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Novel Microbial and Chemical Components of a Specific Black-Band Region in the Cockroach Hindgut

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An area of the hindgut of the cockroach, *Eublaberus posticus*, is characterized by its black color. This area is the site of accumulation of metal sulfides in the lumen next to the gut wall. In addition to the normal hindgut flora, two unusual procaryotic organisms are seen by both scanning and transmission electron microscopy only in this area of the hindgut. They are (i) a large rod (1.2 by 6 μm) with a tuft of polar flagella, many inclusion bodies, and a distinctive complex wall and (ii) an apparently flexible rod with a helically ridged wall. In addition, phagelike particles are described which are apparently infecting gram-positive bacteria attached to the gut wall in the black band area.

Previous studies have demonstrated an extensive bacterial flora concentrated in the hindgut of two types of cockroaches, *Periplaneta americana* and *Eublaberus posticus* (4, 17; D. L. Cruden and A. J. Markovetz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, J25, p. 92). Many common genera of bacteria can be isolated from the hindgut, but electron microscopic studies show an even more diverse population with many attached bacteria. Studies on termites (6) have shown a comparable diversity of bacterial forms in the paunch region of the hindgut. There is a very large surface area for attachment in the cockroach hindgut due to the presence of chitinous spines, and the gut contents are concentrated with a high cell density. No attempts have been made to further localize either morphological or biochemical types within particular regions of the hindgut of insects, except for the attachment of characteristic types of bacteria to the spines in the paunch of the cockroach (17) and of *Actinomycetes* to spines in the posterior hindgut of soil-feeding termites (2).

In the colon of *E. posticus*, we have observed a region which is readily recognized by its black color. This black band was not found in metronidazole-fed *Eublaberus* and was only occasionally present in the hindgut of *P. americana*, another cockroach under examination.

The present study was initiated to determine the nature of the black material in this region and to ascertain whether the microbial components of this area were similar to other regions of the hindgut. Two unusual bacterial forms were seen only in this area of the hindgut. Phagelike particles were readily apparent only in the black band region. Whereas phage have been reported in a pure culture of bacteria isolated from the colon of *Eublaberus* (16), we have found no other reports of phage actively infecting bacteria in the insect gut itself.

MATERIALS AND METHODS

Colonies of *E. posticus* and *P. americana* were maintained in the laboratory as previously described (3). Adult animals were dissected either aerobically or in the anaerobic chamber after anaesthesia by chilling.

Transmission electron microscopy. The midgut and hindgut were removed from the animal into insect Ringer solution. The anterior part of the black band was cut out with a razor blade and slit open. The lumen contents were rinsed out, and the tissue (with many bacteria remaining in the mucous layer lining the wall) was transferred to 2.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2, with 0.15 M sucrose. After fixation for 2 h, the tissue was rinsed briefly and postfixed in 1% OsO_4 for 90 min, dehydrated through acetone, and embedded in Epon. Sections were cut on a Porter-Blum microtome with a diamond knife, mounted on Formvar-carbon-supported 75-mesh grids, and poststained with uranyl acetate and Reynolds lead stain (27). Specimens were examined with a JEOL 100B electron microscope operating at 60 kV.

Low-magnification electron micrographs were taken of one C-shaped section from the black band region (approximately one half the circumference of the gut). The five resulting overlapping micrographs of this section were enlarged to a total magnification of 2,100×, and the total number of bacteria, the large polarly flagellated rods, and the helically ridged rods were counted with the aid of a magnifier.

Negative staining. The black band was dissected out, slit, and rinsed as above. The tissue was then transferred to a tube with 0.5 ml of Ringer solution and agitated with a Vortex mixer for 30 s to suspend the mucous-associated bacteria. A drop of the resulting suspension was allowed to settle on 400-mesh

Formvar-carbon supported grids, rinsed with 1% NH_4 acetate, negatively stained with 2% phosphotungstic acid with 100 μg of bacitracin per ml, and examined immediately after drying.

Scanning electron microscopy. The rinsed, slit black band tissue was divided into four longitudinal sections and fixed in glutaraldehyde for 30 min. Then it was prepared with a modified thiocarbohydrazide-binding technique (21). After critical point drying and coating with gold-palladium, the specimens were examined with a JEOL-35C scanning electron microscope operating at 16 kV.

Chemical analysis. The amounts of sulfide deposited in the areas of the swollen paunch and the black band area of the hindgut of *Eublaberus posticus* were determined. Areas in these regions were ligated to avoid loss of lumen contents, and the area was then removed by dissection. Each section of gut tissue was weighed and placed into a test tube (13 by 100 mm) containing 1 ml of deoxygenated insect Ringer solution, and the tube was sealed with a black rubber stopper. The presence of sulfide was determined by ascending paper chromatography (28). A solvent system of ethanol–pyridine–water–30% NH_4OH (30:30:40:2.5) was used, and sulfide was located by spraying with a solution of 0.1 N $AgNO_3$. Quantitative sulfide content of gut tissue was determined by the methylene blue method (22).

Methane emission by roaches was determined by gas chromatography as previously described (3).

RESULTS

When the alimentary tracts of *E. posticus* were removed, it was noted that a particular region of the hindgut was characterized by a black color. The region was several millimeters long in the last half of the tannish hindgut, posterior to the swollen paunch region. This black region was present in the hindgut of animals emitting methane but was absent from the hindgut of cockroaches not producing methane. In cockroaches fed metronidazole, the dark band area also was absent, as was a significant portion of the anaerobic flora and methane production. The latter two observations were reported previously (3).

Bacteria. Black bands from 10 adult *E. posticus* were examined by transmission electron microscopy, scanning electron microscopy, or negative staining. Other areas of the gut were examined from over 15 adult roaches. The black area contained a heterogenous microflora, with many of the bacteria seen in this area found in other regions of the hindgut (Cruden and Markovetz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, J25, p. 92). However, two procaryotic organisms were unique to the black band area; a large complex rod and a rod with a helically ridged wall.

A large rod with a tuft of polar flagella and a distinctive complex wall structure is seen in the black band region of the hindgut close to the gut wall but not attached to it (Fig. 1). In electron micrographs of cross sections of the gut through the black band region, this organism occasionally is seen throughout the lumen, but the highest numbers are close to the wall in what may be a layer of mucous-type material. This morphotype constitutes approximately 0.27% of the total population of the thin section examined, i.e., 29 of 10,877 bacterial cells counted. The procaryotic cells are 1.15 to 1.25 μm wide and up to 6.5 μm long. The cytoplasm contains an area of nucleoplasm, ribosomes, and large numbers of two kinds of large granules (Fig. 1 to 4). The smaller granules are electron dense and range from 70 to 110 nm in diameter. They are not membrane bound. The larger granules are either very electron dense with electron opaque inclusions or are lost from thin sections. They are up to 0.5 μm in diameter and are bounded by a membrane (Fig. 4). The smaller granules resemble glycogen storage material (15), and the larger resemble polyphosphate granules (20). The cell wall consists of a cytoplasmic membrane, a periplasmic space up to 35 nm wide, a peptidoglycan layer tightly appressed to a unit membrane, and two complex outer layers (Fig. 4). Each of the outer layers is 40 to 50 nm wide. In tangential section (Fig. 2 and 3) the outermost layer appears to be composed of tubule-like structures, 35 nm wide and 45 nm apart, flaring at the ends and extending through the inner layer. The patterns formed by these projections can also be seen in the negatively stained preparation (Fig. 6). The tuft of flagella originates in a depression which is up to 0.4 μm across at one pole of the cell. There are at least 18 flagella in the tuft in the negatively stained preparation (Fig. 6). They are 16 nm in diameter and originate in the classical gram-negative basal structure (Fig. 4). The outer wall layers in the flagellar depression are thinner, more electron dense, and appear less structured. In some sections (Fig. 5), there appears to be a repeating structure in the periplasmic space near the polar region.

A rod with a helically ridged wall was seen first in scanning electron micrographs as an apparently flexible bacterium found close to the gut wall (Fig. 7). There were 39 of these morphotypes out of a total number of 10,877 bacterial cells in the section counted, i.e., 0.36% of the total cells were of the helical type. In examining cross sections of the black band, this morphotype was also found predominantly in what appears to be a layer close to the wall with some organisms seen throughout the lumen. In transmission electron microscopy it can be seen that the cytoplasm extends into the ridge (Fig. 8 and

9). There is no indication of the axial filaments of a spirochete. The rod is 0.3 to 0.4 μm wide, not including the ridge which extends another 0.14 to 0.18 μm. The longest cell measured was 2.65 μm, but extended beyond that on both ends out of the plane of section. In all scanning micrographs, the rod was apparently bent to fit the contour of the wall. The ridge-like extension is 50 nm wide and traces a tight helical pattern the length of the cell. The repeat distance of the helix is 0.25 to 0.28 μm where the organism is not bent. The wall is very thin, but in favorable sections can be seen to be gram negative in structure (Fig. 9, inset). Some of these helically ridged rods contain many electron-dense granules 0.07 to 0.22 μm in diameter similar to those observed in the very large rod (Fig. 8). Other individuals (from a different host) did not appear to contain these granules (Fig. 9).

A modified Hungate technique (19) was employed for growth of anaerobes. Sections of the black band area were used to inoculate PYG medium (18) and RCGSB medium (9) in which cysteine sulfide was replaced by FeS as a reducing agent (7). As yet, neither organism has been isolated. Black band area contents were diluted from 10^{-2} to 10^{-9} in Hungate tubes for *Desulfovibrio* and in the medium described for determining H_2S production from sulfhydryl-containing organic compounds in the Virginia Polytechnic Institute Manual (18). None of the *Desulfovibrio* tubes showed growth or blackening, but bacteria which produced H_2S from organic compounds were isolated from the 10^{-9} dilution.

Phage-like particles. At several places along the gut wall in the black band region in transmission electron micrographs, there were concentrations of electron-dense hexagonal bodies 42 to 48 nm in diameter (Fig. 10). Some were free in the layer of mucous material close to the wall, and others appeared to be partially enclosed in membranes, as from a newly lysed bacterial cell. In some, the suggestion of slender tails could be seen (arrows in Fig. 11). In other micrographs particles of the same size and shape could be seen inside a single type of bacterial cell, as well as free in the lumen (Fig. 11). These bacterial cells were close to the gut wall and often apparently attached to it (Fig. 11 and 12). They are pleomorphic (especially those attached to the wall), gram positive (Fig. 12), and ovoid to rod shaped. They were usually 0.3 to 0.7 μm in diameter, but some reached as wide as 1.2 μm.

When the blended mucous layer was negatively stained, many phage particles could be seen in the preparation (Fig. 13). The heads of these particles are 45 to 50 nm in diameter, approximately the same size as particles seen in thin sections. They have thin simple tails, 140 to 160 nm long with a small knob at the end.

Black material. In transmission electron microscopy the black band material is seen as amorphous electron-dense bodies of variable size and density, often surrounding bacterial cells next to or near the gut wall (Fig. 9 to 11). This amorphous material was found in no other region of the gut of *E. posticus* or *P. americana*. The cockroach tissue in the black band region was indistinguishable from that of other parts of the hindgut. The nature of the electron dense material was thought to be a metal sulfide, and initial analyses on this area were done with an electron probe microanalyzer, but results were equivocal. Sulfide was detected by paper chromatographic analysis, and the results of chemical analyses are given in Table 1. The black band region contained approximately 9 to 85 times more sulfide that detected from the paunch region of the hindgut. Preliminary indications are that the black band region is enriched with ferrous ions, and it may be that FeS is the major constituent of the black material (unpublished data).

DISCUSSION

The black band region of the hindgut appears to be a specialized area high in sulfide and containing some unique morphotypes. What is not known is why sulfide precipitation occurs in this area. It would seem doubtful that this particular area of the hindgut accumulates sulfate or sulfhydryl-containing compounds which are converted to H_2S by microbial action with subsequent precipitation of metal sulfide. It may be that this particular area has a more reducing oxidation-reduction potential (Eh) due to growth of other anaerobes which allows for development of specific H_2S-generating organisms. Once H_2S begins to be produced, FeS could form and a low Eh would then be assured. The observation that some "normal" roaches not producing methane did not exhibit a black band area may indicate in this case that the Eh of the hindgut was not sufficiently reducing to allow for growth of methanogens. The black band was also absent from cockroaches fed metronidazole. Since we had previously demonstrated that metronidazole stopped methane production as well as removing a predominant portion of the obligately anaerobic flora from the hindgut of the cockroach (3), we assumed that the presence of the characteristic black precipitate in this specific area was due to biochemical activities associated with anaerobes.

It is known that methanogenesis in natural ecosystems does not occur when sulfate is pres-

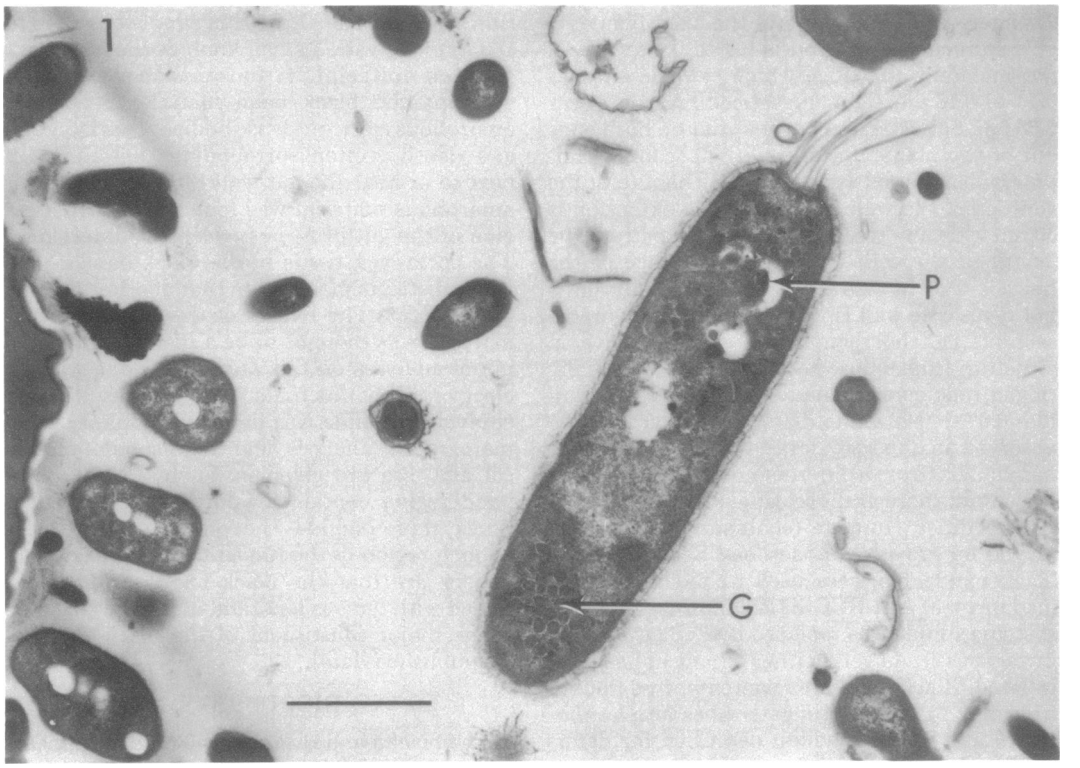


FIG. 1. *Large rod with a polar tuft of flagella near the chitinous wall of the cockroach hindgut. The large granules are polyphosphate (P), and the smaller granules are glycogen inclusions (G). Bar = 1 μm.*

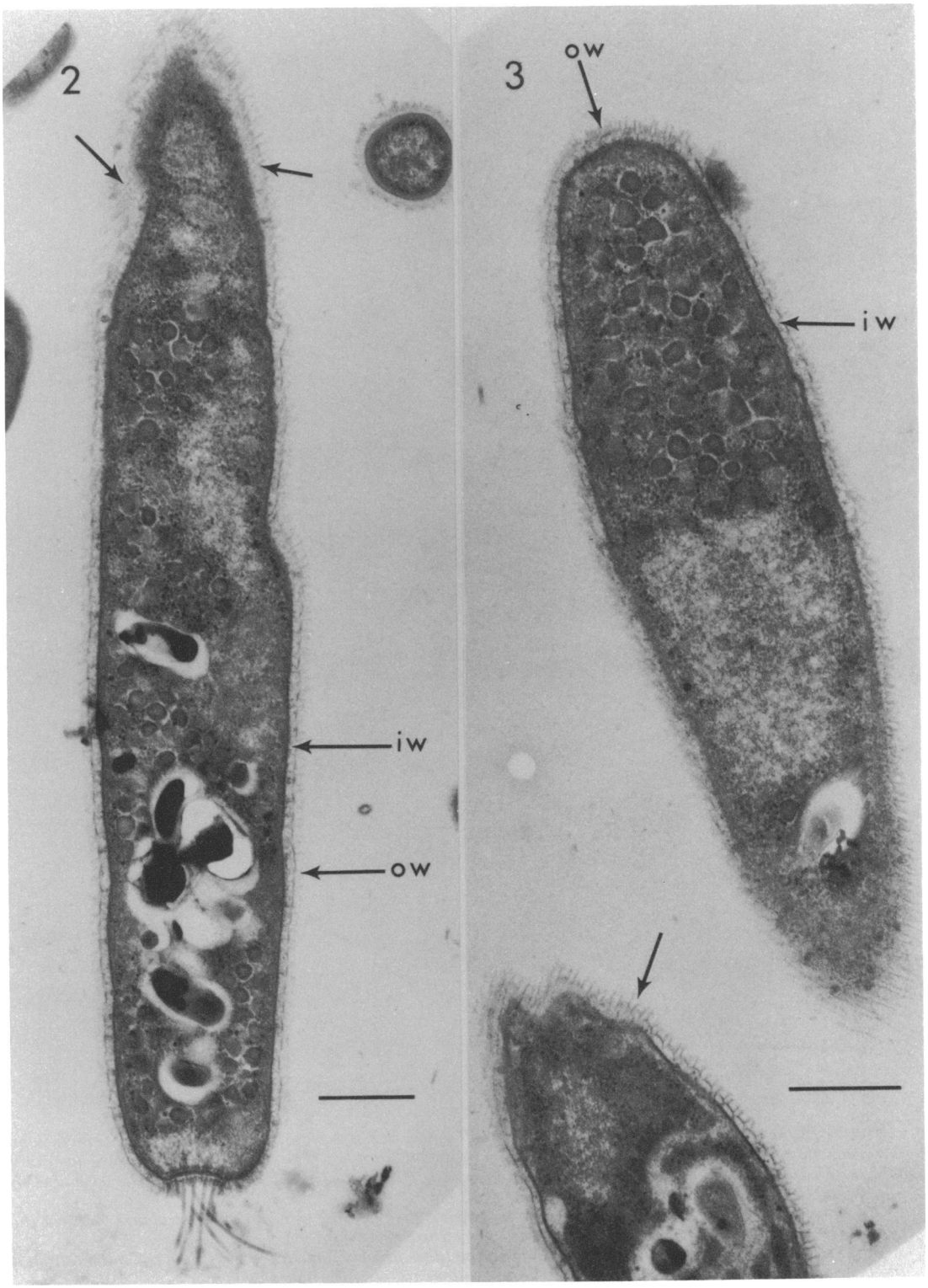
FIGS. 2 and 3. *Thin sections of the large rod showing the structure of the wall. The outer (ow) and inner (iw) wall layers can be seen. In tangential section, the tubule-like projections of the outer layer appear to continue into the inner layer (arrows). In Fig. 2 note the absence of ribosomes and inclusion granules close to the flagellar insertion site. Bar = 0.5 μm.*

ent, and this has been suggested to be due to the sensitivity of methanogens to H_2S produced by sulfate-reducing bacteria utilizing organic compounds (10). Bryant et al. (8) suggest that H_2S is not toxic and that there is a lack of H_2 for growth of methanogens when sulfate is present. In this case, sulfate would probably eliminate the production of H_2 by desulfovibrios from organic precursors, and it is possible that the desulfovibrios would preferentially use any available H_2 produced by other organisms for sulfate reduction. In the cockroach we see *Methanospirillum* in the black band area, and there is a correlation between CH_4 production and possession of a black band. This suggests that either H_2S is not toxic to methanogens or that H_2S is precipitated as metal sulfides before it reaches toxic levels. If sulfate is the precursor of H_2S in the cockroach, then the presence of sulfate (and perhaps *Desulfovibrio*) would appear not to inhibit methanogenesis. However, we have been unable to obtain desulfovibrios by dilution series on enrichment culturing from the black band area. It seems more likely that H_2S originates from sulfhydryl-containing organic compounds.

The black band area may represent an area of the hindgut enriched in iron. Indeed, Day and Powning (13) reported that the middle part of the hindgut may be the site for iron absorption in the cockroach. If this is the case, then any H_2S produced in the hindgut by microbial action would be precipitated as FeS at the specific area of iron absorption. The unique organisms seen in this area may have developed because of the low Eh generated by the presence of FeS.

The large rod described here has been seen in material prepared from the dark band of every *E. posticus* that we have examined by negative staining or transmission electron microscopy. It has not been found in the paunch region of these animals, nor has it been seen in the hindgut of *P. americana* during an extensive electron microscopic survey of its hindgut flora (4).

The size of this rod almost approaches that of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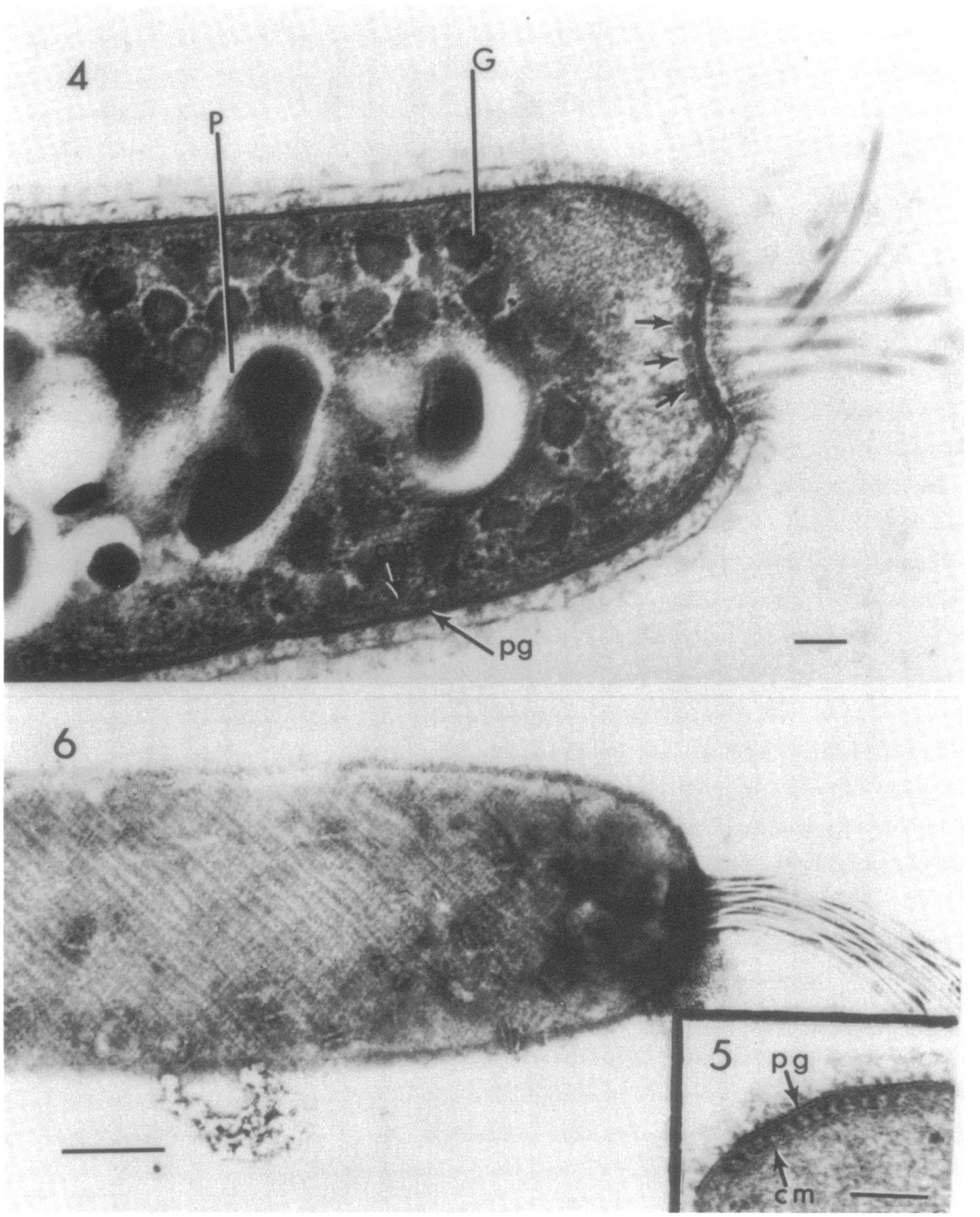

FIG. 4. *Higher magnification micrograph of the cell shown in Fig. 2. Note that the polyphosphate inclusions (P) are membrane bound, whereas the glycogen inclusions (G) are not. The cytoplasmic membrane (cm) and peptidoglycan layer and outer membrane (pg) can be seen. The arrows point to the rings at the base of each of the flagella. The flagella are 16 nm wide. Bar = 0.1 μm.*

FIG. 5. *High magnification of a different cell showing the "polar membrane"-like structure between the cytoplasmic membrane (cm) and the peptidoglycan layer (pg). Bar = 0.1 μm.*

FIG. 6. *Negatively stained preparation showing the large polarly flagellated rod. Note the indentation at the base of the tuft of flagella and the pattern of striations in the wall. Bar = 0.5 μm.*

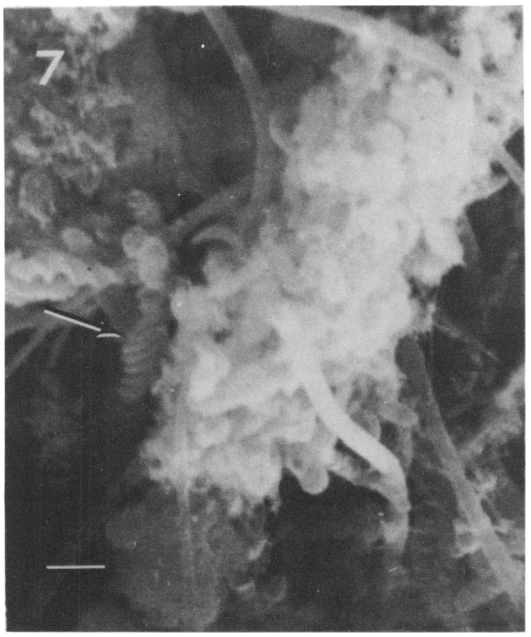

FIG. 7. *Scanning electron micrograph of part of the gut wall in the black band region. Note the apparently flexible helically ridged rod (arrows). Bar = 1 μm.*

the large procaryotic organisms Quin's oval and Eadie's oval found in the rumen (23). The cytoplasm of Eadie's oval is full of glycogen granules which have been isolated and characterized (24). The fine structure of the smaller of the two inclusion granules resembles that of glycogen reserve material (15, 29), whereas that of the larger resembles polyphosphate reserves (20, 29). Eadie's oval has peritrichous flagella, however, and its wall does not resemble that of the rod described here. The presence of storage granules in procaryotes is usually an indication of unbalanced growth due to the presence of some nutrients in the absence of others (12). The presence of many microorganisms in gut and rumen ecosystems with such inclusion bodies, Eadie's oval (23), the large rod, the rod with the helically striated surface reported in this paper, and others from the guts of *Eublaberus* and *Periplaneta* (unpublished data) may be an indication that the gut environment is not one of bounteous plenty, but that, in fact, nutrients become available in local small spurts and that growth is not similar to that in continuous culture (11) but very discontinuous. The presence of significant numbers of sporulating bacteria in vivo (4) may also be an indication of unbalanced growth.

The basal attachment of the flagella of the large rod resembles that of *Escherichia coli* for the individual flagella (14). The fine structure in Fig. 5 resembles the "polar membrane" described by Remsen et al. in *Ectothiorhodospira* (26), but their thin sections do not reveal a depression in which the flagella are seated in that organism.

The helically ridged rod (or an organism very similar to it) has also been reported in both scanning electron microscopy and transmission electron microscopy micrographs in the posterior hindgut of *Periplaneta americana,* a cockroach which is only distantly related to *Eublaberus* (4). We do not know whether their location coincides exactly with the site of metal sulfide deposition because the presence of a recognizable black band in *P. americana* is a variable characteristic.

The phage-like particles seen in negatively stained preparations are in Bradley's category B, with long, simple tails (5). They are different from the phage infecting *Fusobacterium varium* isolated from the gut of the same cockroach in that the latter have an obvious tail sheath and fibers and fit into Bradley's category A (16). In addition, *Fusobacterium* is gram negative, and the particles in our thin sections are in a gram-positive organism. The sizes of the particles seen in thin sections are the same as those in negatively stained preparations and there are suggestions of the presence of tails in some micrographs (e.g., Fig. 11). This, however, constitutes only circumstantial evidence that they are indeed the same type of phage. The organism with which these particles are associated has not been seen in other regions of the hindgut.

The existence of phage in any gut environment is to be expected and Adams et al. have isolated phage from bacteria from the rumen (1). Orpin and Munn reported infection of two rumen bacteria, one of which was Eadie's oval, with phage (25). The phage particles could be seen in cells in vivo, and they also reported that its presence could influence rumen populations of Eadie's oval. We have found no other reports of phage in insect guts and feel that it is significant that the phage-like particles described here are associated with bacteria which are attached to the gut wall and are therefore likely to be autochthonous.

Counts of the large rod and the helically ridged organism are probably low since at the low magnification necessary to count such large numbers of bacteria, cells of either form could only be recognized with certainty in tangential or longitudinal section and not in cross section. The bacteria were found in small localized

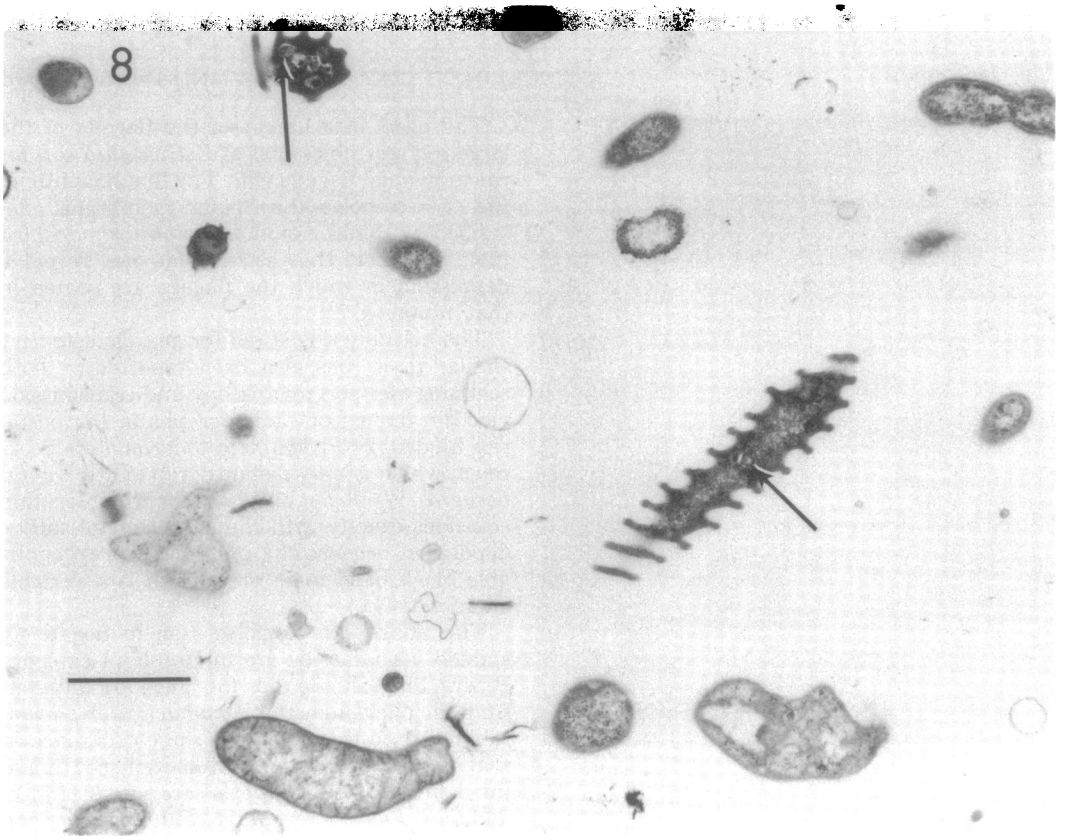

FIG. 8. *Thin section showing two of the helically ridged rods. Note glycogen-like inclusions (arrows). Bar = 1 um.*

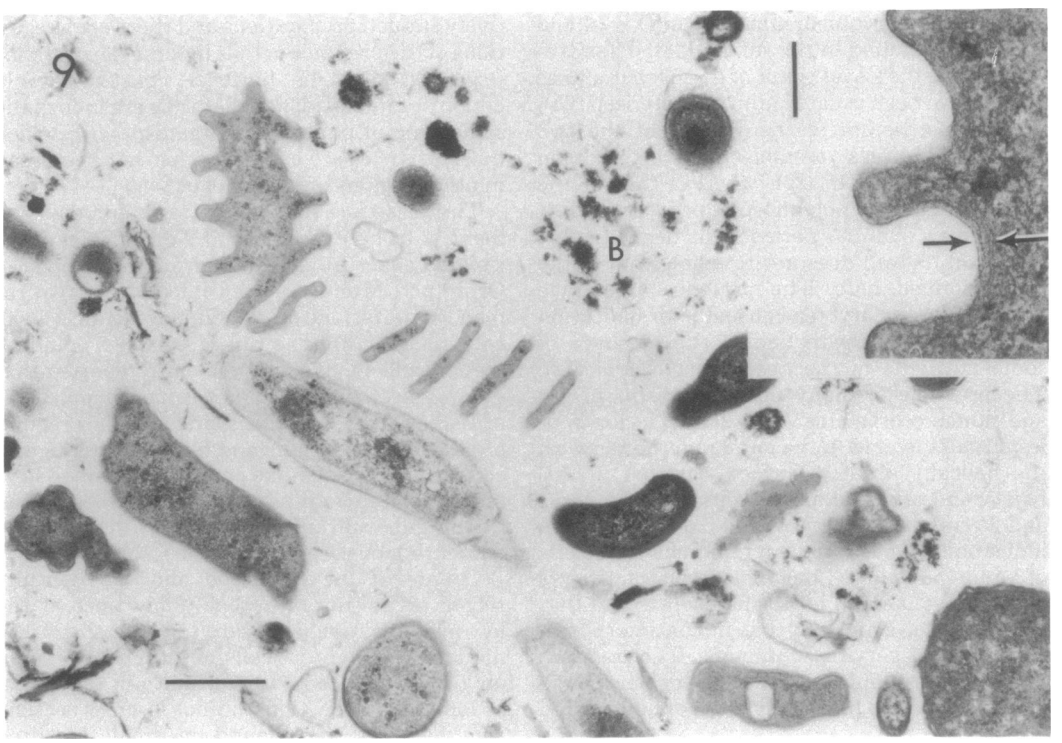

FIG. 9. *Flexible rod with surface ridges. The cytoplasm extends into the ridges, showing that they are not merely wall ornamentation. Small accumulations of amorphous electron-dense material found only in the dark band region close to the wall are present (B). Bar = 0.2 μm. Inset: Higher magnification of part of the cell showing that it is bounded by a gram-negative wall. The arrows indicate the inner and outer membranes. Bar = 0.1 μm.*

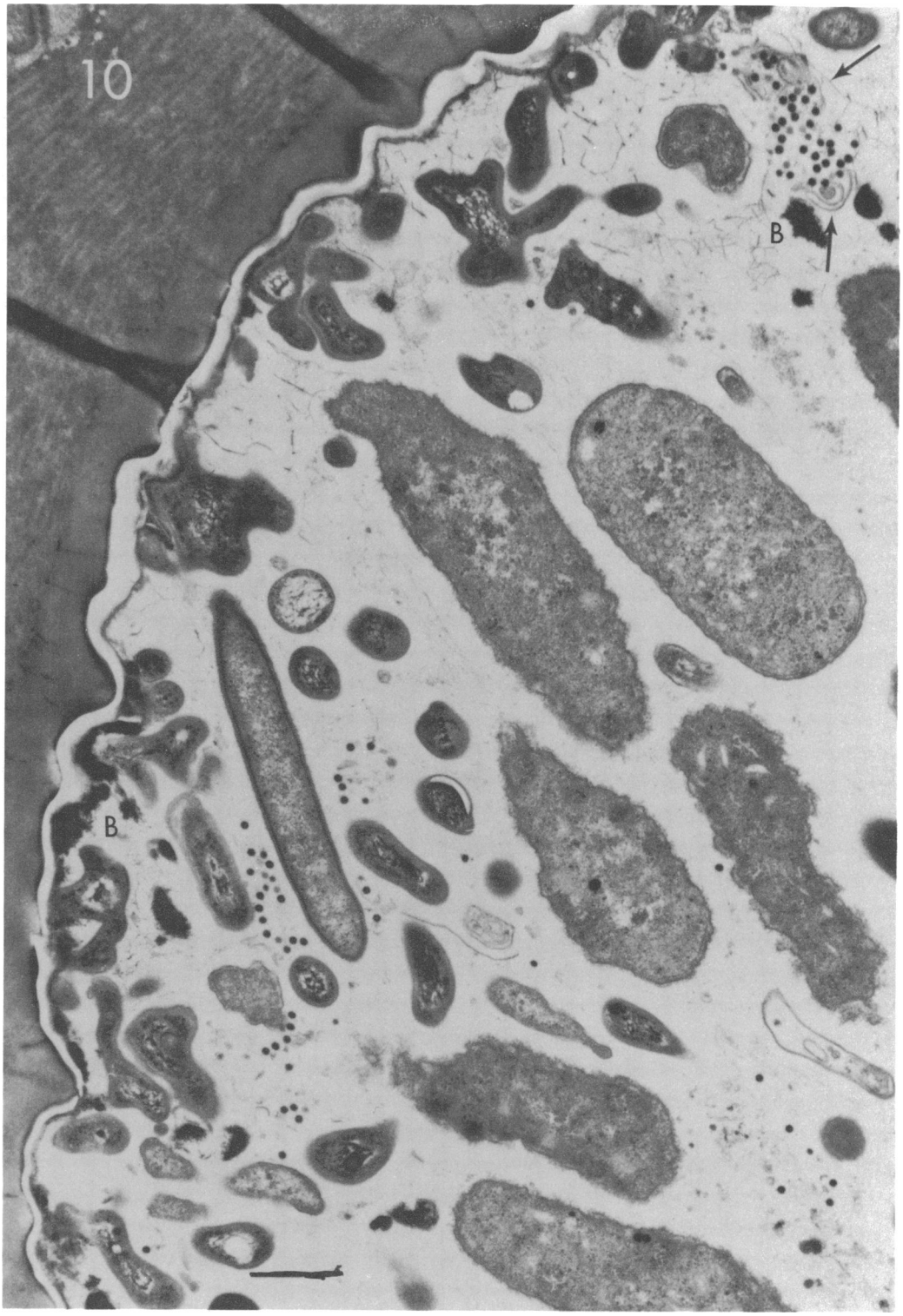

FIG. 10. *Hexagonal phage-like particles close to the colon wall. Some are free while others appear to be partially enclosed by membranes as if just liberated from a lysing bacterial cell (arrows). Amorphous electron-dense material characteristic of the black band region is close to the wall (B). Bar = 0.5 μm.*

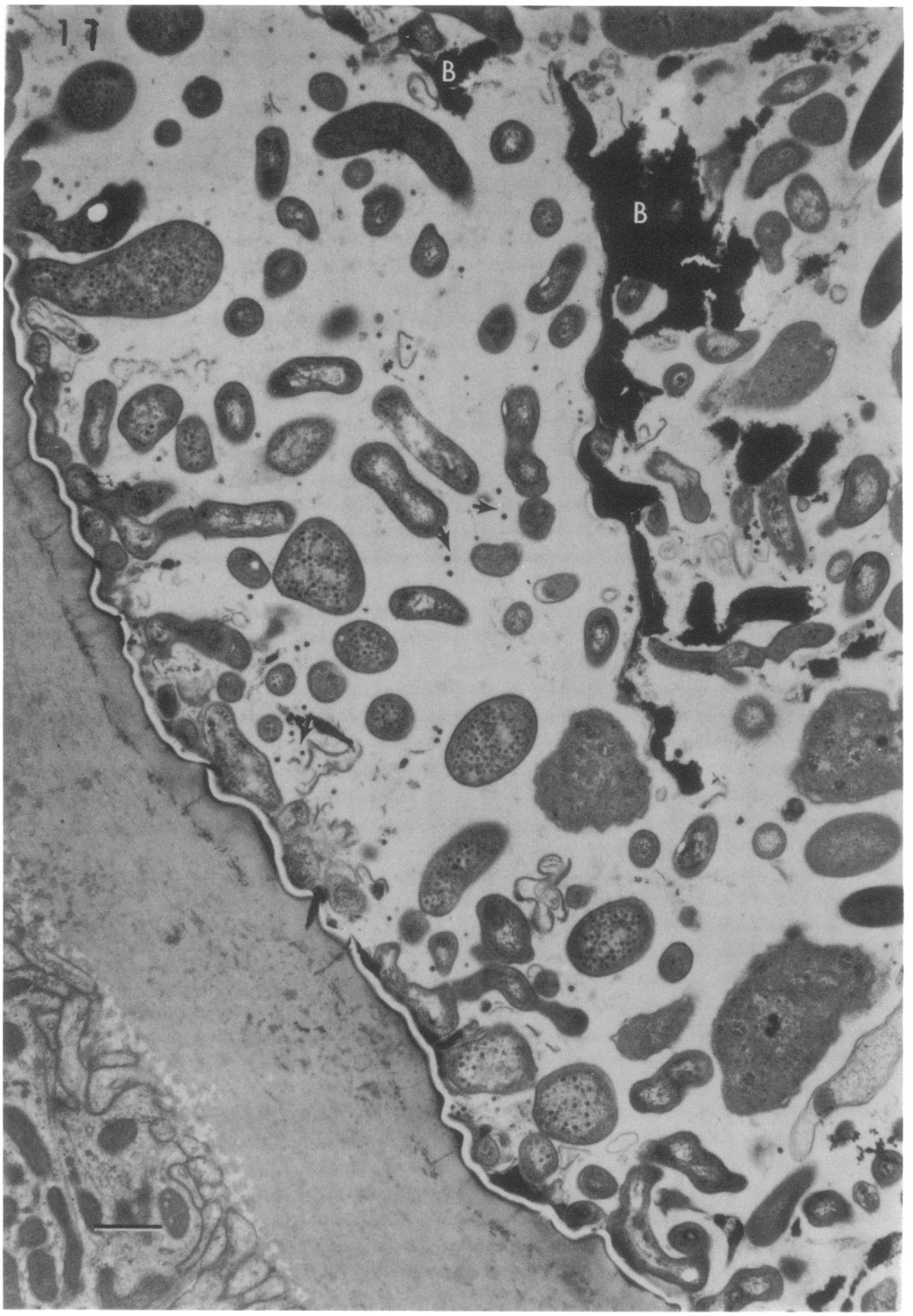

FIG. 11. *Particles packed in irregularly-shaped bacterial cells attached to or close to the gut wall. There are also some scattered free particles. Some of the free particles have tail-like extensions (arrows). Large amounts of electron-dense black band material are present (B), some surrounding bacterial cells. Bar = 0.5 μm.*

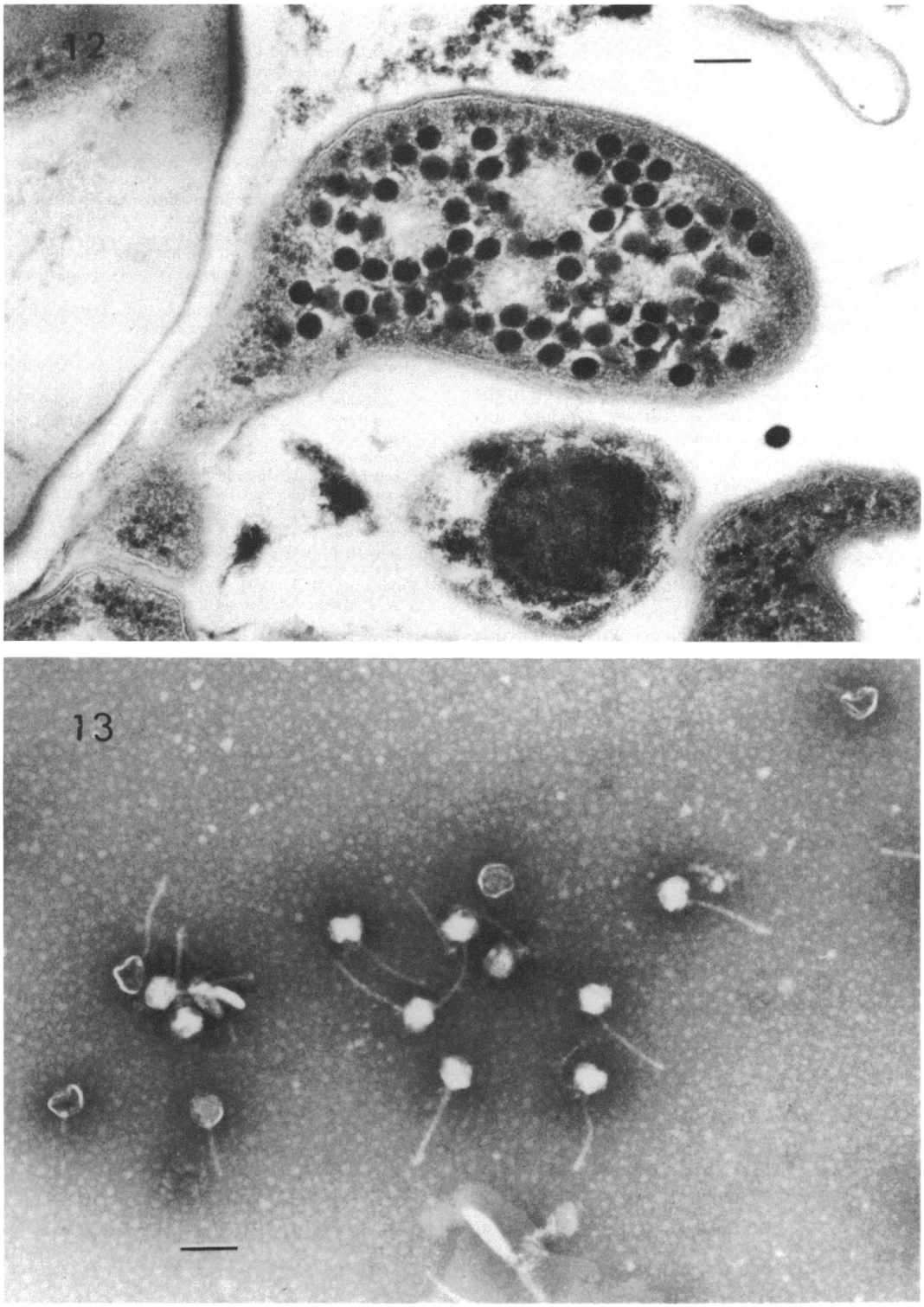

FIG. 12. *Higher magnification of an attached cell packed with particles. Note the gram-positive wall of the bacterial cell. The hindgut is lined with a chitinous cuticle which is difficult to section and almost always pulls away from the lumen and attached organisms. Bar = 0.1 μm.*

FIG. 13. *Negatively stained preparation showing phage from the wall associated material in the black band region. The phage heads appear to be octahedral, and the tails are slender and lack sheaths. Some of the heads are empty. Bar = 0.1 μm.*

TABLE 1. *Accumulation of sulfide in two areas of the hindgut from Eublaberus posticus*

Animal	Amt of sulfide[a] (ng of S^{-2}/mg [wet wt])	
	Paunch	Black band
1	5.6	187
2	14	120
3	2	170

[a] Determined with the methylene blue method (22).

groups throughout the section. Cells containing the phage-like particles could not be recognized at this magnification.

The role of these bacteria in the black band region is unknown. They may be involved in the formation of the metal sulfides or they may merely be taking advantage of the favorable environment provided by the presence of these compounds. However, it appears that both of the unique morphotypes are present in sufficient numbers to be significant components of the microbial flora of this region of the gut.

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