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Bacterial endosymbioses in *Solemya* (Mollusca: Bivalvia)—Model systems for studies of symbiont–host adaptation

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Abstract Endosymbioses between chemosynthetic bacteria and marine invertebrates are remarkable biological adaptations to life in sulfide-rich environments. In these mutualistic associations, sulfur-oxidizing chemoautotrophic bacteria living directly within host cells both aid in the detoxification of toxic sulfide and fix carbon to support the metabolic needs of the host. Though best described for deep-sea vents and cold seeps, these symbioses are ubiquitous in shallow-water reducing environments. Indeed, considerable insight into sulfur-oxidizing endosymbioses in general comes from detailed studies of shallow-water protobranch clams in the genus *Solemya*. This review highlights the impressive body of work characterizing bacterial symbiosis in *Solemya* species, all of which are presumed to harbor endosymbionts. In particular, studies of the coastal Atlantic species *Solemya velum* and its larger Pacific congener *Solemya reidi* are the foundation for our understanding of the metabolism and physiology of marine bivalve symbioses, which are now known to occur in five families. *Solemya velum*, in particular, is an excellent model organism for symbiosis research. This clam can be collected easily from coastal eelgrass beds and maintained in laboratory aquaria for extended periods. In addition, the genome of the *S. velum* symbiont is currently being sequenced. The integration of genomic data with additional experimental analyses will help reveal the molecular basis of the symbiont–host interaction in *Solemya*, thereby complementing the wide array of research programs aimed at better understanding the diverse relationships between bacterial and eukaryotic cells.

Keywords Symbiosis · Sulfur oxidation · Gamma Proteobacteria · Protobranch · Intracellular · Maternal transmission · Y-shaped burrow

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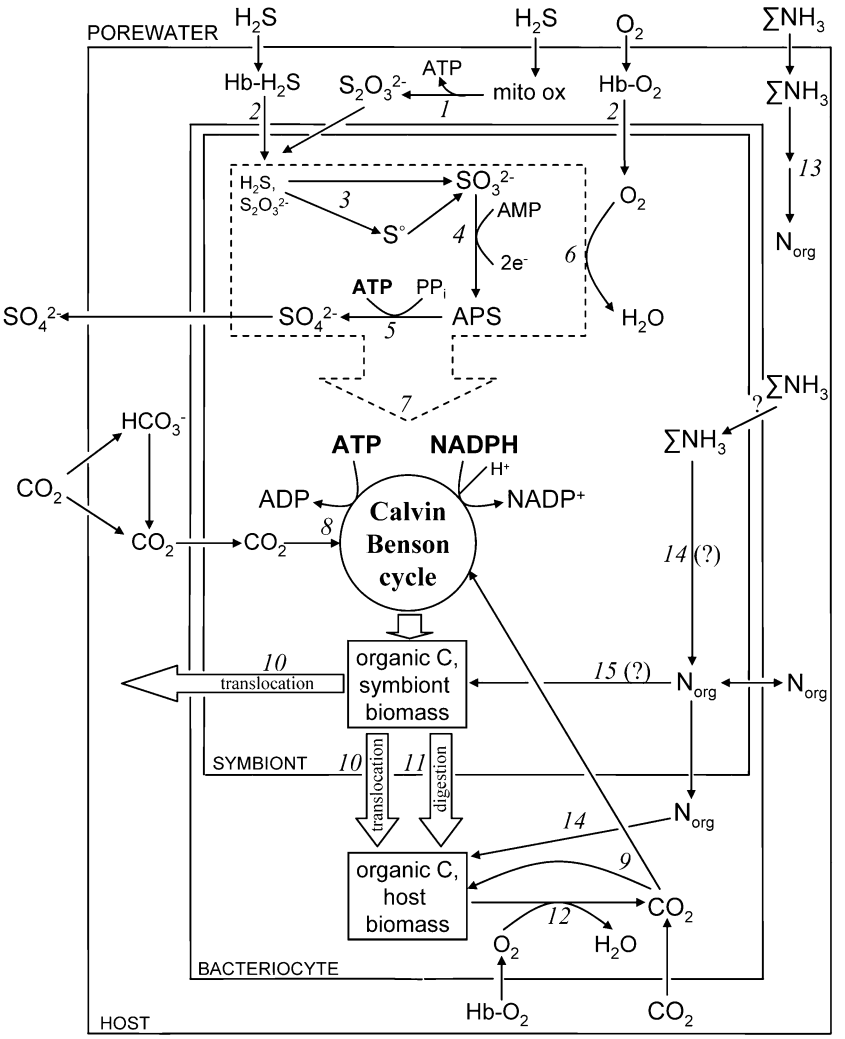
Introduction

Early research prompted by the discovery of deep-sea hydrothermal vents in the 1970s alerted the scientific community to a remarkable biological adaptation: mutualistic symbioses between marine invertebrates and intracellular chemoautotrophic bacteria. In these ''chemosynthetic endosymbioses,'' Proteobacteria living directly within cells of an invertebrate host use the energy of reduced sulfur compounds to fix carbon in support of the metabolic and biomass requirements of both symbiont and host (see model in Fig. 1; Cavanaugh et al. 2005; Stewart et al.



2005). These associations are particularly abundant in the sulfide-rich water emanating from deep-sea vents, where chemosynthesis by bacterial endosymbionts in a diverse array of metazoan hosts forms the basis of primary production. But chemosynthetic endosymbiosis appears to have a long evolutionary history in environments far less hostile than the deep, hot, and pressure-laden waters of hydrothermal vents. Indeed, sulfur-oxidizing endosymbioses are ubiquitous in shallow water habitats (mud flats, eelgrass beds, sewage outfalls) where microbial sulfate reduction in anaerobic sediments ensures a steady supply of sulfide. One of the best studied of these shallow water symbioses occurs between bacterial endosymbionts and protobranch clams in the genus *Solemya* (Fig. 2). This review summarizes past and current research on solemyid symbioses and, in so doing, underscores the biological aspects of these associations that warrant future study and that qualify *Solemya* species as ideal model organisms for the study of chemosynthetic endosymbioses.

Solemyid protobranchs have attracted considerable scientific inquiry. For decades, researchers were particularly captivated by the question of how these organisms, in which the digestive system is either severely reduced or absent entirely (Pelseneer 1891; Reid and Bernard 1980), met their nutritional needs. Following the discovery of chemosynthetic endosymbioses in hydrothermal vent organisms, such as the tubeworm *Riftia pachyptila*, it became apparent that *Solemya* sp. may also harbor intracellular chemoautotrophic

◀**Fig. 1** Proposed model of metabolism in the symbiosis between *Solemya* clams and chemosynthetic sulfur-oxidizing bacteria. Reduced sulfur (represented here as sulfide (H_2S)) and ammonia (as $\sum NH_3$: NH_4^+ and NH_3) enter the clam from the sediment porewater through unidentified transport mechanisms. Carbon dioxide and oxygen enter by diffusion. In peripheral tissue of *S. reidi*, sulfide is initially oxidized to thiosulfate ($S_2O_3^{2-}$) by host mitochondria (*1*; Powell and Somero 1985; Anderson et al. 1987), yielding ATP in the process (Powell and Somero 1986). Thiosulfate then may diffuse into the bacteriocytes or be transported there via an unidentified mechanism for further oxidation. In *S. velum*, sulfide and oxygen bind two separate cytoplasmic hemoglobins (Hb; Doeller et al. 1988) for delivery to the gill symbionts (*2*), which use these substrates for energy-generating sulfide oxidation. Oxygen delivery also may occur via a circulating hemocyanin in both *S. velum* and *S. reidi* (Mangum et al. 1987; Sanders et al. 1998). The mechanism of reduced sulfur oxidation (dashed box) within symbionts is unknown. Sulfide or thiosulfate may be oxidized first to elemental sulfur (S°) or directly to sulfite (SO_3^{2-}; *3*). Enzyme data for *S. velum* and *S. reidi* suggest that sulfite oxidation to sulfate (SO_4^{2-}) may then proceed through the APS pathway via the enzymes APS reductase (*4*) and ATP sulfurylase (*5*; Felbeck et al. 1981; Chen et al. 1987), yielding one ATP by substrate level phosphorylation. Electrons liberated during sulfur oxidation likely pass through an electron transport system, driving oxygen consumption (*6*) and the production of ATP and NADPH (*7*). Enzymmatic, physiological, and molecular data for several species of *Solemya* suggest that fixation of carbon dioxide occurs primarily via ribulose 1,5-bisphosphate carboxylase–oxygenase (RubisCO) in the Calvin Benson cycle (*8*), using ATP and NADPH generated from sulfur oxidation. Anapleurotic pathways in both host and symbiont (*9*) may fix lesser amounts of CO_2. Transfer of organic matter from symbionts to host likely occurs via translocation of simple nutritive compounds (e.g., amino acids) released by the bacteria (*10*; Fisher and Childress 1986), though direct digestion of symbiont cells may also take place (*11*). Host oxygen consumption (*12*) occurs in typical catabolic and anabolic pathways. Ammonia ($\sum NH_3$), the dominant inorganic nitrogen source for the symbiosis, likely diffuses into the host and is then assimilated into organic matter (N_{org}) via a host glutamine synthetase (*13*; Lee et al. 1999). Ammonia assimilation by the symbiont may occur (*14*), though activity of symbiont glutamine synthetase has not been detected. Exchange of organic nitrogen (e.g., amino acids) between host and symbiont also may occur, accompanied by incorporation of N_{org} into symbiont biomass (*15*). Abbreviations: APS, adenosine 5′-phosphosulfate. Modified from Stewart et al. (2005)

bacteria. Observations of the Atlantic species *S. velum*, which digs Y-shaped burrows spanning the oxic–anoxic interface in coastal eelgrass beds, revealed that the clam is ideally situated to provide internal symbionts with the metabolic

Fig. 2 *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

substrates (H_2S, O_2, CO_2) necessary for thioautotrophy (Cavanaugh 1980, 1983). Indeed, microscopical, enzymatic, and physiological data later confirmed that *S. velum*, along with its congeners for which data are available (see refs in Table 1), hosts gram negative, sulfur-oxidizing bacteria directly within cells of its gill filaments. These bacteria, none of which have yet been cultured, cluster evolutionarily with the gamma Proteobacteria, a group that contains the majority of endosymbionts thus far described for other marine invertebrates. DNA sequence data for the well-studied symbioses of *S. velum* and its Pacific congener *S. reidi* demonstrate that each host associates with a single, unique bacterial phylotype, which appears to be transmitted maternally (vertically) between successive host generations via the egg. The initial symbiont innoculum passed by the egg ultimately develops in the adult clam into a metabolically active bacterial population whose carbon fixation supports most if not all of the nutritional requirements of the host. The morphological, behavioral, and physiological adaptations that constitute this remarkable mutualistic association are discussed below. A review of these adaptations is timely given that sequencing of the genome of the bacterial symbiont of *S. velum* is underway (via The Institute for Genomic Research; grant to C. Cavanaugh and J. Eisen) and soon will provide one of the first

Table 1 Known *Solemya* species with their respective geographical distributions and primary evidence supporting the presence of sulfur-oxidizing chemosynthetic endosymbionts

Solemya species	Original description	Geographic distribution	Evidence of endosymbiosis							
			TEM of bacteria[b]	Symbiont 16S rRNA[c]	FISH[d]	Reduced/ absent gut[e]	RubisCO[f]	CO_2 fixation[g]	Sulfide use[g]	Stable isotopes[h]
S. africana	von Martens 1879	Mozambique								
S. australis	Lamarck 1818	W Australia, Indian Ocean	Reid and Brand (1987)			Reid and Brand (1987)				
S. borealis	Totten 1834	NW Atlantic, North America	Conway et al. (1992)			Bernard pers. comm. in Reid (1980)	Conway et al. (1992)	Conway et al. (1992)		Conway et al. (1992)
S. caribbaea	Vokes 1970	W Atlantic, America								
S. grandis	Verrill and Bush 1898	W Atlantic, America								
S. johnsoni (*agassizii*)[a]	Dall 1891	NE Pacific				Reid and Bernard (1980)				
S. mediterranea	Lamarck 1818	Mediterranean								
S. occidentalis	Deshayes 1857	W Atlantic, Caribbean	Krueger et al. (1996a)	Krueger et al. (1996a)			Krueger et al. (1996a)			
S. panamensis	Dall 1908	E Pacific, Panama				Reid and Bernard (1980)				Felbeck et al. (1983)
S. parkinsoni	Smith 1874	New Zealand				Owen (1961)				
S. patagonica	Smith 1885	SW Atlantic								
S. pervernicosa	Kuroda 1948									
S. pusilla	Gould 1861	NW Pacific, Japan	Kuznetsov et al. (1990); Krueger and Cavanaugh (1997)	Krueger and Cavanaugh (1997)	Krueger and Cavanaugh (1997)					

Table 1 Continued

Solemya species	Original description	Geographic distribution	Evidence of endosymbiosis							
			TEM of bacteria[b]	Symbiont 16S rRNA[c]	FISH[d]	Reduced/ absent gut[e]	RubisCO[f]	CO_2 fixation[g]	Sulfide use[g]	Stable isotopes[h]
S. reidi	Bernard 1818	NE Pacific	Felbeck (1983)	Distel et al. (1994); Cary (1994)	Cary (1994)	Reid (1980)	Felbeck et al. (1981)	Felbeck (1983); Anderson et al. (1987)	Felbeck (1983); Anderson et al. (1987)	Felbeck (1983)
S. terraeregina		E Australia	Krueger and Cavanaugh (1997)	Krueger and Cavanaugh (1997)	Krueger and Cavanaugh (1997)					
S. tibai[a]	Kuroda 1948									
S. togata	Poli 1795	Mediterranean, E Atlantic				Reid and Bernard (1980)				
S. valvulus	Carpenter 1864	E Pacific				Reid and Bernard (1980)				
S. velesiana	Iredale 1931	W Australia	Reid and Brand (1987)			Reid and Brand (1987)				
S. velum	Say 1822	W Atlantic, North America	Cavanaugh (1983)	Eisen et al. (1992)	Unpub. data in Krueger et al. (1996b)	Reid and Bernard (1980)	Cavanaugh (1983); Cavanaugh et al. (1988)	Cavanaugh (1983)	Cavanaugh (1983); Chen et al. (1987)	Conway et al. (1989); Conway and Capuzzo (1991)

[a] Also described as belonging to genus *Acharax*; taxonomy unclear

[b] TEM images show gram negative bacteria in bacteriocytes of host gill

[c] Sequence analysis of symbiont 16S rRNA gene indicates single gamma Proteobacterial phylotype, phylogenetically related to endosymbionts of other marine invertebrates

[d] Detection of symbionts via fluorescence in situ hybridization using a symbiont-specific 16S rRNA probe

[e] Reduced or absent gut provides evidence of an alternative feeding strategy and supporting evidence for chemosynthetic endosymbiosis

[f] Ribulose 1,5-bisphosphate carboxylase–oxygenase (RubisCO), the primary carbon-fixing enzyme in autotrophs, detected enzymatically or molecularly

[g] CO_2 fixation or depletion of reduced sulfur in experimental incubations

[h] Analysis of $\delta^{13}C$, $\delta^{15}N$, or $\delta^{34}S$ of symbiont-containing and/or symbiont-free tissue indicates host reliance on symbiont autotrophy

detailed pictures of bacterial adaptation to life in chemosynthetic symbiosis. Studies of these adaptations may help us better understand parallel processes that led to the formation of the first eukaryotic cell or that currently operate in pathogenic bacteria–eukaryotic interactions.

Adaptations to life in symbiosis

Protobranch bivalves of the Family Solemyidae occur ubiquitously throughout the world's oceans (Zardus 2002). The Solemyidae contains ~25 species across two genera, *Acharax* and *Solemya*. While all members of Solemyidae are presumed to harbor chemoautotrophic bacteria, endosymbionts in the deep water genus *Acharax* have been described either molecularly (e.g., symbiont 16S rRNA sequence) or ultrastructurally for only six host clams (Barry et al. 2000; Imhoff et al. 2003), none of which have yet been classified to the species level. Rather, the majority of studies have focused on clams of the genus *Solemya*, several of which have been characterized at the ultrastructural level to definitively show the presence of endosymbiotic bacteria in host gill tissue (see evidence in Table 1). Of these, *S. velum* in the Atlantic and *S. reidi* in the Pacific remain two of the best studied of all symbioses between marine invertebrates and chemosynthetic bacteria. Indeed, many of the concepts discussed here stem from work on these species. Extrapolation of these concepts to as of yet uncharacterized solemyids should be done only in a general sense—details of symbiont and host metabolism, ecology, and evolution undoubtedly vary among distinct species of *Solemya*. Regardless, study of the genus as a whole highlights several remarkable adaptations to life in symbiosis.

Adaptations—host anatomy and bacterial ultrastructure

Solemyids have undergone drastic anatomical adaptations to accommodate life in symbiosis. As far as is known, the digestive system in all solemyids is either greatly reduced (as in *S. velum*) or completely absent (as in *S. reidi*; Reid and Bernard 1980). Similarly, the labial palps, flattened structures that help sort food particles during deposit feeding and that are highly developed in non-symbiotic protobranchs, are reduced in *Solemya* species. In contrast, *Solemya* possesses unusually thick and fleshy gills containing chemoautotrophic symbionts. Indeed, relative to all characterized suspension-feeding bivalves (Zardus 2002), the gills of *S. velum* occupy a disproportionate amount (38%) of total clam weight and have a surface area (measured as cm g^{-1} soft tissue wet weight) greater than that reported for any other marine invertebrate (Scott 2005). Such hypertrophied gills in *Solemya* presumably facilitate access by internal symbionts to necessary metabolic substrates (O_2 and H_2S) present in the environment (Scott 2005).

Our understanding of the ultrastructure and dynamics of the endosymbiont population stems primarily from fluorescence and electron microscopy. Interestingly, electron micrographs depicting the ultrastructure of symbiont-containing gills are remarkably similar across host species (Cavanaugh 1983; Conway et al. 1992; Krueger et al. 1996a; Krueger and Cavanaugh 1997). Enlarged gills of *Solemya* consist of alternating symbiont-free intercalary cells and specialized cells called bacteriocytes, in which reside the chemoautotophic endosymbionts (Cavanaugh 1983; Fig. 3) *Solemya* endosymbionts are generally rod-shaped cells greater than 2 μm in length. These bacteria, which reach densities of $>2.5 \times 10^9$ cells per gram of wet weight (Cavanaugh 1983; Mitchell and Cavanaugh 1983), are encapsulated by membrane-bound vacuoles within the cytoplasm of host bacteriocytes. In most solemyid symbioses studied to date, the symbionts are concentrated apically within the bacteriocytes, presumably to facilitate access to metabolic substrates derived from the external milieu. However, symbiont distribution may vary depending on the size of the host organism. For instance, in the small (1–3 mm) subtropical species *S. occidentalis* symbionts are distributed uniformly throughout the bacteriocyte, presumably due to the relatively small size (~10 μm) of the host cells (Krueger et al. 1996a).

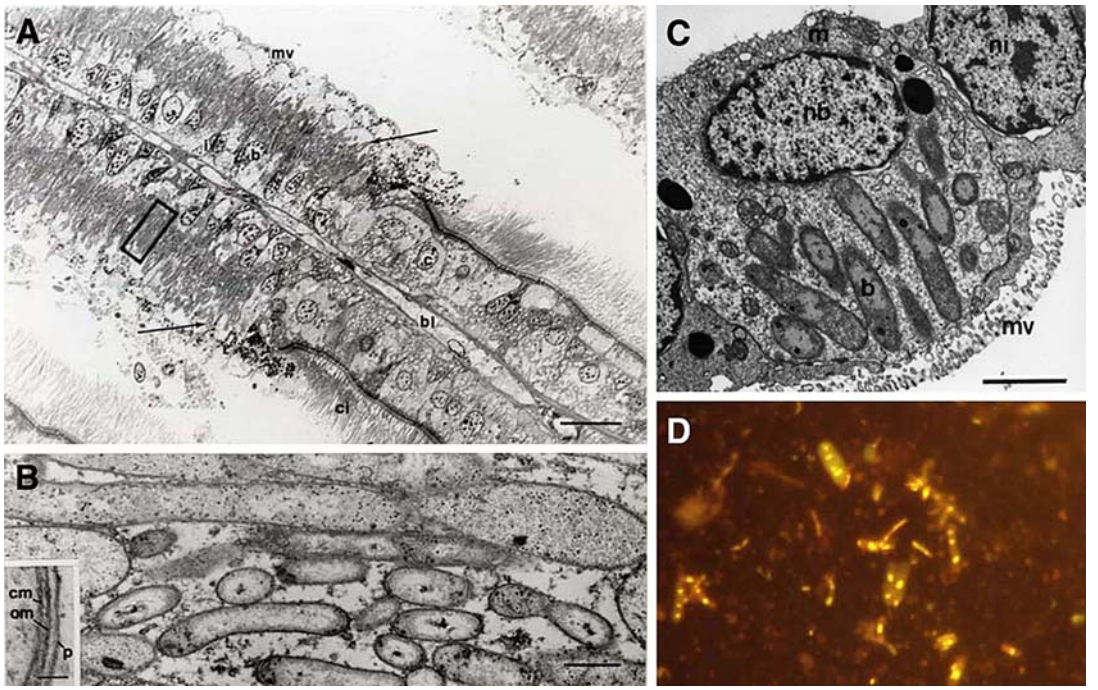


Fig. 3 Panel showing images of *Solemya* symbionts. (**A**) Transverse section of gill filaments of *Solemya borealis* showing intracellular rod-shaped bacteria (arrows, rectangle). Bacteriocytes are confined to the region proximal to the ciliated edge of the gill and are flanked by symbiont-free intercalary 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. Reprinted with permission from *Mar. Biol.* (Conway et al. 1992). (**C**) 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. Scale bar: 3 μm. Reprinted with permission from *Biol. Soc. Wash. Bull.* (Cavanaugh 1985). (**D**) Epifluorescence image of *S. velum* symbionts, showing multiple-nucleoids (per cell) stained with acridine orange

In *S. velum*, the solemyid symbiosis for which perhaps the most visual (microscopy) data are available, symbionts exhibit a high degree of pleomorphism. In adult clams, rod-shaped or filamentous symbiont cells ranging in length from 2 to >10 μm are often interspersed with smaller coccoid cells (Cavanaugh 1983; unpublished observations; Fig. 3). The longer rod-shaped cells often possess multiple nucleoids (Fig. 3), appearing similar to the filamentous phenotype routinely observed in free-living Gram-negative bacteria exposed to suboptimal growth conditions (Mattick et al. 2000), low concentrations of antibiotics (Zak and Kradolfer 1979), or metabolic defects (Mileykovskaya et al. 1998). Similar filamentation has also been documented in *Escherichia coli* cells with mutations in the multiprotein system mediating septum formation and cell division (Margolin 2000), as well as in intracellular pathogenic bacteria (*Salmonella* sp.) inhibited in DNA replication and LPS pathways or exposed to host antimicrobial proteins (Rosenberger and Finlay 2002; Rosenberger et al. 2004; Henry et al. 2005). Interestingly, in adult *S. velum*, actively dividing symbionts are rarely if ever observed via microscopy. A lack of dividing cells and an abundance of multi-nucleoid bacteria indicates potential arrestment of the symbiont cell cycle in *S. velum* and suggests that this symbiosis may be an ideal model system for studying

the dynamics and regulation of endosymbiont population growth.

It would be of particular interest to examine endosymbiont growth in relation to the developmental cycle of the host clam. In contrast to many other marine bivalves whose larvae have a planktonic phase, *S. velum* produces embryos that develop while enclosed in a negatively buoyant gelatinous capsule (Gustafson and Lutz 1992). Upon hatching in late winter or spring, these capsules yield fully formed juveniles that immediately burrow into the sediment where they begin their transition to the adult stage. The bacterial symbiont of *S. velum* appears to be integrated into every aspect of this life cycle. Using symbiont-specific primers for the CO_2 fixation enzyme ribulose 1,5-bisphosphate carboxylase–oxygenase (RubisCO), Krueger et al. (1996b) detected symbionts in the ovaries of *S. velum*, demonstrating that the symbionts are transmitted maternally (without transition to a free-living stage) during fertilization. While the metabolic state of these gonad-associated symbionts is unknown, symbionts in the gill buds of young clams developing within the egg capsule and in filaments of newly hatched juveniles appear to be dividing. These bacteria ultimately develop into a metabolically active population whose carbon fixation supports ~97% of the respiratory budget of the adult host (Krueger et al. 1992; see below). Interestingly, however, symbionts in adult clams no longer appear to be actively dividing. Rather, symbiont populations consist of the elongate, multi-nucleoid cells described above (Cavanaugh personal observation; Cavanaugh 1983). It is hypothesized that the former have been arrested in the process of division. These observations suggest a symbiont life cycle in which population expansion coincides with the developmental growth of the host and division is restricted in clams that have reached adulthood. Determining the molecular or environmental signals that regulate the symbiont–host growth cycle in *S. velum* may suggest corollary mechanisms operating in other bacteria–eukaryote associations. For instance, it would be of medical relevance to determine whether bacterial adaptations to life (and death) in the host cell are conserved across both mutualistic and pathogenic associations.

Adaptations—sulfur acquisition and metabolism

The thioautotrophic metabolism of solemyid symbioses depends on the availability of sulfide in the silty sediments in which these associations occur. Unlike other protobranch bivalves, which reside primarily at abyssal depths, most solemyids occupy continental shelf waters, often thriving in habitats characterized by co-occurring gradients of sulfide and oxygen, such as sewage outfalls, pulp mill effluents, and coastal eelgrass beds. In contrast to hydrothermal vent environments where most sulfide results from the geothermal reduction of seawater sulfate or sulfur-containing rocks, sulfide in shallow water solemyid habitats derives primarily from microbial sulfate reduction. Sulfide concentrations in these zones may vary widely, ranging from ~0.1 μM in intertidal sediments supporting the Australian species *S. velesiana* (Reid and Brand 1987) to several mM in the sewage seepage zones where *S. reidi* occurs (Felbeck 1983; Fisher and Childress 1986).

Unique behavioral strategies for accessing environmental sulfide have evolved in solemyids. For example, *S. velum* digs a unique Y-shaped burrow that spans the oxic–anoxic interface in the sediment (Frey 1967; Stanley 1970). Positioning itself at the triple junction of the Y, the clam alternates between actively pumping oxygenated water from the upper arms of the burrow through the mantle cavity and across the gills and accessing sulfide diffusing up from the anoxic zones below and pumped through a ventral incurrent opening in the mantle (Fig. 4). This strategy provides the clam's symbionts with the necessary substrates (H_2S and O_2) for sulfur oxidation, while at the same time spatially separates these substrates to prevent abiotic oxidation of sulfide by oxygen. But alternative strategies also occur. For instance, *S. reidi* and *S. velesiana* appear to dig U-shaped burrows (Reid 1980; Reid and Brand 1987), whereas species such as *S. togata* and *S. parkinsoni* may not burrow at all (Yonge 1939; Owen 1961). Also, *S. occidentalis*, which inhabits calcareous sands in the tropical Atlantic, may be too small (1–3 mm) to pump water between sulfidic and oxic zones (Krueger et al. 1996a). It is hypothesized that this species instead migrates across the oxic–anoxic interface

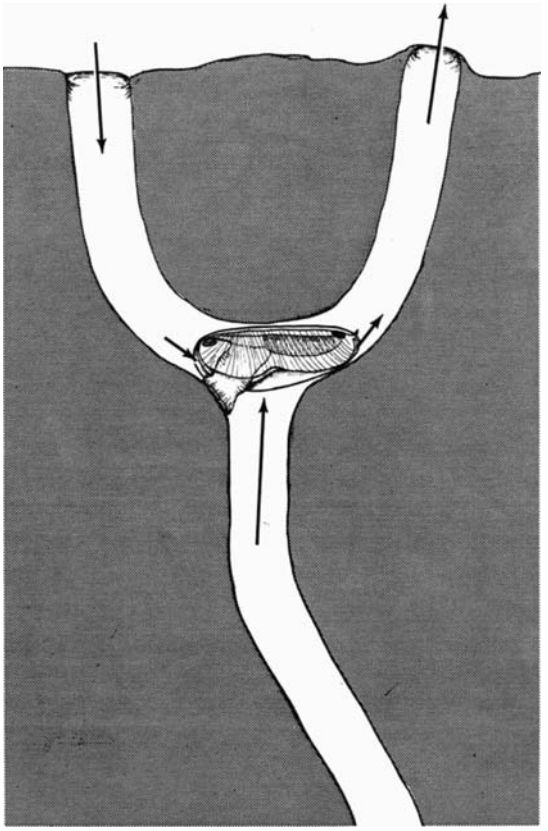


Fig. 4 Schematic diagram of the characteristic Y-shaped burrow dug by *Solemya velum*. The clam positions itself at the junction of the Y, where it can both pump oxygenated water from the upper arms of the burrow through the mantle cavity and access sulfide diffusing up from the anoxic porewater in the stem of the Y. This strategy allows simultaneous acquisition of the substrates (oxygen and sulfide) necessary to support symbiont thioautotrophy. *S. velum* length is ~2 cm. From Cavanaugh 1985 (PhD thesis)

to obtain H_2S and O_2 for symbiont thioautotrophy (Krueger et al. 1996a). This seems feasible given the mobility of *Solemya* bivalves. Indeed, solemyids are capable of swimming by taking in water anteriorly through the mantle cavity and expelling it posteriorly using their flexible valves, suggesting that these clams can relocate to zones with more favorable oxygen or sulfide concentrations (Reid 1980; Reid and Brand 1987). Such mobility may be advantageous given that the symbiotic sulfide demand appears to be quite large. In *S. borealis*, for instance, the daily sulfur requirement may be as high as 23% of the daily sulfide input into the upper (0–16 cm) sediment layers over an area of 1 m^2 (Conway et al. 1992). These authors speculated that this relatively high sulfide demand, particularly in areas where multiple clams reside in close proximity to one another, is met by either processing large quantities of porewater or utilizing partially oxidized sulfur species for symbiont thioautotrophy. Additional ecophysiological studies are necessary to fully quantify the contribution of *Solemya* metabolism to sulfur cycling in shallow water reducing habitats.

The interdependence of symbiont thioautotrophy and host metabolism is the defining adaptation in chemosynthetic symbioses. Indeed, most studies of *Solemya* symbioses have focused on characterizing the metabolism of these associations. Symbionts of *Solemya* species are now known to oxidize reduced inorganic sulfur compounds to obtain energy and reducing power for autotrophic carbon fixation via the Calvin cycle (a schematic overview of symbiont metabolism is provided in Fig. 1). Determination of this metabolic strategy came from studies focusing almost exclusively on two species of solemyids, *S. velum* from eelgrass beds off the coast of Massachusetts and *S. reidi* from sewage-contaminated sites on the Pacific coast. Felbeck et al. (1983) provided evidence of sulfide consumption by documenting a decrease in seawater sulfide concentration over time in incubations containing *S. reidi* relative to incubations without clams. Further analyses demonstrated that exposure to sulfide or thiosulfate stimulated CO_2 fixation and oxygen consumption in both *S. velum* (Cavanaugh 1983) and *S. reidi* (Anderson et al. 1987), providing physiological evidence of sulfide-driven autotrophy.

The process(es) of reduced sulfur transport within *Solemya* symbioses has been the subject of several important studies. In some symbioses sulfide oxidation may first be carried out directly by host mitochondria to obtain energy for oxidative phosphorylation (Powell and Somero 1986). For instance, in *S. reidi*, mitochondria in the body wall of the clam oxidize hydrogen sulfide to the non-toxic intermediate thiosulfate. In other eukaryotes, mitochondrial oxidation of sulfide to thiosulfate in peripheral body tissues is hypothesized to function primarily as a detoxification mechanism (Powell and Somero 1985). Oxidation

in peripheral tissue, followed by the excretion of thiosulfate from the body, prevents exposure of internal aerobic respiratory systems to sulfide, which can inhibit cytochrome c oxidase of the mitochondrial electron transport chain. However, in *S. reidi*, thiosulfate, rather than being excreted from the body, is instead transported to the bacteriocytes for further oxidation by the bacterial symbionts (Sanders et al. 1998).

Cytoplasmic hemoglobin contained in bacteriocytes also may play a role in the delivery of sulfide to internal symbionts of both *S. velum* and *S. reidi*. *Solemya velum* possesses two cytoplasmic hemoglobins, one that reacts only with oxygen and one that, in addition to reacting with oxygen, also reversibly binds sulfide in vivo to form hemoglobin-sulfide (Doeller et al. 1988), as is similar to certain hemoglobins in species of lucinid clams (Kraus et al. 1990, 1996). In contrast, hemoglobin in *S. reidi* gills appears not to accumulate a hemoglobin-sulfide intermediate (Kraus et al. 1992). However, these authors speculate that the inability to detect this intermediate may simply be due to the rate at which hemoglobin reacts with its sulfide ligand. Relative to *S. velum* hemoglobin, the rapid rate at which hemoglobin in *S. reidi* is reduced by sulfide and subsequently transfers reducing equivalents to the symbiont population may prevent accumulation of hemoglobin-sulfide in gill tissue (Kraus et al. 1992).

Interestingly, the sulfur-containing amino acid taurine, which may constitute up to 70% of the free amino acid pool in *S. velum* and is also abundant in *S. borealis* and *S. reidi* (Conway and Capuzzo 1992; Conway et al. 1992; Lee et al. 1997), contributes largely to sulfur cycling in these symbioses. This amino acid, as well as the related compound thiotaurine, may function as a sulfur storage compound, providing reduced sulfur for pathways of symbiont or mitochondrial oxidation when environmental sulfide concentrations are low (Joyner et al. 2003). Alternatively, production of these compounds, which may involve a cysteine intermediate synthesized by the bacterial symbiont, may function as a detoxification mechanism during times when oxygen is not available for sulfide oxidation (Joyner et al. 2003).

While it is clear that symbionts of *Solemya* oxidize reduced sulfur for energy, the biochemical pathway(s) through which this occurs is still unclear. The proposed general model of symbiont thioautotrophic pathways provided in Fig. 1 should be interpreted with caution for *Solemya* symbioses. Relatively few studies have attempted to characterize the enzymes that may mediate the oxidation of sulfide, or another partially reduced intermediate (e.g., thiosulfate), to sulfate in *Solemya* symbionts. Felbeck et al. (1981) detected the enzymes adenosine 5′-phosphosulfate reductase and ATP sulfurylase in the gill tissue of *S. reidi* (Felbeck et al. 1981), suggesting that symbionts of this species may use the energy-conserving APS pathway to oxidize sulfite to sulfate (Fig. 1). But these enzymes are not definitive proof of symbiont thioautotrophy, given that they also function in the reverse direction during sulfate reduction. However, physiological analyses show that *S. reidi* oxidizes sulfide but does not take up and reduce sulfate, suggesting that enzymes of the APS pathway may be functioning only in the direction of sulfide oxidation in this symbiosis (Felbeck 1983). Similarly, Chen et al. (1987) measured activity of APS reductase and ATP sulfurylase in gill tissue of *S. velum*. In addition, these authors also detected activity of thiosulfate sulfurtransferase, an enzyme that catalyzes the oxidation of thiosulfate to sulfite and that may be functioning in the early steps of sulfur oxidation in *Solemya* symbionts. Clearly, forthcoming genomic data for the symbiont of *S. velum* should provide much needed insight into the molecular basis of sulfur oxidation in this and other solemyid symbioses.

Adaptations—carbon metabolism

Internal symbionts of *Solemya* specis appear to use the Calvin cycle for carbon fixation (Fig. 1). Initial evidence for this metabolic strategy came from assays showing high activites of ribulose 1,5-bisphosphate carboxylase–oxygenase (RubisCO), the primary CO_2-fixing enzyme of the Calvin cycle, in gills of *S. velum* (Cavanaugh 1980, 1983) and *S. reidi* (Felbeck et al. 1981). Analyses using immuno-histochemistry later successfully

localized RubisCO to the endosymbionts in *S. velum* gills (Cavanaugh et al. 1988). Further evidence for use of the Calvin cycle comes from TEM images of cellular inclusions resembling carboxysomes (RubisCO storage sites) in symbionts of *S. occidentalis* (Krueger et al. 1996a). As RubisCO is known to occur only in autotrophic organisms, it has been used extensively to test for autotrophy in symbionts. However, other, potentially unique, carbon fixation pathways also may exist in chemosynthetic symbionts of *Solemya*.

The contribution of symbiont carbon fixation to host sustenance has been analyzed via experimental physiological studies, enzymatic characterization, and stable isotope analysis. *Solemya reidi*, which in the adult form completely lacks a gut, is hypothesized to rely almost exclusively on symbiotic bacteria for nutrition (Felbeck 1983). Fisher and Childress (1986) used autoradiography to localize the fixation of carbon (using $NaH^{14}CO_3$) to symbiont-containing gill filaments of *S. reidi* and to follow the movement of fixed carbon into host glandular tissue. Similarly, pulse-chase experiments measuring radioactivity in dissected clam tissues following brief exposure to $NaH^{14}CO_3$ demonstrated that a sizable fraction (>45%) of symbiont-fixed carbon is translocated to the host (Fisher and Childress 1986). Indeed, detailed physiological experiments measuring fluxes of CO_2, O_2, and sulfide in experimental incubations showed that upon exposure to sulfide and under relatively low but constant O_2 concentrations *S. reidi* exhibited a net CO_2 uptake, suggesting that symbiont autotrophy can support the carbon requirements of the symbiosis (Anderson et al. 1987).

Similarly, though *S. velum* does have a vestigial gut, this clam relies almost exclusively on its symbionts for nutrition. Stable isotope analyses suggested that symbiont carbon fixation and biosynthesis may meet up to 98% of the carbon and 100% of the nitrogen requirements of the host clam (Conway et al. 1989). Although *S. velum* may be capable of taking up dissolved amino acids through the epithelium (S. Gallager, personal communication in Conway and Capuzzo 1992), additional stable isotope evidence indicated that endosymbiont biosynthesis provides the bulk of carbon and nitrogen in the total hydrolysable and free amino acid pools in the *S. velum* host (Conway and Capuzzo 1992). Conway and Capuzzo (1991) further demonstrated the importance of symbiont autotrophy to the carbon metabolism of *S. velum* by measuring high concentrations of symbiont-derived *cis*-vaccenic acid in both symbiont-containing and symbiont-free host tissue. Furthermore, $\delta^{13}C$ ratios (–38.4 to –45.3‰) of this and other fatty acids and sterols in *S. velum* were similar to those recorded for a free-living sulfur oxidizing chemoautotroph, suggesting that endosymbiont carbon plays a significant role in host lipid biosynthesis (Conway and Capuzzo 1991). Indeed, *cis*-vaccenic acid later was found to be abundant also within the symbiont-containing gills of *S. borealis* (Conway et al. 1992), indicating that a high concentration of this fatty acid may be an important biomarker of chemosynthetic symbionts in marine invertebrates. Direct experimental measurement of host feeding later confirmed that, while *S. velum* retains a nominal capacity for suspension feeding, ~97% of the respiratory budget of the host clam derives from carbon fixed by its symbionts (Krueger et al. 1992). The remaining 3% can be obtained from planktonic food sources and may supply necessary fatty acids that cannot be synthesized by the symbiont (Krueger et al. 1992).

Recent studies of *Solemya* symbiont metabolism are beginning to provide a more detailed understanding of the enzymatic properties of symbiont chemoautotrophy. This work has focused primarily on the carbon-fixing enzyme RubisCO and its impact on the stable carbon isotope signature of the *S. velum* symbiosis. Comparisons of $\delta^{13}C$ values across chemoautotrophic endosymbioses suggest that the carbon isotopic signature in these associations is driven in large part by the extent to which different types of RubisCO, of which there are eight, discriminate against the ^{13}C isotope when fixing CO_2 (Robinson and Cavanaugh 1995; Cavanaugh and Robinson 1996). *Solemya velum* tissue has a form IA RubisCO (Schwedock et al. 2004) and $\delta^{13}C$ values ranging from –30 to –34‰, which are depleted in ^{13}C relative to photosynthetically-fixed carbon (Goericke et al. 1994) but typical of other bivalves hosting chemoautotrophic symbionts (Robinson and Cavanaugh 1995; Cavanaugh

and Robinson 1996). *Solemya velum* symbiont RubisCO genes have been cloned and expressed in *Escherichia coli* (Schwedock et al. 2004; Scott et al. 2004). Using enzyme purified from these cultures, Scott et al. (2004) demonstrated that symbiont RubisCO fractionates carbon (preferentially assimilates ^{12}C relative to ^{13}C) to a slightly lesser extent than expected based on $\delta^{13}C$ values. Thus, while fractionation by RubisCO likely still plays a large role in determining the $\delta^{13}C$ value of this symbiosis, other factors are contributing to the relatively depleted isotopic signature. Indeed, measurements of the $\delta^{13}C$ of dissolved inorganic carbon in sediment interstitial water suggest that *S. velum* may be assimilating carbon from a source pool that is already isotopically depleted (Scott et al. 2004). In addition, the uniquely large surface area of *S. velum* gills may impact the $\delta^{13}C$. A large gill surface area ensures an exceptionally high rate of CO_2 exchange between the symbiont cytoplasm and the external source pool, allowing intracellular and external pools to reach isotopic equilibrium (Scott et al. 2004; Scott 2005). In the absence of such exchange, CO_2 within the cytoplasm becomes progressively enriched in $^{13}CO_2$ as $^{12}CO_2$ is preferentially fixed by RubisCO. Over time RubisCO draws more and more from the $^{13}CO_2$ fraction of the cytoplasmic pool, thereby enriching the $\delta^{13}C$ of the fixed carbon. However, rapid CO_2 exchange could effectively negate this enrichment, causing the $\delta^{13}C$ of fixed carbon to decrease (Scott 2005). These studies underscore the importance of considering multiple factors when interpreting stable isotopic ratios from living and fossil organisms and provide a model for the enzymatic basis of primary production in chemosynthetic symbioses.

Adaptations—nitrogen metabolism

Due to the autotrophic nature of solemyid symbioses and to the fact that host feeding meets little if any of the metabolic demands of the symbiosis, assimilation of inorganic nitrogen is likely critical to supporting the growth and metabolism of the host and symbiont (Lee and Childress 1994; Lee et al. 1999). Ammonia uptake and ^{15}N tracer experiments demonstrated that for *S. reidi*, inorganic nitrogen is likely assimilated as ammonia (in Fig. 1 as $\sum NH_3$: NH_4^+ and NH_3; Lee et al. 1992; Lee and Childress 1994), which is abundant in the oxygen-depleted, nutrient-rich habitats of the clam. The rate of ammonia incorporation and the activity of ammonia assimilation enzymes 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). In *S. velum* ammonia appears to be first assimilated via a glutamine synthetase synthesized by host tissue (Lee et al. 1999). This contrasts with vent symbioses in which ammonia uptake occurs primarily through the activity of glutamine synthetase belonging to the symbionts. Activity of the host enzyme in *S. velum* is high relative to the activity of glutamine synthetase from non-symbiotic invertebrates (Lee et al. 1999), suggesting that the host is compensating to meet the high metabolic demand for ammonia assimilation imposed by the drastically reduced capacity for filter-feeding in this clam (Krueger et al. 1992). Although low levels of nitrate assimilation have been reported for *S. reidi* (Lee and Childress 1994), *S. velum* does not appear capable of nitrate assimilation (Lee et al. 1999). Indeed, direct utilization of nitrate for growth is likely unnecessary, perhaps due to NH_4^+ cycling between host and symbiont or high levels of ammonia in the surrounding sediment. Further analyses at the molecular and enzymatic level are necessary to fully understand the movement and transformation of nitrogen, as well as all other nutrients required by the symbiosis, within the host–symbiont system.

Symbiont transmission, ecology, and evolution

Transmission

The evolutionary history of *Solemya* symbionts inherently depends on the mechanism by which the symbiont is passed between successive host generations. Data for *S. velum* and *S. reidi* suggest that solemyid symbionts pass directly from the adult host to the larvae via the female reproductive tissue (i.e., vertical or maternal transmission). The gene coding for symbiont

RubisCO was repeatedly PCR-amplified from female ovarian tissue of *S. velum* using symbiont-specific primers (Krueger et al. 1996b). Furthermore, bacteria were observed in the nascent gills of juvenile *S. velum* during larval development within the gelatinous egg capsule, during which time exposure to the external environment is presumably limited. These results suggest that symbionts of *S. velum* are vertically transmitted, presumably via a seed population either attached to the surface or contained within the host oocytes (Krueger et al. 1996b). Similarly, using PCR primers specific to the *S. reidi* symbiont, Cary (1994) amplified symbiont 16S rRNA genes from host ovarian tissue, eggs, and larvae. Successful amplification from eggs following treatment to remove surface-attached bacteria suggest that the symbionts reside directly within the egg capsule. *In situ* hybridization with a symbiont-specific 16S rRNA probe later detected symbionts in the ciliated epithelial test cells surrounding 3-day old larvae. These test cells are ultimately ingested through the mouth of the metamorphosing larvae, suggesting a mechanism by which the inoculation of the internal perivisceral cavity and subsequent transportation to the developing gills occur (Gustafson and Reid 1988a, b; Cary 1994). However, the extent to which symbionts are metabolically active during transmission and early host development remains unclear. Both Cary (1994) and Krueger et al. (1996b) were unable to visualize symbionts on host eggs via *in situ* hybridization, perhaps due to low numbers of ribosomes brought about by a state of quiescence in the bacteria. Further examination of distinct host developmental stages may reveal the physiological or environmental cues that control the activity and growth of the symbiont population during transmission.

Maternal transmission in solemyid symbioses undoubtedly strongly impacts the evolution of the bacterial symbiont. Though not shown definitively, maternally transmitted solemyid symbionts are confined to the host environment, suggesting that the exchange of genetic material is restricted to homologous recombination between members of the putatively clonal, internal symbiont population. Moreover, maternally transmitted populations undergo repeated bottlenecks upon transmission between successive host generations (e.g., Mira and Moran 2002), thereby exacerbating the fixation of near-neutral mutations by genetic drift. As has been suggested for insect endosymbionts, enhanced genetic drift coupled with relaxed selection due to growth in a stable, metabolite-rich host environment effectively enhances the rate of evolution in maternally transmitted lineages (Moran and Wernegreen 2000; Wernegreen 2002). In insect symbioses these factors have contributed to a drastic reduction in genome size relative to the hypothetical symbiont ancestor and to extant free-living bacteria (e.g., Moran and Mira 2001; Moran 2002). Similar processes may be at work in the genomes of solemyid symbionts. Indeed, Peek et al. (1998), by comparing rRNA gene sequences from a variety of sulfur-oxidizing bacteria, including the symbiont of *S. velum*, suggested that nucleotide substitution rates in maternally transmitted symbionts are higher than those in free-living bacteria and environmentally transmitted symbionts. Analysis of the forthcoming genome of the *S. velum* symbiont will help determine whether the predicted effects of maternal transmission on genome evolution (e.g., increased substitution rate, reduced genome size) are applicable across multiple genes in chemosynthetic symbionts.

Evolution

Symbiosis appears to be an ancient evolutionary adaptation in solemyids. The bivalve family Solemyidae is relatively old, with fossil representatives appearing in deposits dating to the Middle Ordivician (460–480 mya; Pojeta 1988). The fact that all extant members of this family harbor symbiotic bacteria suggests that symbiosis is an ancestral condition, having been established prior to the diversification of this group (Distel 1998). Indeed, in trace fossils of ancient solemyids, the presence of Y-shaped burrows, an apparent adaptation to life in symbiosis (see above), further suggests that symbiosis is an ancestral trait in solemyids (Bromley 1996). Assuming that solemyid ancestors were symbiotic at the time of diversification and that symbionts have co-speciated

with their hosts, Krueger et al. (1996a) estimated a substitution rate for *Solemya* symbionts of 1% per 61–63 million years. This rate is similar to those calculated for *Buchnera* endosymbionts of aphids (Moran et al. 1993; Clark et al. 1999) but considerably slower than that calculated for endosymbionts of clams in the family Vesicomyidae (Goedert and Squires 1993; Krueger et al. 1996a) or *Blochmannia* symbionts of ants (Degnan et al. 2004). These data, while based on analyses of only three *Solemya* symbionts, suggest that chemosynthetic endosymbionts do not evolve uniformly across diverse host lineages.

Indeed, evolutionary relationships among solemyid symbionts are complex. Analysis of 16S rRNA gene sequences reveals that these symbionts, like the majority of chemosynthetic symbionts of other marine invertebrates, are gamma Proteobacteria (Eisen et al. 1992; Distel et al. 1994; Krueger et al. 1996a; Krueger and Cavanaugh 1997). However, though only a relatively small subset of solemyid symbiont phylotypes have been characterized (Table 1), phylogenetic analyses suggest that solemyid symbionts do not form a monophyletic group. Solemyid symbionts cluster broadly in a clade with symbionts of lucinid and thyasirid clams and vestimentiferan tubeworms, to the exclusion of symbionts of vesicomyid clams and mytilid mussels (Distel 1998). However, while symbionts of some solemyid species fall within a distinct subclade containing symbionts of lucinid and thyasirid clams (Krueger and Cavanaugh 1997), others do not, suggesting that solemyid symbionts and their respective hosts do not share parallel histories of diversification or that the establishment of symbiosis in solemyids may have involved multiple colonization events (Krueger and Cavanaugh 1997; Distel 1998). Also, it is possible that a symbiont phylotype from a different host species may have replaced an existing symbiont population in a co-occurring host species. In this scenario, the ancestral state of symbiosis would be maintained in the newly colonized host species but the phylogenetic identity of the symbiont would change (Krueger and Cavanaugh 1997). Molecular data for other solemyid symbionts and hosts are necessary to resolve questions of the evolution of symbiosis within this group.

Ecology

Few studies have investigated the impact of solemyid symbioses on the ecological processes of shallow water reducing environments. Indeed, for the majority of *Solemya* species, basic ecological data regarding the distribution and abundance of these clams have yet to be collected. However, some data are available for *S. reidi* and *S. velum*, which both appear to exhibit a patchy distribution. High densities of *S. reidi* have been reported only for specific locations, including a small, sulfide-rich zone at the outlet of a sewage pipe in Santa Monica Bay, CA (Felbeck 1983) and a low-oxygen zone beneath a log boom off of Vancouver Island, where a population density of 16 individuals (~3–5 cm each) per square meter was recorded (Reid 1980). In a detailed study of macrofaunal abundance in Quisset Harbor, Massachusetts, Levinton (1977) found that individuals of *S. velum* (~1–2 cm each), while occurring at relatively low population densities (0.8–3.3 clams per m^2) in deep (3–4 m) channel habitat, occur at exceptionally high densities of up to 253 clams per m^2 in adjacent (~100 m away) eelgrass beds. At such abundances, these clams could play a significant role in the aeration and the cycling of sulfur within soft sediments. In addition, consumption of solemyid clams by other organisms could provide an important link between symbiont primary production and the carbon budget of the benthic food web. Indeed, Rainer and Wadley (1991) showed that an unidentified *Solemya* species (similar to *S. terraereginae*) inhabiting seagrass beds along the western coast of Australia may provide up to 22% of the nutritional requirements of juvenile rock lobsters. Similarly, the tropical solemyid *S. occidentalis* may serve as an important food resource for reef fish (Vokes 1955). However, the long-term stability of *Solemya* populations, and consequently their impact on surrounding organisms, is unclear. Anecdotal evidence for *S. reidi* suggests that when sulfidic zones become oxygenated, which may occur if current patterns shift, *S. reidi* populations soon disappear (Felbeck et al. 1983). Future research should further attempt to characterize links between the unique metabolism of *Solemya* symbioses and the biogeochemical

processes that define shallow-water reducing sediments.

Conclusion

Studies of the mutualistic relationship between sulfur-oxidizing bacteria and *Solemya* clams have provided a foundation for analyses of similar associations in other bivalves. Indeed, chemosynthetic endosymbioses have now been described in bivalves from five families (Solemyidae, Mytilidae, Vesicomyidae, Thyasiridae, Lucinidae) occupying a diverse array of reducing habitats, all of which are characterized by the presence of an oxic–anoxic interface (Cavanaugh et al. 2005; Stewart et al. 2005). Though scientific understanding of these symbioses has increased considerably in recent years, our knowledge of the molecular mechanisms that underlie the host–symbiont interaction, as well as the ecological and evolutionary pressures that impact the association, is still in its infancy. This is due in part to our inability as of yet to culture a chemosynthetic symbiont apart from its invertebrate host. Also, the characterization of symbiont molecular pathways is potentially complicated by the genome reduction that presumably has occurred in maternally transmitted symbionts.

Solemyid clams, however, provide an excellent model system for the study of chemosynthetic endosymbioses. For example, *S. velum* is obtained easily from mudflats off the coast of Massachusetts and can be maintained in flow-through aquaria for periods of weeks, allowing experimental manipulation of environmental conditions (e.g., sulfide or oxygen concentration). Adult *S. velum* have even been induced to spawn in laboratory conditions (Gustafson and Lutz 1992; Krueger et al. 1996b), facilitating characterization of distinct host growth stages. Furthermore, the genome of the bacterial symbiont of *S. velum* is currently being sequenced by The Institute of Genomics Research (TIGR; grant to C. Cavanaugh and J. Eisen). Results of this project will provide a window into the genetic basis of symbiont metabolism, growth, and persistence within the host cell environment, as well as into the selective pressures that have shaped these mechanisms over time. These data will lay the foundation for future analyses of symbiont ecophysiology. Such analyses will complement the impressive body of work that has helped characterize solemyid symbioses over the past two decades and may provide insight into the formation and evolution of other bacteria–eukaryote interactions, including those involving pathogenic bacteria.

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