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Invasion of Bacteroids and BEV Bacterium into Oocytes of the Leafhopper *Euscelidius variegatus* Kirschbaum (Homoptera: Cicadellidae): An Electron Microscopic Study

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Wilkin Wai-Kuen Cheung and Alexander H. Purcell (1999) Invasion of bacteroids and BEV bacterium into oocytes of the leafhopper *Euscelidius variegatus* Kirschbaum (Homoptera: Cicadellidae): An electron microscopic study. *Zoological Studies* **38**(1): 69-75. Electron microscopic studies were carried out on the infective stages of symbiotic bacteroids *a* and *t*, and their chronic pathogen BEV together with ovarioles of the leafhopper *Euscelidius variegatus* Kirschbaum fixed in situ. The bacteroids were carried in cluster of host cells in the hemolymph as an infective mound during the second day of adult emergence. Before entering an ovariole through special wedge cells of the follicular epithelium, they were released as individuals. The BEV bacteria were also carried in infective mounds and were released to the hemolymph for oocyte infection. Host hemocytes (probably plasmatocytes) engulfed bacteroids and BEV bacteria in the hemolymph when they were not protected by host's cell membranes.

Key words: Symbionts, Bacteria, Ovary, Ultrastructure, Insect immunity.

There are 2 types of symbiotic bacteroids, designated by Müller (1949) as *a* and *t*, in the leafhoppers *Euscelidius variegatus* Kirschbaum and *Euscelis* spp. (Hemiptera: Cicadellidae). Körner (1969 1972 1978), Schwemmler (1971 1973 1974), and Louis and Nicholas (1976) have described their fine structure. These prokaryotic microorganisms, named protoplastoids by Schwemmler (1971, Schwemmler et al. 1975), are harbored intracellularly in special organs called mycetomes or bacteriomes (Schwemmler 1974 1983). The infective stages of these bacteroids are carried in host cells (bacteriocytes) or "infective mounds" (Buchner 1965, Körner 1978) and travel to the developing oocytes of young female adults. Within the ovariole, specialized follicular cells called wedge cells mediate the transfer of the bacteroids from the infective mounds to invade the mature oocytes (Körner 1978, Schwemmler 1980a,b 1983). The *a* and *t* symbionts migrate to the posterior pole of the mature oocytes and form a posterior ball of symbiont cells (Schwemmler 1980a,b 1989).

In addition to *a* and *t* symbionts, Schwemmler (1974) described a cultivable, but unidentified, rod-shaped bacterium (designated KRE) as a "facultative symbiont" of the leafhopper *Euscelis incisus* (= *plebejus*). The mutual relationships of these organisms to one another are not fully understood. Purcell et al. (1986) and Cheung and Purcell (1993) also found that a cultivable bacterium (tentatively termed BEV) parasitized *E. variegatus*. This bacterium is transovarially transmitted to the host's progeny and has been shown to reduce the longevity and fecundity of the leafhopper (Purcell and Suslow 1987).

In order to better understand of the migratory mode of these bacteroids in the hemolymph to the oocytes and their relation to BEV, an ultrastructural study was undertaken.

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MATERIALS AND METHODS

Two-day-old adult female *Euscelidius variegatus* (bacteroid migratory phase, Schwemmler 1973) were dissected after decapitation. The ovarioles, lateral oviducts, and migratory bacteroids were fixed in 2.5% glutaraldehdye in 0.1 M sodium cacodylate buffer (pH 7.2) (3 lots of fixation, in triplicate). After fixing 1 h, the severed tissues were briefly washed with cacodylate buffer. They were then post-fixed in 1% osmium tetroxide (pH 7.2) in cacodylate buffer for 1 h. After dehydration in ascending alcohol series and acetone, the tissues were embedded in Spurr resin (Spurr 1969) and polymerized at 60 °C for 48 h. Thin sections were cut by a Reichert OMU2 ultramicrotome and were stained with 2% aqueous uranyl acetate and later 1% lead citrate. Sections were viewed with a JEOL JEM-1200EXII or 100CX electron microscope.

Bacteroids *en bloc*, ovaries and mycetomes were fixed in formol saline, dehydrated, and embedded in paraplast. Sections were cut at 6 μm with a rotary microtome and subjected to the following histochemical tests with or without appropriate enzymic treatments: Pyronin-Y and methyl green for DNA materials (Pearse 1985), mercury-bromophenol blue for proteins (Culling 1963), Sudan black B for lipids (Chieffelle and Putt 1951), and Paraldehyde-Schiff for glycogen materials or mucopolysaccharides (Humason 1979). The microorganisms were also tested with Gram staining (Culling 1963).

RESULTS

Infective stages of *a* and *t* bacteroids had double unit membranes at their periphery, dense matrix, diffuse DNA strands (confirmed by Pyronin Y and methyl green test), and dark inclusion bodies of 1-2.5 μm diameter (Figs. 1-5). These inclusion bodies stained positive for proteins; cell membranes of bacteroids stained positive for proteins, lipids, and mucopolysaccharides. The bacteroids were contained in migratory bacteriocytes, and could move from the bacteriomes to the lower developing oocytes as globular structures that could be termed infective mounds while in the hemolymph. The individual infective bacteroids had different morphologies and osmium-staining properties when compared to their vegetative states (Körner 1972, Schwemmler 1974), but both stained Gram-negative. They were released from the bacteriocytes or infective mounds to invade the follicular cells as individual organisms (Fig. 5). When free in the hemolymph, *a* and *t* bacteroids were not protected by a bacteriocyte membrane (Figs. 5, 6).

The infective *a* bacteroids (Figs. 1-3) measured 7 μm × 8 μm, had division septa, and were heavily osmiophilic owing to an increase of cytoplasmic density (Chang and Musgrave 1975, Körner 1976). When free in the hemolymph, they were oval, 4 μm × 6 μm (only partly shown in Fig.

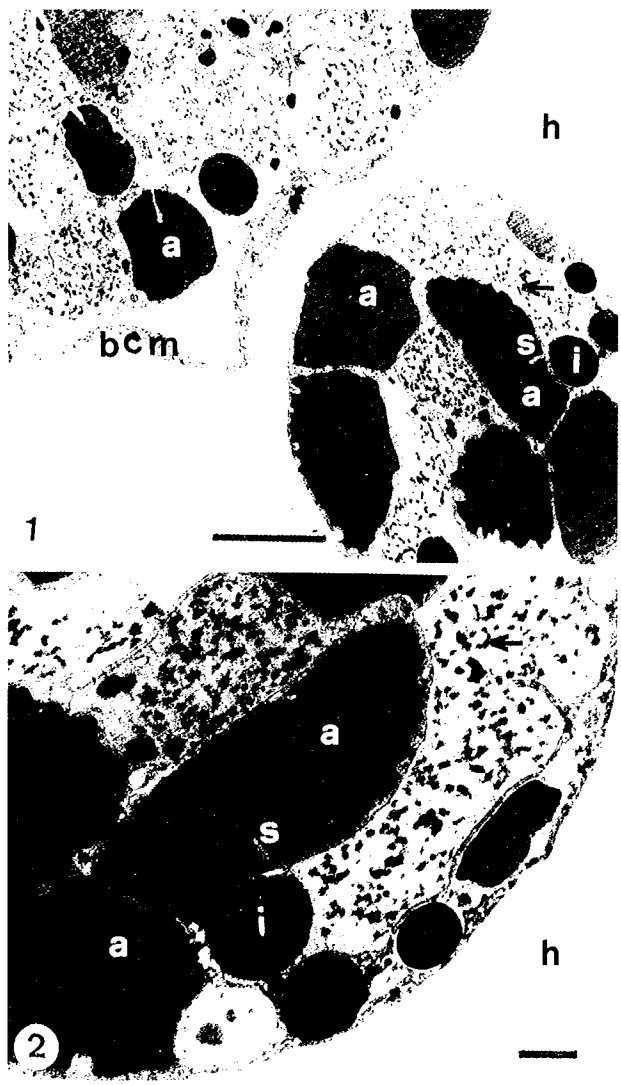

Fig. 1. Transverse section of part of a bacteriome (upper left) of a 2-d-old female adult and a globular infectious mound (lower right) with *a* and *t* bacteroids (a, t), showing division septa (s), inclusion bodies (i), diffuse DNA strands (arrow) in *a* and *t* bacteroids, bacteriome cell membrane (bcm), and hemolymph (h). Bar = 5 μm.

Fig. 2. High magnification of part of an infective mound. Labels as above. Bar = 2 μm.

5). Schwemmler (1980a) called these the 2nd infective stage or non-dividing stage.

Bacteroids of the *t* infective stage (Figs. 1, 2, 4) were 6 μm × 9 μm in size and were less osmiophilic than the *a* bacteroids. They possessed scattered DNA strands and large, oval, dark-staining inclusion bodies which stained positive for proteins by mercury-bromophenol blue. Sometimes division septa were also seen. The free, non-bacteriocyte-bound individuals in the hemolymph (2nd infective stage comparable to *a* bacteroids) were hyaline in appearance and measured 3 μm × 5 μm (Figs. 5, 6). Both *a* and *t* bacteroids could perform phagocytic or vacuole formation activity, resulting in phagosomes or vacuoles in the cytoplasm (Fig. 5).

The follicular epithelial cells of the ovary (Fig. 6) had elongated nuclei, numerous mitochondria (faintly seen), vacuoles, and dense bodies (Cheung 1995). Special follicular cells, called wedge cells, were sites where individual bacteroids penetrated into the developing oocytes to form the posterior symbiont ball (Cheung and Purcell in prep.). The bacteriocytes probably burst, releasing the bacteroids and BEV into the hemolymph from which they infected the wedge cells (Schwemmler

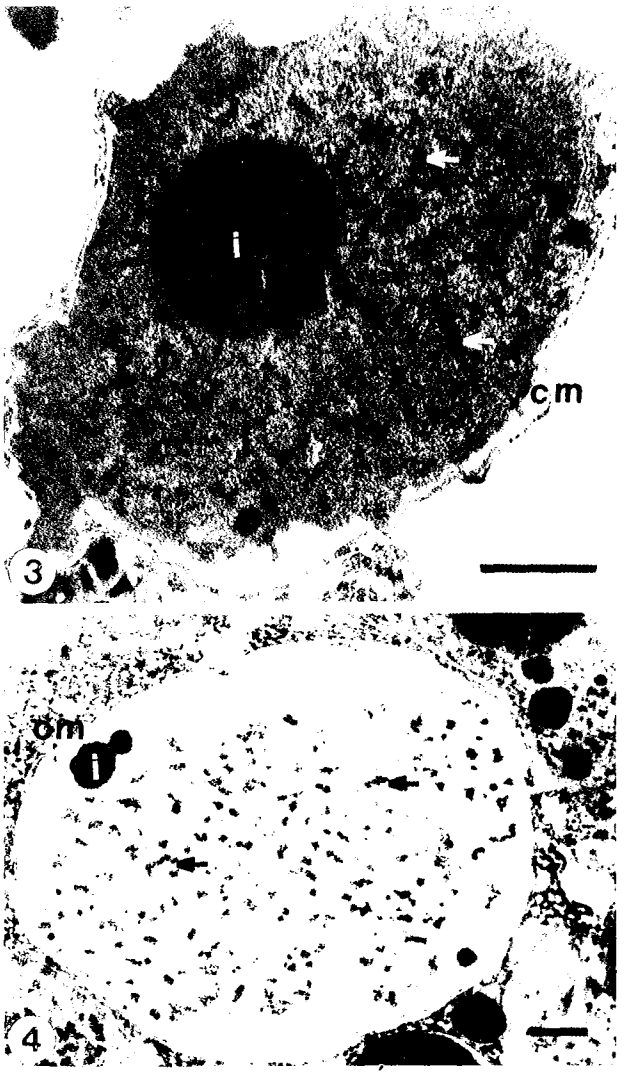

Fig. 3. Transverse section of an infective *a* bacteroid, showing inclusion body (i), DNA materials (arrows), and bacteriocyte membrane (cm). Bar = 1 μm.

Fig. 4. Transverse section of an infective *t* bacteroid, showing inclusion body (i), DNA materials (arrows), and bacteriocyte membrane (cm). Bar = 1 μm.

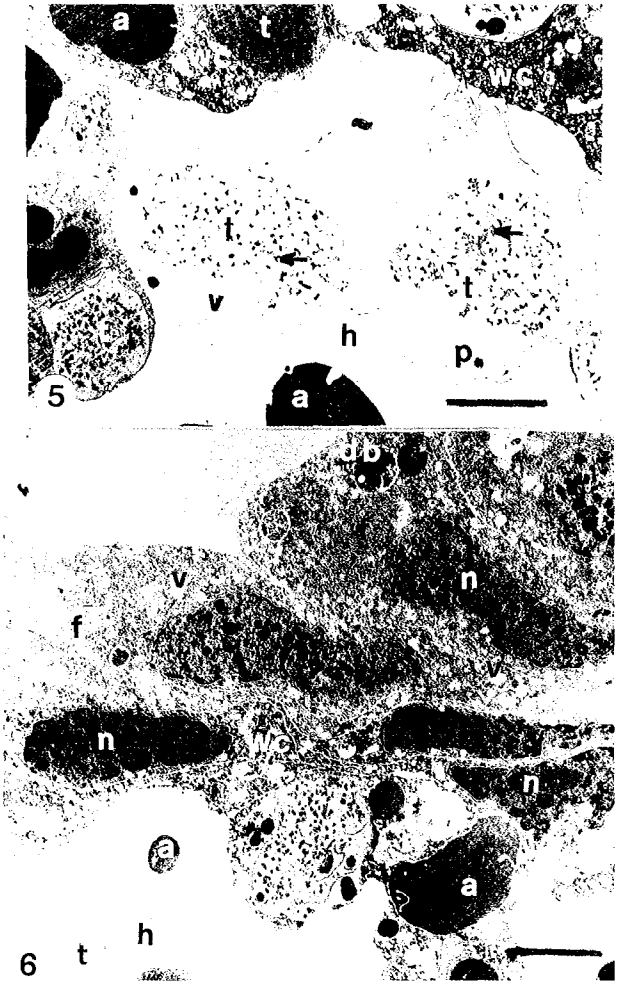

Fig. 5. Transverse section of part of wedge cells (wc) of follicular epithelium with invading bacteroids (a, t) and naked infective *t* and part of *a* bacteroids in the hemolymph (h), showing phagocytic or vacuole formation activity (v), a phagosome (p), and diffuse DNA strands (arrows). Bar = 5 μm.

Fig. 6. Longitudinal section of follicular epithelial cells (f) with wedge cells (wc) invaded by *a* and *t* bacteroids (a, t), showing nuclei (n), vacuoles (v), dense bodies (db), part of a developing oocyte (o), and hemolymph (h). Bar = 5 μm.

1980a).

The rod-shaped bacterium BEV measured 0.5 μm × 5 μm (Figs. 7, 9). It also stained Gram-negative and was observed among *a* and *t* bacteroids in the infective mound, or individually in the hemolymph. Scattered DNA strands were detected in its cytoplasm by using Pyronin Y and methyl green stain, and proteins stained positively using the bromophenol blue test.

Since BEV infected the developing oocytes when released from the infective mounds, it could be seen individually in the hemolymph (Fig. 9). Hemocytes (probably plasmatocytes) were observed in various stages of phagocytosis (Figs. 8, 10). A hemocyte had a large, centrally placed nucleus with dark patches of chromatin-like materials. Various types of vacuoles, dense bodies, and lysosome-like bodies were seen in the ground cytoplasm. The hemocyte in Fig. 8 extended a pseudopodium to engulf foreign bodies or organic debris such as disintegrated cell components (a mitochondrion in this case).

Bacteroids and BEV outside of oocytes were phagocytosed by the hemocytes that engulfed these microbes and lysed them within their lysosomes (Figs. 8, 9). Partially digested BEV and remains of "cell membranes" of ingested materials can be faintly seen in Figs. 9 and 10.

DISCUSSION

It has been proposed that symbionts in insects supply essential nutrients such as steroids, vitamins, and certain proteins for the host (Campell 1989, Douglas 1989). Homopterous insects, which

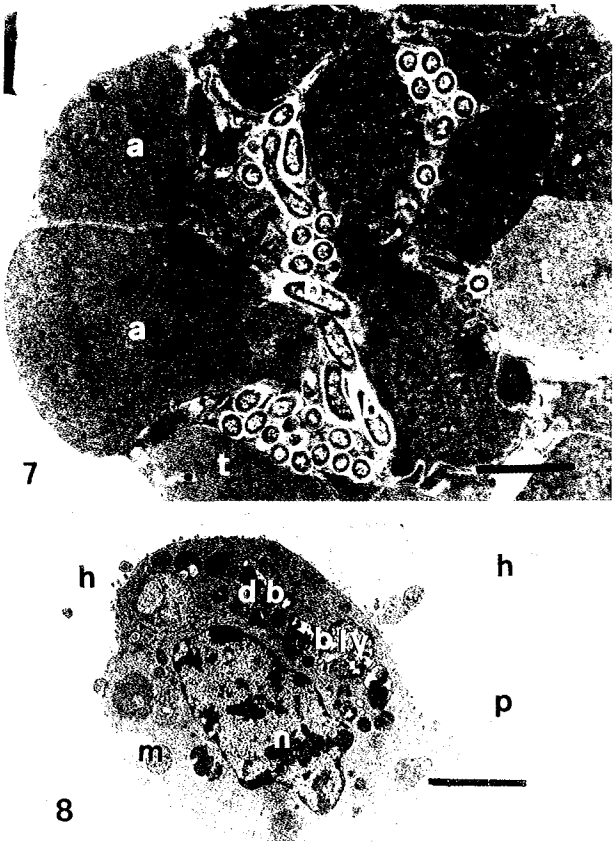

Fig. 7. Transverse section of an infective mound with *a* and *t* bacteroids (a, t) and BEV bacteria (b) inside it. Bar = 2 μm.

Fig. 8. Transverse section of a hemocyte, showing nucleus (n), dense bodies (db), a BEV bacterium undergoing lysis (bly), a mitochondrion (m) to be phagocytosed, and extended pseudopodium (p) in the hemolymph (h). Bar = 2 μm.

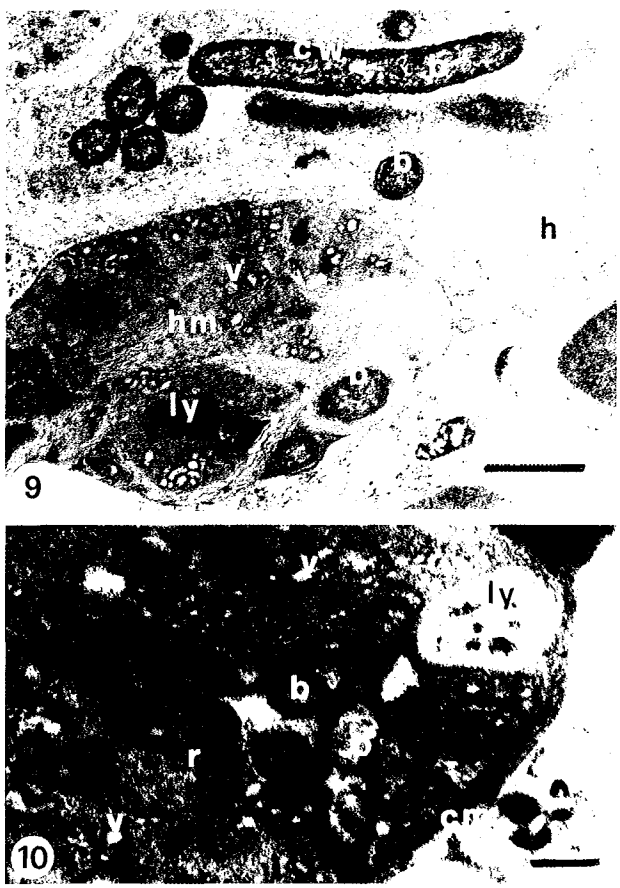

Fig. 9. Transverse and longitudinal sections of BEV bacteria (b) in the hemolymph (h), showing thick bacterial cell wall (cw) and DNA material (arrow), and a hemocyte (hm) with phagocytosed BEV. Note vacuoles (v), lysosome-like body (ly), and remains of "cell membranes" of digested materials (r). Bar = 1 μm.

Fig. 10. High magnification of part of a hemocyte, showing BEV bacteria (b), lysosome (ly), vacuoles (v), hemocyte cell membrane (cm) and remains of "cell membranes" of digested materials (r). Bar = 0.5 μm.

feed on plant sap (such as xylem sap) with extremely poor nutritional contents (Cheung and Marshall 1973) should need food supplements from their harbored symbionts. The same phenomena occur in stored-product insects such as the weevil *Sitophilus granarius* L. (Grinyer and Musgrave 1966) and the head louse *Pediculus humanus* L. (Eberle and McLean 1983).

In *E. variegatus*, the *a* and *t* symbionts are prokaryotic in morphology and their cytochemical properties are comparable to similar forms in other leafhoppers such as *Euscelis incisus* (Schwemmler 1974 1980a,b), *Helochara communis* Fitch (Chang and Musgrave 1972), and *Nephotettix cincticeps* Uhler (Nasu 1965, Mitsuhashi and Kono 1975). They are usually found intracellularly (except as free individuals in the hemolymph), lack organelles and internal compartmentalization, and have diffuse DNA materials and Gram-negative staining properties, etc. The symbionts must adapt to the host's internal environment and be transmitted to the developing oocytes when the female insect is sexually mature. In *E. variegatus*, as in *Euscelis incisus* (Schwemmler 1974 1980a,b), the transmission process is most likely mediated by infective stages carried in bacteriocytes in the hemolymph as an infective mound. When these clustered bacteriocytes burst, the bacteroids are freed within the hemolymph.

Generally, bacteroids in an insect host must withstand the host's antibacterial immune mechanisms such as lysozymes and phagocytic hemocytes (Götz and Boman 1985, Schwemmler and Müller 1986, Gupta 1989 1991). When not protected by host cell membranes, the host's immune system can react to the bacteroids and treat them as foreign bodies. Thus, these prokaryotes are destroyed by host cells or lysozymes unless they reach the specific targets such as the posterior region of lower oocytes at an appropriate time related to suitable female hormone titre (Schwemmler 1974), or by adopting certain strategies to counter the immune reactions of the host (Gupta 1989 1991).

In *P. humanus*, hemocytes were seen to accumulate on the surface of the lateral oviducts so as to engulf the remaining symbionts that failed to penetrate into the developing oocytes (Eberle and McLean 1983). Our observations of *E. variegatus* also show that the hemocytes lysed bacteroids and BEV bacteria that were free in the hemolymph.

As mentioned by Schwemmler and Müller (1986), the production of infectious symbionts takes place only during certain stages of host development (probably the 2nd to the 7th days of adult maturation). The reason may be the fact that a fully grown egg would have a gradually hardened chorion forming a protective covering of the egg (Wigglesworth 1972). This would prevent the easy entry of bacteroids or BEV. Thus the migration of the latter should be synchronized with egg maturation, though egg maturation can be extended over several weeks for some individual leafhoppers (Nault and Rodriquez 1985).

Rod-shaped bacteria have also been reported to be associated with bacteroid symbionts in other leafhoppers such as *Euscelis lineolatus* Brulle (Maillet 1970), *Helochara communis* Fitch (Chang and Musgrave 1972), and *Nephotettix cincticeps* Uhler (Nasu 1965, Mitsuhashi and Kono 1975). The transmission of these microorganisms to their host oocytes may have some similarities that are worthy of investigation.

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REFERENCES

Buchner P. 1965. Endosymbiosis of animals with plant microorganisms. New York: J. Wiley.

Campell BC. 1989. On the role of microbial symbiotes in herbivorous insects. *In* EA Bernays, ed. Insect-plant interactions. Boca Raton, FL: CRC Press, pp. 1-44.

Chang KP, AJ Musgrave. 1972. Multiple symbiosis in a leafhopper, *Helochara communis* Fitch (Cicadellidae: Homoptera): envelopes, nucleoids and inclusions of the symbiotes. J. Cell Sci. **11:** 275-293.

Chang KP, AJ Musgrave. 1975. Conversion of spheroblast symbiotes in a leafhopper, *Helochara communis* Fitch. Can. J. Microbiol. **21:** 196-204.

Cheung WWK. 1995. Ultrastructural studies on the female reproductive system of the leafhopper *Euscelidius variegatus* Kirschbaum (Homoptera: Cicadellidae) II. The vitellarium oocytes and follicular epithelium. Cytologia **60:** 395-405.

Cheung WWK, AT Marshall. 1973. Water and ion regulation in cicadas in relation to xylem feeding. J. Insect Physiol. **19:** 1901-1916.

Cheung WWK, AH Purcell. 1993. Ultrastructure of the digestive system of the leafhopper *Euscelidius variegatus* Kirschbaum (Homoptera: Cicadellidae), with and without congenital bacterial infections. Int. J. Insect Morphol. Embryol. **22:** 49-61.

Chieffelle JL, FA Putt. 1951. Propylene and ethylene glycol as solvents for Sudan IV and Sudan black B. Stain Technol. **26:** 51-56.

Culling CFA. 1963. Handbook of histopathological techniques. 2nd ed. London: Butterworths.

Douglas AE. 1989. Mycetocyte symbiosis in insects. Biol. Rev. **64:** 409-434.

Eberle MW, DL McLean. 1983. Observation of symbiote migration in human body lice with scanning and transmission electron microscopy. Can. J. Microbiol. **29:** 755-762.

Götz P, HG Boman. 1985. Insect immunity. *In* GA Kerkut, LI Gilbert, eds. Comprehensive insect physiology, biochemistry, and pharmacology. Vol. 3. Oxford: Pergamon Press, pp. 453-485.

Grinyer I , AJ Musgrave. 1966. Ultrastructure and peripheral membranes of the mycetomal microorganisms of *Sitophilus granarius* L. J. Cell Sci. **1:** 181-186.

Gupta AP. 1989. Insect host immune system and endocytobionts: their avoidance strategies. *In* W Schwemmler, G Gassner, eds. Insect endocytobiology, morphology, genetics, evolution. Florida: CRC Press, pp. 11-20.

Gupta AP. 1991. Insect immunocytes and other hemocytes. *In* AP Gupta, ed. Immunology of insects and other arthropods. Boca Raton, FL: CRC Press, pp. 19-118.

Humason GL. 1979. Animal tissue techniques. 4th ed. San Francisco: Freeman.

Körner HK. 1969. Zur Ultrastruktur der intrazellulären Symbionten im Embryo der Kleinzikade *Euscelis plebejus* Fall. (Homoptera: Cicadina). Z. Zellforsch. **100:** 466-473.

Körner HK. 1972. Elektronenmikroskopische Untersuchungen am embryonalen Mycetom der Kleinzikade Euscelis plebejus Fall. I. Die Feinstruktur der a-Symbionten. Z. Parasitenk. **40:** 203-226.

Körner HK. 1976. On the host-symbiont cycle of a leafhopper (*Euscelis plebejus*) endosymbiosis. Experientia **32:** 463-464.

Körner HK. 1978. Intraovarially transmitted symbionts of leafhoppers. Zool. Beit. **24:** 59-68.

Louis C, G Nicholas. 1976. Ultrastructure of the endocellular procaryotes of arthropods as revealed by freeze-etching. I. A study of "a"-type endosymbiont of the leafhopper *Euscelis plebejus* (Homoptera, Jassidae). J. Microsc. Biol. Cell **26:** 121-126.

Maillet P. 1970. Présence de particules de type rickettsien dans la salive d'un homoptère vecteur du la phyllodie du trèfle *Euscelis lineolatus* Brullé (Homoptera: Jassidae). Rev. Can. Biol. **29:** 391-393.

Mitsuhashi J, Y Kono. 1975. Intracellular microorganisms in the greenrice leafhopper *Nephotettix cincticeps* Uhler (Homoptera: Deltocephalidae). Jpn. J. Appl. Entomol. Zool. **10:** 1-9.

Müller HJ. 1949. Zur Systematik und Phylogenie der Zikaden Endosymbosen. Biol. Zentralb. **68:** 343-368.

Nasu S. 1965. Electron microscopic studies on transovarial passage of rice drawf virus. Jpn. J. Appl. Entomol. Zool. **9:** 225-237.

Nault LR, JG Rodriquez, eds. 1985. Leafhoppers and planthoppers. New York: J. Wiley.

Pearse AGE. 1985. Histochemistry: theoretical and applied. 4th ed. London: Churchill.

Purcell AH, T Steiner, F Mégraud, J Bové. 1986. *In vitro* isolation of a transovarially transmitted bacterium from the leafhopper *Euscelidius variegatus* (Hemiptera: Cicadellidae). J. Invertebr. Pathol. **48:** 66-73.

Purcell AH, KG Suslow. 1987. Pathogenicity and effects on transmission of a mycoplasma-like organism of a transovarially infective bacterium on the leafhopper *Euscelidius variegatus* (Homoptera: Cicadellidae). J. Invertebr. Pathol. **50:** 285-290.

Schwemmler W. 1971. Intracellular symbionts: a new type of primitive prokaryotes. Cytobiologie **3:** 427-429.

Schwemmler W. 1973. Beitrag zur Analyse des Endosymbiosezyklus von *Euscelis plebejus* F. (Hemiptera, Homoptera, Cicadina) mittels in vitro-Beobachtung. Biol. Zentralb. **92:** 749-772.

Schwemmler W. 1974. Studies on the fine structure of leafhopper intracellular symbionts during their reproductive cycles (Hemiptera: Deltocephalidae). Jpn. J. Appl. Entomol. Zool. **9:** 215-224.

Schwemmler W. 1980a. Principles of endocytobiosis: structure, function, and information. *In* W Schwemmler, HEA Schenk, eds. Endocytobiology: endosymbiosis and cell biology. Vol. 1. Berlin: Walter de Gruyter, pp. 565-583.

Schwemmler W. 1980b. Endocytobiosis: general principles. BioSystems **12:** 111-122.

Schwemmler W. 1983. Analysis of possible gene transfer between an insect host and its bacteria-like endocytobionts. Intern. Rev. Cyto. **14 (Suppl):** 247-266.

Schwemmler W. 1989. Insect endocytobiosis as a model for egg cell differentiation. *In* W Schwemmler, G Gassner, eds. Insect endocytobiosis: morphology, physiology, genetics, evolution. Boca Raton, FL: CRC Press, pp. 37-53.

Schwemmler W, G Hobom, M Egel-Mitani. 1975. Isolation and characterization of leafhopper endocytobiont DNA. Cytobiologie **10:** 249-259.

Schwemmler W, H Müller. 1986. Role of insect lysozymes in endocytobiosis and immunity of leafhoppers. *In* AP Gupta, ed. Hemocytic and humoral immunity of arthropods. New York: J. Wiley, pp. 440-460.

Spurr AR. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26:** 31-43.

Wigglesworth VB. 1972. The principles of insect physiology. 7th ed. London: Chapman and Hall.

類細菌與 BEV 細菌對雜色葉蟬 *Euscelidius variegatus* Kirschbaum（同翅目：葉蟬科）卵母細胞侵染之電鏡研究

張偉權[1] Alexander H. Purcell[2]

本文報導雜色葉蟬 *Euscelidius variegatus* 共生類細菌 *a* 及 *t* 與慢性病原 BEV 對其卵母細胞原位固定後之電鏡研究。在成蟲羽化第二天，類細菌以群狀感染堆在寄主血淋巴中，在未進入卵巢管之卵泡上皮特別楔形細胞前，牠們以個體釋放出去。BEV 細菌也寄載在感染堆中，並同樣在血淋巴中釋放，以便感染卵母細胞。當類細菌與 BEV 細菌沒有受寄主細胞膜保護時，寄主血球（也許是細胞質細胞）在血淋巴中將牠們吞噬。

關鍵詞：共生體，細菌，卵巢，超微結構，昆蟲免疫。

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