Endosymbiotic Bacteria in the Gills of the Littoral Bivalve Molluscs *Thyasira flexuosa* (Thyasiridae) and *Lucinella divaricata* (Lucinidae)

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Abstract

Several authors have recently revealed the presence of intracellular bacteria in the gills of some bivalve molluscs. Initially considered as pathogenic, the presence of prokaryotic microorganisms is now regarded as being synonymous with symbiosis, although the exact bacteria-host relationships are not yet well understood.

In order to elucidate these relationships, structural and ultrastructural studies were conducted on the gill and the digestive tract of two littoral bivalves (*Thyasira flexuosa* and *Lucinella divaricata*) living in hydrogen sulphide-rich substrates.

The gill and various parts of the digestive tract were examined in detail, and the structural modifications observed in these organs are reported. This study reveals that although the gill has a major trophic function through endosymbiont bacterial association, the reduced gut is functional and also plays a trophic role of collecting and digesting organic particles, mainly diatoms.

The activities of the RuBPCase and APS reductase enzymes were also measured in the gill tissues of both species.

1. Introduction

In 1981, the presence of endocellular bacteria was reported in the gill epithelium of the littoral bivalve mollusc *Cerastoderma glaucum* (Henry et al., 1981). Although such bacteria were already known in sponges (Bertrand and Vacelet, 1971) and considered as symbionts, the exact status of prokaryotic organisms discovered in *C. glaucum* was unclear.

During the same period symbiotic bacteria were discovered in the hydrothermal-vent vestimentiferan *Riftia pachyptila* (Cavanaugh et al., 1981; Felbeck, 1981; Felbeck et al., 1981). These authors suggested that the bivalves living in this habitat could have an identical nutritional pathway. Shortly thereafter, the presence of chemosynthetic bacteria in two hydrothermal vents, *Bathymodiolus* sp. and *Calyptogena magnifica*, was confirmed (Le Pennec and Hily, 1984, Fiala-Médioni, 1984).

The hydrothermal vents thus appeared to be an extraordinary ecosystem where reduced inorganic elements were the source of invertebrate nutrition by means of chemosynthetic bacteria. The interest in this type of nutrition contributed to the development of research in littoral bivalves inhabiting reduced sediments, rich in organic matter, where hydrogen sulfide is detectable. Various types of habitats were explored: sea-grass beds, the vicinity of sewer outfalls or pulp mills and sewage-polluted fjords. Several species of bivalves were found to contain autotrophic endosymbiotic bacteria. Most of these species belong to the family Lucinidae (Fisher and Hand, 1984; Schweimanns and Felbeck, 1985; Dando et al., 1986; Southward, 1986).

The general consensus is that the presence of autotrophic symbiotic bacteria in the gill tissue leads to structural modifications of the ctenidial organ. The digestive tract is also more or less altered; in some cases, bivalves with symbiotic bacteria either lack a digestive tract completely (Reid, 1980) or have it greatly reduced, as in the family Lucinidae (Allen, 1958; Felbeck et al., 1981; Berg and Alatalo, 1984; Fisher and Hand, 1984; Schweimanns and Felbeck, 1985; Dando et al., 1986; Southward, 1986).

To better understand the bacteria-host relationships we have conducted structural and ultrastructural investigations of the gill and the digestive tract of two littoral bivalves *Thyasira flexuosa* (Montagu) and *Lucinella divaricata* (Linnaeus). *T. flexuosa* (family Thyasiridae) is distributed from Iceland and Norway to the Canary Islands and the Mediterranean while *L. divaricata* (family Lucinidae) is found from the British Isles to the Canary Islands and south to the Mediterranean and Black Sea (Tebble, 1966).

2.   Materials and Methods

Specimens of *T. flexuosa* were collected by dredging in Brest harbour (Brittany, France). This bivalve inhabits a dark clay mud substrate rich in organic matter: 12.45% at the sediment surface, 11.69% at 2 cm depth and 11.20% at 5 cm depth (Hily, 1984). The Redox Potential Discontinuity Layer was scored at 3 cm depth (Hily, 1984). The Cu content was 60–90 ppm and the PCB (polychlorobiphenyls) was 100 ppb. The area is considered as polluted (Hily, 1984).

Specimens of *T. flexuosa* were collected by digging at low tide in the vicinity of a sea-grass bed. This area contained abundant detritus of plant origin creating anaerobic conditions and the production of hydrogen sulfide.

Gills of the two species were dissected from freshly collected, live animals and the activity of the ribulose — 1,5-biphosphate carboxylase (RuBPCase) and the adenylylsulfate reductase (APS reductase) were measured. For structural studies, gill and gut tissues were fixed with Bouin's solution, embedded in paraffin, sectioned at 5μm and stained using the Masson trichrome technique. For scanning electron microscopy, the tissues were fixed with 7% formalin, dehydrated in a graded alcohol series, critical-point dried, coated with gold and observed using a JSM 35 Jeol microscope. For transmission electron microscopy, the tissues were fixed with 3% glutaraldehyde, postfixed with 2% osmium tetroxide, embedded in Spurr, ultra-sectioned and stained with 7% uranyl acetate and 0.1% lead citrate before examination using a 100 CX Jeol microscope.

3.   Results

RuBPCase and APS reductase enzyme activities were measured in the two bivalves. The values obtained (units per gram of fresh tissue weight) were: 100 in *L. divaricata* and 20 in *T. flexuosa* for RuBPCase; 74 in *L. divaricata* and 36 in *T. flexuosa* for APS reductase.

In *L. divaricata* the gills consist of the inner dimibranchs only. They are large and extend posteriorly below the visceral mass. In *T. flexuosa* the gills are thick and cover the visceral mass. They consist of two dimibranchs, the outer being less developed than the inner. In both bivalves, the gills are homorhabdic. The filaments are arranged in parallel and junctions between them consist of transverse fibrillar bunches. They are formed of a simple epithelium, ciliated in the frontal and abfrontal regions and with voluminous cells in the internal parts. The frontal region presents typical well-defined ciliary bands: frontal, latero-frontal and lateral (Fig. 1A,2A). A

Figure 1. *Lucinella divaricata*

A. Ciliary bands of gill filaments. f, frontal cilia; lf, latero-frontal cilia; l, lateral cilia. Scale bar = 5μm.

B. Microvillous cell with bacteria (b). n, nucleus; p, phagosome; polyphosphate granules (arrows); ic, intercalary cell; h, hemocyte. Scale bar = 1μm.

C. Small bacteria (b) and large bacteria (tb) in the same bacteriocyte. mv, microvilli in the glycocalyx layer. Scale bar = 0.3μm.

D. Storage cell. gr, granular cytoplasm; p, phagosome; db, degenerated bacteria; l, lipid granules. The basal membrane is highly convoluted (arrows). Scale bar = 0.3μm.

E. SEM micrograph of transverse section of intestine filled with particles. cw, cell wall. Scale bar = 20μm.

F. Detail of gut contents, mainly composed of diatom frustules (f). Scale bar = 2μm.

food groove is present at the ventral bend of each demibranch. In the ciliated zones, frontal and latero-frontal cells are covered with microvilli while lateral ciliated cells show no microvilli. All of the cells are elongated and possess a dark nucleus and numerous mitochondria in their cytoplasm. They rest upon a thick, fibrillar and regular basal membrane.

In *L. divaricata* the lateral wall of the filament presents several types of cells. Some of them are large and their cytoplasm contains numerous granules and mitochondria. Their apical pole is densely covered with microvilli. Other microvillous cells are voluminous and their cytoplasm is almost filled with bacteria (Fig. 1B). Their nucleus is located near the basal membrane. The microorganisms are round and envacuolated, with generally one per vacuole. Their average dimensions are $0.4 \times 0.3\mu$m for the smallest and $1.7 \times 1.4\mu$m for the largest (Fig. 1C). The cytoplasm of the bacteria contains numerous relatively electron-dense granules and their cytoplasmic membrane may be invaginated to produce small clear vesicles. Some dark perivesicular granules are frequently observed in the bacteria. Other irregular vacuoles containing an osmiophilic material are associated with the bacteriocytes. The basal membrane of the bacteriocytes is highly convoluted (Fig. 1D).

In the median lateral region of the filaments, the cells are small but the volume of the intracytoplasmic vacuoles containing a relatively electron-dense material increases. The same phenomenon was observed in the bacterial vacuoles, whose volume increases and the dark perivesicular granules become more abundant. The bacteria are progressively degraded within the vacuoles. In some cells all of these vacuoles are gathered together to constitute a large vacuole. Almost all of the cellular volume is occupied by voluminous vacuoles
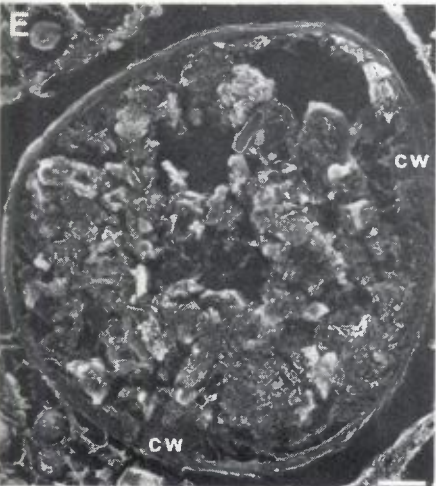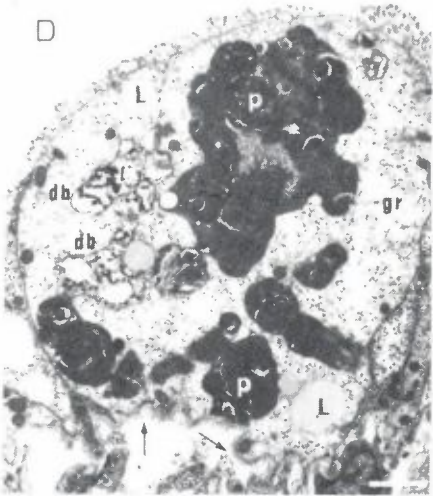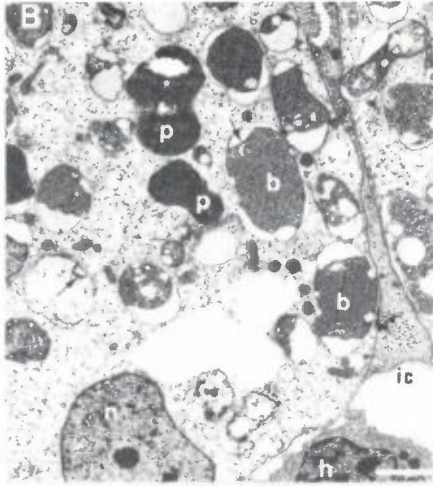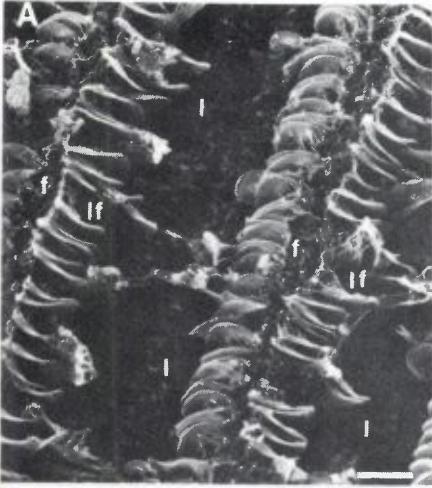
Figure 2. *Thyasira flexuosa*

    A. Ciliary bands of filaments. f, frontal cilia; lf, latero-frontal cilia; l, lateral cilia; mb, mucus balls. Scale bar = 15μm.

    B. Bacteria (b) in bacteriocytes. mv, microvilli; pinocytotic vesicle (arrows). Scale bar = 0.5μm.

    C. Detail of bacteria. Cm, cytoplasmic membrane; nd, nucleoid; t, DNA threads; g, grains (polyphosphate ?); s, sulphur vesicle. Scale bar = 0.1μm.

    D. Lysis of bacteria. pv, phagocytic vacuoles; gr, complex granule (phagosome); bs, blood space with hemocyte (h). Scale bar = 0.5μm.

    E. Secretory cell. n, nucleus; er, endoplasmic reticulum; g, Golgi. Scale bar = 2.5μm.

    F. Digestive cell (dc). n, nucleus; h, heterophagosomes; sc, secretory cell. Scale bar = 2.5μm.

containing an osmiophilic material. Some mitochondria, some lipids and some autophagic lysosomes containing myelin-like structures are also present in the cytoplasm (Fig. 1D).

In *T. flexuosa* the lateral epithelium of the gill filaments is mainly composed of bacteriocytes. They are of various sizes, being voluminous near the ciliary zones and flattened in the central portion of the filament. The largest bacteriocytes are filled with numerous microorganisms occupying three-quarters of the cellular volume (Fig. 2B). The apical membrane is frequently invaginated and numerous pinocytic vesicles can be observed. In the flattened cells the bacteria are arranged in a single row among the microvilli. Mitochondria were visible beneath the bacterial vacuoles.

The bacteria are ovoid and their average dimension is 0.6 $\times$ 0.3μm. Their cytoplasm is finely granular with one or two vacuoles and a small electron-dense nucleoid (Fig. 2C). Dividing bacteria were frequently observed (Fig. 2B). In the basal part of the cells, the cytoplasm presents myelin-like figures, degenerated bacteria and electron-dense globules (Fig. 2D). The basal membrane under the bacteriocytes in convoluted.

Both species present a filamentar lumen containing numerous hemocytes with a cytoplasm more or less filled with electron-dense granules.

The digestive tract of the two species consists of the same four parts classically found in bivalves; however some of these regions are reduced. The labial palps are small and the oesophagus is short, entering a relatively small stomach. This organ possesses a gastric shield and a crystalline style.

In *T. flexuosa* the digestive diverticula are well-developed in the lateral pouches and both types of cells (secretory and digestive) are present (Fig. 2E, 1F). The digestive diverticula open directly on to the ventral floor of the stomach. The intestine leaves the stomach posteriorly, reaches the dorsal
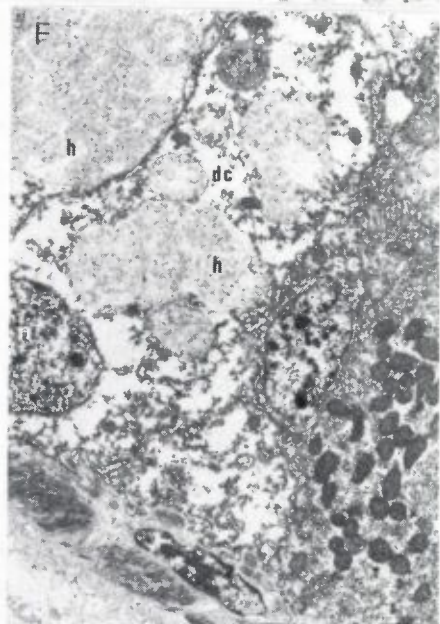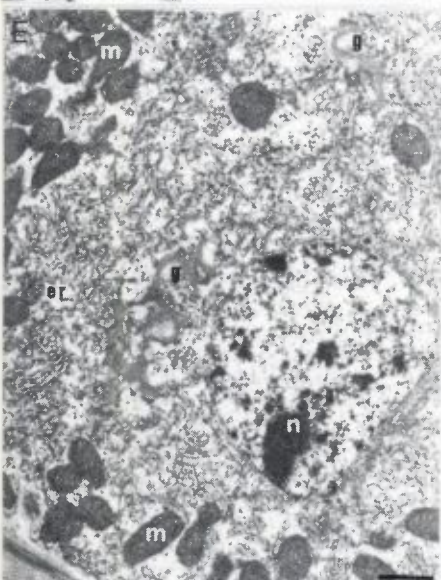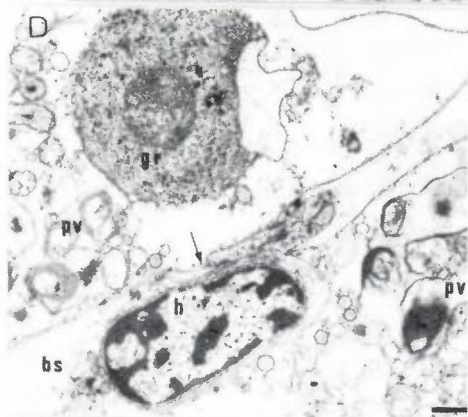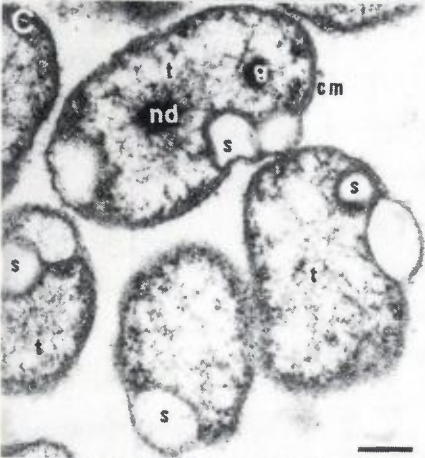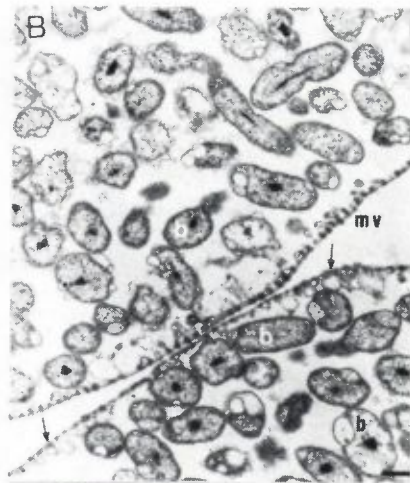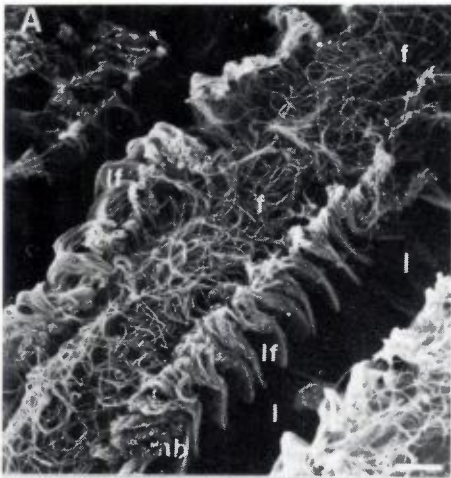
part of the body and opens at the anus, which is situated near the posterior adductor muscle.

The digestive diverticula of *L. divaricata* are mainly located in the lateral part of the stomach where the ducts communicate *via* large apertures. Secretory and digestive cell types are present. the intestine leaves the stomach, forms a simple loop within the visceral mass and runs dorsally to the adductor muscle. It is always filled with the remains of diatoms (Fig. 1E, 1F).

4. Discussion and Conclusion

Structural and ultrastructural investigations reveal that in all bivalves with gill symbionts, relatively major modifications occur in the gill and the gut. Cavanaugh (1985) states that three features now appear to be typical of bivalves which harbour gill symbionts: thick gills, reduced palps, and a short and simple gut. The two littoral species examined in the present study also present these three characteristics.

The gills are thick and light-brown colored. As indicated by Southward (1986), gills containing bacteria are always pigmented. In all bivalves with gill symbionts, only the lateral microvillous cells are able to integrate bacteria. This may occur very early in the life of the bivalve, probably just after metamorphosis, when the gill filaments differentiate (Le Pennec and Herry, 1986). The bacteriocytes become voluminous with few organelles. The bacteria continue to live and to divide for a short period before being progressively degraded by the lysosomal enzymes of the host cells.

All the bacteriocytes are covered with microvilli coated with a glycocalyx incorrectly names a "cuticle" by Southward (1986). The presence of these microvilli indicates that considerable exchanges involving the symbionts occur between the bacteriocytes and the palleal cavity. At the base of the bacteriocytes, the basal membrane is highly convoluted; this may be related to the transport of material from the cell to the vascular lumen where hemocytes are observed with the same granular material in their cytoplasm as in the bacteriocytes.

In *T. flexuosa* there is only one type of small endosymbiotic bacteria with a fine granular cytoplasm. In *L. divaricata*, where the enzymatic activities are more intense than in the thyasirid, two types of symbionts are observed; however, they probably represent different stages of development of the same species. The smallest symbionts are similar to those found in *T. flexuosa*, but the largest have a relatively dense granular cytoplasm containing some dark

perivesicular granules similar to the polyphosphate granules found in some species of lucinids by Dando et al. (1986). Some such granules were also observed in *T. flexuosa*. In both species the bacterial cytoplasm is vacuolated. These vacuoles could be the sites of sulfur particles which constitute the energy source for the bacteria (Fisher and Hand, 1984; Dando et al., 1986; Southward, 1986).

The second morphological modification of these bivalves is the reduced labial palps. However, this feature cannot be generalized to all bivalves with gill symbionts, since in *Bathymodiolus thermophilus*, a deep-sea hydrothermal vents species, the palps are well developed (Hily et al., 1986).

The third modified feature is a short and simple gut. The majority of bivalves with gill symbionts possess a simple digestive tract, often reduced, but still functional. Recent studies of bivalves inhabiting the subduction zones of Japan reveal that all the species of *Calyptogena* found between 3,000 m depth and 6,000 m depth have a functional digestive tract (Le Pennec and Fiala-Médioni, 1986). All these species of *Calyptogena* and also *B. thermophilus* feed both by chemoautotrophy and *via* organic particles (mainly diatoms) sinking from the surface, as well as by ingesting unicellular animals such as foraminifera and coccolithophorida living in their environment. With the exception of some species of *Solemya* which lack a digestive tract altogether (Reid, 1980) and *Calyptogena magnifica* from the Galapagos sites whose simple gut is always empty (Boss and Turner, 1980), the other species from active geological sites and the two littoral species of the present study possess a functional digestive tract.

Chemoautotrophy appears to be more important for the nutrition of *T. flexuosa* than for that of *L. divaricata*. In the thyasirid the stomach is often only partially full, while in the lucinid the gut is always filled with organic particles, mainly various species of diatoms. This difference in nutritional pathways could be related to the different biotopes of these two bivalves. *T. flexuosa* lives frequently in dense populations, in a particular biotope with a restricted assemblage of associated molluscs. In the present study, only two other bivalves were found: *Abra alba* and occasionally *Acanthocardia echinata*. Their gills lack symbionts. It is probable that the symbiotic association of the thyasirids confers upon them a considerable tolerance to the potentially deleterious sediment chemistry which excludes many other infaunal bivalves.

L. divaricata lives near sea-grass beds where several inorganic compounds, mainly hydrogen sulfide, are metabolized by endocellular symbiotic bacteria.

In this habitat, *Tellina tenuis*, which does not contain any bacteria in the gills, is frequently associated with the lucinid.

In both types of habitat, the associated species are less abundant than the bivalves containing gill symbionts and there is no doubt that the lucinid and the thyasirid obtain a substantial proportion of their nutrition from their autotrophic symbionts (half or more of their nutritional needs according to Dando and Southward, 1986). Moreover, Southward (1986) demonstrated that bivalve gill cells are able to control the proliferation of their symbionts, perhaps by rationing their iron supply by means of iron-binding proteins. However, the mechanism involved in this control has not yet been elucidated.

The initial data from the deep-sea bivalves from hydrothermal vents and from the subduction zones of Japan led us to believe that symbiotic relationships could only be established in restricted areas with oxygen-poor or hydrogen sulphide-rich substrates. It is now apparent that the bacteria-bivalve relationships are more complicated than was previously thought. In a given environment bivalves may or may not possess gill symbionts, and may or may not have a functional digestive tract (more or less reduced). In addition, the recent discovery of a littoral bivalve *Spisula subtruncata*, containing gill symbionts and living in well oxygenated sand (Bouvy et al., 1986) raises new questions. Another problem is the origin and temporal evolution of the relationships between the bacteria and the bivalves; this should be the object of future studies.

It is thus apparent that in spite of the recent studies conducted on bivalves containing gill symbionts, many questions concerning the bacteria-host association are not yet resolved and more work is required to better understand this relationship.

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