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Gill filament differentiation and experimental colonization by symbiotic bacteria in aposymbiotic juveniles of *Codakia orbicularis* (Bivalvia: Lucinidae)

OLIVIER GROS, LILIANE FRENKIEL* and MARCEL MOUËZA
Faculté des Sciences Exactes et Naturelles, Département de Biologie, Université des Antilles et de la Guyane, B.P. 592, 97159 Pointe-à-Pitre cedex, Guadeloupe (French West Indies).
Tel. +590 93 87 25; Fax +590 93 87 24; e-mail: liliane.frenkiel@univ-ag.fr

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Summary

Codakia orbicularis is a tropical lucinid harboring gill endosymbionts which are environmentally transmitted from a free living-symbiont form to the new host generation after metamorphosis. Structural changes occurring in the cellular organization from incomplete gill filaments in young aposymbiotic juveniles to full differentiated gill filaments containing bacterial endosymbionts in reared symbiotic juveniles, were analyzed for juveniles from 250 µm to 2 µm shell-length. Aposymbiotic juveniles possess differentiated gill filaments with ciliated, intermediary, and lateral zones similar to those described in wild juveniles, except for the bacteriocytes which are lacking. Granule cells, which progressively differentiate during the morphogenesis of the gill filament, do not appear as a consequence of symbiosis. Experimental colonization of aposymbiotic juveniles by the free-living symbiont form has been obtained through the addition of unsterilized sand collected from the natural habitat of *C. orbicularis*. Two days after exposure to crude sand, symbiosis-competent bacteria enter by endocytosis at the apical pole of undifferentiated cells which progressively differentiate into classical bacteriocytes similar to those found in the adult gill filaments. Undifferentiated cells of aposymbiotic gill filaments remain receptive to bacteria several months after metamorphosis, and become bacteriocytes when aposymbiotic juveniles get contact with the symbiont free-living form. Therefore, the environmental transmission of symbionts does not appear to be restrained to a defined period of time during post-larval development in *C. orbicularis*.

Key words: Endosymbiosis, experimental infection, TEM, transmission mode, bacteria.

Introduction

Adult individuals of the tropical lucinid *Codakia orbicularis* (Linné, 1758), which inhabit shallow water sea-grass beds of *Thalassia testudinum*, live in symbiosis with sulfur-oxidizing chemoautotrophic bacteria (Berg and Alatalo, 1984). These bacteria are housed inside bacteriocytes which occupy only the most superficial one-third of the lateral zone of gill filaments (Frenkiel and Mouëza, 1995). Bacterial chemoautotrophic symbioses (including both ecto- and endosymbionts) are known to be represented at least in seven

*Corresponding author.

different host phyla (Cavanaugh et al., 1981; Felbeck et al., 1981; Fenchel and Finlay, 1989; Fisher, 1990; Felbeck and Distel, 1992; Temara et al., 1993; Cavanaugh, 1994; Polz et al., 1994; Ott et al., 1995; Bauer-Nebelsick et al., 1996). In spite of the increasing number of species known to harbor bacterial endosymbionts, studies on transmission mode are scarce due to the difficulty in cultivating both the symbionts and the host species. So, studies are most based on molecular biological techniques such as PCR and/or *in situ* hybridization (Giere and Langheld, 1987; Cary and Giovannoni, 1993; Cary et al., 1993; Cary, 1994; Krueger et al., 1996; Gros et al., 1998). A crucial condition for any work with symbiont transmission mode is the ability to grow these species under controlled conditions. To date, only four species of bivalves known to harbor gill-endosymbionts have been raised successfully: *Thyasira gouldi* (Blacknell and Ansell, 1974), *Codakia orbicularis* (Alatalo et al., 1984; Gros et al., 1997), *Solemya reidi* (Gustafson and Reid, 1986; 1988a), and *Solemya velum* (Gustafson and Lutz, 1992). The symbiont transmission mode is known for only three of them. Thus, gill endosymbionts are vertically transmitted from parents to offspring in the protobranchs *Solemya reidi* (Gustafson and Reid, 1988b; Cary, 1994) and *S. velum* (Krueger et al., 1996), and environmentally transmitted to the new host generation through a free-living symbiont form in the lucinid *Codakia orbicularis* (Gros et al., 1996b). However, even for these species, informations on (i) morphogenesis of the gill filament in larvae and juveniles and (ii) infection modes of aposymbiotic gill filaments by their endosymbionts are lacking. Gustafson and Reid (1988a; b) proposed that gill buds were infected by symbiotic bacteria in the protobranch *S. reidi* through hemocytes. However, they did not present any data on the gill-cell infection by the symbionts.

Data dealing with morphogenesis of gill filaments during larval and post-larval development in bivalves (with or without endosymbionts) are scarce. Most of them are included in larval development studies (Quayle, 1952; Creek, 1960; Allen, 1961; Ansell, 1962; D'Asaro, 1967; Caddy, 1969; Frenkiel and Mouëza, 1979) and were described without data on ultrastructural modifications occurring during gill organogenesis. Recently, Beninger et al. (1994) have described the gill organogenesis in the filibranch *Pecten maximus* by using scanning electron microscopy (SEM) .

Preliminary studies on *C. orbicularis* have shown that (i) sulfur-oxidizing endosymbionts are environmentally transmitted to the new host generation after metamorphosis through a free living symbiont form probably located in sea-grass bed sand (Gros et al., 1996b) and (ii) the entire development of this lucinid, from fertilization to complete metamorphosis, characterized by the presence of numerous gill filaments and a functional excurrent siphon, occurs without symbiotic bacteria (Gros et al., 1997). Thus, *C. orbicularis* provides a good model to study the initiation and development of sulfur-oxidizing bacterial symbiosis in marine invertebrate species undergoing environmental gill-endosymbiont transmission.

Despite the fact that sulfur-oxidizing gill-endosymbionts remain uncultivable, as all marine invertebrates gill-endosymbionts known to date, we are able to initiate symbiosis and follow the different steps of infection in gill filaments by using symbiosis-competent bacteria located in sea-grass sand which is the natural habitat of *C. orbicularis*. We analyze the evolution of gill ultrastructure in juveniles of *C. orbicularis* before, during, and after the establishment of symbiosis by the free-living form of the chemoautotrophic endosymbiotic bacteria with scanning and transmission electron microscopy.

Materials and methods

Rearing

Aposymbiotic juveniles were obtained as described in a previous paper (Gros et al., 1997). After the beginning of the second phase of metamorphosis character-

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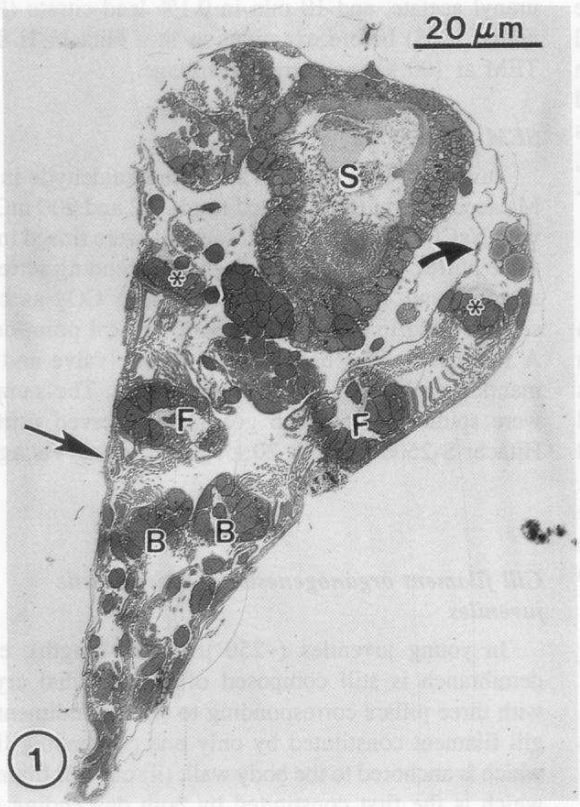
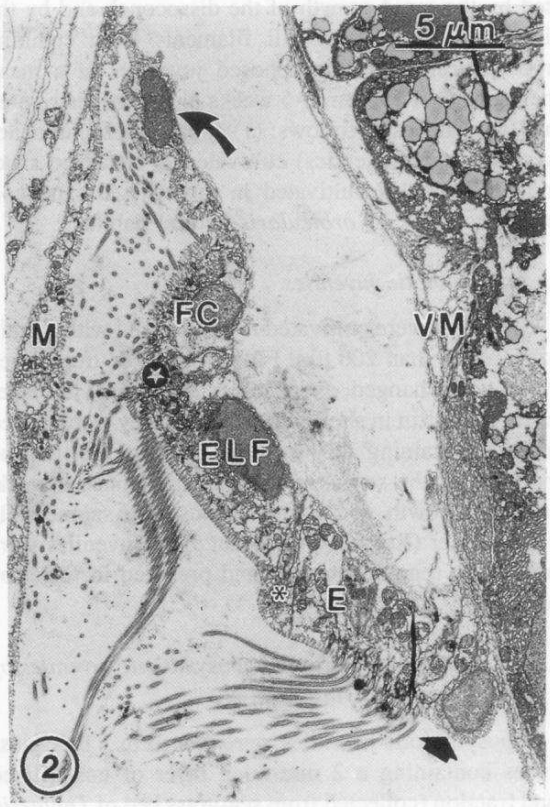
Figs. 1–3. *Codakia orbicularis*, early gills in 250 µm shell-length aposymbiotic juveniles, (transmission electron microscopy).

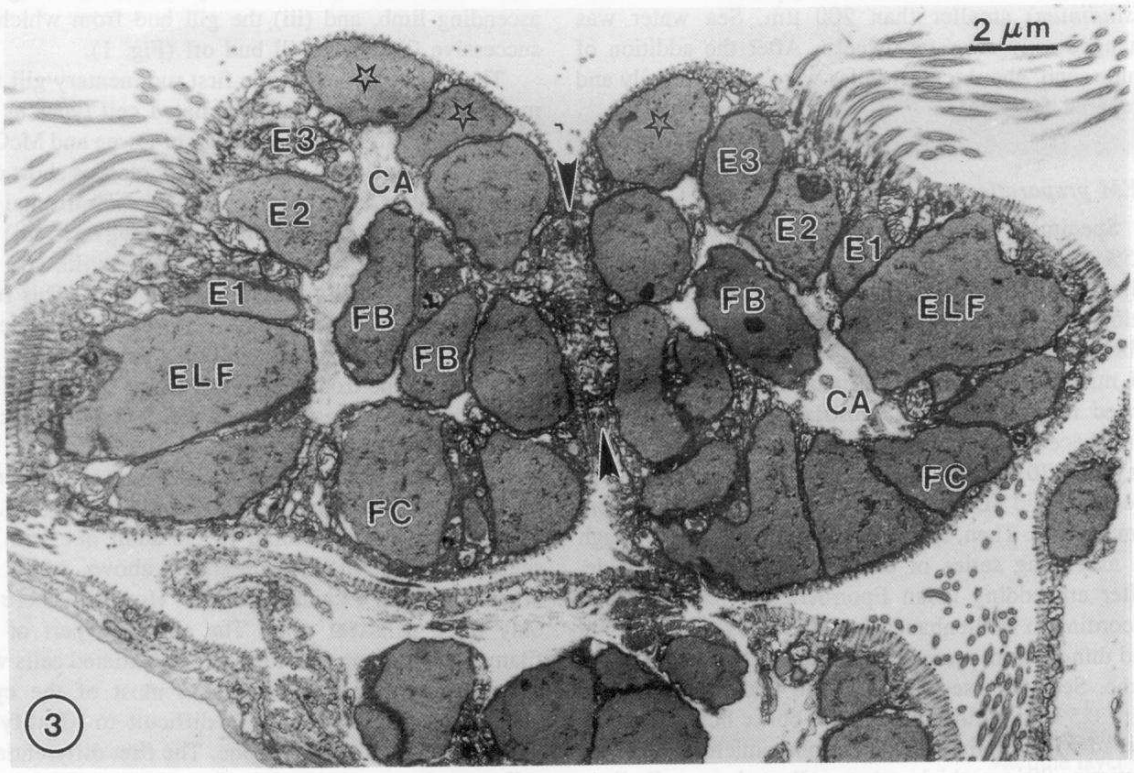
Fig. 1. Transverse section of an aposymbiotic juvenile. Each demibranch is still in the ctenidial crypt stage characterized by three pillars: a rudimentary gill filament (asterisk) anchored to the body wall (curved arrow), a gill filament (F) constituted by both descending and ascending limb, and a gill bud (B) in the posteroventral position. The mantle is indicated by a straight arrow. S, stomach.

Fig. 2. The rudimentary gill filament is composed of a unique lamina made up of five different cell types: a frontal cell (FC) bearing short cilia, an unciliated prolaterofrontal cell (star), an eulaterofrontal cell (ELF) with two rows of long cilia, a prolateral cell (asterisk), and eulateral cells (E) with long single cilia. This rudimentary gill filament is anchored to (i) the mantle (M) in its frontal part by a non-ciliated cell (curved arrow) and to (ii) the visceral mass (VM) by an undifferentiated cell (straight arrow) in contact with the last eulateral cell.

Fig. 3. Gill buds are short and asymmetrically differentiated around a connective axis (CA) containing few fibroblaste-like cells (FB). Their sagittal faces are in close contact through cilia and microvilli (arrow heads). Their differentiated face is composed of frontal cells (FC), eulaterofrontal cells (ELF) with two rows of long cirri, and eulateral cells (E1–E3). The abfrontal part of these gill buds contains only few undifferentiated cells (stars).


20 µm
S
F
F
B
B
1


5 µm
M
FC
VM
ELF
E
2


2 µm
E3
CA
E2
E1
FB
FB
ELF
FC
E3
E2
E1
FB
ELF
CA
FC
3

ized by the rapid growth of the dissoconch and by the differentiation of new gill filaments from ctenidial crypts, recently metamorphosed juveniles at a mean shell-length of 250 µm (~5 weeks old) were distributed into two batches as follows: (1) aposymbiotic juveniles (symbiont-free juveniles) cultivated in sterilized sand, and (2) juveniles cultivated in unsterilized sand collected from the *C. orbicularis* natural habitat.

1. *Aposymbiotic juveniles*

Juveniles were cultivated in sterile sand with a grain size smaller than 200 µm. Filtered and UV-treated sea water was changed every other day until juveniles reached 500 µm in shell-length. Then, they were placed in trays containing a 10 µm-thick layer of sterile sand and 10% of the sea water was changed once a week. Bacterial growth was limited by using a submersible UV sterilizer (Rena UV Compact 9W). Juveniles were sampled at regular intervals and prepared for electron microscopy.

2. *Experimental infection of aposymbiotic juveniles by symbiotic free-living form*

Aposymbiotic juveniles were placed in 1 liter-glass dishes containing a 2 mm-thick layer of unsterilized sand fraction (collected from sea-grass bed of *Thalassia testudinum*) smaller than 200 µm. Sea water was changed only after two weeks. After the addition of crude sand, about ten juveniles were sampled daily and prepared for TEM.

TEM preparation

Specimens were prefixed for one hour at 4°C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 adjusted to 900 mOsM with 0.4M NaCl; 2 mM $CaCl_2$ being added for a better membrane preservation. After a brief rinse, they were fixed at room temperature for 45 min in 1% osmium tetroxide in the same buffer, rinsed in distilled water, postfixed with 2% aqueous uranyl acetate for one additional hour (Silva et al., 1971; Hayat, 1970) and decalcified overnight in 1% aqueous ascorbic acid (Dietrich and Fontaine, 1975) at room temperature. Then, specimens were dehydrated through an ascending series of ethanol and propylene oxide. After embedding in an Epon-Araldite resin mixture, according to Mollenhauer (in Glauert, 1975), semi-thin and thin sections were cut on a Leica Ultracut E microtome. Semi-thin sections, 0.5 µm thick, were stained with 0,5% toluidine blue in 1% borax buffer and observed with a Leica Orthoplan photomicroscope. Thin sections, 80 nm thick, were collected on collodion-coated 100 mesh grids, contrasted 30 min in 2% aqueous uranyl acetate, and 10 min in 0.1% lead citrate (Reynolds, 1963) before examination in a Hitachi H-8000 TEM at 100 kV accelerating voltage.

SEM preparation

Juveniles were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer adjusted to pH 7.2 and 900 mOsM with NaCl. After fixation, all samples were rinsed in the same buffer, dehydrated through an ascending series of acetone, and critical point dried using CO_2 as transitional fluid in a Bio Rad E 3000 critical point drier. A fine needle was used to remove one valve and the mantle to allow the observation of gills. The samples were sputter coated with gold and observed using a Hitachi S-2500 SEM at 20 kV accelerating voltage.

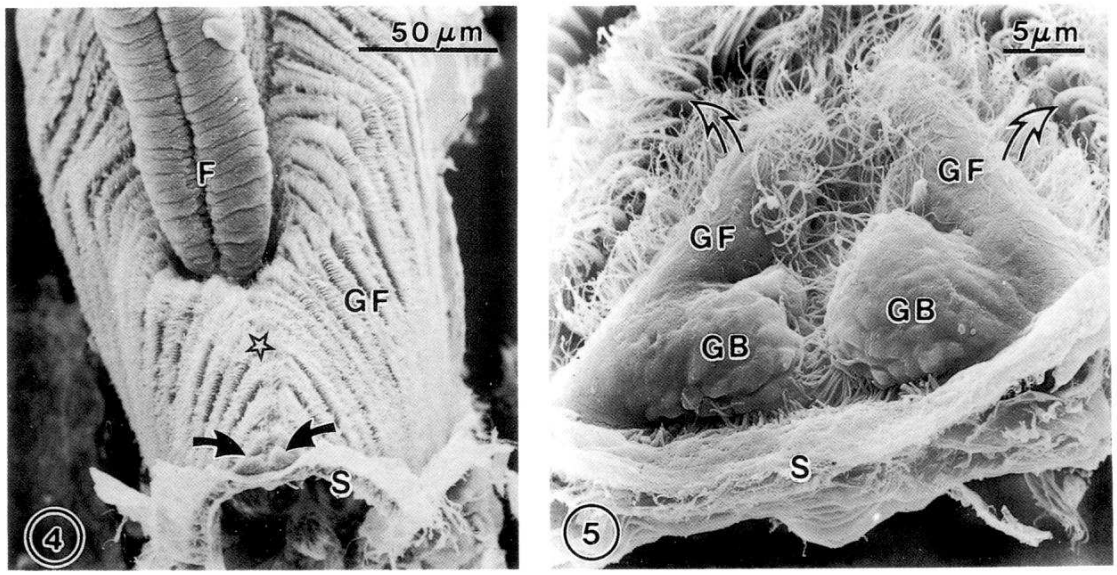
Results

Gill filament organogenesis in aposymbiotic juveniles

In young juveniles (~250 µm shell-length), each demibranch is still composed of the ctenidial crypts with three pillars corresponding to (i) one rudimentary gill filament constituted by only one descending limb which is anchored to the body wall, (ii) one gill filament which is the first constituted by both descending and ascending limb, and (iii) the gill bud from which all successive filaments will bud off (Fig. 1).

The unique lamina of the first rudimentary gill filament is constituted by differentiated cell types as described following the terminology of Owen and McCrae (1976): (i) one frontal cell bearing numerous short cilia without precise orientation, (ii) one prolaterofrontal cell with fused cilia featuring two rows of long cirri, (iii) one unciliated prolateral cell with long microvilli, and (iv) two or three ciliated eulateral cells (Fig. 2). This rudimentary gill filament, which remains incomplete, is attached (i) to the mantle through an unciliated cell bordering on the frontal cell, and (ii) to the wall of the visceral mass through an undifferentiated cell contiguous to the last eulateral cell.

The second gill filament is composed of a differentiated ciliated zone and a short abfrontal zone (Fig. 6). The ciliated zone is similar to the adult one with the same four cell types, as described above, which are organized along a connective axis. However, there are only three eulateral cells. The abfrontal part of the filament is made up of 3 to 4 undifferentiated cells with a voluminous nucleus occupying most of the cytoplasmic volume so that it is difficult to identify an intermediary and a lateral zone. The first differentiated cell type characteristic of the lateral zone of adult gill filaments is a mucocyte.



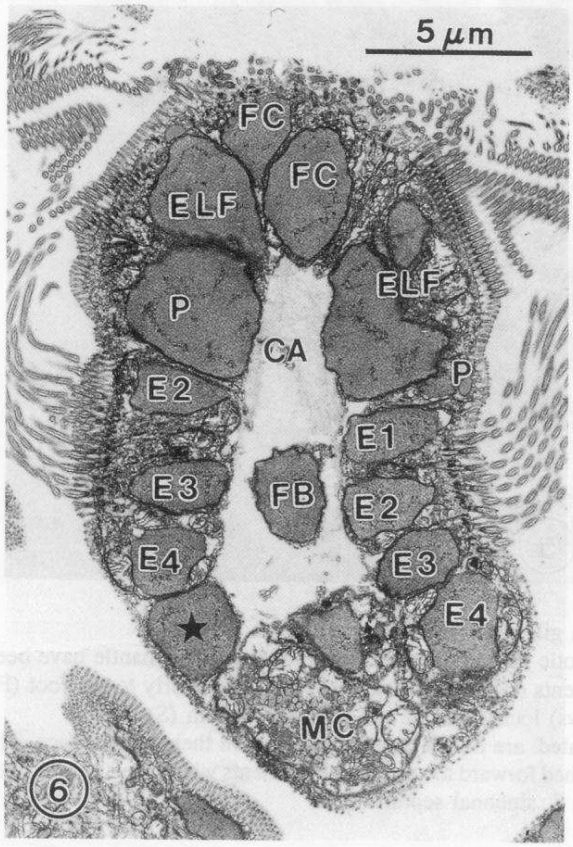
Figs. 4–5. *Codakia orbicularis*, gill-filament formation from gill buds (scanning electron microscopy).
Fig. 4. Posteroventral view of a 2 mm-shell length aposymbiotic juvenile in which one valve and the mantle have been removed. Each demibranch is composed of several gill filaments (GF) linked together (star) posteriorly to the foot (F). New gill filaments bud off from the gill buds (curved arrows) located above the siphonal septum (S).
Fig. 5. Gill buds (GB), the ventral surface of which is unciliated, are linked together by cilia on their sagittal face. The new gill filaments (GF), budding off from the gill buds, are pushed forward towards older filaments which are characterized by the long cirri (curved arrow) of the eulaterofrontal cells. S, siphonal septum.

Gill buds are short and organized around a connective axis containing fibroblaste-like cells. Moreover, they are asymmetrically differentiated and are in contact with each other on their undifferentiated face through cilia and microvilli (Fig. 3). On the other face, four cell types can be identified: (i) frontal cells, (ii) prolaterofrontal cells, (iii) prolateral cells, and (iv) eulateral cells as described in the first rudimentary gill filament. The abfrontal zone is restrained to two undifferentiated cells (Fig. 3). All the cells constituting the gill bud have a voluminous nucleus and large mitochondria mostly located at the periphery of the cell or near the apical pole because of the nucleus volume. These gill buds remain functional in juveniles of several millimeter long which possess numerous gill filaments (Figs. 4,5).

During the post-larval development, new gill filaments bud off from the gill buds increase in number, get longer, and continue their differentiation. The abfrontal part of the gill filament lengthens but the intermediary zone is still not distinct from the lateral zone (Fig. 7). Two new cell-types, characteristic of the lateral zone of adult gill filaments may be identified in 350 µm shell-length juveniles; (i) a "granule cell" characterized by a basal nucleus, few large membrane-bound osmiophilic inclusions, and large dilated granular endoplasmic reticulum profiles, and (ii) an intercalary cell with a narrow base, a nucleus in apical position, scarce organelles mostly mitochondria, and an enlarged apical area covered with microvilli.

From 400 µm to 2 mm shell-length juveniles, the ciliated zone appears similar for each gill filament observed (Fig. 8). The last eulateral cell represents the intermediary zone whereas the lateral zone, whose length varies according to the juvenile-size, is composed of four cell types: (i) mucocytes which are identified at various levels, (ii) granule cells whose osmiophilic inclusion size and density seem to depend on their maturation, (iii) intercalary cells which encroach upon the apical area of adjacent cells, and (iv) undifferentiated more or less cubic cells (Fig. 9) which are characterized by a basal nucleus, an apical pole with short microvilli, and a cytoplasmic volume containing scarce organelles as mitochondria.

In juveniles cultivated in sterile sand, no bacteria are found in the gill filaments. No views of a putative cryptic form of gill-endosymbionts are observed either in gill filaments or in other larval tissues. Addition of either 1 µM sulfide or a sulfide crystal buried at regular


5 µm
FC
FC
ELF
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CA
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E2
E1
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FB
E2
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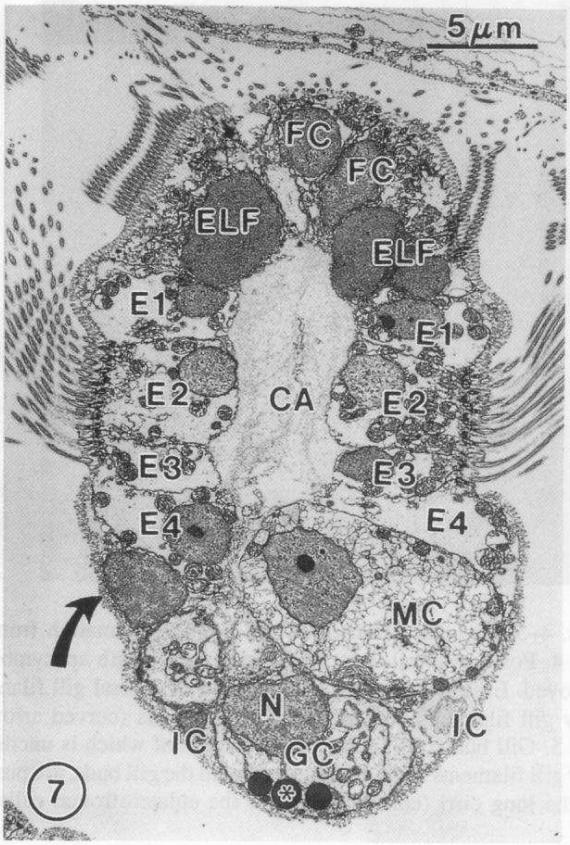
E3
E4
MC
6


5 µm
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FC
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E3
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IC
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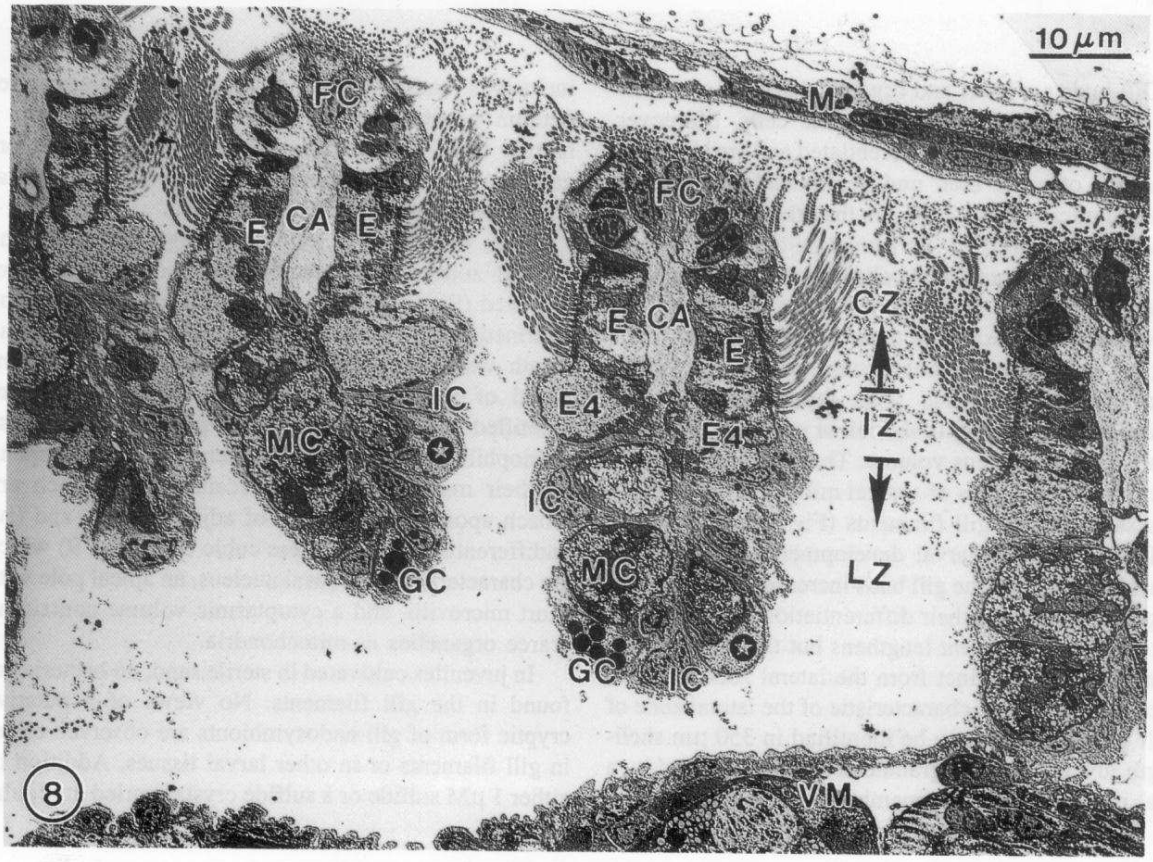
7


10 µm
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E4
E4
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MC
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GC
MC
GC
IC
8
VM

intervals in sterile sand did not permit *C. orbicularis* to acquire symbionts. In 1.5 to 2 mm shell-length aposymbiotic juveniles, gill filaments remain short with a truncated lateral zone devoid of bacteriocytes whereas ciliated and intermediary zones are very similar to those observed in sub-adult specimens collected in sea-grass beds (Fig. 8).

Experimental infection of gill filaments with environmental bacteria

Within 48 hours after exposure to unsterilized sea-grass sand, few symbiosis-competent bacteria enter into the undifferentiated cells located in the lateral zone of aposymbiotic gill filaments (Fig. 10). The symbiotic free-living bacteria appear to enter by endocytosis at the apical pole of the undifferentiated cells (Fig. 11) which progressively differentiate into bacteriocytes. Some plasmic membrane invaginations individually enclose bacteria into endocytic vacuoles. Progressively, the number of endocytosed bacteria increases with a characteristic distribution in the cell. At first step, endosymbionts are mainly distributed at the periphery of the young bacteriocyte and are salient in the microvilli border of the cell whereas the basal part of the bacteriocyte remains free of symbiotic bacteria (Fig. 12). Organelles are scarce, limited to a few mitochondria close to the nucleus. At the same time, no bacteria are detected either in other tissues or in hemocytes which are in close contact to the basal membrane of the bacteriocytes inside the blood lacuna.

Several weeks after exposure with unsterilized sand, the bacteriocyte is almost completely occupied by individually enclosed bacteria (Fig. 13). Bacteria seem to enter at the apical pole of the undifferentiated cell and progress to the basal pole until they fill the cell. At the same time, gill filaments get longer because of the increasing number of the four cell types constituting the lateral zone. In 600 µm shell-length juveniles, gill filaments possess a lateral zone with 3–4 bacteriocytes and some granule cells. In 2 mm shell-length symbiotic juveniles, the lateral zone comprises at least 10 bacteriocytes (Fig. 15); however, no mitotic views of these cells have been detected on the various sections observed. The gill filament structure of juveniles cultivated with unsterilized sand in laboratory (Fig. 13) appears similar to that of gill filaments of 2.5 mm shell length wild juveniles collected in sea grass bed (Fig. 14). As long as the cytoplasmic volume of bacteriocytes is not crowded with bacterial endosymbionts, no partly destroyed bacteria associated with secondary lysosomes were found. Likewise, no bacterial division was found in bacteriocytes conversely to bacteriocytes of adult gill filaments where bacterial dividing-figures are quite common.

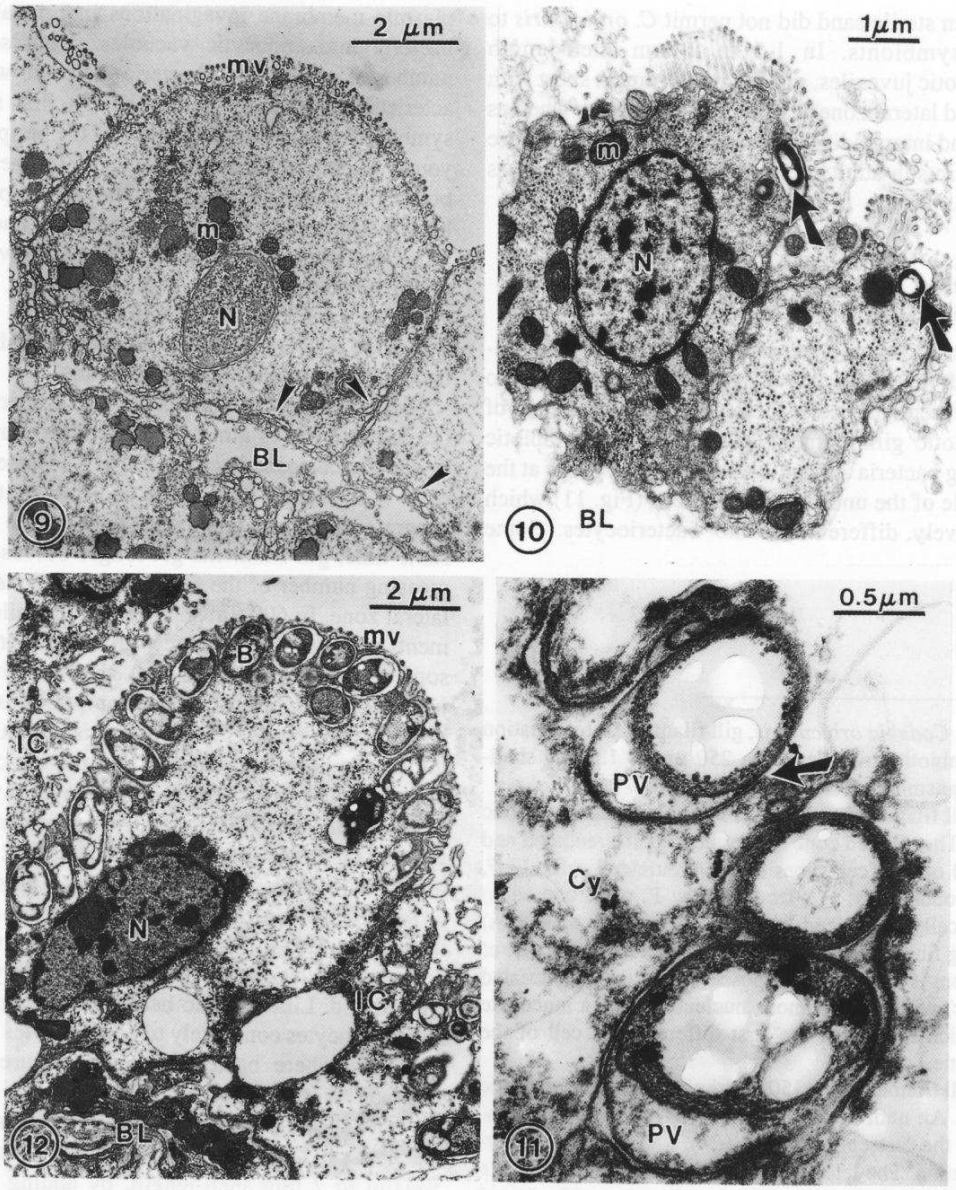
We successfully established symbiosis in several independent batches of aposymbiotic juveniles, from 300 µm to 2 mm shell-length, by adding unsterilized sand into the culture. Undifferentiated cells of aposymbiotic gill filaments remain receptive to symbiosis-competent bacteria several months after metamorphosis, and differentiate as bacteriocytes. In *C. orbicularis*, the environmental transmission of symbionts does not appear to be restrained to a defined period during post-larval development. Moreover, in each case, all the gill filaments were colonized by symbiosis-competent bacteria except the first rudimentary filament. One year after fertilization, laboratory symbiotic juveniles cultivated in sand collected from the *C. orbicularis* habitat reach 4 to 4.5 mm in shell-length whereas aposymbiotic juveniles, issued from the same spawning but cultivated in sterile sand, reach 2 to 2.5 mm in shell-length.

Figs. 6–8. *Codakia orbicularis*, gill filament differentiation in aposymbiotic juveniles from 250 µm to 1.5 mm shell-length (transmission electron microscopy).

Fig. 6. Gill filament in a 250 µm shell-length aposymbiotic juvenile. The ciliated zone is completely differentiated and composed of four cell types (E1–E4, eulateral cells; ELF, eulaterofrontal cells; FC, frontal cells; P, prolateral cells) symmetrically organized along a connective axis (CA) containing fibroblaste-like cells (FB). The abfrontal part of the filament is short and composed of few undifferentiated cells (star) with a voluminous nucleus, and of a mucocyte (MC) which represents the first differentiated cell of the lateral zone.

Fig. 7. Gill filaments in a 350 µm shell-length aposymbiotic juveniles. An undifferentiated cell (curved arrow), in contact with the last eulateral cell (E4), prefigures the intermediary zone. The lateral zone is composed of a mucocyte (MC), a granule cell (GC) with a basal nucleus (N), dilated granular endoplasmic profiles, and few osmiophilic inclusions (asterisk). Intercalary cells (IC), characterized by a narrow base and an apical nucleus, are also identified. CA, collagen axis; E1–E4, eulateral cells; ELF, eulaterofrontal cells; FC, frontal cells.

Fig. 8. Gill filament in a 1.5 mm shell-length aposymbiotic juvenile. Frontal cells (FC), eulaterofrontal cells (ELF), and eulateral cells (E) are the main components of the ciliated zone (CZ), which is organized around a collagen axis (CA). The last eulateral cell (E4), which is partially ciliated, represents the intermediary zone (IZ). Granule cells (GC), intercalary cells (IC), mucous cells (MC), and undifferentiated cells which represent putative bacteriocytes without bacteria (star) are the main components of the lateral zone (LZ). M, mantle; VM, visceral mass.



Figs. 9–12. *Codakia orbicularis*, experimental infection of aposymbiotic juveniles from symbiosis-competent bacteria associated with unsterilized sand, (transmission electron microscopy).

Fig. 9. Putative bacteriocyte in the lateral zone of the gill filament in a 600 µm shell-length aposymbiotic juvenile. These undifferentiated cells have a rounded apical pole with microvilli (mv) linked by a thin glycocalyx, a basal nucleus (N) surrounded by mitochondria (m). Endoplasmic reticulum profiles (arrow heads) are frequent along the lateral and basal cell-membranes of putative bacteriocytes. BL, blood lacuna.

Fig. 10. Lateral zone of the gill-filament in a 400 µm shell-length juvenile 48 hours after contact with unsterilized sand from sea-grass bed. One symbiosis-competent bacterium (arrow) is located at the apical pole of undifferentiated cells which progressively evolve to bacteriocytes. No bacteria are identified at the basal pole of the cell which is in contact with the blood lacuna (BL). m, mitochondria; N, nucleus.

Fig. 11. Endocytosis of symbiosis-competent bacteria at the apical pole of an undifferentiated cell of the gill in a 800 µm shell-length aposymbiotic juvenile placed in crude sand since 48 hours. Gram negative bacteria, with a large periplasmic vesicle (PV), are endocytosed in bacterial vacuoles (arrow) elaborated by the host-cell. Cy, cytoplasm.

Fig. 12. Endosymbiotic bacteria located at the periphery of a bacteriocyte in a 600 µm juvenile cultivated in unsterilized sand since few weeks. B, bacteria; BL, blood lacuna; IC, intercalary cells; mv, microvilli; N, nucleus.

Evolution of the fine structure of bacterial symbionts

The sulfur-oxidizing endosymbionts, which have the typical double-membrane of gram-negative bacteria, are individually enclosed. The nucleoid is identified as a well-defined network. At the beginning of infection, endosymbionts are mostly coccoïd and measure no more than 1 µm (Fig. 12). In old bacteriocytes which are characterized by a cytoplasmic volume crowded by bacterial endosymbionts, only the symbionts located near the apical pole, so recently endocytosed, possess similar size and shape. Conversely, the symbionts located near the basal pole are rod-shaped and measure up to 3.5 mm in 2 mm shell-length juveniles under laboratory conditions (Fig. 13) versus up to 5 mm in 3 mm shell-length wild juveniles and adult specimens (Fig. 14).

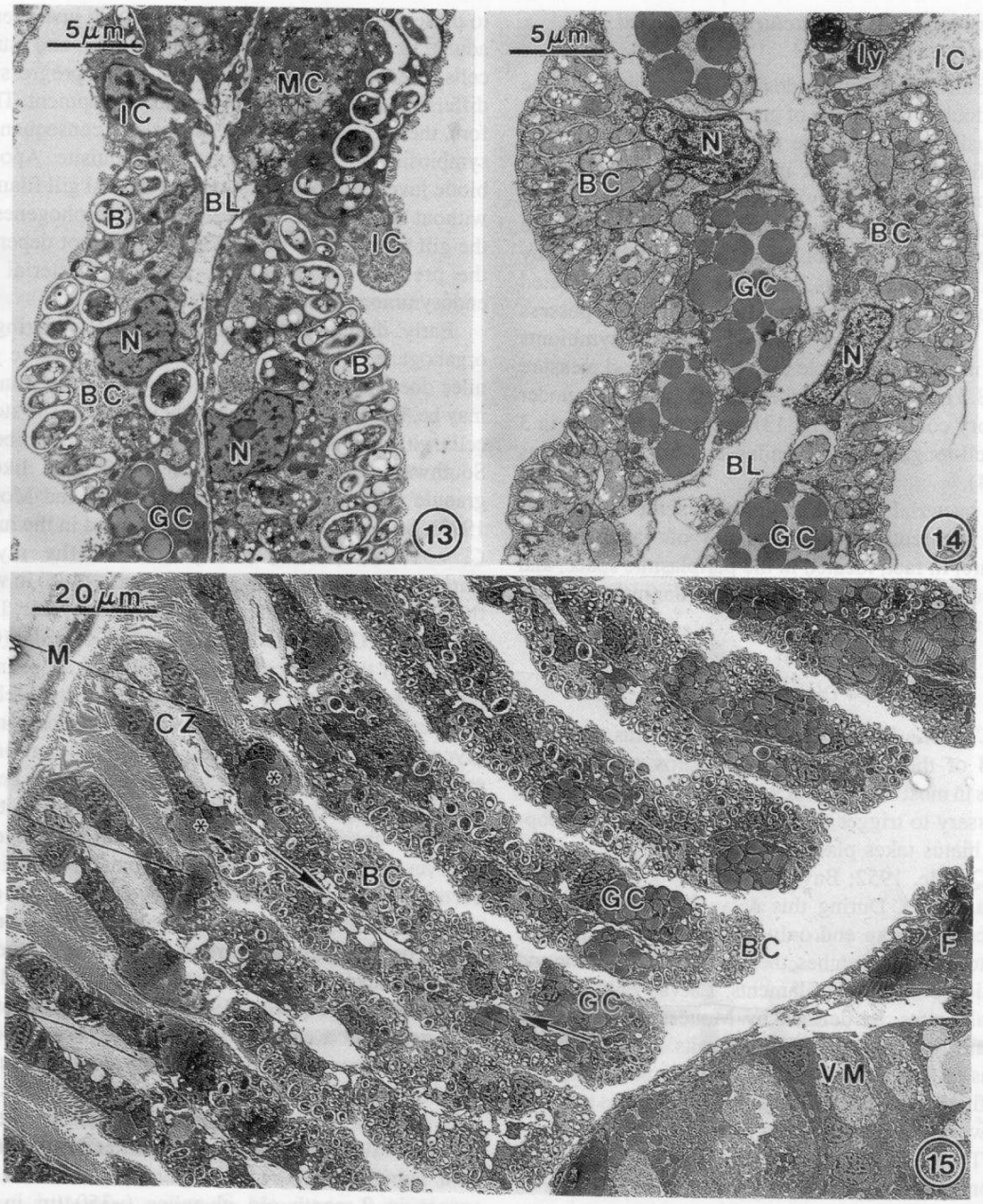
The bacterial cytoplasm contains some membrane-bound, apparently empty, vesicles of variable sizes which are, in fact, located in the periplasmic space, and numerous non-membrane-bound irregular inclusions (25 to 50 nm in diameter).

Discussion

In *C. orbicularis*, a developmental hiatus occurs at the end of the plantigrade stage (Gros et al., 1997) whereas in most bivalves for which an environmental cue is necessary to trigger metamorphosis, such a developmental hiatus takes place at the end of the pediveliger stage (Quayle, 1952; Bayne, 1965, 1976; Frenkiel and Mouëza, 1979). During this delay of metamorphosis, which comes to an end only by addition of a suitable substrate in culture batches, there is no multiplication and elongation of new gill filaments. Larvae only possess ctenidial crypts, as defined by Mouëza et al. (1998), which are not functional gill filaments. Even if sand is added as early as the pediveliger stage, the differentiation of gill filaments does not begin in plantigrades younger than 5 weeks measuring about 250 µm long (Gros et al., 1997). Therefore, the morphogenesis of gill filaments is not a continuous process during the larval and post-larval development as it is in venerid (for review, see Mouëza et al., 1998).

During the second phase of metamorphosis, new gill filaments bud off rapidly from the posterior gill buds. From 250 µm to 500 µm shell-length juveniles, several specific cell types differentiate during gill filament morphogenesis. In aposymbiotic juveniles, the ciliated and intermediary zones of gill filaments appear identical to those described in wild juveniles (Frenkiel and Mouëza, 1995) unlike the lateral zone which remains short, without differentiated bacteriocytes. Nevertheless, this lateral zone is occupied by cell types similar to those observed in gill filaments of wild juveniles and adults, i.e., granule cells, intercalary cells, and mucous cells (Frenkiel and Mouëza, 1995) which progressively differentiate during the post-larval development. Therefore, these cell types do not appear as a consequence of symbiosis, but more likely as a prerequisite. Aposymbiotic juveniles possess several functional gill filaments without bacteriocytes, indicating that morphogenesis of the gill filaments in *C. orbicularis* does not depend on the presence of the sulfur-oxidizing bacterial gill-endosymbionts.

Early differentiation of granule cells during the organogenesis of gill filaments in aposymbiotic juveniles does not allow to define what their real function may be, but allows to say what they are not. The storage cells with osmiophilic proteinic inclusions described by Southward in *Myrtea spinifera* (1986) look like the granule cells of *C. orbicularis* (Frenkiel and Mouëza, 1995). Such cells have been only reported in the lucinid *C. costata*, (Giere, 1985), and in the mytilid *Bathymodiolus sp.* (Fiala-Medioni et al., 1986) in which they have been considered as mucocytes. In *C. orbiculata, C. pectinella,* and *Lucina nassula* (Gros, pers. obs.) they are composed mostly of a cystin-rich protein as well as in *C. orbicularis* (Frenkiel and Mouëza, 1995) definitly different from mucous proteoglycans. The other bivalves known to harbor gill endosymbionts do not appear to possess these granule cells. Herry and Le Pennec (1987) proposed that they could be "modified bacteriocytes" with high levels of reserves originating from the lysosomal destruction of bacteria. This hypothesis can be ruled out because of the appearance of such cells in aposymbiotic juveniles which do not possess symbiotic bacteria. Moreover, data from juvenile gill filaments confirm the conclusion of Frenkiel and Mouëza (1995) indicating that granule cells represent a cell type distinct from bacteriocytes. Because of their appearance in aposymbiotic juveniles, any involvement due to the presence of symbiotic bacteria in the gill should be ruled out. These specific cells could be involved either in a detoxification process or in a nutritional pathway in juveniles. Granule cells appear in 2-month old juveniles (~350 µm in shell length) which are settled since 3 to 4 weeks and consequently are in contact with the sediment. However, before the second phase of metamorphosis, plantigrades do not burrow (Gros et al., 1997), so they are not in contact with the oxic-anoxic zone rich in sulfide compounds that are toxic to larvae (Jørgensen, 1982). So, they probably do not need to be protected against these compounds whereas early juveniles may begin to store sulfur integrated in the cystin-rich protein which is the major component of these granules (Frenkiel and Mouëza, 1995).



Figs. 13–15. *Codakia orbicularis*, gill filaments of reared and wild symbiotic juveniles (transmission electron microscopy).
Fig. 13. Lateral zone of a gill filament belonging to a 1 mm shell-length symbiotic juvenile cultivated in unsterilized sand since several weeks. The cytoplasmic volume of each bacteriocyte (BC) is now crowded by several individually enclosed symbiotic bacteria (B). BL, blood lacuna; GC, granule cells; IC, intercalary cells; MC, mucocytes; N, nucleus.
Fig. 14. Lateral zone of a gill filament of a 3 mm shell-length wild juvenile. The cytoplasmic volume of bacteriocytes (BC) is crowded with endosymbiotic bacteria. BL, blood lacuna; GC, granule cells; IC, intercalary cells; ly, lysosomes; N, nucleus.
Fig. 15. Transverse section of a demibranch from a 2.5 mm shell-length symbiotic juvenile cultivated in unsterilized sand since several weeks. Gill filament ultrastructure is similar to that observed in gills of wild juveniles. The ciliated zone (CZ) is limited by a large clear cell (asterisk) which corresponds to the last eulateral cell. The lateral zone is composed of granule cells (GC) and bacteriocytes (BC) characterized by their endocytosed bacteria. The lateral zone axis is shown by arrows. F, foot; M, mantle; VM, visceral mass.

Some informations about gill filament morphogenesis in other bivalve species in which similar granule cells were observed (Giere, 1985; Southward, 1986; Fiala-Médioni et al., 1986) could be very interesting. Unfortunately, only species harboring environmentally transmitted bacterial symbionts give the opportunity to study organogenesis independently of symbiosis.

Experimental infection of aposymbiotic juveniles with environmental bacteria

We successfully established bacterial symbiosis in a dozen of independent batches of *C. orbicularis* from 1995 to 1997. In all cases, aposymbiotic juveniles acquired gill symbionts and differentiated gill filaments in which the lateral zone contained several bacteriocytes similar to those observed in wild juveniles (Frenkiel and Mouëza, 1995). To date, we do not know how symbionts, which enter by endocytosis in the undifferentiated cells of the gill, move from the apical pole to the basal pole. They may proceed by cell division toward the basal pole of the cell or newly endocytosed bacteria may push the first envacuolated bacteria toward the basal pole while the bacteriocytes enlarge to attain their adult size. From that on, the prokaryotic population inside the bacteriocyte is undoubtedly controlled by the host, but the mechanisms involved in this process are still unclear.

According to Fiala-Médioni et al. (1986) the presence of numerous lysosomes beneath the bacterial zone in symbiotic bivalves suggests a possible lysosomic resorption of the bacterial gill endosymbionts representing either a potential way of organic material transfer from bacteria to host. This lysosomic resorption also could be a means of preventing excessive proliferation of bacterial endosymbionts in the bacteriocyte (Le Pennec et al., 1988). Both hypotheses are supported by observations of large lysosomes within the bacteriocytes of several symbiotic bivalve species studied to date (Southward, 1986; Distel and Felbeck, 1987; Le Pennec et al., 1988; Fiala-Médioni et al., 1989; Herry et al., 1989; Fiala-Médioni et al., 1994; Frenkiel and Mouëza, 1995; Frenkiel et al., 1996; Gros et al., 1996a). In *C. orbicularis*, large secondary lysosomes appear in bacteriocytes in which most of the cytoplasmic volume is occupied by bacteria, in juveniles in which symbiosis is established since several weeks. These data suggest that lysosomes could be involved, directly or not, in mechanisms regulating the bacterial population inside the bacteriocyte by digestion of old symbionts rather than in an unceasing digestion of symbionts. New investigations are necessary to understand the turn over of the symbionts as well as the cellular recognition processes necessary for the selection of the symbiosis-competent bacterial species.

Symbiotic juveniles cultivated in unsterilized sand collected from the *C. orbicularis* habitat are twice as large as aposymbiotic juveniles obtained from the same spawning and reared under the same conditions of salinity, water temperature, feeding, etc... a few months after the establishment of symbiosis. This size difference is likely to be related to the trophic contribution from endosymbiotic bacteria. Therefore, symbiosis appears as beneficial to juveniles unless it is not necessary for metamorphosis (Gros et al., 1997). Berg and Alatalo (1984) demonstrated, through studies of carbon isotope ratios and enzyme activities, that a more likely source of nutrition for adult *C. orbicularis* is endosymbionts and suggested that the simplified gut structure present in adult lucinids is most likely a result of reduced function. Aposymbiotic larvae obtain all their nutrition from ingestion of phytoplankton and possess a functional digestive tract, whereas symbiotic juveniles may rely on a combination of this classical nutritional pathway in bivalves and of chemoautotrophic bacteria.

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