

Chemoautotrophic Bacterial Endosymbiosis in *Spisula subtruncata* (Bivalvia, Mactridae). Ultrastructure, Metabolic Significance and Evolutionary Implications

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Abstract

Two types of endosymbiotic bacteria are observed in the gills of the surf-clam *Spisula subtruncata*. Unlike other bivalves associated with endosymbiotic bacteria, *S. subtruncata* does not show partial atrophy of the gills, siphons, palps and gut. Living in shallow sands and gravels with low organic matter content, *S. subtruncata* evidently subsists mainly by filtering suspended particulate material. Ultrastructural features of the bacteria-*Spisula* association demonstrate an elaborate pattern. Enzymatic and physiological evidence that the endosymbiotic bacteria in *S. subtruncata* possess autotrophic activity and are able to use reduced nitrogen and sulfur compounds as energy sources has been presented. These observations extend the recent discoveries of symbiotic gill bacteria in a range of bivalve molluscs living in sediments containing various levels of reduced compounds. The presence of two distinct types of bacteria in the same host (as is found in *S. subtruncata*) may indicate that the ability of bacteria to become endosymbiotic is more extensive than previously expected and that the establishment of symbiotic relationships is not limited to one bacterial type.

Keywords: Endosymbiosis, bacteria, Bivalvia, Mactridae

1. Introduction

Recent investigations have revealed the constant presence of a relatively small number of two types of intracellular endosymbiotic bacteria in the gills of one of the main species of Bivalvia living on Mediterranean coastal sandy bottoms, *Spisula subtruncata* (da Costa) (Bivalvia, Mactridae) (Bouvy et al., 1986). The discovery of chemoautotrophic bacterial endosymbiosis in a member of Mactridae adds to the recent findings which demonstrate the presence of gill symbionts in a taxonomic range of bivalve molluscs, and reveals that this type of symbiosis may be widespread in marine bivalves living in reducing or non-reducing sediments. In this paper, we summarize the main ultrastructural features of *S. subtruncata* and its symbionts in comparison with other bacterial symbionts in bivalve gills. Moreover, we discuss some of the general implications of this discovery within hypotheses previously proposed for chemoautotrophic function of bacterial symbionts, origin of bacterial symbiosis in bivalves, and bivalve evolution.

2. Ultrastructure

Until now symbiotic bacteria have been described only in five bivalve families: Solemyidae (Cavanaugh, 1983, 1985; Felbeck, 1983; Powell and Somero, 1985), Mytilidae (Fiala-Médioni, 1984; Le Pennec and Prieur, 1984; Fiala-Médioni et al., 1986), Lucinidae (Berg and Alatolo, 1984; Fisher and Hand, 1984; Dando et al., 1985; Giere, 1985; Reid and Brand, 1986), Thyasiridae (Reid and Brand, 1986; Southward, 1986) and Vesicomysidae (Cavanaugh, 1983, 1985; Fiala-Médioni, 1984; Fiala-Médioni and Métivier, 1986). The discovery of endosymbiotic bacteria in gill filaments of *S. subtruncata* now adds the family Mactridae to the range of the bivalves with symbiotic bacteria.

Methods and detailed ultrastructural observations of symbiosis in *S. subtruncata* have already been published (Bouvy et al., 1986). Our results are summarized in Table 1 and 2 and Fig. 1.

The comparison of the main ultrastructural patterns of bacterial endosymbiosis in bivalve species (Table 2) points out that *S. subtruncata* shows one of the most elaborate types of bacterial association in bivalves. The two distinct morphological types of Gram-negative bacteria are included in differentiated cells, or bacteriocytes, strictly located at the basis of the inner part of the gill filament. Within the bacteriocyte, more than two hundred bacteria per section are packed into a spherical assemblage, or "bacteriophore", surrounded

Table 1. Ultrastructural comparison of the two types of endosymbiotic bacteria in *Spisula subtruncata*

| | | TYPE 1 | TYPE 2 |
|---------------|-----------|---|--|
| Bacteria | Shape | comma-shape (vibrios) | ovoid (bacilla) |
| | size | 1x3 μm | 0.8x1.2 μm |
| | membranes | slightly pleated outer membrane peptidoglycan layer thin cytoplasmic boundary | very pleated outer membrane peptidoglycan layer inner cytoplasmic membrane |
| | nucleoid | arch-shaped filaments condensed area | tangled nucleofilaments extended area |
| | cytoplasm | dense numerous ribosomes | small area, compact numerous ribosomes |
| Bacteriophage | | packed assemblages of more than 200 bacteria per section surrounded by a single membrane granular matrix | packed assemblages of more than 200 bacteria per section surrounded by a single membrane clear matrix |

by a membrane. The membrane of the bacteriophage may derive from the hypertrophy of rough endoplasmic reticulum as described for some associations between intracytoplasmic bacteria and their protist hosts (Chesnick and Cox, 1986). It must be noted the presence of two nuclei in numerous observed bacteriocytes. Cells adjacent to the bacteriocytes bear microvilli, which cover them partially or totally. Bacteriocytes and adjacent cells contain numerous granules and lysosomes. Bacteria are often observed in division. Southward (1986) has suggested that the bacterial symbionts may be more stable in the lucinids than in the thyasirids, "with the bacteria being 'milked' of excess production rather than being 'eaten'" (Southward, 1986, p. 910). In our opinion, the numerous division stages observed in *S. subtruncata* and the absence of bacterial lysis may well indicate that the association has been evolutionary more stable than the associations with the lucinids.

However, on several occasions and in different specimens, we have observed bacteriocytes with type 1 bacteria which are two or three times larger than normal (Fig. 2A). Higher magnification revealed that the hypertrophied bacteria are infected with a large bacteriophage virus lacking a tail. Numerous polyhedral virions (100 nm in diameter) are visible within bacteria and evidently provoke partial lysis (Fig. 2B). The bacterial nucleoid, still visible, becomes displaced to the periphery of the bacterial cell. Viral infection of the type 2 bacteria was never observed. The presence of a bacteriophage virus in endosymbiotic bacteria of a marine invertebrate has never before been recorded.

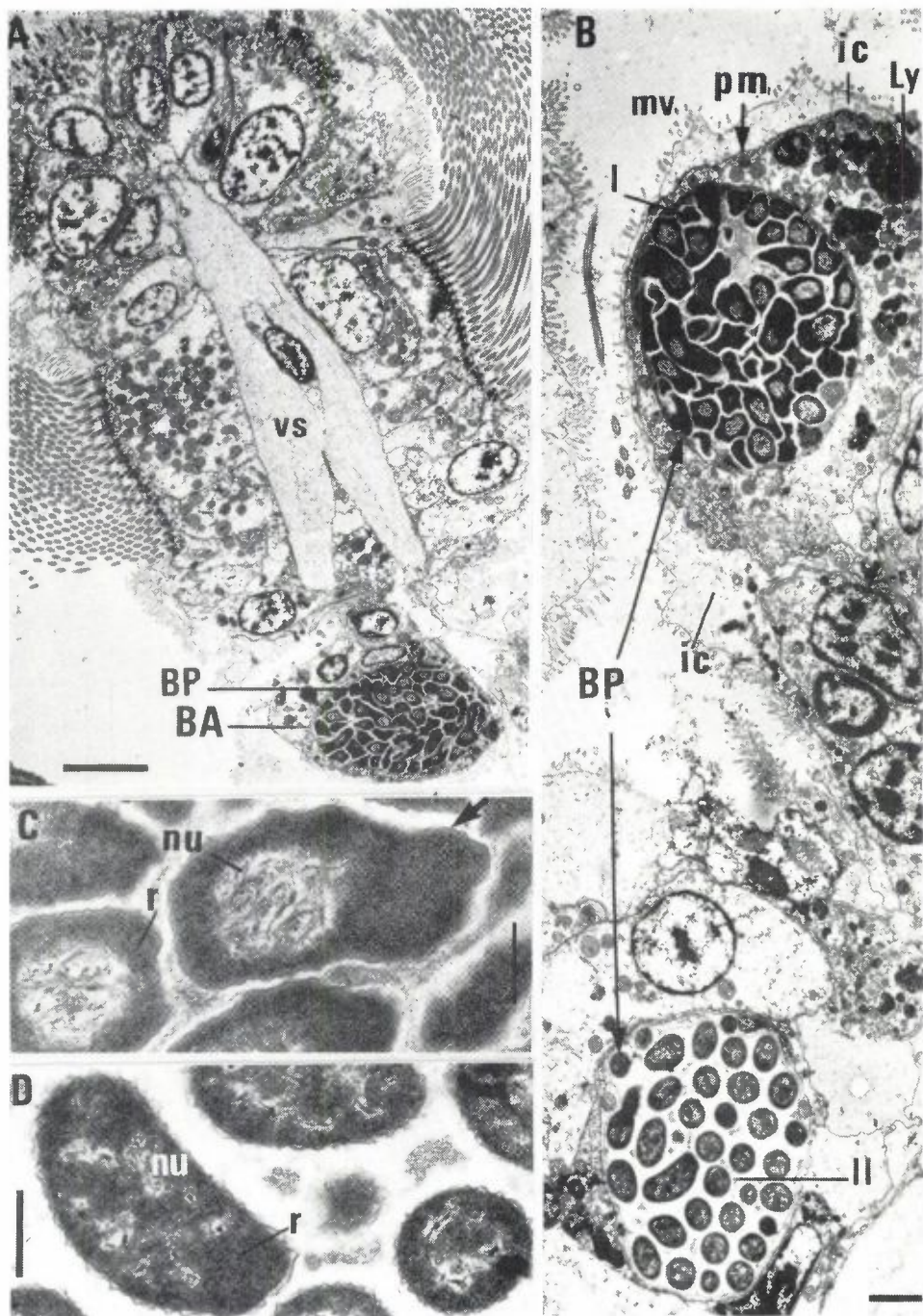


Figure 1. Fig. A: Gill Filament. A bacteriophage (BP) within a bacteriocyte (BA) can be seen in the posterior part of the gill filament. Note the ciliated cells with numerous small mitochondria (VS: vascular space). $\times 2500$. bar = $5 \mu\text{m}$. Fig. B: Ultra-thin section showing bacteriocytes with two different types of bacteria (I-II). Note the homogeneity of each bacteriophage (BP) content (ic: intercalary cell; mv: microvilli; pm: plasma membrane; Ly: lysosome). $\times 5000$. bar = $2 \mu\text{m}$. Fig. C: Detail of type 1 bacteria. Observe the arch-shaped nucleoid filament organization (nu). The dense bacterial cytoplasm with numerous ribosomes (r) is surrounded by a double membrane (arrow). $\times 25000$. bar = $0.5 \mu\text{m}$. Fig. D: Detail of type 2 bacteria. The tangled nucleoid (nu) is dispersed in a dense cytoplasm with numerous ribosomes (r), surrounded by a double folded membrane (arrow). $\times 24000$. bar = $0.5 \mu\text{m}$.

3. Metabolism and Ecological Significance

Symbiotic bacteria, sulfide-oxidizing enzymes and enzymes of the Calvin-Benson cycle were identified in thermal vent bivalves (Cavanaugh, 1983; Fiala-Médioni, 1984; Le Pennec and Prieur, 1984; Belkin et al., 1986) and in other bivalve species more widely distributed in shelf and slope sediments where dissolved sulfide concentrations were elevated (Felbeck et al., 1981; Cavanaugh, 1983, 1985; Felbeck, 1983; Berg and Alatolo, 1984; Dando et al., 1985; Schweimanns and Felbeck, 1985; Reid and Brand, 1986). More recently, it has been demonstrated that several of these symbiotic associations can exist in habitats where the dissolved sulfide concentrations are relatively low (Dando et al., 1985; Dando and Southward, 1986; Dando et al., 1986a; Dando et al., 1986b).

Generally, the presence of endosymbiotic bacteria in the gills of bivalvia leads authors to suppose some trophic exchange is occurring between the mollusc and the endosymbiotic bacteria. The bacteria are chemoautotrophs, deriving energy from geothermal sources such as hydrothermal vents, from decaying organic matter or from decaying seagrass leaves. Recently, a methane-based symbiosis between intracellular bacteria and a mussel (Childress et al., 1986; Cavanaugh et al., 1987) and a pogonophoran (Schmaljohann and Flügel, 1987) has been demonstrated. These findings suggest that the associated bacteria may provide a substantial proportion of organic compounds to the host.

The discovery of two spatially separated types of endosymbiotic bacteria in the gill tissue of *S. subtruncata* suggests that these bacteria might differ in metabolism or use different sources of reduced inorganic compounds. Bacteria chemoautotrophic activity has been demonstrated by the detection of ribulose biphosphate carboxylase, a major CO_2 fixation enzyme of the

Table 2. Ultrastructural characteristics of symbiosis between bivalves and endosymbiotic gill bacteria. This table compares 10 of the 22 bivalves species currently known to house such bacteria. The species listed were selected to exemplify the main ultrastructural patterns. Methanotrophic bacteria bivalve symbiosis is not included.

| | Ref. | BACTERIA | | BACTERIOCYTE | | | SULFUR VESICLES | | INTERCALARY CELLS |
|---|---|---|---|--------------------------|---|-----------------|-----------------|---|-------------------|
| | | Number of bacteria type per bivalve species | Appearance within bacteria type | Lysis divisions observed | Location | Apical membrane | Lysosomes | Shape* | |
| Subclass PROTobranchia | | | | | | | | | |
| Order Solemyoidea | | | | | | | | | |
| Superfamily Solemyiacea | | | | | | | | | |
| Family Solemyidae | | | | | | | | | |
| <i>Solemya reidi</i> Bernard | Feilbeck (1983) | 1 | Vast number enclosed in vacuoles | ? | MBSR | Microville | + | Partially cover the bacteriocyte | |
| Subclass LAMELLibranchia | | | | | | | | | |
| Suborder Ptenoidea | | | | | | | | | |
| Superfamily Mytilacea | | | | | | | | | |
| Family Mytilidae | | | | | | | | | |
| <i>Bathymodiolus</i> sp. | Fiala-Médioni et al. (1986) | 1 | Several packed in clear space | + | >> | | + | Thin and enlarged at their basis | |
| Superorder Veneroidea | | | | | | | | | |
| Superfamily Lucinacea | | | | | | | | | |
| Family Lucinidae | | | | | | | | | |
| <i>Lucina foridana</i> (Conrad) | Fisher & Alatolo (1984) | 1 | Packed assemblages numerous, | ? | >> | ? | + | ? | |
| <i>Myrica spinifera</i> (Montagu) | Dando et al. (1985) | 1 | | ? | >> | Microville | + | Partially or totally cover the bacteriocyte | |
| <i>Parvilucina tenuisculpta</i> (Carpenter) | Reid & Brand (1986) | 1 | Enclosed in vacuoles | ? | >> | >> | + | >> | |
| <i>Anodonta philippiana</i> Reeve | Giere (1985) | 1 | aggregation of bacteria enclosed in vacuoles | ++ | >> | >> | ? | Conical structures | |
| Family Thyasiridae | | | | | | | | | |
| <i>Thyasira flezuosa</i> (Montagu) | Southward (1986) | 1 | Apical mass, confined under <<cuticle>> | + | >> | >> | + | Single ciliated cells among the bacteriocytes | |
| <i>Thyasira</i> T1 | " | 2 | made up of tips of microvilli | + | >> | >> | - | >> | |
| Superfamily Macruraea | | | | | | | | | |
| Family Macruridae | | | | | | | | | |
| <i>Spisula subtruncata</i> (Da Costa) | Bouvy et al. (1986) | 2 | Packed in bacteriophore surrounded by a single membrane | ++ | FBSR | Plasma membrane | - | Partially cover the bacteriocyte | |
| Superfamily Glossacea | | | | | | | | | |
| Family Vesicomidae | | | | | | | | | |
| <i>Calyptrigena magnifica</i> (Boss & Turner) | Fiala-Médioni & Métyvier (1986) Cavanaugh (1985) | 1 | Dense concentrations isolated or grouped in spaces | + | All gill filament, except for the apical part | Microville | + | Intermediate cells among bacteriocytes | |

*Original terminology from literature citation because no standard terminology is now in use. **Observed in bacteria or bacteriocyte or other adjacent cells. + present ++ abundant, MBSR = many bacteriocytes, sublamellar region, FBSR = few bacteriocytes, sublamellar region

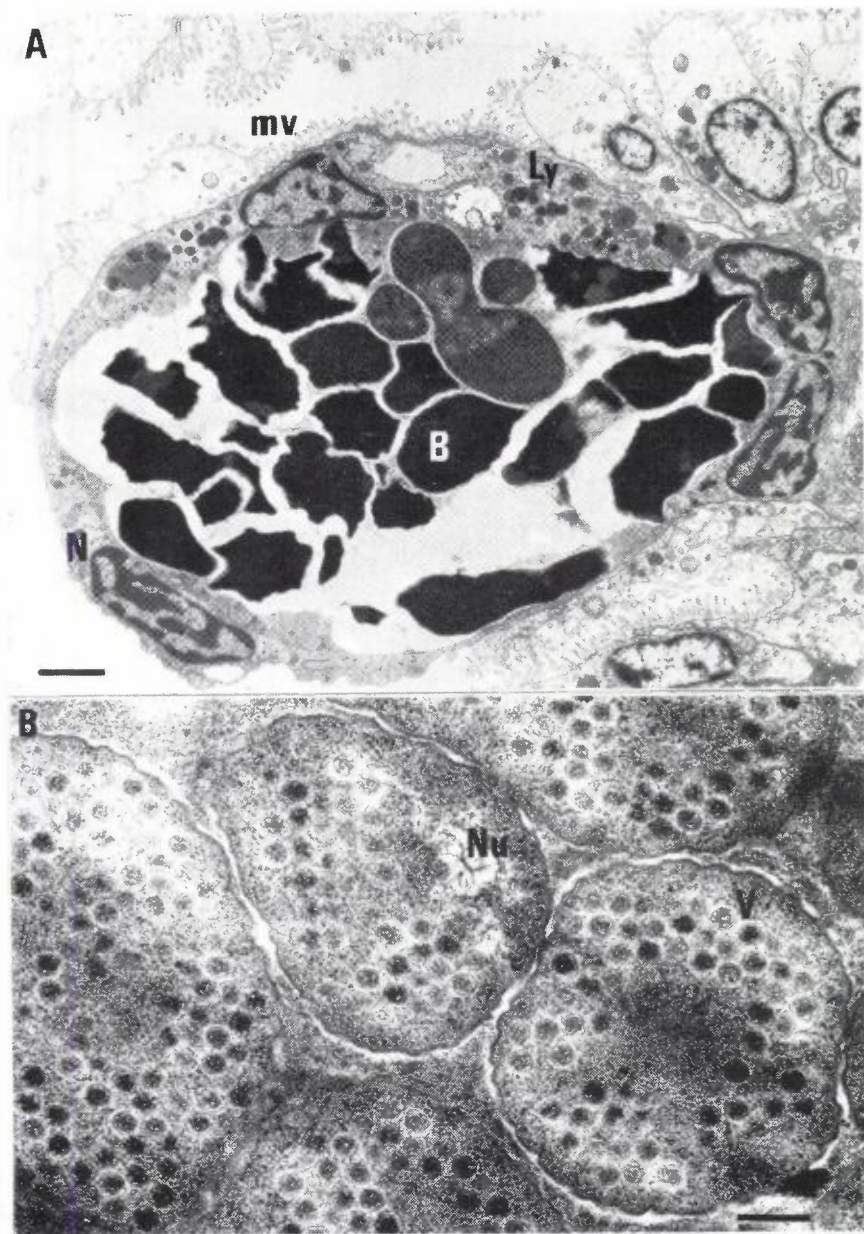


Figure 2. Fig. A: Type 1 bacteriocyte infected with bacteriophage. The size of bacteria (B) increased three or four times (mv: microvilli; Ly: lysosome; N: nucleus). $\times 8000$. bar = $1 \mu\text{m}$. Fig. B: High magnification of type 1 bacteria with numerous polyhedric virions (V). Observe the residual nucleoid (Nu). $\times 50000$. bar = $0.2 \mu\text{m}$.

Calvin-Benson cycle, and by measurement of the $^{14}\text{CO}_2$ fixation by gills exposed to $\text{NaH } ^{14}\text{CO}_3$ in the absence or presence of sodium thiosulfate and ammonium chloride. The finding that fixation is increased in *S. subtruncata* in the presence of both reduced nitrogen and sulfur compounds is very interesting because of the key role that sulfur metabolism commonly plays in associations between bacteria and invertebrates (Cavanaugh et al., 1981; Felbeck, 1981; Felbeck et al., 1981; Cavanaugh, 1983; Felbeck, 1983; Fisher and Hand, 1984; Vetter, 1985; Dando et al., 1985; Powell and Somero, 1985; Reid and Brand, 1986; Belkin et al., 1986; Dando and Southward, 1986; Dando et al., 1986a; Dando et al., 1986b; Southward et al., 1986; Fisher and Childress, 1986). In contrast, the utilization of nitrogen compounds (e.g., nitrite or ammonia) by endosymbiotic bacteria is only suspected (Felbeck et al., 1981; Fisher and Hand, 1984). Southward et al. (1986) suggested that the endosymbiotic bacteria of *Siboglinum atlanticum* (Pogonophora) could utilize reduced inorganic compounds other than sulfur and would thus be nitrifiers. In a recent paper, Dando et al. (1986b) have been unable to detect any evidence of nitrification in the symbiont-containing tissues. Our results in *S. subtruncata* provide the first evidence of a possible oxidation of ammonia as energy source by symbiotic bacteria. To confirm this, it would be necessary to attempt isolation experiments on the two types of bacteria from *S. subtruncata* and to study their specific metabolic properties.

Gills of *S. subtruncata* show generally lower values of ribulose biphosphate carboxylase activity and CO_2 uptake than those recorded in other symbiotic bivalve species. These low values could be related to the small number of endosymbiotic bacteria and suggest that the relative contribution to total nutrition made by chemolithotrophically-generated CO_2 fixation should be low.

Ecological studies show that *S. subtruncata* lives on beaches where wave energy is high. This clam is widespread in shallow sandy bottoms of the European coasts (Tebble, 1966; Massé, 1972). It occupies light silty sands or clean sands and gravels (Pérès and Picard, 1964; Guille, 1970; Massé, 1971, 1972), with low organic matter content but high turbidity (Massé, 1972). Furthermore, *S. subtruncata* can exploit suspended particulate material and has a high metabolism and is greatly sensitive to anoxia (Kiorboe and Mohlenberg, 1981; Mohlenberg and Kiorboe, 1981). It is therefore likely that suspended particulate is usually the main source of energy. But the balance between autotrophic and heterotrophic processes may be strongly related to the environmental conditions (Dando et al., 1986a; Dando et al., 1986b; Fiala-Médioni et al., 1986).

4. Implications for Bivalve Evolution

No direct evidence on the origin of symbiosis of this type is available, although bivalve groups (such as Solemyacea and Lucinacea) with chemoautotrophic bacteria have long geological histories (Allen, 1985) and "may be relics from times when the earth was largely anaerobic" (Allen, 1985, p. 371).

Looking at the bivalve families with bacterial symbioses, there is no clear relationship between the symbioses and the paleontological record of the family. Fossil forms of these families are first recorded from different geological times: Silurian for the Lucinidae, Devonian for the Solemyidae and the Mytilidae, Triassic for the Thyasiridae, Cretaceous for the Mactridae (Moore, 1969–1971) and Oligocene for the Vesicomidae (Boss and Turner, 1980). Even so, there are common primitive morphological features in the superfamilies Solemyacea and Lucinacea (Allen, 1985). Hickman (1984) identifies a distinct *Thyasira* — *Lucinoma* — *Solemya* community from Cenozoic deep-water deposits, similar to the extant one we observed. According to Reid and Brand (1986), sulfide-oxidizing symbiosis would provide the major evolutionary drive for the emergence of the Solemyacea and Lucinacea.

Paedomorphic events involving gills, palps, siphons and guts (Cavanaugh, 1985; Reid and Brand, 1986; Southward, 1986) are characteristic of five symbiont-containing bivalve families: Solemyidae, Mytilidae, Lucinidae, Thyasiridae and Vesicomidae. *S. subtruncata* belongs to the family Mactridae, one of the three major lineages of the superorder Veneroida within the adaptative radiation that has occurred since the Mesozoic (Allen, 1985). This species has conspicuously well-developed gills, palps or gut (Yonge, 1948). Nevertheless, the few bacteriocytes are located at the base of the inner part of the gill as in most other symbiotic bivalve species. Ultrastructural features suggest a high degree of integration of the endosymbiotic bacteria. Another lineage of the superorder Veneroida has an endosymbiotic association: Henry et al. (1981) described an association between bacteria and the cardiacean *Cerastoderma glaucum*. These two records suggest that this type of symbiosis may be widespread in marine bivalves not living in reducing sediments. It is however difficult to determine if such symbiosis developed as an adaptation to life in reducing sediments or if they might be considered as relics of an ancient history. Neither the anatomical features and the feeding habits of *S. subtruncata* nor the sediment chemistry and the characteristic of the environment can explain the occurrence of chemoautotrophic bacteria in the gills of this species.

5. Implications for the Origin of Bacteria-Bivalve Endosymbiosis

The occurrence of separated types of endosymbiotic bacteria in the same host, like in *S. subtruncata*, is unusual: generally only one type of bacteria is observed. However two morphological types of symbionts have been recorded in deep-sea thyasirids (Southward, 1986), apparently different species, mingled together in the same bacteriocyte. In their review of sulfide-oxidizing symbiosis in Lucinaceans, Reid and Brand (1986) postulated that only one, or a small number of free-living species of sulfide-oxidizing bacteria seems to have had the potential for endosymbiosis with sedentary marine invertebrates. In support of this view, Wilkinson (1984) demonstrated by immunological analysis that bacterial symbionts in sponges are closely related to one another within the phylum Porifera and argued that the endosymbiosis must have been an unique Precambrian event. From the results of ribosomal RNA sequences analysis, Stahl et al. (1984) concluded that the bacterial symbiont populations of the two clams *Calyptogena magnifica* and *Solemya velum* and those of the vestimentifera tube worm *Riftia pachyptila* were at least 90 percent homologous. Nevertheless the DNA base composition of symbionts in the vent bivalve *Bathymodiolus thermophilus* differs from that of the symbionts of *Riftia* (Belkin et al., 1986). The presence of two distinct types of bacteria in the same host like in *S. subtruncata* may indicate that the ability of bacteria to become endosymbiotic is more extensive than previously expected and that several bacterial strains are potential endosymbionts. The mechanisms of transmission of the gill symbiotic bacteria are unknown in bivalves. Three hypotheses may be advanced: (1) acquisition during the ontogenetic development of the clam; (2) acquisition of a mixed population of bacteria from the ambient environment followed by selection of only certain species or strains; (3) ancestral acquisition before the evolution of bivalves into the present-day classes and orders. However, Gustafson (1985, cited in Reid and Brand, 1986) assumes that in *Solemya reidi* transmission would be holobiotic, that is the eggs are already infected.

The number of known endosymbiotic relationships between bacteria and invertebrates has increased drastically since the original discoveries of Cavanaugh et al. (1981) and Felbeck (1981). Such symbioses occur in several species belonging to an increasing range of bivalve families. These species inhabit various environments with a large range of reduced compounds concentrations. The energy source for the symbiosis seems to be provided by different reduced compounds (sulfide, ammonia, nitrite, methane). But bivalve species with or without endosymbiotic bacteria can coexist in the same

environment. Further investigations should be extended to the widest possible spectrum of bivalves in particular (and even of invertebrates in general) (Thiriôt-Quévieux and Soyer, 1986) to clarify the evolutionary implications of endosymbiosis.

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