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COMPARATIVE ULTRASTRUCTURE OF DIGESTIVE DIVERTICULAE IN BATHYMODIOLIN MUSSELS: DISCOVERY OF AN UNKNOWN SPHERICAL INCLUSION (SIX) IN DIGESTIVE CELLS OF A SEEP MUSSEL

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ABSTRACT Mussels in the genus *Bathymodiolus* host endosymbiotic bacteria in their gills, from which the mussel derives much of its nutrition. Bathymodiolin mussels also have functional digestive systems and, as in shallow-water mytilid mussels, cells of the digestive diverticulae are of two types: basophilic secretory cells and columnar digestive cells. Cellular contents of secretory and digestive cells of *Bathymodiolus thermophilus* and *Bathymodiolus brevior* from deep-sea hydrothermal vents are comparable to cellular contents of these cell types observed in shallow-water mytilids. In the seep mussel *Bathymodiolus heckerae*, cellular contents of columnar cells were anomalous, being dominated by an unknown cellular inclusion herein called spherical inclusion unknown or SIX. SIX was observed in all digestive cells and some basophilic cells of *B. heckerae* examined with TEM. It is a large (2–10-μm diameter) and abundant (7 ± 1.5 inclusions per epithelial cell section) inclusion, with a double external membrane and stacked internal lamellae. No microbial DNA was detected in digestive tubules of *B. heckerae* using molecular probes, preferential DNA amplification techniques, or DAPI staining, suggesting that SIX is not a unicellular parasite or symbiont. The ubiquity and abundance of SIX within cells of the digestive diverticula suggest that it has an important cellular function (positive or negative), yet to be determined.

KEY WORDS: seep mussel, *Bathymodiolus*, digestive diverticula, lysosomal progression

INTRODUCTION

Bathymodiolin mussels have been found at vent sites on all major ridge systems of the oceans and at seep sites on continental margins, where they often represent the dominant invertebrate biomass (Sibuet & Olu 1998, Van Dover 2000, Levin 2005). Vent mussels were initially assumed to be suspension feeders like their shallow-water relatives, relying on photosynthetically derived detritus (Lonsdale 1977, Enright et al. 1981). Evidence of chemoautotrophic primary production at deep-sea vents and seeps and ultrastructural and enzymatic studies of mussel gill tissue led to the conclusion that bathymodiolin mussels depend on chemoautotrophic endosymbiotic bacteria (thiotrophic and/or methanotrophic, Cavanaugh 1983, Fisher et al. 1993, Distel et al. 1995, Fiala-Médioni et al. 2002) for all or most of their nutrition requirement, with digestion of bacteria or organic detritus fulfilling any remaining energy requirements (Rau 1981, Cavanaugh 1983, Childress & Fisher 1992).

Uptake of radiolabeled bacteria by *Bathymodiolus thermophilus* Kenk & Wilson demonstrate that this species can clear and assimilate particulate organic matter. *In situ*, mussels could ingest bacteria sloughed from their gills, bacteria from the environment, or organic detritus (Page et al. 1991). Vent and seep mussels are thus considered to be mixotrophic organisms, capable of obtaining nutrition from chemoautotrophic gill bacteria and from normal trophic processes (Le Pennec et al. 1990).

Unlike endosymbiont-bearing vestimentiferan tubeworms and vesicomyid clams, which have reduced or nonexistent digestive systems (Boss & Turner 1980, Jones 1981), digestive systems of bathymodiolin mussels appear to be anatomically and enzymatically complete (Kenk & Wilson 1985, Le Pennec et al. 1990, Le Pennec et al. 1995, Von Cosel et al. 1994). Grooves on the gills, well-developed labial palps and the presence of both secretory and digestive cells in the digestive diverticulae suggest that morphologically, *B. thermophilus* contains a functional digestive system (Kenk & Wilson 1985, Le Pennec et al. 1990).

Ultrastructurally, cells of the digestive diverticulae of vent mussels *B. thermophilus* from the East Pacific Rise (Hily et al. 1986a, Hily et al. 1986b) are similar to those of the shallow-water mussel *Mytilus edulis* Linnaeus (Owen 1972, Owen 1973). Basophilic secretory cells in both species are filled with endoplasmic reticulum for synthesis and secretion of digestive enzymes into the lumen of the tubule. Digestive columnar cells in *B. thermophilus* and *M. edulis* are more numerous than secretory cells and contain lysosomal bodies in various stages of the cellular digestive process.

This study focuses on the ultrastructure of epithelial cells in the digestive diverticulae of the seep mussel, *Bathymodiolus heckerae* Turner, Gustafson, Lutz & Vrijenhoek, from the Blake Ridge methane hydrate site. Digestive diverticulae of *Bathymodiolus* cf. *thermophilus* from a vent site along the Pacific Antarctic Ridge and of *Bathymodiolus brevior* Cosel, Métivier & Hashimoto from North Fiji back-arc basin vents were also examined to determine the scope of ultrastructural variation in representative *Bathymodiolus* species. The shallow-water mussel *Geukensia demissa* Dillwyn was used as a reference. As reported here, digestive cells of *B. heckerae* lacked the lysosomal progression observed in the other mussel species and instead contained abundant, large, unidentified inclusions.

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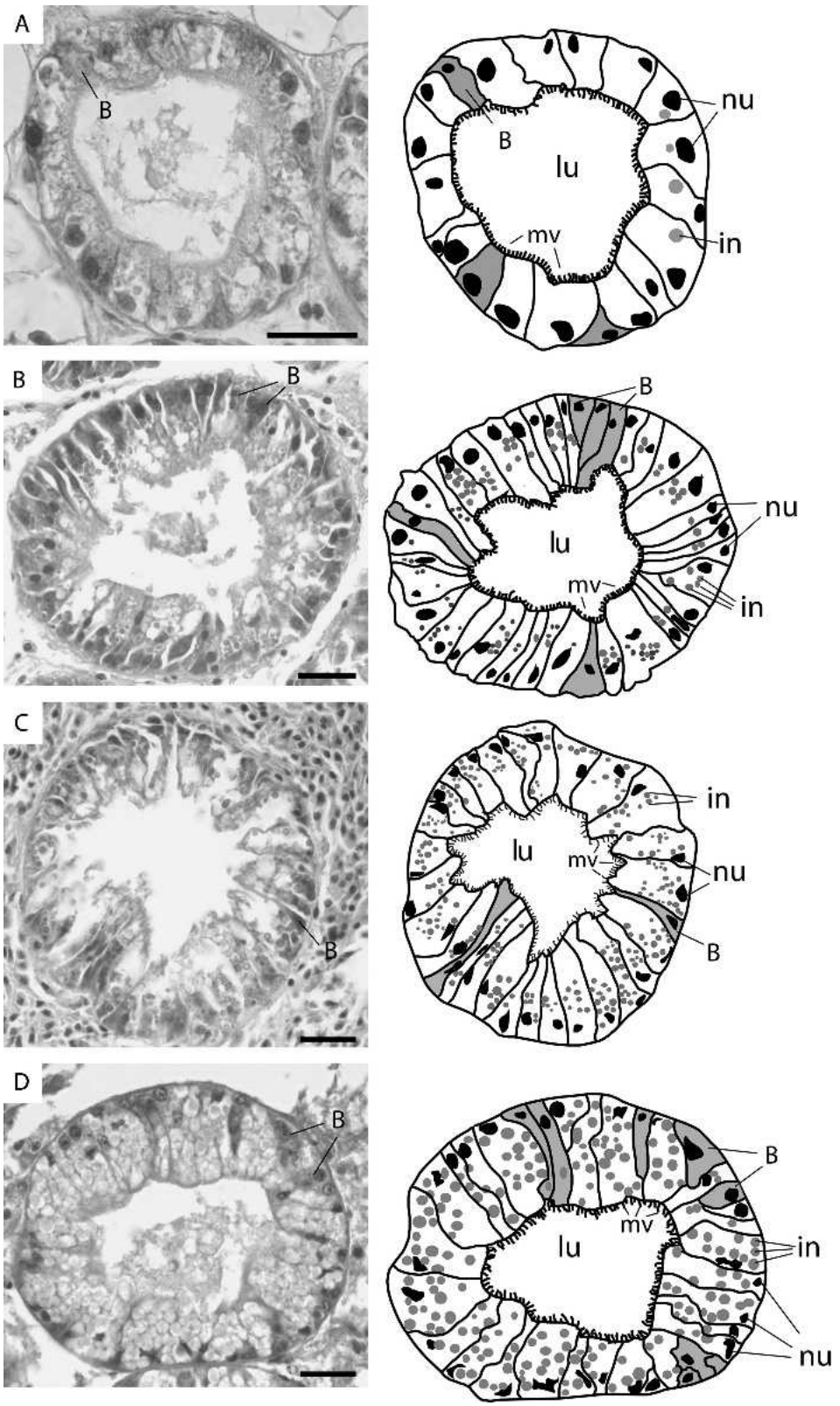

Figure 1. Digestive diverticulae of mussels (light microscopy, ×40). (A) *Geukensia demissa*; (B) *Bathymodiolus thermophilus*; (C) *Bathymodiolus brevior*; (D) *Bathymodiolus heckerae*. Scale bars: 25 μm. B: basophilic cell; in: inclusion; lu: lumen; nu: nucleus; mv: microvilli.

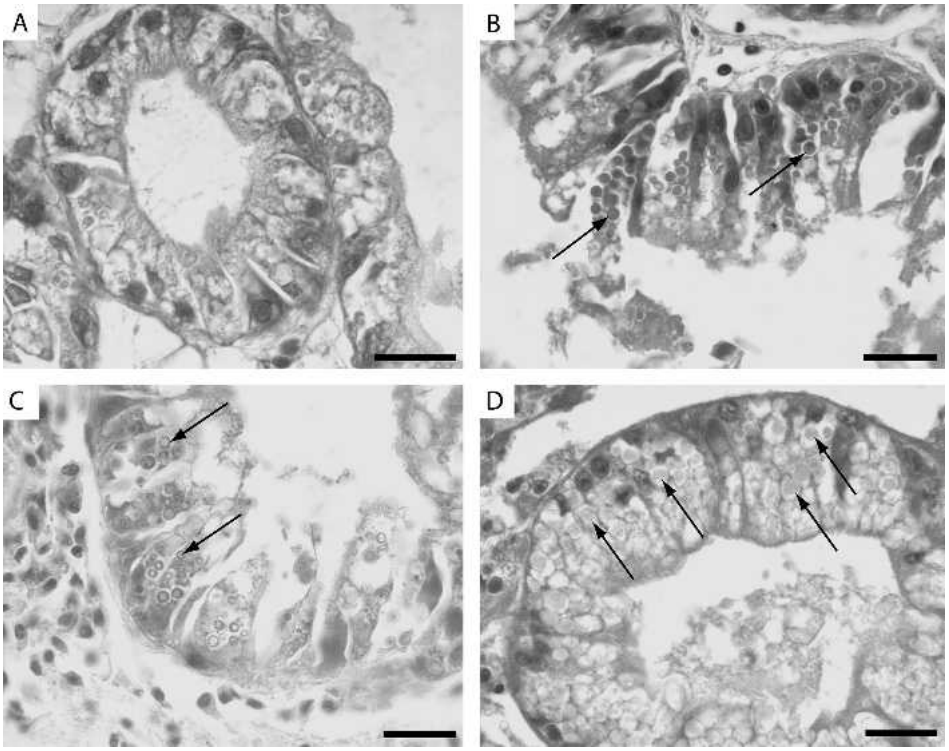

Figure 2. Digestive diverticulae of mussels (light microscopy, × 100). (A) *Geukensia demissa*; (B) *Bathymodiolus thermophilus*; (C) *Bathymodiolus brevior*; (D) *Bathymodiolus heckerae*. Scale bars: 25 μm. Arrows in B, C point to examples of brown-staining inclusions; arrows in D point to examples of light-purple-staining inclusions.

MATERIALS AND METHODS

Sample Collection

Bathymodiolus heckerae were collected from the Blake Ridge methane hydrate seep off the coast of South Carolina (32°32′N, 76°12′W, 2,155 m) in September 2001 and July 2003 by DSV *Alvin*. *Bathymodiolus* cf. *thermophilus* (referred to hereafter as *B. thermophilus*) collections were collected from hydrothermal vent fields on the Pacific-Antarctic Ridge (37°47′S, 110°54′W, 2,230 m) by ROV *Jason II* in March 2005. *B. brevior* were collected from the Mussel Hill vent of the North Fiji back-arc basin (16°59′ S, 173° 55E, 1990 m). Shallow-water mussels (*Geukensia demissa*) were collected from Felgate's Creek, York River, VA in August 2005.

Light Microscopy

Mussels were fixed whole or in parts in a 10% formalin-seawater solution or in Davidson's Solution for 24 h, then stored in 70% ethanol. For *Bathymodiolus heckerae*, the digestive gland was bisected, with half the tissue fixed for light microscopy, the other half fixed for transmission electron microscopy (TEM). Formalin-fixed samples were dehydrated in an ethanol series and embedded in paraffin, and sectioned (4–6-μm thick). Stains included Gill's hematoxylin and eosin (*B. heckerae* $n = 62$, *B.* cf. *thermophilus* $n = 2$, *B. brevior* $n = 2$, *Geukensia demissa* $n = 2$) (Stevens 1990), DAPI for DNA ([*B. heckerae* $n = 4$, *B.* cf. *thermophilus* $n = 2$] [Coleman et al. 1981]), and the Brown-Hopps method for gram-positive (blue staining) and gram-negative bacteria (red staining) ([*B. heckerae* $n = 3$, *B.* cf. *thermophilus* $n = 1$; *Geukensia demissa* $n = 1$] [Luna 1968]).

In Situ Hybridization

To determine whether the inclusions in digestive cells of *B. heckerae* were bacterial, *in situ* hybridization was carried out on *B. heckerae* ($n = 2$) paraffin-embedded digestive tissue sections using UNI16S-1, a peptide nucleic acid (PNA) probe "universal" in specificity for prokaryotes (Gauthier, unpublished) that was synthesized with a digoxigenin label. PNA is a DNA mimic that has a high affinity for complementary DNA/RNA sequences because of a neutrally charged polyamide backbone. Negative (no probe) and positive (methanotrophic and thiotrophic symbionts in *B. heckerae* gill tissue) controls were included in *in situ* hybridization experiments.

Transmission Electron Microscopy

Digestive tissues from all four mussel species (1 mm^3) were fixed for 2 h in 2% glutaraldehyde and 0.1 M phosphate buffer with 0.25 M sucrose (pH 7.4) (*B. heckerae* $n = 8$, *B.* cf. *thermophilus* $n = 2$, *B. brevior* $n = 2$, *Geukensia demissa* $n = 2$). *Bathymodiolus heckerae* samples were stored in the same buffer for up to 20 mo at 4°C before postfixation. The other samples were immediately postfixed in 1% osmium tetroxide in the same buffer and stored in 70% ethanol. Samples were dehydrated in an acetone series, *en bloc* stained in 70% acetone-2% uranium acetate, and embedded in Embed 812 epoxy

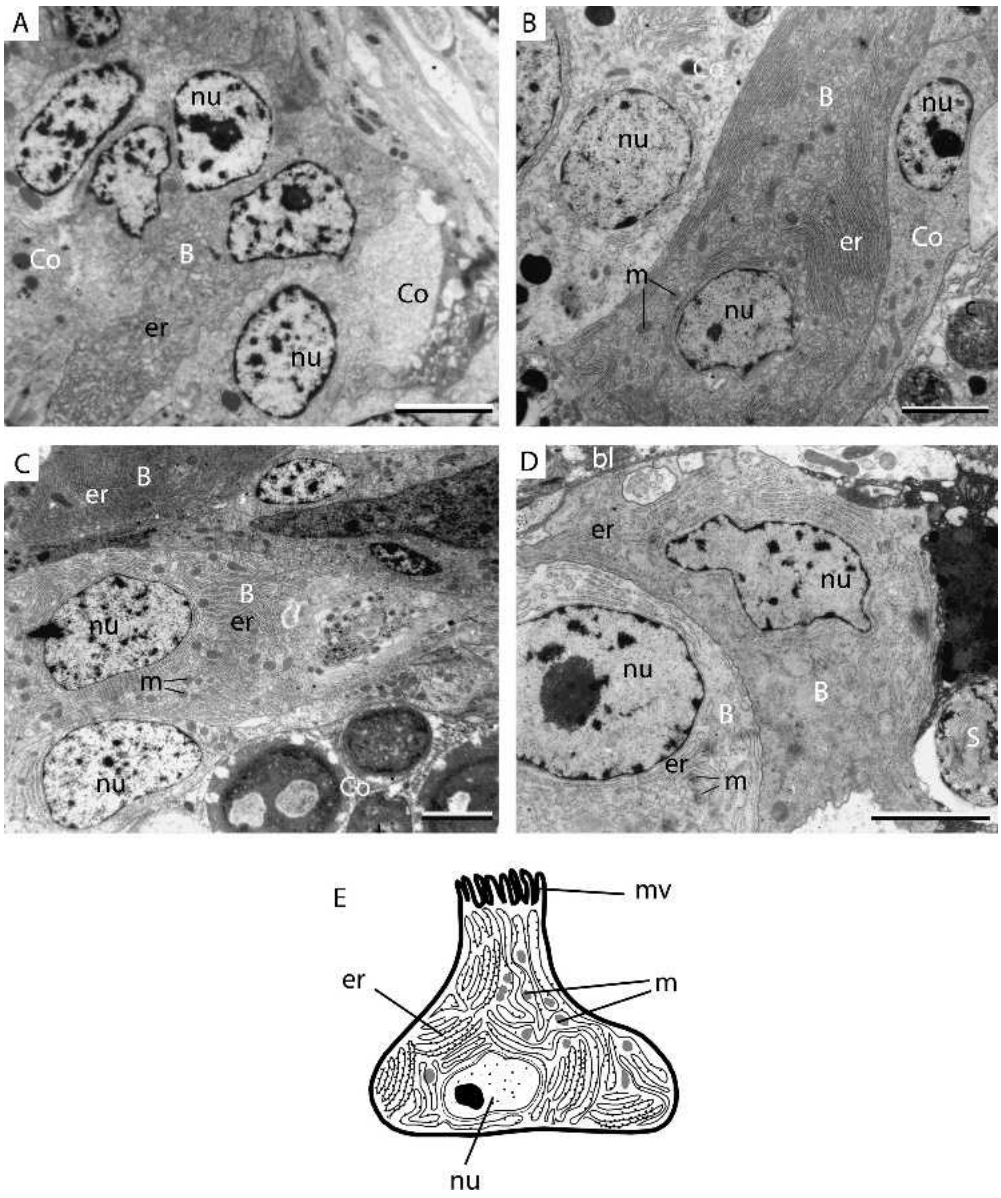

Figure 3. Basophilic cells of digestive diverticulae (transmission electron microscopy). (A) *Geukensia demissa*; (B) *Bathymodiolus thermophilus*; (C) *Bathymodiolus brevior*; (D) *Bathymodiolus heckerae*; (E) Schematic of a typical basophilic cell. Scale bars: 3 μm. B: basophilic cell; bl = basal lamina; Co = columnar cell; er = endoplasmic reticulum; m: mitochondria; nu: nucleus; S: SIX.

resin embedding medium. Sections (~90 nm thick) were stained briefly with lead citrate and examined on one-hole formvar grids with a Zeiss EM 109 transmission electron microscope.

DNA Extraction and PCR

Digestive, foot, mantle, and gill tissues of mussels were fixed in 95% EtOH (*B. thermophilus, n* = 1), or whole mussels were immediately frozen at –70°C (*B. heckerae*, *n* = 3). DNA was extracted from mussel tissue using a QIAamp DNA Stool Mini Kit (QIAGEN Inc., Valencia, CA) on digestive tissue from *B. heckerae* mussels collected in 2001 and *B. thermophilus* mussels collected in 2005. Preferential PCR was used to target non-metazoan DNA present in the digestive tissue for amplification. An SSU rDNA region was amplified using primers targeting sites that are highly conserved across Archaea, Bacteria, and nonmetazoan eukaryotes, as published previously (Bower et al. 2004). Reaction volumes of 25 μL included PCR buffer at ×1 concentration, $MgCl_2$ at 2.5 mM, nucleotides at 0.2 mM, primers (581F and 1134R; Bower et al. 2004) at 0.05 μM, Platinum *Taq* polymerase at 0.05 units/μL, and 100–200 ng of template DNA. Each experiment included no-probe treatments and bacterial-inhabited tissues (gills) as negative and positive controls, respectively. All reagents, including the synthesized primers, were obtained from the Invitrogen Corporation (Carlsbad, CA). Temperature cycling began with a denaturation at 95°C for 7 min, followed by 35 cycles of denaturation at 95°C, annealing at 49°C, and extension at 72°C, followed finally by a 7-min final extension at 72°C. Products were visualized

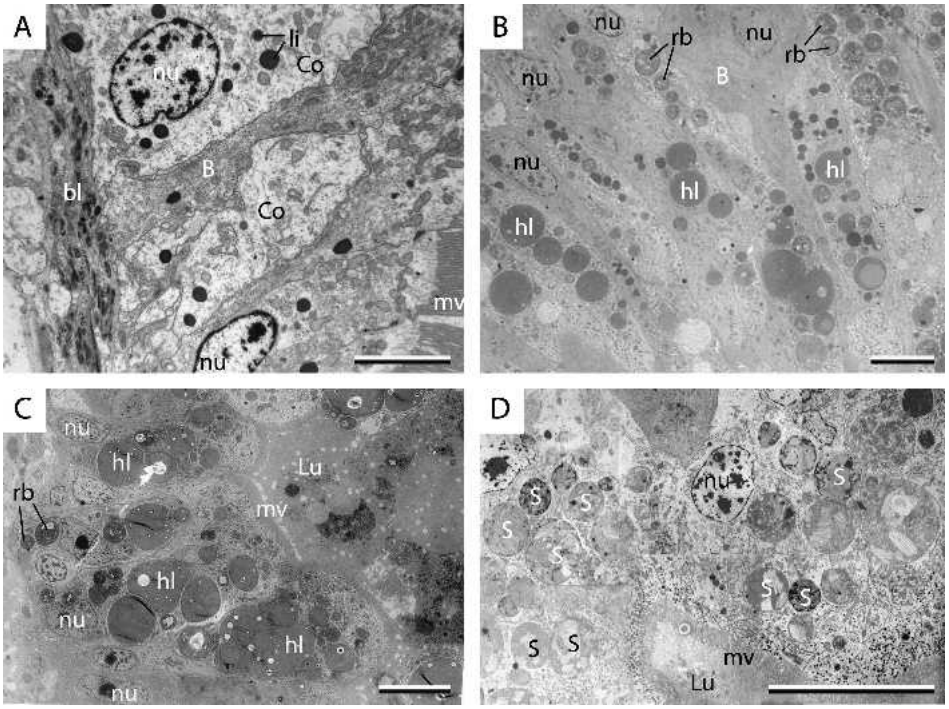

Figure 4. Digestive cells of digestive diverticulae (transmission electron microscopy). (A) *Geukensia demissa* (scale bar: 5 μm); (B) *Bathymodiolus thermophilus* (scale bar: 10 μm); (C) *Bathymodiolus brevior* (scale bar: 10 μm); (D) *Bathymodiolus heckerae* scale bar: 10 μm). B: basophilic cell; bl: basal lamina; Co: columnar cell; hl: heterolysosome; li: putative lipid droplet; Lu: lumen; m: mitochondria; Mv: microvilli; N = nucleus; rb: residual body; S: SIX inclusion.

under UV light after electrophoresis in agarose gels and staining with ethidium bromide.

RESULTS

Characterization of Digestive Tubules in Mussels from Vents and Seeps

Light Microscopy

Digestive tubules in all mussel species examined were lined by two types of epithelial cells: dark-staining basophilic cells (typically triangular in cross section) and light-staining, columnar digestive cells (Fig. 1). The columnar digestive cells of mussels from chemosynthetic environments contained numerous cellular inclusions that were not evident in the shallow-water mussel (Fig. 2). These inclusions were similar in size (2–4 μm) and staining characteristics (brown) in the two vent mussel species, *Bathymodiolus thermophilus* and *B. brevior*; inclusions in the seep mussel, *B. heckerae*, were larger (~3–5 μm) and stained light purple.

Transmission Electron Microscopy (TEM)

Basophilic secretory cells contained extensive rough endoplasmic reticulum and numerous Golgi bodies; their ultrastructure did not differ among species (Fig. 3). Columnar digestive cells of *Geukensia demissa*, *Bathymodiolus thermophilus*, and *B. brevior* contained spherical, membrane-bound, granular lysosomal bodies (Figs. 4–6), often in various stages of the digestive progression (including lysosomes, heterolysosomes, residual bodies). Lysosomal bodies range from 2 μm (lysosomes) to 10 μm (heterolysosomes) in diameter and were far less common in *G. demissa* columnar digestive cells than in those of vent mussels (Figs. 5, 6). Brown inclusions under light microscopy corresponded to lysosomal residual bodies in *B. thermophilus* and *B. brevior*.

Lysosomal bodies were not observed in columnar digestive cells of *Bathymodiolus heckerae* (Fig. 7). Instead, columnar digestive cells of *B. heckerae* were filled with large (4.1 μm), double-membrane-bound (in best preparations) spherical inclusions (Fig. 8A), herein referred to as SIX, containing stacked lamellae embedded in variably electron-dense material (Fig. 8B). Large, light-purple inclusions under light microscopy corresponded to SIX in *B. heckerae*. SIX often appeared to deform the nucleus (Fig. 8C). All digestive gland tissues of *B. heckerae* examined under TEM contained bacteria-like inclusions (hereafter referred to as bacteria based on ultrastructure) in addition to SIX. In four individuals, bacteria were only present in the basal lamina and tissues surrounding the tubules. The other three individuals contained bacteria infecting epithelial cells of the digestive diverticulae. Bacteria were typically free in the cell cytoplasm (Fig. 8D) or, more rarely, associated with stacked internal lamellae of SIX. Epithelial cells lining the stomach and intestines of *B. heckerae* did not contain SIX.

Characterization of SIX in Bathymodiolus heckerae

DNA Tests

Nuclei of the columnar digestive cells in *B. heckerae* stained with DAPI, but the numerous cellular inclusions evident in light

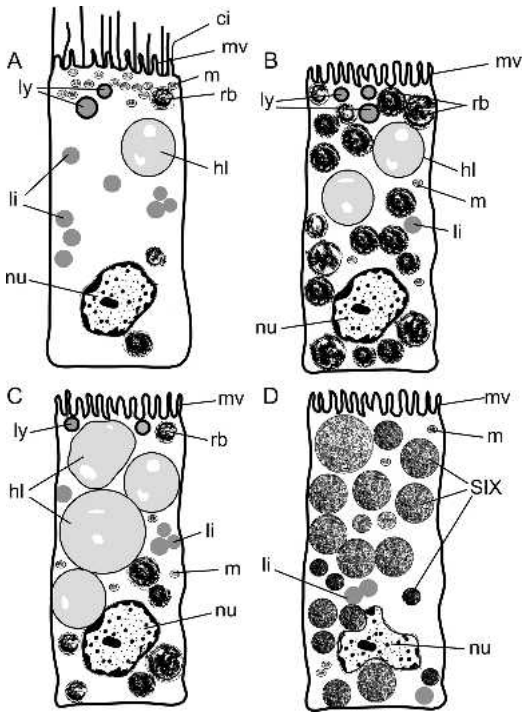

Figure 5. Comparative schematic illustrations of digestive cells. (A) *Geukensia demissa*; (B) *Bathymodiolus thermophilus*; (C) *Bathymodiolus brevior*; (D) *Bathymodiolus heckerae*. B: basophilic cell; ci: cilia; ly: lysosome; hl: heterolysosome; li: putative lipid droplet; m: mitochondria; Mv: microvilli; nu: nucleus; rb: residual body; SIX: "spherical inclusion unknown".

microscopy (Figs. 1D, 3D), interpreted to be SIX, did not stain with DAPI (Fig. 9). Bacterial symbionts in gill tissue of *B. heckerae* reacted positively using the UNI16S-1 universal probe for prokaryotes (Archaea and Bacteria; Fig. 10A), but SIX in the columnar digestive cells did not react with the probe (Fig. 10B).

PCR amplicons likely representing bacterial symbionts were generated from gill tissue extractions of *B. heckerae* (Fig. 11A) and *B. thermophilus* (Fig. 11B), but no strong bands plausibly attributable to nonmussel DNA were amplified from *B. heckerae* digestive diverticulae or foot tissues (Fig. 11A). These results suggested that no symbiont or parasite was present in abundance in these tissues.

Brown-Hopps Stain for Bacterial Membranes

Cellular inclusions of epithelial cells of the digestive diverticulae of *Geukensia demissa* and *Bathymodiolus thermophilus* did not react with the Brown-Hopps stain for gram-negative cell wall material. SIX inclusions in digestive diverticulae and symbiont-bearing gill tissues of *Bathymodiolus heckerae* were similar in color, suggestive of being Brown-Hopps positive (light purple to red), but this interpretation is subjective.

DISCUSSION

The intracellular digestive process in shallow-water bivalves was described by Owen (1972), and begins with food uptake at the apical region through endocytosis, followed by merger with membrane-bound heterophagosomes (2 μm in diameter). Digested material is released into blood sinuses of the mussel, and extraneous material is transformed into heterolysosomes (up to 10 μm in diameter). As material is digested and condensed, the heterolysosome shrinks to form a smaller (5-μm diameter) residual body. Residual bodies and mature heterolysosomes collect at the basal end of the cell and residual bodies are cyclically released into the lumen of the digestive tubules and eliminated *via* the stomach and intestine.

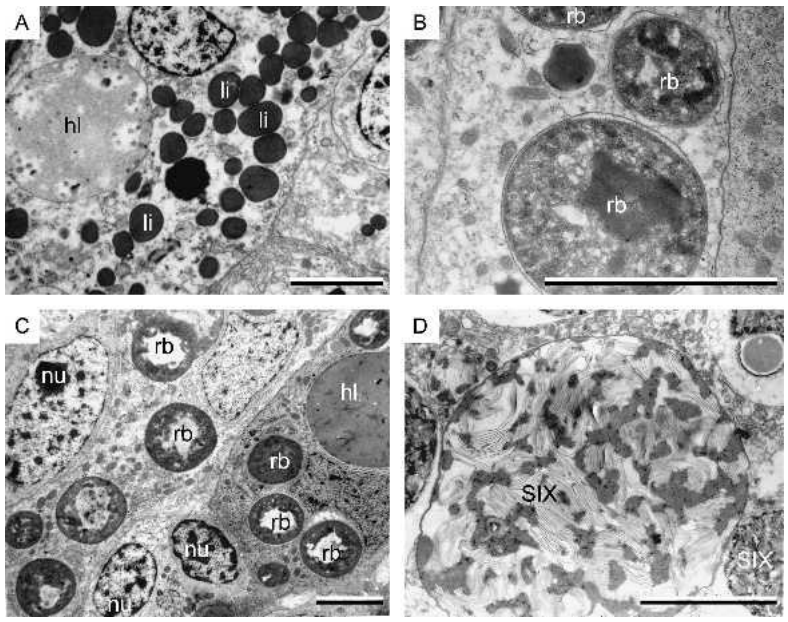

Figure 6. Cellular inclusions in digestive cells (TEM). (A) *Geukensia demissa*; (B) *Bathymodiolus thermophilus*; (C) *Bathymodiolus brevior*; (D) *Bathymodiolus heckerae*. Scale bars: 3 μm. hl: heterolysosome; li: lipid droplet; nu: nucleus; rb: residual body; SIX: spherical inclusion unknown.

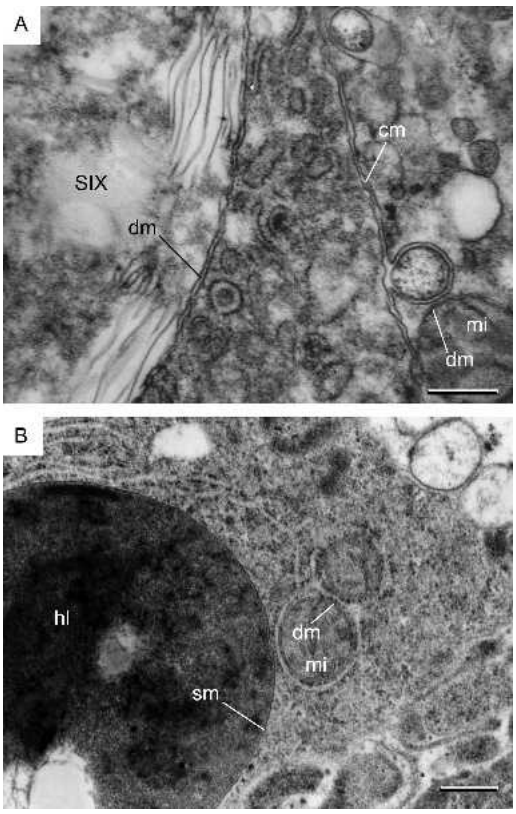

Figure 7. Detail of SIX and heterolysosome structure (TEM). (A) SIX in *Bathymodiolus heckerae*; (B) heterolysosome in *Bathymodiolus thermophilus*. Scale bars: 0.5 μm. cm: cell membrane; dm: double membrane; hl: heterolysosome; mi: mitochondrion; sm: single membrane.

The major elements of cellular digestion have been observed in digestive cells of *Bathymodiolus thermophilus* (Hily et al. 1986a, Hily et al. 1986b, this study) and *B. brevior* (this study) from deep-sea hydrothermal vents. Digestive cells of the seep mussel *B. heckerae* appeared to lack the typical components of the cellular digestive system of mussels. Instead of heterolysosomes and residual bodies, *B. heckerae* digestive cells were filled with large (~2–10 μm), double-membraned inclusions, referred to as spherical inclusions unknown (SIX). SIX inclusions vary in electron density, but all SIX inclusions contain whorled lamellae. These lamellae are reminiscent of the stacked lamellae of the methanotrophic bacteria found in the gills of *B. heckerae*. Tests for bacterial and archaeal DNA within digestive cells of *B. heckerae* were negative or inconclusive; the Brown-Hopps histochemical test for bacterial cell wall material provided subjectively positive but inconclusive evidence for a microbial origin for SIX without a second, independent assay. Despite the double membrane, which is restricted to cellular organelles such as plastids, nuclei, bacterial endosymbionts, and mitochondria, the evidence collected to date suggests that it is unlikely that SIX are endosymbiotic or parasitic microorganisms, although we cannot eliminate the possibility that they are a derivative of a microorganism.

In some respects, SIX resembles sulfide-oxidizing bodies (SOBs), reported first in gills of the symbiont-bearing and gutless clam *Solemya reidi* Bernard (Powell & Somero 1985) and subsequently in gill bacteriocytes of a lucinid calm (Liberge et al. 2001), in gills of the vent shrimp *Rimicaris exoculata* Williams (Compere et al. 2002), and in the cloacal epithelium of the echiurans worm *Urechis caupo* Fisher & Macginitie (Menon & Arp 1993). SOBs are generally (but not always, as in the case of SOBs in *U. caupo*) associated with sulfur-oxidizing endosymbionts and are coupled to ATP production in *S. reidi* (Powell & Somero 1985). There are important differences between SIX and SOBs: SIX are double- (rather than single-) membraned inclusions, SIX occurred in tissues that are remote from the seawater-tissue interface where sulfide oxidation is an important element of the cellular sulfide detoxification system, and SIX were not closely associated with mitochondria. Prevalence of SIX was uncorrelated

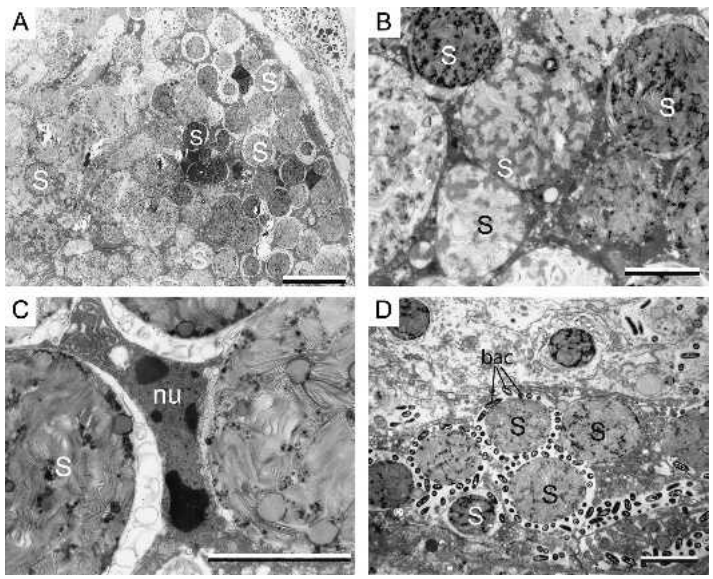

Figure 8. SIX in *Bathymodiolus heckerae* digestive cells (TEM). (A) low magnification (scale bar: 10 μm); (B) SIX inclusions of various electron densities (scale bar: 2 μm); (C) SIX apparently deforming nucleus of the digestive cell (scale bar = 2 μm) (D) bacteria (arrows) associated with SIX (scale bar 2 μm). nu: nucleus; S = SIX.

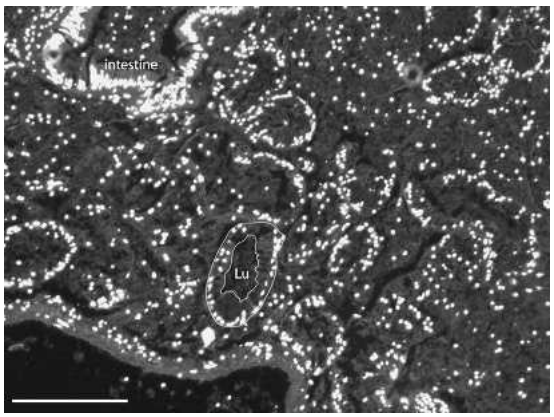

Figure 9. Digestive gland, *Bathymodiolus heckerae*, with the cross-section of one digestive diverticula outlined; DAPI stain. Scale bar: 300 μm. Lu: lumen of digestive tubule

with the presence of bacterial-like inclusions, suggesting they are independent phenomena.

The absence of the normal components of cellular digestion in the seep mussel is surprising, particularly, because

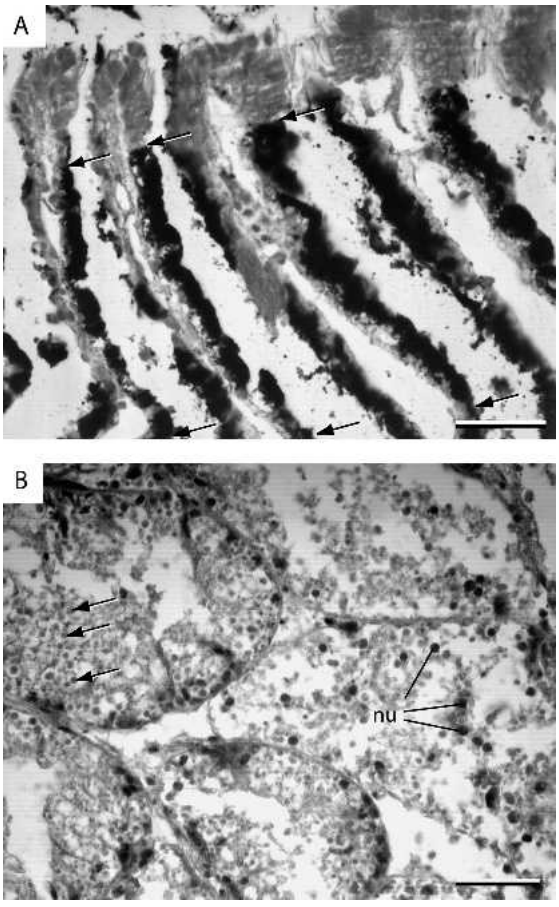

Figure 10. *In situ* hybridization using UNI16S-1 probe for prokaryotes in *Bathymodiolus heckerae*. (A) Positive control using endosymbiotic bacteria in gills (arrows point to darkly staining bacteriocytes); (B) Digestive diverticula showing no evidence of prokaryotic DNA (arrows point to SIX; nu: mussel cell nuclei). Scale bars: 15 μm.

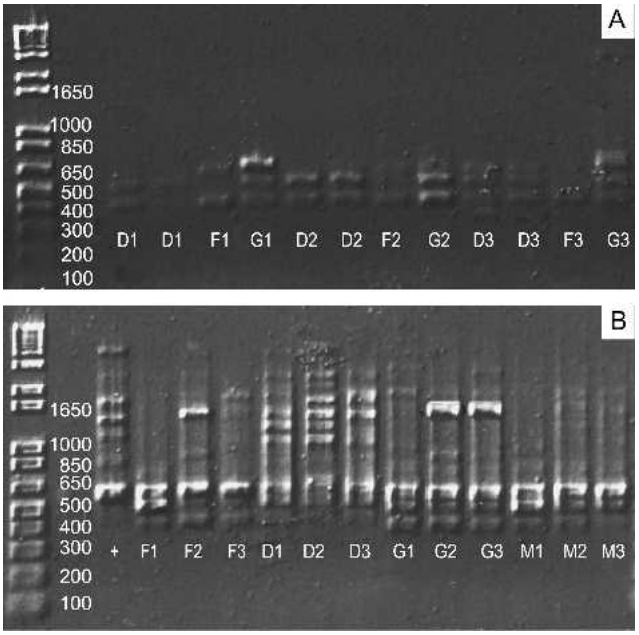

Figure 11. Gel electrophoresis of extracted and amplified SSU rDNA. (A) *Bathymodiolus heckerae* $n = 3$ individuals, with duplicate lanes for digestive tissue; (B) *Bathymodiolus thermophilus* $n = 3$ individuals. D: digestive tissue; G: gill tissue (positive control); F: foot tissue (negative control); M: mantle tissue; +: *Crassostrea virginica* Gmelin (oyster) digestive tissue. Units for numbers in ladder bands: bp

seep mussels elsewhere are postulated to depend on phytodetritus to synchronize their reproductive cycles (Tyler et al. 2007). Without further study, it is impossible to determine whether the presence of SIX is an anomalous condition or whether it represents a novel cellular organelle involved in digestion or other aspects of cellular physiology. Further studies, including cytochemical assays to identify reactions taking place in SIX and lipid analysis to constrain the origin of the SIX membranes and lamellae, should help to determine the role that SIX plays in the digestive cells of the seep mussel *Bathymodiolus heckerae*.

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