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Vesicomyid Symbioses from Monterey Bay (Central California) Cold Seeps

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Abstract

Symbioses between sulfide-oxidizing bacteria and vesicomyid clams dominate invertebrate assemblages at cold seeps worldwide. We provide here microscopic and biochemical information concerning the symbionts and symbiont-containing tissues in seven vesicomyids from Monterey Bay. For all seven species, this represents the first microscopic documentation of prokaryotic symbionts, presumably sulfide-oxidizers based on the presence of sulfur vesicles, previous estimates of elemental sulfur, and molecular evidence showing affinities to known thiotrophs. Gill morphology and ultrastructure were similar to previously examined vesicomyids, including the presence of bacteria and host cell inclusions, such as vacuoles and mucus cells. We found interspecies variability in elemental sulfur content for five vesicomyids measured. In addition, comparison of *Calyptogena kilmeri* and *C. pacifica*, the two predominant clams at Monterey Bay cold seeps, revealed both intra- and interspecific differences in symbiont size and abundance in cross sections of bacteriocytes, as well as differences in the activity of the hydrolytic enzymes acid phosphatase and lysozyme. Comparisons between closely-related symbioses provides further evidence for potentially diverse physiological capabilities among unique invertebrate-bacterial associations.

Keywords: Vesicomyid, chemoautotrophic, symbiont, Monterey Bay, microscopy

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1. Introduction

In the late 1970s scientists discovered novel deep-sea ecosystems based on chemosynthetic processes, fueled primarily by reduced compounds, such as hydrogen sulfide, rather than photosynthetic carbon input (Corliss et al., 1979). Despite the poisonous nature of sulfide, numerous metazoans, including molluscs and annelids, inhabit sulfide-rich habitats (see Fisher, 1990; Childress and Fisher, 1992 for review). Many sulfide-rich cold seeps, abundant with chemosynthetically supported life, are found along the California coast. In the 1980s, communities dominated by invertebrate-bacterial symbioses were first discovered in Monterey Bay in association with sea floor seepage near 3200 m depth (Embley et al., 1990). Research has since focused on chemosynthetic communities in Monterey Bay that occur between 600 m and 3400 m depth (Barry et al., 1996, 1997). The two predominant animals at Monterey seep habitats are the vesicomyid clams *Calyptogena kilmeri* BERNARD 1974 and *C. pacifica* DALL 1891 (Barry et al., 1996). Three additional vesicomyid species, *C. packardana* BARRY, KOCHEVAR, BAXTER, and HARROLD 1997, *Vesicomya stearnsii* DALL 1895, and *V. gigas* DALL 1896, are also common in Monterey Bay, but lower in abundance among shallow seep habitats (Barry et al., 1996, 1997). *Ectenagena extenta* KRYLOVA and MOSKALEV 1996 forms nearly monospecific aggregations at seeps between 3000–3400 m depth (Embley et al., 1990). In addition, one individual of *C. aff. angulata* DALL 1896, previously known only from Costa Rica, was recently discovered at a depth of 2950 m in the Monterey Canyon.

The family Vesicomyidae is mostly confined to deep water, low oxygen environments, such as hydrothermal vents, cold seeps and other sulfide rich habitats. All known species are thought to rely exclusively on chemoautotrophic bacteria for nutrition and, in fact, this association with sulfide-utilizing bacteria is suggested to have profoundly influenced the course of evolution for vesicomyids (Turner, 1985; Distel, 1998). Like all known vesicomyids, Monterey Bay clams have morphological specializations that presumably enable their chemosynthetic life style, including hypertrophied gills and a large volume of extracellular fluid that concentrates sulfide (Dall, 1896; Boss and Turner, 1980; Childress et al., 1991, 1993; Krylova and Moskalev, 1996). The presence of symbionts in a few vesicomyid species from Monterey has been shown via a number of indirect methods. Previous measures of delta ^{13}C values of –35–36‰ for gill tissues of both *C. kilmeri* (Barry et al., 1996) and *C. pacifica* (Barry, unpublished data) provides evidence for the presence of chemoautotrophic symbionts within these Monterey vesicomyids (Rau and Hedges, 1979; Fisher, 1990). Likewise, similar ^{13}C values measured for both symbiont-containing and host-only tissue suggest a reliance of the host upon the productivity of chemoautotrophic endosymbionts for nutrition. Molecular

techniques have also been used to successfully identify unique symbiont 'species' found in 5 species of Monterey vesicomyids (except *C. packardana* and *C. angulata*; Peek et al., 1998). All symbionts identified clustered tightly within a group of sulfide-utilizing gamma Proteobacteria (Distel et al., 1988; Peek et al., 1998).

Although all vesicomyids are assumed to exist in symbiotic relationships with chemoautotrophic bacteria, there is evidence that the functioning of both host and symbiont varies considerably. Physiological variation among vesicomyid hosts, which is likely influenced by symbiont physiology, includes metabolic potentials, growth rates, ion regulation, and uptake of metabolites (Childress and Mickel, 1982; Childress et al., 1991, 1993; Barry and Kochevar, 1998; Goffredi and Barry, 2002). Physiological variation among symbiotic bacteria may include the types of sulfur compounds fueling ATP synthesis, production of organic compounds, energetic efficiencies, use of nitrate as an alternate electron acceptor, and facultative heterotrophic capabilities, as well as variation in the transfer of organic carbon from symbiont to host (Nelson and Fisher, 1995; Nelson and Hagen, 1995). Investigation of congeners within seep habitats provides an opportunity to compare adaptations among closely-related species inhabiting similar habitats, but displaying different strategies for survival. To date, there have been no microscopic studies concerning the symbionts in Monterey Bay vesicomyids. We present here a comparison of seven vesicomyid clam species from Monterey Bay seep habitats, with emphasis on electron microscopy of the gills, elemental sulfur analysis, and the potential for symbiont digestion based on the measure of hydrolytic enzymes. Such information may contribute to our understanding of the potential physiological diversity of unique symbiont types, and serve as a basis for comparative studies on the influence of this diversity on host physiology and ecology.

2. Methods

Collection

Animals were collected in 1997–1999 from cold seeps in Monterey Bay, CA (USA) using MBARI's ROV's *Ventana and Tiburon* aboard the R.V.'s *Pt. Lobos* and *Western Flyer*, respectively. Species used in this study are included in Table 1. Efforts were focused on six sites within Monterey Bay (Fig. 1). Mt. Crushmore and Tubeworm City are located in a vertically fractured region at 600 m depth along the walls of the Monterey Canyon. Invert Cliff is located on a tectonically exposed portion along the flanks of the Monterey Canyon at ~1000 m. Clam Field is located near outcrops in the hydrocarbon-bearing Monterey formation at ~950 m depth. Clam Flat is located near an

accretionary-like prism on the continental slope of the Monterey Bay at ~1000 m depth. Axial Valley is an erosional site at the axis of the Monterey Canyon at ~3200 m depth.

Table 1. Habitat description for seven species (phylum Mollusca, family Vesicomyidae) used in this study.

Species	Site*	Depth (m)
Calyptogena pacifica	Mt. Crushmore	600
	Tubeworm City	650
	Clam Field	1000
Calyptogena packardana	Mt. Crushmore	600
Calyptogena kilmeri	Clam Field	950
	Clam Flat	1000
Vesicomya stearnsii	Invert Cliff	1100
Vesicomya gigas	Invert Cliff	1100
Ectenegena extenta	Axial Valley	3000
Calyptogena angulata	Axial Valley	3000

Animals were collected in 1997–1999 from cold seeps in Monterey Bay. *Refer to text for site descriptions.

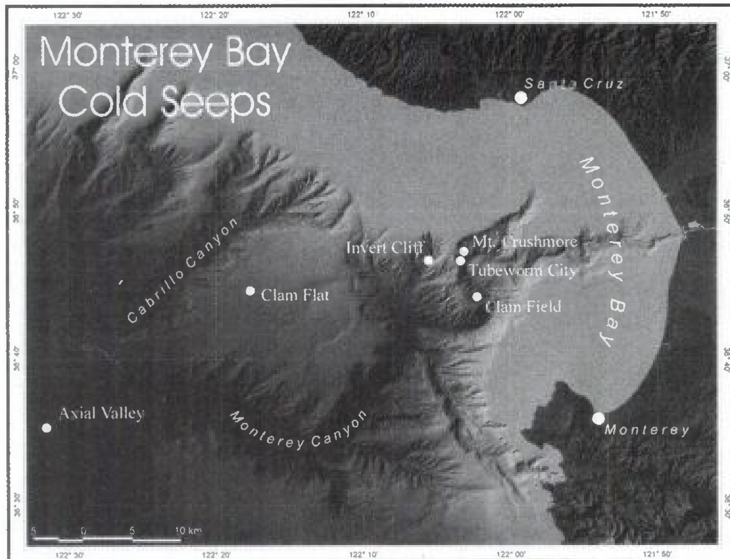

Figure 1. Map of Monterey Bay showing cold seep locations as described in the methods section (modified from Naehr et al., 2000, with permission).

Microscopy

Animals were kept in ice-cold seawater upon recovery. Dissection and preservation of specimens were performed within several hours of collection. Small pieces of excised gill (the symbiont-harboring tissues involved primarily in the nutrition of these symbioses) were preserved in 2% glutaraldehyde in filtered seawater, buffered with 0.1 M cacodylate, (4°C). In some cases (where noted), *Calyptogena kilmeri* and *C. pacifica* were kept in captivity in "mock" seep tanks for 2–9 d prior to dissection. All results showing changes over time were calculated from measurements made on these captive animals.

Samples for transmission electron microscopy (TEM) were post-fixed in 1% osmium tetroxide, ethanol dehydrated, infiltrated and polymerized using microwave techniques (Giberson et al., 1997). Thick sections were cut for light microscopy and thin sections, stained with lead citrate were placed on formar-coated grids for viewing in either a JEOL 100B or 1200EX. To determine variability within an individual, we serially sectioned tissue samples from 6–8 places along the gill of a single animal (only for *C. kilmeri* and *C. pacifica*). For interspecies comparison, we took a single gill demibranch per individual, and analyzed several TEM preparations (grids) from that sample, including multiple bacteriocytes (for bacterial abundance measurements = abundance in cross sections of bacteriocytes) and bacteria (for bacterial size = the largest dimension measured in cross section of the bacteria, rather than the actual size of the bacteria). Thus, in all cases the animal sample number is not the same as the observation sample number (i.e. bacteriocyte or symbiont n, see Table 2). For example, if more than one TEM grid (serially sectioned) was measured from a single animal, the resulting sample n's are larger than the animal n.

Bacterial abundance in cross sections of bacteriocytes (as the number of symbionts/mm^2 bacteriocyte) was determined from thin sections via the methods described in Santavy et al. (1990). Abundances were measured per bacteriocyte and only in grids where the entire bacteriocyte cross section could be visualized. While we acknowledge the 2-dimensional and static nature of this technique, fluorescent methods, such as DAPI and in situ fluorescence hybridization, have proven difficult and error prone due to high auto fluorescence and high mucus load of the tissues. Semi-quantitative intra- and interspecific comparisons between *C. kilmeri* and *C. pacifica* were estimated with animal sample sizes ≥ 3. For all other species, only qualitative microscopic observations could be made due to low sample size.

Samples for scanning electron microscopy (SEM) were post-fixed with osmium tetroxide, dehydrated via a graded series (10% increments) of increasing acetone concentration, critical point dried, and viewed on an ISI LaB6 SEM.

Elemental sulfur

Sulfur was measured via colorimetric cyanolysis, modified from Bartlett and Skoog (1954) and Schedel and Truper (1980). Elemental sulfur reacts rapidly and quantitatively with cyanide to produce thiocyanate, determined colorimetrically by the addition of a ferric ion solution. Tissue (40–100 mg, initially frozen at –80°C) was weighed and homogenized (1:500 and 1:1000) in de-ionized water to generate S° concentrations <1 mg ml^{-1}, and filtered through 0.2 µm polycarbonate filters. Filters were placed in 15 ml vials with 3 ml 0.025 M NaCN (in 50% acetone), incubated for 10 min at 90°C, vortexed, and allowed to cool at room temperature. After cooling, 6.5 ml DI and 0.5 ml 0.75 M $Fe(NO_3)_3$ (in 20% HNO_3) were added, mixed, and the absorbance read at 460 nm. Absorbance values of samples were compared against known S° standards. Sulfur values are expressed as a % of gill wet weight. To determine variability within an individual, we took tissue samples from 10 places along the gill of a single animal (only for *C. kilmeri*, n = 4 individuals). In *C. kilmeri*, there was up to 0.5× variation in sulfur content within individuals along a single gill demibranch (2.8 ± 1.3% S°). For this reason, single whole gill demibranchs were homogenized and measured for comparative analyses.

Digestive enzymes

Hydrolytic enzyme activities (for both lysozyme and acid phosphatase) were measured according to the method of Barret and Heath (1972) and McHenery et al. (1979). For both assays, tissue (100–300 mg) was homogenized (5–20×), on ice, in 0.1 M acetic acid, containing 1% Triton-X, 10 mM EDTA, and 0.05% bovine serum albumin. In the lysozyme assay, quantitative measurements of *Micrococcus luteus* cell wall degradation by lysozyme in the sample were made (McHenery et al., 1979). Agarose plates were made by autoclaving 200 ml 0.06 M phosphate buffer (pH 6.4), 0.17 M NaCl, and 0.8% agarose. When cool, 0.6 mg ml^{-1} *Micrococcus luteus* (freeze dried cells) was added and the plates were poured. When solidified, 3–4 mm diameter wells were cut, approximately 6 per plate. To start the reaction, 20 µl samples (or standards) were dispensed into the wells and incubated for 20 h at 20°C. After incubation, the diameters of lysis zones were measured and compared to zones of lysis produced via egg-white lysozyme standards (10–190 mg ml^{-1}). Boiling completely abolished all measurable activity. Acid phosphatase activity was measured via a spectrophotometric method in which phosphatase was allowed to hydrolyze p-nitrophenol phosphate and activity was measured as the liberation of p-nitrophenol (Barret and Heath, 1972). 0.3 ml sample was incubated in 1.2 ml sodium acetate mix (0.1 M acetic acid, pH 5.0, with 0.2 M sodium acetate and 10 mM EDTA) for 10 min at 37°C. 0.5 ml 32 mM p-nitrophenol phosphate (8 mM

final concentration) was added to start the reaction. After 30–60 min at room temperature, 2 ml ice-cold Tris phosphate solution (1 M Tris-HCl, pH 8.5, with 0.4 M K_2HPO_4) was added to stop the reaction. The sample was read immediately at E_{420} and absorbances were compared to known standards. Standards were used by replacing the sample with 0.3 ml nitrophenol at concentrations of 64–225 μg p-nitrophenol ml^{-1}. Appropriate blanks containing substrate and buffer alone (no enzyme) or enzyme and buffer alone (no substrate) were used. In both cases, the enzyme or substrate was added with the stop solution.

The Mann-Whitney U test was used to test for significant differences in measured parameters between *C. kilmeri* and *C. pacifica.*

Molecular analysis

The DNEASY kit (Qiagen, Valencia, California) was used to extract total DNA from frozen gill tissue, following the manufacturer's protocol. A fragment of 16S rRNA gene was amplified using Taq polymerase (Promega, Madison, Wisconsin), the bacteria specific primers 27F and 1492R, and the following protocol: initial denaturation at 94°C/3 min; 30 cycles of (94°C/1 min, 55°C/1 min, and 72°C/1 min); and a final extension at 72°C/7 min. PCR products were sequenced directly using an ABI 3100 capillary sequencer (Perkin Elmer/Applied Biosystems, Foster City, CA), according to the manufacturers recommended protocols. Both forward and reverse strands were sequenced and aligned. Sequences were aligned and proofread using Sequencher v 4.1 (Gene Codes Corp., Ann Arbor, MI). Both new sequences for *C. packardana* and *C. aff angulata* symbionts were deposited in GenBank (accession numbers: AY310507 and AY310508). Kimura 2-parameter distances were performed using PAUP*4.0b10 ADDIN ENRfu (Swofford, 1998).

3. Results

General microscopy

The anatomy of vesicomyid clams has been described in detail by others (Boss and Turner, 1980; Cavanaugh, 1983; Fiala-Medioni and Métivier, 1986). The major adaptations include reduction of the digestive system and enlargement of symbiont-containing gills, both of which are features of vesicomyids observed in this study. The gills can account for up to 25% of their total soft tissue weight, and are often cream-colored due to the storage of bacterially-produced sulfur compounds (Fig. 2). Each of two gills has paired demibranches, which consist of numerous ciliated filaments (Figs. 2, 3).

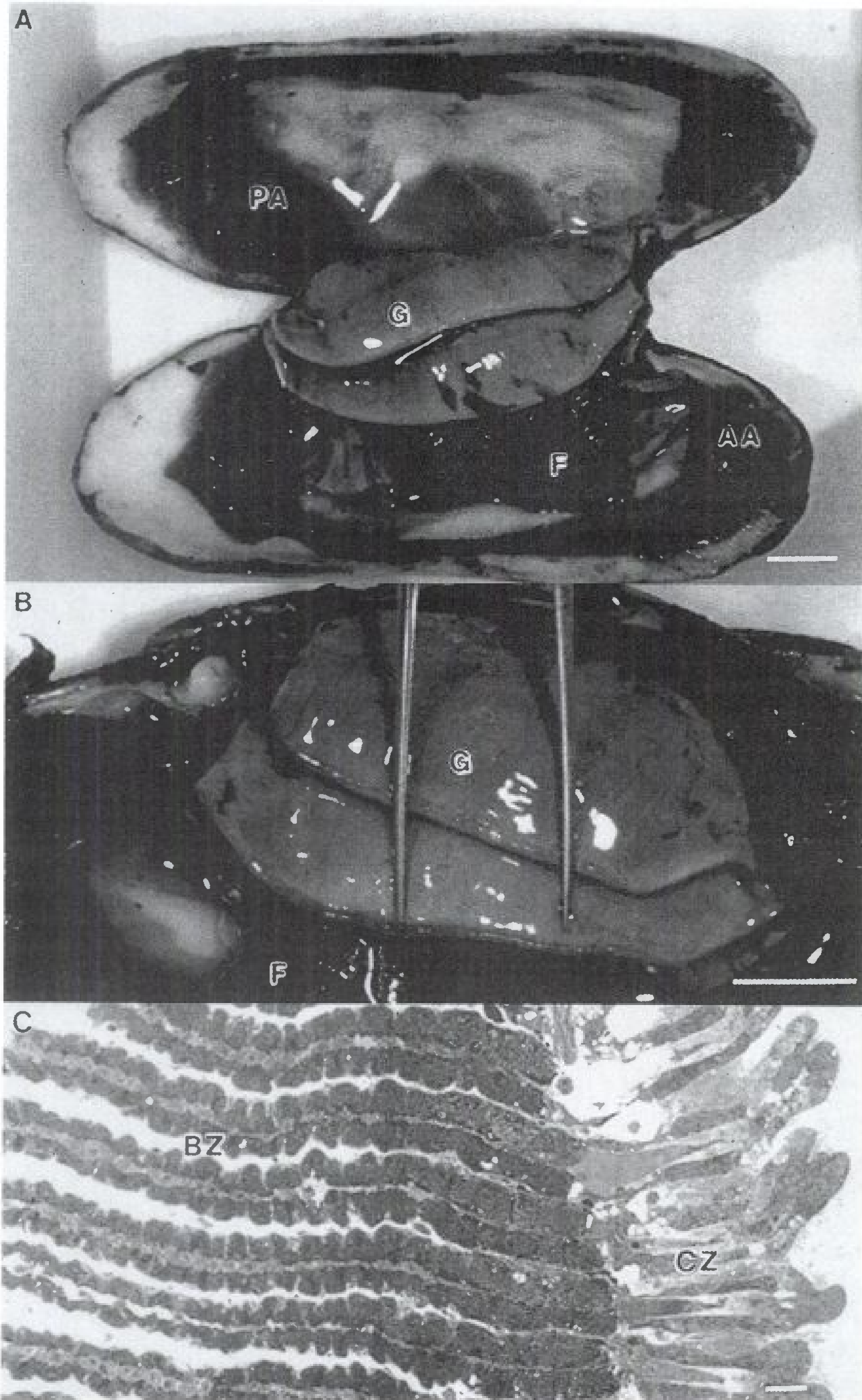

Figure 2. See legend on next page.

Although cilia aid in filter feeding in most bivalves, the ciliary feeding groove on the ventral margin of the gills is reduced in vesicomyids, which results in a decreased ability to process particulate matter (Fiala-Medioni and Métivier, 1986; Fiala-Medioni and Le Pennec, 1988). In order to reject particulates during respiration, vesicomyids have enlarged projections lining the outer end of the incurrent siphon to prevent particles from entering. Although the external cilia appear similar to other bivalves, ciliary movement in vesicomyids is thought to primarily facilitate increased CO_2 and O_2 uptake from seawater (Fig. 3).

In general, gill filaments in vesicomyids are composed of parallel sheets of cells, separated by a hemolymph space, and surrounded by an outer seawater space (Fig. 2C). The close proximity of bacteriocytes to both the medium and blood presumably allows for effective exchange of CO_2, O_2, and H_2S metabolites (Distel and Felbeck, 1987). The apical region of paired rows (in cross section of the sheets) consists primarily of ciliated cells (40–100 μm^2 in area) filled with mitochondria and a nucleus (Fig. 4A). The central region of the filament is composed primarily of symbiont-containing epithelial cells (bacteriocytes), ranging from 150–300 μm^2 in cross section, and surrounded by a vacuolar membrane, presumably of host origin (Fig. 4B). Bacteriocytes were sometimes separated by symbiont-free intercalary cells, which often contained more mitochondria than the actual bacteriocytes (Fig. 4B). Bacteriocytes were also often covered by microvilli along cell margins in contact with the seawater canal (Fig. 4B). These microvilli may greatly increase the surface area of the bacteriocytes and also presumably aid in gas exchange from the surrounding medium.

In *C. pacifica*, small (1–3 μm^2) homogeneous bodies were observed adjacent to bacteriocytes and were similar in appearance to mucus-secreting cells. Mucus cells, which probably aid in normal housekeeping processes, have been observed in the gills of some chemosynthetic bivalves (the mussel *Bathymodiolus* sp., the vent clam *C. magnifica*, and various lucinids), but not in

Figure 2. A) Dissection of the vesicomyid clam *Calyptogena kilmeri* (Clam Field). Lateral view of whole animal showing gross anatomical features including the foot (F), gill (G), anterior adductor muscle (AA), and posterior adductor muscle (PA). B) Same, enlarged view. C) Transverse thin section through the gill of *Vesicomya stearnsii* (Invert Cliff), showing parallel sheets (planes) of cells, including the ciliated (CZ) and bacterial zones (BZ). Light microscopy. Scale bars: A: 10 cm, B: 1 cm, C: 1 mm.

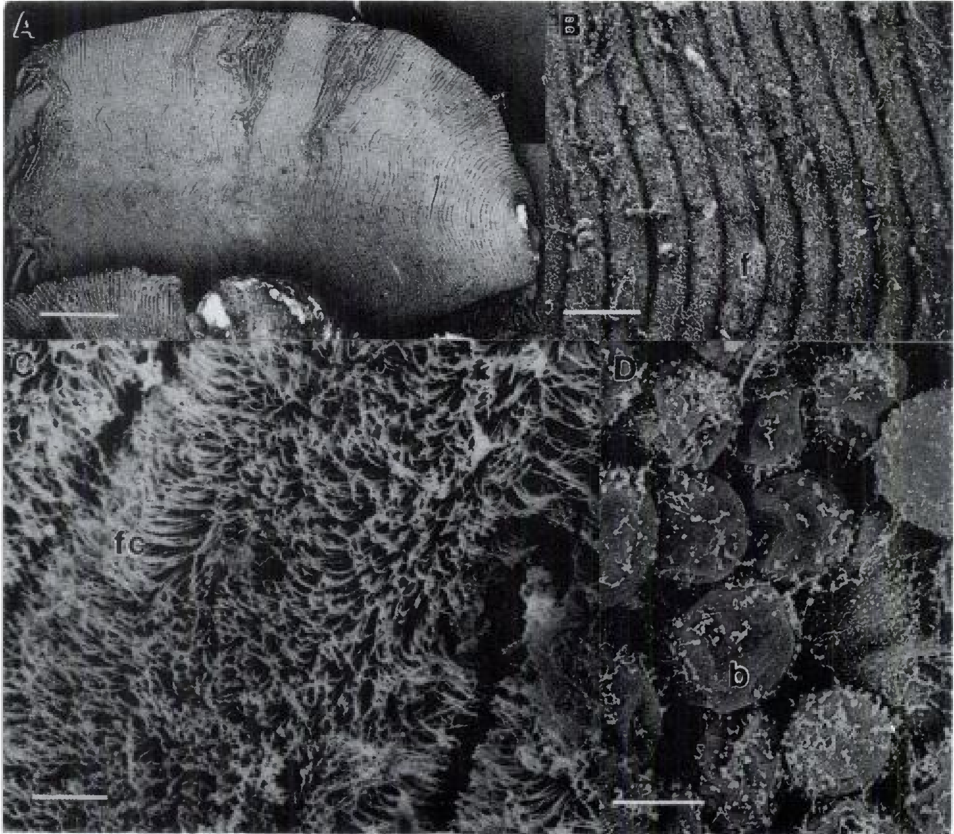

Figure 3. SEM. A) External gill surface of *Calyptogena pacifica* (Clam Field). B) External ciliated face, showing even distribution of ciliary rows. C) Same, higher magnification, showing dense ciliation. D) Endosymbionts (b). f, ciliary filaments; fc, frontal ciliation. Scale bars: A: 1 mm, B: 100 µm, C: 10 µm, D: 2 µm.

others (*C. laubieri* and *C. phaseoliformis*; Fiala-Medioni, 1984; Fiala-Medioni and Métivier, 1986; Fiala-Medioni et al., 1986; Fiala-Medioni and Le Pennec, 1988; Frenkiel et al., 1996). Our observation of mucus-producing cells only in *C. pacifica*, which does not preclude their occurrence in other vesicomyids, may suggest an alternative function in this species. More strikingly, *C. pacifica* and *V. stearnsii* possessed large (<10 µm diameter) heterogeneous vacuoles inside the bacteriocytes that typically occupied as much as 50% of the total bacteriocyte (in cross section; Figs. 5C, 7C) and, in the case of *C. pacifica,* appeared to be in the process of engulfing symbionts.

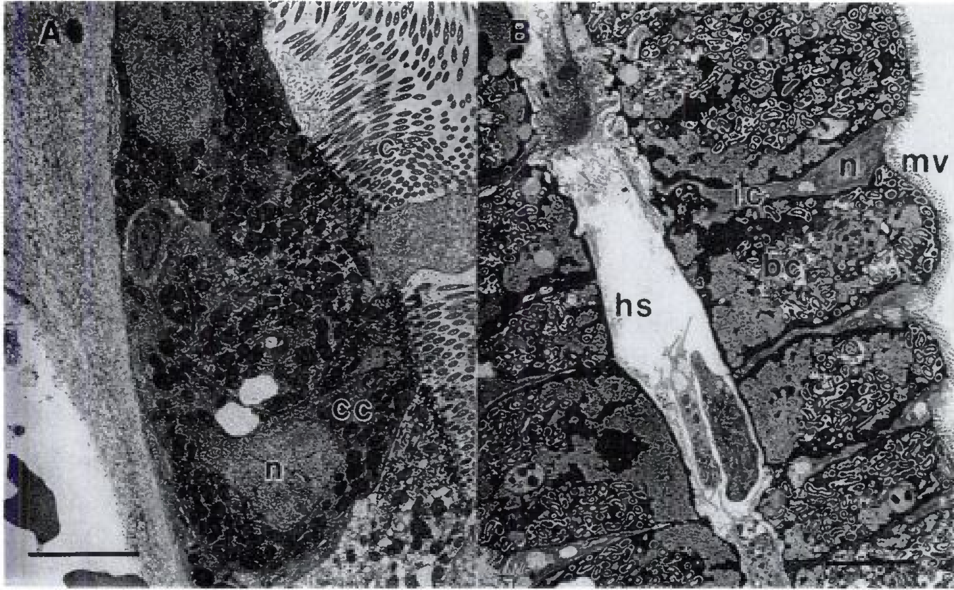

Figure 4. TEM sections through a vesicomyid gill. A) Ciliated cell showing dense Type II mitochondria. (*Calyptogena packardana* from Mt. Crushmore). B) Bacteriocytes and intercalary cells (*Calyptogena pacifica* from Clam Field). cc, ciliated cell; c, cilia; n, nucleus; ic, intercalary cell; bc, bacteriocyte; hs, haemal space; mv, microvilli. Scale bars: 5 µm.

Endosymbionts were observed in all species examined and were generally similar to those described for other vesicomyids (Figs. 3D, 5, 6–8; Cavanaugh, 1985; Fiala-Medioni and Métivier, 1986; Fiala-Medioni and Le Pennec, 1988). Most symbionts examined were polymorphic, irregular cocci-shaped, and 0.6–1.1 µm in diameter, with the exception of the deep-living *E. extenta* and *C. angulata*, with bacteria 1.5–1.6 µm in size (Figs. 6C/D, 8). The variable size of bacterial symbionts may represent species-specific differences, as was likely for the larger *E. extenta* and *C. angulata* symbionts, differences in physiological state or developmental stage of the bacteria, or artifacts with the serial sectioning of the samples. In some species, bacterial division was common and may account for intraspecific variation in size (although bacteria thought to be in the process of dividing were not included in size estimates). In addition, symbionts in *V. stearnsii* were enclosed in separate secondary membranes within the larger bacteriocyte membrane, which is similar to what has been seen in lucinid clams (Fig. 7D; Distel and Felbeck, 1987; Frenkiel et al., 1996).

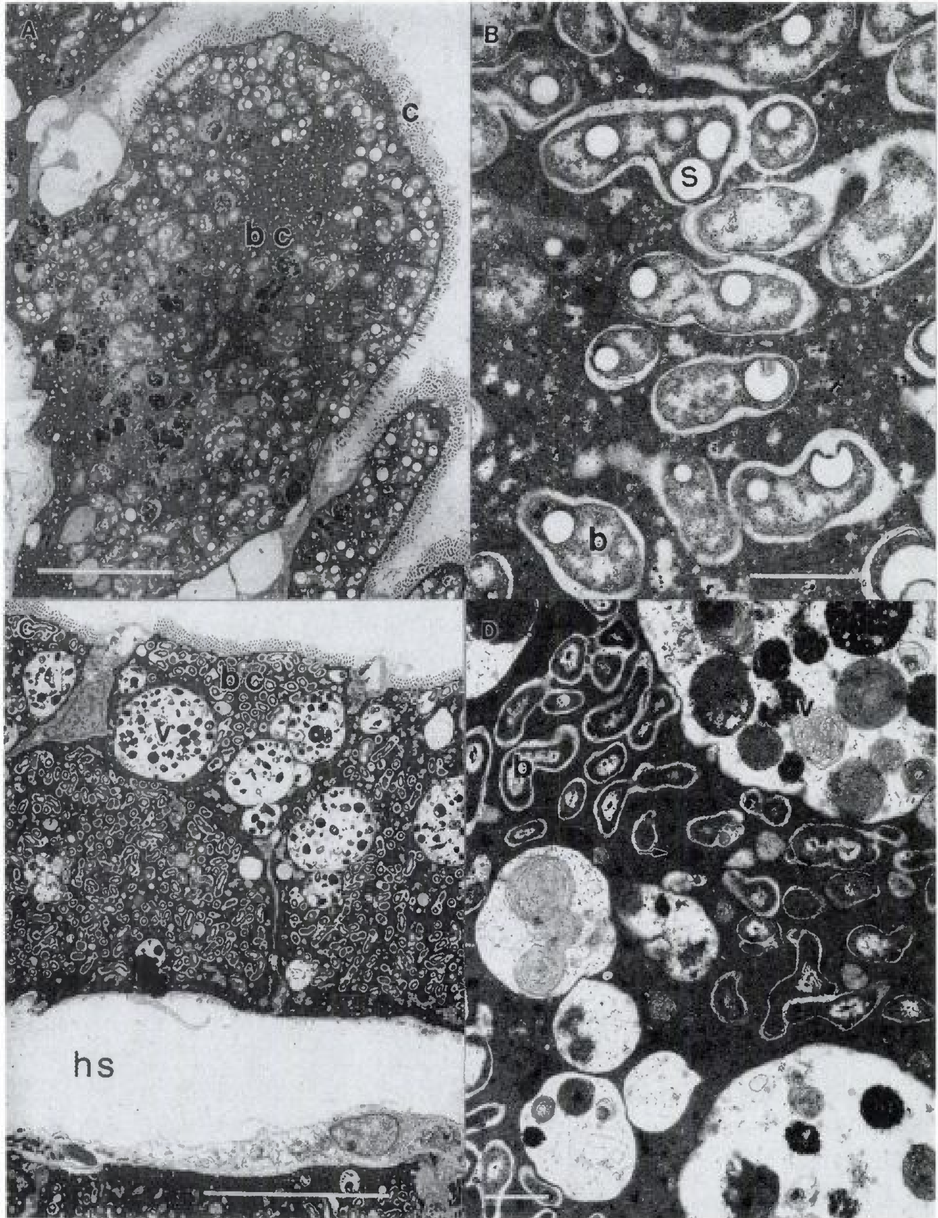

Figure 5. TEM. A) Bacteriocytes within the gill tissue of *Calyptogena kilmeri* (Clam Field). B) Same, higher magnification, showing individual bacteria. C) Bacteriocytes within the gill tissue of *Calyptogena pacifica* (Clam Field). D) Same, higher magnification. Arrow shows Type I mitochondria. bc, bacteriocyte; b, bacteria; s, sulfur vesicle; v, vacuole; hs, haemal space. Scale bars: A: 10 μm, B: 1 μm, C: 10 μm, D: 1 μm.

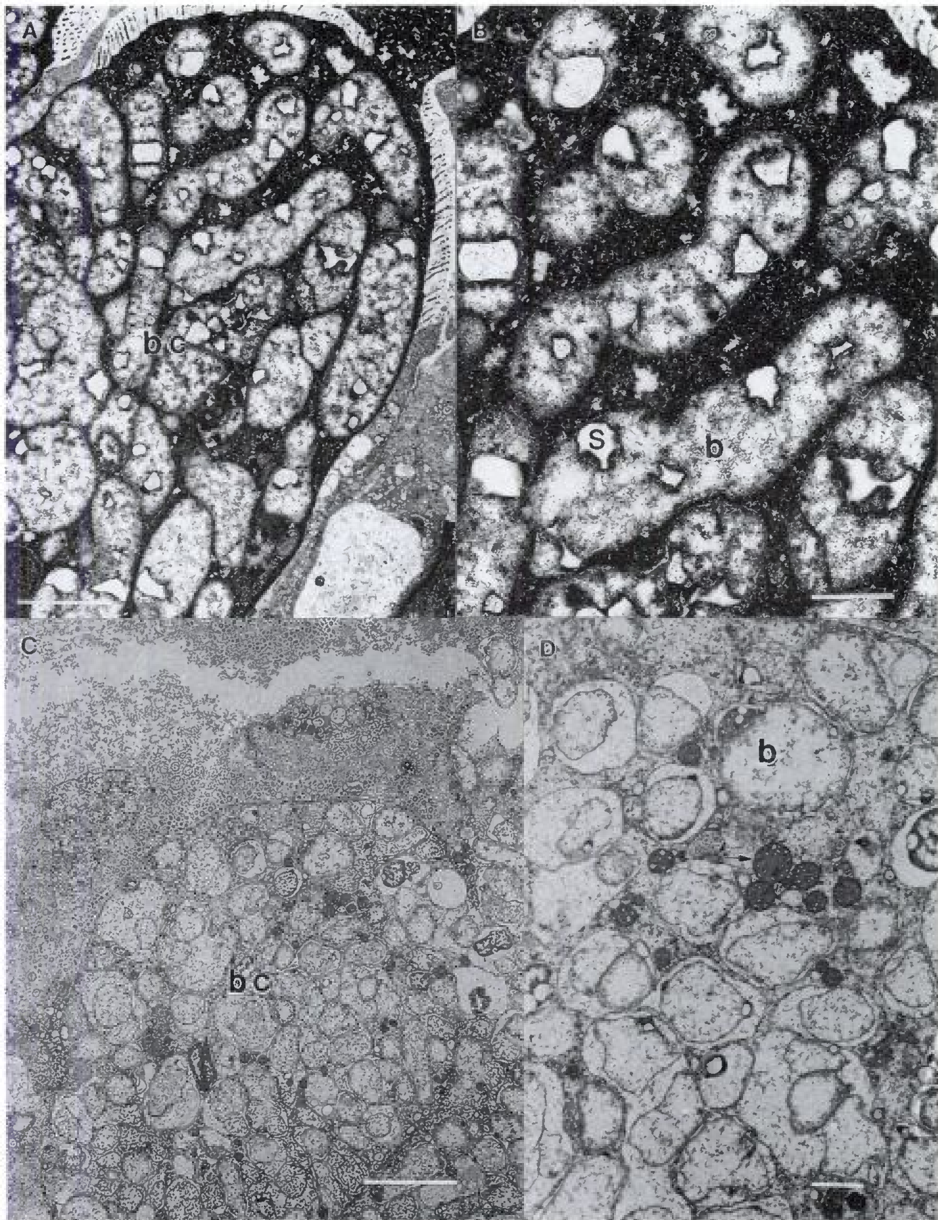

Figure 6. TEM. A) Bacteriocytes within the gill tissue of *Calyptogena packardana* (Mt. Crushmore). B) Same, higher magnification. C) Bacteriocytes within the gill tissue of *Calyptogena aff. angulata*. D) Same, higher magnification. Arrow shows Type I mitochondria. bc, bacteriocyte; s, sulfur vesicle; b, bacteria. Scale bars: A: 2 µm, B: 1 µm, C: 5 µm, D: 1 µm.

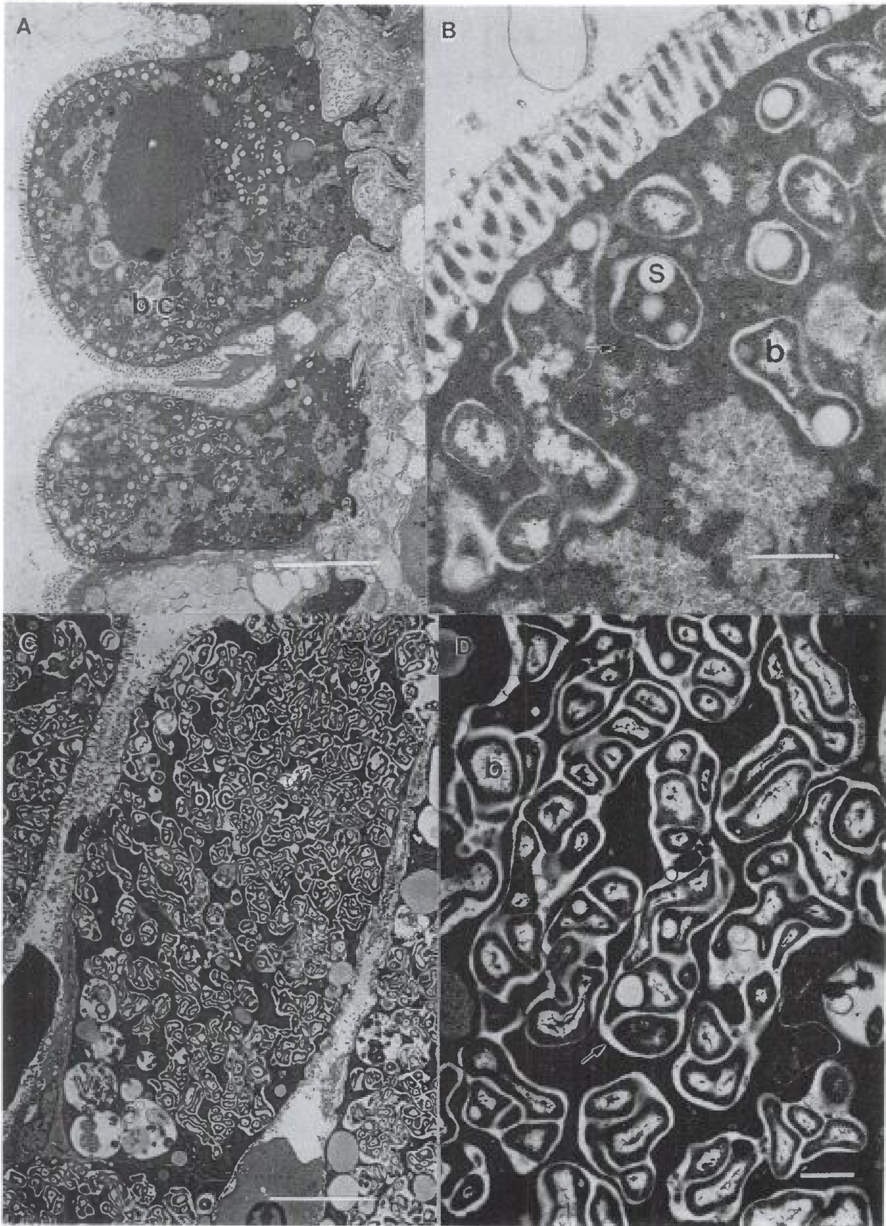

Figure 7. TEM. A). Bacteriocytes within the gill tissue of *Vesicomya gigas* (Invert Cliff). B) same, higher magnification. Arrowhead indicates Type I mitochondria. C) Bacteriocytes within the gill tissue of *Vesicomya stearnsii* (Invert Cliff). D) same, higher magnification. Note the secondary membrane surrounding groups of symbionts (arrow). s, sulfur vesicle; b, bacteria; v, vacuole. Scale bars: A: 5 µm, B: 1 µm, C: 5 µm, D: 1 µm.

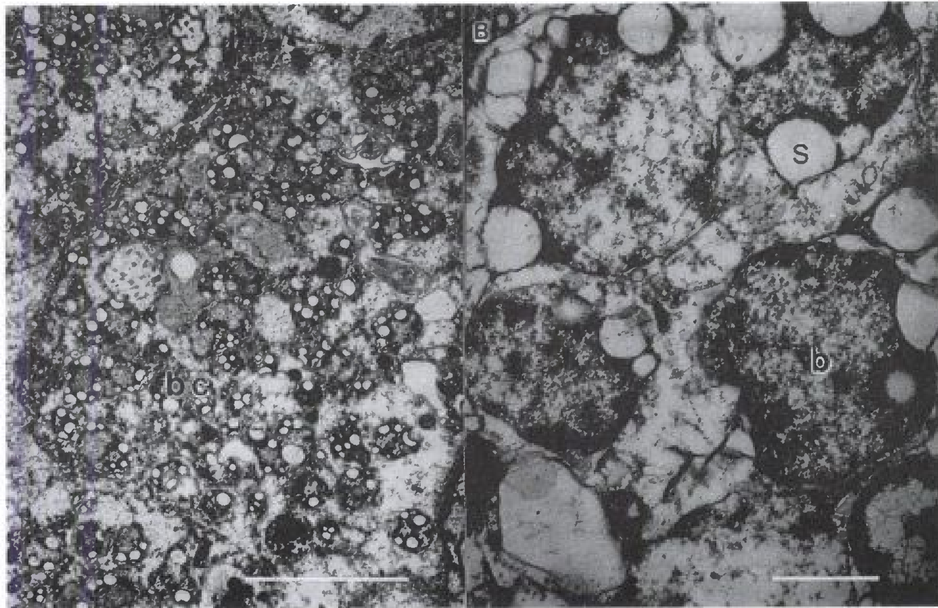

Figure 8. TEM. A). Bacteriocytes within the gill tissue of *Ectenagena extenta* (Axial Valley). B) same, higher magnification. bc, bacteriocyte; b, bacteria; s, sulfur vesicle. Scale bars: A: 5 µm, B: 1 µm.

In general, symbiotic bacteria lacked cytoplasmic structures, including carboxysomes. Carboxysomes, which have been reported in many microscopic studies of autotrophic bacteria, are thought to contain ribulose 1,5-bisphosphate carboxylase (RuBPCO), the key enzyme involved in CO_2 fixation via the Calvin-Benson cycle. The absence of carboxysomes in clam symbionts in this study, however, may suggest the utilization of Form II RuBPCO, which is not contained within carboxysomes (Shively et al., 1998).

The appearance of 0.1-0.7 μm^2 vesicles that appeared "empty" was common for all species. These vesicles are thought to be remnants of elemental sulfur, polysulfides, or polythionates, and the "empty" appearance is a result of dissolution of these compounds by polar solvents, such as ethanol or acetone, during dehydration for TEM preparation (Vetter, 1985; Shively et al., 1988). In most cases, sulfur vesicles were membrane bound, presumably by invaginations of the bacterial cell membrane, which is consistent with reports for other sulfur-utilizing bacteria (Nelson, 1981; Vetter, 1985). A recent Raman X-ray study has confirmed that these inclusions in *C. kilmeri* consist primarily of elemental sulfur (Pasteris et al., 2001).

In addition, two distinct types of mitochondria were evident in TEM preparations from many clam species. Direct comparison of mitochondrial types

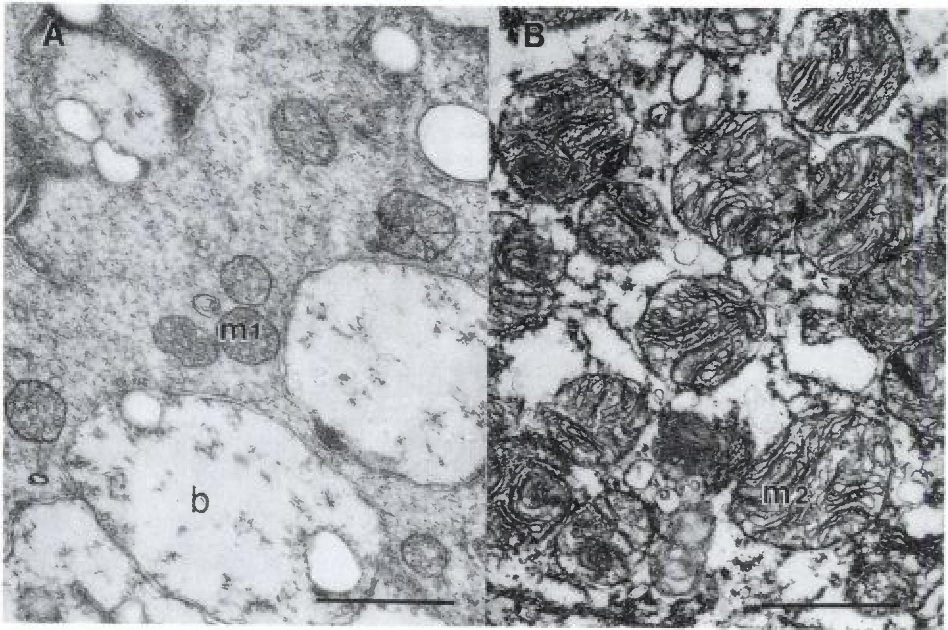

Figure 9. TEM. A) Bacteriocyte in *Calyptogena kilmeri* (Clam Flat), showing Type I mitochondria (m1). B) Ciliated cell in *C. kilmeri* (Clam Field), showing Type II mitochondria (m2). b, bacteria. Scale bars: 1 μm.

was possible only for *C. kilmeri*, *C. pacifica*, and *V. stearnsii*. One mitochondrial form, found within the bacteriocytes, was small (0.3–0.6 μm in largest dimension) and sparsely distributed among symbionts (1:7 mitochondria:symbiont ratio; Fig. 9A). Another mitochondrial type, however, was larger (0.8–1.1 μm in largest dimension), densely populated within ciliated cells of the apical region of gill filaments (Fig. 9B).

Comparisons between C. kilmeri and C. pacifica

Within an individual animal, *C. pacifica* appeared to have more variability in bacterial abundance in cross sections of bacteriocytes along a single gill demibranch (~40% variability; $10.0 \pm 3.8 \times 10^5$ symbionts/mm^2, n = 8 gill sections), than *C. kilmeri* (~20% variability; $5.2 \pm 1.0 \times 10^5$ symbionts/mm^2, n = 6 gill sections). In addition, intraspecific differences in symbiont abundance in cross sections of bacteriocytes differed among locations for both *C. pacifica* and *C. kilmeri*. *C. pacifica* from Mt. Crushmore had lower symbiont abundances (40% and 25%, respectively) than those from both Tubeworm City and Clam Field (Table 2). Likewise, *C. kilmeri* from Clam Field had lower symbiont

Table 2. Morphology and sulfur biochemistry of the symbiont-harboring gill tissue of seven vesicomyid species.

Species	Site	Animal (n)	Symbiont abundance ($\times 10^5/mm^2$)	bacteriocyte (n)	Symbiont size (µm)	symbiont (n)	S° (% of gill wt)	sample (n)
C. pacifica	Mt Crushmore	1	5.0 ± 0.4	n = 5	0.98 ± 0.07	n = 20	1.3 ± 0.7	n = 4
	Tubeworm City	1	6.7 ± 0.6	n = 2	0.64 ± 0.07	n = 30	nm	
	Clam Field	3	8.3 ± 0.9	n = 8	0.65 ± 0.06	n = 29	2.4 ± 1.1	n = 8
C. packardana	Mt Crushmore	1	5.7 ± 1.4	n = 4	1.12 ± 0.07	n = 20	4.6 ± 1.6	n = 6
C. kilmeri	Clam Field	3	4.6 ± 0.4	n = 5	0.84 ± 0.04	n = 52	4.7 ± 0.8	n = 11
	Clam Flat	3	6.5 ± 0.1	n = 3	0.94 ± 0.05	n = 62	5.9 ± 1.1	n = 5
	Clam Flat (2d in captivity)	1	1.4 ± 0.2	n = 2	1.35 ± 0.09	n = 20	nm	
V. stearnsii	Invert Cliff	1	11.5	n = 1	0.86 ± 0.08	n = 30	nm	
V. gigas	Invert Cliff	3	4.1 ± 0.6	n = 4	0.78 ± 0.06	n = 27	3.3 ± 1.2	n = 4
E. extenta	Axial Valley	4	2.2 ± 0.3	n = 5	1.59 ± 0.09	n = 35	9.3 ± 3.3	n = 4
C. angulata	Axial Valley	1	0.3	n = 1	1.55 ± 1.0	n = 49	nm	

Symbiont size (largest dimension in cross sections of bacteria) and abundance (in cross sections of bacteriocytes) were determined via examination of transmission electron micrographs. For most species, only qualitative inferences from microscopic observations could be made due to low sample size. Elemental sulfur (as % of gill wt) was determined via colorimetric cyanolysis. All values are x ± se. nm = not measured.

abundances (40%) than those from Clam Flat (Mann-Whitney P = 0.025). These differences may be due to inter-individual variation in C. *pacifica* (i.e. within 40%) however the differences exceeded (i.e. greater than 20%) inter-individual variation for C. *kilmeri*. Intraspecific differences in symbiont size were not significant between sites for either species.

A single individual of C. *pacifica* held in captivity for 9 d had a markedly lower bacterial abundance (not possible to quantify via TEM), when compared to C. *pacifica* dissected immediately following recovery (Fig. 10A/B). Likewise, the individual C. *kilmeri* ("f") kept in captivity for 2 d demonstrated a lower bacterial abundance in cross sections of bacteriocytes (1.4×10^5 symbionts/mm^2), when compared to C. *kilmeri* dissected immediately following recovery (6.5×10^5 symbionts/mm^2; Table 2). C. *kilmeri* ("f") kept in captivity for 2 d also appeared to have larger symbionts (1.35 μm in largest average dimension), when compared to C. *kilmeri* dissected immediately following recovery (0.94 μm in largest average dimension), possibly suggesting a decrease in symbiont division due to substrate (sulfide) limitation in captivity.

Although the abundance of bacteria in the gill tissues varied considerably (~20–40%) within individual and within species, clear interspecific differences in symbiont loads were detected. C. *pacifica* from Clam Field had a significantly higher overall bacterial abundance in cross sections of bacteriocytes (8.3×10^5 symbionts/mm^2 of bacteriocyte) than C. *kilmeri* from Clam Field (4.6×10^5 symbionts/mm^2 of bacteriocyte; Mann Whitney P = 0.0083). Bacterial abundance in cross sections of bacteriocytes in E. *extenta* (with an animal $n \geq 3$) appeared lower than all other species measured (2.2×10^5 symbionts/mm^2; Table 2), while symbiont abundance in V. *gigas* was not different than other species measured with an animal $n \geq 3$ (Table 2). E. *extenta* coincidentally also had the largest symbionts (1.6 μm in diameter), which may suggest trade-offs between symbiont abundance and symbiont size.

Differences in the amount of sulfur among clam species was apparent both visually and biochemically (Table 2). E. *extenta* and C. *kilmeri* (C. Flat) contained the highest percentage of elemental sulfur (by mass, 9.3 ± 3.3% and 5.9 ± 1.1% of the gill, respectively) and were significantly different (Mann-Whitney P<0.1) from all other species measured except C. *packardana* (4.6 ± 1.6%). C. *pacifica* had significantly lower gill sulfur levels (1.3–2.4%, Table 2; Mann-Whitney P<0.03) when compared to other clams in this study. For comparison, two symbiont-free clams (*Macoma nasuta* CONRAD 1837 and *Protothaca staminea* CONRAD 1837) that live in mildly reduced sediments (<1 mM sulfide, C. Sakamoto, Monterey Bay Aquarium Research Institute, unpubl. data) measured 0–0.4% in gill sulfur content.

One individual of C. *kilmeri* (labeled "f") kept in captivity for 2 d demonstrated lower S° in the gills, observed visually in electron micrographs, than animals dissected immediately following collection (data not shown).

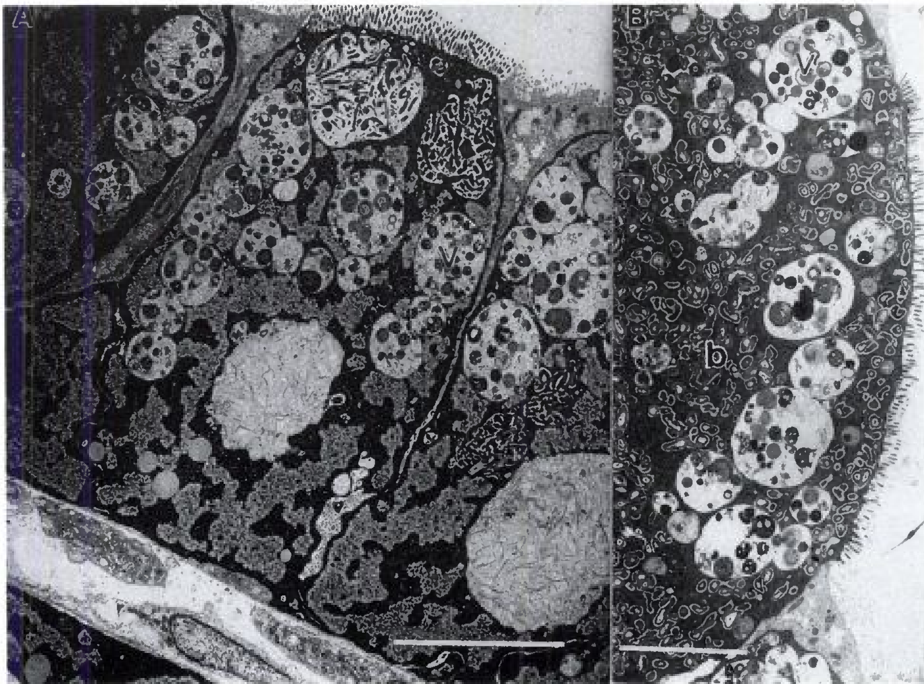

Figure 10. TEM. A) Bacteriocytes within the gill tissue of *Calyptogena pacifica* (Mt. Crushmore), kept in captivity for 9 days. Note abundant and enlarged vacuoles and very few symbionts. B) *C. pacifica* (Mt. Crushmore), dissected and visualized immediately upon recovery. b, bacteria; v, vacuole. Scale bars: 10 µm.

Biochemical measurements of additional *C. kilmeri* (n = 4) kept in captivity revealed 9–30% lower S° levels than animals dissected immediately following collection (n = 16), which equaled a loss of ~6–8 mg S° day^{-1}. Likewise, additional *C. pacifica* kept in captivity (n = 9) had S° levels ~80% lower (a loss of ~1–2 mg S° day^{-1}), than those dissected immediately following collection (n = 13). Intraspecific differences in sulfur content were not significant among sites for either species.

Measurements of hydrolytic enzyme activity indicated that *C. pacifica* had a higher potential for intracellular enzymatic digestion. *C. pacifica* from Clam Field possessed significantly higher lysozyme activity in gill tissue (52.6 ± 4.9 µg gill wet wt^{-1}) than *C. kilmeri* from Clam Field (29.9 ± 7.7 µg gill wet wt^{-1}; Mann-Whitney P = 0.021, Table 3). Likewise, acid phosphatase activity was also significantly higher in *C. pacifica* (49.3 ± 23.7 mg gill wet $wt^{-1}h^{-1}$) than *C. kilmeri* (13.6 ± 4.5 mg gill wet $wt^{-1}h^{-1}$; Mann-Whitney P=0.006, Table 3).

Table 3. Activities of hydrolytic enzymes (mean ± sd) in the gill tissue of *C. pacifica* and *C. kilmeri* from Clam Field.

Species	Lysozyme (μg g^{-1} gill wet wt)		Acid phosphatase (μg g^{-1} gill wet wt h^{-1})	
C. pacifica	52.6 ± 4.9	n = 4	49.3 ± 23.7	n = 5
C. kilmeri	29.9 ± 7.7	n = 4	13.6 ± 4.5	n = 6
P value*	0.021		0.006	

*Mann-Whitney U-test P values are shown to demonstrate significant interspecific differences in enzyme activity.

4. Discussion

Monterey seep habitats are unique in the high diversity of congeneric vesicomyid species found living in close association, yet possessing distinct microhabitat distributions and physiological capabilities. Morphological and ultrastructural studies of closely-related species provide further evidence for potentially diverse physiological capabilities among unique invertebrate-bacterial associations. Knowledge gained from these studies can be applied to our understanding of species-specific differences in physiological functioning of both the host and symbiont, and the influence of these differences on the distribution of vesicomyid species along continental margins.

Despite an overlap in habitat type and close taxonomic affinity, the life styles of unique vesicomyid species can vary considerably, suggesting different strategies to thrive in the cold seep environment. Within the phylogenetic tree of vesicoymid clams, constructed via molecular DNA sequences, *C. kilmeri* and *C. pacifica* are relatively divergent compared to the rest of the group (Peek et al., 1998). This divergence is observed in ecological and physiological comparisons as well (Goffedi and Barry, 2002). *C. kilmeri* is metabolically poised for high sulfide tolerance and rapid growth, while *C. pacifica* grows very slowly, requiring many decades to reach adult size (Barry and Kochevar, 1998). Endosymbiont functionality, in such a tightly coupled symbiotic system, surely has direct influence over the physiological capabilities of the host. Therefore, the obvious differences observed in physiological divergence seen in these associations may be reflected in, or even likely caused by, differences in symbiont functionality.

To date, there have been no microscopic studies concerning the symbionts of Monterey Bay vesicomyids. TEM imagery revealed the presence of bacterial symbionts (presumably sulfide-oxidizers) in all species examined, which is

consistent with previous demonstrations of symbionts via molecular and biochemical measures. Symbionts were polymorphic and were densely packed intracellularly in filaments positioned between the blood and seawater spaces. To augment previous molecular studies, the symbionts of *C. packardana* and *C. aff. angulata* were sequenced and added to the GenBank database. Similar to all vesicomyid symbionts, those of *C. packardana* and *C. aff. angulata* demonstrated 1–4% divergence in 16S rRNA sequence from other symbionts within the family Vesicomyidae (Peek et al., 1998, 2000).

We found that intraspecific symbiont abundances and elemental sulfur content were positively correlated with external sulfide conditions (previously measured in Barry et al., 1996, 1997; Goffredi and Barry, 2002). For example, *C. kilmeri* from Clam Flat (with environmental sulfide values of ~10 mM) demonstrated higher bacterial abundance in cross sections of bacteriocytes ($6.5 \pm 0.1 \times 10^5$ symbionts/mm^2) and elemental sulfur (5.9 ± 1.1%) than *C. kilmeri* from Clam Field ($4.6 \pm 0.4 \times 10^5$ symbionts/mm^2 and 4.7 ± 0.8%, respectively), with environmental sulfide values of ~4 mM). Likewise, *C. pacifica* from Clam Field demonstrated higher bacterial abundance in cross sections of bacteriocytes ($8.3 \pm 0.9 \times 10^5$ symbionts/mm^2) and elemental sulfur (2.4 ± 1.1%) than *C. pacifica* from Mt. Crushmore ($5.0 \pm 0.4 \times 10^5$ symbionts/mm^2 and 1.3 ± 0.7%, respectively), with environmental sulfide values of ~0.1 mM). This suggests that environmental conditions, particularly sulfide, have an influence over internal symbiont populations and internal storage of sulfur.

Interspecific differences in symbiont characteristics were also noted. For example, bacterial abundance in cross sections of bacteriocytes appeared higher (2×) in *C. pacific*a from Clam Field than *C. kilmeri* from Clam Field. *C. pacifica* has previously demonstrated a greater sulfide-utilizing potential than *C. kilmeri*, based on measures of sulfide oxidase and APS reductase activity, both of which are involved in sulfur metabolism of the symbionts (Goffredi and Barry, 2002). Together, these results suggest an elevated potential for bacterial productivity in *C. pacifica*.

C. pacifica, C. kilmeri, and *V. stearnsii*, demonstrated marked differences between mitochondrial populations within the bacteriocytes and the peripheral ciliated cells. Morphological and distributional differences among mitochondrial types may suggest differing functional roles in these associations. It is possible that mitochondria within the bacteriocytes have the ability to harness energy from sulfide oxidation, which could result in additional ATP resources for the intact associations (Powell and Somero, 1986; OíBrien and Vetter, 1990). On the other hand, mitochondria-filled ciliated cells are not uncommon in bivalves, and probably aid primarily in gas exchange (Morse and Zardus, 1997).

For all species examined, microscopy revealed what appeared to be "empty" inclusions within individual symbionts. These inclusions in *C. kilmeri* have

been shown (via Raman spectroscopy) to consist primarily of microcrystalline elemental sulfur rather than "liquid" organic sulfur compounds (Steudel, 1989; Pasteris et al., 2001).

Biochemical measures of elemental sulfur (S°) have revealed high, but variable, levels in many invertebrate hosts, including vestimentiferan worms (4–6% S° (w/w) of trophosome; deBurgh et al., 1989) and the vent clam *C. magnifica* (1.2% S° (w/w) of gill; Fisher et al., 1988; Vetter and Fry, 1998). Our biochemical assay revealed 1–6% S° (w/w of gill) for four species of vesicomyid from Monterey Bay. The deepest living species, *E. extenta,* contained up to 9.3% S° in its gill tissue, which was reflected in the very "creamy" coloration of the gills, compared to other species in this study.

Sulfur containing compounds are important storage products for these symbioses due to the possibility of additional oxidation and energy yield for carbon fixation in the absence of environmental sulfide. After only a few days in the absence of external sulfide in captivity, *C. kilmeri* and *C. pacifica* contained lower S° levels presumably due to symbiont utilization of the stored sulfur or to environmental stress, tissue autolysis, or pH decrease and subsequent chemical (rather than metabolic) dissolution. Putative sulfur vesicles in the free-living sulfide-oxidizing bacterium, *Beggiatoa alba,* were shown to expand and collapse in the presence and absence, respectively, of exogenous sulfide (Lawry et al., 1981). Based on previous studies, in which a loss of sulfur was observed in invertebrate-bacterial associations (*C. elongata* and *C. magnifica*) in the absence of sulfide and attributed to metabolite consumption of S°, we also believe that endosymbiont S° utilization is responsible for the observed reduction in sulfur stores in our study (Vetter, 1985; Vetter and Fry, 1998; Childress et al., 1991, 1993).

Endosymbionts were the dominant sub-cellular structures in *C. kilmeri,* whereas *C. pacifica* (and *V. stearnsii*) also contained heterogeneous vacuoles within the bacteriocyte cytoplasm (up to ~50% of bacteriocyte area in freshly collected animals). Some of these vacuoles appeared to be engulfing bacterial symbionts, suggesting that digestion of bacteria may be an important mode of organic material transfer from symbiont to host. In addition, mucus cells were observed exclusively in *C. pacifica.* Because vesicomyids typically have little need for digestive mucus, the presence of these cells in *C. pacifica* may suggest a digestive function. Biochemical measures of hydrolytic enzymes involved in possible digestion of symbionts, including acid phosphatase and lysozyme, were used to support the microscopic studies. High lysosomal enzyme activities (above non-symbiotic species levels) have been demonstrated in both vent mussels and clams, implicating intracellular bacterial digestion in these animals (Fiala-Medioni et al., 1990, 1994). Increased activities of both lysozyme and acid phosphatase in *C. pacifica* also implicate digestion as a possible strategy for carbon transfer in this symbiosis. The relative importance

of digestion versus direct translocation has not been established, and it remains a necessary endeavor to determine the mode of carbon transfer from symbiont to host. Evidence gained from microscopy, along with biochemical indices, has suggested the potential for divergent strategies of carbon transfer from symbiont to host in *C. kilmeri* and *C. pacifica*. It is possible that the pathways by which carbon is transferred in these two symbioses are different and that these differences may not only reflect the environments in which they are found but also have a strong influence on host productivity.

5. Conclusion

Chemosynthetic communities continue to receive intensive scientific study. There are, however, many unanswered questions concerning the unique invertebrate-bacterial associations that are integral components of these communities. For instance, it is currently unknown how differences in, and influences upon, symbiont metabolism affect the divergent physiological ecology of the hosts. This study revealed that despite the specialized lifestyle of vesicomyid associations, there appears to be room for variation. All seven species of vesicomyid clam from Monterey Bay were shown to have symbionts. Symbiont abundance, elemental sulfur content, and hydrolytic enzyme activity, however, were variable between the two dominant vesicomyids, *C. pacifica* and *C. kilmeri*. These differences, which have direct impacts upon these symbiotic associations as a whole, and are likely reflected in the overall ecological physiology of these animals, may be the result of environmental, ecological, or evolutionary differences.

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