

Ultrastructure studies of two chemosynthetic invertebrate-bacterial symbioses (*Lamellibrachia* sp. and *Acharax* sp.) from the Hatsushima cold seeps in Sagami Bay, Japan

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Transmission electron micrographs prepared from the symbiont-containing tissues of two cold seep metazoans were used to explore the ultrastructure of trophosome and gill tissues from a vestimentiferan (*Lamellibrachia* sp.) and a protobranch bivalve (*Acharax* sp.) from cold seeps in Sagami Bay, Japan. Interpretation of cell ultrastructure focused on the potential modes of chemosynthesis by symbiotic bacteria and method of carbon transfer from symbionts to the host tissue. The characteristics of symbiont ultrastructure were similar to sulfide-oxidizing symbionts reported from other invertebrate-bacterial symbioses, with little evidence of symbiont morphologies typical of methane-oxidizing bacteria. Two different symbiont types were observed in trophosome tissue from a single individual of the vestimentiferan *Lamellibrachia* sp. Both symbiont types were ovoid to coccoid with sizes near 0.5 to 1 μm and 3 to 5 μm . Symbiont morphology of one type (type A) was very similar to sulfide-oxidizing symbionts reported from other vestimentiferans. Dense cytoplasm and much smaller size characterized the second symbiont type from *Lamellibrachia* sp. Gill tissue from an individual of the protobranch bivalve *Acharax* sp. had large bacteriocytes, packed densely with pleomorphic rod-like symbionts to 5 μm long. The size, shape, and cytoplasmic appearance of these symbionts are similar to sulfide-oxidizing symbionts from chemoautotrophic protobranch in the genus *Solemya*. For both symbioses, closely related species have been shown to release carbon products directly from symbiont to host, and may not rely on intracellular digestion of symbionts for symbiont carbon assimilation. Vacuoles in symbiont containing tissues of both host metazoans examined are unlike those observed in chemosynthetic species of vesicomyid clams and mytilid mussels, which may rely primarily on symbiont digestion as a means of carbon transfer. Degradation of symbionts in bacteriocytes in some trophosome regions in *Lamellibrachia* sp. suggests that symbiont digestion may also be important. Although our limited sample of TEM images from both seep metazoans are consistent with cell structure reported for other sulfide-oxidizing symbionts, additional study, including enzymatic and isotopic analyses, as well as greater replication of ultrastructural studies, are required to determine if sulfide-oxidation is the sole chemosynthetic mode of these species. Multiple chemosynthetic modes for the two symbiont types observed in *Lamellibrachia* sp. is also possible, as documented for symbionts in some chemosynthetic mytilid mussels from cold seep and hydrothermal vent locations.

Key words : ultrastructure, symbioses, cold seep, Sagami Bay, vestimentiferan, protobranch bivalve

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1. INTRODUCTION

Chemosynthetic symbioses involving bacteria and various metazoan phyla are common at hydrothermal vents, cold seeps, and other reducing environments. Although several reduced organic or inorganic compounds potentially support chemosynthetic invertebrate-bacterial symbioses, only reduced sulfur compounds and C_1 compounds, are documented to be involved in energy acquisition in such symbioses (Fisher, 1990). Sulfur-based symbioses are by far the most common chemosynthetic symbioses reported from natural systems, both in terms of taxonomic diversity and zoogeographic breadth. Sulfide-oxidizing bacteria are involved in symbioses with many molluscan species, particularly bivalves in the families vesicomidae, lucinidae, thyrsaridae, mytilidae, and solemyidae. All vestimentiferan tubeworms and most pogonophorans investigated are thought to have sulfide-oxidizing endosymbionts. C_1 -based symbioses are known only from about 7 metazoan species, all of which are thought to oxidize methane or methanol to support chemosynthetic carbon fixation.

The disparity between the frequency of sulfide and methane based symbioses worldwide, and the absence of chemosynthetic symbioses based on other reduced compounds is probably related to several factors. The similar oxidation potential of methane and sulfide (Anthony, 1982; Nelson and Hagen, 1995), but rarity of methane-based symbioses suggests that constraints on C_1 -based symbioses are linked to factors other than energy yield, such as the absolute concentration of methane in the environment or its biological availability. Energetic limitations of some compounds, owing to either low energy yield to support chemosynthesis or high energetic costs of transport, storage, or elimination of oxidation products may be important. Environmental concentrations of these reduced compounds must be sufficiently high and persistent to support symbiont and host throughout the host life span. Toxicity of sulfide or other compounds may reduce their utility for some symbioses. Unlike many sulfide-oxidizing species that have evolved abilities to bind sulfide, no metazoans are known that can bind methane, or otherwise concentrate methane above ambient environmental levels. For sulfide-binding species, this capability provides internal stores of sulfide that may function to buffer fluctuations in environmental sulfide that could otherwise limit symbiont productivity, as well as detoxifying sulfide. Lack of

methane-binding compounds reduces the capacity for a methane-buffering system and exposes methanotrophs to environmental fluctuations in methane supply, potentially limiting the range of suitable habitats worldwide (Barry et al., 1997).

For many chemosynthetic invertebrate-bacterial symbioses, however, little investigation of symbiont morphology or function has been performed, and for others, multiple symbiont types, including some with differing chemosynthetic pathways, have been shown to inhabit the same metazoan hosts. For example, both sulfide-oxidizing symbionts and methanotrophic or methylotrophic symbionts have been identified from the same individuals of *Bathymodiolus heckerae* and *B. brooksi* from the Gulf of Mexico (Gustafson et al., 1998), and *B. boomerang* from the Barbados accretionary complex (von Cosel and Olu, 1998), while other individuals of these same species may have only a single symbiont type. Dual symbiont types have also been detected in a hydrothermal vent gastropod (*Olgaconcha tufari*; Gal'chenko et al., 1992) and two species of vestimentiferans (*Ridgeia phaeophiale* and *R. piscesae*) from the NE Pacific hydrothermal vents (Burgh et al., 1989).

In this report, we investigate the ultrastructure of symbiont-containing tissues in two chemosynthetic symbioses from the Hatsushima cold seeps in Sagami Bay, Japan. Transmission electron micrographs (TEM) from the trophosome of *Lamellibrachia* sp. and gill tissues of *Acharax* sp. are used to assess symbiont morphology and other cytological features relevant in evaluating aspects of chemosynthesis at Sagami Bay cold seeps.

2. METHODS

Specimens of a vestimentiferan tubeworm, *Lamellibrachia* sp., and a protobranch bivalve mollusk, *Acharax* sp., used during this study were collected from the Hatsushima cold seeps, located at 1200 m depth in Sagami Bay, Japan (34° 59.9' N, 139° 13.6' E) during dives of the *Dolphin 3K* ROV, operated by JAMSTEC. The Hatsushima cold seeps have been described in detail by Hashimoto et al. (1989), Fujikura et al. (1995a,b), and Tsunogai, et al. (1996). The fauna of Sagami Bay cold seeps includes a diverse assemblage of chemosynthetic metazoans. Vesicomid clams (mainly *Calyptogena soyoeae*) are most abundant, covering many hectares of the sea floor. Mytilid mussels (*Bathymodiolus platifrons* and

other congeners; Hashimoto & Okutani, 1994), protobranch bivalves (*Acharax johnsoni*, and perhaps other congeners), and vestimentiferan tubeworms (*Lamellibrachia* sp. and *Escarpia* sp.) are the most common chemosynthetic species. Many species of non-chemosynthetic benthic megafauna are also found at these seeps.

Specimens of *Lamellibrachia* sp. were collected during ROV dives from groups of tubeworms near aggregations of vesicomyid clams or near rocky outcrops where chemosynthetic mytilid mussels were found. *Acharax* sp. are generally found beneath the sediment surface and were probably mixed with other species collected with vesicomyid clams. For each collection, animals were placed in the sample drawer and covered with sediment to insulate them from warm surface temperatures during recovery of the submarine. Upon recovery of the ROV to the ship, specimens were sorted, dissected, and preserved for microscopic investigation.

2.1 Electron Microscopy

Following dissection, samples of gill tissue from a single individual of *Acharax* sp. and trophosome tissue from a single *Lamellibrachia* sp. were preserved in cold 0.1M cacodylate-buffered glutaraldehyde (2%) in filtered seawater for ultrastructural studies. Tissues prepared for transmission electron microscopy (TEM) were treated using microwave techniques to expedite and enhance processing (Giberson et al., 1997).

3. RESULTS & DISCUSSION

3.1 *Lamellibrachia* sp.

TEM images of trophosome tissue from *Lamellibrachia* sp. revealed a heterogeneous distribution of muscle, blood vessels, and symbiont-containing bacteriocytes and accessory cells. Some regions of the trophosome were dominated by muscle, with few or no bacteriocytes. Adjacent areas have bands of bacteriocytes and blood vessels or other haemolymph space. Two distinct symbiont types were present in high densities within different bacteriocytes. In some bacteriocytes, the cell margin adjacent to blood spaces was microvillous. The margins of other bacteriocytes were adjacent to apical cells with numerous vacuoles filled with glycogen-like material apparently resulting from carbon transfer from symbionts or symbiont digestion. These apical cells were juxtaposed with a microvillous membrane of haemolymph. Numer-

ous lysosome-like structures (dark bodies in Figure 1) and large vacuoles were also common in some bacteriocytes or within adjacent intercalary cells in some regions of the trophosome. These lysosome-like bodies were most common near vacuoles containing a homogeneous electron translucent material unlike that observed in apical cells filled with glycogen-like material, but which may also be involved in carbon transfer from symbiont to host.

Two types of endosymbiotic bacteria were apparent in trophosome tissues. The most abundant and largest symbionts (designated type A) were coccoid to ovoid symbionts 3 to 5 μm in diameter, present in high densities in bacteriocytes distributed in some sections of trophosomal tissues (Figures 1a, b). Smaller ($\sim 1 \mu\text{m}$) ovoid symbionts (designated type B) occupied bacteriocytes in other regions of the trophosome (Figures 1c, d). We did not observe three symbiont types, as reported for trophosome tissues of *Lamellibrachia* sp. collected from the same site by Naganuma et al. (1997), perhaps due to the limited scope and sampling size of our studies. Nucleated bacteriocytes for both symbiont types were most abundant near haemolymph spaces.

Type A symbionts have a granular and fairly uniform cytoplasm (Figure 1b) with a few light membrane-bound vesicular structures or invaginations, and dark bodies. As suggested for other chemoautotrophic bacterial symbionts (Childress et al., 1993), these vesicular invaginations were probably filled with elemental sulfur deposits prior to processing for TEM. Membranous filamentous structures visible inside bacteriocytes are very likely degradation products, resulting from intracellular digestion of symbionts. Dark, fine-grained material in the cells are very similar in appearance to material thought to be glycogen in other vestimentiferans (Burgh et al., 1989), but probably also include ribosomes or ribosomal proteins. Similar structures have been observed in several other chemoautotrophic symbioses (e.g. Fisher and Childress, 1992).

Smaller symbionts (type B) were enclosed in different bacteriocytes than type A symbionts, but were also found near haemolymph spaces or blood vessels near type A bacteriocytes (Fig. 2). These symbionts were small ($\sim 1 \mu\text{m}$), and distributed throughout bacteriocytes as single cells or aggregated in bundles of cells. Symbionts had generally finely grained and mostly homogeneous cytoplasm with fine-grained dark bodies and finely divided stacked membranes near the margins of some cells.

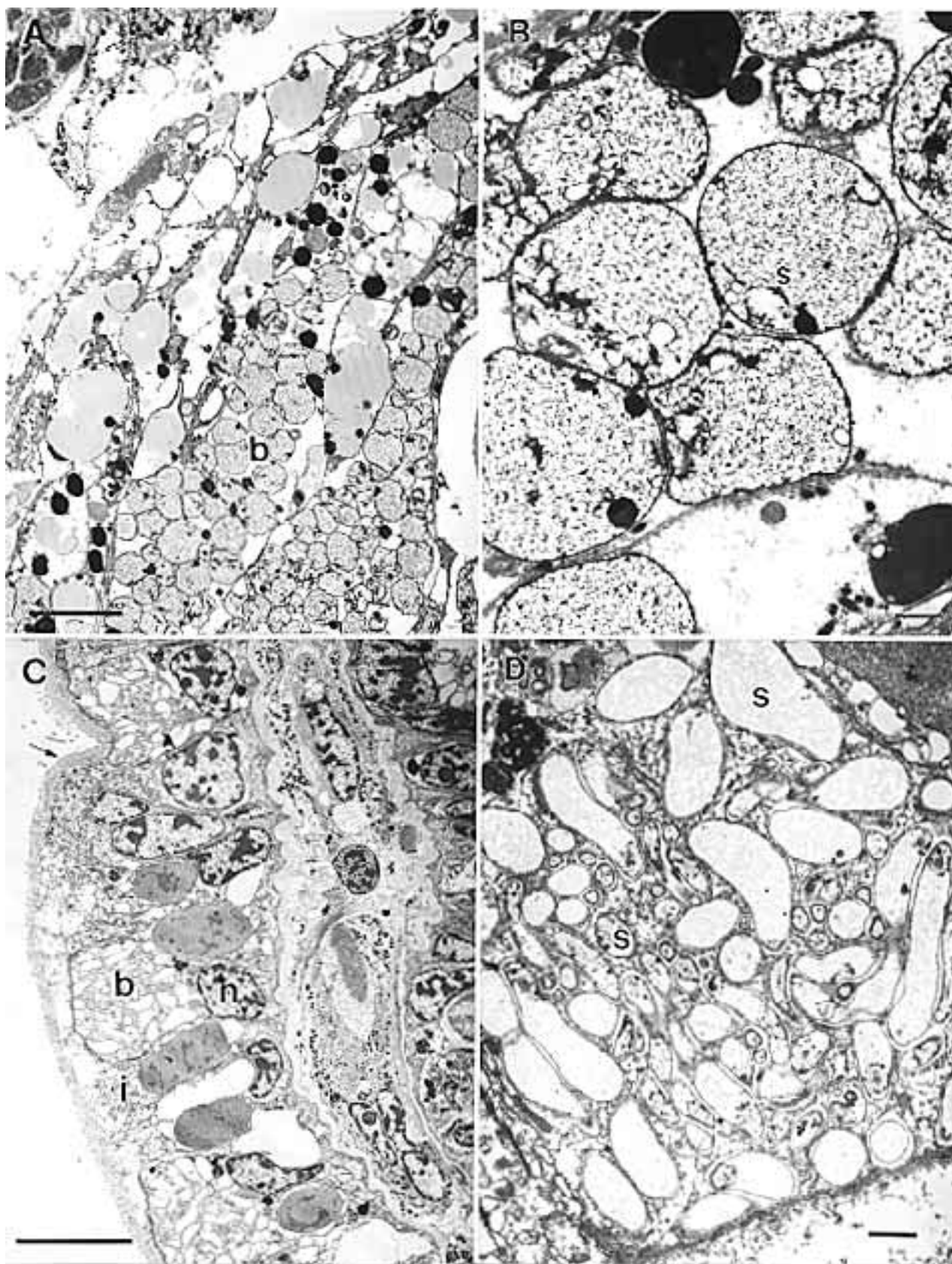


Fig. 1 Transmission electron micrographs of *Lamellibrachia* sp. and *Acharax* sp. from the Hatsushima cold seeps in Sagami Bay, Japan. A & B. *Lamellibrachia* sp. A. Trophosome with two bacteriocytes (b) containing type A symbionts. B. Type A endosymbiotic, chemoautotrophic bacteria (s) inside bacteriocyte. C & D. *Acharax* sp. C. Cross section of *Acharax* sp. gill illustrating bacteriocytes (b), their respective nuclei (n), intercalary cells (i) and microvilli (arrow). D. Close view of bacteriocyte showing cocci-shaped endosymbiotic bacteria (s). Scale bars = 10 μ m for Figs. A & C, 1 μ m for Figs. B & D.

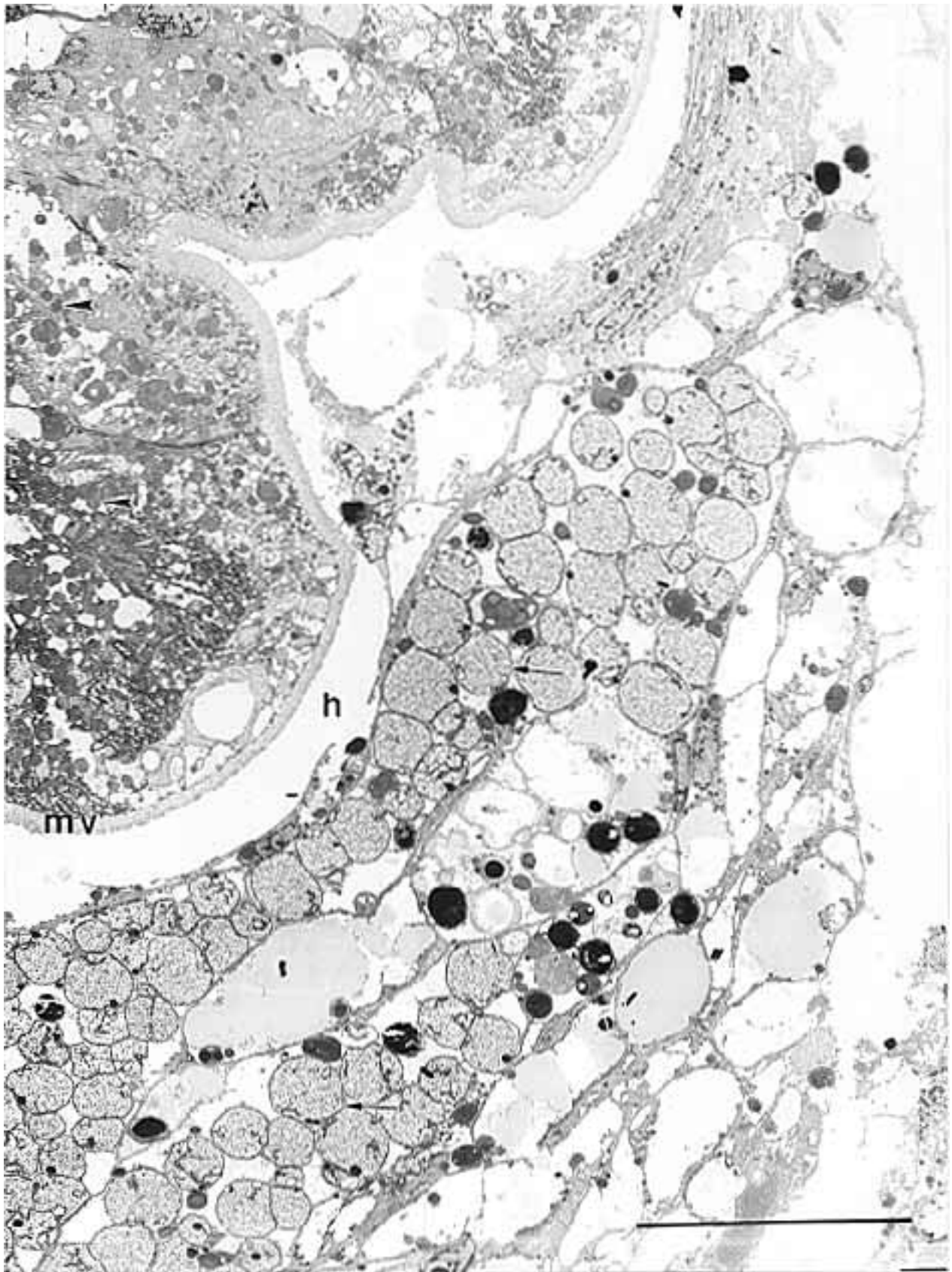


Fig. 2 Transmission electron micrograph of a section of the *Lamellibrachia* sp. trophosome. This section includes different trophosomal regions housing bacteriocytes with type A symbionts (arrows) and type B symbionts (arrowheads) separated by the haemolymph space (h) with microvilli (mv) along one edge. Scale bar = 10 µm.

Bacteriocytes housing type B symbionts often contained large vacuoles (Fig. 2).

Type A symbionts from *Lamellibrachia* sp. are very similar in general appearance to sulfide-oxidizing symbionts documented from *Escarpia spicata* (Cavanaugh, 1985) and *Riftia pachyptila* (Cavanaugh et al., 1981). The smaller (2–3 μm) symbionts of *Escarpia* also have a bacterial cell envelope similar to that observed in *Lamellibrachia* sp. *R. pachyptila* symbionts are also ovoid, with a granular cytoplasm containing a few vesicular invaginations of the bacterial cell wall. Stacked intracytoplasmic membranes reported from *R. pachyptila* symbionts (Cavanaugh et al., 1981) were not observed in type A symbionts in *Lamellibrachia*. Such membranous structures were, however, observed in type B symbionts of *Lamellibrachia* sp.

Several features of the ultrastructure of trophosome tissue of the vestimentiferan *Lamellibrachia* sp. observed in TEM images were similar or analogous to those reported for other vestimentiferans. Although we observed two symbiont types, neither has the appearance of free-living or symbiotic methanotrophic or methylotrophic bacteria, suggesting that sulfide oxidation is the principal energetic pathway to support chemosynthesis. It remains possible, however, that the physiology of type B symbionts differs from that reported for most vestimentiferan symbionts. The presence of two symbiont types has also been reported from *Ridgeia piscesae* from the Endeavor Segment of the Juan de Fuca ridge (Burgh et al., 1989). One symbiont from *R. piscesae* is generally similar to the type A symbionts we observed in *Lamellibrachia* sp. Although the scope of our survey of cell structure was very limited, we observed considerable variation in the size of both symbiont types, and did not detect a gradation of increasing symbiont size towards the central region of the trophosome or the larger haemolymph space. Small type B symbionts were observed adjacent to the microvillous margin of some bacteriocytes. Changes in the character of bacteriocytes, including regions of possible symbiont degradation or accumulation of glycogen or other metabolic products, were observed near the microvillous margin of some haemolymph spaces or blood vessels.

3.2 *Acharax* sp.

Gutless protobranch bivalves in the family solemyidae depend on endosymbiotic chemoautotrophic bacteria for

their nutrition. These animals inhabit methane- and sulfide-rich habitats such as cold seep muds, sewage effluent sites, and sea grass beds where they burrow into the sediment. At cold seeps in Monterey Bay, California, USA and Sagami Bay, Japan, solemyid bivalves are rare compared to vesicomyid clams, and often more cryptic owing to their deeper life position in the sediment. Their deep position in the sediment places them in close association with high sulfide levels of interstitial fluids, while maintaining contact with oxygenated seawater through mucous-lined burrows (Reid, 1980).

Sulfur-based chemoautotrophic symbiotic bacteria have been documented from gill tissues of all species of solemyids investigated to date. Unlike vesicomyid bivalves, a capacity to bind sulfide, thereby detoxifying sulfide and elevating internal sulfide levels to support chemoautotrophy, has not been demonstrated for protobranch bivalves. Instead, some species (e.g. Solemyidae - *Solemya reidi*; Lucinidae-*Lucinoma floridana*) oxidize sulfide to a non-toxic oxidation product, thiosulfate (Powell and Somero, 1985; Anderson, 1993), which is then transported to gill tissues for further oxidation to support bacterial chemosynthesis.

TEM images of gill tissues from *Acharax* sp. from Sagami Bay are very similar in appearance to gill tissue preparations from other solemyids (Cavanaugh, 1985; Krueger et al., 1996; Krueger & Cavanaugh, 1997). All have pleomorphic, but mostly rod-shaped symbionts approximately $1 \times 5 \mu\text{m}$ in size, held in bacteriocytes near microvillous membranes at the margin of the gill filament (Figures 1c, d). No ultrastructural evidence suggesting chemosynthetic pathways other than sulfide oxidation were observed. As observed in *Solemya pusilla*, microvillous surfaces adjacent to bacteriocytes in *Acharax* sp. are extensions of intercalary cells nearly surrounding bacteriocytes. Symbionts are most abundant and densely packed within bacteriocytes near the outer portion of the cell (toward the microvillous surface) and the cell nucleus is generally distal (Figure 1c). The largest symbionts are filled with moderately dense, clumped, material that may be glycogen, as suggested for other solemyids (Krueger and Cavanaugh, 1997). Lysosomes and peroxisomes appear to be less abundant or perhaps more highly localized in bacteriocytes of *Acharax* sp., compared to *Lamellibrachia* sp. trophosome.

3.3 Modes of Carbon Transfer from Symbiont to Host

There are two potential modes of carbon transfer from symbionts to host in chemosynthetic symbioses, which may be employed simultaneously. First, symbionts may transfer intermediate carbon fixation products directly to the host via excretion. Second, harvesting of symbiotic bacteria by intracellular digestion may provide nutrition to the host. Direct measurements via time series tracking of the uptake and transfer of radiolabeled carbon is rare (e.g. Fisher and Childress, 1986, 1992), owing largely to the difficulty in tracking carbon transfer. Without such measurements, however, some inference concerning modes of carbon transfer may be possible from the ultrastructure of the symbiont-containing tissues.

The general structure of symbiont containing tissues and the cellular machinery to process and transfer carbon between symbiont and host are expected to be quite similar among closely related taxa (e.g. *Lamellibrachia* sp. and other vestimentiferans). However, similar ultrastructure among distantly related taxa suggests that related cellular processes are involved. Ultrastructural details of the trophosome from *Lamellibrachia* sp. differ in many ways from symbiont-containing tissue from chemoautotrophic clams (*Calyptogena* sp.; Fiala-Médioni et al., 1993) or methanotrophic mytilid mussels (Childress et al., 1986), but to our surprise, had some similarities to *Acharax* sp. gill tissue. This pattern may be related to potential overlap in modes of carbon transfer.

For both vestimentiferans and solemyid protobranch bivalves, direct carbon transfer may be the dominant nutritional link between symbiont and host. Although the sample size is very low, at least one species in each taxon is thought to release anabolic products directly. Rapid carbon transfer from symbionts to host in *Solemya reidi* has been measured using radio-labeling experiments (Fisher and Childress, 1986), and direct release of glutamate and perhaps other anabolic compounds has been detected in *Riftia pachyptila* (Felbeck and Jarchow, 1998). The large intracellular vacuoles in bacteriocytes or intercalary cells in *Lamellibrachia* sp. and *Acharax* sp. that contain fine-grained material are similar to structures observed in confamilial species, but have no clear analog in vesicomysids or chemosynthetic mytilid mussels, and may function in carbon transfer. The large vacuoles along the apical margins of bacteriocytes or intercalary cells of *Lamellibrachia* sp. or *Acharax* sp. may be storage areas

for anabolic compounds from symbionts, prior to translocation to host tissues. If direct carbon transfer, rather than intracellular symbiont digestion, is the dominant mechanism of carbon transfer from symbiont to host, lysosome densities within bacteriocytes may be expected to be low compared to species relying principally on symbiont digestion. Thus, low lysosome densities and other general ultrastructural similarities between *Acharax* sp. and *Solemya reidi* suggests a similar mode of carbon transfer.

Alternatively, these sites may function to store catabolic products of intracellular symbiont digestion, or perhaps products from both metabolic sources. Although we did not observe large areas of bacteriocytes dominated by symbiont digestion in *Acharax* sp., degradation products of symbionts were evident within bacteriocytes and may also be transferred to marginal vacuoles, prior to translocation to other host tissues. Bosch and Grassé (1984) and Burgh et al. (1989) proposed for some vestimentiferan species that harvesting of symbiont populations is the primary nutritional mode. Bacteriocytes in some regions of *Lamellibrachia* sp. trophosome were filled with 'healthy' symbionts, while other regions have much lower symbiont densities, higher densities of lysosome-like bodies and secondary lysosomes containing degrading symbionts, supporting the idea that symbiont digestion is nutritionally important. Moreover, the apical vacuoles in *Lamellibrachia* sp. may also result from efficient digestion of symbionts - lack of obvious degradation products such as membranous structures is not sufficient evidence to discount symbiont digestion as an important nutritional mode. Thus, although some evidence for direct carbon transfer exists, symbiont digestion may be equal or greater in importance to energy transfer between symbiont and host.

Unlike *Lamellibrachia* sp. and *Acharax* sp., the ultrastructure of gill tissues from *Bathymodiolus childressi*, a methanotrophic mussel thought to depend entirely on symbiont digestion (Fisher and Childress, 1992), and several species of vesicomysid clams (Barry et al., unpublished), have relatively large regions of bacteriocytes involved in symbiont digestion. Bacteriocytes have vacuoles or secondary lysosomes filled with degraded or degrading bacteria, but lack large, marginal vacuoles filled with glycogen-like material or other compounds more likely associated with direct carbon transfer. The similar patterns of large areas of bacteriocytes in the process of intracellular symbiont digestion in *B. childressi*, other *Bathymodiolus*

sp. and vesicomyid clams, suggest common carbon uptake mechanisms that contrast with features observed in *Lamellibrachia* sp. and *Acharax* sp.

4. CONCLUSIONS

For both *Acharax* sp. and *Lamellibrachia* sp., the ultrastructure of symbiont-containing tissues supports the hypothesis that chemosynthesis in both groups is supported by sulfur-based chemoautotrophy, rather than methane-based. Further study is required to determine whether these metazoans are involved with symbioses based on other modes of chemosynthesis. Additional specimens from more than one site should be examined, preferably with studies of environmental chemistry to determine causes of variability, if any, in these invertebrate-bacterial symbioses. A more thorough investigation of differences in the function and importance of dual symbionts in *Lamellibrachia* sp. will also require additional study of numerous individuals. Inferring the function of ultrastructural features in organisms such as *Lamellibrachia* sp. and *Acharax* sp. is wrought with uncertainty. These rare creatures from extreme environments with unusual anatomy and physiology have not been studied in detail, and until more comprehensive studies involving a spectrum of physiological, molecular, environmental and ultrastructural measurements and observations is complete, conclusions concerning based on the limited observations reported here remain speculative.

The large vacuoles within trophosome tissues in *Lamellibrachia* sp. filled with homogeneous glycogen-like material or other compounds, suggest a potential role in direct transfer of carbon fixation production from symbiont to host. Other features of *Lamellibrachia* sp. bacteriocytes, such as numerous degrading symbionts within bacteriocytes, support the hypothesis that symbiont harvesting may be important in carbon transfer. Gill tissues of *Acharax* sp. are very similar to other solemyids studied, and may rely largely on direct carbon transfer, even though the products of symbiont digestion are evident in small areas throughout bacteriocytes.

5. ACKNOWLEDGEMENTS

We would like to thank JAMSTEC for dive time and logistic support in relation to this project. Assistance from the ship's crew of the R/V *Natsushima* and the pilots and operators of the *Dolphin 3K* ROV were very helpful dur-

ing dive operations. Additional funding was provided by the Monterey Bay Aquarium Research Institute.

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(Manuscript received 17 December 1999)