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CHAPTER 2.5

Marine Chemosynthetic Symbioses

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Introduction

Bacteria and marine eukaryotes often coexist in symbioses that significantly influence the ecology, physiology and evolution of both partners. De Bary (1879) defined symbiosis as "the living together of differently named organisms," implying that the term encompasses both positive (e.g., mutualism) and negative (e.g., parasitism) associations. Many researchers now view symbiotic interactions as those that persist over the majority of the lifespan of the organisms involved and that provide benefits to each partner beyond those obtained in the absence of association. This chapter describes such symbioses, specifically those between marine invertebrate and protist hosts and chemosynthetic bacterial symbionts.

These bacteria, which cluster primarily within the Gammaproteobacteria (Fig. 1), are chemoautotrophs or methanotrophs. In both chemoautotrophic and methanotrophic symbioses, the hosts, through an astonishing array of physiological and behavioral adaptations, provide the symbiont access to the substrates (i.e., electron donors and acceptors) necessary for the generation of energy and bacterial biomass. In exchange, a portion of the carbon fixed by the symbiont is used, either directly or indirectly, for host energy and biosynthesis. These symbioses thereby increase the metabolic capabilities, and therefore the number of ecological niches, of both the host and the bacterial symbiont.

In those symbioses for which the electron donor has been explicitly identified, sulfide and other inorganic reduced sulfur compounds (e.g., thiosulfate) fuel energy generation by the chemoautotrophic symbionts, serving as electron sources for oxidative phosphorylation. In these symbioses, the ATP produced in electron transport fuels autotrophic CO_2 fixation via the Calvin cycle. In contrast, bacteria in marine invertebrate-methanotroph symbioses use methane (CH_4) as an energy, electron, and carbon source. Unlike their protist or metazoan hosts, chemoautotrophs and methanotrophs share the ability to use reduced inorganic compounds or methane for energy generation and carbon dioxide or methane for carbon fixation and utilization. On the basis of these unique biosynthetic capacities, notably the ability to synthesize C_3 compounds from C_1 compounds, we refer collectively to these bacterial symbionts as "chemosynthetic."

Given the sulfide-rich habitats in which chemoautotrophic symbioses occur, researchers infer that the bacterial symbionts oxidize reduced inorganic sulfur compounds to obtain energy and reducing power for autotrophic carbon fixation. While some endosymbionts (such as those in the protobranchs *Solemya velum* and *S. reidi*; Cavanaugh, 1983; Anderson et al., 1987) utilize thiosulfate ($S_2O_3^{\mu}$), an intermediate in sulfide oxidation, hydrogen sulfide is inferred to be the preferred energy source in a variety of symbioses (see review in Van Dover, 2000). But for many symbioses the actual energy source has not been identified definitively; rather, only an autotrophic metabolism has been confirmed. Indeed, chemosynthetic bacteria utilizing other energy sources (e.g., hydrogen or ammonia) could also serve similar nutritional roles in symbiotic associations. In this review, bacterial symbionts that have been shown to use reduced sulfur compounds (H_2S, HS^{μ}, $S^{\mu 2}$, $S_2O_3^{\mu}$, S^{o}) for energy metabolism are referred to as thioautotrophs, while the more general term "chemoautotroph" is used to describe symbionts for which data supporting autotrophic CO_2 fixation exist but for which the lithotrophic energy source is unknown.

History

The discovery of deep-sea hydrothermal vents and the flourishing ecosystems associated with them significantly advanced scientific understanding of chemosynthetic symbioses. Oceanographers in the research submersible *Alvin* discovered hydrothermal vents along the Galapagos Rift in 1977. In stark contrast to perceptions of the deep benthos as a cold, food-limited habitat incapable of supporting substantial biomass, hydrothermal vents are oases, characterized by high concentrations of free-living

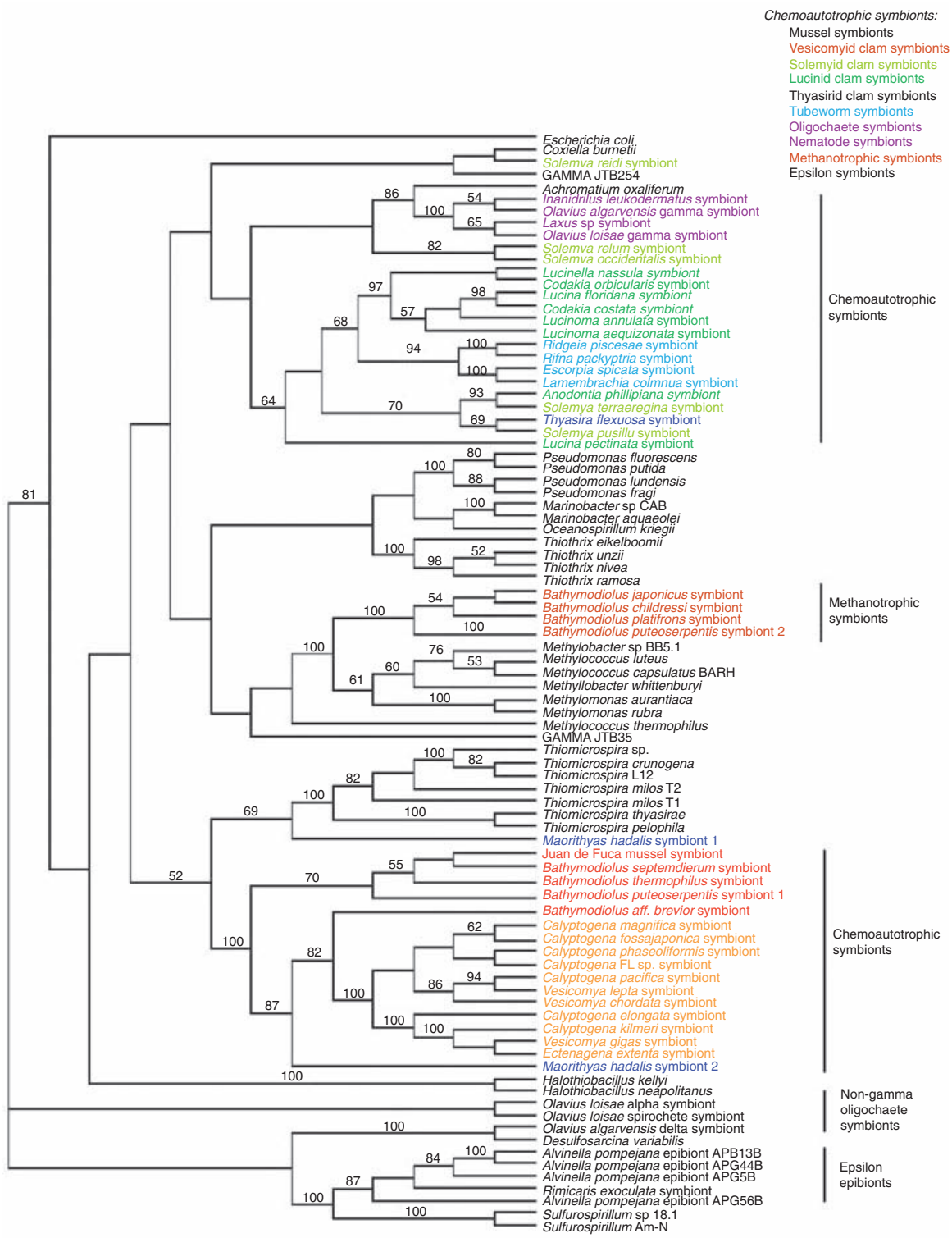

Fig. 1. Phylogeny showing the strict consensus of 46 trees obtained via parsimony analyses of 16S rRNA gene sequences (1456 bp) from symbiotic and free-living bacteria. Results greater than 50% from a 500 replicate bootstrap analysis are reported above respective branches. Chemosynthetic symbiont taxa are color-coded (see key on Figure).

Fig. 2. Symbiont-containing host organisms from hydrothermal vents and cold seeps. (A) *Calyptogena magnifica* shell; courtesy of Emilio Jorge Power. (B) Filamentous bacteria on sulfide deposits from the East Pacific Rise. (C) *Bathymodiolus childressi* from the Gulf of Mexico; courtesy of the National Oceanic and Atmospheric Administration. (D) Riftia pachyptila at the East Pacific Rise.

microorganisms and dense aggregations of invertebrates (Lonsdale, 1977; Grassle, 1985; Van Dover, 2000). Researchers first argued that vent invertebrates achieved high densities by filtering organic matter, which was presumably transported to vent sites in hydrothermally-driven convection cells (Lonsdale, 1977). A second hypothesis suggested that the invertebrate community fed directly on locally dense populations of free-living chemoautotrophic bacteria (Lonsdale, 1977; Corliss et al., 1979).

However, studies of the giant tubeworm, *Riftia pachyptila* (Fig. 2), whose lack of mouth and gut precludes suspension feeding, suggested that sulfide-oxidizing endosymbiotic bacteria might contribute substantially to the vent food web. Cavanaugh et al. (1981) proposed that symbiotic chemosynthetic bacteria occurred in *R. pachyptila*. Microscopic and biochemical evidence indicated Gram-negative bacteria were present, packed within the tubeworm trophosome (Cavanaugh, 1981; Fig. 3), a highly vascularized organ in the tubeworm trunk in which activities of enzymes involved in sulfide oxidation and carbon fixation were also detected (Felbeck, 1981a). In addition, Rau (1981) used stable isotope signatures to show a nonphotosynthetic carbon source for *R. pachyptila*, implying a role for chemoautotrophy in tubeworm metabolism. Following confirmation of a chemosynthetic endosymbiosis within the giant tubeworm, researchers questioned the putative reliance on filter feeding by other vent invertebrates. Ultimately, anatomical, enzymological, and isotopic analyses revealed the presence of sulfur-oxidizing bacterial symbionts either within the tissues (endosymbiotic) or attached to the surfaces (episymbiotic) of most vent taxa, including vesicomyid clams, mytilid mussels, shrimp, and polychaete worms (Cavanaugh, 1994; Nelson and Fisher, 1995a; Van Dover, 2000).

Recognizing the ubiquity of chemoautotrophic symbioses at hydrothermal vents, researchers searched for and discovered similar symbiotic associations in other marine habitats, including coastal and subtidal reducing sediments (e.g., Felbeck et al., 1981b; Southward et al., 1981; Southward, 1982; Cavanaugh, 1983; Giere, 1985; Bauer-Nebelsick et al., 1996), brine and hydrocarbon seeps (Sibuet and Olu, 1998), and whale skeletons (Bennet et al., 1994; Smith and Baco, 2003), thereby extending the host taxa to include solemyid and lucinid bivalves, pogonophoran tubeworms, echinoids and ciliates. In addition, methanotrophic bacteria were detected in a marine sponge (Vacelet et al., 1995), a pogonophoran tubeworm (Schmaljohann and Flügel, 1987), and in vent and seep mussels, sometimes co-occurring with sulfur-oxidizing chemoautotrophs in a "dual symbiosis" (Childress et al., 1986; Cavanaugh et al., 1987; Cavanaugh, 1993).

Habitat Chemistry

The seemingly disparate ecological niches where these symbioses are found all possess a chemical gradient, or chemocline, which chemosynthetic bacteria exploit for energy production. Chemoclines form where reduced, high-energy compounds such as sulfide or methane (typically produced in anoxic habitats, including vent fluids and sediments) underlie an oxic water column. Chemosynthetic microorganisms must bridge the oxic-anoxic interface to access both the reduced compounds (e.g., H_2S) used as an energy source and the oxygen to which electrons are shuttled in aerobic energy metabolism.

The source of reduced compounds used in chemoautotrophic energy metabolism differs among habitats. In marine sediments microbial sulfate reduction (in which $SO_4^{\mu 2}$ is used as an electron acceptor during the oxidation of organic matter) dominates, and the sulfide utilized by thioautotrophic symbioses (e.g., involving lucinid clams or solemyid protobranchs) in these habitats is of biogenic origin. In contrast, sulfide at

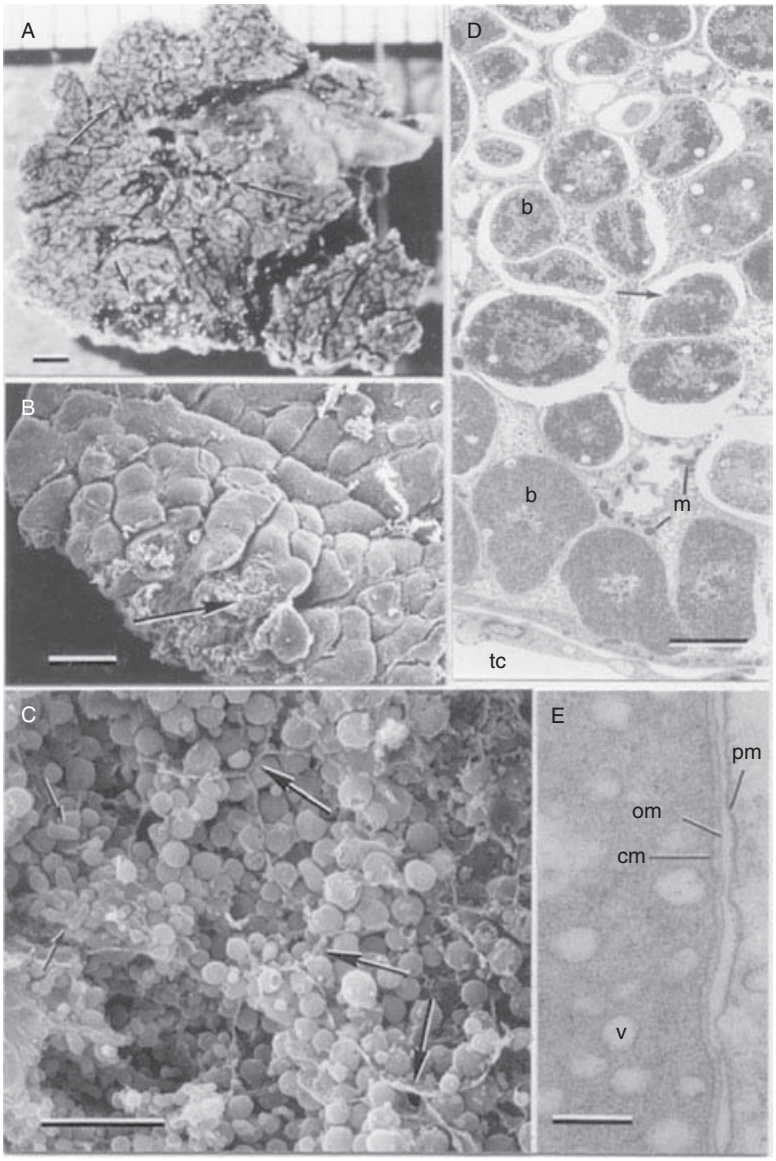

Fig. 3. *Riftia pachyptila* Jones (A–C: Galapagos Rift; D, E: 21°N, East Pacific Rise). (A) Photograph showing elemental sulfur crystals (arrows) scattered throughout trophosome; courtesy of M. L. Jones. (B) Scanning electron micrograph showing lobules of trophosome; arrow indicates area of C (below) where surface epithelium was removed to reveal symbionts within trophosome. (C) Same, higher magnification, showing symbionts within trophosome; note spherical cells as well as rod-shaped cells (small arrows); large arrows indicate likely host cell membranes. (D) Cross-section of portion of trophosome lobule showing variable fine structure of symbionts, including membrane-bound vesicles in many cells; all symbionts contained within membrane-bound vacuoles, either singly or in groups of two or more; arrow: dividing bacterium; b: bacteria; m: mitochondria; tc: trunk coelomic cavity. (E) Same, higher magnification, showing cell envelope of symbiont (resembling that of Gram-negative bacteria), intracytoplasmic vesicles, and peribacterial membrane; v: vesicle; cm: symbiont cytoplasmic membrane; om: symbiont outer membrane; pm: peribacterial membrane. Scale bars: A, 1 μm; B, 250 μm; C, 10 μm; D, 3 μm; and E, 0.2 μm. From Cavanaugh (1985), with permission.

hydrothermal vents is produced by the geothermal reduction of seawater sulfate and by the interaction between geothermally heated water and sulfur-containing rocks (e.g., basalt; Alt, 1995; Elderfield and Schultz, 1996; Rouxel et al., 2004). Seawater that percolates into the developing crust becomes heated and reacts with oceanic basalt, becoming enriched with metals and sulfide and charged with volcanic gases such as methane and carbon dioxide. Heated vent water then exits with concentrations of reduced compounds orders of magnitude higher than in ambient seawater. Hydrothermal effluent is hot (temperatures up to 350–400°C), acidic (pH ~ 3), anoxic, and can contain 3–12 mmol/kg of H_2S, 25–100 μmol/kg of CH_4, and 0.05–1 mmol/kg of H_2, as well as 360–1140 μmol/kg of Mn and 750–6500 μmol/kg of Fe (Elderfield and Schultz, 1996). As it exits the seafloor and mixes with the ambient bottom oxygenated seawater (pH, ca. 8; temperature = 1.8°C; $[O_2]$, ca. 110 μM), metallic sulfides precipitate out resulting in "black smokers" (reviewed in Elderfield and Schultz, 1996). Vent organisms are typically found clustered around more diffuse or low flow vents, which are caused by ambient seawater mixing in the shallow subsurface with vent fluid. These vents are characterized by a higher pH (ca. 6), lower temperatures (1.8 to ca. 40°C), and, consequently, lower concentrations of reduced chemicals (Van Dover, 2000).

The relative acidity of vent fluid (black smokers: pH, ca. 3; diffuse flow vents: pH, ca. 6) significantly impacts the concentration of inorganic

chemicals available to chemosynthetic symbioses. For example, carbon dioxide (CO_2), bicarbonate (HCO_3^{μ}), and carbonate ($CO_3^{\mu 2}$), the three distinct chemical species of the dissolved inorganic carbon (DIC) pool, vary in relative abundance depending on pH; pK_a values for these compounds are 6.4 for $CO_2 : HCO_3^{\mu}$ and 10.3 for $HCO_3^{\mu} : CO_3^{\mu 2}$ at 25°C (Stumm and Morgan, 1996). Thus CO_2, which diffuses freely across biological membranes and is the DIC species fixed by chemoautotrophic symbionts utilizing the Calvin cycle, is readily available at vents (Cavanaugh and Robinson, 1996; Goffredi et al., 1997b). In addition, sulfide exists at three levels of dissociation (H_2S, HS^{μ}, and $S^{\mu 2}$) depending on pH, with pK_a values of 7.0 for $H_2S : HS^{\mu}$ and 12.9 for $HS^{\mu} : S^{\mu 2}$ at 25°C (Stumm and Morgan, 1996). Therefore, in the relatively acidic vent fluids sulfide occurs predominantly as H_2S. In such effluent, total sulfide (H_2S, HS^{μ}, and $S^{\mu 2}$) concentration correlates positively with temperature (Johnson et al., 1988); conversely, higher temperatures (>30°C) may facilitate reactions between sulfide and other dissolved elements (such as iron) that reduce free sulfide availability (Luther et al., 2001). The chemical environment (i.e., concentrations of CO_2, O_2, H_2S, CH_4, H^+, and dissolved metals) is therefore expected to significantly influence the ecology and evolution of chemosynthetic symbioses.

Methods for Studying Chemosynthetic Symbioses

To date, the bacteria involved in these symbiotic associations have not yet been isolated and grown in pure culture—perhaps because the unique environment encountered in situ by chemosynthetic symbionts has not been recreated or because a reduced genome, characteristic of many endosymbionts, has precluded growth outside of the host. Symbiotic bacteria are therefore studied indirectly, using methods to assess their physiology, ecology and phylogeny within the context of the intact symbiosis. Traditionally, researchers identify chemosynthetic symbioses using a combination of microscopy (light, confocal, scanning and transmission electron), which provides visual information on the location, morphology and ultrastructure of symbionts, and enzyme assays, which detect and quantify the activity of key proteins involved in chemoautotrophic (e.g., ribulose 1,5-bisphosphate carboxylase-oxygenase) or methanotrophic (e.g., methanol dehydrogenase) metabolism. In addition, tracing the incorporation of radiolabeled substrates (e.g., carbon dioxide, methane and nitrogen species) within the host helps define the physiology of the host-bacteria partnership. Such physiological assays, in conjunction with analyses of stable isotope signatures of symbiont-containing and symbiont-free host tissue, provide valuable insight into the trophic dynamics of symbiont-based communities. Molecular approaches, such as polymerase chain reaction (PCR)-based gene probing, 16S rRNA gene analysis, and fluorescent in situ hybridization (FISH), are increasingly used to characterize the systematic relationships of symbiont and host species (e.g., Distel et al., 1995; Peek et al., 1998; Dubilier et al., 1999) and the metabolism and gene flow of the bacterial symbionts (Robinson et al., 1998; Lee et al., 1999; Millikan et al., 1999; Podar et al., 2002). Molecular techniques have also been used to detect symbiont transmission modes (Cary and Giovannoni, 1993a; Krueger, 1996a) as well as symbiont abundance (Polz and Cavanaugh, 1995).

Summary

This chapter reviews symbiotic associations between chemosynthetic bacteria and marine invertebrate and protist hosts. A bias toward symbioses between chemoautotrophic bacteria and hydrothermal vent invertebrates is evident, primarily because our knowledge of marine bacterial symbioses stems largely from studies of vent fauna done in the 27 years following the discovery of these unique organisms. But despite this impressive body of research, much about these marine symbioses remains to be revealed. In conjunction with several earlier reviews that provide a thorough and thoughtful treatment of symbioses occurring at hydrothermal vents and cold seeps (Fisher, 1990; Felbeck and Distel, 1991; Childress and Fisher, 1992; Cavanaugh, 1994; Nelson and Fisher, 1995a), the following chapter presents an overview of the ecology, physiology and evolution of chemosynthetic symbioses.

Host Diversity

Chemosynthetic bacteria are known to associate with a diversity of invertebrate hosts (six phyla), as well as with ciliate protists (Table 1 and references therein). To date, the majority of the symbionts characterized via 16S rRNA phylogenetic analyses fall within the Gammaproteobacteria division (Fig. 1; discussed further below). The intimacy of these associations varies among taxa. The bacterial partners may be episymbionts living on the surface of the host (e.g., on shrimp, nematodes, sponges, limpets and ciliates; Figs. 4–6), or endosymbionts living either extracellularly within host tissue (e.g., in oligochaetes; Fig. 7) or in specialized host cells and organs (e.g., in bivalves and

Table 1. List of invertebrate taxa hosting chemoautotrophic or methanotrophic bacterial symbionts.[a]

Group	Common name	Symbiont-containing tissue	Location	Habitat	Symbiont type	References
Protozoa						
Class Ciliata	Ciliate	NA	Epibiotic	Cold seeps, mangrove swamp	Chemoautotroph	Bauer-Nebelsick et al., 1996 Ott et al., 1998 Buck et al., 2000 Fenchel and Finlay, 1989
Porifera						
Class Demospongiae Family Cladorhizidae	Sponge	NA	Extracellular	Cold seeps	Methanotroph	Vacelet et al., 1995, 1996
Nemata						
Subfamily Stilbonematinae	Nematode	Cuticle	Epibiotic	Reducing sediments	Chemoautotroph	Schiemer et al., 1990 Polz et al., 1992
Mollusca						
Class Bivalvia						
Subclass Protobranchia						
Family Solemyidae	Clam	Gills	Intracellular	Reducing sediments, hydrothermal vents,[b] cold seeps[c]	Chemoautotroph	Cavanaugh, 1983 Fisher and Childress, 1986 Conway et al., 1989
Subclass Heterodonta						
Family Lucinidae	Clam	Gills	Intracellular	Reducing sediments, cold seeps[c]	Chemoautotroph	Giere, 1985 Schweimanns and Felbeck, 1985
Family Thyasiridae	Clam	Gills	Intracellular	Reducing sediments, cold seeps[c]	Chemoautotroph	Dando and Southward, 1986 Herry and Le Pennec, 1987
Family Vesicomyidae	Clam	Gills	Intracellular	Hydrothermal vents, cold seeps	Chemoautotroph	Cavanaugh, 1983 Rau, 1981
Subclass Pteriomorphia						
Family Mytilidae	Mussel	Gills	Extracellular Intracellular	Hydrothermal vents, cold seeps	Chemoautotroph and/or methanotroph	Childress et al., 1986 Cavanaugh et al., 1987 Fisher et al., 1988 Cavanaugh et al., 1992
Class Gastropoda						
Family Provannidae	Snail	Gills	Intracellular	Hydrothermal vents	Chemoautotroph	Stein et al., 1988 Endow and Ohta, 1989

Family Lepetodrilidae	Limpet	Gills	Epibiotic	Hydrothermal vents	Chemoautotroph	de Burgh and Singla, 1984 Fox et al., 2002 Bates et al., 2004
Annelida[d]						
Class Polychaeta	Worm	Dorsal surface	Epibiotic	Hydrothermal vents	Chemoautotroph	Desbruyeres et al., 1983, 1985 Cary et al., 2003
Family Alvinellidae						
Family Siboglinidae (Vestimentifera and Pogonophora)	Tubeworm	Trophosome	Intracellular	Deep-sea hydrothermal vents, cold seeps, reducing sediments, fjords	Chemoautotroph	Cavanaugh et al., 1981 Felbeck, 1981 Southward et al., 1981 Brooks et al., 1987 Schmaljohann and Flügel, 1987 Southward and Southward, 1988 de Burgh et al., 1989
Class Clitellata						
Subfamily Phallodrilinae	Oligochaete	Subcuticular	Extracellular	Coralline sands	Chemoautotroph[e]	Felbeck et al., 1983 Giere, 1981, 1985 Giere and Langheld, 1987
Arthropoda						
Class Crustacea						
Family Alvinocarididae	Shrimp	Carapace	Epibiotic	Hydrothermal vents	Chemoautotroph	Van Dover et al., 1988 Polz and Cavanaugh, 1995 Polz et al., 1999
Echinodermata						
Class Echinoidea	Sea urchin	Gut	Extracellular	Reducing sediments	Chemoautotroph[f]	Temara et al., 1993 Brigmon and de Ridder, 1998

Abbreviation: NA, not applicable.

[a]Chemosynthetic status of symbionts inferred from ultrastructural, physiological, enzymatic, and molecular data.

[b]A solemyid protobranch, *Acharax alinae*, has been described from the Lau Basin hydrothermal vents, but the symbiosis has not been characterized (Metivier and Voncosel, 1993).

[c]Solemyid clams have been collected from cold seeps in the eastern Pacific, and lucinid and thyasirid clams have been collected from cold seeps in the Gulf of Mexico, Sagami Bay, and Barbados Prism, though the presence of symbionts has not been formally described (Sibuet and Olu, 1998).

[d]Though it is now accepted that the pogonophoran and vestimentiferan worms are not separate phyla but members of phylum Annelida, they are listed as separate groups for identification purposes.

[e]The oligochaete *Olavius algarvensis* has been shown to have an additional symbiont which is a sulfur reducing bacterium and member of the delta Proteobacteria (Dubilier et al., 2001). *Olavius loisae* has been shown to host an alpha proteobacterium and a spirochete as well as a chemoautotroph (Dubilier et al., 1999).

[f]This symbiont has been described as *Thiothrix*-like on the basis of morphology, physiology, and immunological assays (Temara et al., 1993; Brigmon and de Ridder, 1998).

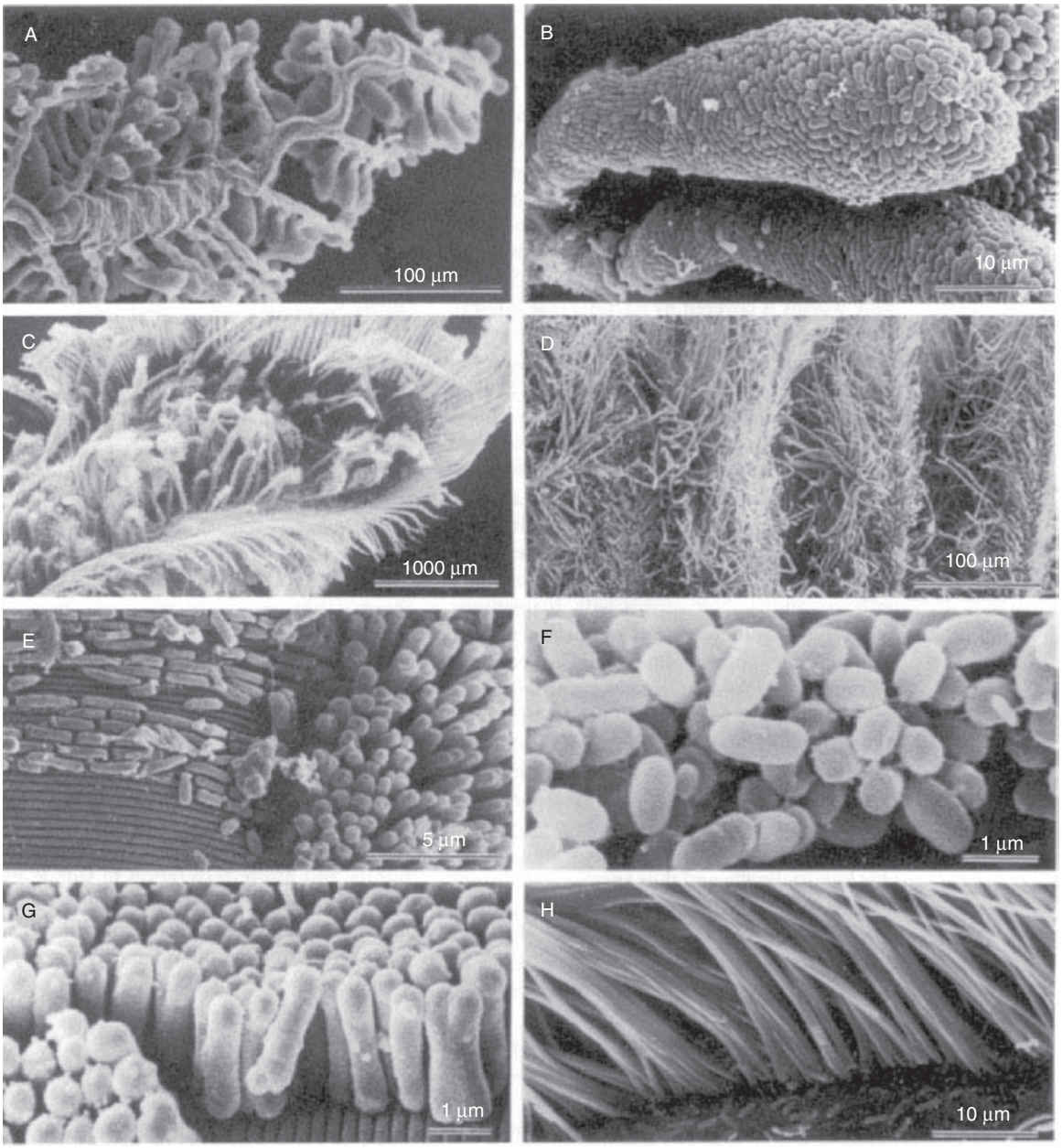

Fig. 4. Scanning electron micrographs showing the morphological diversity of ectosymbiotic bacteria on the colonial ciliate *Zoothamnium niveum* (A, B), the shrimp *Rimicaris exoculata* (C, D), and the nematodes within the subfamily Stilbonematinae (E-H). (A) Entire ciliate colony with zooids attached to a common stem, and (B) bacterial epigrowth on an individual zooid. (C) Shrimp appendage covered by dense arrays of filamentous bacteria, and (D) detail of the hair-like bacterial covering. Epigrowth on different species of nematodes showing (E) irregular epigrowth of two morphological types on *Robbea* sp., (F) coccoid bacteria forming multilayers on *Stibonema* sp., (G) upright standing, longitudinally dividing rods on *Laxus oneistus*, and (H) dense array of nonseptate filaments that can reach up to 100 mm in length on *Eubostrichus dianae*. From Polz et al. (2000), with permission.

vestimentiferan tubeworms; Figs. 2, 3, and 8). In intracellular endosymbioses the symbionts are housed within specialized host cells called "bacteriocytes" and are contained within a host-derived membrane bound vacuole (Cavanaugh, 1983; Cavanaugh, 1994; Fisher, 1990). Host morphology clearly suggests a nutritional benefit from these intimate associations. Indeed, as in the giant vent tubeworms, the digestive system of many endosymbiont-containing marine invertebrates is either reduced (e.g., in coastal solemyid protobranchs) or absent altogether (e.g., in oligochaetes and vestimentiferan and pogonophoran tubeworms), consistent with

host dependence on the symbiont for part or all of its nutrition.

All of the members of the tubeworm family Siboglinidae examined to date, including the vestimentiferan and the smaller pogonophoran tubeworms, contain intracellular symbionts. Most of these symbionts are inferred to be chemoautotrophic, but methanotrophs have been found in one host species (*Siboglinum poseidoni*; Schmaljohann and Flügel, 1987). The vent tubeworm *Riftia pachyptila* and other vestimentiferan and pogonophoran tubeworm species possess a unique morphological adaptation to accommodate their symbionts. Tubeworm bacteria reside within a lobular and highly vascularized organ (the trophosome) that occupies most of the tubeworm trunk and functions specifically to house bacteria (Cavanaugh, 1981; Felbeck, 1981a; Figs. 3 and 9). The symbiosis is obligate for these worms, as they are mouthless and gutless as adults and depend on their internal bacteria for their nutrition.

Chemosynthetic symbioses are widespread within the Mollusca and have been detected in five bivalve and two gastropod families (Table 1). In mollusk symbioses the bacteria occur only in the gills; bacteria have been found within gill epithelial cells of solemyid protobranchs (Cavanaugh, 1983; Krueger et al., 1996b; Figs.

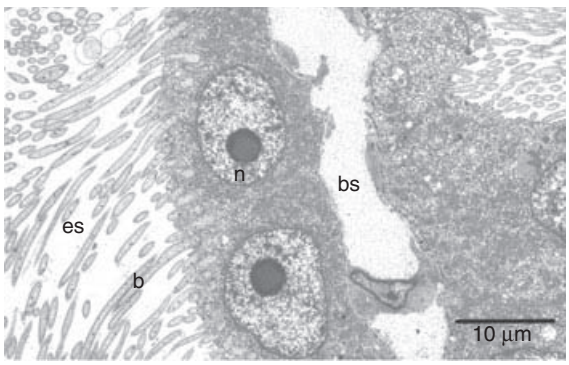

Fig. 5. *Lepetodrilus fucensis*. Transverse section of gill tissue from the hydrothermal vent limpet showing episymbiotic filamentous bacteria partially embedded in the host epithelium. b, bacteria; es, extracellular space; n, nucleus; bs, blood space. Courtesy of Amanda Bates.

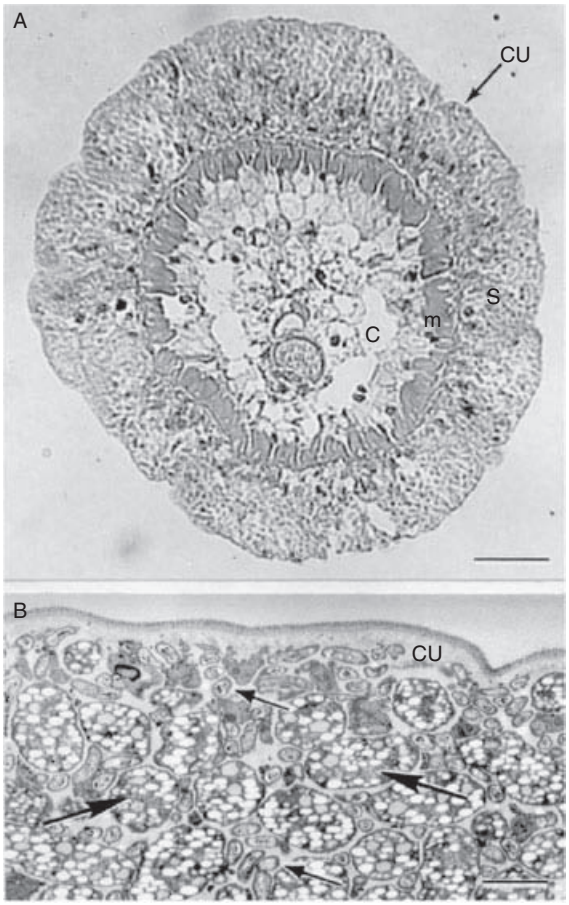

Fig. 7. *Inanidrilus leukodermatus*. (A) Light micrograph of a cross section of an oligochaete worm. (B) Transmission electron micrograph of symbiont-containing region just below the cuticle. Note smaller and larger symbiont morphotypes (smaller and larger arrows, respectively). c, coelomic cavity; m, muscle tissue; s, symbiont-containing region between cuticle and epidermis; cu, cuticle. Scale bars: A, 20 µm; B, 2 µm. From Dubilier et al. (1995), with permission.

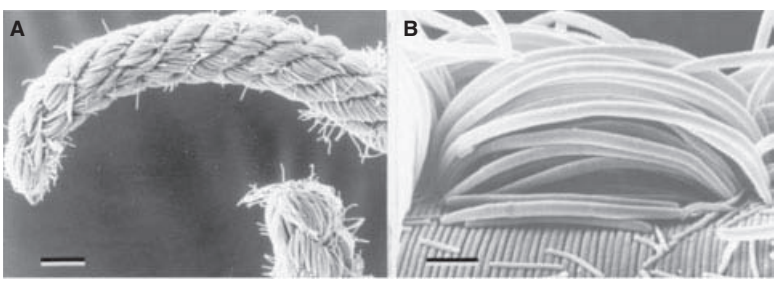

Fig. 6. *Eubostrichus* cf. *parasitiferus*. Scanning electron micrographs showing the symbiotic bacteria on the surface of the nematode. (A) Anterior (bottom) and posterior (top) end with symbionts arranged in a characteristic helix. (B) Higher magnification. Bacteria are attached with both ends to the worm's cuticle. Note the increasing length of the cells from proximal to distal along the worm's surface. Scale bars: A, 20 µm; B, 2 µm. From Polz et al. (1992), with permission.

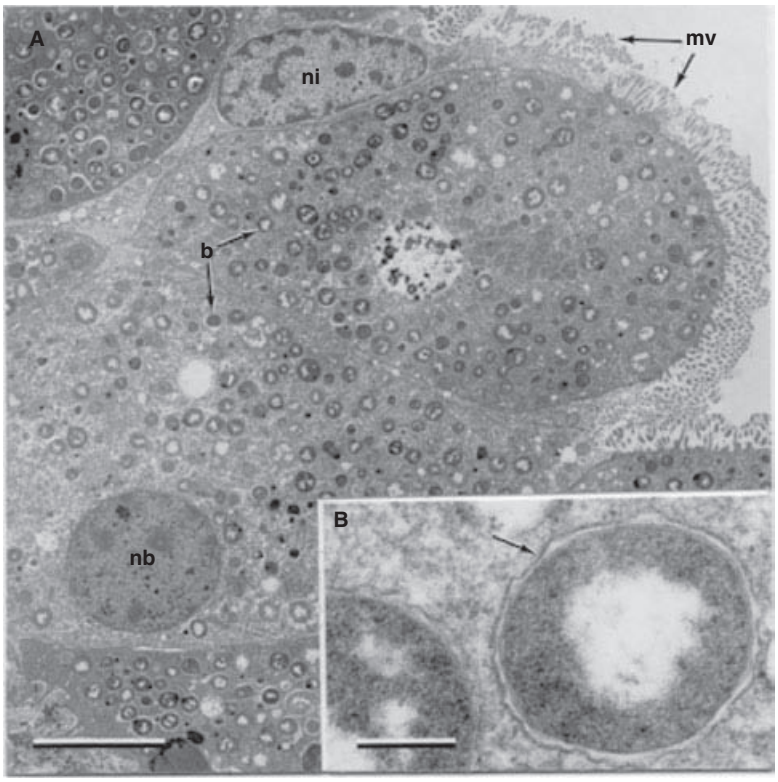

Fig. 8. *Calyptogena magnifica* Boss and Turner (21°N East Pacific Rise). (A) Transmission electron micrograph of slightly oblique transverse section of gill filament, showing coccoid-shaped bacteria within gill bacteriocyte and intercalary cells lacking symbionts; b: bacteria; mv: microvilli (of both cell-types); nb: nucleus of bacteriocyte; ni: nucleus of intercalary cell. (B) Same, higher magnification, transverse section of coccoid-shaped symbionts, showing cell ultrastructure typical of Gram-negative bacteria and peribacterial membrane (arrow). Scale bars: A, 5 μm; B, 0.25 μm. From Cavanaugh (1985), with permission.

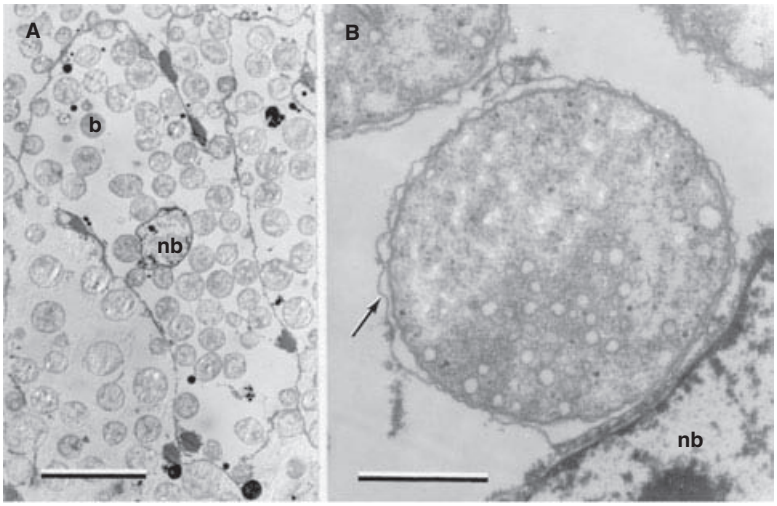

Fig. 9. Escarpia spicata Jones (San Clemente Fault). (A) Transmission electron micrograph, portion of trophosome lobule showing numerous coccoid- to ovoid-shaped bacterial symbionts, some of which appear intracellular. (B) Same, higher magnification, showing bacterial cell envelope (resembling that of Gram-negative bacteria) and intracytoplasmic membrane-bound vesicles; arrow: peribacterial membrane; b: bacteria; nb: nucleus of bacteriocyte. Scale bars: A, 10 μm; B, 1 μm. From Cavanaugh (1985), with permission.

10–13), lucinid clams (Cavanaugh, 1983; Felbeck, 1983a), thyasirid clams (Felbeck et al., 1981b; Cavanaugh, 1983; Arp et al., 1984), vesicomyid clams (Boss and Turner, 1980; Rau, 1981; Arp et al., 1984; Fig. 8), mytilid mussels (Fiala-Médioni, 1984; Le Pennec and Hily, 1984; Figs. 2 and 14), and provannid gastropods (Stein et al., 1988; Windoffer and Giere, 1997). Within certain mollusk families (e.g., Solemyidae, Lucinidae and Thyasiridae), all species examined form symbioses with chemoautotrophic bacteria. In other families, such as the Mytilidae, chemoautotrophic symbionts have been detected only in members of the subfamily Bathymodiolinae, which are found exclusively in the deep-sea. Further, dual symbioses involving both methanotrophs and chemoautotrophs are restricted to species of deep-sea bathymodioline mussels collected from methane seeps and hydrothermal vents (Cavanaugh, 1994; Nelson and Fisher, 1995a; Van Dover, 2000; Fig. 14).

Chemosynthetic bacteria also occur as episymbionts on marine invertebrates (Table 1; Fig. 4). These symbionts include the Epsilonproteo-

Fig. 10. *Solemya* sp. (right hand) collected from deep-sea vent sites (2380 m depth) along the subduction zone off Oregon, and *Solemya velum* (left hand) collected from subtidal reducing sediments (<1 m depth, mean low tide) of Massachusetts eelgrass beds. Photo courtesy of Dr. Ruth D. Turner.

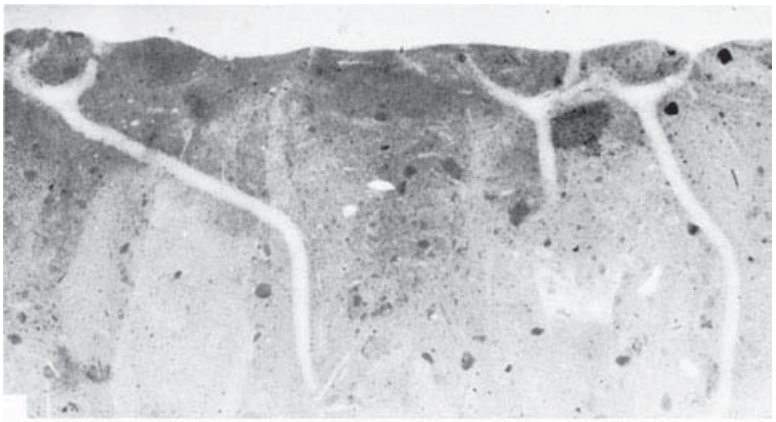

PLATE 3. SOLEMYA

Fig. 11. *Solemya velum*. Characteristic Y-shaped burrows dug by the coastal protobranch clam to bridge the oxic-anoxic interface and access both reduced sulfur (from below) and oxygen (from above). From Stanley (1970), with permission.

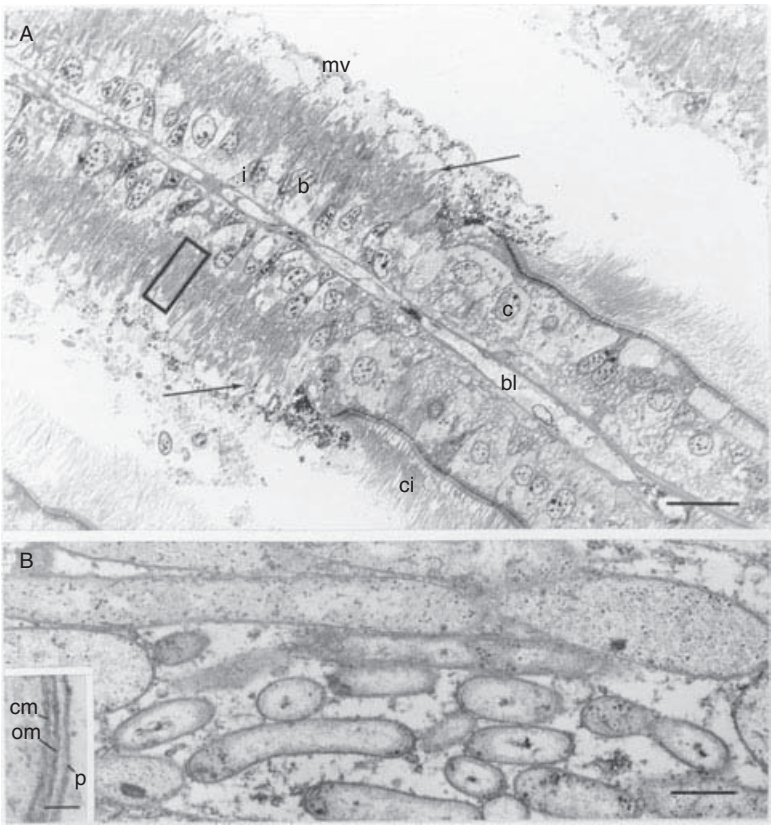

Fig. 12. Solemya borealis. (A) Transverse section of gill filaments showing intracellular rod-shaped bacteria (arrows, rectangle). Bacteriocytes are confined to the region proximal to the ciliated edge of the gill and are flanked by symbiont-free intercalary cells that appear to comprise the microvillar surface of the gill filament. Light micrograph. b: bacteriocyte nucleus; c: ciliated cell nucleus; i: intercalary cell nucleus; bl: blood space; ci: cilia; mv: microvilli. (B) Higher magnification of symbionts showing cell ultrastructure typical of Gram-negative. Inset: Detail of symbiont cell envelope and peribacterial membrane. p: peribacterial membrane; cm: cell membrane; om: outer membrane. Scale bars: A, 20 µm; B, 1 µm; inset, 0.05 µm. From Conway et al. (1992b), with permission.

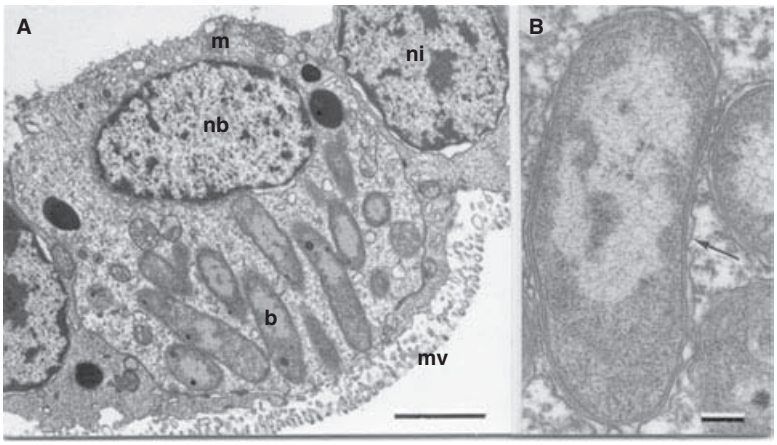

Fig. 13. *Solemya velum*. (A) Transmission electron micrograph, transverse section of gill filament, showing rod-shaped bacteria within gill bacteriocyte and intercalary cells lacking symbionts; b: bacteria; mv: microvilli; nb: nucleus of bacteriocyte; ni: nucleus of intercalary cell. (B) Same, higher magnification, transverse section of rod-shaped bacterium, showing cell ultrastructure typical of Gram-negative bacteria and peribacterial membrane (arrows). Scale bars: A, 3 μm; B, 0.2 μm. From Cavanaugh (1985), with permission.

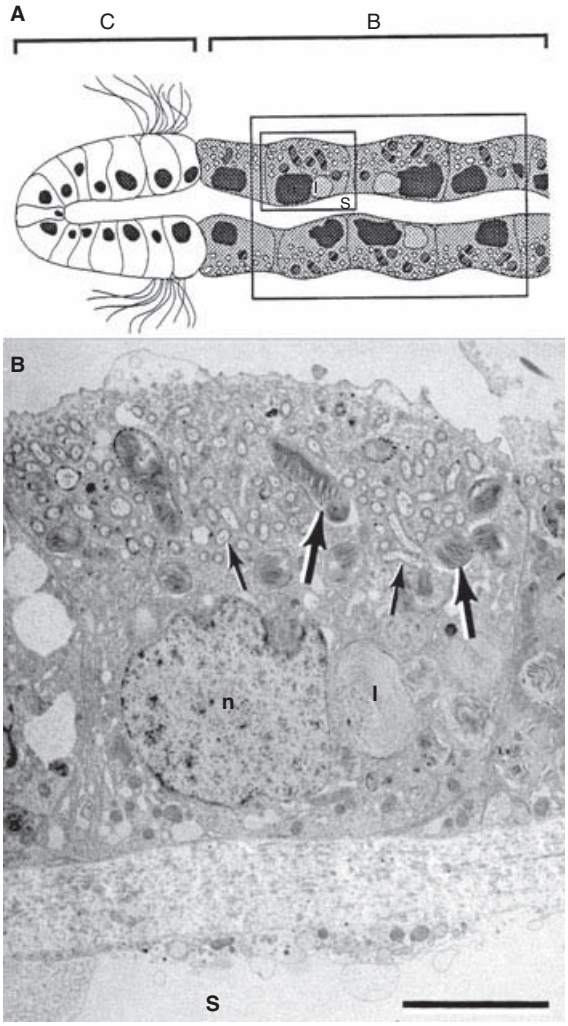

Fig. 14. *Bathymodiolus puteoserpentis*. Transverse section of Mid-Atlantic Ridge (MAR) mytilid gill filament, showing symbiont-containing gill epithelial cells (bacteriocytes). (A) Diagram of gill filament. Bacteriocytes are confined to the region proximal to the ciliated border of the gill. Small box shows positions of Figs. 14B. B: symbiont-containing bacteriocyte region; and C: symbiont-free ciliated region. (B) Transmission electron micrograph. Large and small symbionts (large and small arrows, respectively) are located in the apical region of the cells, while nuclei and lysosomal residual bodies occupy the region closest to the blood sinus. Note centrally stacked intracytoplasmic membranes in large symbionts. 1, Lysosomal residual body; n, bacteriocyte nucleus; and s, blood sinus. Scale bar: 5 μm. From Distel et al. (1995), with permission.

bacteria that cover the cuticle of *Rimicaris* shrimp, dominant members of the metazoan fauna at vents on the Mid-Atlantic Ridge (MAR; Polz et al., 1998) and the Central Indian Ridge (CIR; Van Dover et al., 2001; Van Dover, 2002a), and the surfaces of alvinellid polychaetes (Desbruyères et al., 1985; Cary et al., 1997). Chemosynthetic episymbionts also associate with nematodes (Weiser, 1959; Ott et al., 1991; Polz et al., 1992; Polz et al., 1994) and ciliates

(e.g., Fenchel and Finlay, 1989; Bauer-Nebelsick et al., 1996). In addition, methanotrophic episymbionts have been found living on a deep-sea sponge (Vacelet et al., 1995; Vacelet et al., 1996). Vent limpet-bacteria associations seem to be an intermediate between epi- and endosymbioses; bacteria exist partially embedded in the limpet gill epidermis and may be endocytosed or fed on by the host (de Burgh and Singla, 1984; Bates et al., 2004; Fig. 5). Some epibiont communities, like those residing on the *Rimicaris* shrimp and the nematode *Laxus* sp., are dominated by a single phylotype (Polz et al., 1994; Polz and Cavanaugh, 1995), while others are quite diverse (Polz et al., 1999; Campbell et al., 2003). But given that morphological plasticity often belies the phylogenetic identity of symbionts (Polz et al., 1999; Giere and Krieger, 2001), symbiont diversity estimates are only appropriate when putative symbiont phylotypes are confirmed using hybridization methods (e.g., FISH).

Symbiont Diversity

Morphology and Ultrastructure

Symbiont morphology varies among functional types (chemoautotroph vs. methanotroph), among phylotypes within the same functional group, and among individuals in a population of a single phylotype. The symbionts all have a Gram-negative cell envelope but range from small (ca. 0.25 μm diameter) coccoid endosymbionts within mussel gills (Cavanaugh, 1985; Dubilier et al., 1998) to large (>10 μm length) rod-shaped and filamentous episymbionts on vent shrimp (Hentschel et al., 1993b; Polz and Cavanaugh, 1995; Fig. 4). Some bathymodioline mussels host two metabolically distinct symbionts: small (<0.5 μm) chemoautotrophs and larger (1.5–2.0 μm) methanotrophic bacteria possessing stacked intracytoplasmic membranes, which are typical of Type I methanotrophs (e.g., Childress et al., 1986; Cavanaugh et al., 1987; Cavanaugh et al., 1992; Fiala-Médioni et al., 2002; Pimenov et al., 2002; Fig. 14). Morphological diversity can also occur throughout monospecific populations within a single host animal. For example, populations of sulfur-oxidizing chemoautotrophic symbionts in the tubeworm *Riftia pachyptila* contain distinct morphotypes that vary in abundance depending on location within the trophosome lobule (Bright et al., 2000); small, rod-shaped bacteria occur primarily in the innermost zone of the lobule nearest the host's axial blood vessel, while small and large cocci (1.6–10.7 μm diameter) occupy zones nearer the periphery of the trophosome (Bright et al., 2000). Such variability may relate to differences in the lifecycle stage and metabolism among symbiont cells (Bright et al., 2000).

Symbiont Phylogeny

While chemoautotrophic symbionts have consistently evaded culture, the suite of cellular and molecular methods used to characterize these bacteria has revealed startling evolutionary trends. Investigators have successfully sequenced 16S rRNA genes from symbiont-containing tissue and subsequently confirmed the symbiont origin of these sequences via hybridization with symbiont-specific probes. In contrast to the wide diversity of host taxa involved in these symbioses, chemosynthetic symbionts cluster primarily within a single bacterial division, the Gammaproteobacteria, on the basis of 16S rRNA gene sequences (Distel and Cavanaugh, 1994; Dubilier et al., 1999; McKiness, 2004). Such analyses have also shown that most host species typically form a relationship with a unique symbiont phylotype. But this clearly is not always the case. For example, while strain level variation may occur, vestimentiferan tubeworms belonging to the genera *Riftia*, *Tevnia*, and *Oasisia* appear to share a single, or very similar, symbiont phylotype based on 16S rRNA gene sequences (Feldman et al., 1997; Laue and Nelson, 1997; Di Meo et al., 2000; Nelson and Fisher, 2000; McMullin et al., 2003), as do some species of tropical lucinid clams (Durand and Gros, 1996a; Durand et al., 1996b).

Recently, McKiness (2004) reported phylogenetic analyses of 16S rRNA gene sequences from chemosynthetic symbionts within the Gammaproteobacteria. This study represented the most comprehensive analysis of chemoautotrophic symbionts to date. It included 39 symbiont sequences and over 30 sequences from free-living bacteria representatives of chemoautotrophs, methanotrophs, and marine bacterioplankton. Here, an expanded phylogenetic analysis is presented that includes the Epsilonproteobacteria symbionts of shrimp and alvinellid worms (Fig. 1). This consensus tree illustrates the strong level of resolution afforded by the 16S rRNA gene and shows that almost all of the chemosynthetic symbionts for which sequence data are available cluster into two main clades. The first clade includes symbionts of lucinid and thyasirid clams, solemyid protobranchs, tubeworms, nematodes, and oligochaetes, and the second clade includes the mytilid mussel and vesicomyid clam symbionts.

Though the first clade as a whole has relatively low bootstrap support, smaller clusters within the first clade are strongly supported. For example, the monophyletic cluster of nematode and oligochaete symbionts has 100% bootstrap sup-

port. Similarly, the vestimentiferan tubeworm symbiont clade also has high bootstrap support, corroborating prior evidence that these worms share a single or very similar symbiont phylotype, which is consistent with environmental transmission of symbionts (Feldman et al., 1997; Laue and Nelson, 1997; Di Meo et al., 2000; Nelson and Fisher, 2000; McMullin et al., 2003; see the section Ecology and Evolution in this Chapter). In contrast, the clam symbionts exhibit a more complicated relationship. The lucinid clam symbionts form a paraphyletic group; some are sister to the tubeworm symbionts while others group with thyasirid and solemyid symbionts. The solemyid symbionts show similarly complicated relationships, as they are polyphyletic and scattered throughout the first clade. *Solemya velum* and *S. occidentalis* symbionts cluster with the nematode and oligochaete symbionts, while *S. terraeregina* and *S. pusilla* symbionts cluster with lucinid and thyasirid clam symbionts. Thus, this disjointed distribution does not suggest cospeciation between host taxa and symbionts in this first clade and indicates that there were multiple initiations of symbiosis within the solemyid and lucinid clams.

The position of the *S. reidi* symbiont within this first main group is curious; this symbiont falls at the base of this first main group, clustering with an intracellular pathogen, *Coxiella burnetti*, and an environmental clone from a Japanese cold seep, Gamma JTB254 (discussed further below). This symbiont sequence has held a basal position in other analyses (see Bayesian analysis in McKiness, 2004) and provokes questions concerning the nature of symbiosis in protobranch bivalves. As additional sequences become available, it will be necessary to reassess the position of the *S. reidi* symbiont with respect to other chemoautotrophic symbionts.

The second clade of symbionts, which includes the mytilid mussel and vesicomyid clam symbionts from vents and cold seeps, shows 100% bootstrap support (support for the mussel and vesicomyid clades being 70% and 99%, respectively). The symbiont from the Central Indian Ridge mussel, *Bathymodiolus* aff. *brevior*, falls at the base of the vesicomyid clam symbionts, with 82% bootstrap support. In contrast to the first symbiont clade (Fig. 1, top), evidence suggests that an ancestral symbiont initiated symbioses with both the vesicomyid clams and the bathymodioline mussels and predated the split between these bivalve lineages. On the basis of 16S rRNA gene sequence data, the divergence of the clam and mussel symbionts has been dated to 125–300 million years ago (Mya). This is corroborated by the fossil record, which dates the bathymodioline mussel hosts to 150 Mya and the vesicomyid clams to 100 Mya (Distel, 1998).

The symbionts of the thyasirid clam *Maorhithyas hadalis* occupy unique positions in this phylogenetic framework. On the basis of 16S rRNA sequence data and in situ hybridization, Fujiwara et al. (2001) described two different symbionts within this clam. One of the symbionts shows evolutionary relatedness to the bathymodioline mussel and vesicomyid clam symbionts, occurring basal to the clade containing these bacteria, and the other symbiont clusters with the free-living *Thiomicrospira* spp. This free-living "symbiont" phylotype, however, may be a contaminant. Difficulties with in situ hybridization have precluded attempts to describe the microdistribution of the two symbiont types (Fujiwara and Uematsu, 2002), bringing into question the phylogenetic identity of the clam symbionts.

The phylogenetic positions of the methanotrophic endosymbionts and the filamentous epibionts are shown also in Fig. 1. The methanotrophic symbionts characterized to date all belong to the Gammaproteobacteria, forming a clade with 100% bootstrap support. The sister group of this clade consists of free-living Type I methanotrophs (*Methylococcus*, *Methylobacter* and *Methylomonas* spp.). Given their monophyly, the mussel symbionts apparently arose from a common ancestor. But the question of whether these symbionts subsequently cospeciated with their hosts remains unanswered.

The episymbionts in this analysis include the sulfur-oxidizing Epsilonproteobacteria found on the Mid-Atlantic Ridge shrimp *Rimicaris exoculata* and the eastern Pacific polychaete *Alvinella pompejana.* Interestingly, the shrimp epibiont clusters with the polychaete epibionts despite the fact that *R. exoculata* occurs on the Mid-Atlantic Ridge while alvinellid polychaetes inhabit vents in the eastern Pacific Ocean.

Free-living microorganisms can potentially provide insight into the ancestral form of endosymbionts. For instance, the evolution of insect endosymbionts (e.g., *Wolbachia* and *Buchnera* spp.) is commonly studied by comparative analyses with free-living, closely related microbes (Wernegreen, 2002; Moran, 2003). But until recently, the chemoautotrophic symbiont clades have not included any free-living bacteria. Figure 1 includes two species of bacteria that are not chemoautotrophic symbionts (*Coxiella burnetti* and *Achromatium oxaliferum*) and an environmental clone (JTB254). All three of these sequences fall within the first symbiont clade. *Coxiella burnetii* is an intracellular pathogenic bacterium (Woldehiwet, 2004) and the Gamma JTB254 clone was recovered from a deep-sea cold seep in the Japan Trench (Li et al., 1999). They both fall in a cluster with the *S. reidi* symbiont. Both *C. burnetii* and the *S. reidi* symbionts are able to maintain an intracellular existence in

eukaryotic hosts. *Coxiella burnetii*, however, is capable of growth in animal cell lines (e.g., Woldehiwet, 2004) and pathogenically infects a wide range of hosts (Niemczuk and Kondracki, 2004; Watanabe, 2004; Woldehiwet, 2004). In addition, *A. oxaliferum*, a freshwater sulfur-oxidizing bacterium, falls out with the nematode and oligochaete symbionts clade. *Achromatium oxaliferum* occurs in freshwater sediments along the redox zone where it has access to sulfide and oxygen (Head et al., 1996; Glockner et al., 1999; Gray et al., 1999). As cultivation methods improve and sequences are added to the 16 rRNA gene database, other free-living bacteria that are closely related to symbionts will likely be identified. Indeed, recent studies incorporating 16S rRNA gene sequences from unidentified environmental clones into phylogenetic analyses of free-living and symbiotic bacteria suggest that chemosynthetic symbionts may in fact resolve into three distinct clades (N. Dubilier, personal communication; Duperron et al., 2004). Free-living relatives of chemosynthetic symbionts should reveal much about the ecological and evolutionary constraints on the symbiont as well as about the potential for gene loss during the transition from the free-living to the symbiotic state.

Symbiont Characterization

Enzyme Activities

Researchers routinely demonstrate chemoautotrophy or methanotrophy in symbionts by the activity or presence of diagnostic enzymes. Indeed, given the inability to culture chemoautotrophic symbionts, detection of such enzymes is often the only evidence used to infer symbiont metabolism. This characterization often involves physiological assays using tissue or purified protein extract, immunodetection, or PCR-based gene probing. Such studies were initially conducted on the tubeworm *Riftia pachyptila*. For example, Felbeck (1981a) assayed tubeworm trophosome tissue for the activities of key enzymes of the Calvin cycle, the CO_2-fixing enzyme, ribulose 1,5-bisphosphate carboxylase-oxygenase (RubisCO), and phosphoribulose kinase (PRK) as well as enzymes associated with the oxidation of reduced inorganic sulfur compounds. The activity or presence of RubisCO has subsequently been used to diagnose symbiont autotrophy in a diversity of host species, including all of the chemoautotroph-harboring invertebrates listed in Table 1 (excluding alvinellid polychaetes; see below): shallow water solemyid and lucinid bivalves, vent and seep tubeworms and bivalves (including mussels hosting both methanotrophic and chemoautotrophic symbionts), nematodes, oligochetes, shrimp, sea urchins, and ciliates (Felbeck et al., 1981b; Cavanaugh, 1983; Cavanaugh et al., 1988; Polz et al., 1992; Johnson et al., 1994; Nelson et al., 1995b; Bauer-Nebelsick et al., 1996; Krieger et al., 2000; Elsaied et al., 2002; Fiala-Médioni et al., 2002). While RubisCO has not been detected in the alvinellid epibionts, genes encoding citrate lyase, a key enzyme of the reductive tricarboxylic acid (TCA) cycle, recently have been detected via analyses of symbiont DNA sequences, suggesting that the Epsilonproteobacteria symbionts of alvinellid worms fix carbon via this pathway (Campbell et al., 2003).

Enzymes involved in chemosynthetic energy generation have also been used to characterize these symbionts. Although the sulfur metabolism enzymes are not unique to sulfur oxidation, certain enzymes such as ATP sulfurylase, when detected in high activities, have been used to infer sulfur-based chemolithotrophy (Felbeck, 1981a; Fisher et al., 1993b; Laue and Nelson, 1994). As methane monoxygenase, the enzyme that catalyzes the first step in the oxidation of methane in aerobic methanotrophs, is notoriously labile (Prior and Dalton, 1985; Cavanaugh, 1993), methanol dehydrogenase (MeDH), the enzyme that catalyzes the second oxidation step (i.e., methanol to formaldehyde) and is known to occur only in methylotrophs, has been used extensively to diagnose methanotrophy. MeDH has been detected in gill extracts of mussels hosting methanotrophs or both methanotrophs and thioautotrophs (Cavanaugh et al., 1992; Fisher et al., 1993b; Robinson et al., 1998; Fiala-Médioni et al., 2002; Pimenov et al., 2002; Barry et al., 2002) and in a deep-sea sponge (Vacelet et al., 1996). Such enzymatic evidence strongly suggests methanotrophy, particularly when coupled with ultrastructural observations showing symbionts with the complex intracytoplasmic membranes that are characteristic of Type I methanotrophs.

Stable Isotope Signatures

Carbon Isotopes In addition to enzymology, stable isotope data provided some of the first evidence in support of chemoautotrophy in marine invertebrate-bacteria symbioses (e.g., Rau and Hedges, 1979; Spiro et al., 1986) and continue to be useful in assessing symbiont metabolism and tracking energy and carbon transfer in chemosynthetic symbioses (Colaco et al., 2002; Levin and Michener, 2002; Van Dover, 2002a; Robinson et al., 2003; Scott et al., 2004). Because enzymes involved in distinct carbon fixation pathways discriminate differently against the use of the heavier carbon isotope (^{13}C), the stable

carbon isotope ratio comparing ^{13}C to ^{12}C ($\delta^{13}C$) can be used to help distinguish different autotrophic metabolisms. For example, whereas $\delta^{13}C$ values of marine phytoplankton typically vary between μ18‰ and μ28‰ (Fry and Sherr, 1984; Gearing et al., 1984; Goericke et al., 1994), carbon derived chemosynthetically at vents is either considerably lighter (enriched in ^{12}C), with $\delta^{13}C$ values from μ27‰ to μ35‰, or heavier (depleted in ^{12}C), with values from μ9‰ to μ16‰ (Childress and Fisher, 1992; Robinson and Cavanaugh, 1995; Robinson et al., 2003). Depending on the source of methane, symbioses between mussels and methanotrophic bacteria may be even more depleted in ^{13}C, with $\delta^{13}C$ ranging from μ37‰ to μ78‰ (Cavanaugh, 1993; Nelson and Fisher, 1995a; Barry et al., 2002).

Because consumers generally retain the carbon isotopic signature of their food (i.e., "you are what you eat"; DeNiro and Epstein, 1979), comparisons between $\delta^{13}C$ signatures of symbiont-containing host tissue and symbiont-free host tissue can be used to study the transfer of symbiont-derived carbon to the host. For example, $\delta^{13}C$ values (μ30.8‰ to μ35.8‰) in symbiont-containing gill tissue from the western Pacific vent mussel *Bathymodiolus brevior* were significantly lower than values from symbiont-free foot tissue, suggesting that *B. brevior* supplements its diet via filter feeding on photosynthetically derived carbon (Dubilier et al., 1998). In contrast, other studies show a high dependence on symbiont carbon by host species, including coastal solemyid protobranchs (Fisher and Childress, 1986; Conway and Capuzzo, 1991), thyasirid clams (Dando and Spiro, 1993; Fiala-Médioni et al., 1993), and vestimentiferan tubeworms (Kennicutt et al., 1992), as well as suggest differences in the contribution of methanotrophs and chemoautotrophs to host carbon in mussels containing dual symbioses (Cavanaugh, 1993; Trask and Van Dover, 1999; Fiala-Médioni et al., 2002; Yamanaka et al., 2003).

Stable carbon isotope signatures have also been used to detect chemosynthetic symbioses in fossil bivalves of the clam family Lucinidae, whose extant members all host chemosynthetic symbionts. CoBabe (1991), by determining the $\delta^{13}C$ values of organic matrix material extracted from lucinid fossils dating to ca. 120,000 ya, showed that fossilized lucinid (*Epilucina* sp.) shells ($\delta^{13}C$ = μ25‰) were about 5‰ lighter than values from other bivalves collected in the same deposit (Pt. Loma, CA). Also, the organic matter of lucinid fossils was similar to that from modern samples, implying that the fossil organic matrix did not decay or change significantly over time. These results, along with strong evidence showing that shell $\delta^{13}C$ values are reasonable proxies for tissue values in extant species, suggest that the fossil lucinid hosted chemosynthetic symbionts ca. 120,000 ya (CoBabe, 1991). Thus, stable carbon isotope analysis may be an effective tool for tracing the evolution of chemosynthetic symbioses in the fossil record.

One of the main factors affecting stable carbon isotope signatures of chemoautotroph-invertebrate symbioses appears to be the form of RubisCO used by the symbionts. While $\delta^{13}C$ values for vent bivalves hosting sulfide-oxidizing symbionts cluster between μ27‰ and μ35‰ and resemble values for free-living chemoautotrophic bacteria, values for vent tubeworms, shrimp episymbionts, and many free living bacterial mats at vents are significantly heavier, ranging from μ9‰ to μ16‰ (Childress and Fisher, 1992; Van Dover and Fry, 1994; Robinson and Cavanaugh, 1995; Cavanaugh and Robinson, 1996; Robinson et al., 2003). The difference between these groups relates to the form of RubisCO used to fix CO_2 by the symbionts, with form I RubisCO occurring in most members of the isotopically lighter group and form II in all members of the heavier tubeworm group (Robinson and Cavanaugh, 1995). Corroborating this hypothesis, Robinson et al. (2003) showed that the kinetic isotope effect (ε value), the relative rate of $^{13}CO_2$ to $^{13}CO_2$ fixation ($^{12}k/^{13}k$) and a measure of discrimination against ^{13}C by the purified RubisCO enzyme in vitro, is significantly lower for form II RubisCO from *Riftia pachyptila* symbionts (ε = 19.5‰) than for the form I enzyme (ε = 22–30‰). Such variation may have arisen from evolution under differing concentrations of CO_2 and O_2 (Robinson et al., 2003).

But isotopic discrimination by RubisCO does not fully account for ^{13}C-enrichment in these symbioses. For example, to explain the discrepancy in *R. pachyptila* biomass $\delta^{13}C$ values, Scott (2003) used a mass balance model to show that steep gradients in [CO_2] among symbiont, host, and environmental pools may drive ^{13}C enrichment. RubisCO, which occurs in the symbiont cytoplasm, preferentially fixes $^{12}CO_2$, leaving $^{13}CO_2$ behind. If fixation is rapid, CO_2 equilibration between the isotopically lighter host cytoplasm and the isotopically heavier symbiont cytoplasm cannot occur, causing RubisCO to draw from a more enriched $^{13}CO_2$ pool and accounting for the relatively heavy $\delta^{13}C$ of tubeworm biomass. Further, stable carbon isotope values are also affected by the $\delta^{13}C$ of the environmental carbon pool (Fisher, 1995; Colaco et al., 2002). Scott et al. (2004) demonstrated that the "light" $\delta^{13}C$ values of the symbionts of the coastal protobranch clam *Solemya velum* are explained not only by the kinetic isotope effect of symbiont form I RubisCO (ε = 24.5‰) but also by the $\delta^{13}C$ value of the CO_2 in the sediment.

Similarly, the source of methane production can significantly impact the isotopic signature of methanotroph symbioses (Fisher, 1995; MacAvoy et al., 2002). The $\delta^{13}C$ of methane varies considerably depending on whether it is produced thermogenically ($\delta^{13}C$ of CH_4 >µ45‰) or biologically by methanogens ($\delta^{13}C$ of CH_4 <µ60‰; Lilley et al., 1993; Fisher, 1995), and this variability is reflected in the $\delta^{13}C$ values of chemosynthetic symbioses (Fisher, 1995). Therefore, in instances where the $\delta^{13}C$ of the source methane is unknown, conclusions about the contribution of methanotrophic symbionts to host $\delta^{13}C$ values should be interpreted with caution (Fisher, 1995). In addition, interpreting $\delta^{13}C$ signatures may be especially problematic for dual symbioses in which both methanotrophic and thioautotrophic symbionts co-occur in the same host cell. In these symbioses the $\delta^{13}C$ values of the symbionts and the host reflect a mix of methanotrophic and thioautotrophic metabolism (Fisher, 1995). These signatures are potentially confounded in instances when the thioautotrophic symbionts use the CO_2 respired by the methanotrophs, resulting in a second discrimination against an already light pool of CO_2 and an anomalously light tissue $\delta^{13}C$ value (Fisher, 1993a).

Thus, while $\delta^{13}C$ values often provide the first evidence that chemoautotrophic or methanotrophic symbioses occur in certain animal species, researchers must recognize that $\delta^{13}C$ is inherently responsive to physical, environmental and enzymatic factors. Stable carbon isotope signatures therefore should not be used apart from other corroborating evidence (e.g., physiological and enzyme activity assays and genetic characterization) to identify carbon fixation pathways or methane oxidation in chemosynthetic symbioses (Fisher, 1995; Scott, 2003; Scott et al., 2004).

Sulfur and Nitrogen Isotopes In addition to carbon isotopes, stable isotopes of sulfur and nitrogen are also used to study sources and metabolism of these elements in symbioses. The extent to which different sources of reduced sulfur—geothermal production in vent fluid or microbial sulfate reduction in bottom sediment—support thioautotrophic metabolism has been inferred from the $\delta^{34}S$ value of biological samples. Such analyses revealed hydrothermally derived sulfide as the dominant sulfide source for deep-sea vent symbioses (Fry et al., 1983; Yamanaka et al., 2003). In contrast, symbiotic bacteria within a shallow water vestimentiferan tubeworm, *Lamellibrachia satsuma* (Miura et al., 2002), and the protobranch, *Solemya velum* (Conway et al., 1989), rely predominantly on sulfide derived from microbial sulfate reduction.

Similarly, $\delta^{15}N$ values, because they vary predictably and largely between producer and consumer trophic levels (increase of ca. 3.4 per level), are particularly useful markers for studying aquatic food web interactions (Minagawa and Wada, 1984). In general, $\delta^{15}N$ values of chemoautotrophic organisms are significantly lighter (<0‰; Van Dover and Fry, 1994) than values for photosynthetic organisms (>6‰; see Michener and Schell [1994] and Fisher [1995]). Researchers have used this discrepancy and the predictable trophic level fractionation of ^{15}N to show host reliance on symbiont-derived organic matter in a number of symbioses including the coastal clams *Solemya velum* and *S. borealis* (Conway et al., 1989; Conway et al., 1992b) and in vent mussels from the Mid-Atlantic Ridge (MAR) and the Galapagos Rift (Fisher et al., 1988; Trask and Van Dover, 1999). In addition, $\delta^{15}N$ values have been used extensively in conjunction with $\delta^{13}C$ values to show the flow of chemosynthetically derived organic matter through vent food webs, including those on the MAR (Vereshchaka et al., 2000; Colaco et al., 2002), the Central Indian Ridge (Van Dover, 2002a), and the Galapagos Rift (Fisher et al., 1994). As with $\delta^{13}C$ data, $\delta^{15}N$ values vary considerably among sites; $\delta^{15}N$ may depend in part on the $\delta^{15}N$ of the dissolved inorganic nitrogen (DIN) pool, the proportions and $\delta^{15}N$ values of different components (NH_4^+, $NO_3^{\mu 2}$, NO_2^{μ}, and urea) in the DIN pool (Waser et al., 1998; Colaco et al., 2002), the uptake kinetics of different DIN assimilation pathways (Waser et al., 1998; Krueger, 1996a), and, as shown for vent shrimp (Vereshchaka et al., 2000) and mussels (Trask and Van Dover, 1999), the ontogenetic stage of the host. Therefore, as noted above with stable carbon isotopes, in the absence of additional enzymatic, genetic and environmental data, caution must be used when comparing $\delta^{15}N$ values from different habitats and species.

Ecophysiology

Symbioses between chemosynthetic bacteria and marine invertebrates must acquire all of the substrates necessary for chemosynthetic metabolism: reduced sulfur or methane, oxygen, dissolved inorganic carbon (DIC, as CO_2 or CH_4), and other nutrients (e.g., nitrogen and phosphorus) for use in biosynthesis. In particular, to support energy generation, these symbioses must obtain substrates from both oxic and anoxic environments. To meet these demands, the host-symbiont association relies on specialized biochemistry, physiology and behavior. These adaptations are best studied in thioau-

totrophic endosymbioses and are discussed primarily within this context below.

Spanning the Oxic-Anoxic Interface

Access to both oxygen and reduced chemicals is necessary for aerobic respiration by chemosynthetic symbionts. Specifically, thioautotrophs shuttle electrons from reduced sulfur (e.g., sulfide) to a terminal electron acceptor during oxidative phosphorylation, generating a proton gradient that drives ATP synthesis. Though some thioautotrophic symbionts (such as those in the tubeworm *Riftia pachyptila* [Hentschel and Felbeck, 1993a] and the clam *Lucinoma aequizonata* [Hentschel et al., 1993b]) may use nitrate as an electron acceptor during periods of anoxia, most thioautotrophic symbionts typically use molecular oxygen for respiration. Similarly, methanotrophs must obtain oxygen for respiration as well as methane for both energy generation (via methane oxidation) and carbon assimilation (Anthony, 1982).

This dual requirement for oxygen and reduced compounds poses unique problems for thioautotrophs and methanotrophs. First, these organisms must obtain energy substrates from mutually exclusive environments—oxygen is absent or at very low levels in the anoxic zones from which sulfide or methane is typically obtained. Second, sulfide, the predominant energy source for thioautotrophy, spontaneously reacts with oxygen to form less-reduced sulfur compounds (S^0, $S_2O_3^{\mu 2}$, or $SO_4^{\mu 2}$; Zhang and Millero, 1993), thereby decreasing the availability of substrates for thioautotrophy. Though such abiotic oxidation may be several orders of magnitude slower than biological sulfide oxidation (Millero et al., 1987; Johnson et al., 1988), thioautotrophic symbioses must still compete with oxygen for free sulfide. Also, in habitats containing both sulfide and methane, abiotic oxidation of sulfide may limit the oxygen available for methanotrophy. These limitations force free-living thioautotrophs and methanotrophs into microaerophilic zones at the interface, or chemocline, between oxic (e.g., water column) and anoxic (e.g., vent fluid and sediment pore water) habitats. Such free-living bacteria demonstrate unique mechanisms to support life at the oxic-anoxic interface; these adaptations may be behavioral (e.g., tracking the chemocline via gliding by *Beggiatoa*), anatomical (e.g., keeping cells in the chemocline via "veil" formation by *Thiovulum* or creation of a filamentous sulfur matrix by *Arcobacter*), biochemical (e.g., internal or external sulfur deposition that serves as an electron source or sink when sulfide or oxygen is limiting, as by *Beggiatoa* and *Arcobacter*), or developmental (e.g., resting stage formation by methanotrophs; Table 2 and references therein).

Table 2. Adaptations of thioautotrophs and methanotrophs for life at oxic-anoxic interfaces.[a]

Adaptation	Example
Attachment	*Thiothrix*
Motility, chemotaxis	*Beggiatoa Thioploca*
Elemental sulfur deposition	*Beggiatoa Thiothrix*
Nitrate and sulfur storage	*Thiomargarita Thioploca*
Create own interface	*Thiovulum*
Filamentous sulfur production	*Arcobacter* sp.
Resting cysts	Methanotrophs
Associate with eukaryote	Thioautotroph and Methanotroph symbionts

[a]From Anthony (1982), Jørgensen and Postgate (1982), Cavanaugh (1985), Schulz et al. (1999), and Wirsen et al. (2002).

Symbiosis thus may be viewed as an adaptation to simultaneously obtain sulfide (or methane) and oxygen from anoxic-oxic interfaces, allowing thioautotroph or methanotroph symbionts, via association with a eukaryotic host, to circumvent many of the problems of sulfide acquisition (Cavanaugh, 1985). Similarly to free-living sulfur bacteria, thioautotrophic symbioses use specialized behavioral, anatomical or physiological mechanisms, either to spatially or temporally bridge sulfidic and oxic zones or to simultaneously sequester sulfide and oxygen (Cavanaugh, 1994; Fisher, 1996; Polz et al., 2000). For instance, the cold seep vestimentiferan tubeworm *Lamellibrachia* cf. *luymesi* acquires oxygen via its anterior plume while extending a posterior section of its tube (the root) deep into the sediment to acquire sulfide (Julian et al., 1999; Freytag et al., 2001). Similar burrowing tactics occur in some species of symbiont-containing thyasirid clams, which possess a superextensile foot (up to 30 times the length of the shell) that burrows into the sediment to access hydrogen sulfide (Dufour and Felbeck, 2003), and in protobranchs of the genus *Solemya*, which dig Y-shaped burrows in reducing sediments to allow simultaneous pumping of oxygenated water from above and sulfide-rich pore water from below (Stanley, 1970; Cavanaugh, 1983; Fig. 11). Also, shrimp, nematodes and oligochaetes migrate vertically along the oxygen-sulfide gradient or between separate oxic and anoxic zones, thereby enabling their symbionts to simultaneously access both energy substrates or to store reduced sulfur compounds for later oxidation (Polz et al., 2000).

The vent tubeworm *Riftia pachyptila* possesses a remarkable biochemical adaptation to simultaneously acquire sulfide and oxygen. *R. pachyptila* produces coelomic and vascular hemoglobins that, in contrast to most invertebrate and verte-

brate hemoglobins, can bind oxygen in the presence of sulfide (Arp et al., 1985; Arp et al., 1987; Childress et al., 1991; Zal et al., 1996). *R. pachyptila* appears to preferentially take up HS^{μ} from the surrounding fluid, despite a large H_2S gradient from tubeworm blood to the environment (Goffredi et al., 1997a). The HS^{μ} diffuses across the plume of the worm (Goffredi et al., 1997a) and then binds reversibly and independently of O_2 at two free cysteine residues, each located on a distinct globin type (Zal et al., 1997; Zal et al., 1998; Bailly et al., 2002). These residues are well conserved in both symbiont-containing and symbiont-free annelids from sulfidic environments but are absent in annelids from sulfide-free habitats (Bailly et al., 2002; Bailly et al., 2003). Bailly et al. (2003) suggest that the sulfide binding function may have been lost via positive selection, if the sulfide-binding cysteine residues react disadvantageously with other blood components in the absence of sulfide.

Extracellular hemoglobins that simultaneously bind sulfide and oxygen are absent in most other marine invertebrates that host sulfide-oxidizing symbionts (Weber and Vinogradov, 2001); such organisms have evolved other mechanisms for regulating sulfide toxicity and delivery. For instance, the vesicomyid clam *Calyptogena magnifica* synthesizes a di-globular, non-heme molecule that readily binds free sulfide within the blood serum, perhaps via zinc residues (Arp et al., 1984; Zal et al., 2000). Also, several thioautotroph-containing species, including the vent mussel *Bathymodiolus thermophilus* and the coastal clam *Solemya velum*, appear to mediate detoxification in part by storage of sulfur in amino acids (e.g., taurine and thiotaurine; Conway and Capuzzo, 1992a; Pruski et al., 2000a; Joyner et al., 2003; Pruski and Fiala-Médioni, 2003). Indeed, thiotaurine may be used effectively as a biomarker of thioautotrophic symbioses (Pruski et al., 2000b).

Other host organisms, including some bivalve mollusks, apparently avoid sulfide toxicity via mitochondrial oxidation of sulfide. Powell and Somero (1986) first demonstrated mitochondrial sulfide oxidation in the coastal protobranch *S. reidi*. The authors showed that mitochondria isolated from the gill and foot of *S. reidi* exhibit ADP-stimulated oxygen uptake and ATP synthesis following the addition of sulfide. On the basis of the effects of cytochrome and reduced nicotinamide adenine dinucleotide (NADH) oxidase inhibitors, electrons from sulfide oxidation appear to enter the respiratory chain at cytochrome *c* in *S. reidi* mitochondria (Powell and Somero, 1986). Further characterization of this system using ^{35}S showed that sulfide is oxidized exclusively to thiosulfate (O'Brien and Vetter, 1990), a nontoxic intermediate that can function as the energy source in symbiotic carbon fixation. Subsequently, researchers have demonstrated mitochondrial sulfide oxidation across a wide range of organisms, including polychaete worms, clams, fishes and chickens (Grieshaber and Volkel, 1998; Yong and Searcy, 2001). These data lend credence to the hypothesis that mitochondria evolved from sulfide-oxidizing endosymbiotic bacteria (Searcy, 1992).

Readers should consult several additional reviews (e.g., Cavanaugh, 1994; Fisher, 1996; Polz et al., 2000) for a more extensive discussion of the remarkable adaptations used by chemoautotrophic symbioses to sequester both oxygen and reduced chemicals across oxic-anoxic zones.

Carbon Uptake and Transport

In addition to oxygen and reduced sulfur compounds, thioautotrophic symbionts utilizing the Calvin cycle require CO_2 for autotrophic carbon fixation. Acquisition of CO_2 is not trivial given that relative concentrations of the three distinct chemical species (CO_2, HCO_3^{μ} and $CO_3^{\mu 2}$) in the dissolved inorganic carbon (DIC) pool can vary considerably depending on pH (pK_a of 6.4 for $CO_2 : HCO_3^{\mu}$ at 25°C; see the section Habitat Chemistry in this Chapter). In general, the majority of DIC in seawater (pH ~ 8.0) is HCO_3^{μ}. But at vents the typically lower pH of the mixed vent fluid and ambient bottom water generates higher concentrations of CO_2, giving organisms that use the Calvin cycle a distinct advantage.

The tubeworm *Riftia pachyptila* provides an interesting model in which to study the uptake and transport of DIC. Goffredi et al. (1997b) demonstrated that for *R. pachyptila*, pH plays an important role in DIC uptake. The acidity of diffuse vent fluid (pH ca. 6) around tubeworms ensures that CO_2 (pK_a of 6.1 at in situ temperature and pressure of ca. 10°C and 101.3 kPa; Dickson and Millero, 1987) is the dominant DIC form in the vent environment. This contrasts with the vascular fluid of the worm, which has an alkaline pH of 7.1–7.5, apparently because of the action of H^+-ATPases (Goffredi et al., 1999; Goffredi and Childress, 2001; Girguis et al., 2002). The alkaline pH inside *Riftia* results in rapid conversion of CO_2 to HCO_3^{μ}, which, because of its negative charge, cannot diffuse out of the worm; this in effect creates a bicarbonate "trap" (Childress et al., 1993). Thus, a gradient of higher external [CO_2] to lower internal [CO_2] develops across the tubeworm plume and drives diffusion of DIC into the blood (Childress et al., 1993; Goffredi et al., 1997b; Scott, 2003). Following diffusion into the plume, DIC (as CO_2 and HCO_3^{μ}) is transported by the vascular system to the symbiont-containing trophosome. Here, carbonic anhydrase, the enzyme that reversibly con-

verts CO_2 into HCO_3^{μ} in both prokaryotes and eukaryotes, may play a role in converting HCO_3^{μ} into CO_2, the DIC species used by RubisCO (Kochevar and Childress, 1996; De Cian et al., 2003a; De Cian et al., 2003b). As discussed above for *Riftia*, DIC incorporation into symbiont biomass occurs via CO_2 fixation by a form II RubisCO of the Calvin-Benson cycle. Rapid CO_2 fixation rates create steep internal $[CO_2]$ gradients between symbiont and host cytoplasm that may, in combination with the relatively low discrimination of form II RubisCO against ^{13}C, result in a ^{13}C-enriched signature of symbiont and host biomass (Robinson et al., 2003; Scott, 2003).

In chemosynthetic endosymbioses the host benefits by obtaining part or all of its nutrition from the symbiont, via two potential transfer mechanisms: the host may assimilate autotrophically fixed carbon that has been released by the symbiont and translocated to host cells in the form of soluble organic molecules, or the host may directly digest bacterial cells. Radiotracer analysis and microscopy have proven particularly useful in studying host nutrition. For example, Fisher and Childress (1986) showed a rapid (within hours) appearance of radiolabeled carbon in the symbiont-free tissues of the host clam *Solemya reidi* following exposure to ^{14}C-labeled bicarbonate, suggesting release of fixed carbon by the symbiont population. In contrast, a slow (1–5 days) transfer of labeled organic carbon from methanotroph-containing tissue to symbiont-free tissue of a seep mussel exposed to ^{14}C-labeled methane was inferred to be due to initial $^{14}CH_4$ incorporation by the symbionts with host digestion of symbionts occurring later (Fisher and Childress, 1992). Electron microscopy showing symbionts being degraded in the basal region of bacteriocytes in other methane-based and dual chemoautotroph-methanotroph mussel symbioses supports this interpretation (Cavanaugh et al., 1992; Barry et al., 2002), as does the detection of lysosomal enzymes in the gills of the vent bivalves *Calyptogena magnifica* and *Bathymodiolus thermophilus* (Fiala-Médioni et al., 1994; Boetius and Felbeck, 1995) and the shallow water clam *Lucinoma aequizonata* (Boetius and Felbeck, 1995).

In the *R. pachyptila* tubeworm symbiosis, the transfer of carbon from symbiont to host appears to occur via both translocation and digestion (Bright et al., 2000). Felbeck (1985) and Felbeck and Turner (1995) documented a rapid (within seconds) appearance of labeled succinate and malate in trophosome tissue and in vascular and coelomic blood following exposure of whole worms (in pressure vessels) and plumes to ^{14}C-bicarbonate. Subsequently, Felbeck and Jarchow (1998) showed that succinate, malate, and several other organic acids and sugars were excreted by purified suspensions of *R. pachyptila* symbionts, suggesting that these simple organic compounds might be important intermediates in the transfer of fixed carbon from symbionts to host. Corroborating these data, Bright et al. (2000), using pulse labeling analysis, showed that the bulk of organic carbon assimilated into *R. pachyptila* tissue is first released by metabolically active bacteria at the center of a trophosome lobule. However, these authors also showed that a smaller fraction of host carbon is obtained by digestion of bacterial cells at the lobule periphery (Bright et al., 2000). This evidence for digestion is supported by prior studies showing degenerative stages of bacteria within the *R. pachyptila* trophosome (Bosch and Grassé, 1984; Hand, 1987). In addition, relatively high lysozyme activity in *Riftia* tissue further suggests that digestion of symbionts plays a role in tubeworm nutrition (Boetius and Felbeck, 1995).

Nitrogen

The partners in a symbiosis must also acquire all of the other macro- and micronutrients, particularly nitrogen and phosphorus, for use in the biosynthesis of organic compounds. Currently, very little is known about how various forms (inorganic and organic) of phosphorus are transferred to and among different pools within chemoautotrophic endosymbioses. Most studies have focused on nitrogen metabolism, using a combination of enzyme characterizations and physiological experiments to elucidate nitrogen assimilation pathways. Nitrate ($NO_3^{\mu 2}$), which is abundant at vents (in situ concentrations of ~40 μM; Johnson et al., 1988), appears to be the predominant nitrogen source for vent symbioses. For example, Lee et al. (1999) demonstrated the activity of nitrate reductase, a bacterial enzyme involved in converting nitrate to ammonia for either assimilatory or respiratory purposes, in the vent tubeworms *Riftia pachyptila* and *Tevnia jerichonana* and the mussel *Bathymodiolus thermophilus*. In addition, the ammonia assimilation enzymes glutamine synthetase (GS) and glutamate dehydrogenase (GDH) were detected in these symbioses, and almost all GS activity in symbiont-containing tissue was shown to be due to enzyme produced by the bacterial symbiont and not the host (Lee et al., 1999). Supporting these data, physiological experiments on *R. pachyptila* kept in pressurized chambers showed that the symbiont population reduces nitrate to ammonia not for respiratory purposes but for incorporation into both symbiont and host biomass (Girguis et al., 2000). However, in *R. pachyptila,* high GS activity also occured in symbiont-free branchial plume tissue, suggesting

that the host may also be involved in assimilation of ammonia from the vent environment (Minic et al., 2001). But further enzymatic characterization of *Riftia* tissues demonstrated that the tubeworm depends on its symbionts for the de novo synthesis of pyrimidine nucleotides (Minic et al., 2001) as well as for the biosynthesis of polyamines (Minic and Herve, 2003), suggesting that the trophosome is a primary site for nitrogen assimilation and metabolism.

In contrast, in the thioautotrophic symbiosis involving the shallow-water clam *Solemya reidi*, inorganic nitrogen is readily assimilated in the form of ammonia (Lee and Childress, 1994), which is abundant in the shallow water, nutrient-rich habitats of the clam (e.g., sewage outfalls). Ammonia incorporation rates are highest in the symbiont-containing gill tissue, and the sulfur-containing amino acid taurine appears to be a major end product of ammonia assimilation (Lee et al., 1997). The mechanisms by which chemosynthetic symbionts, particularly those contained within the cells of invertebrate hosts (such as *Solemya* and *Riftia*), acquire all of the other macro- and micronutrients for biosynthesis have yet to be characterized.

Ecology and Evolution

History

Prior to the use of molecular techniques, researchers considered vent taxa to be relic species. These organisms, whose strange morphologies suggest a primitive state, purportedly survived past extinction events due to the relative isolation of vents from the photic zone (McArthur and Tunnicliffe, 1998). This perception of vents as ancient ecosystems is supported by the fossil record, which shows that over 80% of vent species are found only at vent sites (Tunnicliffe, 1991; Tunnicliffe, 1992; Little et al., 1997; Little and Vrijenhoek, 2003) and that the oldest vent site dates to the Silurian (~430 Mya; Little et al., 2004). But the fossil record for vents is relatively poor. There are only 19 known fossilized vent sites on the planet, perhaps because calcium carbonate structures dissolve relatively quickly in vent fluids (Hunt, 1992; Kennish and Lutz, 1999). Also, studying vent fauna evolution based on the morphological characters of fossils is problematic if much of the specimen has degraded or if the preserved character is plastic or isomorphic. In particular, vestimentiferan tubeworms are known for the phenotypic plasticity of their tubes (Southward et al., 1995; Black et al., 1998).

In contrast, molecular evidence suggests that vent taxa evolved more recently (22–150 Mya; later-Mesozoic and Cenozoic) and suggests an alternative hypothesis to vent taxa as living relics: vents were recently populated from shallow seeps or whale falls (Van Dover et al., 2002b; Hurtado, 2002). Indeed, the communities most similar to those of vents occur at seeps. Compared to the spatially and temporally patchy distribution of vent fossils (with most being concentrated in the Silurian and Devonian rocks of the Ural mountains), seep fossils are ubiquitous (Little and Vrijenhoek, 2003). At least 50, and perhaps as many as 200, fossilized seep sites dating from the Devonian to the Pleistocene have been uncovered. These specimens are much better preserved than most vent fossils and include extant vent taxa not yet uncovered at fossil vent sites (e.g., vesicomyids, thyasirids, mytilids and solemyids; Little and Vrijenhoek, 2003). This greater diversity supports the seeps-to-vents hypothesis. However, opponents argue that the vent fossil record has been greatly affected by high calcium carbonate dissolution rates (Little and Vrijenhoek, 2003).

While the discrepancy between the evolutionary histories suggested by the fossil and molecular evidence needs to be resolved, it must also be stressed that these data are not evidence for chemosynthetic symbioses. In a unique study, CoBabe (1991) was able to deduce a chemosynthetic symbiosis by analyzing the organic matrix from fossil lucinid shells using stable carbon isotopes. This result is encouraging and suggests that both the age of these organisms and their symbiosis can be addressed using current methods.

Organism Interactions

In the relatively featureless and nutrient poor deep sea, vent and seep environments are ecological oases (Laubier, 1989). Initially, free-living chemoautotrophic bacteria were hypothesized to provide the bulk of primary production in these communities (Lonsdale, 1977). Indeed, at some vent sites, suspended bacteria or bacteria in surface-attached mats are a large food source for higher trophic levels (Humes and Lutz, 1994; Van Dover, 2000). But the dominant strategy for the major vent and seep fauna is symbiosis with chemoautotrophic bacteria (Cavanaugh, 1994), and these symbioses significantly influence the ecology of the nonsymbiotic community. Not only are chemosynthetic symbioses a major and stable source of organic carbon (Sarrazin and Juniper, 1999), but as biogenic structures, they also provide living space for a diversity of species in an otherwise two-dimensional landscape of basalt or sediment (Bergquist et al., 2003). For example, the tubes of chemosynthetic vestimentiferans support mussels, sponges and limpets,

many of which host their own chemosynthetic symbionts (Yamamoto et al., 2002; Bergquist et al., 2003; Bates et al., 2004).

Vent symbioses may also significantly impact the free-living bacterial community by providing increased surface area for attachment. Free-living bacteria that cluster phylogenetically with known chemoautotrophic and heterotrophic groups have been isolated from tubeworm surfaces (Lopez-Garcia et al., 2002; Yamamoto et al., 2002). On the Mid-Atlantic Ridge, a single phylotype of shrimp episymbionts, which appear to be transmitted among hosts via the environment, represented over 60% of the free-living bacteria (Polz and Cavanaugh, 1995). This suggests that the host inoculates inanimate surfaces continuously, increasing the probability of symbiont attachment relative to the free-living community (Polz and Cavanaugh, 1995). Such environmental inoculation may also occur in tubeworm and lucinid clam symbioses, in which the symbionts also appear to be transmitted environmentally (Durand and Gros, 1996a; Durand et al., 1996b; Di Meo et al., 2000; Nelson and Fisher, 2000; McMullin et al., 2003).

Transmission Strategies and Effects on Symbiosis

The transmission strategy of a symbiosis reveals much about the evolutionary dynamics between host and symbiont. Symbiont transmission can occur environmentally (through a free-living population of symbiotic bacteria), horizontally (between contemporary organisms sharing the same habitat), or vertically (from parent to offspring). Vertically transmitted endosymbionts are effectively disconnected from their free-living counterparts. These symbionts experience elevated rates of mutation and fixation of slightly deleterious alleles because of genetic drift (Wernegreen, 2002). For the most part, these evolutionary effects are due to a vastly different selective regime inside the host and a severely depreciated population size (Ohta, 1973); endosymbionts undergo a population bottleneck upon host colonization and another upon transmission (Mira and Moran, 2002). But the asexuality and lack of recombination in endosymbionts exacerbate these genetic problems through what is known as "Muller's rachet" (Muller, 1964; Moran, 1996). In Muller's ratchet, wildtype recombinants cannot be introduced into the endosymbiont population (Moran and Baumann, 1994; Dale et al., 2003); genetic drift therefore occurs quickly, and the population cannot recover after fixation of deleterious alleles.

In contrast, symbiont populations that are environmentally transmitted are effectively larger and more genetically heterogeneous than populations transmitted vertically. Comparisons of 16S rRNA gene evolution between free-living bacteria, in which significant recombination occurs (Dykhuizen and Green, 1991; Levin and Bergstrom, 2000), and symbiotic chemosynthetic bacteria revealed unexpected differences in rates of evolution depending on mode of transmission (Peek et al., 1998). While chemoautotrophic, maternally transmitted endosymbionts did exhibit rapid evolutionary rates, consistent with their small population sizes, environmentally transmitted symbionts evolved more slowly than their free-living counterparts (Peek et al., 1998). The authors suggest that this slower rate of evolution could be caused by purifying selection in a large population. These results, however, were based on one gene across many lineages; a true genomic analysis of evolution in chemosynthetic endosymbionts is necessary to extend these findings.

Because chemosynthetic symbionts have yet to be cultured and their hosts are difficult to maintain in the laboratory, the transmission strategy of a symbiosis has been inferred by phylogenetic analysis or PCR-based detection of bacteria in host reproductive tissues or gametes. If the symbionts are maternally transmitted and the symbioses stable, congruence of host and symbiont phylogenies should occur (e.g., Chen et al., 1999; Thao et al., 2000; Degnan et al., 2004) and bacterial symbionts should be found in ovaries or oviducts of the host. Using these techniques, vertical transmission has been proposed for the solemyid protobranchs (Cary, 1994; Krueger et al., 1996b) and vesicomyid clams (Endow and Ohta, 1990; Cary and Giovannoni, 1993a; Peek et al., 1998; Hurtado et al., 2003). Interestingly, although bacteria have been detected via PCR in the gonads of female hosts, this does not necessarily imply direct bacterial endocytic localization in host eggs. Indeed, in *Solemya reidi*, the internal contents of oocytes do not contain bacteria, and instead the transmission mechanism is thought to occur via ingestion; the larvae ingest the bacteria, which are then engulfed by hemocytes in the larval perivisceral cavity and transported to the developing gill (Gustafson and Reid, 1988). The oligochaetes also exhibit an interesting mechanism of vertical transmission. During oviposition, the eggs appear to be infected with the symbiotic bacteria via the adult's genital pad (Giere and Langheld, 1987). During the development of the larvae, many of the bacteria exist intracellularly, but as the animal matures, the symbionts take their primarily extracellular form.

However, of the putatively vertically transmitted symbioses, only associations involving vesicomyid clams show phylogenetic congruence

between host and symbiont (Peek et al., 1998; Hurtado et al., 2003). Cospeciation does not appear to have occurred in the solemyid protobranchs (Durand et al., 1996b; Krueger and Cavanaugh, 1997) or in the mytilid mussels (McKiness, 2004). When evaluating phylogenetic congruence, however, other factors that influence a phylogenetic reconstruction, such as geographic constraints, must be taken into account. Also, robust phylogenies with adequate taxa sampling for both host and symbiont are necessary; incomplete phylogenies may be hindering analyses of the solemyid and mytilid symbioses.

Lack of PCR-based evidence and phylogenetic incongruence has been used to infer an environmental mode of transmission for several of the chemosynthetic symbioses. For instance, the lucinid clams exhibit environmental transmission (Durand and Gros, 1996a; Gros et al., 1996; Gros et al., 1998; Gros et al., 2003a; Gros et al., 2003b). Researchers have even been able to exchange symbionts between lucinid species without affecting the development of the juvenile animal (Gros et al., 2003a). In addition, vent tubeworms appear to acquire their symbionts from the environment (Distel and Cavanaugh, 1994; Feldman et al., 1997; Laue and Nelson, 1997; Di Meo et al., 2000; Nelson and Fisher, 2000; McMullin et al., 2003), as evidenced in part by the presence of functional genes for sensing and responding to the environment as well as a flagellin gene in the *Riftia* symbiont (Hughes et al., 1997; Hughes et al., 1998; Millikan et al., 1999). Indeed the 16S rRNA phylotype has been detected in vent environments via both PCR and in situ hybridization, suggesting the vent tubeworm symbionts are environmentally transmitted (Harmer et al., 2004). While environmental transmission of tubeworm symbionts seems to be a potentially risky strategy, given the stochastic nature of environmental transmission and the complete dependence of the adult tubeworms on their symbionts for nutrition, detection of "wild" symbionts in conjunction with the phylogenetic evidence supports environmental transmission in this species.

The mechanism of transmission for the mytilid mussels remains largely unresolved; on the basis of varying evidence, researchers have suggested both vertical and environmental transmission. Vertical transmission in *Bathymodiolus thermophilus*, the thioautotroph-hosting mussel, was suggested in 1993, but evidence supporting this report is not yet published (Cary and Giovannoni, 1993a). In contrast, a recent study based on genetic and ultrastructural data of the chemoautotrophic symbionts of *B. azoricus*, a MAR mussel hosting both thioautotrophs and methanotrophs, indicated environmental acquisition of the chemoautotrophic symbionts (Won et al., 2003a; DeChaine et al., 2004). In addition, McKiness (2004) provided the first assessment of cospeciation between symbiont and host in *Bathymodiolus* mussels, analyzing molecular data for both methanotrophic and chemoautotrophic symbionts and testing phylogenetic congruence with the hosts. The results showed weak support for vertical transmission of the chemoautotrophic symbionts but provided no evidence for vertical transmission of the methanotrophs.

Biogeography and Population Genetics

The view of hydrothermal vents as deep-sea islands frames questions of vent biogeography and population genetics. Compared to the relatively uniform and stable environment of the abyssal deep sea, hydrothermal vents are ephemeral, dynamic and geographically fragmented. A chain of vents along a mid-ocean ridge resembles a chain of islands in an archipelago. However, genetic data for many vent species do not cleanly fit an "island" or "stepping-stone" model of biogeography (Vrijenhoek et al., 1998). Some host taxa do exhibit a decline in gene flow with increasing distance between sites (Black et al., 1994), as a stepping-stone model would predict (Kimura and Weiss, 1964), while others show a more widespread gene flux (Karl et al., 1996) or appear to encounter barriers to dispersal other than distance (Black et al., 1998). These differences should be resolved with a greater understanding of the major variables affecting vent biogeography, including larval development and dispersal, symbiont distribution, oceanic flow, and past and current bathymetry. This section focuses predominantly on host biogeography because research on the population genetics and biogeography of bacterial symbionts is lacking. Understanding host population dynamics, however, does provide valuable insight into the distribution of the chemosynthetic symbionts to which most vent fauna are tightly linked.

Vent habitats are highly ephemeral and sensitive to variations in tectonic activity, hydrothermal inputs, and geologic events. Consequently, the persistence of vent organisms, which are predominantly sessile as adults, depends on successful larval dispersal to new sites. The dispersal strategy of larvae can significantly impact the biogeography of the adult organism (Lalou and Brichet, 1982; Fustec et al., 1987). On the basis of laboratory studies and comparisons with shallow water species, researchers infer that some vent larvae are planktotrophic while others are lecithotrophic (Lutz et al., 1980; Turner et al., 1985; Young et al., 1996; Marsh et al., 2001). Although both forms are pelagic, planktotrophic larvae are positively buoyant and feed in the

water column while lecithotrophic larvae are negatively buoyant and nonfeeding (Poulin et al., 2001). Larvae of the large vesicomyid clam *Calyptogena magnifica* typify a planktotrophic dispersal strategy successfully exploiting the vent plume to carry them many kilometers (Pradillon et al., 2001; Mullineaux et al., 2002). Although planktotrophic larvae risk being carried off the ridge axis by cross currents, *C. magnifica* apparently encounters no significant barriers to dispersal across the equator on the East Pacific Rise (EPR; Karl et al., 1996). Conversely, lecithotrophic larvae are less affected by cross currents but, because they are non-feeding, have relatively short larval stages and therefore limited time for dispersal. For example, in laboratory studies, larval *Riftia pachyptila* exhibit a lecithotrophic strategy, surviving a maximum of 38 days (Marsh et al., 2001). Assuming flow rates characteristic of EPR currents, this interval suggests a maximum dispersal distance of 100 km (Marsh et al., 2001); however, the in situ dispersal distance is unknown given that *Riftia* larvae have not been detected in the wild.

The geology and tectonic activity associated with mid-ocean ridges also impact the biogeography of vent organisms. For instance, Iceland, an active site of crust formation, rises out of the ocean along the northern MAR, forming a barrier that prevents dispersal along the ridge axis (Tyler and Young, 2003). Given that Iceland interrupted the MAR approximately 55 Mya, the ridge axis north of Iceland constitutes one of the most isolated vent systems on the planet, perhaps representing a new biogeographic province (Bilyard and Carey, 1980; Dunton, 1992; Svavarsson et al., 1993). Similar dispersal barriers are seen among vent fields abutting the Azores Rise in the Atlantic (Tyler and Young, 2003) and also evident between the EPR and the Northeast Pacific vent fields (Tunnicliffe, 1988; Tunnicliffe and Fowler, 1996). These barriers are insurmountable and may provide the conditions for allopatric speciation of both host and symbiont.

Research on EPR bathymodiolid mussels and their symbionts provides a good example of how larval dispersal strategy, current regime, and bathymetry interact to structure biogeography (Lonsdale, 1977; Corliss et al., 1979). Except at Northern Pacific sites, which are separated from the EPR by the North America landmass, vent communities on the Pacific ridge axis appear relatively uniform. For example, the mussel *Bathymodiolus thermophilus*, which undergoes a planktotrophic larval stage, occurs over a distance of 4900 km (from 13°N to 32°S) on the EPR. Mussel populations along 13°N and 11°S are genetically indistinguishable, indicating no population subdivision (Craddock et al., 1995; Won et al., 2003). Deep ocean currents that flow primarily NNW and SSE along the axis (Marsh et al., 2001) may facilitate dispersal of larval *B. thermophilus*, contributing to the homogeneity observed along the EPR. However, there is some genetic structure in the EPR mytilid populations; the westward currents across the ridge axis at 15°N and the Easter Microplate are obstacles for planktotrophic larvae. At 15°N, a westward current flows across the ridge axis, partially isolating the 17°S population from the other northern populations. Further south, at the Easter Microplate, mussel populations are severely divergent (Won et al., 2003). Although morphologically indistinguishable, mussels north and south of the Microplate are genetically distinct (Won et al., 2003). The Easter Microplate therefore appears to be a significant topographic obstacle for larval dispersal. Such a feature can produce cross-axis currents, like those at 15°N, that may sweep bathymodiolid larvae (which are positively buoyant) off the ridge axis (Fujio and Imasato, 1991; Mullineaux et al., 1995). The degree to which such barriers also impact the genetic diversity and biogeography of chemosynthetic symbiont populations remains an open question.

Summary

Scientific understanding of chemosynthetic symbioses continues to expand. The spectacular discovery of hydrothermal vents highlighted the importance of chemosynthetic bacteria both in food webs and in symbioses with eukaryotes and provided the impetus to examine less exotic environments for such associations. As other oxic-anoxic environments (e.g., freshwater) and the invertebrates and protists that inhabit them are explored, new symbioses will undoubtedly be discovered. Further, chemosynthetic bacteria that use other sources of energy (e.g., hydrogen) may be found in similar associations.

Current studies of these fascinating symbioses involve a range of experimental and diagnostic tools, including physiological assays in specialized growth chambers, enzyme characterizations, immunodetections, and stable isotope analyses. Increasingly, molecular techniques, such as PCR-based gene probing, FISH, and 16 rRNA phylogenetic analysis, are used to complement traditional methods. These studies provide valuable insight into the population dynamics, evolutionary history, and carbon and nutrient metabolism of symbionts. In addition, projects are currently underway to sequence the genomes of some of the chemosynthetic symbionts described in this review (e.g., symbionts of *Riftia pachyptila* and *Solemya velum*). Genomic analysis, in conjunction with new technologies to manipulate symbioses under in situ conditions

(e.g., via vascular catheters; Felbeck et al., 2004) and to sample the physical environment (e.g., electrochemical sampling; Luther et al., 2001), will contribute significantly to our understanding of symbiont biology. Scientists are now poised to reveal how interactions with the host and the abiotic environment impact symbiotic chemosynthetic bacteria over both ecological and evolutionary timescales.

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