closer together and became more electron dense, whereas the cortex thickened and the plaques of spore coat material became more numerous (Fig. 23). As the cortex continued to increase in thickness, the dense plaques developed into a thin confluent layer of inner spore coat, and a new layer of less dense outer spore coat was added (Fig. 24). Thickening of the outer spore coat and clearing of the cortex were seen in spores in which the contents were no longer stainable (Fig. 25). Presumably, the lack of staining resulted from the failure of fixatives to penetrate the walls of mature spores.

DISCUSSION

The general morphology of the filamentous, segmented microbe and its distribution and attachment to the intestinal mucosa were first described in mouse ileum by Hampton (8) and Hampton and Rosario (9). Reimann (11) observed morphologically similar filamentous organisms attached to the ileal mucosa of rats. His figures included sections of filaments containing sporelike bodies, which he suggested might be developmental stages. Recently, a more detailed life cycle has been proposed (S. Erlandsen, D. Chase, G. Wendelschafer, and J. Rolston, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, G188, p. 57). Some support for this life cycle was provided by Davis and Savage (3), who presented figures of intrasegmental bodies that resembled holdfasts. However, these authors expressed reservations about accepting the proposed life cycle without more evidence and particularly without culturing the microorganism.

Sufficient data have been presented here to construct a detailed life cycle with no major gaps. This life cycle answers at least the more obvious demands of the environment in which the microbe is consistently found. Thus, if these organisms are indigenous to the murine ileum, as suggested by the observations of Davis and Savage (3), some means must exist for maintaining the microbial population on a substrate that turns over continuously. This requirement is clearly met by holdfast production. The appearance of filamentous microbes in young mice within a few weeks after birth suggests the existence of a microbial stage capable of surviving outside the host. This function is most probably served by the spores. Thus, the completeness of the data and the internal and teleological consistency of the interpretations support the validity of the proposed life cycle.

A clue to the duration of the life cycle is gained from the observation that filaments of all stages can be found on the floor of the intervillar space (Fig. 2). This means that a filament can progress to an advanced stage before the epithelial cell to which it is attached can migrate onto a villus. Thus, unless attachment

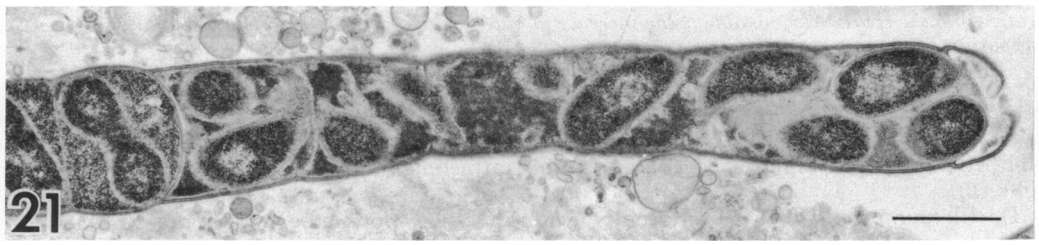


FIG. 21. *Portion of a filament in the process of releasing holdfasts. The cell wall at the free end is ruptured. The transverse septa of about the last six segments, which contained mature individual holdfasts, are no longer evident. Just proximal to this group are two segments with intact septa containing developing holdfasts. Scale = 500 nm.*

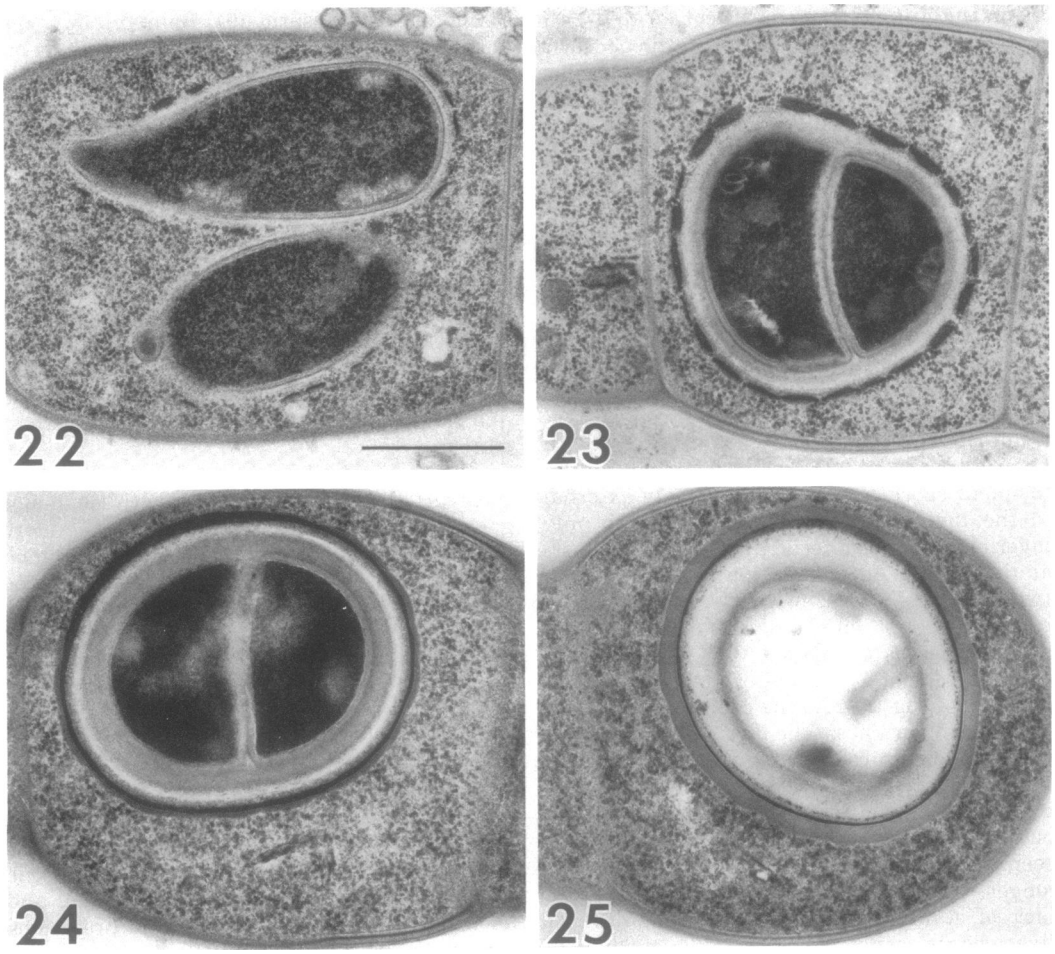


FIG. 22. *Early stage in spore formation. Inner spore coat material is seen as thin, very dense plaques in the mother cell cytoplasm around the holdfasts. Immediately outside the cell wall of the holdfasts is a thin lucent layer assumed to be incipient cortex. The density of the holdfast cytoplasm is greater than that of mother cell cytoplasm. Scale = 500 nm.*

FIG. 23. *Slightly more advanced stage in spore formation. Changes in the amount of spore coat material, in thickness of the cortex, and in the density of the spore contents are evident. A round plaque of tangentially sectioned spore coat material surrounded by a single layer of evenly spaced, ribosome-sized granules is seen at the left edge of the figure.*

FIG. 24. *Later stage in spore formation showing thick cortex, compact spore content, continuous inner spore coat, and the addition of an outer spore coat.*

FIG. 25. *Mature spore in which the maturation of the spore wall evidently prevented the penetration of fixative into the spore. Also notable is the increase in outer spore coat thickness relative to earlier stages. No exosporium is present.*

retards or inhibits cell migration, microbial maturation should occur well within the 20- to 30-h villous transit time of rodent epithelial cells (10).

The filamentous microbe was originally identified in mice as *Streptobacillus moniliformis*, an agent of rat bite fever (8, 9). Despite its similarity to the microbe described by Hampton, Reimann (11) concluded that the filamentous organism he saw in rat ileum was probably a fungus. Davis and Savage (3) noted that the filamentous microbes and their characteristic attachment sites were morphologically identical in rats and mice and established that the microbes were indigenous to the ileum in many strains of these hosts. These findings, combined with their inability to culture the filamentous microbe, led Davis and Savage (3) to conclude that it is not *S. moniliformis* but, rather, is a member of the *Arthromitaceae*, a group of fila-

mentous, segmented sporeformers described many years ago from the intestines of certain arthropods and amphibians (see reference 2, p. 835). The observations presented here, particularly the demonstration of spore formation, provide strong support for this conclusion.

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