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Ultrastructural Studies of the Fat Body and Bacterial Endosymbionts of *Cryptocercus punctulatus* Scudder (Blattaria: Cryptocercidae)

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Abstract

Transmission electron microscopy of ultra-thin sections and freeze-fracture replicas of the fat body of the wood feeding cockroach *Cryptocercus punctulatus* show that the protein and lipid stores in trophocytes are substantially smaller than in those of cockroaches that are not xylophagous. The urocytes exhibit needle-shaped deposits of urate arranged in concentric layers around central cores. Bacteriocytes harbor in their cytoplasm Gram-negative bacterial endosymbionts enclosed in a vacuolar membrane. In the vacuolar space, vesicles formed by a blebbing process were observed detaching from the symbionts' outer membrane. These processes suggest the presence of metabolite exchange between host cell and endosymbionts. Among adjacent bacteriocytes, trophocytes, and urocytes, cell membranes exhibit junctional complexes that may allow for metabolic interaction. Transmission of the bacterial endosymbionts occurs *via* transovarial transmission, as in other cockroaches.

Keywords: *Cryptocercus punctulatus*, endocytobiosis, symbiotic bacteria, fat body, cockroaches

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1. Introduction

Wood feeding cockroaches of the genus *Cryptocercus* are regarded as key organisms for understanding the origin and evolution of termite symbioses and social organisation (Seelinger and Seelinger, 1983; Nalepa, 1984, 1991, 1994). In addition to their wood diet, these cockroaches are subsocial and harbor a community of interacting symbiotic microorganisms in a hindgut paunch. The need for the transmission of these gut symbionts from parents to offspring *via* anal trophallaxis is hypothesized to have been one of a complex of life history traits that characterized termite ancestors (Nalepa, 1994).

Cryptocercus spp. are unique among cockroaches in that they have two categories of symbionts: the above mentioned gut microorganisms, typical of lower termites (reviewed by Breznak, 1982), and intracellular bacterial endosymbionts in the fat body, typical of cockroaches. Only the termite *Mastotermes darwiniensis* has the same double set of symbionts (Jucci, 1952; Grassé and Noirot, 1959). In view of their large size, intrinsic interest, and relevance to social evolution in termites, it is not surprising that several studies since the pioneering work of Cleveland et al. (1934) have focused on the flagellated gut protozoans of *Cryptocercus*. The fat body endosymbiotic bacteria of this cockroach genus, however, are less well known.

Functionally, the symbiosis with bacterial endosymbionts in cockroaches is expressed in many metabolic pathways. The best studied of these is the degradation of uric acid stored in the host fat body (Wren and Cochran, 1987). The majority of cockroaches build up internal deposits of uric acid when their diet is rich in nitrogen. Later, *via* their fat body endosymbionts, they mobilize and use these urate stores when their diet lacks nitrogen or when their nitrogen requirements increase (Cochran and Mullins, 1982; Mullins and Cochran, 1987). This storage-mobilization nitrogen physiology may be one basis for the success of cockroaches in evolutionary time, as it may allow for several traits exhibited by the taxon, including reproductive versatility (Nalepa and Bell, 1997).

The importance of characterizing the relationship of *Cryptocercus* to its endosymbiotic bacteria stems from the role of this genus as our best extant model of a subsocial termite ancestor. Because it has been proposed that the unequal distribution of nitrogenous materials among family members was a key factor in the evolution of termite eusociality (Nalepa, 1994), the role of bacterial endosymbionts in mobilizing stored nitrogenous reserves may have been a crucial one. A phylogeny of cockroach bacterial endosymbionts based on the genes encoding for the small subunit ribosomal rRNA (16S rDNA) provides evidence for a deep branching of the evolutionary line leading to the endosymbionts of *Cryptocercus*. Furthermore, analysis of the endosymbionts of

the termite *Mastotermes darwiniensis* indicates that the symbiotic association with fat body bacteria was already established in an ancestor of cockroaches and termites prior to the divergence of these two taxa (Bandi et al., 1994, 1995). Subsequently, fat body endosymbionts should have been lost in termite lineages, with the exception of the lineage which gave rise to *M. darwiniensis*.

We had two major goals in initiating this study. The first was to analyze the ultrastructural morphology of the fat body of *C. punctulatus*, focusing in particular on urocytes, the cells specialized for storing urates, and on bacteriocytes, the cells containing symbiotic bacteria. The fat body of the termite *Zootermopsis nevadensis* (Hagen) was analyzed for comparison. The second goal was to observe in *C. punctulatus* the process of transovarial transmission. In cockroaches phylogenetically distant from the Cryptocercidae (Sacchi et al., 1988), the crucial steps of this process are known to occur at the interface between the follicular cells and the previtellogenic egg.

2. Materials and Methods

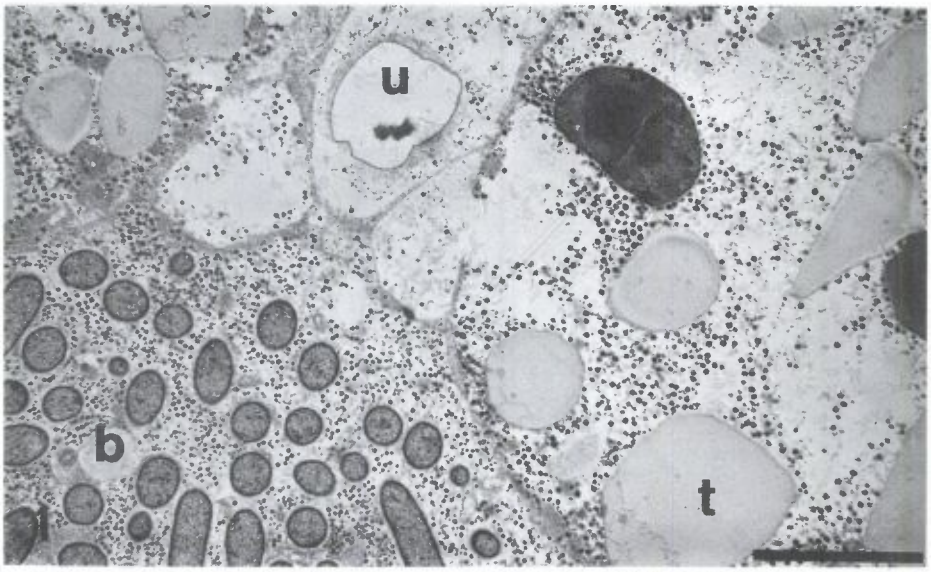
Subadult nymphs of *C. punctulatus* were collected at Mountain Lake Biological Station, Giles County, VA, USA. Pseudergates of *Zootermopsis nevadensis* were obtained from a culture maintained in Raleigh, NC by C. A. N.

Samples of abdominal visceral fat body and ovary were fixed for 2 h in a cold mixture of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), rinsed in the same fixative, post-fixed in 1% buffered OsO_4, dehydrated in ethanol and embedded in an Epon-Araldite mixture.

Freeze-fracturing was performed on fragments of the fat body fixed in Karnovsky's fixative for 30 min, cryoprotected in 10, 20 and 30% glycerol in 0.1 M cacodylate buffer (pH 7.2), frozen in Freon 22 and stored in liquid nitrogen. Freeze-fracturing was carried out with a Balzer unit. Freeze-fracture replicas and stained sections were examined and photographed with a Philips EM 400 and a Zeiss EM 900.

3. Results

As in other cockroaches (Cochran et al., 1979), there are three characteristic cell types in the fat body of *C. punctulatus*: (1) trophocytes, containing lipids and other reserves, (2) urocytes, containing crystalloid spherules of urates, and (3) bacteriocytes, cells specialized to house the symbiotic bacteria. The three types of cells adhere firmly to each other, and the membranes of adjacent cells are parallel over a wide area (Fig. 1).



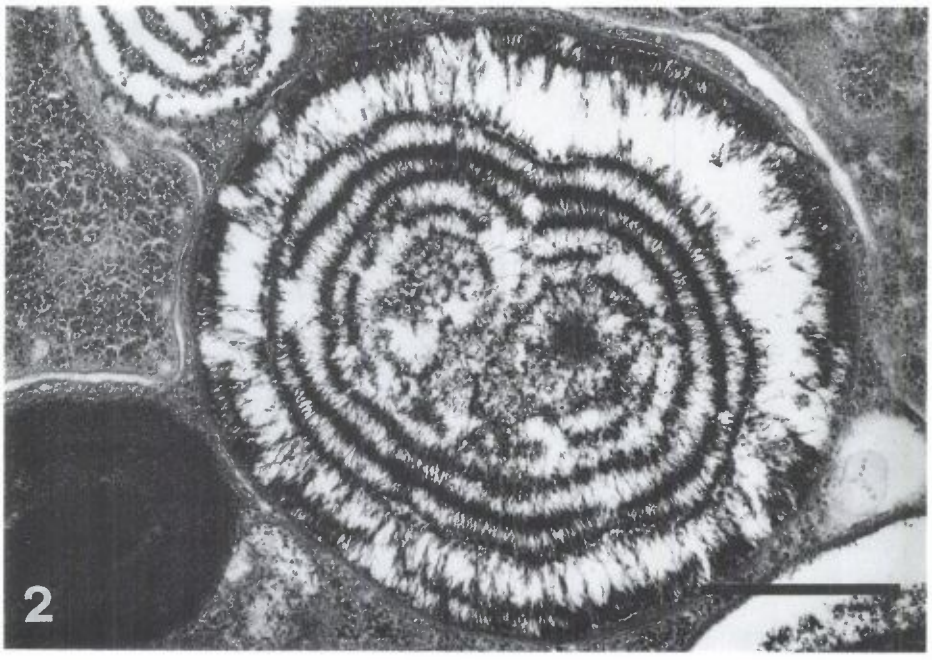
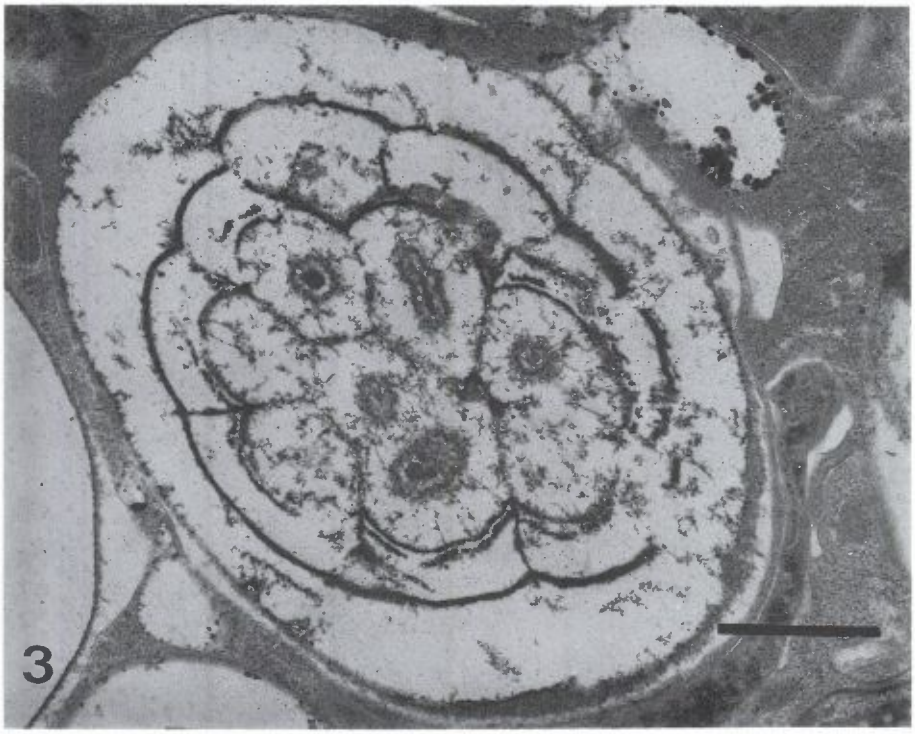


Figure 1. Characteristic cell types in the fat body of *Cryptocercus punctulatus*. Abbreviations: (t) trophocyte; (u) urocyte; (b) bacteriocyte. Bar = 5 µm.

Figure 2. Urocyte of *C. punctulatus*: note the crystalloid subunit arranged concentrically around dark cores of urate structural units. Bar = 0.8 µm.



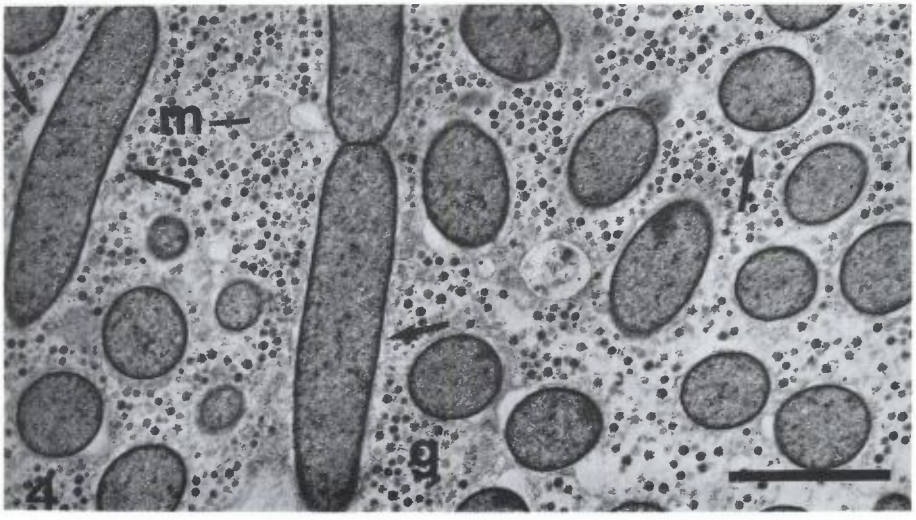


Figure 3. Urate structural unit of *C. punctulatus* showing urate deposition around a cluster of dark cores. Bar = 1.2 µm.

Figure 4. Bacteriocyte of *C. punctulatus* with cytoplasm filled by symbiotic bacteria. Abbreviations: (arrows) vacuolar membrane; (g) glycogen granules; (m) mitochondrion. Bar = 2.2 µm.

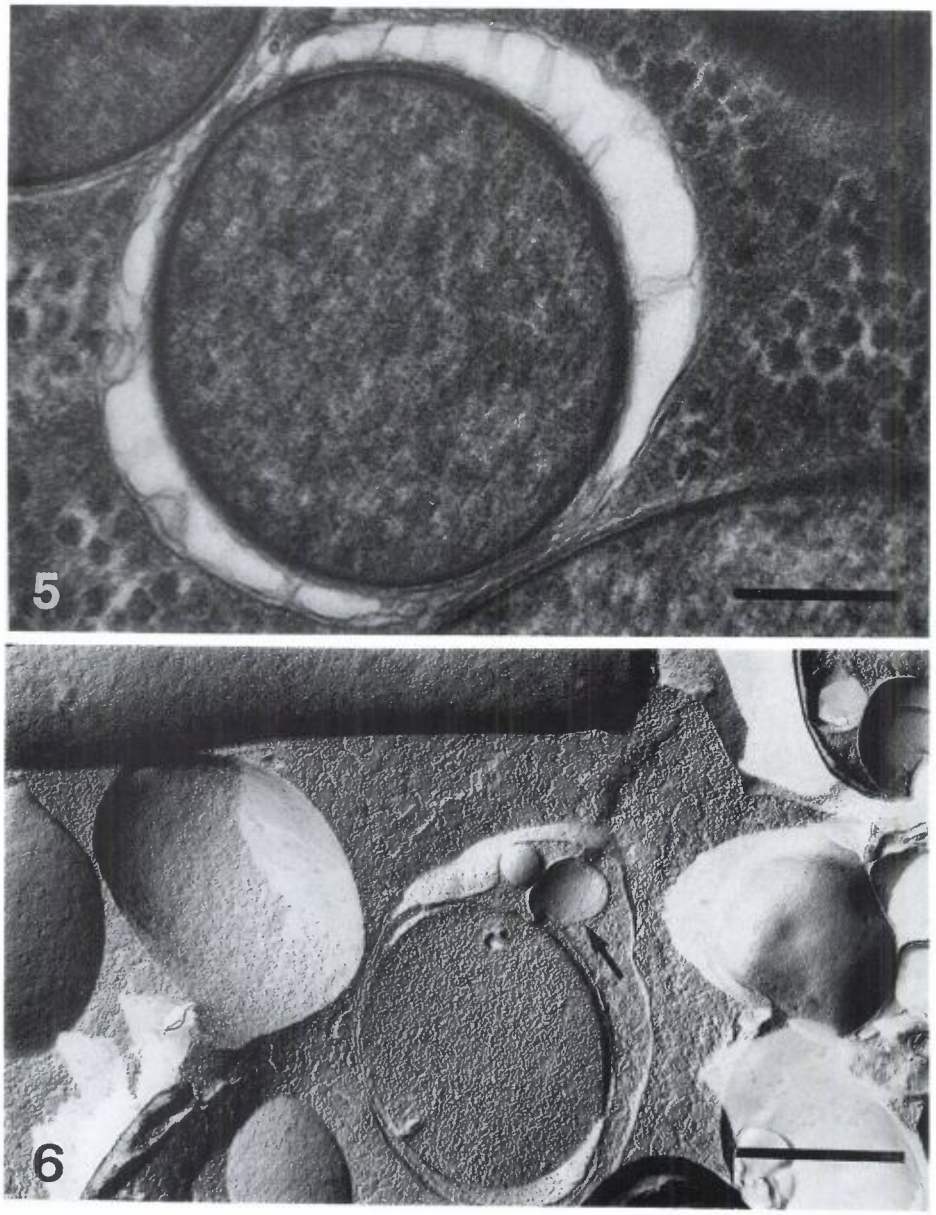


Figure 5. Detail of the vacuolar space showing an intense blebbing of the symbiont outer membrane. Bar = 0.3 μm.

Figure 6. Freeze-fracture replica of the vacuolar space showing the blebbing of the symbiont outer membrane. Two vesicles are present within the vacuolar space: the larger (arrow) is emerging from the symbiont outer membrane. Bar = 0.5 μm.

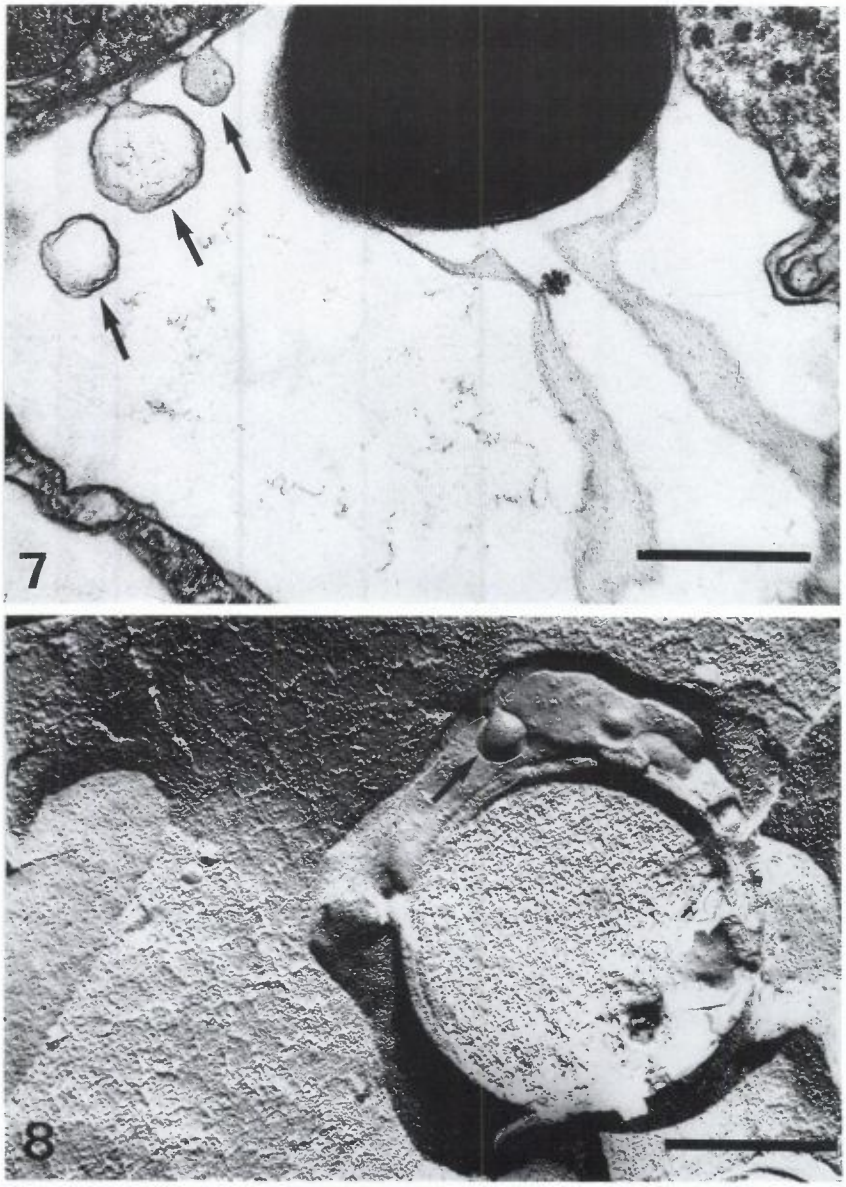


Figure 7. Detail of a bacteriocyte: in the vacuolar space a sequence of vesicles (arrows) is becoming detached from the vacuolar membrane. Bar = 0.5 μm.

Figure 8. Freeze-fracture replica of a bacteriocyte showing a roundish vesicle becoming detached from the vacuolar membrane (arrow). Bar = 0.56 μm.

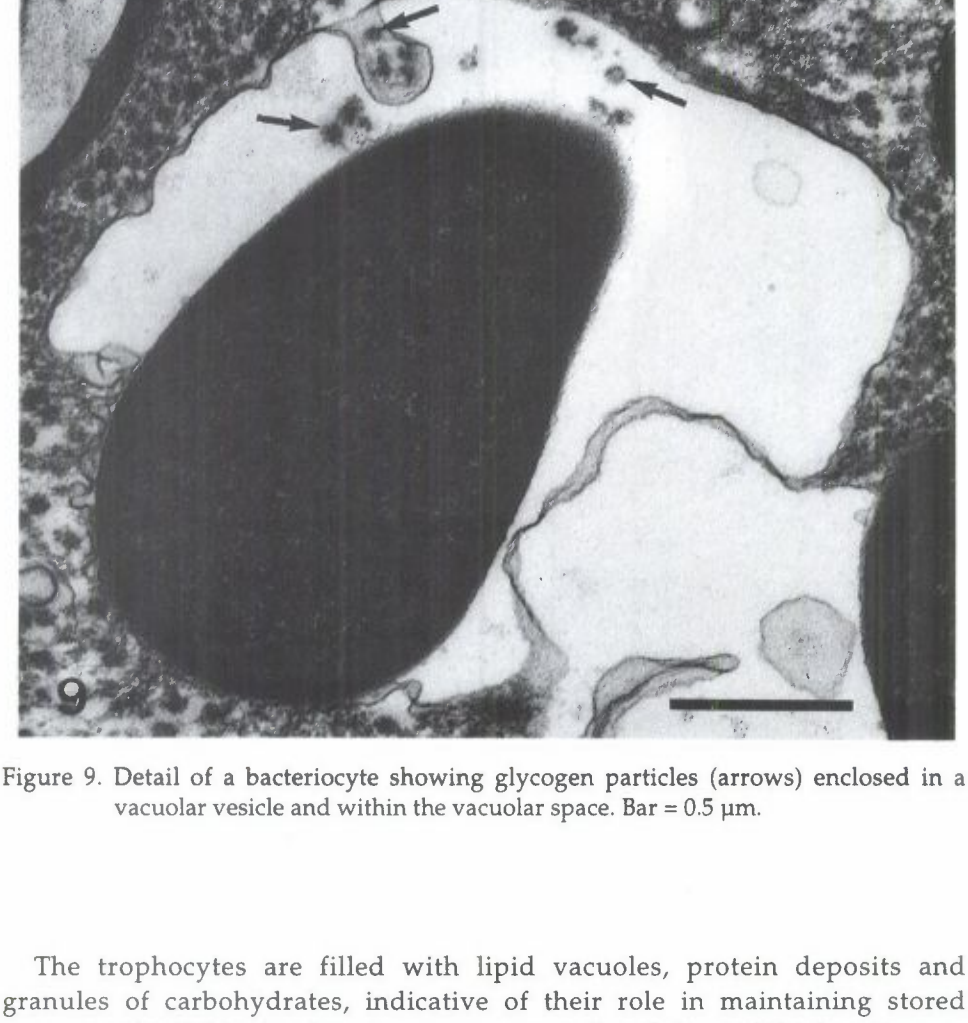


Figure 9. Detail of a bacteriocyte showing glycogen particles (arrows) enclosed in a vacuolar vesicle and within the vacuolar space. Bar = 0.5 μm.

The trophocytes are filled with lipid vacuoles, protein deposits and granules of carbohydrates, indicative of their role in maintaining stored reserves. The lipids droplets, however, are small and few, while the glycogen granules are abundant (Fig. 1).

The urocytes are rich in glycogen and contain typical urate structural units. These units are vacuolar bodies bounded by a double layered structure; they have a central dark region which may be the seed around which urate synthesis or deposition occur (Cochran et al., 1979). Fig. 2 shows the typical concentric arrangement of urate spherules around a dark core. Some urate units show a more complex structure, with urate crystalloid subunits aggregated around clusters of dark cores (Fig. 3); this might be a result of a cross section through a continuous but convoluted structure.

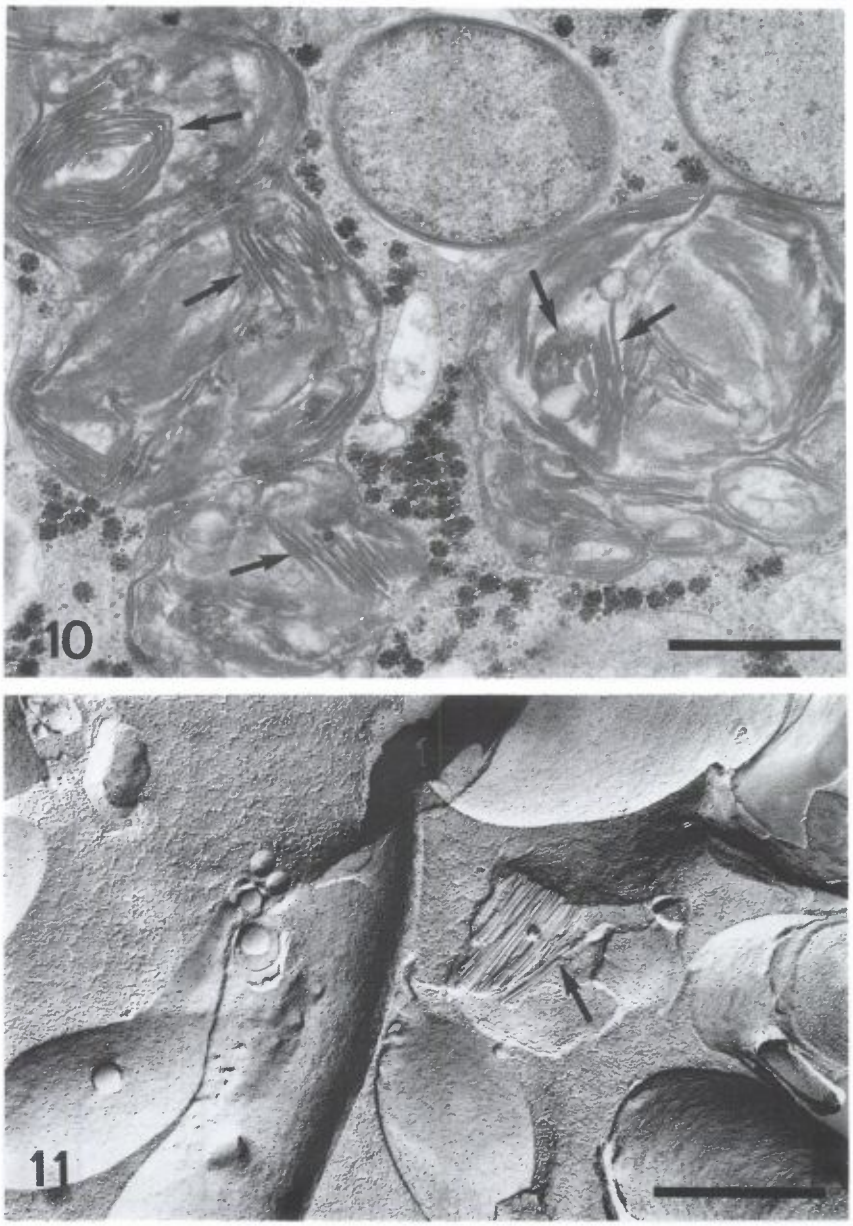


Figure 10. Detail of a bacteriocyte showing vacuoles encircling a packed set of tubular structures (arrows). Bar = 0.8 µm.

Figure 11. The freeze-fracture technique evidences in the bacteriocyte the presence of an envelope encircling the densely packed tubular structures (arrow). Bar = 0.5 µm.

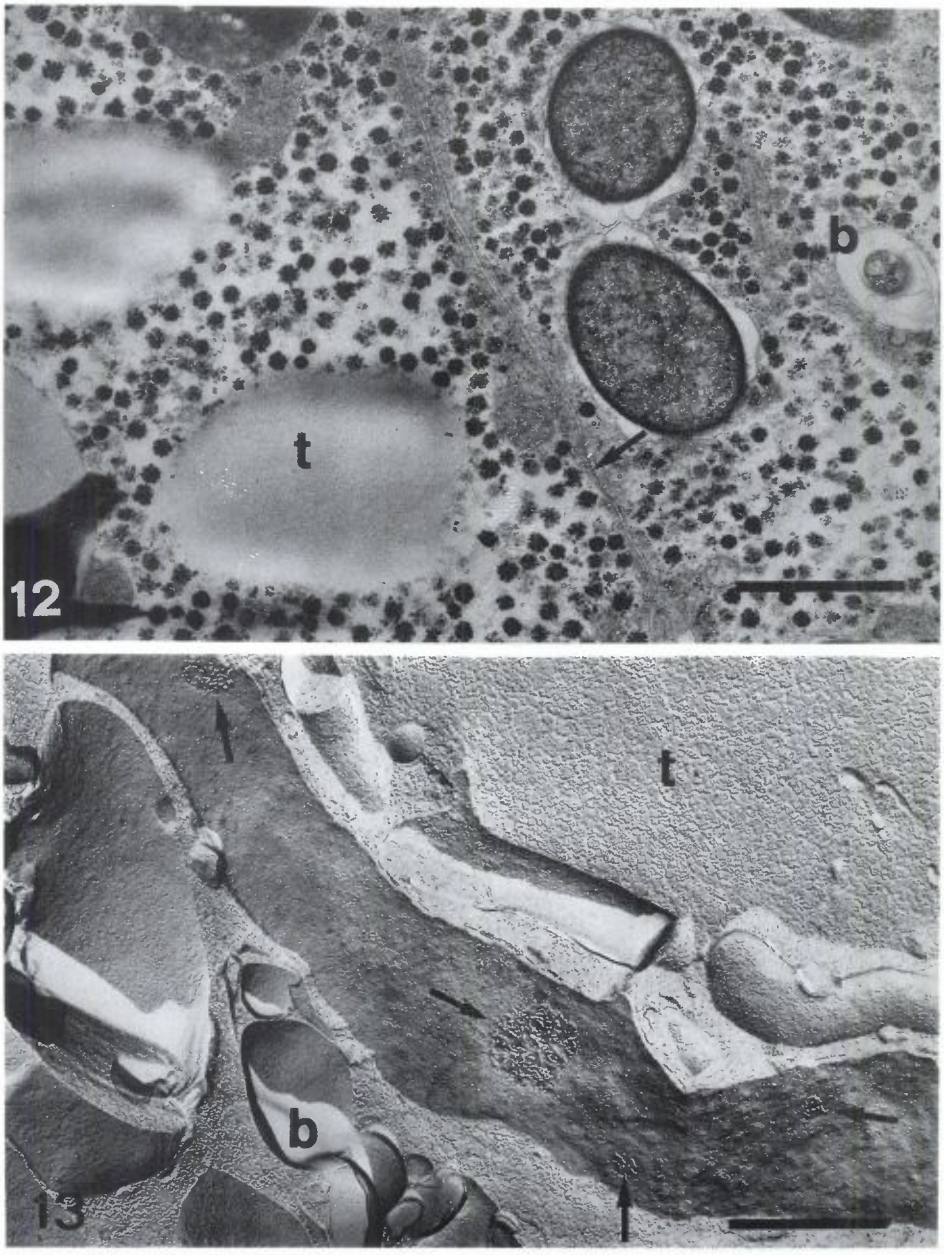


Figure 12. A junctional complex (arrow) between bacteriocyte (b) and trophocyte (t) cell membranes. Bar = 1.2 µm.

Figure 13. Freeze-fracture replica showing the plasma membrane organisation of adjacent bacteriocyte (b) and trophocyte (t): numerous randomly distributed clusters of E-face connections are visible (arrows). Bar = 0.5 µm.

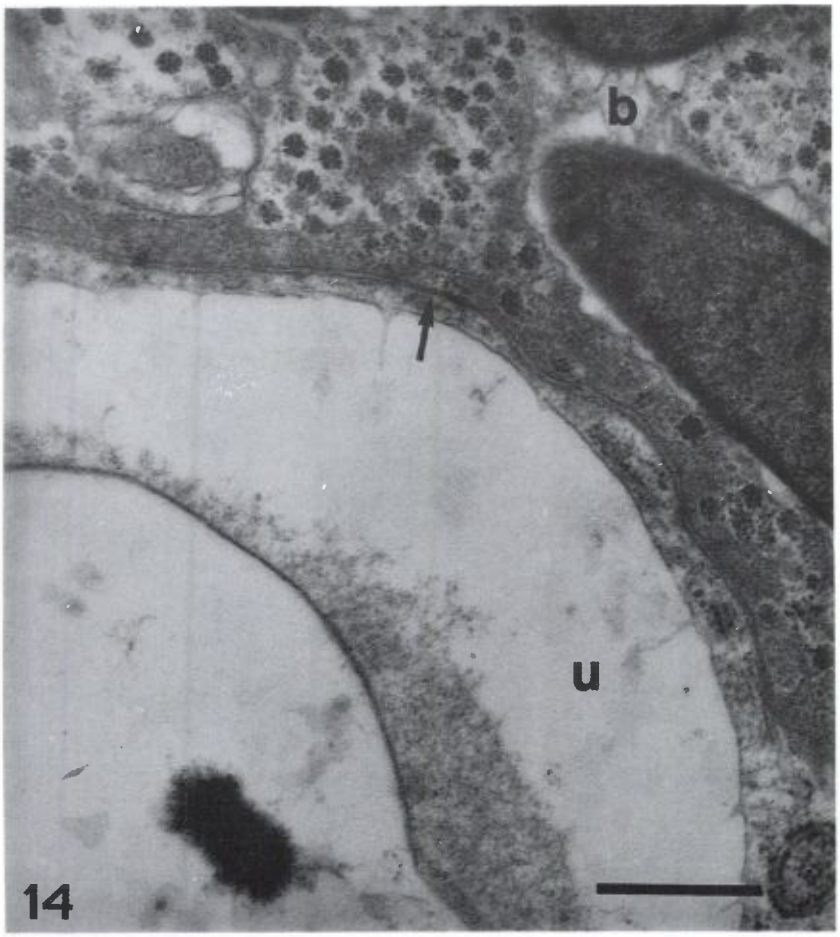


Figure 14. Junctional complex (arrow) between bacteriocyte (b) and urocyte (u) cell membranes. Bar = 0.8 μm.

In most features the bacteriocyte of *C. punctulatus* is similar to that of other cockroaches. Bacteria are surrounded by a vacuolar membrane which separates the prokaryote from the host cell cytoplasm (Fig. 4). The symbionts have a structure typical of Gram-negative bacteria, consisting of a thin cytoplasmic membrane surrounded by a cell wall and by an outer membrane. In some vacuole-symbiont units, blebbing of the outer membrane is seen (Figs. 5–6). In the vacuolar space, a sequence of vesicles becoming detached from the vacuolar membrane and pushing inward can be observed (Figs. 7–8). Some of the vacuolar vesicles enclose glycogen granules, and a few such granules are detectable in the

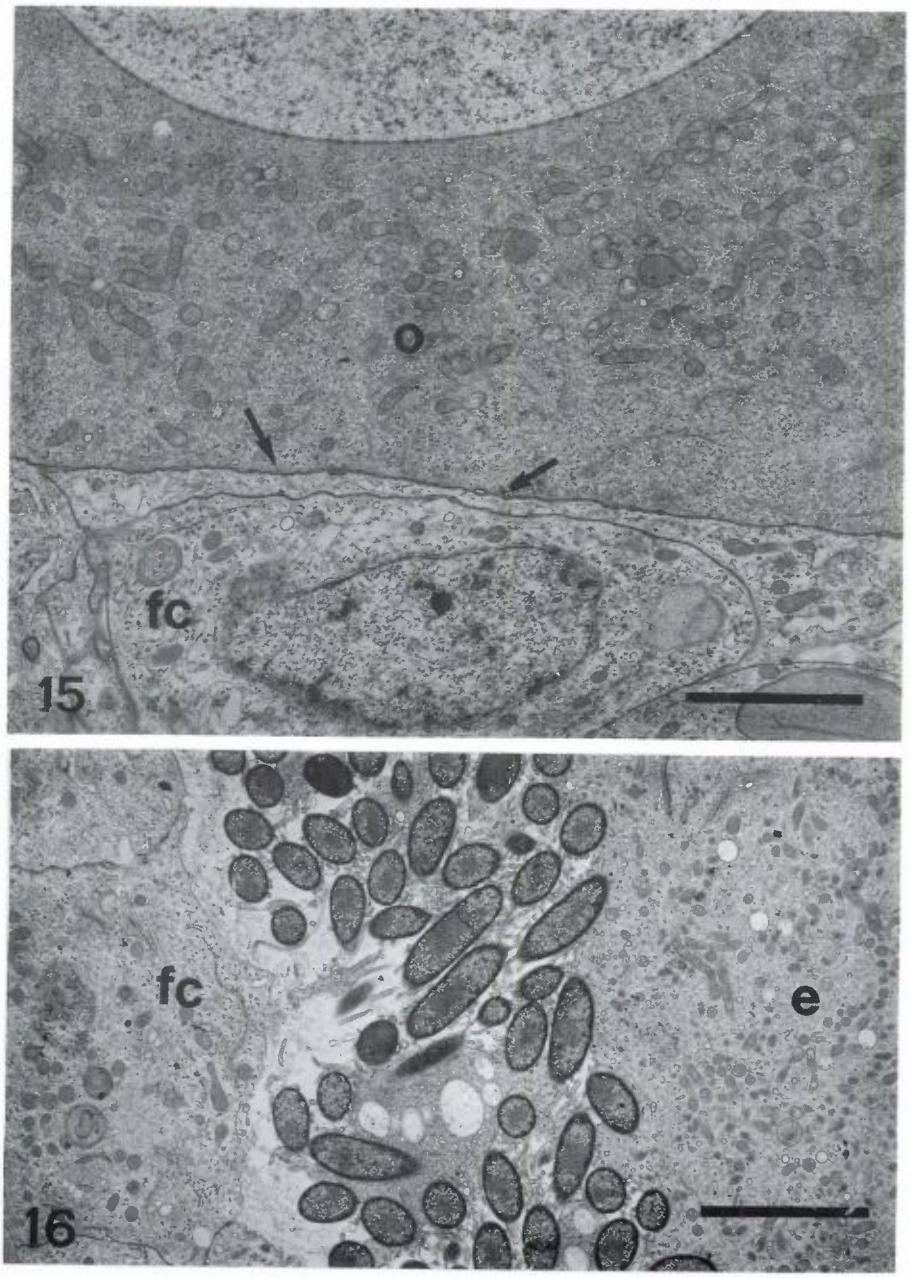


Figure 15. Young oocyte of *C. punctulatus*. No symbiotic bacteria are present at the interface (arrows) between the oocyte (o) and follicle cells (fc). Bar = 3.4 µm.

Figure 16. Previtellogenic oocyte showing symbionts located between egg (e) and follicle cells (fc). Bar = 5 µm.

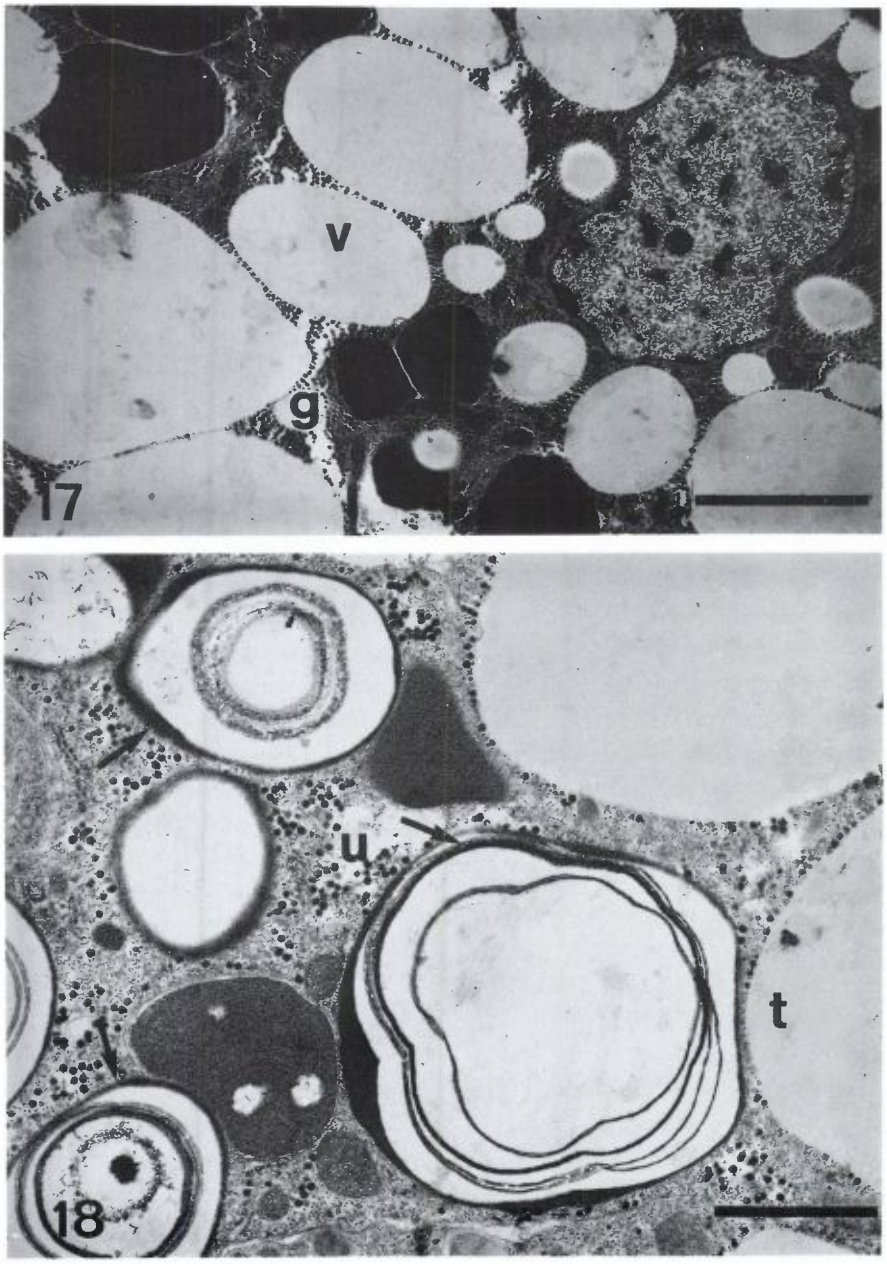


Figure 17. Trophocyte of *Zootermopsis nevadensis* with numerous variously sized lipid vacuoles (v) and glycogen granules (g). Bar = 5 µm.

Figure 18. Urate cell of *Z. nevadensis*: note several urate structural units (arrows) with crystalloid subunits arranged concentrically around dark cores. Abbreviations: (t) trophocyte; (u) urocyte. Bar = 2.2 µm.

vacuolar space (Fig. 9). In some bacteriocytes, microtubules connected with the bacteria are observed. Vacuoles containing close-packed sets of tubular, crystal-like structures are evident both in thin sections (Fig. 10) and in freeze-fracture replicas (Fig. 11).

Cell membranes have junctional complexes between adjacent bacteriocytes and trophocytes that are visible both in thin sections and in freeze-fracture replicas. In thin sections, apposed membranes of adjacent cells, with 2–3 nm gaps, are parallel over a wide area (Fig. 12). In freeze-fracture replicas, clusters of E-face connections are recognizable and associated with the membranes of adjacent trophocytes and bacteriocytes (Fig. 13). The same intermembrane communication was seen between trophocytes and urate cells and between bacteriocytes and urocytes (Fig. 14).

In the ovary of *C. punctulatus* young oocytes are surrounded by a layer of follicular cells but no symbiotic bacteria are seen in the follicular cell-oocyte interface (Fig. 15). In the previtellogenic oocyte the symbionts are located between the egg and the follicular epithelium, and are enmeshed in the microvillar border close to the symbiotic cell wall (Fig. 16).

Only two cell types are found in the fat body of the termite *Zootermopsis nevadensis*: trophocytes and urocytes. Thin sections of trophocytes show abundant lipid vacuoles, variously sized, and a great number of glycogen granules, often loosely packed. Mitochondria are numerous and small. The spherical nucleus, with the chromatin evenly distributed, is located at the lobular periphery (Fig. 17). The urocytes are characterized by vacuolar bodies delimited by a double layer structure and by urates arranged concentrically around dark cores (Fig. 18).

4. Discussion

The trophocytes of *C. punctulatus* are comparable to those of other cockroaches in that they contain stored reserves. Nevertheless, the protein and lipid deposits appear smaller and less numerous than those seen in cockroaches that do not have cellulose-based diets. Ultrastructural features of the urate cell differ from descriptions of the urate structural unit of *Periplaneta americana* (Cochran et al., 1979) and *Blattella germanica* (De Piceis Polver et al., 1986). In particular, the urates of *C. punctulatus* are deposited in concentric rings (or layers) around one or more dark cores, which probably act as seeds around which urate deposition occurs (Cochran et al., 1979). It is noteworthy that when *Periplaneta americana* is fed a cellulose diet, large deposits of urates appear in the fat body which sometimes have a concentric ring structure (Cochran et al., 1979). In *C. punctulatus*, the urate deposits around the central

core appear needle-shaped. Needle-like projections have also been observed by scanning electron microscopy on cockroach urate spherules isolated in aqueous media. These projections have been regarded as artifacts resulting from prolonged exposure to these media (Mullins, 1979). It is however unlikely that sample preparation for transmission electron microscopy could generate similar artifacts.

The amount of urates in cockroach urocytes and the size and shape of the structural units are known to be greatly influenced by the kind and quantity of food available (Cochran et al., 1979). Experimental evidence indicates that the bacteriocyte-bacteria system in cockroaches actively participates in mobilizing the waste nitrogen stored in the urocytes (Brooks and Richards, 1956; Malke and Schwartz, 1966); they therefore facilitate the maintenance of an optimum nitrogen balance in the host (Cochran, 1975). The adhesion sites among the plasma-membranes of bacteriocytes, trophocytes and urocytes may allow for direct metabolic interaction between these fat body cells. Urate units similar to those of *C. punctulatus* were observed in the termite *Z. nevadensis*, supporting the hypothesis that the features of these units are related to a wood diet. Bacteriocytes, however, are absent in this termite, indicating that, like most other termites (Potrikus and Breznak, 1980; Slaytor and Chappell, 1994), *Z. nevadensis* accumulates reserves of urates in the fat body, but is unable to mobilize the nitrogen in them for use on an individual basis (see also Potrikus and Breznak, 1981, for the role of gut microorganisms in nitrogen recycling in termites). The exception to this pattern may be the termite *Mastotermes darwiniensis*, which has retained its symbiotic association with fat body bacteria.

As in other cockroaches, the endosymbionts in *C. punctulatus* are enclosed in a membrane-bound vacuolar space within the bacteriocytes. They show the typical cell boundary profile of Gram-negative bacteria, characterized by a layer of peptidoglycane (the cell wall) and by an outer membrane. Our results, then, confirm the Gram-negative profile of the bacteria in *C. punctulatus* published by Wren et al. (1989). In *C. relictus*, however, Gromov and Mamkaeva (1980) reported that the symbiotic bacteria in the fat body had a Gram-positive profile. It should be noticed that the symbiotic bacteria of *C. punctulatus* and *C. clevelandi* have been assigned to the flavobacteria-bacteroides on the basis of 16S rDNA sequence analysis (Fig. 19). The flavobacteria-bacteroides group is one of the main lineages of the eubacteria and encompass a variety of Gram-negative bacteria (Gherna and Woese, 1992), including the symbiotic bacteria of the termite *Mastotermes darwiniensis* and all cockroach bacteria examined to date (Bandi et al., 1994, 1995; Nalepa et al., 1997).

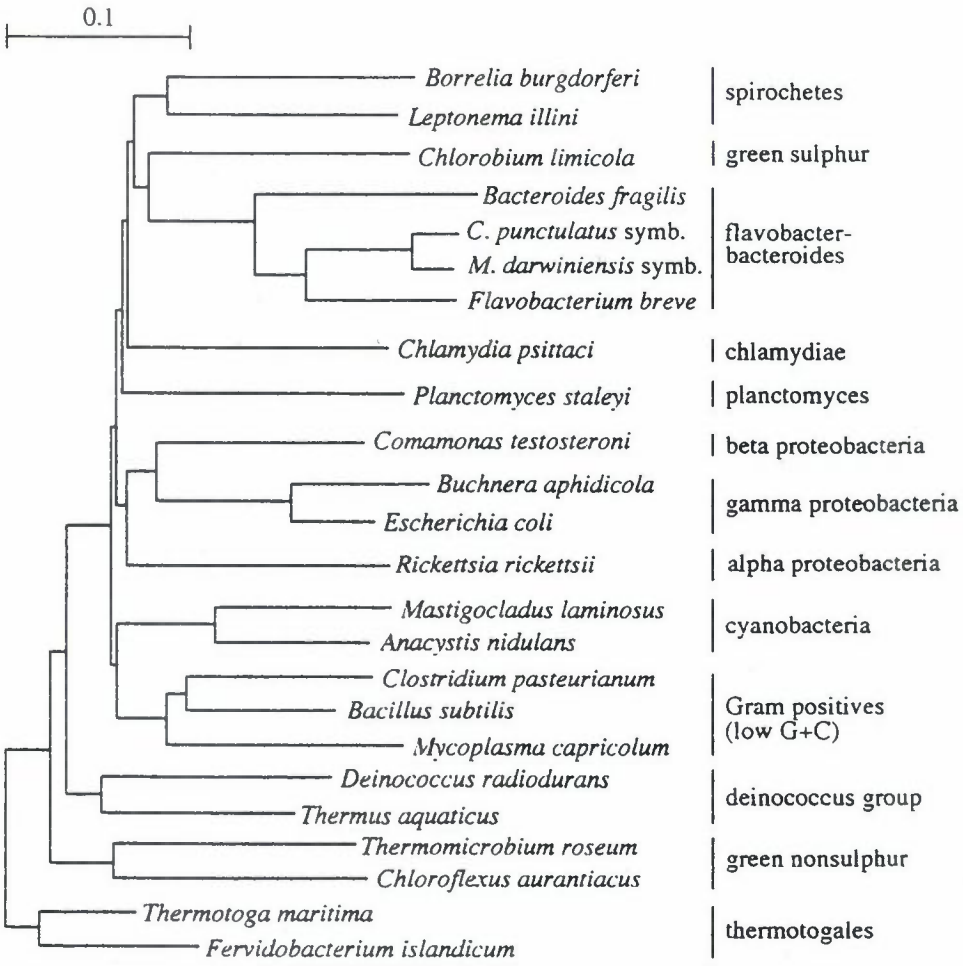


Figure 19. Distance matrix tree (16S rDNA sequence data; Jukes & Cantor correction; neighbour joining method) showing the endosymbiont of *C. punctulatus* in relation to selected representatives of the main eubacterial lineages. The endosymbiont of *M. darwiniensis* is included. The scale bar indicates the distance in substitutions per nucleotide. The tree is based on an alignment in which 16S rDNA variable regions and insertions/deletions are excluded. Outgroup: *Thermococcus celer* (not shown).

The ultrastructural features of the vacuolar membrane and the symbiont envelopes, like those already described for cockroaches in the Blattellidae (Bigliardi et al., 1989) and Blaberidae (Bigliardi et al., 1991), suggest exchanges of metabolites between the host cell cytoplasm and the bacterial

endosymbiont. This is indicated by the formation of numerous vesicles that protrude from the vacuolar membrane towards the vacuolar space, the glycogen granules both in the vesicles and free in the vacuolar space, and the blebbing observed in the outer membrane of the symbiont. These observations agree with those of Griffiths and Beck (1975) and Akhtar and van Emden (1994) for aphid bacteriocytes, and confirm the findings for *C. punctulatus* previously reported by Bigliardi et al. (1995). Douglas (1996) recently suggested that nutrient acquisition by intracellular symbionts is controlled primarily by the transport properties of the surrounding host membranes. As suggested by the blebbing of the outer membrane of the symbionts, this might be true also for the export of bacterial metabolites to the host cell.

Acknowledgements

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