Ultrastructural Morphology of Some Prokaryotic Microorganisms Associated with the Hindgut of Cockroaches

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Scanning electron microscopy and transmission electron microscopy have been used to visualize the morphology and ultrastructure of two types of microorganisms in the hindgut of the cockroach Blaberus posticus. Both organisms, designated as either short or long rods, are attached to chitinous projections from the gut wall. Micrographs suggest that the organisms are prokaryotic with a cell wall complex characteristic of gram-negative bacteria. However, certain differences were noted between the cell wall complex of the two types. Two forms of the long-rod type were noted, with one form appearing to be a "degenerate" or "transitional" cell. In the degenerate cells, vesicles are observed that often are contiguous with the cytoplasmic membrane. There are indications that the long-rod type may divide by longitudinal fission. Neither the short- nor long-rod type has been cultivated in its respective recognizable form.

Numerous kinds of microorganisms have been reported to occur in the gut of cockroaches (9). Some have simply been observed microscopically and others have been isolated and identified. In murine guts, some autochthonous bacteria attach to or associate with the gut tissue (5, 6, 7, 11, 12). During a study of the strict anaerobic bacteria of the hindgut of the cockroach Blaberus posticus scanning electron microscopy (SEM) and transmission electron microscopy (TEM) examinations of the hindgut were undertaken in an attempt to ascertain whether any of the identified anaerobes could be located morphologically in intimate association with the hindgut, perhaps at specific areas of the gut. The work reported here illustrates the morphology and ultrastructure of the microorganisms attached to spine-like projections emanating from the cuticular layer of the insect's hindgut. Correlation of the scanning and transmission micrographs indicates two predominant types of attached bacteria.

MATERIALS AND METHODS

General preparation for SEM. For the initial scanning electron micrographs of guts, cockroaches were dissected to expose the alimentary canal, and the hindgut was ligated and perfused with a 1% osmium tetroxide-2.5% glutaraldehyde fixative in 0.1 M cacodylate buffer, pH 7.2. After 30 min, the ligated portion was excised and placed in the above fixative for an additional 30 min. After fixation, the tissue was washed in buffer for 1 h and dehydrated to 70% ethanol. The tissue was cut into doughnut-shaped sections, sliced in half to give two C-sections, dehydrated to 100% ethanol, and placed in amyl acetate. After critical-point drying, the specimens were mounted on SEM disks, coated with gold-palladium, and viewed in a Kent Cambridge Stereoscan S4 scanning electron microscope operated at accelerating voltages of 5 to 20 kV and recorded on Polaroid type 55 P/N film.

Preparation for TEM. For subsequent microscopy examinations reported in this paper, the hindgut was removed, slit longitudinally, placed in a small amount of phosphate buffer (pH 7.2), and spun vigorously in a Virtis tissue homogenizer for 2 min. Some of the material was prepared, as above, for SEM examination. The remaining material was fixed and prepared for thin sectioning and TEM examination. The fixed specimen was dehydrated through increasing propylene oxide concentrations and embedded in Epon 812. Blocks were cut with a diamond knife on an LKB-Huxley ultramicrotome, and sections were collected on 300-mesh copper grids with a carbon-coated parlodion supporting film. Sections were stained with 7% uranyl acetate for 30 min and with Reynold lead stain (10) for 1 min. Sections were examined and photographed by using a JEOL 100B transmission electron microscope operated at 60 kV.

RESULTS

Ultrastructure as revealed by SEM. Scanning electron micrographs of C-sections from the hindgut revealed a multitude of bacterial-like organisms ranging from spirichetes to short-rod forms. Numerous organisms, long filaments, and larger spines appeared to be associated with the gut wall. However, it was difficult to ascertain whether organisms were free in the
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Fig. 1. Low-magnification SEM micrograph of the cockroach hindgut (bar represents 10 μm). Note the many small filaments and prominent spine-like projections (spines) emanating from the cuticular floor. The floor appears to be carpeted with short rods; these rods can also be seen adhering to the spines (white arrows, center).

Fig. 2. High-magnification SEM micrograph of the region of Fig. 1 indicated by the thick arrow on the lower right. Note the short, rough rods attached to the floor and one of the spines. Note also the long smooth rods apparently attached to the floor. The bar represents 2 μm.
lumen or were associated with any structural elements.

In an attempt to remove unattached organisms and debris from the gut, the hindgut was removed, slit, spun in a Virtis mixer, and examined by SEM as described above. Figure 1 shows a portion of the gut wall obtained after this treatment. Since the cockroaches were fully adult, the hindgut wall was covered by a rigid cuticular layer—an invagination of the exoskeleton material. Numerous small filaments and large prominent spines can be seen projecting away from or emanating out of the cuticular layer. The layer itself is covered with a mat of bacterial-like structures. These short rods are frequently attached to the spines either singly or in clusters (Fig. 1, arrows). The spines themselves have an average width of 1.10 μm: 20 were measured and the variation was from 0.63 to 1.52 μm. From many other scanning micrographs (not shown), the spines reach a length of ~50 μm, taper slightly (15 to 20% from base to tip), and terminate with a blunt end.

Figure 2 is a higher magnification of a portion of Fig. 1. The short rods covering the floor and packed onto the base of a spine are somewhat irregular in appearance and exhibit a rough appearance, suggesting the presence of a capsule or slime layer. They vary from 0.3 to 0.5 μm in width and 0.7 to 1.0 μm in length. Also seen in Fig. 2 is a “spray” of long tapering rods. These are more dramatically shown in Fig. 3 and 4 where they are clustered in their attachment to the spines. In Fig. 4 they are very tightly clustered and several appear to be dividing longitudinally. From the scanning micrographs, these microorganisms are 2.0 to 2.5 μm in length and 0.5 to 0.7 μm in width.

Ultrastructure as revealed by TEM. Considerably more information is revealed concerning the organisms described above from transmission electron micrographs of thin sections of the cockroach hindgut. These ultrastructural details are shown in Fig. 5 through 12. Figure 5, a longitudinal section through a spine, has both of the previously described organisms attached. Before a detailed description of the ultrastructure of the organisms is presented, the spines warrant some comment. In Fig. 5 through 12, the average diameter is 0.9 μm with a range of 0.6 to 1.4 μm, which is consistent with the dimensions observed by SEM. (Assuming the

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**Fig. 3, 4.** SEM micrographs of long smooth rods attached to spines in clustered arrays. The rods are similar in size and shape to those shown in Fig. 2. Those in Fig. 4 are closely packed around the spine, and at least one (arrow) has a split suggestive of longitudinal fission. The bar represents 2 μm.
spines are circular in cross sections, the shortest distances were measured to allow for error possible in tangential cuts.) The interiors of the spines have an amorphous structure consistent with their proteinaceous-chitinous composition. The presence of chitin is further suggested by the tearing artifacts in Fig. 8, 11, and 12 and by the peripheral gaps showing in most of the figures. The spine is usually surrounded by a fibrillar border 80 ± 10 nm thick. Since the spines are probably secreted extensions of the cuticular layer of the hindgut, which is itself a secreted product, it is not likely that these borders are produced by the spines. Their morphology suggests that they are polysaccharide in nature and may be a residuum from previously attached bacteria.

The attached bacteria were termed short rods and long rods in keeping with the SEM observations. The short rods are shown in Fig. 5 through 8. They are usually closely packed around the spine. Sections of the short rods are usually round or ovoid as in Fig. 8, but irregular shapes can be seen in Fig. 5 through 7. Their size is about 0.6 μm in diameter with variations from 0.4 to 0.9 μm. The length of the long rods (Fig. 5; 9–12) is about 1.8 μm, with variations from 1.3 to 2.2 μm. All these dimensions are consistent with those obtained from SEM.

The ultrastructure of the short rods is described as follows. The large central region of the cytoplasm is electron transparent and reticulated in appearance, typical of bacterial nucleoplasm containing deoxyribonucleic acid. Ribosomes and other dense material are confined to the periphery of the cytoplasm. The cytoplasmic membrane can be seen in the Fig. 8 insets. The cell wall is complex and characteristic of gram-negative bacteria (4). There is no discernible "periplasmic space," and a distinct peptidoglycan layer is not readily distinguishable as such. The outer membrane of the envelope is seen in Fig. 8 insets as a transparent line.

Fig. 5. Rare TEM micrograph of a thick longitudinal section through a spine showing two types of microorganisms attached. The smaller organisms in the lower half of the micrographs are probably analogous to the short, rough rods of Fig. 1 and 2 (see Fig. 6 through 8); the larger, more-electron-dense rods in the upper half probably correspond to the long, smooth rods of Fig. 2 through 4 (see Fig. 9 through 12). Note the appearance of the spine in this and succeeding figures: it is usually "pulled away" in many places from the surrounding portion of the section, an artifact due to its chitinous structure. The magnification bar in this and all subsequent figures represents 1 μm; the inset bars, 0.1 μm.
Fig. 6, 7. Cross sections of spines with the smaller microorganisms attached (morphologically identifiable with those in the lower half of Fig. 5). Note their typical procaryotic bacterial morphology: complex cell wall, lack of conspicuous internal organization, and electron-transparent areas indicative of nuclear material. The periphery of the cells is outlined by a prominent "coat" consisting of a moderately dense outer corona, a thick transparent layer, and another dense layer contiguous with the outer membrane of the wall (see inset). The corona and transparent layer of the coat lose their morphological integrity and are "replaced" by long fibrillar material (capsular?) especially where attachment to the spines is apparent. An exception is indicated by the arrow (see inset) in Fig. 7.

Fig. 8. Cross section similar to Fig. 6 and 7 but showing cells closely packed and more regular in shape. The same features can be seen, but the outer corona of the coat has a more extensive mass of material. The transparent layer is again prominent except where the long fibrillar substance emanates to attach to the spine. Insets are higher magnifications of the cell wall regions indicated by the two arrows which show the inner cytoplasmic membrane and the outer membrane of the cell wall, the later being contiguous with the complex coat.
running parallel to the transparent line of the cytoplasmic membrane. The distance between the two membranes is 15 to 18 nm. Contiguous with the outer membrane is a prominent coat (probably lipopolysaccharide) 25 to 30 nm thick and consisting of a very dense, compact zone, a prominent transparent zone, and another dense zone (Fig. 7, inset). This distinctive coat outlines the cells nicely even when an additional fuzzy layer (20 to 25 nm) is present as seen in Fig. 8. The integrity of the primary coat structure is lost only in those areas where the cells attach to the spine: fibrillar material stretches from the zone adjacent to the outer membrane to the spine (Fig. 6–8) or to the layer bordering the spine (not shown), the distance varying from 0.11 to 0.26 μm. The only exception to this rule is noted in Fig. 7 (and inset), where a 0.15-

![Fig. 9. Longitudinal section of the tip of a spine with long rods attached. Two variants of the cells can be observed: the minority variant is not as heavily stained, shows finely dispersed nuclear regions (electron transparent), and has a smooth and even cell wall; the other variant is more densely stained, is full of small electron-dense vesicles, often has electron-transparent vacuoles, and is contained by an undulating or irregular cell wall. Note the attachment to the spine via the prominent fibrillar layer coating the spine. Note also the cell apparently undergoing division; the arrow points to an invagination of the cell wall about 0.2 μm in length.](http://jb.asm.org/)

to 0.21-μm fibrillar layer emanates from one side of the cell (it could be attached, however, to another spine not seen in the section).

The long rods, besides being a different size and shape, differ considerably from the short rods in their ultrastructure. Two types of long rods can be seen in Fig. 9 through 12. The minority class, which we call healthy cells, is less densely stained. This is particularly evident in Fig. 10. The majority class is degenerate cells or transitional cells. The healthy cells have sparse regions of nucleoplasm scattered throughout the cytoplasm. These gram-negative cells have a very smooth, continuous cell wall; the cytoplasmic membrane and outer membrane of the wall (Fig. 11, insets) are the same distance apart as in the short rods described above. The coat, however, differs in the following respects. The first zone, immediately contiguous with or part of the outer envelope, is seen as a very dense line (Fig. 10, inset; Fig. 11, insets). The next zone (transparent) is not prominent as with the short rods and is sometimes non-existent. The outer zone is a thin, fuzzy band. The entire coat is 18 to 24 nm thick (compared with the 25- to 30-nm primary coat of the short rods). In Fig. 9, one of the cells appears to be dividing longitudinally; this is supported further by a 0.2-μm invagination (arrow) proceeding distally from the basal attachment. See Fig. 4 for similar evidence from SEM.

The transitional and degenerate long rods differ from the healthy cells in possessing little or no discernible nucleoplasm as such, many unit membrane vesicles (Fig. 9 through 12), and, often, very-electron-transparent vacuoles (Fig. 10, 11). They have progressively irregular wall outlines with extensive invaginations. These are particularly noticeable in Fig. 12 where the plane of the section cuts through several different cells at an angle. Some vesicles

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**Fig. 10.** Cross section of a spine with long rods attached. Most of the same features described for Fig. 9 can be discerned; moreover, the difference in staining density between the two variant cell types is much more apparent. The inset is a higher magnification of the distal end of the cell indicated by the arrow. The inset arrow points to a particularly dense line which is contiguous with or part of the outer envelope of the cell wall.
Fig. 11. Serial cross sections of long rods attached to a spine, indicating a close packing arrangement analogous to that seen in one of the scanning micrographs (Fig. 4). Again the variant types can be seen although not as dramatically as in Fig. 10. The insets show details of the cell wall structure from one of the smooth-walled cells. The white refractile lines represent the lipid bilayer of the inner cytoplasmic membrane and the outer membrane of the cell wall. Occasionally, vesicles can be seen in this cell type, detectable only by their being delineated by a unit membrane (white arrows, upper left). The other inset shows the cell wall and its attachment to the fibrillar layer associated with the spine.
Fig. 12. Cross section of spine with attached long rods, predominantly of the type with very irregular outlines and filled with many small vesicles. The lower portion of the main figure indicates that the tangential plane of the section has passed through parts of attached cells having a different orientation with respect to the spine than those in the upper part of the figure. Cross sections of other, smaller cells can be seen in the upper and extreme right-hand portions of the main figure. Several aspects of cell wall structure of the long rods and smaller cells can be seen in insets a and c. The arrow points to membranes stacked beneath the outer coat of the cell wall. Inset b shows several vesicles possibly associated with a cytoplasmic membrane.
can be seen with difficulty in the early transitional (or perhaps healthy) cell in Fig. 11 (upper left inset). In the more degenerate stages, the vesicles are darker inside (Fig. 12). They are often contiguous with the cytoplasmic membrane (Fig. 12b); some vesicles are as long as .017 µm. They probably arise as extensive mesosomal-like invaginations of the cytoplasmic membrane. Simultaneously, the peptidoglycan layer is probably undergoing severe changes or even breakdown as evidenced by the effects on the rigidity of the cell wall: the inner and outer membranes are no longer smooth, and the very dense line of the outer coat is irregular, erratic, and sometimes disjointed (Fig. 12a, b).

As suggested by Fig. 4 (SEM), the long rods are very closely packed around the spine. This is particularly evident from observing carefully the serial sections of Fig. 11. They can also be found at the tips of the spines (Fig. 9). Their attachment to the spines differs from the short rods in their apparent requirement for the fibrillar border and the much shorter attachment length (48 nm; range 20 to 80 nm). That the different long rods represent different stages in a life cycle is probable but must remain speculative until the organisms are isolated and studied in pure culture.

**DISCUSSION**

Savage and Blumershine (12) pointed out that organisms attached to the gut would have a survival advantage in a moving environment. Our ultrastructural studies revealed an intimate association between microorganisms and cockroaches at specific sites in the gut, i.e., that portion of the alimentary tract bearing spines. There is some indication from Fig. 1 and 2 that organisms similar in size and shape may attach to the wall as well as the spines. The culturing and identification of these organisms remain to be accomplished. Morphologically they do not resemble any of the strict anaerobes we have cultured. Whether or not these are obligate anaerobes is not known, but it seems reasonable to assume they are at least facultative organisms since the reported redox potential of the colon of another cockroach, *Periplaneta americana*, averaged −170 mV with a range of −84 to −241 mV (D. Warhurst, Ph.D. thesis, Univ. Leicester, Leicester, England, 1964).

The structure of these gram-negative rods is also intriguing. The short rods have never been observed to undergo division, and their function as attached organisms must remain highly speculative. The longer rods appear to undergo morphogenesis. Their ultrastructure at earlier stages is not greatly different from species of *Bacteroides* (2, 3), *Acetobacter* (1), and some rumen (3) and aquatic (8) bacteria. The identification of the attachment material as polysaccharide would require staining with ruthenium red.

The apparent longitudinal fission of the long rods may be very unique. It does make sense, however, in that, as degenerative cells may ultimately be released, longitudinal fission of the healthy cells would replace them and keep the spines closely packed. The function of the vesicles and vacuoles remains to be established.

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