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TEM Observation Studies on the Chemoautotrophic Symbiotic Bacteria of Invertebrates Inhabiting at Vents and Seeps

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Abstract: Chemoautotrophic symbiotic bacteria of organisms inhabiting the hydrothermal vent and cold seep communities in the deep-sea were histologically examined using a transmission electron microscopy on symbionts of *Calyptogena* sp. A from the site east off Miyako (water depth at 1,700m), *Calyptogena* sp. B from the *Calyptogena* Site , vestimentiferan tube worm *Lamellibrachia* sp. A from Sagami Bay *Lamellibrachia* sp. B from *Calyptogena* Site of the Iheya Ridge, pogonophoran tube worms from Sagami Bay and *Calyptogena* Site of the Iheya Ridge, *Bathymodiolus* spp. from Sagami Bay, the Iheya Ridge and the North Fiji Basin. Based on the morphological microscopic observations, two species of *Calyptogena* from Miyako and the Iheya Ridge, two species of vestimentiferan tube worms from Sagami Bay and the Iheya Ridge, and pogonophoran tube worms from Sagami Bay and the Iheya Ridge observed to host sulfur-oxidizing symbiotic bacteria. The occurrence of chemosynthetic symbionts in these organisms was expected beforehand based on the ecological observations of their habitats. Other members of these groups from the world oceans, and the recent advances in the symbiosis at vents and seeps were reviewed.

1. Introduction

Endosymbiotic association of bacteria with eukaryotic hosts is a widely displayed survival mechanism and well known in nature. Since the discovery of hydrothermal vents in 1970 , chemoautotrophic and methanotrophic bacteria have been added to the collection of bacterial endosymbionts (Felbeck 1981; Cavanaugh *et al* 1981, 1987; Fisher *et al* 1994; Nealson and Fisher 1995).

The entry of non-pathogenic or parasitic bacteria into host cells largely relies on phagocytic ability of the host cells (Smith 1979). And, De Burgh and Singla (1984) first found the phagocytic activity in the gill epithelial cells of an exosymbiont-bearing hydrothermal vent limpets from the Juan de Fuca Ridge. Southward (1986) reported the phagocytic incorporation of exosymbiotic bacteria in the gill epithelial cells of several thyasirid bivalves. In both cases, bacteria engulfed by host cells had rapidly undergone destruction by lysosome fusion, and stable endosymbiotic associations could not be established.

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The chemolithoautotrophic and methanotrophic symbiosis are associations established between these bacteria and marine invertebrate hosts. It appears that the bacterial symbionts in all of the described chemoautotrophic associations use some forms of reduced sulfur as an energy source, albeit there is no a priori reason not to suppose that other types of chemoautotrophic symbiosis may be involved.

It must also be noted that since no confirmed chemoautotrophic or methanotrophic symbionts have yet been cultured in vitro, perhaps the most unique animal found in this ecosystem was the giant tube-worm, *Riftia pachyptila*, It is a gutless animal which first lead investigators to look for the presence of symbiotic chemoautotrophs in this animal. The assumption made was that autotrophic symbionts would contribute to the host carbon needs. In 1981, Felbeck (1981) reported on the presence of high activities of several enzymes from the Calvin-Benson cycle and bacterial sulfur metabolism in the trophosome of *R. pachyptila*. In addition, Cavanaugh *et al.* (1981) reported that this organ was packed full with intracellular prokaryotic cells of chemoautotrophic sulfur bacteria. These two papers represented the first demonstration of chemoautotrophic bacterial symbiosis with a marine invertebrate. In 1986, the first documented methanotrophic symbiosis was reported from a mussel, *Mytilidae* gen. sp., from hydrocarbon seeps in the Gulf of Mexico (Childress *et al.* 1986).

Bacterial endosymbionts using reduced sulfur compounds as energy sources and fixing CO_2 by means of Calvin-Benson cycle have been suggested as primary producers for the food web of the deep-sea hydrothermal vent community. These bacteria are found in the gills of several bivalves and within the trunk of the vestimentiferan tube worms that inhabit near the vents (Cavanaugh 1983; Fisher 1996). Since the initial discovery, such symbioses have been found in a variety of other taxa (Cavanaugh 1985; Felbeck *et al.* 1981) as well as in a variety of other habitats (Schweimanns and Felbeck 1985; Paull *et al.* 1984; Kennicutt II *et al.* 1985) characterized by the availability of both reduced sulfur compounds and O_2. Shortly after the initial discovery of these symbioses, investigators began to look for symbiosis based on other reduced compounds found in these environments. This paper presents evidences of sulfur and methane-based symbiosis in hydrothermal vent and cold seep between animals and intracellular bacteria collected from the Iheya Ridge off Okinawa and from the Miyako Ridge, northern Japan.

2. Materials and Methods

Calyptogena spp. were sampled by the deep-sea submersible HINKAI 2000 during the dive #480 (16 May, 1990; 273.0 ; 1268.2 E: 1,360-1,405m) from the Iheya Ridge off Okinawa (Fig. 1) and R.V. Tansei Maru KT-90-08 (17 June, 1990, 1399.13 ; 14250.75 : 1,698-1,715m) from the east off Miyako, northeast Japan.

Samples of vestimentiferan tube worms were collected during Dives #381 and #515 from the Hatsushima Site, Sagami Bay (for the detailed explanations of the site, see Hashimoto *et al.* 1989; Ohta 1990a) and Dive #410 from the *Calyptogena* Site n the Iheya Ridge, Okinawa Trough (Ohta 1990b) by the HINKAI 2000 (Fig. 2). Mussels were sampled during Dives #481 and #542 from the Okinawa Trough and the Minami Ensei Knoll also by the HINKAI

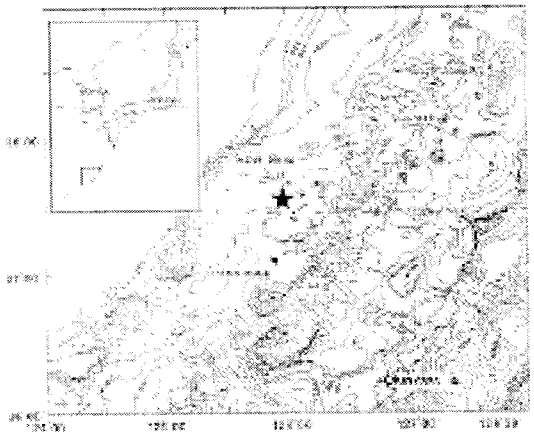

Fig. 1. Bottom configuration of the Okinawa Trough in Japan. Bottom contour intervals are 200 m.

2000 (for the detailed explanations of these sites, see Hashimoto *et al* 1990).

The provannid gastropods, *Ifremeria nautilei*, were collected during Dive #STARMER10 of the French submersible Nautile, and Dives #77 and #80 of the HINKAI 6500 on a central rift system in the North Fiji Basin at a depth around 1,980 m (Table 1).

Gill samples were fixed with 2.5% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4. After rinsing in the same buffer, they were post-fixed in osmium tetroxide (final concentration 1%). Specimens were dehydrated in a series of graded ethanol followed by propylene oxide, embedded in Epon 812, and sectioned for light and transmission electron microscopy (TEM). The sectioned material was stained with Toluidine Blue for light microscopy and by uranyl acetate and lead citrate for transmission electron microscopy. Jeol 100CX transmission electron microscope was used for observations.

3. Results

Calyptogena sp. A from the Iheya Ridge

The seemingly hypertrophy or enlargement of the gills, related to the development of the bacteriocyte parts appeard to be a general feature of the genus *Calyptogena*. Observation on the transverse thin sections through the gill filaments of this species confirmed the general

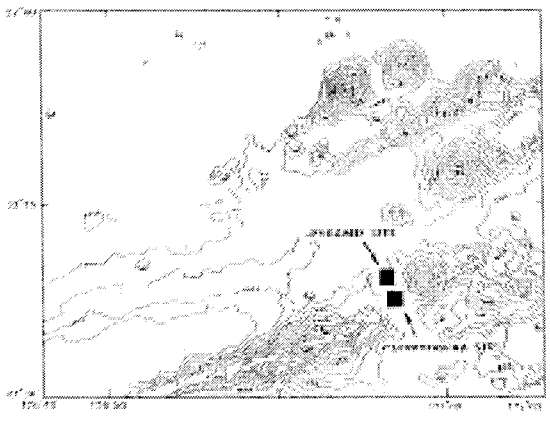

Fig. 2. Detailed bathymetry map of the Iheya Ridge, Mid-Okinawa Trough, Japan.

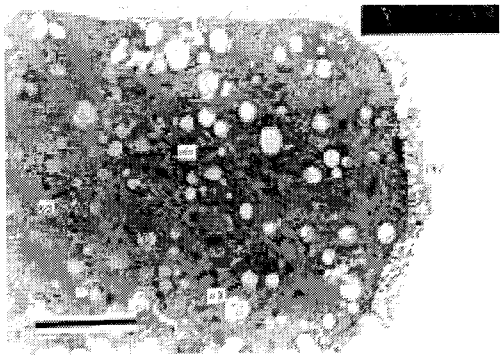

Photo 1. TEM micrograph of the bacteriocyte in the gill tissue of *Calyptogena* sp. from the Iheya Ridge. ed: electron-dense material; mv: microvilli; sb: symbiont bacteria; va: vacuole. scale bar: 5 μm.

structure which was similar to those of *C. magnifica* (Fiala-Medioni and Metivier 1986).

In cross-sectional views, the gills of *Calyptogena* sp. A were composed of two distinct parts, i.e., both extremities of the filament comprised of ciliated cells and some mucus cells, and major parts were occupied by dense cells bearing symbionts. Fine sections of the extremity of a filament revealed a typical distribution of the frontal cilia and the lateral cilia emerging from fusiform cells. These cells had a well developed nucleus and high density of mitochondria. Most of the central part of the gill filaments were composed of a unicellular row of bacteriocytes filled with bacteria (Photo 1). Intermediate cells were sometimes found among the bacteriocytes. External surfaces of each bacteriocyte were fringed by well developed microvilli. These bacteriocytes were nearly filled with coccoid Gram-negative type of bacteria, i.e., double cell walls. Although the endocellular bacteria of the gills had rather irregular outlines due to numerous vacuoles, they were basically alike in size, structure and cell division mode, of the sulfur-oxidizing bacteria found in *C. soyoae* (Endow *et al.* 1987; Kim unpublished data). The presence of abundant elemental sulfur crystals in the gills observed under the light microscopy supported the hypothesis of an active sulfur-oxidizing metabolism associated with these bacteria. They were elliptical in shape

Table 1. List of samples observed by transmission electron microscopy (TEM) and light microscopy.

Station or Dive No.	Vessel or Submersible	Date	Locality	Position	Depth	Specimen
SR.127	R/V Tansei Maru	17 June 1990	E off Miyako Sanriku	39°18.73 N 142°50.3 E	1700m	*Calyptogena* sp. A
# 381	SHINKAI 2000	05 Nov. 1988	Hatsushima Sagami Bay	35°00.0 N 139°13.5 E	1170m	*Lamellibrachia* sp.
# 381	SHINKAI 2000	05 Nov. 1988	Hatsushima Sagami Bay	35°00.0 N 139°13.5 E	1170m	Pogonophora gen. sp.
# 410	SHINKAI 2000	11 June 1989	Calypt. Site Iheya Ridge	27°32.94 N 126°58.20 E	1400m	*Lamellibrachia* sp. B
# 480	SHINKAI 2000	16 May 1990	Calypt. Site Iheya Ridge	27°32.94 N 126°58.20 E	1400m	*Calyptogena* sp. B
# 480	SHINKAI 2000	16 May 1990	Calypt. Site Calypt. Site	27°32.94 N 126°58.20 E	1400m	*Calyptogena* sp.
# 480	SHINKAI 2000	16 May 1990	E off Miyako Iheya Ridge	27°32.94 N 126°58.20 E	1400m	Pogonophora gen. sp.
# 481	SHINKAI 2000	17 May 1990	Pyramid Site Iheya Ridge	27°33.03 N 126°58.15 E	1400m	*Bathymodiolus* sp.
# 515	SHINKAI 2000	06 Nov. 1990	Hatsushima Sagami Bay	35°00.0 N 39°15.14 E	1170m	Pogonophora gen. sp.
# 542	R/V Tansei Maru	26 May 1991	Minami Ensei Okinawa Tr.	28°23.5 N 127°38.5 E	700m	*Bathymodiolus* sp. B
STA10	NAUTILE	05 July 1989	North Fiji B.	16°58.67 S 173°54.96 E	1975m	*Ifremeria nautilei*
# 77	SHINKAI 6500	07 Sep. 1991	North Fiji B.	16°58.67 S 173°54.96 E	1975m	*Ifremeria nautilei*
# 80	SHINKAI 6500	11 Sep. 1991	North Fiji B.	16°59.46 S 173°54.87 E	1965m	*Ifremeria nautilei*

and measured about 0.9 to 4.3 ㎛ in longer diameter (transverse sections). Some of the bacteria contained electron-transparent vacuoles in their periplasms and within the cells. Electron-dense materials were also observed in the periplasm among some bacteria.

Calyptogena sp. B from the Miyako Ridge

The gills of this species were also hypertrophied, and the cells within the gills were mostly composed of bacteriocytes with low densities of coccoid Gram-negative bacteria (Photo 2). They were also ellipsoid in shape and measured 0.8 to 3.7 ㎛ in longer diameter. However these bacteria contained no electron-transparent vacuoles in their periplasms. They grouped inside peribacterial membranes, indicating similar internal structures. They seemed to divide in a similar manner as that previously observed in *C. magnifica* (Fiala-Medioni and Metivier 1986).

Vestimentiferan Tube Worm (*Lamellibrachia* sp.) from Sagami Bay

Trophosome organs occupied the major part of the coelom of the observed vestimentiferan species, and reproductive organs were also embedded in these tissues. Symbiotic bacteria were enclosed (usually individually) within a membrane that was presumably produced by the host cell, forming a vacuole-like structure (Photo 3). These membranes were often seen as extra layers around the bacterial cell wall and not generally seen in the observed vestimentiferan as compared with small pogonophorans from the Okinoyama Site. Symbionts of these tube worms had Gram-negative type cell walls.

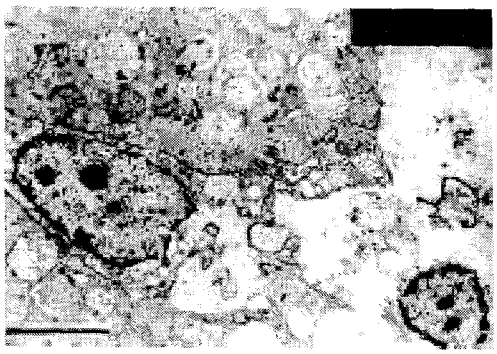

Photo 2. TEM micrograph of the bacteriocyte in the gill tissue of *Calyptogena* sp. off Miyako, Northeastern Japan. mi: globular mitochondria; n: nucleus of the bacteriocyte; sb: symbiont bacteria. scale bar:

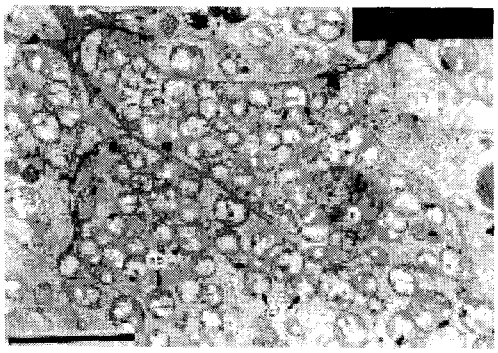

Photo 3. TEM micrograph of the trophosome of vestimentiferan tube worm (*Lamellibrachia* sp.) from Sagami Bay. Symbiont bacteria in this photo are round and smaller than those of the following photo. ed: electron-dense materials; n: nucleus of the bacteriocyte; pf: pale fenestrations; sb:

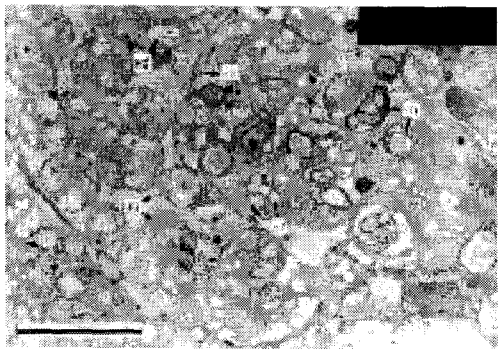

Photo 4. TEM micrograph of the trophosome of the same individual of the vestimentiferan tube worm (*Lamellibrachia* sp.) from Sagami Bay. The symbiont bacteria have lamellate membrane structure (1 m). ed: electron-dense material; gg:

Symbionts within the cells were categorized morphologically into two distinct types (Photo 3 and 4). The first class of symbionts were larger, basically round bacteria, and tightly-packed in groups of by threes or fives in a bacteriocyte and in the shape of polygonal. They were, if liberated from the cells, coccoid in shape ranging from 2.5 to 7.3 μm in diameter. Glycogen-like granules were uniformly scattered and pale fenestrations were seen (pf in Photo 4) relatively smaller. Larger types of this species had dense, elaborate internal membrane systems reminiscent of lysosomes.

Another type of the symbionts was smaller than aforementioned ones and round in shape ranging from 1.0 to 3.8 μm in diameter. Pale fenestration occupied relatively wider areas in cross sections than those of the former.

However the distinction between two types might have been two extremes, and possibly, they could be contiguous of the same type. It seemed that smaller and new trophosome cells were produced in the center of the lobules near the longitudinal blood vessels and gradually displaced radially, increasing in size and developing clear globular vesicles, toward the outside of the lobules where bacteria were degraded and the cells assume a larger excretory function (Photo 5).

Bacterial symbionts showing concentrations of glycogen-like particles (Photo 4) and possible carboxysomes (containing CO_2-fixing enzymes) within the bacterial cells may contain a lipid storage compound, poly-B-hydrobutyrate (PHB) (Jensen and Sicko 1973; Shively 1974), even though Vetter (1985) found that similar granules in gill symbionts of bivalves were deposits of elemental sulfur. These granules were generally seen in most of the bacteriocytes of this species.

Production of glycogen-like granules appeared to occur in association with lamellate membrane structures (Photo 4). In many symbionts, glycogen accumulations were extremely dense. Glycogen might also exist free-forms in the bacterial cytoplasm without membrane boundaries.

Large electron-dense bodies were found in both types of bacteria, and of bacteriocytes. Photo 4 showed lamellar internal membranes in a bacterial cell. The electron-dense inclusions might possibly be the polyphosphate bodies that function in phosphate storage and energy production (Faure-Fermiet and Rouiller 1958; De Burgh *et al.* 1989; Photo 4 of the present study). Isolated packets of glycogen-like granules, often enclosed by more than one membrane layer, and presumably originating from the bacteria, were commonly observed in the bacteriocyte cytoplasm. Similar granules were also frequently scattered in the host cell cytoplasm (Photo 4). These free granules might have originated from the bacteria or could be storage products of the trophosome organs.

Pogonophoran Tube Worm from Sagami Bay

Bacteriocytes were present anterior to the septum separating the opisthosome from the posterior end of the trunk, but were absent from the opisthosome. All the bacteria were enclosed in host cell membranes in close-packed state (Photo 5). The symbiotic bacteria were coccoid in shape ranging 1.3 to 4.1 and 2.5 to 6.3 ㎛ in diameter, and had glycogen-like granules scattered in the cytoplasms (Photo 6). These coccoids were distinguishable from organelles of eukaryotes by their boundary membranes, cytoplasm and nuclear region which were typical of prokaryotes, especially of Gram-negative cells. The cytoplasm in these symbionts was moderately electron-dense and sometimes contained electron-lucent vacuoles (Photo 6). These electron-lucent vacuoles were found more frequent in smaller symbionts than larger ones.

The plasma membrane extended into intracytoplasmic membranes which can cross the cells (Photo 5). The luminal boundary of the bacteriocyte was a simple membrane without microvilli or cilia. The number of bacteria per cell was difficult to estimate from the sections; they ranged from rare to abundant in different cells in the same section.

Photo 5 suggested that new trophosome cells were produced in the center of the lobules and gradually displaced toward the outside of the lobules where bacteria were degraded and the cells assumed an excretory function. Moving radially from the interior to the exterior of the trophosome lobules the symbionts increased in size, and they develop the membrane structures and clear globular vesicles within the bacteriocytes.

Pogonophoran Tube Worm from Iheya Ridge

The intracellular bacteria were rather irregular

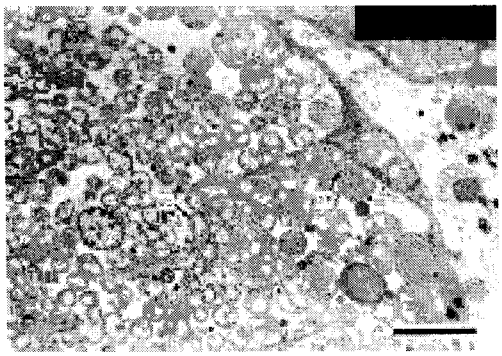

Photo 5. Trophosome of a pogonophore from the Okinoyama Bank, Sagami Bay. Note the incremental series of cell size from left bottom to upper right. Note in the larger symbionts membrane structure (pm), reminiscent lamellae of the larger vestimenti- feran symbionts. n: nucleus of bacteriocyte; sb:

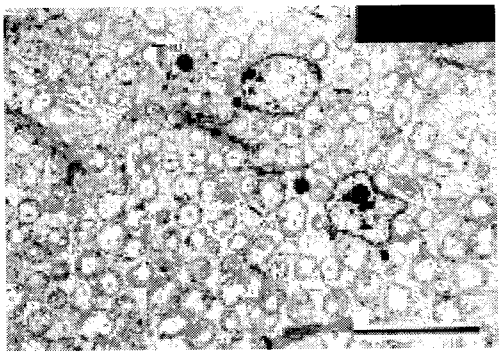

Photo 6. TEM micrograph of the another portion of trophosome of the same individual of Photo 5. Note the occurrence of vacuoles (va) and electron-dense materials (ed) in the symbionts. Glycogen-like granules (gg) are dispersed in the cytoplasm of the bacteriocyte. N: nucleus of

Photo 7. TEM micrograph of the trophosome of a pogonophoran from the *Calyptogena* Site, Iheya Ridge. ed: electron dense materials; gg: glycogen-like granules; mi: mitochondria; n: nucleus of bacteriocyte; pf: pale fenestration.

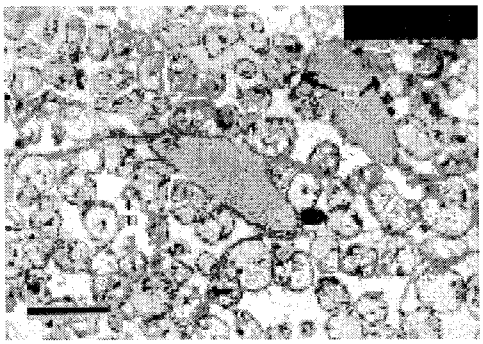

Photo 8. TEM micrograph of the another portion of trophosome of the same individual of Photo 7. Note the occurrence of light cores containing some electron-dense strands (es) in the symbionts. gg: glycogen-like granules; mi:

coccoids in shape of about 1.5-6.0 μm in diameter (Photo 7). The cell wall consisted of an outer layer, an intermediate layer and plasma membrane, typical of a Gram-negative bacterium.

The cytoplasm of the symbiont was moderately electron-dense, however there was a lighter core containing some electron-dense strands (Photo 8). Intracellular bacteria were usually surrounded by host cell membrane, either individually or in groups, and there were lysosome-like organelles containing bacteria. Pinocytotic vesicles occurred frequently inside and outside the bacterium containing vacuoles, suggesting there might be metabolic exchanges between the bacteria and the host cells.

Bacteriocytes contained the structures composed of electron-dense layers (Photo 8). The cytoplasm contained only a few mitochondria.

4. Discussion

Calyptogena

Species belonging to the genus *Calyptogena* were characterized by large fleshy gills, short simple gut, small labial palps, and a reduced feeding groove on the ventral margin of the gills. The large size and structure of the gills clearly indicated that these were the organ mainly involved in the nutritional process. The enlargement of the gills related to the development of the bacterial part appeared to be a general feature of the genus *Calyptogena*.

The external structure of the gill filaments, with a well developed normal ciliation, did not differ from that of standard filter feeders. Despite the evident production of mucus, the typical appearance of the ciliation and, especially, the presence of well developed compound latero-frontal cilia, the gills did not appear to be involved in any active filtering process. The groove was visible at the ventral margin of the demibranchs. Digestive tubes were reduced and obviously not adapted for the transfer of sufficient food material, as would be expected in a large type of the bivalve. The dissection of 13 specimens of *C. magnifica* revealed the gut to be generally empty, confirming previous observations (Boss and Turner 1980). This special adaptation of the gills probably characterized the entire family of Vesicomyidae, which was widely distributed in the Pacific Ocean, explaining its ability to densely colonize oligotrophic deep-sea zones abundantly supplied with chemical energy.

It was generally thought that along with its main function in respiration, the latero-frontal cilia act as filters which retain suspended particles in the passing currents and transferred them onto the frontal surface of the gill filaments. The presence of well developed compound of frontal cilia in the absence of filtering process would suggest that their function was

related to creating surface movements of water as suggested by the hydromechanical theory of Jorgensen (1981, 1983).

In addition, the obvious ability of the clam to produce abundant mucus could be explained by the necessity to keep the gills and other organs from being congested.

The histological and ultrastructural observations on the endocellular bacterial-host cell association in the present study clearly demonstrated the importance of TEM as a research tool. Except for the frontal area occupied by the ciliary cells, the gills were predominantly composed of bacteriocytes with abundantly reproducing bacteria.

This observations, together with the evidence indicating that the clams benefit metabolically as well as from their symbiotic relationships with their endocellular bacteria, corroborated the ecological observations on the populations which strictly dependent upon the hydrothermal fluid emissions (Hessler and Smithey 1983; Laubier and Desbruyeres 1984; Hessler *et al*1985). The general sulfide-based metabolic scheme of the relationships among bacteria with a number of molluscs and other invertebrates was also observed in sulfide-rich habitats (Cavanaugh *et al* 1981; Felbeck *et al* 1983; Southwards 1986). *Calyptogena* spp. have been collected from the habitats apparently characterized by at least moderate levels of sulfide contained in, or issuing from, the substrate. These habitats included hydrothermal vents, subduction zones, sulfide seeps, hydrocarbon seeps and other deep-sea reducing sediments (Fisher 1990). The sulfide remainder in the mud (Boulegue *et al.* 1987) might be absorbed through the foot of the deep buried clams and carried to the bacteria via the bloodstream as suggested by Arp *et al* (1984).

Isotopic analysis has suggested that the organic carbon and nitrogen of the clam tissues originated predominantly from chemoautotrophic processes (Rau 1981; Saino and Ohta 1989) supporting the hypothesis that a nutritional need of the clams was met through their endocellular symbionts in their gill.

TEM micrographs have revealed a number of structures that might be related to the transfer of the energy source substrates to the bacteria and the subsequent transfer of organic molecules to the clam tissues. The abundant microvilli covering the external surface of the bacteriocytes were undoubtedly related to a direct uptake of sulfides and oxygen, and possibly dissolved organic molecules through the epithelial membranes. Organic molecules could then be transferred to the tissues of the clams through the abundant blood supplies to the gills.

The intermediate cells observed among the bacteriocytes probably represented the cells which may be contaminated by bacteria and transformed into new bacteriocytes. The occurrence of a number of vacuoles in these bacteriocytes might be related to their endocytotic activities.

The abundance of fingerprint-like mitochondria in the ciliated cells attested to particularly high metabolic activities.

Vestimentiferan Tube Worms

Morphological comparison of the symbionts revealed that similar intracellular bacteria were found in different vestimentiferan species and that one or two bacterial types existed within single individuals of these vestimentiferan tube worms.

Studies on the giant vestimentiferans *Riftia pachyptila*, which inhabit near the sulfide-rich hot vents on the axis of the East Pacific Rise and Galapagos Rift, showed an abundance of globular bacteria, 3 - 5 ㎛ in diameter, in the spongy organs of trophosome, inside the main trunk of the animal. The bacterial volume in the trophosome of *R. pachyptila* has been ranged in 15 and 35% of the total volume of the trophosome (Powell and Somero 1985). Particles of elemental sulfur were seen to occur inside the cells containing these bacteria, and the bacteria are thought to be sulfide-oxidizing chemoautotrophs which might contribute substantially to the nutrition of their hosts. TEM photographs of the usual technique including the present study did not demonstrate concentrations of sulfur, however, the elemental sulfur could be lost

from the tissues during the alcohol dehydration and embedding phase of the sample preparation (Vetter 1985).

All vestimentiferans have been found to harbor chemo- synthetic symbionts in their trophosomes. The trophosome organs were unusual in that its major function is the containment of symbionts, and presumably, its function may be related to the nutrient production and storage. Perhaps its most striking attributes were its simplicity and the amorphous nature of its arrangement. The relative extent of bacteria-containing tissue is much greater in *R. pachyptila*, *Lamellibrachia luymesi*, and vestimentiferans observed in the present study than in the pogonophora (Jones 1981; Southward *et al.* 1981; Southward 1982, 1988), where bacteria only occurred in the posterior trunk region and where the proportion of cross-sectional area occupied by them was relatively small (Southward 1982, 1988). In all trophosome organs so far described, symbiotic bacteria were observed in vacuoles or sacs within cells; the vacuolar membranes being separate from the plasma membranes (Southward 1982; Southward *et al.* 1981). The ubiquity of this arrangement suggested that the membrane surrounding the bacterium might play an important role in regulating the nutrient or metabolite transfer between the host and the bacterium, or in protecting the bacterium from host enzymes. This observation of separate junctions among bacteriocytes in the trophosome organs was interesting, however difficult to interpret in terms of the normal function of this type of cell junction.

Southward (1988), in a study on development of a vestimentifera from the Northeast Pacific, found that newly-settled stages had a functional gut which disappears as bacterial symbionts appear in the trophosome. Bacteria were first observed in the gut cavity and within digestive vacuoles of cells of the gut lining. The bacteriocytes, which later developed into characteristic lobules, were apparently derived from the gut epithelium (Southward 1988). The mechanism of nutrient exchanges in the vestimentiferan-bacterium relationship is not clear yet. nevertheless, Felbeck (1985) suggested that excretion of nutrient molecules (e.g., small carbohydrates, amino acids and polypeptides) from symbionts to host cells was the most likely means of bacterial contribution. The host supplies CO_2, O_2, and H_2S through blood circulation and cellular metabolism; the symbionts might also utilize other cell metabolites, particularly nitrogen compounds. The apparent glycogen reserves seen in many bacteria including the present study might constitute a potential energy supply for both bacteria and host cell metabolism, as has been shown in *R. pachyptila* (Childress *et al.* 1984). Bosch and Grasse (1984) concluded that *R. pachyptila* obtained nutrients from its symbionts by lysosomal digestion of senescent bacterial cells, although the quantitative importance of this process has never been determined. They proposed that new trophosome organ cells were produced in the center of the lobules near the longitudinal blood vessels and gradually displaced toward the outside of the lobules where bacteria were degraded and the cells assumed an excretory function (see Photo 5). Moving radially from the interior to the exterior of the trophosome lobule the symbionts increased in size and develop the membrane structures and clear globular vesicles within the bacteriocytes. In the next step, globular vesicles disappeared and glycogen emerged. Symbionts gradually disintegrated allowing glycogen particles to be passed on to the bacteriocyte cytoplasm. Nutrient transfers would occur mainly through digestion of aging bacterial cells by the bacteriocytes.

Felbeck (1981) found in the trophosome organs, high activities of enzymes that were involved in generating ATP from the oxidation of reduced sulfur compounds, such as, thiosulfate sulfur-transferase (rhodanese), APS reductase and ATP sulfurylase. The prokaryotic cells made up a major portion of the trophosomes in *Riftia*. This strongly suggested that they were chemoautotrophic symbiotic bacteria which were capable of generating ATP by way of sulfide oxidation and reducing CO_2 for organic matter.

The first described organism that demonstrated a symbiotic association between chemoautotrophic bacteria and an animal host was *R. pachyptila*, the giant hydrothermal vent tube worm. When this animal was first described, it was placed in the subphylum Obturata in the phylum Pogonophora (Jones 1981). The Vesti- mentifera are currently being considered by Jones to constitute a separate phylum (Jones 1981, 1988). All of the vestimentiferans were found in habitats characterized by active venting or seepage of pore waters. The reasons for this restricted habitat might be explained by the animal anatomy. Unlike bivalves or the smaller pogonophora and annelids, the symbionts in the vestimentiferans were situated deeply in the interior of a relatively large animal with no close connection to the outside environment. Therefore, the supply of all the nutrients to the endosymbiotic bacteria had to be by way of bloodstream, which contained an abundant hemoglobin that bond both oxygen and sulfide simultaneously and also reversibly (Arp and Childress 1981; Arp *et al.* 1987). Also unlike most other animals with endosymbiotic chemoautotrophs, the vestimentiferans were presumably immobile once they have settled. The vestimentiferans must be exposed, therefore, to both sulfide and oxygen simultaneously. As sulfide and oxygen did not persist when both were present in a static environment the tube worms had to live in an environment where both substances were constantly being supplied, or form a living bridge between reduced and oxic environments.

In another vestimentiferan pogonophora, Lamelli-brachia, acuoles were described (Van der Land and Norrevang 1977), which seemed to match the bacteria observed in *Riftia* by Cavanaugh *et al.* (1981). In the vestimentiferans *Lamellibrachia* and *Riftia* the symbiotic bacteria were found in an extensive spongy organs called the trophosomes which surrounded the gonads and almost fills the trunk (Van der Land and Norrevang 1977; Jones 1981). Light microscopy showed that the trophosomes of *Lamellibrachia* were penetrated by capillaries from the dorsal and ventral blood vessels, and that its cells were of two kinds. The cells close to the capillaries contained acuoles 28 m in diameter, staining with gallocyanin, toluidine blue and para-aminosalicylic acid, while outer cells contained dark pigment granules (Van der Land and Norrevang 1977). The acuole seemed to match the bacteria 35 in diameter observed in electron micrographs of the trophosomes of *Riftia* by Cavanaugh *et al.* (1981). The presence of two cell types in association with blood vessels suggested a homology with the bacterial cylinder of small perviate pogonophores, and it was very probable that the trophosomes of vestimentiferans were elaborate and hypertrophic forms of the simpler tissue of small pogonophores.

The hydrothermal vent vestimentiferans apparently took up both sulfide and oxygen across its plume surface, but might not be the case with some of the seep vestimentiferans. According to investigators working with vestimentiferans associated with cold seeps (Ohta 1990a), hydrocarbon seeping on the Louisiana slope of the Gulf of Mexico have been unable to detect sulfide around their plumes at most sites, and the highest concentration of sulfide detected near their plumes was 3 M (MacDonald *et al.* 1989). It was possible that these worms were taking up sulfide across their tube walls since they were often buried by reduced sediments that were over 1 meter high. The highest level of sulfide that has been detected in the blood of freshly collected seep vestimentiferans was 147 M (Childress *et al.* 1986). They could, therefore, acquire sulfide across their plume from very low environmental concentrations (in an equilibrium situation at low sulfide concentrations, free sulfide in their blood would approximate the environmental concentration) or took it up across their tube and body wall. Hand (1987) noted an increase in the average size of the symbionts of *R. pachyptila* outward dimension from the interior of a lobule. This was true not only for *R. pachyptila*, but also for several species of vestimentiferans from the Juan de Fuca Ridge (De

Burgh *et al* 1989) and for two species of vestimentiferans from the Louisiana Slope, Gulf of Mexico. The symbionts within a single species of vestimentiferans may range in diameter from 1 to 9 (De Burgh 1989). The possibility that the size gradients within lobules were due to some interactions with the host cells (and not unique to a specific symbiont) was suggested by the reasoning of the morphological dissimilarity in the symbionts of different vestimentiferan species. In these associations, there was the same basic spatial arrangement of small and large symbionts within a single lobule. Whether this was due to a metabolic gradient as suggested by Hand (1987) or evelopmental stages of the symbionts as suggested by Bosch and Grasse (1984), remains to be answered.

Pogonophoran Tube Worms

Phylum Pogonophora is unique among free-living Metazoa and devoid of an internal digestive system at any stage in their life history. The taxonomy of this group is currently in dispute. While Jones (1988) considered the Pogonophora and Vestimentifera to be two separate phyla, Southward (1988) continued to follow the original classification of the Pogonophora into two subphyla; the Perviata or m a l l pogonophoran and the Obturata or large vestimentiferan worms. The small pogonophores were anatomically distinct from their larger relatives. They were thin and slender. Over 100 species of small pogonophores, thus far, have been described. They inhabited tubes partially buried in sediments or in rotting wood, and have been collected at depths ranging from 20 to 9,950 m. Owing to their small size and lack of a mouth and gut, these worms were previously thought to depend on dissolved organic matter for nutrition (Southward and Southward 1968, 1981).

Symbiotic bacteria have been described from eight species of the smaller pogonophorans referred to as Perviata (Southward 1982; Southward *et al* 1981; Fisher 1990). In all eight species thus far examined were found in host cells (bacteriocytes) resembling that of Gram-negative type, and in all but one of these species, the symbionts are clearly enclosed within host cell membranes or vacuoles (Flugel and Langhof 1983; Southward 1982). Southward (1973) suggested that the extensive glycogen stored in the trunk of the pogono-phoran *Siboglinum atlanticum* might serve as an energy source during respiration.

After the demonstration of chemoautotrophic symbiosis in the closely related Vestimentifera, the nutrition of this group was re-examined, and chemoautotrophic bacterial symbionts were discovered only in the post-annular region of several species. The symbionts were housed in bacteriocytes in a tissue homologous to the vestimentiferan trophosomes. The volume occupied by the trophosome organs was estimated to be about 10% of the total volume of that area in *Siboglinum fiordicum* (Southward 1982).

Discovery of the CO_2 fixing enzyme RuBP carboxylase in small pogonophores, particularly in the post-annular region of *S. fiordicum*, also points to chemoautotrophy in the bacterial symbionts of this species (Southward *et al* 1981). Transfer of useful metabolites from bacteria to host cells might be a form of xcretion by the bacteria and uptake by the host cell membranes, however, digestion of bacteria by the host cells may occur as well. Whether they were likely the energy sources used by the bacteria or not in the small pogonophores is not yet known.

Although the problem of whether the present specimen of pogonophoran tube worms from Sagami Bay and from the Iheya Ridge should be included either into *Pogonophora* or into *Vestimentifera* is still open, they house symbiotic bacteria within their trophosomes. The general plan section of the bacterial cylinder nearby blood supply and storage cells was the same in all the small pogono-phores examined. A center-to-periphery gradient of increasing bacterial size was clearly discernible in the trophosome lobules of the vestimentiferans. Further more, symbionts containing large pole granules were notably smaller than those containing abundant glycogen-like particles. These symbionts could thus correspond to the early and late periods of

the stage 2 proposed by Bosch and Grasse (1984). The presence of glycogen-like inclusions within bacterial cells could also reflect local biochemical conditions.

Even within the pogonophoran trophosomes, symbiotic bacteria may sometimes be relatively rare. The blood of all species of small pogonophores examined thus far contained hemoglobin with a very high affinity for oxygen (Wells and Dales 1976; Schmaljohann and Flugel 1987) which has been postulated to function in transporting oxygen to the symbionts in the deeply buried posterior end of the worms.

Immature and female pogonophores had much more of the bacteria-containing tissue than did males, in which most of the space in the post-annular region was filled with gonads and developing gametes (Southward *et al* 1981).

Bakke (1977) has shown that the embryos of *Siboglinum fiordicum* were brooded inside the tube of the female until they were ready to settle and form their own tubes, at which stage they burrow vertically several centimeters into the sediment. During development of the juveniles, the post-annular regions of the trunk lengthen more rapidly than the pre-annular regions, which suggested that the post-annular regions were more important to the juveniles and supported the hypothesis that the symbiotic bacteria inhabiting inside made a useful contribution to nutrients for the animal.

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References

Arp, A. J. and J. J. Childress. 1981. Blood function in the hydrothermal vestimentiferan tube worm. *Science*, 213, 342-344.

Arp, A. J., J. J. Childress, and R. Fisher. 1984. Metabolic and blood gas transport characteristics of the hydrothermal vent bivalve *Calyptogena magnifica*. *Physiol. Zool.*, 57, 648-662.

Bakke, T. 1977. Development of *Siboglinum fiordicum* Webb (Pogonophora) after metamorphosis. *Sarsia*, 63, 65-73.

Bosch, C. and P. P. Grasse. 1984. Cycle partiel des bacteries chimiautotrophs symbiothiques et leurs rapport avec les bacteriocytes chez Riftia pachyptila Jones (Pogonophora, Vestimentifere), II. L volution des bacteries symbiotiques et des bacteriocytes. *C. R. hebd. Seanc. Acad. Sci. Paris*, 299, Ser. III, No. 9, 413-419.

Boss, K. J. and R. D. Turner. 1980. The giant white clam from the Galapagos Rift, *Calyptogena magnifica* species novum. *Malacology*, 20, 161-194.

Boulegue, J., E. L. Benedetti, D. Dron, A. Mariotti, and R. Letolle. 1987. Geochemical and biogeochemical observations on the biological communities associated with fluid venting in Nankai Trough and Japan Trench subduction zones. *Ear. Planet. Sci. Lett.*, 83, 343-355.

Cavanaugh, C. M. 1983. Symbiotic chemoautotrophic bacteria in marine invertebrates from sulfide-rich habitats. *Nature*, 302, 58-61.

Cavanaugh, C. M. 1985. Symbioses of chemoautotrophic bacteria and marine invertebrates from hydrothermal vents and reducing sediments. *Bull. Biol. Soc. Wash.*, 1985(6), 373-388.

Cavanaugh, C. M., S. L. Gardiner, M. L. Jones, H. W. Jannasch, and J. B. Waterbury. 1981. Prokaryotic cells in the hydrothermal vent tube worm *Riftia pachyptila* Jones: possible chemoautotrophic symbionts. *Science*, 213, 340-342.

Cavanaugh, C. M., P. R. Levering, J. S. Maki, R. Mitchell, and M. E. Lidstrom. 1987. Symbiosis of methanotrophic bacteria and deep-sea mussels.

Nature, 325, 346-348.

Childress, J. J., A. J. Arp, and C. R. Fisher. Jr. 1984. Metabolic and blood characteristics of the hydrothermal vent tube-worm *Riftia pachyptila*. *Mar. Biol.*, 83, 109-124.

Childress, J. J., C. R. Fisher, J. M. Brooks, M. C. Kennicutt II, R. R. Bidigare, and A. E. Anderson. 1986. A methanotrophic marine molluscan (Bivalvia: Mytilidae) symbiosis: mussels fueled by gas. *Science*, 233, 1306-1308.

De Burgh, M. E. 1989. Evidence for a physiological gradient in the vestimentiferan trophosome: size-frequency analysis of bacterial populations and trophosome chemistry. *Can. J. Zool.*, 64, 1095-1103.

De Burgh, M. E. and C. L. Singla. 1984. Bacterial colonization and endocytosis on the gill of a new limpet species from a hydrothermal vent. *Mar. Biol.*, 84, 1-6.

De Burgh, M. E., S. K. Juniper, and C. L. Singla. 1989. Bacterial symbiosis in Northeast Pacific Vestimentifera: a TEM study. *Mar. Biol.*, 101, 97-105.

Endow, K., H. Sakai, and S. Ohta. 1987. A simple detection method for symbiotic bacteria in epoxy resin-embedded tissues using acridine orange staining. *JAMSTEC Deepsea Res.*, 3, 61-68.

Faure-Fermiet, E. and C. Rouiller. 1958. Etude au microscope electronique d ne bacterie sulfureuse, *Thiovulum majus* Hinze. *Exp. C. Res.*, 14, 29-46.

Felbeck, H. 1981. Chemoautotrophic potential of the hydrothermal vent tube worm, *Riftia pachyptila* Jones (Vestimentifera). *Science*, 209, 336-338.

Felbeck, H. 1985. CO_2 fixation in the hydrothermal vent tube worm *Riftia pachyptila* Jones. *Physiol. Zool.*, 58, 272-281.

Felbeck, H., J. J. Childress, and G. N. Somero. 1981. Calvin-Benson cycle and sulfide oxidation enzymes in animals from sulfide-rich habitats. *Nature*, 293, 291-293.

Felbeck, H., J. J. Childress, and G. N. Somero. 1983. Biochemical interactions between molluscs and their algal and bacterial symbionts. p. 331-358. In *The Mollusca*, Vol. 2. *Environmental biochemistry and Physiology*, ed. by P. W. Hochachka. Academic Press, New York.

Fiala-Medioni, A. and C. Metivier. 1986. Ultrastructure of the gill of the hydrothermal vent bivalve *Calyptogena magnifica*, with a discussion of its nutrition. *Mar. Biol.*, 90, 215-222.

Fisher, C. R. 1990. Chemoautotrophic and methanotrophic symbioses in marine invertebrates. *Aqu. Sci.*, 2, 399-436.

Fisher, C. R. 1996. Ecophysiology of primary production at deep-sea vents and seeps. p. 311-334 In *Deep-sea and extreme shallow-water habitats: Affinities and adaptations*. ed. by R. Uiblein, J. Ott, and M. Stachowtish. Biosystematics and Ecology Series 11.

Fisher, C. R., J. J. Childress, S. A. Macko and J. M. Brooks. 1994. Nutirtional interactions in Galapagos Rift hydrothermal vent communities: inferences from stable carbon and nitrogen isotope analysis. *Mar. Ecol. Prog. Ser.*, 103, 45-55.

Flugel, H. and I. Langhof. 1983. A new hermaphroditic pogonophore from the Skagerrak. *Sarsia*, 68, 131-138.