Symbiosis, 29 (2000) 293–317
Balaban, Philadelphia/Rehovot

Sulfur-Oxidizing Endosymbiosis in *Divaricella quadrisulcata* (Bivalvia : Lucinidae): Morphological, Ultrastructural, and Phylogenetic Analysis

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Received September 24, 2000; Accepted December 5, 2000

Abstract

Based on light and electron microscopy, the cellular organization of the gill filament of *Divaricella quadrisulcata* is described and compared with other gill filaments of lucinids examined to date. TEM observations revealed a dense population of Gram-negative bacteria located within bacteriocytes in the lateral zone of the gill filament which looks similar to that of *Codakia orbicularis* with typical "granule cells". The digestive tract of this shallow-water lucinid species is less modified than in other lucinid species. The stomach has a well developed gastric shield, a cristalline style protruding in the stomach from a typical style sac, and active digestive diverticula. The mid gut is coiled through the visceral mass. Therefore, *D. quadrisulcata* appears to be at least partially dependent on filter-feeding for nutrition. Only one type of bacterial 16S rRNA gene was PCR-amplified from symbiont-containing gill tissue of two specimens, indicating a symbiont population composed of a single species. Phylogenetic analysis showed that sequences of *D. quadrisulcata*- and *C. orbicularis*-symbiont were 100% identical at all nucleotide positions determined, suggesting that this other tropical lucinid species harbors the same bacterial symbiont species as the previously analyzed *C. orbicularis*. Thus, *D. quadrisulcata* appears as the fifth tropical bivalve

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0334-5114/2000/$05.50

colonized by the same symbiont species even though it lives in a different habitat as the four other ones. The symbiont transmission mode was investigated by PCR amplifications from mature ovaries and testes. The *C. orbicularis*–specific 16S rDNA primer set was unsuccesful in amplifying DNA target for all individuals investigated suggesting that the gill-endosymbionts are environmentally transmitted to the new host generation as for all lucinids examined to date.

Keywords: Symbiosis, Lucinidae, gill ultrastructure, phylogeny, 16S rRNA gene

1. Introduction

Chemoautotrophic bacteria have been known as symbionts in marine invertebrates for years. Since the discovery of sulfur-oxidizing bacteria housed in the trophosome of the hydrothermal vestimentifera *Riftia pachyptila* (Cavanaugh et al., 1981; Felbeck et al., 1981), thioautotrophic endosymbionts have been described in several invertebrate species living in various habitats (from highly reducing mud and hydrothermal vents to low sulphide sediments in shallow-water sea-grass beds). Up to date, species harboring chemoautotrophic symbionts have been described from Annelida, Mollusca, Nematoda, Pogonophora, and Vestimentifera (Fisher, 1990; Felbeck and Distel, 1992; Cavanaugh, 1994). However, the greatest diversity of this symbiotic association is found among Bivalvia (Fisher, 1990; Reid, 1990; Distel et al., 1994) especially in the family Lucinidae in which all the species examined to date harbor gill endosymbionts (Gros, 1997).

With the goal to have a better understanding of the relationships between tropical lucinids and chemoautotrophic bacteria, a new tropical lucinid species was analyzed, belonging to the subfamily Divaricellinae, which inhabits slightly reduced sediment nearby *Thalassia testudinum* beds known to harbor several lucinid species such as *C. orbicularis*.

The cellular organization of gill filaments of this new examined lucinid was studied regarding the relationships between intercalary cells and bacteriocytes, the number of cellular types in the lateral zone, and were compared with those of other tropical lucinid gill filaments already described (Giere, 1985; Distel and Felbeck, 1987; Frenkiel and Mouëza, 1995; Frenkiel et al., 1996; Gros et al., 1996a). We have also studied the digestive tract (gut, stomach, and digestive diverticula) of this lucinid bivalve from histological sections in order to put forward some hypotheses on its digestive ability. In addition, we have identified and characterized the bacterial endosymbiont of *D. quadrisulcata* by using 16S rDNA (DNA encoding rRNA) sequencing, and examined the phylogenetic relationships of this symbiont with the sulfur-oxidizing chemoautotrophic bacterial endosymbionts known yet.

2. Materials and Methods

Organisms collection

Specimens of *Divaricella quadrisulcata* (Orbigny, 1842), identified according to Abbott (1974) and Warmke and Abbott (1962), were collected in Guadeloupe from low-sulphide sediment nearby sea-grass beds (*Thalassia testudinum* environment). Adult individuals (up to 13 mm in shell-length) were kept alive in 5 µm-filtered sea water until fixation and DNA extraction, which were performed within 48 hours.

Histology and histochemistry

An overall view and some histochemical information were obtained from paraffin sections. Whole animals were fixed in Bouin's fluid prepared in sea water and embedded in paraplast after standard dehydration. Sections (7 µm thick) were stained by various histological techniques performed according to Gabe (1968) and Martoja and Martoja (1967). We used Goldner's trichrome for morphological observation, Alcian blue staining (pH 3) to discriminate the type of mucosubstances and periodic acid Schiff (PAS) reaction to reveal glycoconjugates. The tetrazoreaction according to Dianelli (in Gabe 1968) was used to ascertain the presence of proteinic inclusions. The DDD (2,2'-Dihydroxy-6,6'-Dinaphtyl Disulfide) reaction according to Barrnet and Seligman (in Martoja and Martoja, 1967) was used to detect sulfhydryl groups in proteins; disulfur bonds were detected after reduction of the S-S groups by a 5% aqueous ammonium sulfide before the DDD reaction.

Enzymological analysis

Twenty to 25 mg of digestive gland were dissected, taking care to not cut the stomach, from adult specimens of *D. quadrisulcata* and from two classical filter-feeding bivalves such as the venerids *Anomalocardia brasilliana* and *Chione cancellata*. Digestive gland samples were crushed in 2.5 ml of sterile distilled water in order to check for the presence of 18 enzyme activities by using the semi-quantitative micromethod kit API ZYM (Biomérieux, France). Each cupule of the API ZYM strip was inoculated with 65 microliters of the diluted specimens. After inoculation, each strip was incubated at 37°C for 4 hours before revelation according to the manufacturer instructions.

Scanning Electron Microscopy (SEM) preparation

Both intact and delaminated gill tissues were fixed in 2.5% glutaraldehyde

in sea water for 24 hours at room temperature. After a rinse in 0.1 M pH 7.2 cacodylate buffer adjusted to 900 mOsM by NaCl, gill pieces were dehydrated in a graded acetone series and critical point dried using CO_2 as transitional fluid. Then, they were sputter coated with gold before observation in a SEM Hitachi S-2500 at 20 kV.

Transmission Electron Microscopy (TEM) preparation

Small pieces of gills were fixed for two hours at 4°C in 2.5% glutaraldehyde in 0.1 M pH 7.2 cacodylate buffer adjusted to 900 mOsM with NaCl. After a brief rinse, they were postfixed for one hour at room temperature in 1% osmium tetroxide in the same buffer, then rinsed in distilled water and treated with aqueous 2% uranyl acetate for one more hour. Pieces were then rinsed in distilled water, dehydrated through a graded ethanol series, and embedded in Epon-Araldite according to Mollenhauer (in Glauert, 1975). Sections were cut using an Ultracut E Leica ultramicrotome; semi-thin sections (0.5 µm thick) were stained with 0.5% toluidine blue in 1% borax and thin sections (60 nm thick) were contrasted with aqueous uranyl acetate and lead citrate before being examined in a TEM Hitachi H-8000 at 100 kV.

DNA extraction, PCR amplification and sequencing

Nucleic acids from symbiont-containing gill and symbiont-free foot tissues (negative controls) were independently extracted from two specimens as described elsewhere (Durand and Gros, 1996; Durand et al., 1996). 16S rDNA sequences were determined directly from PCR amplification products. PCR amplifications were performed using 0.25 mM each dNTP, 5 µl of 10x reaction buffer, 0.2 µg of each universal primers 27f and 1492r which recognize the 16S rRNA gene from Bacteria (Lane, 1991), 0.1 µg of template DNA, and 2.5 units of Taq polymerase (Promega), in a total volume of 50 ml. Samples were amplified under the following regime: initial denaturation at 95°C for 4 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 2 minutes, followed by a final elongation time of 7 minutes at 72°C. Negative controls were always run as a precaution against laboratory-derived contamination.

PCR products of three independent amplifications were pooled for each sequencing reaction in order to reduce nucleotide misincorporation errors introduced by Taq polymerase, then separated from primers and unincorporated dNTPs by using the QIAquick PCR Purification Kit (Qiagen Inc., Germany) according to the manufacturer's protocol. PCR products were directly sequenced by using the Taq DyeDeoxy™ Terminator Cycle Sequencing method (ABI Prism

sequencer, Perkin-Elmer, USA), according to the manufacturer's instructions. Sequencing reactions were electrophoretically separated on a Perkin-Elmer ABI 373A DNA Sequencer.

Phylogenetic analysis

Sequences were manually aligned with published sequences from previously described thioautotrophic symbionts by using the ae2 sequence editor. Nucleotide positions which were undetermined were eliminated from consideration. A total of 764 nucleotide positions were utilized in this analysis. Phylogenetic analyses were performed by using the programs contained in the Phylip 3.5 package (Felsenstein, 1989). Evolutionary distance were estimated by using DNADIST with Jukes and Cantor correction (1969). Phylogenetic trees were constructed using NEIGHBOR (Saitou and Nei, 1987). Maximum parsimony analysis was performed using DNAPARS. Bootstrap values based on the analysis of 100 trees were calculated using the programs SEQBOOT and CONSENSE. Bootstrap values greater than 50% are given but are considered to support the grouping of organisms in an associated node only at values greater than 75% (Zharkikh and Li, 1992).

PCR amplification of C. orbicularis symbiont specific target

In order to confirm the results obtained from sequencing, *C. orbicularis* symbiont target DNA were amplified from *D. quadrisulcata* gills by using the specific primer set Symco1-1492r as previously described (Gros et al., 1996b). PCR amplification was performed in a 25 µl reaction volume using 200 µM of each deoxynucleoside triphosphates (dNTP), 2.5 µl of 10x reaction buffer (Appligene®Oncor, France), 25 pmoles of each primer, 0.5 unit of Taq DNA polymerase (Appligene®Oncor), and 25 ng of DNA. Samples were amplified using a GeneAmp® PCR System 2400 (Perkin Elmer Inc, Norwalk, USA) under the following conditions: initial denaturation at 94°C for 8 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1.5 min, followed by a final elongation time of 7 min at 72°C.

Moreover, crude DNA were extracted from ripe gonads as previously described (Gros et al., 1996b; 1998b) in order to study the symbiont transmission mode. Samples of DNA were independently obtained from 6 mature specimens (3 males and 3 females) during the spawning period. The presence of *C. orbicularis* symbiont target DNA was checked from gonads by using the specific primer set Symco1-1492r as described above. To test for PCR inhibitors, 25 ng of gill DNA were mixed with duplicate samples before the PCR amplifications.

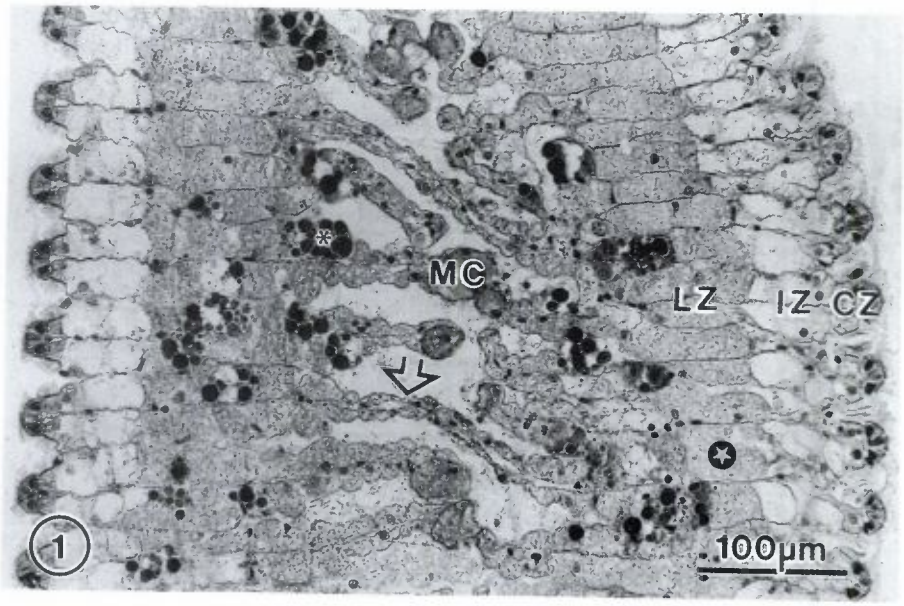


Figure 1. Light micrograph of a histological section of the gill of *D. quadrisulcata*. Each gill consists of a single inner demibranch constituted by a descending and an ascending lamellae linked by lacunar tissular bridges (open arrow). Each gill filament is composed of 3 distinct regions: a short ciliated zone (CZ), an intermediary zone (IZ) and a lateral zone (LZ) containing the chemoautotrophic symbionts. Four cell types can be observed in the lateral zone: bacteriocytes (star), intercalary cells, "granule cells" (asterisk) which are PAS positive, and mucocytes (MC) which are alcian blue positive.

Restriction fragment analysis

The amplification products were digested using the endonuclease *Hinf* I (Appligene®Oncor) according to the supplier's instructions. Restriction fragment pattern resulting from *Hinf* I activity is specific to the *C. orbicularis* gill-endosymbiont sequence, as determined by a search of the 16S rRNA sequences of all the other chemoautotrophic symbionts. The positions (*E. coli* numbering) of the restriction sites are: 836; 1034; 1338, 1355, 1475. They generate fragments of the following size: 304 bp, 198 bp, 198 bp, 120 bp, 35 bp and 17 bp. Reactions were performed in a 10 µl volume with 6 µl of amplified DNA, and compared by 8% acrylamide gel to the restricted gill amplification product obtained from *C. orbicularis*.

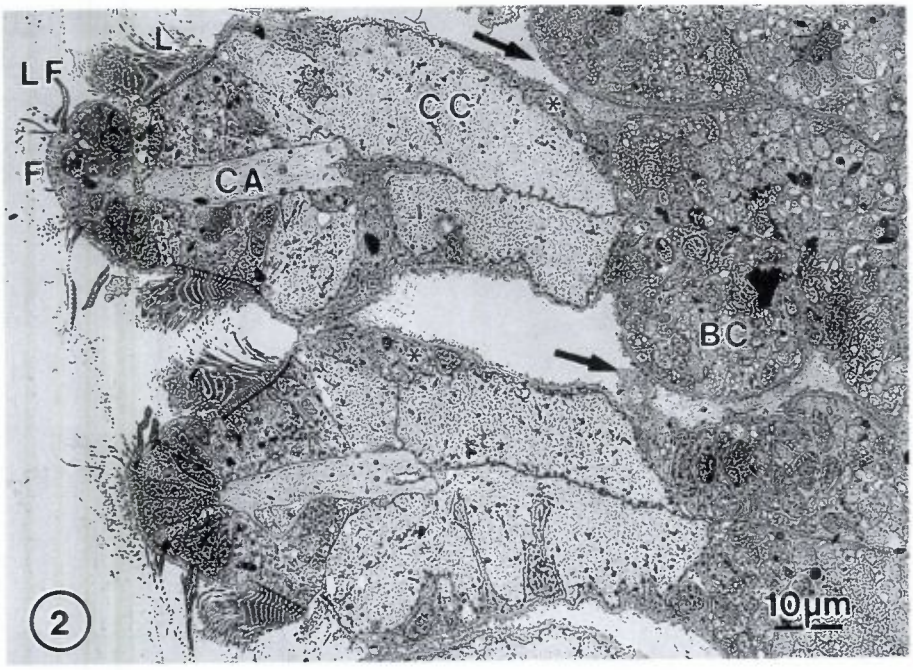


Figure 2. TEM of the ciliated and intercalary zones. Frontal (F), laterofrontal (LF) and lateral (L) ciliated cells are the main cell types composing the ciliated zone which is organized long a collagen axis (CA). The apical surface of the large clear cells (CC) constituting the intermediary zone of the gill filament is covered by unciliated cells (asterisks) which delimit, together with those of adjacent filaments, a narrow canal which directs the sea water flow (arrows) to the bacteriocyte channel. BC: bacteriocytes.

3. Results

Morphology

The creamy-beige demibranches of *D. quadrisulcata* consist of a single inner demibranch covering the visceral mass on both sides as described in various Lucinidae. A faint groove is present along the ventral margin of the homorhabdic gills. Observed on paraffin sections, the descending and ascending lamellae linked by lacunar tissular bridges delimit an "empty" interlamellar space suggesting that it does not contain stainable secretory products (Fig. 1).

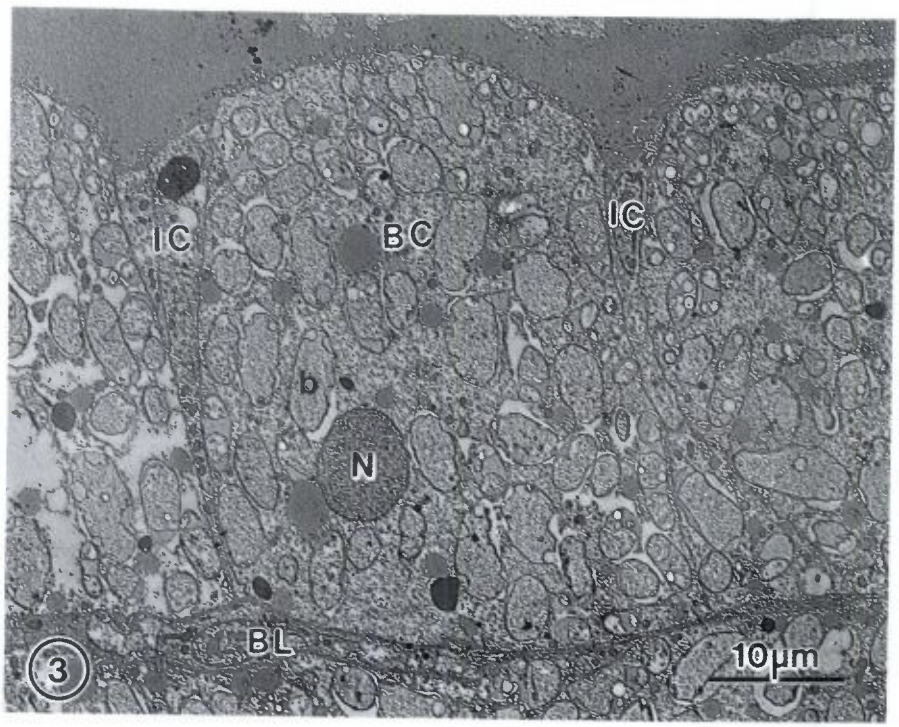


Figure 3. TEM. Bacteriocytes (BC), which are the most prevalent cells in the gill filament, have a basal nucleus (N) near the blood lacuna (BL) of the filament axis, a cytoplasm crowded by envacuolated bacteria (b), and a rounded apical pole developing a broad contact with pallial sea-water. Intercalary cells (IC), characterized by a trumpet shape, are regularly interspersed among bacteriocytes.

Gill filament structure

Each filament is composed of 3 distinct regions: a short ciliated zone (~30 µm), a relatively large intermediary zone (up to 50 µm long), and a lateral zone which contains the bacterial symbionts (Figs. 1 and 2). Each gill filament consists of a simple epithelium, which is in contact with a connective axis at the level of the ciliated zone, and encloses a blood lacuna in the lateral zone (Fig. 2).

The ciliated zone is similar to that previously described in other lucinid species with typical frontal, latero-frontal, and lateral ciliated cells (Fig. 2). The intermediary zone which appears longer than the ciliated zone is composed of up to four large electron lucent cells containing numerous

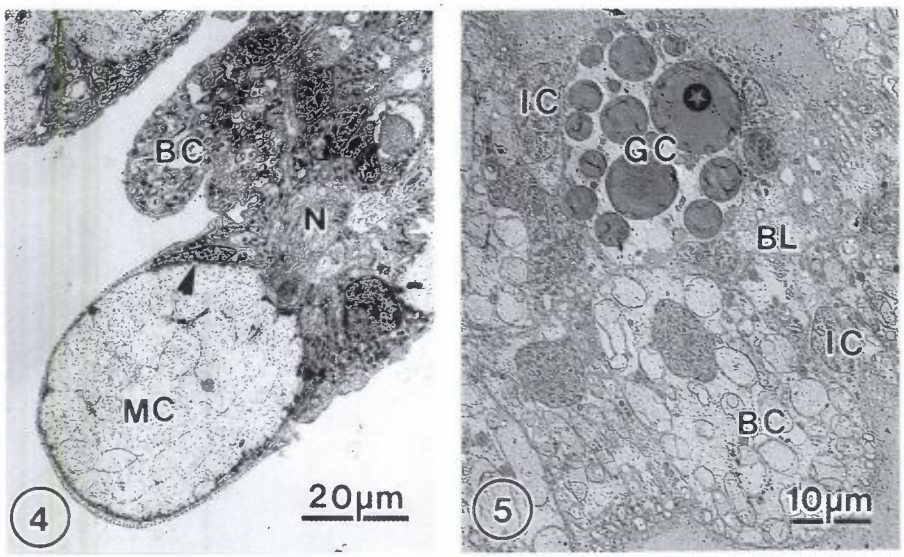


Figure 4. TEM view of the mucocyte cell-type (MC) presents in the abfrontal region of the gill filament. The nucleus (arrow head) is pressed against the cell membrane due to the fact that all the cytoplasmic volume is occupied by proteoglycans. Bacteriocytes (BC) are contiguous with a nerve (N).

Figure 5. TEM view of the granule cell-type (GC) intermingled with bacteriocytes (BC) and intercalary cells (IC) in the lateral zone of the gill filament. "Granule cell" has a narrow apical pole (arrow), a basal nucleus generally in contact with the blood lacuna (BL) of the gill filament. Most of the "granule cell" volume is occupied by large homogeneous proteinic granules (star).

mitochondria. These cells together with those of adjacent filaments constitute a narrow canal allowing a regular sea water flow along the lateral zone which contains the bacteriocytes (Fig. 2).

The lateral zone, which constitutes the main part of the gill filament, is organized as a simple epithelium in contact with a blood lacuna. Four cell types can be observed in the lateral zone: bacteriocytes, intercalary cells, "granule cells", and mucocytes.

Bacteriocytes, which are the most prevalent cells in the gill filament, possess a basal nucleus and a cytoplasm crowded with envacuolated bacteria (Fig. 3). Organelles are relatively scarce except of a few mitochondria and lysosomes. The most conspicuous inclusions of the bacteriocytes are the bacterial symbionts. The apical surface, which differentiates regular microvilli linked by a fibrous glycocalyx, is rounded and develops a broad contact with pallial sea-water (Figs. 3 and 5).

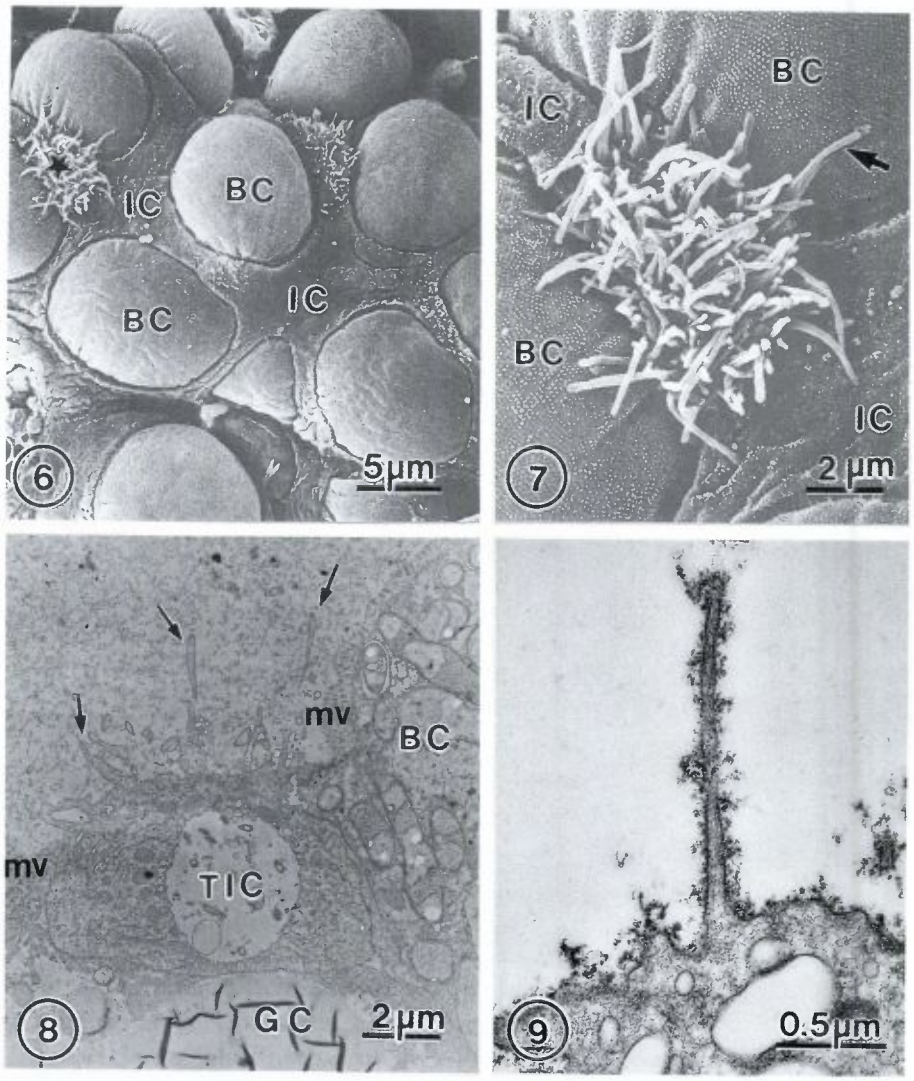


Figure 6. SEM. Intercalary cells (IC) and bacteriocytes (BC) make up an intricate array of small and large apical surfaces respectively, all covered with microvilli. Tufty intercalary cells (star).

Figure 7. SEM. Apical surface of the tufty intercalary cells which are characterized by long narrow protusions (arrow) much longer than microvilli from adjacent cells such as bacteriocytes (BC) and intercalary cells (IC).

Figure 8. TEM. A tufty intercalary cell (TIC) characterized by its long narrow protrusions (arrows) compared to the microvilli (mv) from the adjacent bacteriocyte (BC) and "granule cell" (GC).

Figure 9. TEM. A higher magnification of a long narrow protrusion from a tufty intercalary cell in the lateral zone of *D. quadrisulcata* gill filament. Note that these cytoplasmic expansions are devoid of any ciliary axonemal structure.

In *D. quadrisulcata* two types of intercalary cells can be distinguished. Some, characterized by a trumpet shape with a narrow basis and an enlarged apical area covered by microvilli, are regularly interspersed with bacteriocytes (Figs. 3 and 5). They have an elongated nucleus in an apical or lateral position and a large cytoplasmic area with numerous mitochondria and conspicuous reticulum profiles. TEM and SEM observations show that these intercalary cells encroach upon the apical area of the bacteriocytes and restrict their contact with pallial sea water (Figs. 3 and 6). The other type of intercalary cells, which also possesses a narrow basis and an apical nucleus, is characterized by enlarged irregular expansions of the apical pole. These cells differentiate on their apical part long narrow protrusions which give them a tufty aspect (Figs. 6 and 7). These protrusions are much longer than microvilli (Figs. 7 and 8) but without any ciliary axonemal structure (Fig. 9).

Intermingled with bacteriocytes and intercalary cells, two secretory cell types can be observed. The first one, which is generally found in the abfrontal region of the gill filament (Fig. 1) is characterized by electron lucent granules showing red metachromasia with toluidine blue on semithin sections. They are negative to the PAS reaction whereas they are strongly positive to the alcian blue. Thus, these cells which elaborate typical proteoglycans or sulfomucins, are mucocytes (Fig. 4). The second one is characterized by a cytoplasmic volume filled with large, membrane bound, osmiophilic inclusions which are stained orthochromatically with toluidine blue on semithin sections (Fig. 5). By using histological techniques such as Goldner triple staining or Mann Dominici, the granules appear to be acidophilic; they are PAS positive and alcian blue negative at pH 3 as well as at pH 0.5. Therefore, they do not contain proteoglycans and are not mucous platelets. Their staining by the Danielli's tetrazoreaction demonstrates their proteinic content, so these cells contain glycoproteins. The weakly positive DDD reaction without reduction of disulfur bonds and strong positive DDD reaction after reduction of the disulfur bonds by ammonium sulfide strongly suggests that these proteinic granules are rich in cystine residues. Only a few of these "granule cells" can be observed in the lateral zone of each gill filament even if they are present all along the two gills (Fig. 1).

Bacterial symbiont ultrastructure

Bacterial endosymbionts which are rod-shaped with the typical double-membrane of Gram-negative bacteria, are individually enclosed, dividing occasionally within vacuoles (Figs. 3 and 5). Due to the fact that the symbionts appear to be rod-shaped in crushed gill and/or from SEM observations, the ovoid-shaped figures are probably due to the section orientation. The bacterial

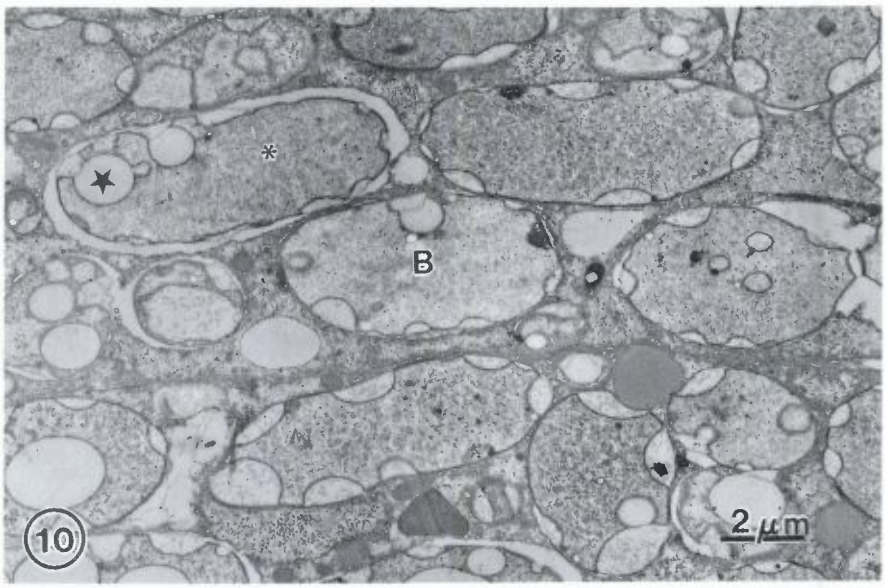
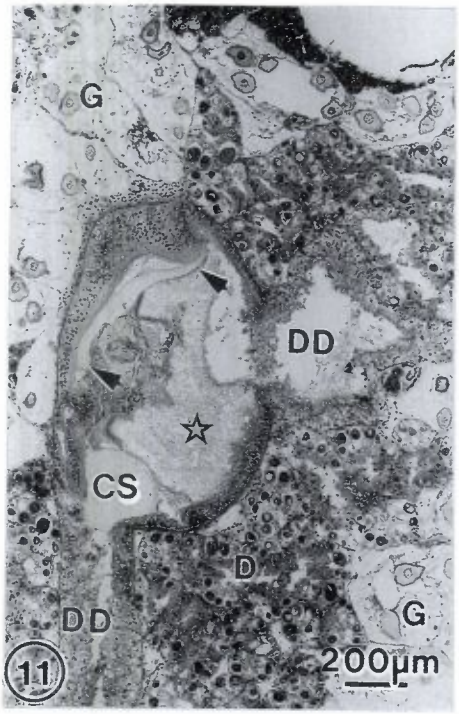


Figure 10. TEM of bacterial endosymbionts (B), which are rod-shaped with the typical membrane of Gram negative bacteria, tightly enclosed within bacteriocyte-vacuoles. All endosymbionts contain periplasmic membrane bound vesicles (star) considered as sulfur storage globules and numerous non-membrane-bound slightly polygonal inclusions (asterisk) considered as glycogenic storage granules.

cytoplasm contains some periplasmic membrane-bound vesicles considered as sulfur globules and numerous non-membrane-bound slightly polygonal inclusions (Fig. 10) considered as storage granules (glycogen-like particles) due to their strong positive PAS reaction.

Digestive tract structure

The digestive tract of this shallow-water lucinid species appears to be functional as suggested by histological sections. The stomach has a well developed gastric shield, a cristalline style protruding from the style sac, and active digestive diverticula (Fig. 11). The food content of the stomach appears to be mixed with amoebocytes. Digestive diverticula comprise large ducts, the cells of which are crowded with yellow lipofuscin granules and the acini comprise well defined secretory and digestive cells (Fig. 12). Whereas secretory cells are scarce, the numerous digestive cells are filled with large inclusions stained blue with alcian blue (Fig. 12). The cristalline style stained by the PAS



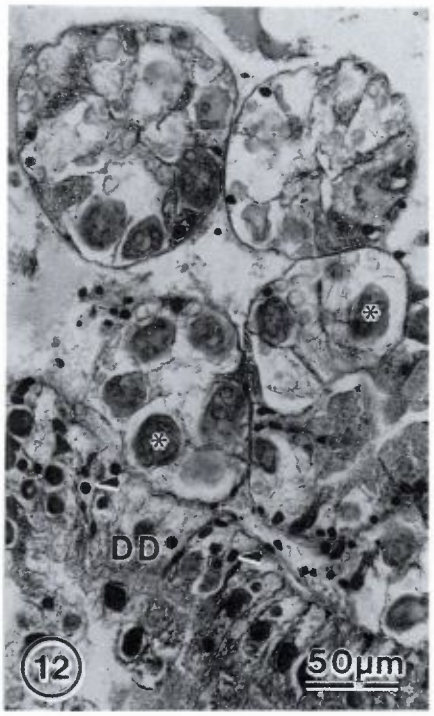


Figure 11. The stomach contains a typical gastric shield (arrow heads) and a solid cristalline style (CS) surrounded by an alcian blue positive liquid (star). It receives 3 large digestive diverticula ducts (DD); only 2 of them can be observed due to the section orientation. D: digestive diverticula. G: female gonad.

Figure 12. The digestive diverticula are composed of several acini characterized by large digestive cells filled with typical blue inclusions (asterisks) after alcian blue staining. The epithelial cells constituting the digestive diverticula duct (DD) are characterized by numerous lysosome-like inclusions (arrow heads).

reaction is surrounded by a liquid product that is also alcian blue positive (Fig. 13) all along the style sac. The style sac is not clearly individualised but constitutes a prolongation of the posterior part of the stomach which connects the stomach and the initial portion of the mid-gut. The mid-gut is coiled through the visceral mass and harbors a typical typhlosolis (Fig. 14). All of these aspects are characteristic for a functional digestive tract.

Enzymological analysis

Fourteen enzymes, which are probably involved in the digestion process,

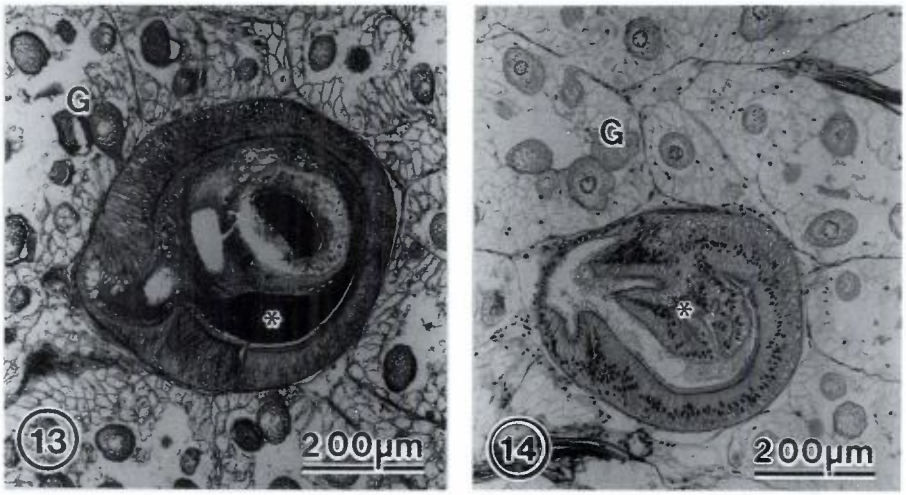


Figure 13. The style sac contains a solid cristalline style (asterisk) strongly positive to PAS reaction conversely to other lucinids in which the cristaline style is liquid or even absent. G: female gonad.

Figure 14. The ciliated epithelium of the mid gut differentiates a well developed typhlosolis (asterisk) similar to those found in filter feeding bivalves. G: female gonad.

were detected from crushed digestive glands of *Divaricella quadrisulcata* (Table 1) against fifteen for the filter-feeding venerids. The most important enzymes are phosphatase acid, β-galactosidase, α-mannosidase, and glucosaminidase. On the other hand, α glucosidase, β glucosidase, α galactosidase, and phosphatase alcaline were not detected (or with a weak activity) while they present strong activities in the two venerids analyzed in this study (Table 1).

Symbiont phylogeny

PCR amplifications performed with universal eubacterial 16S rDNA primers on gill tissue produced DNA fragments of the expected size (about 1,500 bp long, as determined by agarose gel electrophoresis). No amplification products were obtained with foot DNA as template. Direct sequence analysis indicated that the gill PCR products contained a single detectable sequence. Each of the symbiont 16S rDNA samples was then sequenced independently. A total of 764 nucleotides were sequenced from the *D. quadrisulcata* symbiont, corresponding to 8 to 370 and 1108 to 1510 of the *Escherichia coli* nomenclature (Brosius et al., 1981). In each case, 16S rDNA sequences from the symbionts of two specimens

Table 1. Enzymes detected from digestive gland of the lucinid *Divaricella quadrisulcata* and from the two venerids *Anomalocardia brasiliana* and *Chione cancellata*

Enzyme assayed for	*Anomalocardia brasiliana*	*Chione cancellata*	*Divaricella quadrisulcata*
Phosphatase alcaline	5	5	1
Esterase lipase (C8)	3	3	2/3
Lipase (C14)	0	0	0
Leucine arylamidase	5	5	5
Valine arulamidase	5	5	3
Cystine arylamidase	2	2	1
Trypsin	0	0	0
α Chymotrypsin	0	0	0
Phosphatase acid	5	5	5
Naphtol-AS-BI-phosphohydrolase	5	5	5
α Galactosidase	3	3	0
β Galactosidase	5	5	5
β Glucuronidase	5	5	3
α Glucosidase	4	4	1
β Glucosidase	3	3	1/2
N-acetyl-β-glucodaminidase	5	5	5
α Mannosidase	3	3	3
α Fucosidase	5	5	5

Quantity of hydrolysed substrate. 0: 0 nanomoles; 1: 5 nanomoles; 2: 10 nanomoles; 3: 20 nanomoles; 4: 30 nanomoles; 5: $\geq$ 40 nanomoles.

were identical at all positions determined indicating that the symbiont population in this newly examined tropical lucinid is composed entirely, or at least predominantly, of a single symbiont species.

Sequence analysis of the 16S rDNA of *D. quadrisulcata* symbiont showed it to be identical at all 764 nucleotides positions determined with the previously examined *C. orbicularis* symbiont and, consequently, to the *C. orbiculata- C. pectinella-* and *Linga pensylvanica-* symbiont (Fig. 15). Thus, it is highly probable that this symbiont represents a single bacterial species (Fox et al., 1992).

Data obtained from partial 16S rDNA sequence analysis were confirmed by PCR amplification and restriction fragment length polymorphism analysis. The specific primer set for *C. orbicularis* symbiont (Symco1 [*Escherichia coli* positions 638-656] - 1492r) strongly amplifies the target sequence from DNA

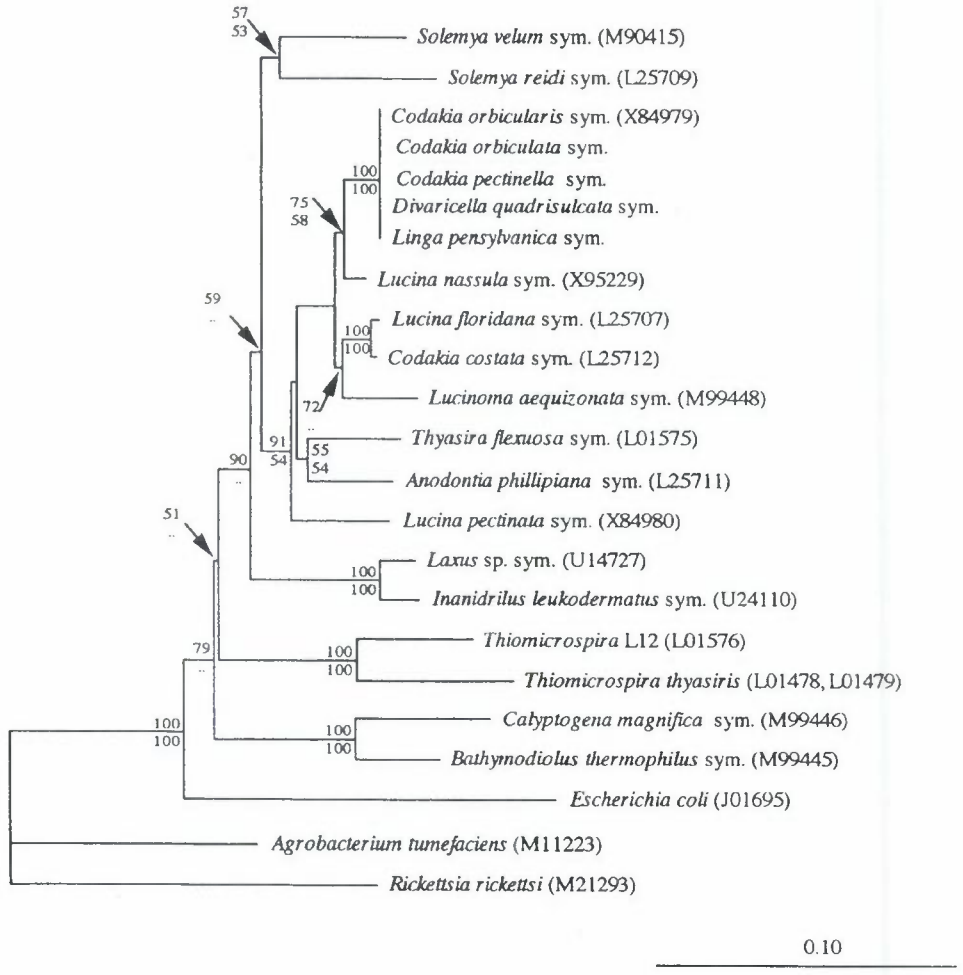


Figure 15. Phylogenetic tree based on partial sequences of 16S rDNA from lucinid gill-endosymbiotic bacteria, selected thioautotrophic bacterial symbionts, and representative members of the class Proteobacteria. Bootstarps values for selected nodes which are supported in more than 50 of 100 trees by distance analysis (upper numbers) and parsimony analysis (lower numbers) are shown. The scale bar indicates 0.1 nucleotides substitutions per sequence position.

extracted from the gills of *D. quadrisulcata*. Moreover, the restriction fragment pattern resulting from PCR amplifications indicated that the products amplified from *D. quadrisulcata*-gill were similar to those obtained from *C. orbicularis*-gill with three major bands (Fig. 16). The results strongly suggest

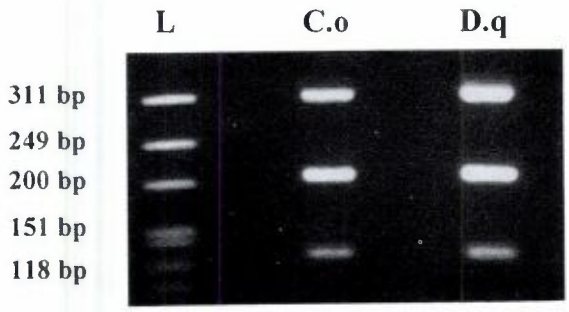
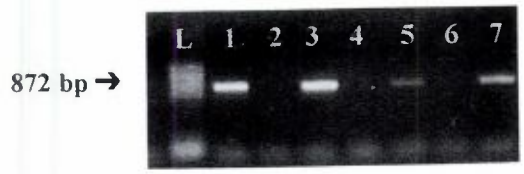
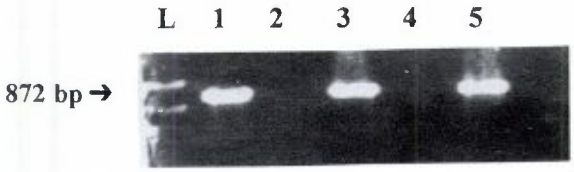


Figure 16. Agarose gel electrophoresis of *Hinf* I-digested fragments from the PCR primer set Symco 1-1492r. Lanes M, DNA marker; 1, *C. orbicularis* gill; 2, *D. quadrisulcata* gill.



A.



B.

Figure 17. PCR detection of the 872 bp *C. orbicularis* symbiont target in ovaries (A) and testes (B). Arrow points to the specific 872 bp fragment.
A: Female gonads: L: ladder; 1: gill; 2: ovary #1; 3: ovary + 25 ng of gill DNA; 4: ovary #2; 5: ovary #2 + 25 ng of gill DNA; 6: ovary #3; 7: ovary #3 + 25 ng of gill DNA.
B: Male gonads: L: ladder; 1: gill; 2: testis #1; 3: testis + 25 ng of gill DNA; 4: testis #2; 5: testis #2 + 25 ng of gill DNA.

that both bivalve host species harbor the same bacterial chemoautotrophic symbiont.

Symbiont transmission mode

PCR amplifications performed with *C. orbicularis* symbiont-specific primer set produced a single band of the expected size (~872 bp) from gill and positive controls (samples with gill DNA added) ruling out the hypothesis of PCR

inhibitors co-extracted with DNA. Despite repeated attempts, no amplification products were detected from ovaries and testes (Fig. 17).

4. Discussion

Ecology

Divaricella quadrisulcata is a silt-sand dwelling species living essentially in large depressions scattered all over the seagrass meadows. Conversely to the other common tropical lucinid species (except *Lucina pectinata* which lives burrowed in black reduced mud in mangrove) which are known to live in contact with the roots of *Thallassia testudinum, D. quadrisulcata* individuals were never found in seagrass beds. This small species lives in an environment characterized by (i) finer and silter sediments than those encountered in the seagrass bed, (ii) a low sulphide concentration associated with a very short oxic/anoxic zone because of a residual vortex in these depressions when compared with the seagrass bed, and by (iii) an accumulation of fine particles of organic detritus.

Gill filament

The ciliated and intermediary zones of the gill filaments of *Divaricella quadrisulcata* are very similar to those previously described in various lucinid species (Frenkiel and Mouëza, 1995; Frenkiel et al., 1996; Gros et al., 1996a) with the same ciliated cell types. The intermediary zone is longer than that found in *Linga pensylvanica,* which was known to have the longest intermediary zone described in lucinids up to date (Gros et al., 1996a). Intermediary cells appear to form the narrow aperture of the bacteriocyte channel controlling the water-flow along the apical pole of bacteriocytes from the mantle cavity into interlamellar space as described by Distel and Felbeck (1987) in *Lucinoma aequizonata.*

The most discriminating features in the gill filament organization of *D. quadrisulcata* are observed in the lateral zone and involve the various identified cell types. Mucocytes are relatively scarce when compared with the gill structure of other lucinids. They are only located at the abfrontal end of the gill filaments instead of being intermingled with bacteriocytes and granule cells as in *C. orbicularis* in which they are distributed throughout the lateral zone (Frenkiel and Mouëza, 1995) or even at both end of gill filaments as in *Linga pensylvanica* (Gros et al., 1996a) and *L. pectinata* (Frenkiel et al., 1996). In *D. quadrisulcata,* bacteriocytes are distributed all along the lateral zone intermingled with intercalary cells as described in other lucinids (Frenkiel et al., 1996; Gros et al., 1996a). However in *C. orbicularis,* bacteriocytes occupy

only the most superficial one-third of the lateral zone, the remaining two-third being occupied by large "granule cells". This cell type has been observed in few species (Giere, 1985; Southward, 1986; Fiala-Medioni et al., 1986) even if they were inappropriately considered as "storage cells" or "modified bacteriocytes". Frenkiel and Mouëza (1995) have strongly suggested that the abundant "granule cells" in *C. orbicularis* represent a cell type distinct from the other cells commonly found in the lateral zone such as bacteriocytes, mucocytes and intercalary cells. Recent observations performed on aposymbiotic juveniles (from 250 µm to 2.5 mm long) obtained in laboratory culture (Gros et al., 1997) have definitively confirmed that "granule cells" represent a distinct cell type, probably involved in detoxification processes, appearing before the establishment of the bacterial symbiosis which occurs in all adults and/or juveniles in the wild (Gros et al., 1997; Gros et al., 1998a). However, the "granule cells" found in *D. quadrisulcata* present some histochemical variations suggesting physiological differences that could be related to environmental chemical parameters (such as sulfide concentration in sediments) or to the host physiology rather than to the symbiosis due to the fact that *C. orbicularis* and *D. quadrisulcata* harbor the same bacterial species.

Digestive tract

According to Allen (1958) the course of the alimentary canal is quite simple in the superfamily Lucinacea. However, the representative member of the family Lucinidae, *Loripes lucinalis*, has a much more simplified digestive tract than the representative member of the family Ungulinidae, *Diplodonta punctata*. The most obvious difference is a coiled midgut in *D. punctata* versus a simple inflexion in *L. lucinalis*. Accordingly, the stomach typical of the superfamily is type V for Purchon (1987) but this type is only valid for species belonging to the family Ungulinidae whereas the stomach is regressed to type IVb in the families Lucinidae and Thyasiridae. Both regressions are now considered as correlated with the development of bacterial symbiosis in all the members of the family Lucinidae but not in the family Ungulinidae. However all the Lucinidae are not similar and the digestive tract of *D. quadrisulcata* is much similar to the previously described one of the Ungulinidae. The stomach and style sac which constitutes its posterior part appear as well differentiated with a typical ciliated epithelium. Even if the crystalline style is peculiar, with 2 distinct parts heavily stained with PAS surrounded by a mucous fluid stained by Alcian blue, it is a real crystalline style. A coiled midgut with a large dorsal typhlosolis may neither be considered as really simplified. Moreover, the digestive diverticulae appear as fully active with well differentiated cell types as well of the secretory as the digestive type.

These histological data were confirmed by classical enzymes analyses. The results obtained have suggested that *D. quadrisulcata* digestive diverticula complex possesses several enzyme activities such as phosphatase acid, β-galactosidase, α-mannosidase, and glucosaminidase. So, *D. quadrisulcata* appears to possess an enzymological equipment quite similar to those encountered in classical filter-feeding bivalves. Moreover, in the temperate lucinid *Loripes lucinalis*, cells from the digestive diverticula ducts have been shown to possess numerous lysosomes suggesting that they are actively involved in absorption and intracellular digestion of food particles (Johnson et al., 1996). As shown in this study, *D. quadrisulcata* also possess numerous secondary lysosomes in its digestive diverticula ducts and could present a similar process of absorption and intracellular digestion of food particles. The fact that *D. quadrisulcata* lives in seagrass depressions known to accumulate fine particles of organic detritus strengthens this hypothesis.

Research is in progress to check this potential lysosomal activity by cytochemical analysis. Moreover, previous carbon isotope analysis have shown that shallow water lucinids like *Codakia orbicularis* do not rely entirely on direct utilisation of carbon derived from endosymbiont bacterial autotrophy (Berg and Alatalo, 1984) conversely to hydrothermal vent invertebrates which do not possess a functional digestive tract (Rau, 1981; Brooks et al., 1987).

Thus, shallow water lucinids such as *D. quadrisulcata*, are characterized by a mixotrophic diet in which energy can be obtained by (i) digestion of organic particles from the water column and/or sediments and by (ii) carbon derived from gill-endosymbiont autotrophic activity.

In the family Lucinidae, *Divaricella quadrisulcata* may be considered as the less modified type of digestive tract encountered which retains a full competence for all the usual digestive processes of normal suspension feeding eulamellibranch bivalves without assistance of a symbiotic nutrition.

Bacterial phylogenetic analysis

Comparative 16S rDNA gene sequence analysis of the *D. quadrisulcata*-symbiont with representative members of the Bacteria indicated that they were related to the gamma subdivision of the Proteobacteria. More specifically, these sequences fall within the distinct cluster containing 16S rRNA sequences of all symbionts from bivalves of the superfamily Lucinacea. This phylogenetic congruence among hosts and symbionts has already been described by Distel et al. (1994). One exception has been reported concerning a hydrothermal vent mussel in which the co-existence of a thioautotrophic and a methanoautotroph endosymbiont was demonstrated by using molecular techniques (Distel et al., 1995).

Previous investigations have also shown that the specificity of the association between host bivalve and chemoautotrophic symbionts was unique to each host species (Distel et al., 1994): one bacterial symbiont species for one invertebrate host. Nevertheless, our results show that at least five species of lucinid bivalves representing three genera (*Codakia, Divaricella,* and *Linga*) harbor the same bacterial species inside their bacteriocytes. So, these results do not support the previous concept of a monospecific association between marine invertebrate hosts and their chemoautotrophic symbionts. Other associations between marine animal hosts and non chemoautotrophic symbiotic microorganisms examined to date display this moderate level of specificity (see for review Gros et al., 1998b). Previous studies based on DNA-DNA hybridization, have suggested that the two vestimentiferans *Riftia pachyptila* and *Tevnia jerichonana* harbor the same bacterial species (Edward and Nelson, 1991). More recently, these results were confirmed because the chemoautotrophic symbionts from different genera (*Oasisia, Riftia, Ridgea,* and *Tevnia*) collected from various hydrothermal vent sites presented identical 16S rRNA gene sequences (Feldman et al., 1997; Laue and Nelson, 1997). However, Di Meo et al. (2000) have recently shown, by using bacterial genomic fingerprint generated with rep-PCR, that each bacterial endosymbiont displayed a unique pattern showing strain level genetic variations among vestimentiferan symbionts.

Research is in progress to assess the genetic diversity of gill endo-symbionts colonizing these five tropical lucinids by using genetic fingerprint methods as rep-PCR, box-PCR or ERIC-PCR known to be powerful tools for bacterial population diversity analysis (Louws et al., 1999).

Gill endosymbiont transmission mode

The gill-endosymbiont transmission mode in *D. quadrisulcata* was investigated with a protocole used in previous studies (Gros et al., 1996b; 1998b; 1999). No amplification products were obtained from both male and female mature gonads, strongly suggesting that gametes are devoid of symbiotic bacteria. However, because early life stages of *D. quadrisulcata* were not available for developmental studies, it was impossible to negate the hypothesis of an endospore-like stage which could be present in sperm and/or in oocytes (as well as in cells constituting gonads as follicle cells in ovaries). This stage could be resistant both to nucleic acid extraction and to the PCR conditions. Nevertheless, these molecular data are consistent with previous studies on the transmission mode of lucinid symbionts (Gros et al., 1996b; 1998b; 1999) suggesting that bacterial gill-endosymbionts are transmitted to the new host generation from an environmental stock of a free-living form. Up to date,

the environmental transmission mode has only been demonstrated in the family Lucinidae in Bivalvia and strongly suggested in the hydrothermal vestimentiferans *Riftia pachyptila* and *Ridgea piscesae* (Cary et al., 1993). Previous studies on other bivalve families harboring sulfur-oxidizing bacteria in their gill cells, have only shown a vertical transmission mode in the solemyids *Solemya reidi* (Cary, 1994) and *S. velum* (Krueger et al., 1996), and in the vesicomyids *Calyptogena magnifica*, *C. phaseoliformis*, and *C. pacifica* (Cary and Giovannoni, 1993; Cary et al., 1993). Thus, the results presented in this paper confirm the hypothesis by Gros et al. (1998b) stating that the transmission mode of gill-endosymbionts in bivalves is specific for a family and "environmental" in Lucinidae and "vertical" in Solemyidae and Vesicomyidae.

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