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NOTES

Bacteria Associated with the Ectoperitrophic Space in the Midgut of the Larva of the Midge *Xylotopus par* (Diptera:Chironomidae)†

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An ectoperitrophic association of bacteria with the midgut of *Xylotopus par* larvae was investigated by scanning electron microscopy and transmission electron microscopy. The bacteria attached to the epithelium as a well-defined band in the posterior midgut. They were morphotypically uniform and formed short filaments with endosporelike structures. The consistent presence and well-defined location of the bacteria in a region of the insect digestive tract usually void of microbes indicates a highly evolved symbiotic association, the nature of which is unknown at present.

The association of microorganisms with the intestinal tracts of insects is varied and widespread. Reports of insect-microbe associations usually describe bacterial populations in the hindgut (2, 7, 11). Colonization of midgut tissue surfaces by bacteria is much less common and is generally restricted to insects which lack a peritrophic membrane (4). The peritrophic membrane in insects is a chitinous sheath which envelops the food bolus and forms a continuous tube separating ingested particles from the epithelium throughout the length of the midgut (13). It is thought that one function of the membrane is to exclude microorganisms from midgut tissue (1, 5); however, ectoperitrophic colonization of the midgut has been shown to occur in termites (3, 4, 6). To our knowledge, the studies of microbes of termite intestines constitute the only previous descriptions of this type of association in insects.

We report here a unique ectoperitrophic association in the midgut of the larva of the midge *Xylotopus par*. *X. par* larvae inhabit and feed upon submerged, decayed wood in eastern North American streams (12; M. Kaufman, M.S. thesis, Central Michigan University, Mount Pleasant, 1983). Meitz (A. Meitz, M.S. thesis, Michigan State University, East Lansing, 1978), using phase-contrast microscopy, first noted a conspicuous band of bacteria localized in the posterior midguts of the larvae. We examined the association in further detail by scanning electron microscopy and transmission electron microscopy.

Fourth-instar larvae were collected from logs in Augusta Creek (Kalamazoo County, Michigan). Guts to be observed by scanning electron microscopy were excised from the animal under 0.1 M phosphate buffer and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer. Tissues were then rinsed in 0.1 M phosphate buffer and dehydrated in a graded ethanol series. Cross sections through the colonized portion of each midgut were obtained by freeze-fracturing in liquid nitrogen.

Some additional tissues were examined in the following manner. A small section of the midgut, containing the bacterial band, was cleaved from an excised gut. The peritrophic membrane generally was slightly extruded from the rest of the section at one end of the cleaved section. Ultrafine-tip forceps were then used to gently pull the membrane and enclosed contents from the encircling epithelium. This technique left a small, hollow cylinder consisting of the midgut tissue and the associated bacterial band. This tissue was then sliced longitudinally and vortexed vigorously in 0.1 M phosphate buffer. Fixation and dehydration were

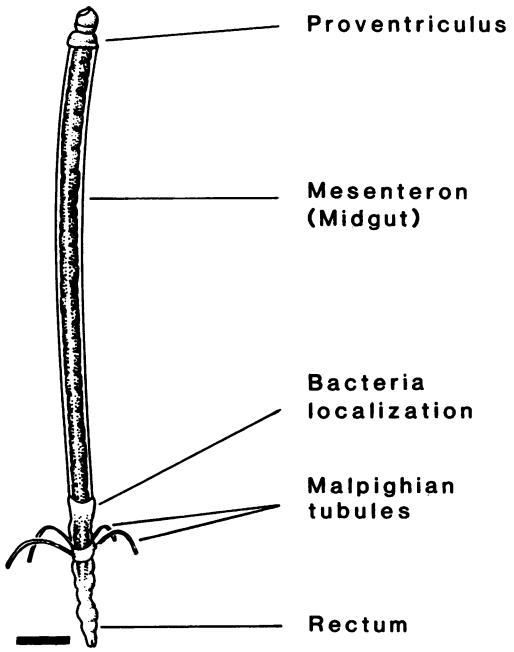

FIG. 1. Diagram of the digestive tract of *X. par* larvae. Bar = 1 mm.

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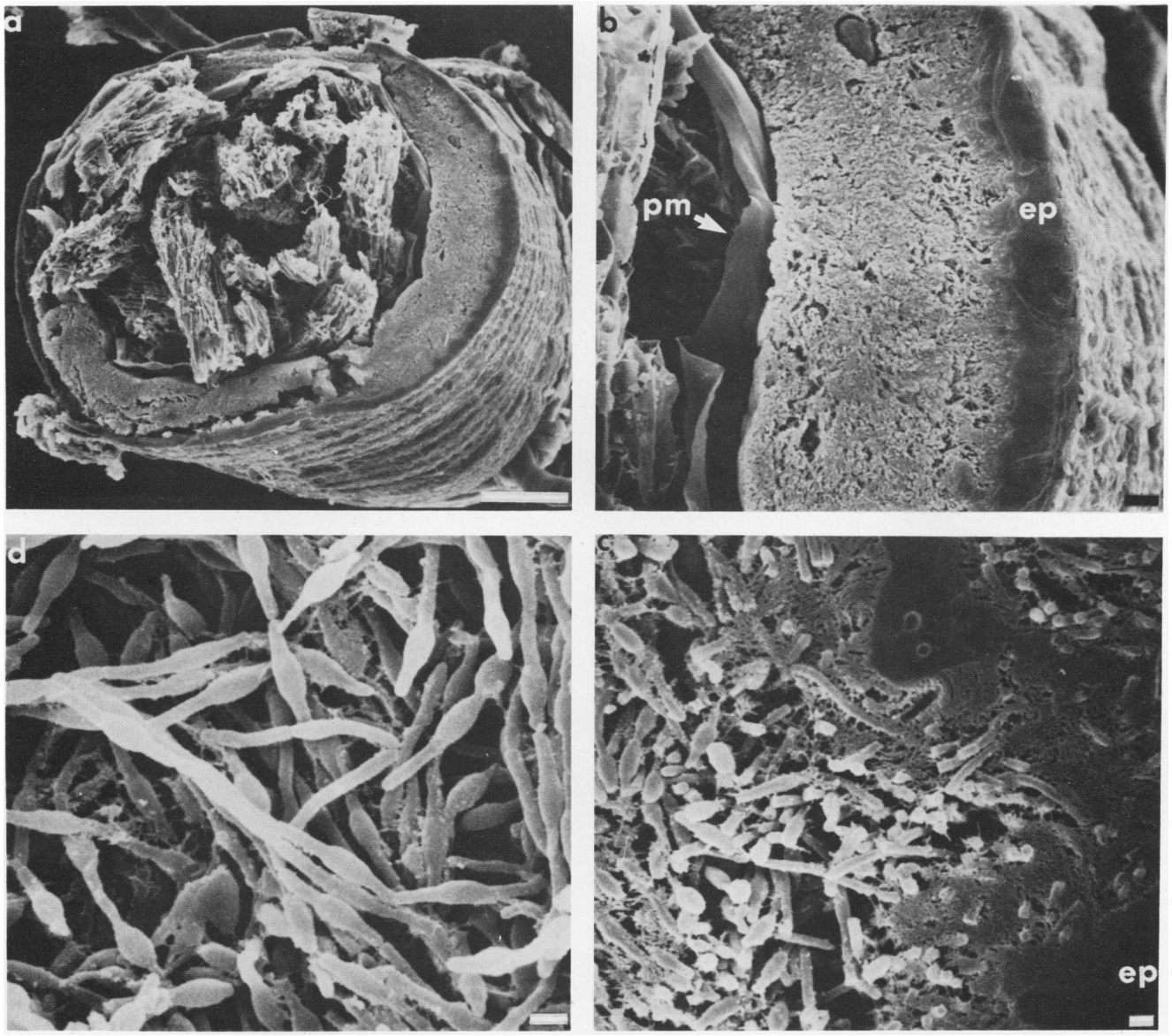

FIG. 2. (a) Scanning electron micrograph of transverse section through posterior midgut of *X. par* larvae. The central region shows gut contents (wood particles). Bar = 100 μm. (b) Higher magnification of panel a, showing the band of bacteria between the peritrophic membrane (pm) and midgut epithelium (ep). Bar = 10 μm. (c) Bacterial/epithelial cell interface, showing a thick adhesive matrix between the bacterial cells and the epithelium (ep). Bar = 1 μm. (d) Surface morphology of midgut bacteria. Bar = 1 μm.

done as described above. Dehydrated tissues were critical-point dried in CO_2, coated with gold, and examined with a Super III scanning electron microscope (International Scientific Instruments, Inc., Santa Clara, Calif.).

Dissected gut tracts were prepared for transmission electron microscopy by fixation in 2.5% glutaraldehyde. After being rinsed with 0.1 M phosphate buffer, the tissues were post-fixed with osmium tetroxide and dehydrated in a graded ethanol series. Alternatively, some specimens were fixed in a tannic acid-glutaraldehyde-saponin mixture, essentially by the method of Maupin and Pollard (10). Dehydrated specimens were embedded in either VCD/HXSA or DER epoxy resin (PolyScience Corp., Niles, Ill). The tannic acid fixation, in combination with the VCD/HXSA embedding, yielded the best results (see Fig. 2 and 3). Embedded specimens were thin-sectioned with a diamond knife (Du Pont Co., Wilmington, Del.) mounted on an Ultrotome III (LKB Instruments, Inc., Rockville, Md.) and post-stained with uranyl acetate and lead citrate. Micrographs were taken with a Philips EM300 transmission electron microscope.

The gut morphology of *X. par* larvae and localization of the midgut bacteria are illustrated in Fig. 1. Bacteria were found attached to the gut wall exclusively within the band area. Meitz (M.S. thesis), however, previously reported the occasional attachment of rods to the hindgut in some larvae examined by phase-contrast microscopy. In our study, transmission electron microscopy examinations of hindgut transverse sections revealed no bacteria. The midgut bacteria formed a continuous band that lay on the lumen side of the epithelium and encircled the peritrophic membrane (Fig. 2a and b). In late-fourth-instar larvae, the bacteria occupied a zone which averaged 0.75 mm in length (standard deviation 0.15; $n = 20$), the posterior edge of which was located 0.94 mm (standard deviation = 0.13; $n = 20$) anterior to the insertion of the Malpighian tubules (midgut/hindgut junction). This association of bacteria with a distinct narrow

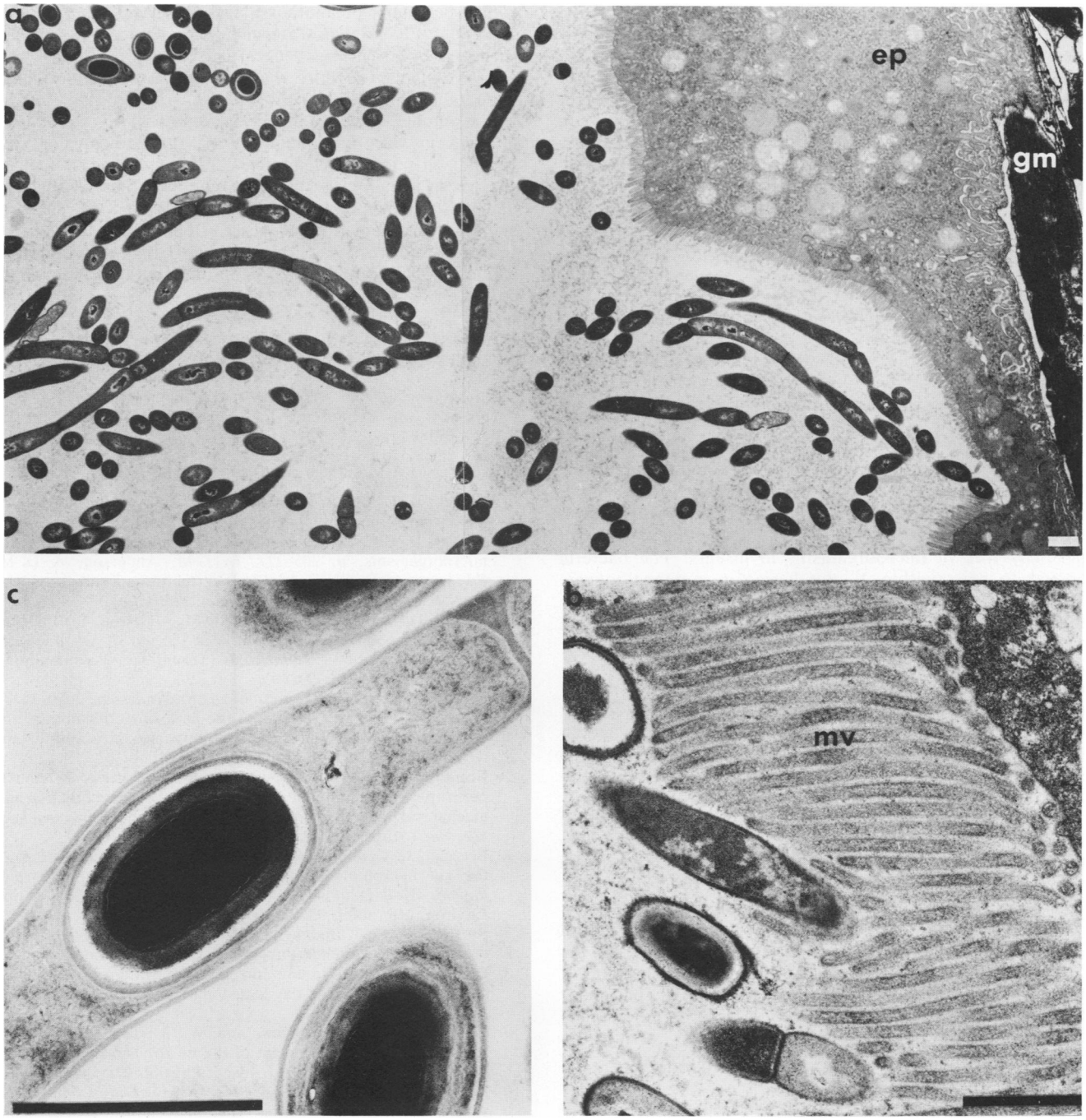

FIG. 3. (a) Transmission electron micrograph of a transverse section through the bacterial/midgut epithelium interface in *X. par* larvae. Abbreviations: ep, midgut epithelium; gm, gut wall muscle. The adhesive matrix appears as dark, granular material between the bacterial cells. (b) Interdigitation of bacteria with microvilli (mv). The adhesive matrix is also apparent. (c) Detail of the spore morphology of associated bacteria. Bar = 1 μm in all micrographs.

zone of the midgut is, to our knowledge, a novel occurrence in insects. The physiological characteristics of the region that make it suitable for colonization have not been determined, but examinations of uncolonized regions posterior and anterior to the band of bacteria revealed no marked change in midgut tissue morphology.

The microorganisms apparently do not attach to the peritrophic membrane, as has been reported for the actinomycetes inhabiting the ectoperitrophic space in the termite *Cubitermes severus* (4). Instead, examination of vortexed gut tissue showed a firm attachment en masse to the midgut epithelium. Ectoperitrophic midgut bacteria in the termites *Procubitermes aburiensis*, *Reticulitermes flavipes*, and *Coptotermes formosanus* adhere in close physical proximity to microvilli or epithelial cell surfaces or both (3, 6). The bacteria in *X. par* larvae may not attach by such intimate association with epithelial cells. Examinations of the epithelium/microbe interface only rarely revealed bacterial cells between microvilli (Fig. 3b), and no bacteria were observed near the epithelial cell surfaces at the bases of the microvilli. The mode of attachment appears to be a granular matrix (Fig. 2c and 3a and b) that may serve as an adhesive

to the insect gut tissue and to other bacterial cells. The composition of the matrix is unknown, and its origin is assumed to be the bacteria themselves.

In addition to its well-defined localization, the band of bacteria is apparently composed of a single morphotype. Figures 2d and 3a illustrate the filamentous, sporeforming morphology characteristic of the bacteria. Branching was not observed, and most filaments appeared to consist of fewer than five cells. The bacteria show gram-positive or gram-variable staining characteristics and are at least facultatively anaerobic, because O_2 microelectrodes detected no oxygen in the lumen of the posterior midgut (unpublished data).

The sporeforming characteristic (Fig. 2d and 3c) of the midgut bacteria was generally observed more frequently in cells closest to the lumen side of the band. Spores were also most common in the terminal cell of the filament. The significance of these structures is unclear, but, interestingly, midgut bacteria in worker termites are also reported to form endosporelike structures (6, 7).

The band of bacteria was found in examinations of younger (second- and third-instar) larvae and occupied the same gut region. All *X. par* larvae examined to date (>500) contained this extremely localized, morphotypically consistent mass of bacteria in a region of the digestive tract normally free of microorganisms in insects. The bacteria appear to exist within *X. par* larvae in a nonpathogenic relationship. The considerable mass of the bacteria relative to the gut tissue in the region (Fig. 2a and b) seem to indicate a significant, if local, effect on the digestive system of *X. par*. A mutalistic, nutritional contribution to *X. par* is an appealing hypothesis, yet the location of the bacteria, outside the peritrophic membrane and thus isolated from direct contact with ingesta, seems to preclude significant polymer degradation. Furthermore, no cellulolytic activity has been detected in midgut homogenates of *X. par* larva gut tracts (unpublished data).

The location of the bacteria within the ectoperitrophic space also raises interesting questions regarding fluid movement and digestive processes in the midgut. Fluid in the ectoperitrophic space moves counter to the overall peristalsis in the midgut (1, 8, 9); liquid and dissolved material move anteriorly from the Malpighian tubules. Terra and Ferreira (14) have also shown the space to be important in the partitioning of digestive processes and the conservation of enzymes. Perhaps the bacteria are exploiting the particular properties of this stream of materials; however, the consequences to *X. par* of the bacterial mass within the ectoperitrophic space and the effects of its metabolic activities are unknown at this time.

The origin and transmission of the bacteria also are unexplained. The gut tract degenerates in pupal and adult chironomids, and it is unlikely that the midgut bacteria survive the metamorphosis to be carried over to eggs and subsequent generations of larvae. It is probable that the bacteria recolonize from the submerged wood habitat and somehow circumvent the peritrophic membrane after ingestion. The endosporelike structures may play a part in this dispersal; however, no spores were observed in examinations of fecal material by phase-contrast microscopy.

The significance of a wood-feeding habit to the presence of midgut bacteria is unclear, but it is interesting to restate the point that termites harbor midgut bacteria also. Additionally, some specimens of another dipterous insect, *Epiphragma* (Tipulidae), have been observed with a band of bacteria in the posterior midgut (unpublished observations). *Epiphragma* larvae bore into rotting wood in flood plain habitats.

The unique, sharply delineated location of this association of bacteria with the midgut of *X. par* indicates a very specialized microorganism-insect relationship. Our lack of success in isolating the bacteria on general media suggests that the organisms have fastidious nutritional, physical, or chemical requirements which are supplied through this association. Efforts are currently in progress to isolate the bacteria and elucidate their role in the feeding ecology of *X. par*.

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