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Microbiota Associated with the Gastrointestinal Tract of the Common House Cricket, *Acheta domestica*†

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The location and morphology of the bacteria associated with the gastrointestinal tract of *Acheta domestica* were studied, and these bacteria were partially characterized. Bacteria were associated with the peritrophic membrane in the midgut and with the gut wall and cuticular structures of the hindgut. No bacteria were associated with the fat bodies. Colony-forming unit determinations indicated that there were three times more cultivatable bacteria in the hindgut than in the midgut. Of these bacteria, 40 to 85% cleared uric acid anaerobically, and 90 to 100% cleared uric acid aerobically. Of the 25 isolates obtained, 21 belonged to the genera *Citrobacter*, *Klebsiella*, *Yersinia*, *Bacteroides*, and *Fusobacterium*.

A variety of associations between microorganisms and insects have been described (5). Many of these associations, especially in cases of endosymbiosis, are thought to be correlated with the diets of the animals (5). Animals which feed on an incomplete diet generally contain endosymbionts and require the metabolic functions of these endosymbionts, whereas animals which feed on complete diets fulfill their requirements from their diets.

Cockroaches, which have an omnivorous feeding behavior, fall in the former category and contain endosymbionts. These endosymbionts, which are associated with the fat bodies in a number of cockroaches, provide critical growth factors for these animals (3, 4, 11). Recently, a distinctive array of bacterial morphotypes was found attached to cuticular structures in the hindguts of *Eublaberis posticus* (7) and *Periplaneta americana* (1, 9, 10). Although the role of these microorganisms in the nutrition of the animals has not been determined, the density and attached nature of the bacteria suggest that they are not transient inhabitants of the gut.

The purpose of this study was to determine whether the house cricket (*Acheta domestica*), whose feeding behavior is similar to that of cockroaches, has similar microbial associations. This cricket has a gut morphology similar to that of cockroaches, and presumably its digestive processes are also similar to those of cockroaches (8). This paper describes the morphology and location of the microorganisms associated with this animal and partially characterizes these microorganisms.

MATERIALS AND METHODS

Animals. House crickets (*A. domestica*) of known age were obtained from Top Hat Cricket Farm, Kalamazoo, Mich. Chicken starter scratch containing no antibiotics or supplements was used as a rearing diet. Wild crickets of the same species were trapped on the premises of the Michigan State University W. K. Kellogg Biological Station.

Preparation for microscopy. In preparation for scanning electron microscopy, insects were anesthesized with CO_2 and dissected in 0.1 M potassium phosphate buffer (pH 7.2) containing 3.0% glutaraldehyde. The gut of each animal was removed and cut open longitudinally. Microorganisms not firmly attached to the gut wall were removed by vigorous blending in a Vortex mixer for several washes of 0.1 M phosphate buffer. Gut segments were fixed overnight at 4°C, rinsed in three 10-min changes of 0.1 M phosphate buffer, and postfixed in the same buffer containing 1.0% OsO_4 for 1 h. They were then rinsed three times with distilled water, dehydrated in a graded ethanol series to 100%, and critical point-dried with carbon dioxide. Samples were mounted on stubs, sputter-coated with gold, and examined with a Cambridge scanning electron microscope.

Preparations to be used for phase-contrast microscopy and transmission electron microscopy were treated as described above, but after dehydration they were cleared with propylene oxide and embedded in Epon 812 (14). Thick and thin sections were cut by using glass knives and an LKB-Huxley ultramicrotome. Thick sections (0.5 μm) were stained with toluidine blue for light microscopy. Thin sections for electron microscopy were stained with 2.0% aqueous uranyl acetate for 20 min (20) and then with Reynolds lead citrate stain for 3 min (18). Thick sections were examined and photographed with a Zeiss Universal phase-contrast photomicroscope. Thin sections were

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examined and photographed with a Siemans 1-A transmission electron microscope, which was operated with an accelerating voltage of 80 kV.

Bacterial media. Tryptone yeast extract and Sweet E media were used for determinations of colony-forming units (CFUs) and to obtain isolates of organisms growing under anaerobic conditions (12). Tryptone yeast extract medium contained 1.0% tryptone, 1.0% yeast extract, 0.2% (vol/vol) vitamin K-hemin, 0.0001% resazurin, 0.05% cysteine-hydrochloride, and 1.5% agar in an inorganic salts solution which was modified from the salts solution of Holdeman and Moore (12) and contained the following: K_2HPO_4, 0.52 g; KH_2PO_4, 0.28 g; Na_2CO_3, 0.40 g; $MgSO_4$, 0.10 g; $(NH_4)_2SO_4$, 0.20 g; NaCl, 0.48 g; $FeCl_3$, 5 mg; $CaCl_2$, 0.6 g; and distilled water to 1 liter. Sweet E medium was made as described by Holdeman and Moore, except that rumen fluid was omitted and the above-described salts solution was added.

The utilization of uric acid by gut isolates was tested by using an agar overlay medium. We prepared petri dishes in which tryptone yeast extract medium (with tryptone and yeast extract reduced to 0.5% each) was used as a base and the same medium amended with 1% uric acid was used as a thin overlay. An overlay medium for cellulolytic enrichment was prepared, which contained 0.1% yeast extract, 0.1% peptone, 0.01% cellobiose, 0.05% cysteine hydrochloride, 0.0001% resazurin, and 2.0% agar; plates were prepared with this medium as a base and the same medium containing 0.4% cellulose (ball-milled Whatman no. 1 filter paper) as an overlay. All media were transferred into an anaerobic glove bag (Coy Manufacturing) with an atmosphere containing 85% N_2, 10% H_2, and 5% CO_2 and reduced before use. Media for aerobic isolations, CFU counts, and uric acid utilization studies were prepared as described above, except that cysteine hydrochloride and resazurin were omitted. All media were adjusted to pH 7.8 before autoclaving.

Determination of CFUs. Crickets were anesthetized with CO_2, rinsed several times with sterile distilled water, and irradiated for 2 min externally with ultraviolet light (4 W) before they were dissected in order to minimize contamination of gut preparations. The insets were dissected through the ventral side of the abdomen to expose the gastrointestinal tract. The posterior midgut and hindgut were ligated with nylon sutures. Gut sections were removed, measured, and added to a 10-ml anaerobic dilution blank under anaerobic conditions (12). The inorganic salts solution described above was substituted in the dilution blanks. The blanks were then transferred to the glove bag and vigorously blended with a mixer; Vortexed 10-fold dilutions were made of the midgut exclusive of the gastric ceca, of the hindgut wall exclusive of the rectum, and of the hindgut contents. Each dilution (0.1 ml) was plated onto tryptone yeast extract and Sweet E agar plates. Duplicates were made for both aerobic and anaerobic incubations. Freshly defecated feces from animals were weighed, diluted, and plated as described above for the gut tissue.

Detection of uric acid utilizers. Uric acid plates were inoculated as described above. Uric acid utilization was determined by observing the clearing of uric acid directly beneath the colonies after 3 and 7 days of incubation.

Cellulolytic enrichment. Cellulose agar plates were inoculated as described above and incubated both aerobically and anaerobically in the glove bag for 4 weeks at room temperature. Controls, which consisted of a similar plating of bovine rumen contents, cleared cellulose within this time period. Clearing of cellulose was scored as positive cellulose hydrolysis.

Bacterial isolates. Representatives of each colony morphotype growing under aerobic and anaerobic conditions were picked from the same dilution; the dilution selected was one which yielded 50 to 200 colonies. Cultures were considered to be pure after three successive passages on streak plates.

Characterization of isolates. Anaerobes were identified to genus by the methods described by Holdeman and Moore (12). Acid fermentation products were extracted from cultures grown anaerobically in peptone yeast extract broth (prepared in the same manner as tryptone yeast extract medium) and identified by gas chromatography. Additional biochemical characteristics were determined by utilizing the API 20A system for the identification of anaerobes (Analytab Products, Inc.). The presence or absence of flagella was determined by negatively staining young broth cultures with 1.0% phosphotungstic acid and examining them by transmission electron microscopy. Gram-negative facultative bacteria were identified to genus by utilizing the API 20E system. Presumptive identification of streptococci was based on Gram staining, morphology, and catalase reactions.

Uric acid determinations. The percent dry weight of uric acid was determined for the peripheral fat bodies, malpighian tubules, midgut, hindgut tissues, and feces of the cricket by using method of Cochran (6) in conjunction with the Sigma Chemical Co. Uric Acid Kit 292-UV. The tissues and feces from six animals were pooled and assayed for uric acid.

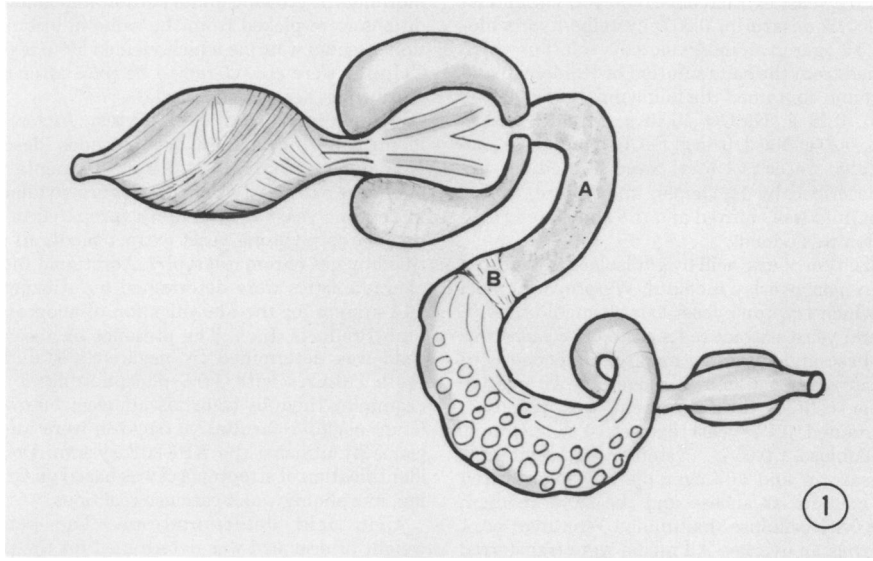
RESULTS

Morphology of the gut. The major regions of the gastrointestinal tract are shown in Fig. 1. The foregut region consists of a buccal cavity, esophagus, crop, and proventriculus. The midgut region is divided into the gastric ceca and the posterior midgut. The hindgut consists of the ileum, colon, and rectum. Below, the term midgut refers to the midgut region exclusive of the gastric ceca, and hindgut refers to the hindgut region anterior to the rectum.

Distribution of microbiota: phase-contrast microscopy. Phase-contrast microscopy of wet mount preparations of gut contents and walls indicated dense populations of bacteria in both the midgut and the hindgut, with low densities anterior to these areas. In the midgut and hindgut preparations the following three bacterial morphotypes were dominant: gram-negative rods 0.5 by 1.0 μm, gram negative rods 0.5 by 5.0 to 7.0 μm long, and gram-positive cocci. Similar morphotypes were observed in preparations from both wild and domestically raised crickets. No other microorganisms (i.e., protozoans or trichomycetes) were observed. Scanning elec-

tron microscope probes through the gut regions confirmed the low density of microbes anterior to the midgut and the abundance of microbes in the migdut and hindgut.

The pouch-shaped area of the hindgut contains bulb-shaped structures (Fig. 2) on the ventral surface of the hindgut (Fig. 1, area C). Projections (bristles) give these structures a tufted appearance (Fig. 3). Toluidine blue-stained 0.5-μm sections through these structures revealed an extremely dense association of bacterial cells with the protruding bristles (Fig. 4, arrow A). The structures themselves appear to be hollow, with the inner region appearing to be



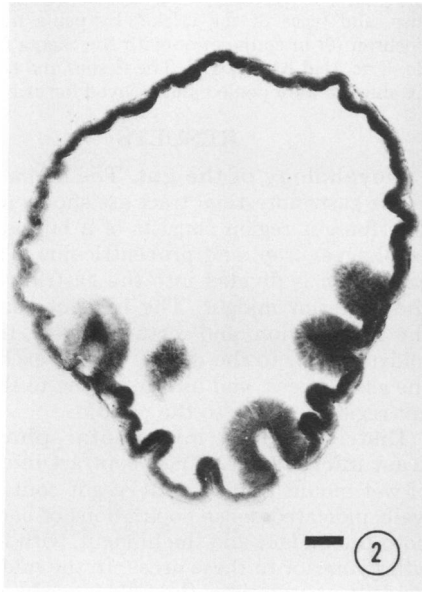


FIG. 1. *Morphology of the excised gut tract. The major regions are the posterior midgut (A), the ileum (B), and the colon (C).*

FIG. 2. *Phase-contrast photomicrograph of a section through the hindgut. The bulb-shaped structures are visible. Bar = 100 μm.*

FIG. 3. *Scanning electron micrograph of the ventral interior hindgut of a terminal instar cricket. Bar = 100 μm.*

continuous with the hemocoel (Figure 4, region B).

Bacterial localization revealed by electron microscopy. The bacteria in the midgut region were attached to the lumen side of the peritrophic membrane and associated with the gut contents. Since the peritrophic membrane disintegrated under very mild blending in a Vortex mixer, it was difficult to distinguish the nature of the attached bacteria. For this reason no further attempts were made to describe the morphology of these bacteria. The bacteria in the hindgut were associated predominantly with the bulb-shaped structures (Fig. 3 through 5). The protruding bristles, which were described by Buchner (5) as "chitinous bristles and telodendrons," resembled paintbrushes; they were joined at the base and feathered out to fine fibers at the tip (Fig. 6 and 7). Oval rods 0.5 by 0.75 μm and rods 0.5 by 1.25 μm were the predominant associated bacteria. These organisms formed chains along the bristles (Fig. 5 and 7, arrow B) or were attached end-on (Fig. 7, arrow A).

The hindgut wall areas across from and adjacent to the bulb-shaped structures also contained a dense, firmly attached population of bacteria. The bacteria associated with the wall across from the bulb-shaped structures were of the same general morphology as those associated with the bulb-shaped structures. In addition, numerous rods up to 10 μm long were observed (Fig. 8). The wall area between the bulb-shaped structures was dominated by one morphotype of bacterium (Fig. 9). Only occasional bacteria remained on the bulb-shaped structures and wall after the animal molted, and presumably these were transients not attached to the gut wall (Fig. 10).

Packets of dense-staining granules were observed intracellularly in the tissue of the cuticular structures (Fig. 11, arrow A). These granules were often in close proximity (within 1 μm) of the lumen. The composition of these granules remains unknown; however, granules similar to these have been observed in termite gut wall tissue (2). The bacteria associated with the cuticular structures were surrounded by a dense-staining material of unknown composition (Fig. 11, arrow B).

Microscopy of the peripheral fat bodies. Phase-contrast microscopy of toluidine blue-stained 0.5-μm sections of the fat bodies revealed no recognizable bacterial cells. Electron microscopy confirmed the absence of associated bacteria.

CFU. Table 1 shows the number of CFUs obtained by plating midgut contents, hindgut contents, and homogenates of hindgut wall from 4-week-old crickets. No significant difference was found between the numbers obtained under aerobic and anaerobic conditions; however, a significant difference was observed between the midgut and hindgut contents under both incubation conditions.

Bacterial isolates from midguts, hindguts, and feces of crickets. Of the 25 bacterial isolates obtained, 11 were identified as members of the following three genera: *Citrobacter*, *Klebsiella*, and *Yersinia*. A summary of the biochemical characteristics of these isolates is given in Table 2. Four of the isolates were strict anaerobes and belonged to the genera *Bacteroides* and *Fusobacterium*; the biochemical characteristics of these are shown in Table 3. Based on presumptive tests, six isolates (two each from midgut contents, hindgut contents, and hindgut wall) were identified as streptococci. Three gram-negative, facultatively anaerobic rods and one gram-positive, catalase-negative facultatively anaerobic rod were not identified.

Clearing of uric acid. A total of 40 to 85% of the colonies on anaerobic CFU replicate plates cleared uric acid; 90 to 100% of the colonies on aerobic CFU replicate plates cleared uric acid. The total numbers of colonies growing on these plates did not vary significantly from the numbers on the CFU plates of the same dilution. Of these isolates, *Citrobacter*, *Klebsiella*, *Yersinia*, one *Streptococcus* isolate, and the unidentified gram-negative bacteria all cleared uric acid. The *Bacteroides* and *Fusobacterium* isolates and the unidentified gram-positive rod failed to clear uric acid.

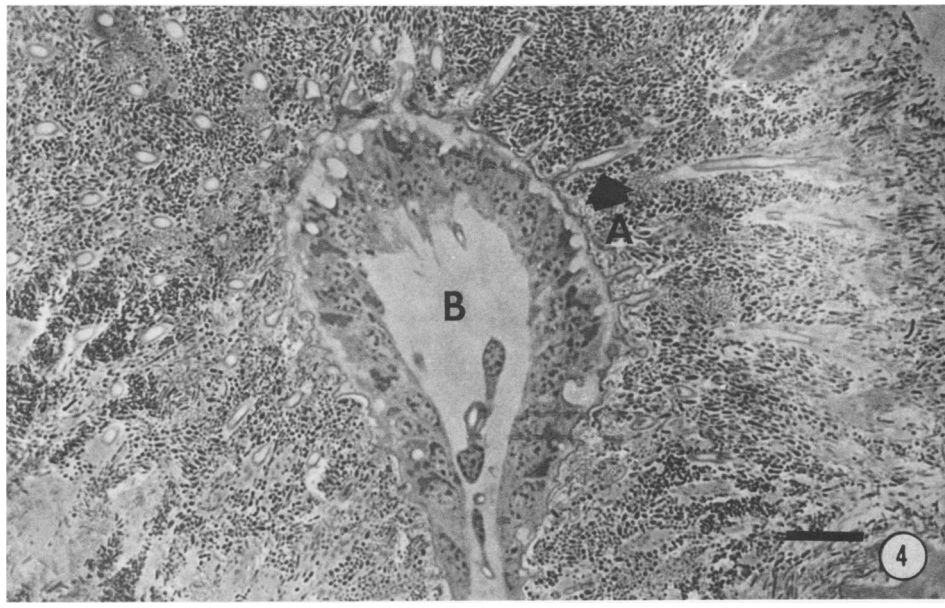
Uric acid in crickets. The percent dry weights of body tissues, fat bodies, and feces that were composed of uric acid in crickets were 2.0, 53.0, and 79%, respectively.

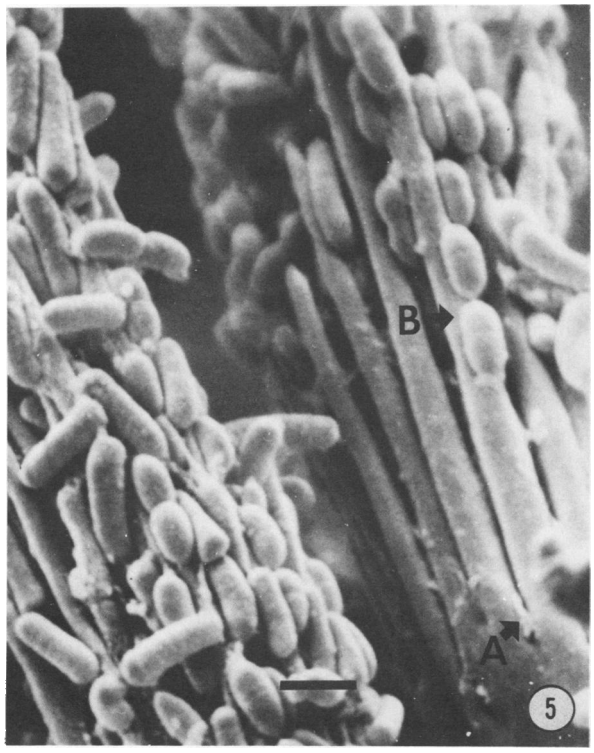
Cellulolytic enrichment. The cellulolytic enrichment plates showed no sign of cellulose clearing after 4 weeks of incubation.

DISCUSSION

Observations made by scanning electron microscopy and transmission electron microscopy demonstrated that bacteria were attached to the peritrophic membranes in the midgut and gut wall and to cuticular bristles in the hindgut. Further determinations of the nature of the bacteria attached to the peritrophic membranes were not made due to the fragile nature of the membranes.

The failure to clear the wall when vigorous blending in a Vortex mixer was used demonstrated the firm attachment of the bacteria to the hindgut wall and cuticular bristles. Coloni-





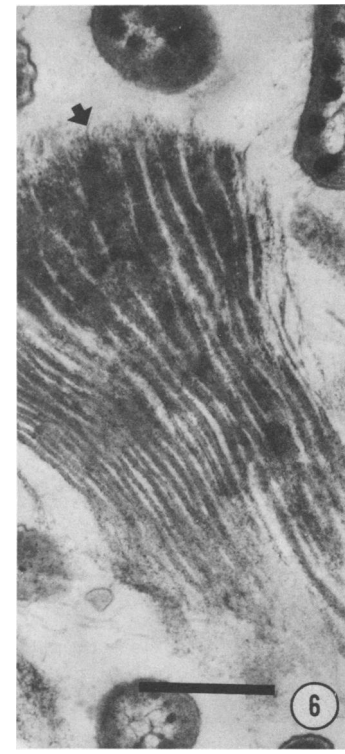


FIG. 4. *Phase-contrast photomicrograph of a cross-section through a hindgut bulb-shaped structure (Fig. 3). Numerous bristles (arrow A) protrude from the bulb surface and are densely surrounded by bacteria. The center of the bulb (region B) is thought to be continuous with the hemocoel (Fig. 2). Bar = 10 μm.*

FIG. 5. *Scanning electron micrograph of bristles protruding from the surface of a bulb-shaped structure. Each bristle is shaped like a paintbrush, with spines branching out from a base (arrow A). Bacteria (arrow B) are attached to the bristles. Bar = 1.0 μm.*

FIG. 6. *Transmission electron micrograph of a section through the tip of one of the bristles, showing its division into fine fibers (arrow). Bar = 0.5 μm.*

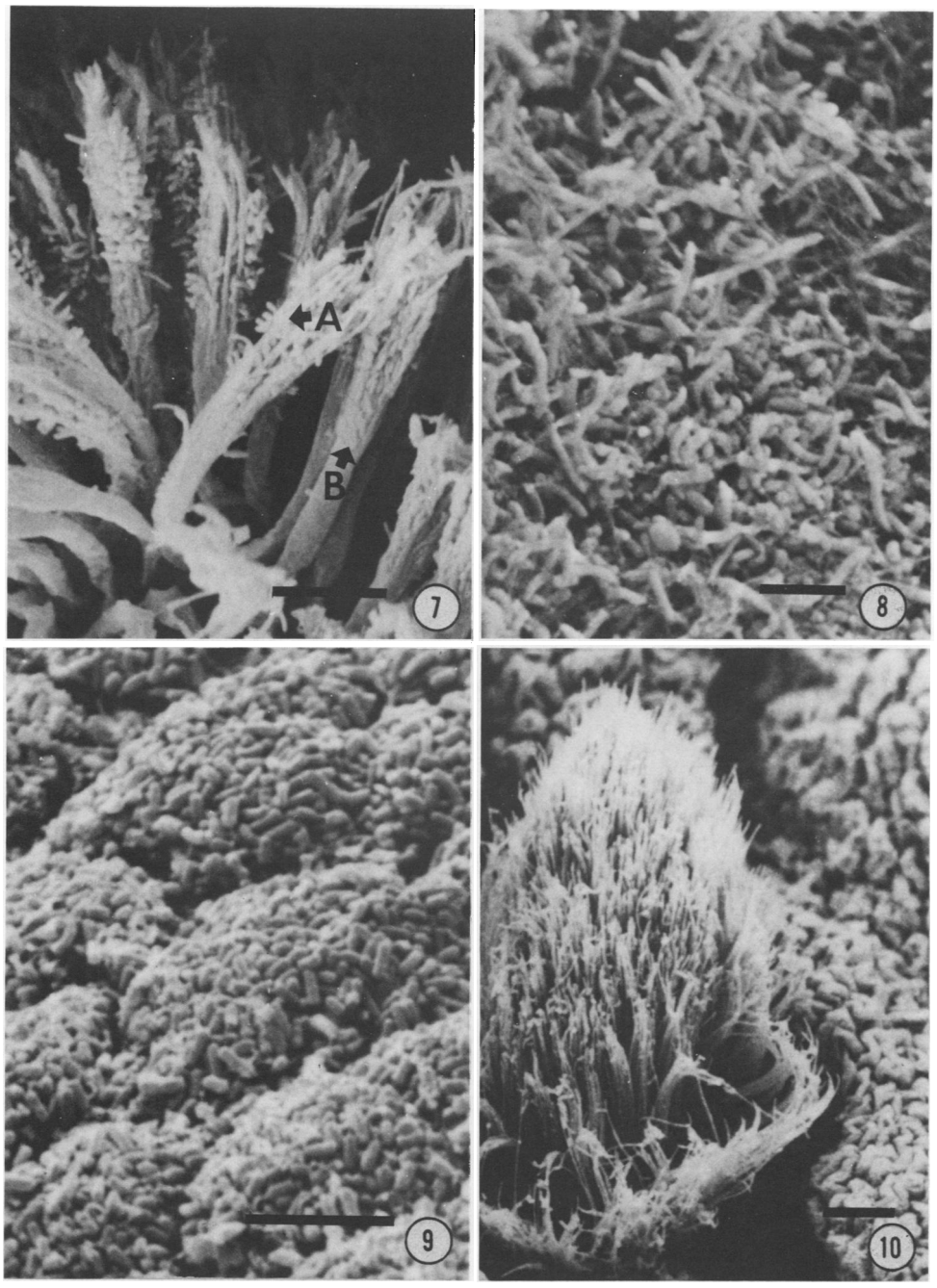


FIG. 7. *Scanning electron micrograph of a hindgut bulb-shaped structure in the hindgut of a 4-week-old cricket. The following different types of bacterial attachment are present: end-on (arrow A) and chaining (arrow B). Bar = 10 μm.*

FIG. 8. *Scanning electron micrograph of the wall area across the lumen from the bulb-shaped structures in the hindgut. Bar = 2.5 μm.*

FIG. 9. *Scanning electron micrograph of the hindgut wall area between the bulb-shaped structures. Bar = 5 μm.*

FIG. 10. *Scanning electron micrograph of a hindgut bulb immediately after molting. Bar = 10 μm.*

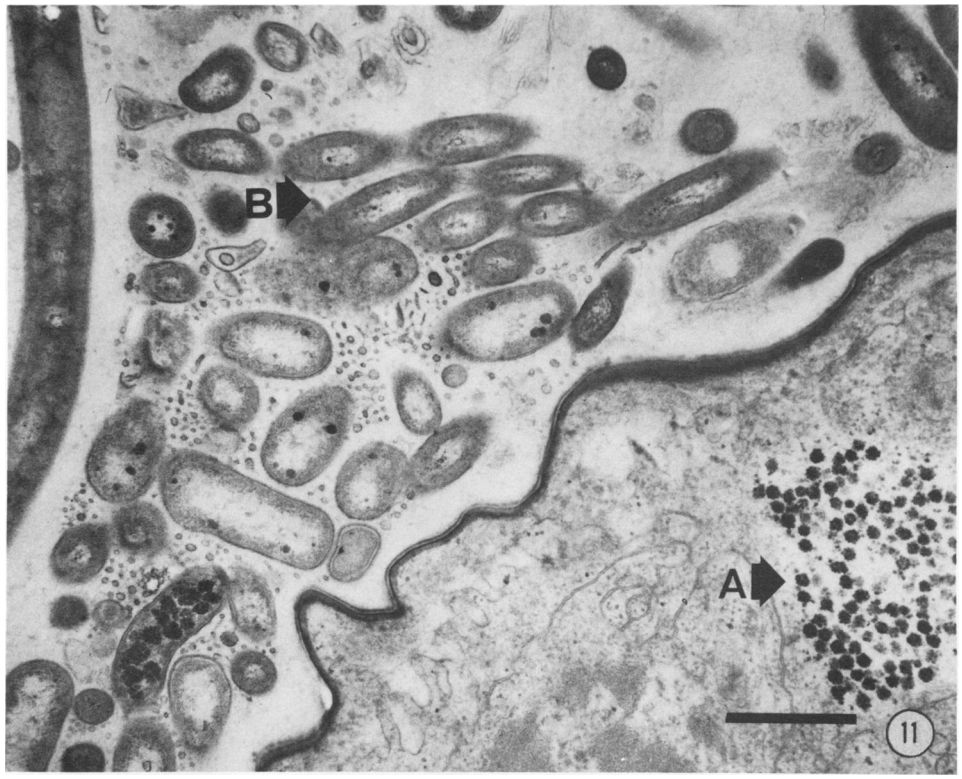


FIG. 11. *Transmission electron micrograph of the hindbut bulb-lumen interface. Dense-staining granules are in close proximity to the lumen (arrow A). A microcolony of bacteria surrounded by a dense-staining matrix is also present (arrow B). Bar = 1.0 μm.*

zation of the cuticular bristles was similar to the colonization of the cuticular spines in the hindgut of cockroaches (1, 7, 10). However, the bacteria had no apparent holdfast structures, as were observed associated with bacteria attached in the hindguts of cockroaches (1, 7, 10) and paunches of termites (2). Attachment appears to be through the dense-staining material observed surrounding the bacterial cells (Fig. 11, arrow B). Compared with the studies on cockroaches and termites, no filamentous bacteria and a general lower diversity of morphological forms were observed in crickets.

Bacteria alone seem to populate the gut, as no protozoans or fungi were observed in any preparation. Light microscopic observations of wild crickets demonstrated the same bacterial associations. Aerobic and anaerobic CFU counts of bacteria in the midgut and hindgut were nearly identical. Since the same media were used for both sets of cultures, these data suggest that most of the cultivatable microorganisms are facultative bacteria. This hypothesis is supported by the number of facultative bacteria isolated (Table 2). The counts also indicated that there

TABLE 1. *Number of CFUs of bacteria obtained after aerobic and anaerobic incubations of the contents and walls from various regions of the gut on tryptone yeast extract agar*

Location	Avg no. of anaerobic CFUs/mg (dry wt)[a]	Avg no. of aerobic CFUs/mg (dry wt)[a]
Midgut	4.9×10^8 (1.2×10^8)[b]	5.0×10^8 (3.1×10^8)
Hindgut	2.9×10^9 (3.2×10^9)	2.1×10^9 (2.2×10^9)
Hindgut wall	1.6×10^9 (1.2×10^9)	1.3×10^9 (5.0×10^8)

[a] $n = 5$.

[b] Numbers in parentheses are standard deviations (95% level of confidence).

are three times more culturable bacteria in the hindgut than in the midgut. The larger number of bacteria in the hindgut may be due to retention of the bolus in the hindgut by the bristle structures.

General morphologies of the facultative bacteria isolated coincide with those of the bacteria observed by light and electron microscopy. Based on morphological and biochemical characteristics, the unidentified gram-negative bacteria appear to be members of *Enterobacteria-*

ceae. Presumptive tests indicate that the unidentified gram-positive rod is a member of the genus *Lactobacillus.*

Anaerobic and facultative bacteria similar to our isolates from crickets were also found in the termite *Reticulitermes flavipes* (17, 19). Our failure to recover any strict anaerobes by random isolations from midgut CFU plates indicates that anaerobes either represent a small portion of the midgut population or are absent.

The ability to clear uric acid was a common characteristic of a high percentage (40 to 90%) of the CFUs. Similarly, Potrikus and Breznak (17) have shown high populations of uricolytic bacteria in the hindgut of the termite *R. flavipes.* Uric acid made up 1 to 45% of the dry weight of termites (16), with the majority associated with the fat bodies. Feces of these same animals contained a small percentage of uric acid. Similar findings were observed for a number of cockroach species (6, 15).

TABLE 2. *Characteristics of gram-negative, facultative rods isolated from guts of crickets, as determined by the API 20E test system*

Characteristic	*Klebsiella*[a]	*Citrobacter*[b]	*Yersinia*[c]	Unidentified strain 1[d]	Unidentified strain 2[e]
ONPG[f]	+[g]	+	+	+	−
Arginine	−	−, (+)	−	−	−
Lysine	−	−	−	−	−
Ornithine	−	+	+	−	−
Citrate	−	−, (+)	−	−	−
H_2S	−	−	−	−	−
Urea	−	−	−	−	−
Tryptophane	−	−	−	−	−
Indole	+	+	−	−	−
Voges-Proskauer	+	−	−	−	+
Gelatin	−	−	−	−	−
Glucose	+	+	+	+	+
Mannitol	+	+	+	−	−
Inositol	+	−	−	−	−
Sorbitol	+	+	+	−	−
Rhamnose	+	+	+	−	−
Sucrose	+	−	+	−	−
Melibiose	+	−	+	+	+
Amygdalin	+	+	+	−	+
(+)-*l*-Arabinose	+	+	+	+	−
Oxidase	−	−	−	−	+
NO_2	+	+	+	+	
Gas	−	−	−	−	−
Motility[h]	+	−	−	+	

[a] Two isolates from the midgut and one isolate from the hindgut contents.
[b] Two isolates from the midgut, two isolates from the hindgut wall, one isolate from the hindgut contents, and one isolate from the feces.
[c] One isolate from the hindgut contents and one isolate from the feces.
[d] One isolate from the hindgut contents and one isolate from the feces.
[e] One isolate from the hindgut wall.
[f] ONPG, *o*-Nitrophenyl-β-D-galactopyranoside.
[g] +, Positive; −, negative; (+), positive for one isolate.
[h] Determined by the hanging drop method.

TABLE 3. *Characteristics of anaerobes isolated from guts of crickets, as determined by the API 20A system and gas chromatography*

Characteristic	*Fusobacterium* strain 1[a]	*Fusobacterium* strain 2[a]	*Bacteroides*[b]
Indole	+	−	−
Urea	−	−	−
Glucose	+	−	−
Mannitol	−	+	−
Lactose	+	−	−
Saccharose	+	−	−
Maltose	+	+	−
Salicin	−	+	−
(+)-*d*-Xylose	+	−	−
(+)-*l*-Arabinose	+	−	−
Gelatin	−	−	+,−
Esculin	−	−	−
Glycerol	−	+	−
Cellobiose	−	−	−
Mannose	+	−	−
Melezitose	+	−	−
Raffinose	+	−	−
Sorbitol	−	−	−
Rhamnose	+	−	+,−
Trehalose	+	−	−
Catalase	−	−	−
Gram stain	−	−	−
Acid fermentation products	B,V[c]	A,B[c]	A,P,I[c]
Flagella	−	Peritrichous	−

[a] This strain was isolated from the hindgut wall.
[b] Two isolates from the hindgut contents.
[c] A, Acetate; B, butyrate; P, proprionate; V, valerate; I, isovalerate.

The higher concentration of uric acid in the fat bodies than in the feces of termites (16) and cockroaches (6, 15) suggests recycling of uric acid. It has been hypothesized that the gut microbiota associated with the hindguts of cockroaches (7) and termites (13) may be involved in this recycling. The finding of a high percentage of uricolytic bacteria in the hindgut of *R. flavipes* (17), the absence of animal uricase activity (16), and the uricolytic activity of fresh gut contents (17) certainly support this hypothesis.

The reported ability of a high percentage of the CFUs from crickets to clear uric acid and the high percentage of uric acid in the feces of this animal do not support the hypothesis of uric acid recycling in this animal. Dietary differences may account for these results; however, little difference was found in the percentages of uric acid in the feces of a number of cockroach species fed on a variety of diets (15).

Although similarities exist in the diets and gut morphologies of crickets and cockroaches, each species appears to harbor distinctly different

bacterial morphotypes. Furthermore, the failure to demonstrate bacteria associated with the fat bodies of crickets suggest evolution away from the harboring of endosymbionts. The significance of these findings to the dietary requirements of crickets are currently under investigation.

ACKNOWLEDGMENTS

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