Marine Biology (1996) 125: 511–524

L. Frenkiel · O. Gros · M. Mouëza

Gill structure in *Lucina pectinata* (Bivalvia: Lucinidae) with reference to hemoglobin in bivalves with symbiotic sulphur-oxidizing bacteria

Received: 5 October 1995/Accepted: 23 December 1995

Abstract *Lucina pectinata* is a large tropical Lucinidae which is characterized by abundant tissue hemoglobin in its deep-red gills. In the present paper, hemoglobin is described as being located in cytoplasmic dark patches of the bacteriocytes together with a cystine-rich protein. Large microbodies contain a non-hemoglobin heme-compound which is identified with a previously described non-protein-bound hematin; however, it has not been established whether this heme is involved in a sulphur-oxidizing system or represents a catabolic by-product of hemoglobin. Electron-lucent vesicles are associated with the basal microbodies but their function is, so far, unknown. In addition, the bacteriocytes have been observed to have direct contact with sea water, modulated by large intercalary cells which overlap the bacteriocytes on their margin. Such relationships between bacteriocytes and intercalary cells, as well as their cytological features, are different from those observed in lucinid species inhabiting sea-grass beds, but very similar to those observed in *Calyptogena magnifica*. From the congruence between the shallow-water Lucinidae *L. pectinata*, inhabiting mangrove swamps, and the deep-sea Vesicomyidae *C. magnifica*, found at hydrothermal vents, we conclude that such features are likely to be adaptative to high-sulphide environments, notwithstanding the phylogenetic distance.

Introduction

The large lucinid clam *Lucina pectinata* (Gmelin, 1791) (= *Phacoïdes pectinatus* Gmelin) lives deeply burrowed in black sulphide mud in mangrove swamps, ranging from the Caribbean area (Warmke and Abbott 1962) to Brazil (Rios 1985). Its deep-red gills were noticed by Read (1962) who demonstrated their high content of three types of hemoglobin (Read 1965). In tests under low salinity, high temperature and stagnant conditions, Read (1964) established that, in the high-stress, shallow-water mangrove environment, *L. pectinata* is one of the most resistant species.

The discovery of endocellular symbiotic bacteria, first described in deep-sea fauna associated with hydrothermal vents (Cavanaugh et al. 1981; Felbeck et al. 1981), was rapidly followed by the identification of a similar symbiosis in coastal bivalve species inhabiting reduced sediment, such as *Solemya reidi* (Cavanaugh 1983; Felbeck 1983), and sea-grass beds, such as *Codakia orbicularis* (Berg and Alatalo 1982, 1984). Similar types of symbioses have been found in all examined species of the family Lucinidae and in some species of the related family Thyasiridae; both temperate (Dando et al. 1985, 1986; Dando and Southward 1986; Southward 1986; Herry and Le Pennec 1987; Le Pennec et al. 1988a, b; Diouris et al. 1989; Herry et al. 1989) and tropical species (Fisher and Hand 1984; Giere 1985; Schweimanns and Felbeck 1985; Reid and Brand 1986; Distel and Felbeck 1987) are involved. Such symbiotic associations led Reid (1990) to consider that the onset of symbiosis was the most important factor in the subsequent evolution of the Lucinacea. The occurrence of hemoglobin, although considered to be an obligate component of many symbiotic associations between animals or plants and intracellular bacteria by Wittenberg (1985), appears to be very variable in the gill tissues of bivalves which harbour symbiotic bacteria; the concentration of tissue hemoglobin ranges from a few μmol kg^{-1} tissue up to a maximum of 1.5 mmol in *Lucina pectinata*. However, the presence of hemoglobin has not been investigated in every species having symbiotic bacteria, and it is likely that some species with light-beige gills may even be devoid of hemoglobin. In *L. pectinata*, Kraus and Wittenberg (1990)

Communicated by O. Kinne, Oldendorf/Luhe

L. Frenkiel (✉) · O. Gros · M. Mouëza
Biology Department, Faculty of Sciences,
BP 592-97159 Pointe à Pitre Cedex, Guadeloupe F-97159,
French West Indies

confirmed that three different hemoglobins can be isolated from the gills and demonstrated that the monomeric hemoglobin, Hb I, reacts with hydrogen sulphide to form ferric hemoglobin sulphide. However, no ultrastructural study on *L. pectinata* gills has been undertaken, and the location of tissue hemoglobin within the gills of various species is controversial. Read (1962) detected occult iron in numerous yellow-brown pigment granules in the gill cells of *L. pectinata* and considered this to be proof of the occurrence of hemoglobin in these granules. Fisher and Hand (1984) identified iron in similar pigment granules of *Lucina floridana*, but did not consider the presence of iron to be sufficient proof of the occurrence of hemoglobin in these granules. Dando et al. (1985) postulated that, in *Myrtea spinifera*, hemoglobin might occur in the nuclei of bacteriocytes. A number of other lucinid species which host abundant, endosymbiotic, sulphur-oxidizing bacteria have little, if any, hemoglobin, e.g. *Lucinoma borealis* the gill extract of which only contains cytochrome C which is known to be associated with thiobacilli and other sulphur-oxidizing bacteria (Dando et al. 1986). In *L. pectinata*, Wittenberg (1985) presumed that hemoglobin may be in solution in the cytosol, but did not exclude the possibility that it could be within the peribacterial sacs. In most lucinids, the concentration of hemoglobin appears to be so low that it is not detected in situ by histochemical techniques; for example, the hemoglobin concentration in the gills of *Codakia orbicularis*, which is 20-fold lower than in *L. pectinata* (Wittenberg 1985; Gros personal observation), is not detected by the diaminobenzidine (DAB) reaction (Frenkiel personal observation). Therefore, *L. pectinata* appears to be a most interesting species for the actual localization of tissue hemoglobin in gill cells.

The aim of the present paper is to clarify the status of hemoglobin in *Lucina pectinata* and to compare the cellular organization of gill filaments in this species with the organization prevalent in related and unrelated species which have developed symbiosis with sulphur-oxidizing bacteria; and, therefore, estimate the adaptative value of hemoglobin and symbiosis.

Materials and methods

Adult specimens of *Lucina pectinata* (30 to 60 mm shell-length) were collected by hand in the black mud of mangrove swamps in Guadeloupe (1.5 m average depth of water). Whole gills, fixed in various seasons – together with the visceral masses which were fixed for a study of the reproductive cycle – were examined and showed no obvious variations. Gills from individuals collected in various seasons were dissected in sea water; one gill was bisected for histological, histochemical or scanning electron microscopy (SEM) fixations, the other was cut into small pieces for transmission electron microscopy (TEM) fixation. Some starved individuals – kept for several weeks in the laboratory with algal food but no sulphur supplementation – were also fixed, but their gills were not included in the present study.

TEM preparation

Small pieces, dissected from various locations in the gills of freshly collected individuals, were fixed for TEM, for 2 h at 8 °C in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer adjusted to pH 7.2 and 1000 mOs*M* with a saline solution designed for marine invertebrates (Hernandez-Nicaise and Amsellem 1980); they were post-fixed for 1 h at 4 °C in 1% osmium tetroxide in the same buffer, treated with 2% aqueous uranyl acetate at room temperature and dehydrated through ascending ethanol. After embedding in epoxy (Epon-Araldite) or in acrylic (LR White) resins, sections were cut on an Ultracut E ultramicrotome. Semi-thin sections (0.5 µm thick) were stained with 0.5% toluidin blue in 1% borax; thin sections (60 nm thick) mounted on copper grids with pyroxylin film were contrasted with aqueous uranyl acetate and lead citrate before being observed in a Hitachi H-8000.

SEM preparation

Whole gills from small adult individuals (30 mm shell-length) were prefixed, for 24 h at room temperature in 2.5% glutaraldehyde either in sea water or in 0.1 *M* cacodylate buffer adjusted to pH 7.2 and 1000 mOs*M*. After a rinse in the same cacodylate buffer, small pieces of delaminated gill lamellae were cryoprotected in 25 and 50% buffered glycerol solutions, rinsed, and frozen in liquid propane cooled by liquid nitrogen, then immersed in liquid nitrogen and fractured with a liquid-nitrogen-chilled scalpel. The fragments were thawed in 50% buffered glycerol, rinsed and fixed, for 1 h at room temperature in 1% osmium tetroxide in the same buffer. Then, either the dissected pieces or the cryofractured fragments were dehydrated in acetone series, critical-point-dried using CO_2 as transitional fluid and sputter-coated with gold before observation in a Hitachi S-2500.

Histochemistry and cytochemistry

Histological methods were performed according to Gabe (1968) and Pearse (1985). Gill tissues were fixed in Bouin's and Regaud's fluids, as well as in 4% formalin in sea water and in a ferricyanide-formalin fixative designed to stabilize hemoglobin as methemoglobin. They were embedded in paraplast, sectioned (6 µm thick), and stained by various methods summarized in Table 1 to identify mucosubstances, proteins, and pigments. Alcian blue staining (pH 1 or 3) and periodic acid Schiff (PAS) reaction allow identification of various mucosubstances. The general identification of proteins by Danielli's tetrazoreaction and Barrnett and Seligman's diamino-dihydrodinaphtyl (DDD) reaction performed with and without reduction of -S-S- into -S-H either by ammonium sulphide or by thioglycerol, following Gabe's (1968) rationale and technique, allows discrimination of cysteine- or cystine-rich proteins. Fautrez and Lambert's staining with cyanol buffered at pH 6.4 was used to identify the location of a protein with high pI, which is likely to be hemoglobin. The rationale behind this technique (Lison 1960; Gabe 1968) is that the pI of hemoglobin (6.8) is much higher than the pI of other cytoplasmic proteins; therefore, the acidophily of hemoglobin remains effective at higher pH levels of the staining solution than for other proteins. According to Pearse (1985), the histological detection of hemoglobin is divisible into two parts, that of the globin and that of the hematin. Heme identification through its pseudoperoxidasic activity is considered the most reliable identification of hemoglobin; however this method does not distinguish hemoglobin from various other heme-compounds, because the peroxidase reaction is positive for hemoglobin in its various forms as well as for globin-free compounds such as hematin, heme and hemin. Moreover, the catabolic products of hemoglobin, with the exception of hemosiderin, are also DAB-reactive. Therefore, DAB oxidation indicates the presence of

Table 1 *Lucina pectinata.* Histochemical characterization of gill cells (+ + and + are indicative of strong or light reaction; – is indicative of no reaction). Mucocytes 1 are located near the intermediary zone and Mucocytes 2 along the interlamellar space. Red metachromasia with toluidin blue together with paraldehyde-fuchsin and Alcian blue in various acidic conditions characterize lubricating mucus mostly composed of glycosaminoglycans. PAS + and PAS + /Alcian blue lilac staining indicate the presence of sialomucins. Green metachromasia with toluidin blue on resin sections is indicative of hemoglobin. Danielli's tetrazoreaction indicates the presence of proteins which are more precisely identified as a cystine-rich protein by Barrnett and Seligman's DDD (diamino-dihydro-dinaphtyl) reaction, and as hemoglobin by Fautrez and Lambert's cyanol at pH 6.4 and by DAB (diaminobenzidine) reaction. As for the large bacteriocyte microbodies, the positive Schmorl's reaction together with a green coloration after Perls' reaction are indicative of putative lysosomal residual bodies; however, their strong reaction with DAB – typical of heme – may indicate an oxidizing function

Method	Mucocytes 1	Mucocytes 2	Bacteriocyte	
			Cytoplasm	Microbodies
Goldner's trichrome	green	green	orange	–
Paraldehyde-fuchsin	+ +	+ +	–	–
Toluidin blue on semi-thin section	meta red	meta red	meta green	–
Alcian blue, acetic (pH 3)	+	–	–	–
Alcian blue, chlorhydric (pH 1)	+ +	+ +	–	–
PAS	+	–	–	–
PAS–Alcian blue–Naphtol yellow	lilac	blue	–	–
Danielli's tetrazoreaction	–	–	+ +	+
Barnett and Seligman's DDD	–	–	–	–
DDD after reduction	–	–	+ +	–
Schmorl's adapt. Adam's	–	–	–	+
Perls' after oxidization	–	–	–	+
Fautrez and Lambert's	–	–	+ +	–
DAB oxidizing (with H_2O_2)	–	–	+	+ +

heme whether associated with proteins or not. The histological detection of hemoglobin by characterization of its proteinic part is thus a useful complement to its detection by pseudoperoxidasic activity; then, Fautrez and Lambert's method, which is only indicative when used alone, allows a reliable localization of hemoglobin when associated with heme identification.

Heme was identified by incubation with diaminobenzidine (Sigma DAB tablets) at a concentration of 2 mg ml^{-1} in Tris-HCl buffer adjusted to 7.6, with a final concentration of 0.03% H_2O_2, according to Goldfischer et al. (1970). Incubation was performed for 0.5 h at room temperature with paraffin sections of gill tissue fixed by formalin in sea water or by ferricyanide-formalin fixative; and for 2 h at 37 °C with 100 µm thick slices, obtained with a tissue-slicer, of gill tissue fixed in a 1% paraformaldehyde/2% glutaraldehyde mixture in cacodylate buffer. Three different controls were used: (i) incubation in complete medium without H_2O_2; (ii) incubation of tissues heated up to 80 °C; (iii) incubation with 3-amino,1,2,4-diaminotriazole as inhibitor of catalase. After incubation, and extemporaneous light microscopy control, slices were postfixed for 1 h in 1% osmium tetroxide, dehydrated and embedded as usual in Epon-Araldite. Sections (100 nm thick) were observed without further contrast. The rationale behind this method developed by Lewis and Knight (1992) emphasizes the conditions of incubation with DAB to discriminate enzymatic peroxidasic activity and pseudoperoxidasic non-enzymatic oxidation of DAB which allows identification of heme compounds.

Results

In *Lucina pectinata*, gills consist of single inner demibranchs covering the visceral mass on both sides as described for various Lucinidae. A faint groove is present along the ventral margin of the homorhabdic gills (Fig. 1) which are characterized by their deep-red color. The descending and ascending lamellae, which may easily be delaminated, are loosely linked by tissue bridges occupied by blood lacunae (Fig. 2). On frontal sections of the gill, the lateral, or bacteriocyte, zone appears approximately three times as thick as the ciliated and intermediary zones together (Figs. 2, 3).

Gill filament structure

Ciliated and intermediary zones

The ciliated zone is similar to that described in other lucinid species with typical frontal, latero-frontal, and lateral ciliated cells (Figs. 2, 4). In the ciliated zone, the core of each filament is occupied by a collagen axis which encloses a few fibroblasts, whereas, in the lateral zone, it is occupied by connective tissue and a blood lacuna. Some neural and muscular tissues are enclosed inside this axis, at the abfrontal part of the filament, lining the interlamellar space.

The intermediary zone (Figs. 4, 5) is made up of a large clear cell, a few narrow ones, and some mucous cells. The first cell, only ciliated on the side adjacent to the lateral ciliated cells, is characterized by a large area of electron-lucent cytoplasm. Its nucleus varies in aspect, being more or less elongated and in an apical, medial or basal position; numerous mitochondria are its most abundant organelles. Elongated nuclei belong to unciliated cells which are so compressed between the large ciliated cell and the mucous cells that it is difficult to describe them. The mucous cells are located between adjacent filaments at the junction of the intercalary and

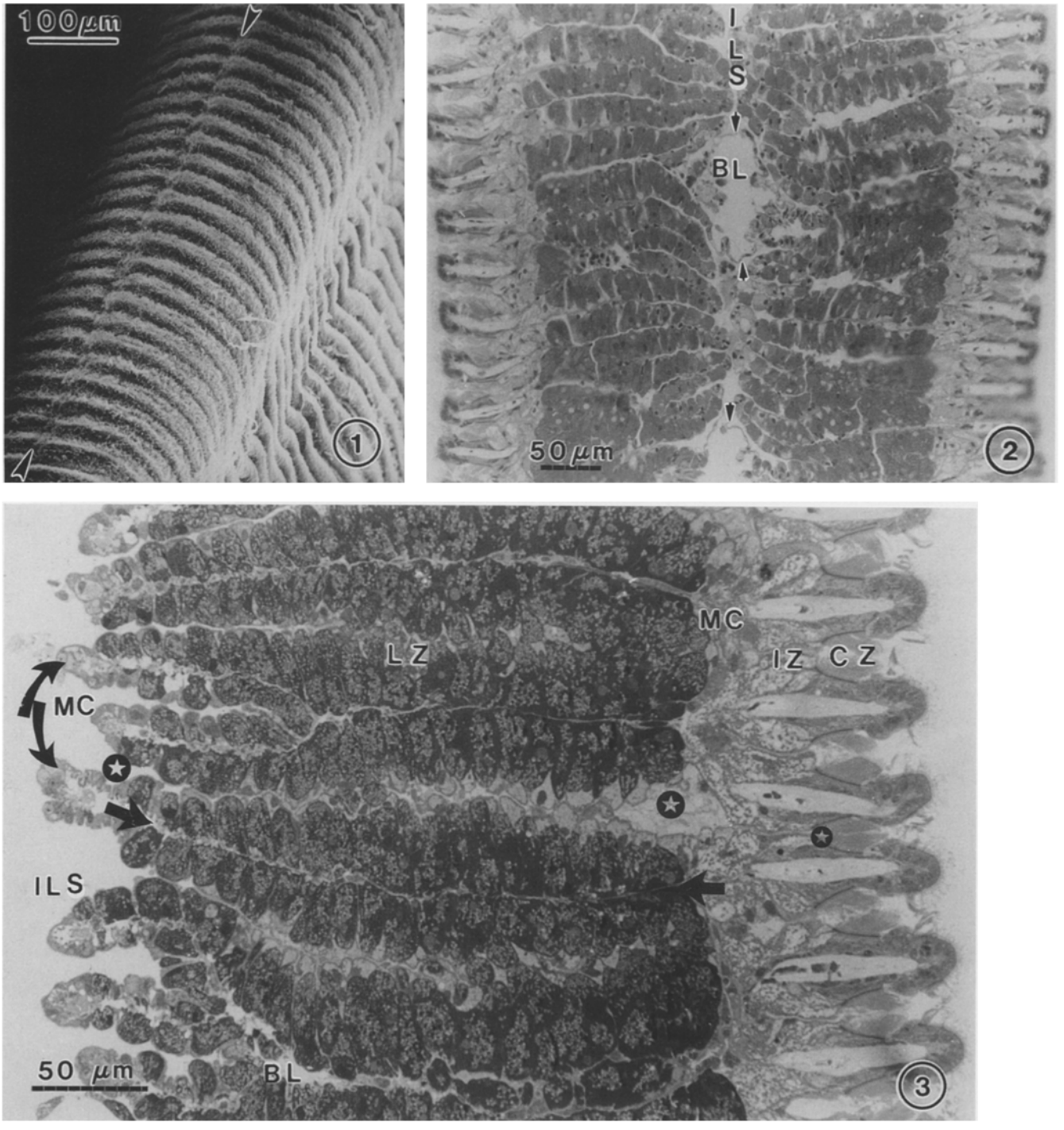


Fig. 1 *Lucina pectinata.* SEM, general view of gill. Faint marginal groove along the ventral edge of the homorhabdic gill

Fig. 2 *Lucina pectinata.* LM, general view of gill. Frontal section of outer and inner laminae united by interlaminar bridges (*arrows*) occupied by a large blood lacuna (*BL*). The translucent interlaminar space (*ILS*) contains circulating sea water

Fig. 3 *Lucina pectinata.* LM, general view of gill. Frontal semi-thin section of the outer lamina showing ciliated zone (*CZ*), intermediary zone (*IZ*), lateral zone (*LZ*). *Straight arrows* indicate the filament axis occupied by connective tissue and blood lacunae (*BL*); *stars* indicate the sea water flow along the bacteriocytes from the ciliated heads of filaments to the interlaminar space (*ILS*). Bacteriocytes are heavily stained by toluidin blue, while the metachromatic mucous cells (*MC*) are clear at each end (*curved arrows*) of the bacteriocyte zone

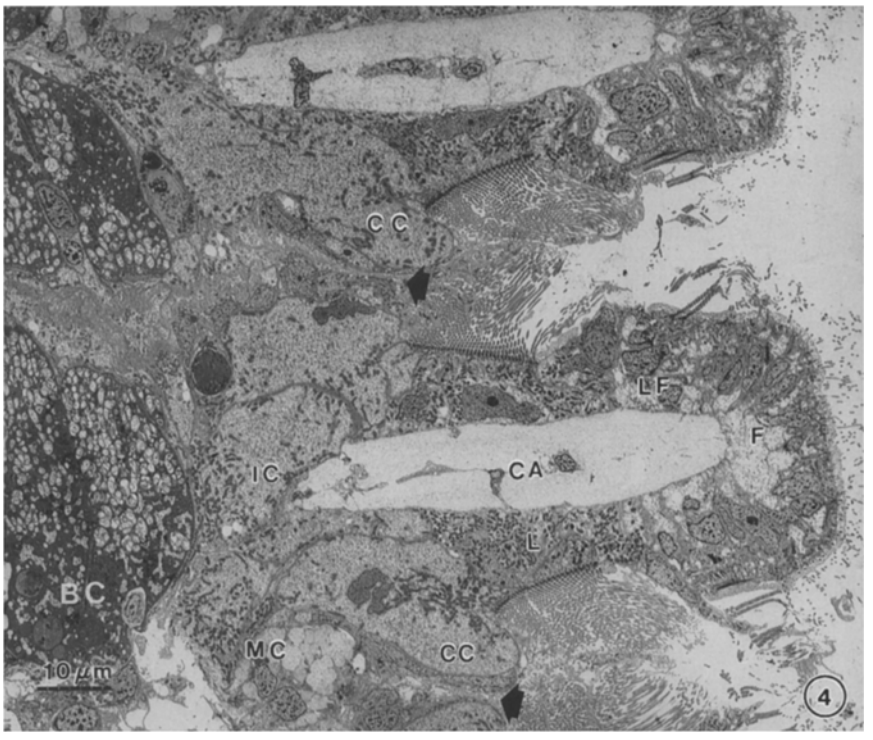


Fig. 4 *Lucina pectinata.* TEM. Ciliated and intermediary zones, and the adjacent part of the lateral zone with a few dark bacteriocytes (*BC*). Frontal (*F*), latero–frontal (*LF*), and lateral (*L*) ciliated cells are the main cell-types of the ciliated zone organized along a collagen axis (*CA*). The large clear cells (*CC*) constitute the narrow aperture (*arrows*) which directs sea water to the bacteriocyte channel. Together with the mucous cells (*MC*) and some unciliated intermediary cells (*IC*), they constitute the intermediary zone

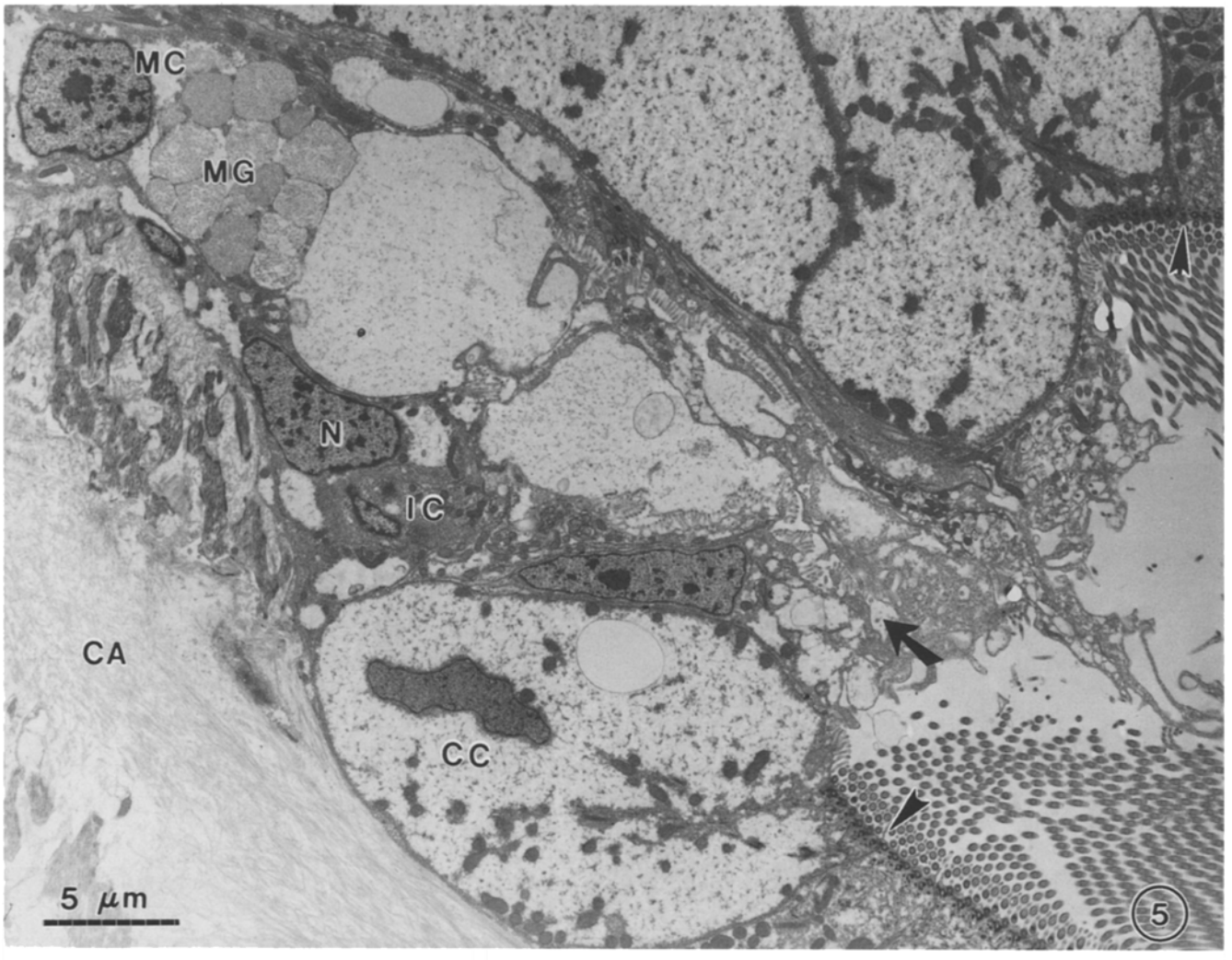
lateral zones. Some of them are well inside the intermediary zone, and others border on the lateral zone.

Lateral zone

The lateral zone is quite simple in appearance; bacteriocytes and intercalary cells are organized in a uniform pattern throughout its entire thickness. On the SEM micrograph of fractured gill filaments (Fig. 6), as well as on transverse sections (Fig. 7), the lateral zone appears to be organized in cylindrical channels. Mucous cells are located at each end of these channels, at the junction of the intermediary and lateral zones, and along the interlamellar space (Fig. 3). According to the histochemical methods summarized in Table 1, the mucous cells located near the intermediary zone (Mucocytes 1) produce sialomucins while the mucous cells located along the interlamellar space (Mucocytes 2) produce glycosaminoglycans.

TEM observations show that bacteriocytes and intercalary cells (Fig. 8) are covered with microvilli which are regularly distributed and linked by a glycocalyx on bacteriocytes, and irregularly distributed, without any glycocalyx, on intercalary cells. The bacteriocytes (20 to 30 µm in height and 10 to 15 µm in width) have convoluted basal membranes and roundish apical poles (Figs. 8, 9). They are characterized by patchy cytoplasm with finely granular electron-dense areas, and electron-lucent areas with coarse inclusions that may be glycogen particles (Fig. 9). Each bacteriocyte has a basal nucleus 5 µm in diameter which appears small when compared to the overall cell size (Fig. 8). Various inclusions, distributed in a specific pattern from apical pole to base, occupy most of the cell volume. The apical cytoplasmic area contains mainly bacterial vacuoles and mitochondria (Fig. 10). Smooth reticulum profiles are frequent along the plasma membrane (Fig. 16), and may be observed also along the bacterial vacuoles (Fig. 19). In the basal part of the bacteriocytes, bacteria become scarce and one or two conspicuous microbodies or lysosome-like vesicles occur next to the nucleus (Figs. 11, 12). The microbodies vary in size from 1 to 9 µm, and contain dense granules and whorls of membranes as observed on sections (Figs. 11, 17) and on fractured gill tissue (Fig. 16). Near these microbodies, clear vesicles, approximately 0.5 to 2 µm in diameter, are more or less abundant according to individuals.

Trumpet-shaped intercalary cells have narrow elongated bases and enlarged irregular apical poles (Figs. 8, 9). Each one has an elongated nucleus in an apical or lateral position and a large cytoplasmic area with numerous mitochondria and conspicuous reticulum and Golgi profiles. Their most peculiar features are irregular expansions of their apical poles. From SEM, further information has been obtained on the relationship between bacteriocytes and intercalary cells (Figs. 13, 14). Intercalary cells have prominent apical surfaces covered by small microvilli and by long, narrow protrusions which give them a tufty aspect (Fig. 15). They encroach upon adjacent bacteriocytes which have small apical poles, situated at a lower level. Thus, contact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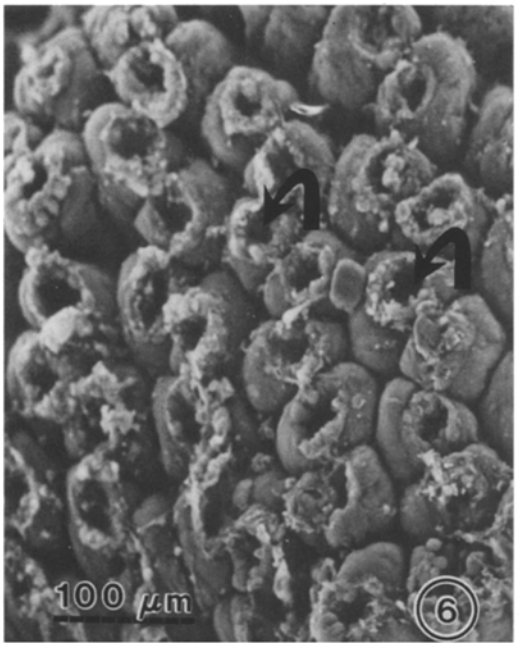
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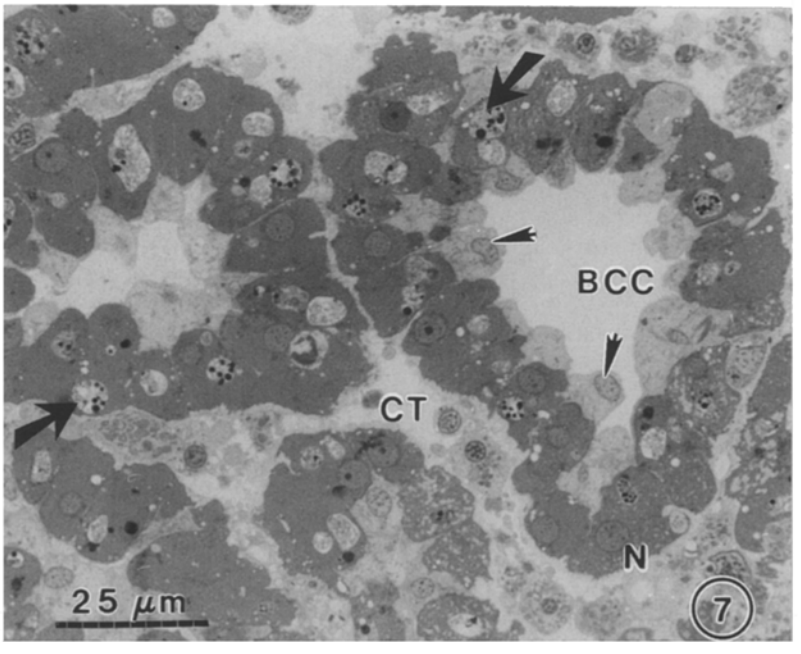
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25 μm
7

between the bacteriocytes and sea water is limited according to the extention of intercalary cells.

Characteristics of bacterial endosymbionts

Bacteria up to 3 μm in length and 1 μm in diameter are enclosed within bacterial vacuoles occupied most often by a single bacterium (Figs. 17–19), but occasionally by two or four, probably the result of recent divisions (Fig. 20). These rod-shaped endosymbionts have the typical double membrane of gram-negative bacteria; most of their volume is occupied by a clear nuclear area with tenuous DNA filaments, while their cytoplasm contains unidentified electron-dense granules. Membrane-bound periplasmic inclusions appear to be frequent at each end of the rod. While the cytoplasmic organelles of the bacteriocytes are well preserved (Fig. 10), many of these bacteria appear partly destroyed.

Location of hemoglobin and other proteins

Some information has been obtained on the bacteriocytes, by histological and histochemical staining (Table 1). The fact that the basal cytoplasmic region of the bacteriocytes is stained by Orange G in Goldner trichrome is indicative of the presence of very acidophilic proteins; dark blue staining by Fautrez and Lambert's cyanol reagent buffered at pH 6.4 shows that its acidophily is maintained at least up to pH 6.4, a reaction indicative of hemoglobin. Perls' ferricyanide reaction, applied after unmasking occult iron by H_2O_2 or by ammonium sulphide, stains the large microbodies, located near the nucleus, green but does not stain the cytoplasm. Both large microbodies and cytoplasm were DAB-stained when H_2O_2 was added to the incubation buffer, on both paraffin sections and 100-μm thick slices incubated before inclusion. The control, which was negative in the absence of H_2O_2, proves the specificity of the reaction. The unmodified reaction on heated sections shows the non-enzymatic oxidation of DAB which is specific to heme-compounds whether associated with proteins or not. The fact that the control with diaminotriazole added appears to be positive both in microbodies and cytoplasmic areas dismisses the hypothesis that these microbodies may contain catalase.

At the ultrastructural level, DAB detection of hemoglobin appears to be positive in the dark cytoplasmic areas and even more strongly positive in the microbodies, but negative in the clear vesicles located nearby. Mitochondria are also reactive (Fig. 12); however, their reaction, which is not heat-resistant, is due to cytochrome-oxidase according to Lewis and Knight (1992). The test reaction without H_2O_2 was negative in all cases. The Barrnett and Seligman's DDD reaction, which is negative when applied alone, is positive after reduction with ammonium sulphide or thioglycerol, indicating the absence of sulphydryl groups and the presence of disulphide bonds in proteins; therefore, the basal region of the bacteriocytes contains a cystine-rich protein, in addition to hemoglobin.

Fig. 5 *Lucina pectinata.* TEM, intermediary zone and bacteriocyte channels. The apical area of the clear cell (*CC*) is occupied by cilia where it is adjacent to the ciliated eulateral cells. *Arrowheads* indicate the junction complex between the eulateral ciliated cells and the clear large intermediary cell. Unciliated cells are compressed between the others; their nuclei (*N*) are the only obvious organelles. A mucous cell with a rounded nucleus secretes clear mucous granules (*MG*) through a canal (*arrow*) near the ciliated cells. These mucous cells are part of the intermediary zone; others are part of the lateral zone, being located at the aperture of the bacteriocyte channels (*CA* collagen axis; *IC* intermediary cell; *MC* mucous cell)

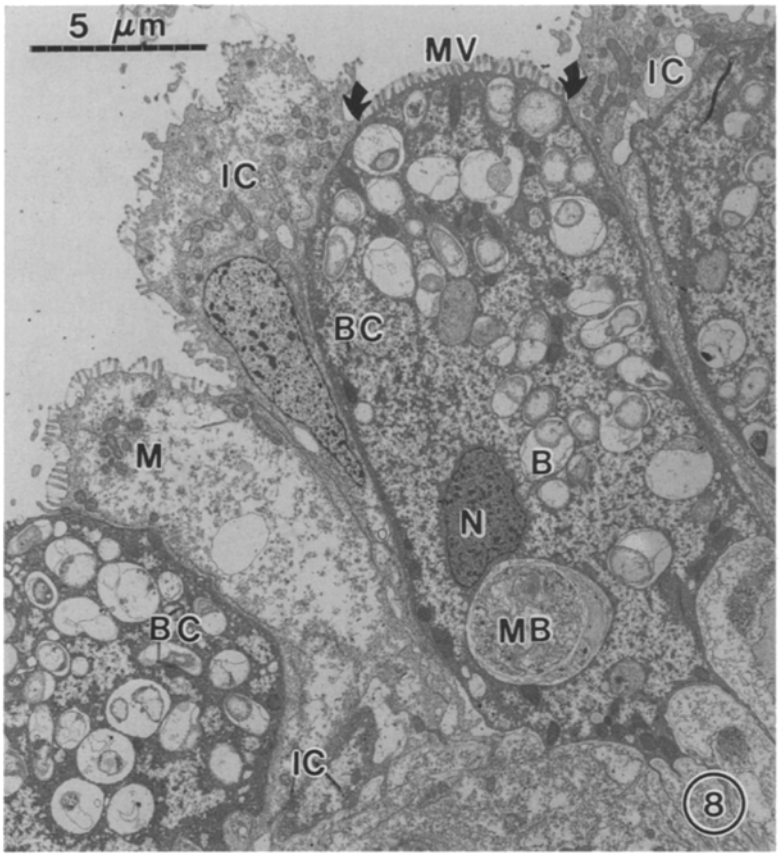
Fig. 6 *Lucina pectinata.* SEM, bacteriocyte channels viewed from the outer aperture (*curved arrows*) surrounded by mucus; the ciliated zone has been fractured off; the enlarged walls of the channels are made up of bacteriocytes and intercalary cells

Fig. 7 *Lucina pectinata.* LM, semi-thin transverse section of bacteriocyte channels (*BCC*). The filament axes are modified into a continuous connective and lacunar tissue (*CT*). The bacteriocytes are dark cells with conspicuous microbodies (*arrows*) as large as the grey nuclei (*N*). The clear cells with an apical nucleus are intercalary cells (*arrowheads*)

Discussion and conclusions

Structural relationship between cells

The ciliated and intermediary zones of the gill filaments of *Lucina pectinata* are very similar to those described for *Codakia orbicularis* (Frenkiel and Mouëza 1995), with a similar, large clear cell which, being ciliated, is associated with the lateral ciliated cell group; however that peculiar cell represents the most conspicuous part of the intermediary zone. The main function of these cells is apparently to constitute the narrow aperture of large bacteriocyte channels. These bacteriocyte channels, similar to those described by Distel and Felbeck (1987) in *Lucinoma aequizonata*, appear to be an arrangement which allows regular water-flow along the bacteriocytes from the mantle cavity into the interlamellar space. Most discriminating features in the gill filament organization of *L. pectinata*, when compared with the organization prevalent in *C. orbicularis* and other Lucinidae, are observed in the lateral zone and involve the various identified cell types. In *L. pectinata*, mucous cells are limited to well defined points – bordering on the intermediary zone, on one hand, and along the interlamellar space, on the other – whereas they are distributed throughout the lateral zone in *C. orbicularis* (Frenkiel and Mouëza 1995) and are scarce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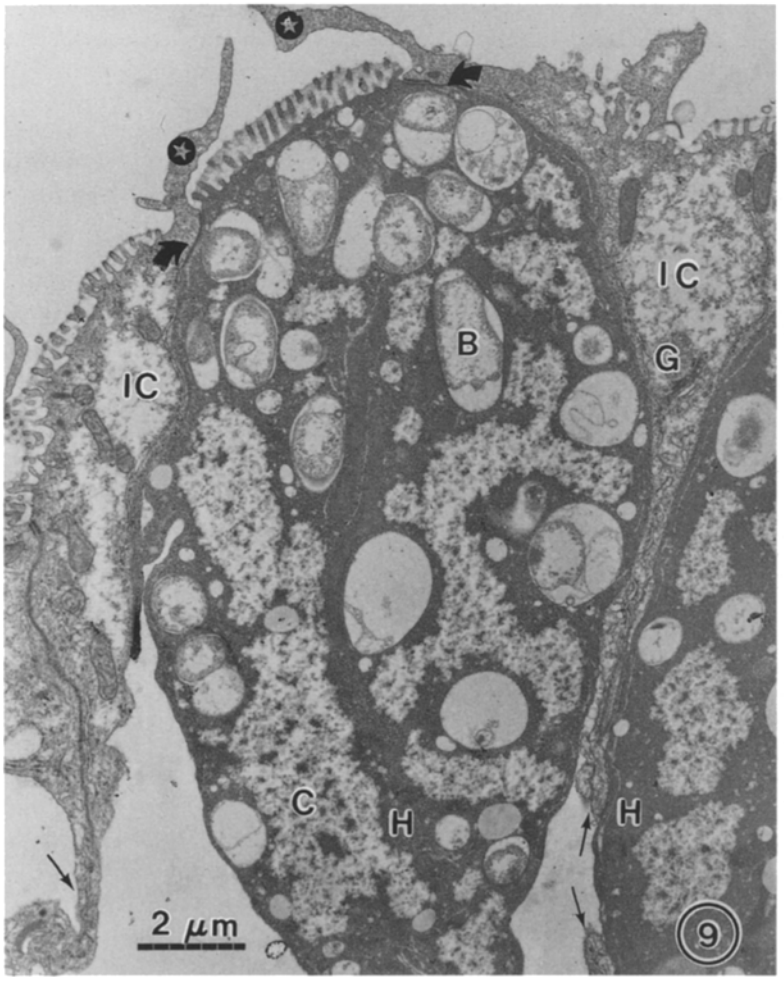
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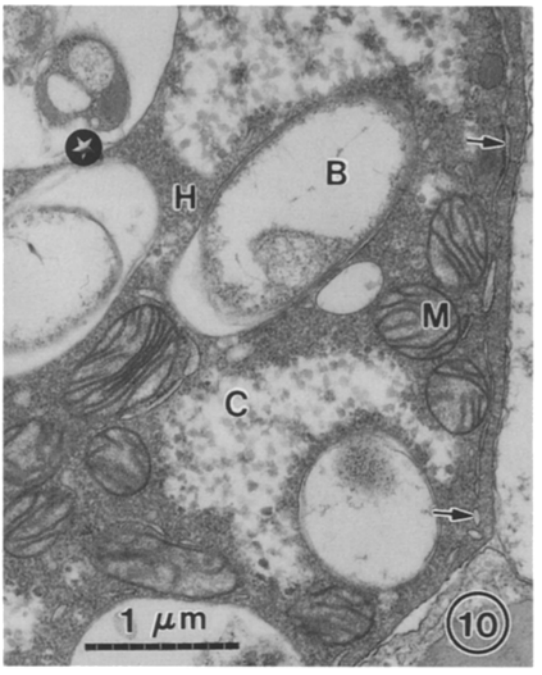
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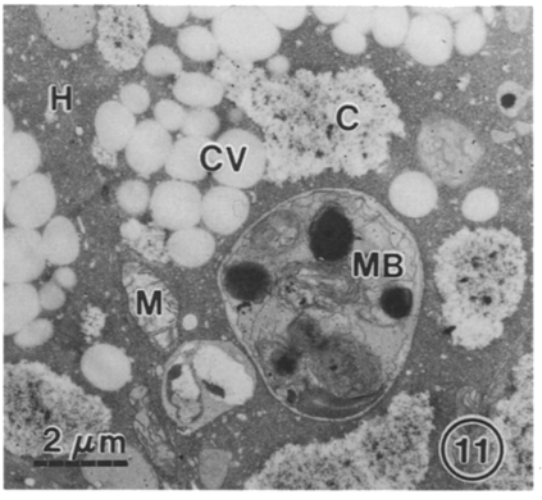
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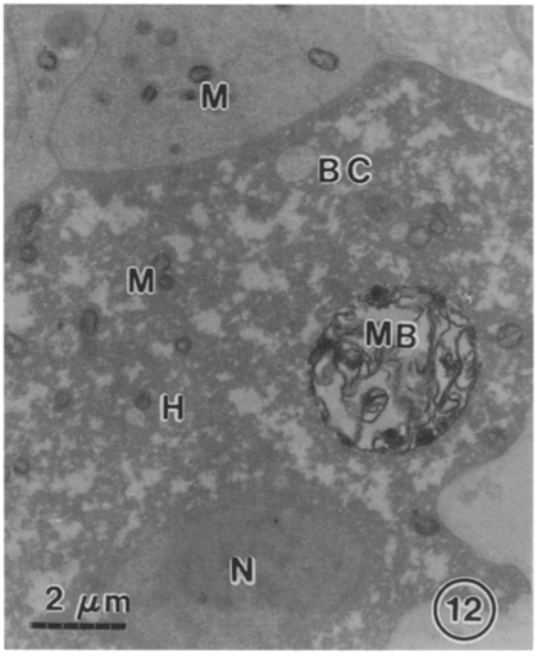
M
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2 μm
12

or absent in various other Lucinidae (Dando et al. 1985; Southward 1986).

Most significant differences involve the relationships between bacteriocytes and intercalary cells. A question raised by several research workers (Felbeck 1983; Fisher and Hand 1984; Giere 1985; Distel and Felbeck 1987) was the situation of the bacteriocytes with regard to their contact with circulating sea water. From their study, Distel and Felbeck (1987) concluded that the organization of bacteriocytes, shielded from direct contact with sea water by a thin epithelial sheet, such as described for *Lucinoma aequizonata*, was pervasive throughout the Lucinidae. A similar interpretation may be deduced from the schematic drawing of *Calyptogena magnifica* gill filament by Fiala-Medioni and Le Pennec (1987); although the original micrograph of the same gill filament (Fiala-Medioni and Metivier 1986) does not lead to the same conclusion. Moreover, Giere (1985) showed that, in most cases, such apparently complete overlapping of intercalary cells on the bacteriocytes was due to obliquous planes of section.

Fig. 8 *Lucina pectinata.* TEM, bacteriocytes (*BC*) and intercalary cells (*IC*) in the most superficial part of the lateral zone near the intermediary zone. The bacteriocytes have a rounded apical pole with microvilli (*MV*), a basal nucleus (*N*) and an adjacent large microbody (*MB*); the bacteria (*B*) appear to be small with an enlarged periplasmic space. Intercalary cells with elongated nucleus have an irregular prominent apical pole with numerous mitochondria (*M*)

Fig. 9 *Lucina pectinata.* TEM, in the innermost part of the lateral zone, bacteriocytes are often pear-shaped, and the intercalary cells (*IC*) have an elongated basal end which may be considered as an axon (*thin arrows*); the bacteriocyte has patchy cytosol with dark hemoglobin-containing areas (*H*) and clear granular areas (*C*); marginal flanges of intercalary cells (*stars*) expand over the bacteriocyte apical microvilli; junctional complexes are indicated by *curved arrows* (*B* bacteria; *G* glycogen particles)

Fig. 10 *Lucina pectinata.* TEM, smooth reticulum (*arrows*) is frequent along the lateral membrane of bacteriocytes; mitochondria (*M*) with longitudinal cristae are abundant in the hemoglobin-rich dark cytosol (*H*); whereas the clear cytosol (*C*) contains storage granules. Vacuoles contain bacteria apparently undergoing progressive lysis (*star*)

Fig. 11 *Lucina pectinata.* TEM, at the base of the bacteriocytes, a large lysosome-like microbody (*MB*) is characterized by dense iron-rich granules and membrane whorls; surrounding these microbodies are numerous electron-lucent, apparently empty vesicles (*CV*). (*C* clear cytosol; *H* hemoglobin-rich dark cytosol; *M* mitochondria)

Fig. 12 *Lucina pectinata.* TEM, after DAB reaction without any further contrast, mitochondria (*M*) appear DAB + in any type of cell, which is due to cytochrome oxidase. In the bacteriocyte (*BC*) the dark cytoplasmic patches and microbodies are both DAB +; this positive heat-resistant DAB reaction identifies heme-compounds which are hemoglobin (*H*) in the cytosolic dark patches and a non-protein-bound heme in the microbody (*MB*) whereas the cell in the upper part of the micrograph, in which only the mitochondria are DAB-reactive, constitutes a negative test (*N* nucleus)

Fisher (1990) noted conflicting reports concerning the same species and difficulties in obtaining a clear view of the relationship between bacteriocytes and intercalary cells. SEM observations of the apical surface and of fractured gills allow better interpretation of TEM results. With both microscopes, it is possible to show that, in *Lucina pectinata*, large intercalary cells develop numerous protrusions and a circular flange overlapping the apical microvilli of the bacteriocytes. The extent of overlap may be variable, but it never covers the apical surface of the bacteriocytes. Therefore, the bacteriocytes are in contact with a restrained flow of sea water. On the contrary, in *Codakia orbicularis* the bacteriocytes are in contact with sea water through a large apical area covered with regular microvilli and a well developed glycocalyx (Frenkiel and Mouëza 1995), whereas the intercalary cells are very narrow cells with an apical nucleus and a small apical area covered by irregular microvilli, similar to those of *Loripes lucinalis* (Herry et al. 1989). In most species studied so far, intercalary cells are not characterized by typical organelles but have been credited with several potential functions. They have been considered to be new bacteriocytes designed to replace the old ones (Fiala-Medioni and Metivier 1986; Reid and Brand 1986); or possibly to play a role in the evacuation of residual bodies, in view of some large lysosomal residual bodies extruding from their apical pole (Distel and Felbeck 1987).

In some species, less modified intercalary cells retain a basal nucleus and apical cilia; such is the case of *Thyasira equalis* (Southward 1986) and of *Bathymodiolus* sp. (Fiala-Medioni et al. 1986). The most remarkable features of the intercalary cells of *Lucina pectinata* are numerous protrusions of their apical surface, much longer than microvilli but without any ciliary axonemal structure. An apical surface view of these intercalary cells is suggestive of chemoreceptive sensory buds; thus, intercalary cells may be able to detect some vital parameters of sea water such as sulphide and oxygen concentrations, and to reduce or increase the contact of bacteriocytes with sea water. These intercalary cells appear more differentiated in *L. pectinata* than in most lucinids inhabiting sea-grass beds, which suggests that they may be more developed in species living in high-sulphide environments. In high-sulphide environments the need to apprise sulphide and oxygen availability may be vital, whereas fairly constant levels of sulphide and oxygen are available in the sea-grass bed environment, and so this differentiation would be unnecessary. Some neural and axonal profiles have been identified at the abfrontal end of the filaments facing the interlamellar space; however, their relationship with the basal part of intercalary cells is unclear. Moreover, as the sensory receptors are mostly primary receptors in bivalves, axonal processes may travel to the visceral ganglion without any synaptic differentiation (Welsch and Storch 1976).

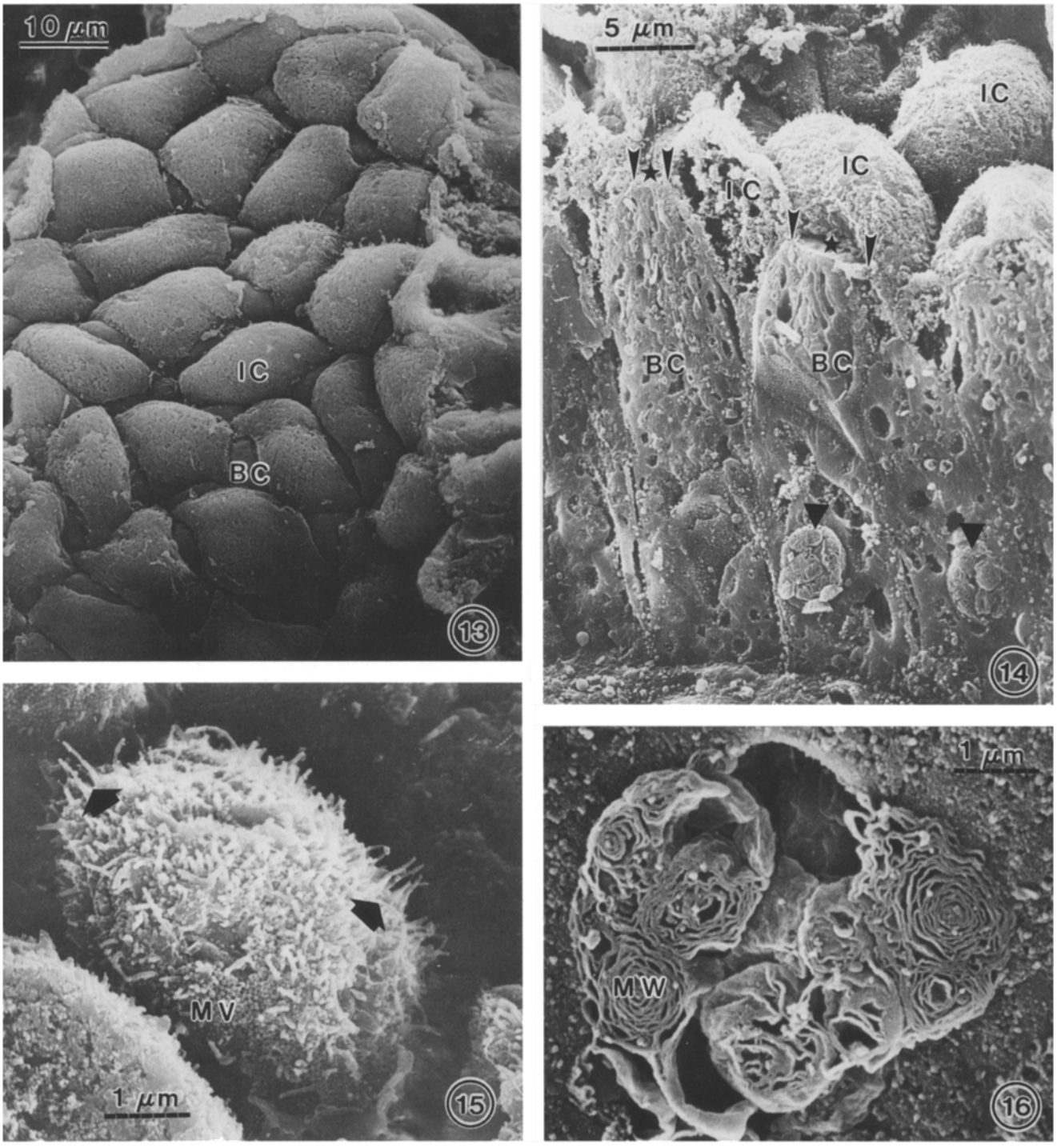


Fig. 13 *Lucina pectinata.* SEM, the bacteriocytes (*BC*) and intercalary cells (*IC*) constitute an intricate array of small and large apical surfaces, respectively

Fig. 14 *Lucina pectinata.* SEM, fractured bacteriocytes (*BC*) and intercalary cells (*IC*). The flattened apical surfaces of bacteriocytes (*stars* and *arrowheads*) are at a lower level than the roundish apical surfaces of intercalary cells; inside the bacteriocytes, the large basal inclusions are lysosome-like microbodies (*inverse triangles*) identical to Fig. 16

Fig. 15 *Lucina pectinata.* SEM, tufty surface of an intercalary cell; microvilli (*MV*) are shorter and more regular than the slender protrusions which characterize the intercalary cells (*arrows*)

Fig. 16 *Lucina pectinata.* SEM, fractured microbody; membrane whorls (*MW*) are more conspicuous on a fractured cell than on thin sections; conversely, the dense granules are not obvious, having been dissolved during some phase of preparation or being in other types of microbodies; the hypothesis of two different types of microbodies cannot be excluded

The relationship between intercalary cells and bacteriocytes is very similar in both *Calyptogena magnifica* (Fig. 4 in Fiala-Medioni and Metivier 1986) and *Lucina pectinata*, notwithstanding their taxonomic status and the differences between their respective environments. Such similarities are likely to be related to high-sulphide environments, suggesting that they constitute an adaptative feature. The bacteriocytes are maintained in direct but restricted contact with sea water in some species and in open contact in others; the oversimplifying idea that Lucinidae all follow the same pattern, as advocated by Distel and Felbeck (1987), is therefore most questionable.

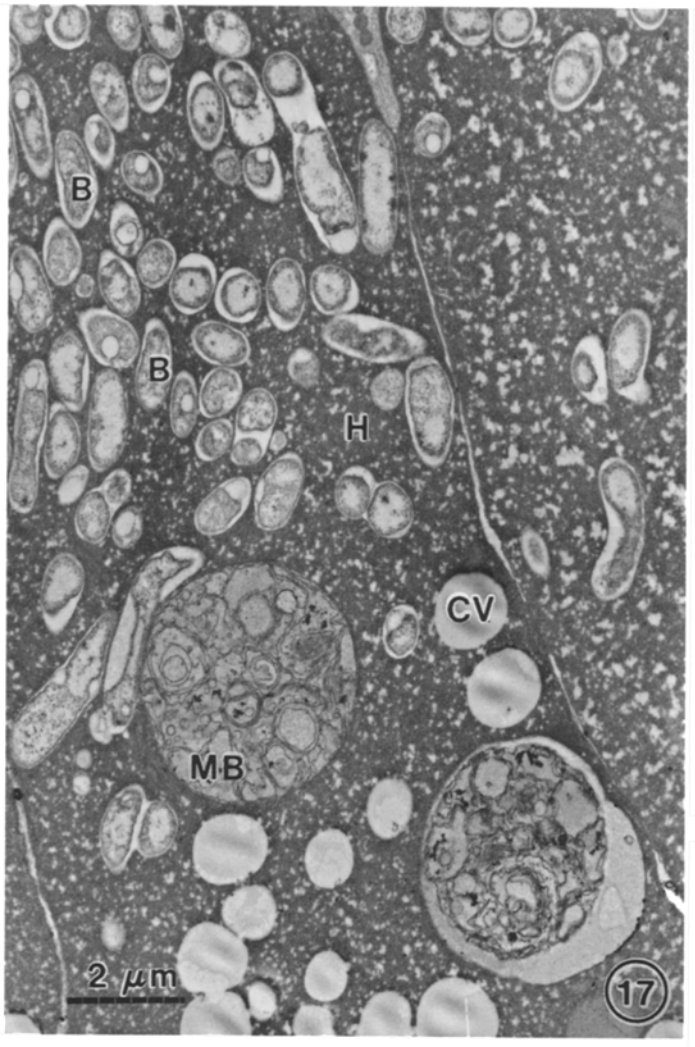
Bacteriocyte ultrastructural and cytochemical features

In *Lucina pectinata*, certain cytological features of the bacteriocytes are quite different from those observed in other Lucinidae, above all, the make-up of the cytoplasm and the abundance of large microbodies or lysosome-like inclusions. The unusual aspect of cytoplasmic dark and clear patches, which has not been observed, so far, in other Lucinidae, appears variable among different individuals (Figs. 9, 17), and may be correlated with their metabolic status. Both clear and dark patches may be large enough to be obvious on semi-thin sections, or the clear areas may disappear almost completely. A similar pattern exists in the bacteriocytes of *Calyptogena magnifica*, where the granular clear areas were assumed to represent the result of bacteria lysis (Fiala-Medioni and Metivier 1986). Such an interpretation is questionable, as no membrane separates such areas from the dark cytoplasmic areas and no bacterial residue has been identified to support that hypothesis. On the contrary, the clear patches are likely to be normal cytoplasmic areas, while the dark patches are similar to the finely granular, dark cytoplasm of mammalian red blood cells which constitute the standard for hemoglobin-rich cells. This hypothesis has been checked by two different techniques. When observed on histological sections, the cytoplasmic basal region of the bacteriocytes is stained blue by Fautrez and Lambert's method, which is indicative of the presence of hemoglobin; moreover, the non-enzymatic oxidation of DAB indicates the presence of heme-compounds in the same cytoplasmic area. The conjunction of both reactions makes it possible to conclude that the dark cytoplasmic patches constitute the cytoplasmic localization of hemoglobin in *L. pectinata* and most probably also in *C. magnifica*.

The strong non-enzymatic oxidizing activity observed in the numerous large microbodies of *Lucina pectinata* is difficult to interpret. These large inclusions were considered by Read (1962) to be hemoglobin locations, based on the detection of occult iron which is, in fact, more prone to prove the existence of heme-compounds issued from the catabolism of hemoglobin than the existence of functional hemoglobin. Similar large microbodies were assumed by Giere (1985) and by Distel and Felbeck (1987) to be lysosomal residual bodies in various other species. In *L. pectinata*, these microbodies are detected by their DAB-oxidizing activity, but not by Fautrez and Lambert's method. Therefore, the proteinic part of hemoglobin is lacking, and the DAB reaction remains to demonstrate the presence of one or more non-protein-bound heme, which may be either hemoglobin catabolic products or active free hematin. This seems to be in accordance with Kraus and Wittenberg's (1990) analysis showing the existence of a yellow iron compound distinct from the three identified hemoglobins in the gill tissue of *L. pectinata*. The presence of non-protein-bound hemes in these microbodies is also compatible with the fact that twice as much iron as hemoglobin-iron was detected by Wittenberg (1985) in symbiotic gill tissues.

These microbodies may also correspond to the sulphur-oxidizing bodies described by Powell and Arp (1989) in the bacteriocytes of other bivalves associated with symbiotic sulphur-oxidizing bacteria; however, we failed to identify sulphur-oxidizing activity in the microbodies of *Lucina pectinata*. These may, conversely, be characterized as lipofuscin granules by Schmorl's and Perls' reactions and as lysosomal residual bodies by ultrastructural observations. We have checked that they are not catalase-containing peroxisomes and noted that DAB-reactive heme-compounds are not exceptional in lysosomal residual bodies (Essner 1975; Pearse 1985), to which the Perls' reaction endows a green color. Their abundance in *L. pectinata* may be due to an important turn-over of cytoplasmic hemoglobin. These hemes may be mixed with lipoproteins issued from cell and bacterial membranes, in accordance with authors who suppose that microbodies observed in the basal part of bacteriocytes of various species are lysosomal residual bodies resulting from the regulation of the bacterial load (Giere 1985; Distel and Felbeck 1987; Fiala-Medioni et al. 1989). The lysosomal resorption of bacteria has been observed in *Codakia orbicularis*; however, in *L. pectinata* it remains hypothetical, as partly destroyed bacteria are frequently observed inside bacterial vacuoles but their inclusion in lysosomal bodies has not been observed as yet.

The detoxification process does not appear to follow similar pathways in *Lucina pectinata* and *Calyptogena magnifica*, as neither the complement of three hemoglobins nor the cystine-rich protein and yellow iron-compound identified in *L. pectinata* by Kraus and Wittenberg (1990) have been identified in *C. magnifica*. Whereas, according to Powell and Arp (1989), *C. magnifica* oxidizes sulphide in various tissues and in sulphur-oxidizing bodies, in *L. pectinata* sulphide is transported by a sulphide-reactive cytoplasmic hemoglobin (Kraus and Wittenberg 1990). The electron-lucent vesicles, which are abundant near the large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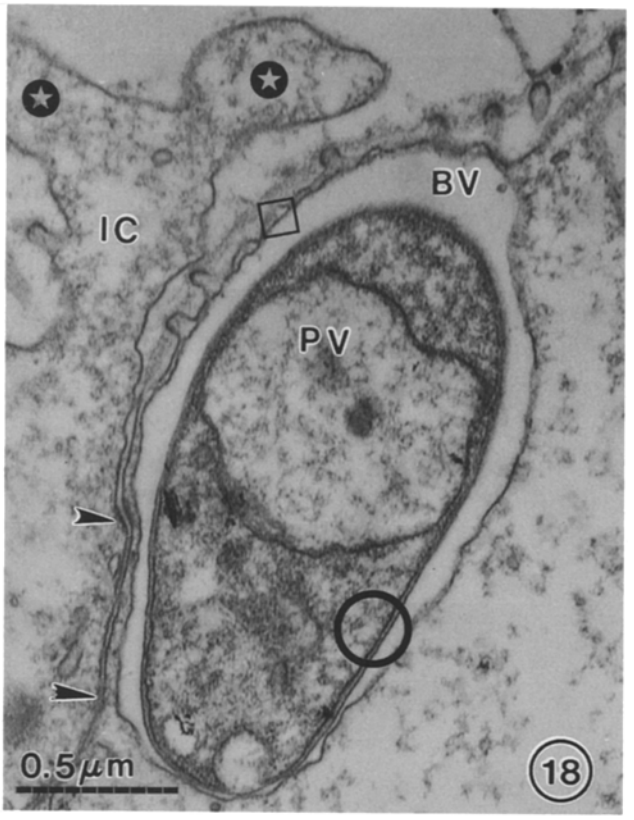
B
H
CV
MB
2 μm
17


BV
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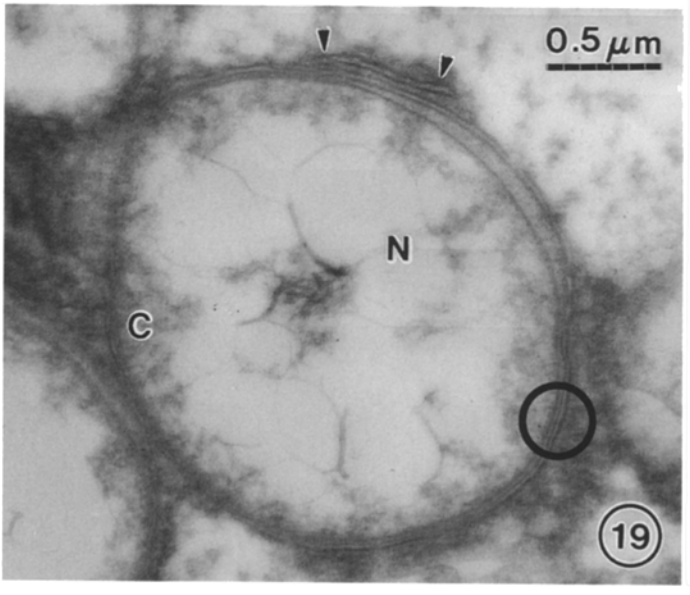
18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C
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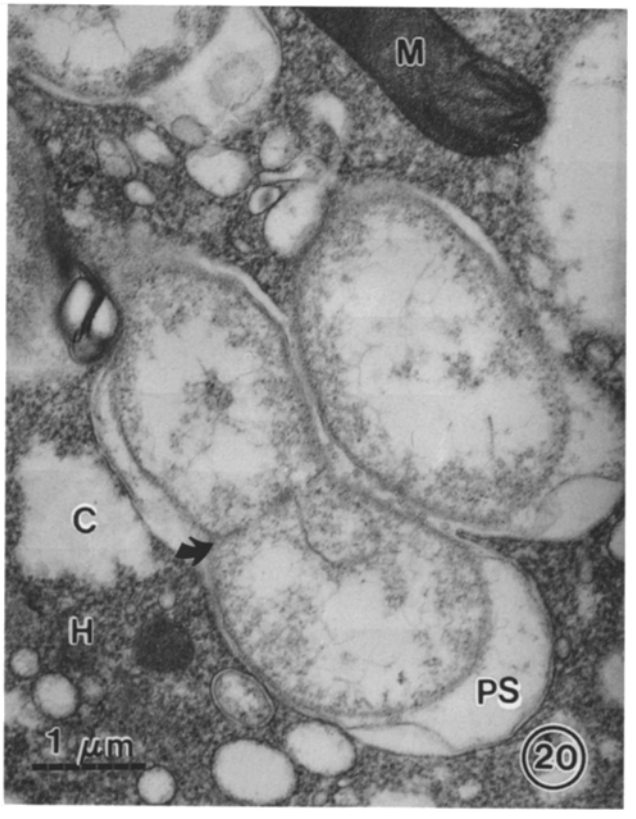
M
C
H
PS
1 μm
20

microbodies, may also have an unknown function in detoxification processes. A cyto-enzymological study is in progress to ascertain whether lysosomal and sulphur-oxidizing activities are sequential in a given type of microbodies or simultaneous in various types.

Conclusion

In *Lucina pectinata*, hemoglobin is located in dark cytoplasmic patches of the bacteriocytes. The cystine-rich protein identified by Kraus and Wittenberg (1990) appears to be mixed with hemoglobin in the cytoplasmic basal region. The large pigment granules, considered by Read (1962) to contain hemoglobin, are complex microbodies containing a non-hemoglobin heme-compound which corresponds to the yellow iron fraction isolated by Kraus and Wittenberg (1990). They might be similar to the brown granules containing hematin (defined as an oxidized heme-compound without associated protein) described in *Calyptogena magnifica* by Powell and Arp (1989). In *L. pectinata*, these microbodies oxidize DAB non-enzymatically, but sulphur-oxidizing activity has not been detected yet. Their function is, therefore, still uncertain.

Similar features of intercalary cells and similar cytoplasmic hemoglobin status in the bacteriocytes emphasize the congruence between the shallow-water mangrove species *Lucina pectinata* and the hydrothermal vent species *Calyptogena magnifica*. On the other hand, the numerous granule cells containing cystine-rich proteinic granules, identified in *Codakia orbicularis*, appear to be lacking in *L. pectinata*. From these features, we conclude that *L. pectinata* shares closer adaptative cytological structures with the Vesicomyidae *C. magnifica* than with the related Lucinidae *C. orbicularis*. This point of view supports the hypothesis of convergent adaptative features to the high-sulphide occurrence prevalent in mangrove muddy bottoms and in deep-sea hydrothermal vents. These conclusions concur with the fact that *L. pectinata* hosts a symbiont which has been typified by its 16S rRNA gene sequence (Durand et al. 1996) as being the most divergent microorganism from the cluster of Lucinacea symbionts.

Acknowledgements This study was carried out at the SIMAG (Service Interrégional de Microscopie des Antilles et de la Guyane). We are grateful to all those who have supported the foundation of SIMAG which has received generous funding from the local authorities (Regional Council and General Council of the French West Indies), from the French Ministries of Education, Research and DOM (FIDOM) and from the EEC (FEDER). We thank K. Lacoste for reviewing the English text.

Fig. 17 *Lucina pectinata.* TEM, bacteriocyte base with numerous bacteria (*B*), electron-lucent vesicles (*CV*), and large microbodies (*MB*) with membrane whorls; the cytosol is made up mostly of the dark hemoglobin-rich patches (*H*), interspersed with very small clear ones

Fig. 18 *Lucina pectinata.* TEM, the junctional complex (*arrowheads*) is situated under a large flange (*stars*) of the intercalary cell (*IC*) which overlaps the adjacent part of the bacteriocyte. A gram-negative symbiont (typical membrane surrounded by a *circle*) is included in a bacterial vacuole (*BV*) and, in the region of the bacteriocyte which is protected by the adjacent intercalary-cell flange, this bacterial vacuole adheres to the plasmic membrane (*square*); the bacterium has a large periplasmic vesicle (*PV*) with granular content quite different from the usual appearance of sulphur globules

Fig. 19 *Lucina pectinata.* TEM, bacterium transverse section is at a level where the nuclear area (*N*) occupies most of the cell section, cytosolic areas (*C*) being reduced; the bacterial-vacuole membrane adheres not only to the bacterium but also to a reticulum profile (*arrowheads*)

Fig. 20 *Lucina pectinata.* TEM, dividing bacteria in a single vacuole; *curved arrow* indicates the dividing plane between two of them. One has a periplasmic enlarged apical space (*PS*), independent of osmolality adjustment of the fixative, which is frequently observed in *L. pectinata* symbionts (*C* cytosol; *H* hemoglobin-rich cytosol; *M* mitochondria)

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