

# On the evolutionary ecology of symbioses between chemosynthetic bacteria and bivalves

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**Abstract** Mutualistic associations between bacteria and eukaryotes occur ubiquitously in nature, forming the basis for key ecological and evolutionary innovations. Some of the most prominent examples of these symbioses are chemosynthetic bacteria and marine invertebrates living in the absence of sunlight at deep-sea hydrothermal vents and in sediments rich in reduced sulfur compounds. Here, chemosynthetic bacteria living in close association with their hosts convert CO<sub>2</sub> or CH<sub>4</sub> into organic compounds and provide the host with necessary nutrients. The dominant macrofauna of hydrothermal vent and cold seep ecosystems all depend on the metabolic activity of chemosynthetic bacteria, which accounts for almost all primary production in these complex ecosystems. Many of these enigmatic mutualistic associations are found within the molluscan class Bivalvia. Currently, chemosynthetic symbioses have been reported from five distinct bivalve families (Lucinidae, Mytilidae, Solemyidae, Thyasiridae, and Vesicomysidae). This brief review aims to provide an overview of the diverse physiological and genetic adaptations of symbiotic chemosynthetic bacteria and their bivalve hosts.

**Keywords** Clams · Hydrothermal vents · Methane · Sulfur · Oxidizing bacterial symbionts · Chemoautotrophy

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## Introduction

The term “symbiosis” was coined to describe associations in which different species live closely together, in relationships ranging from mutualisms to parasitism. Symbiosis has played a major role in shaping the evolution and diversity of eukaryotic organisms, and it is now clear that eukaryotic organelles such as chloroplasts and mitochondria evolved via ancient endosymbiotic events (Margulis 1970). Chemosynthetic endosymbiosis is a nutritional strategy that loosely parallels photosynthesis in higher plants. Whereas chloroplasts convert light energy into chemical energy, in the form of ATP and NADPH, chemosynthetic endosymbionts harness chemical energy stored in reduced sulfur compounds or methane. In chemosynthetic symbioses, the host provides access to reduced compounds (e.g., hydrogen sulfide) and oxygen that the bacterium uses to drive the formation of fixed carbon from single carbon molecules (either CO<sub>2</sub> or CH<sub>4</sub>). The symbionts support a substantial fraction, if not all, of the host’s nutrition (Cavanaugh et al. 2006). Although known to occur within several invertebrate groups, it is within the class Bivalvia that this nutritional strategy is most widespread, both phylogenetically and geographically. Here we aim to review the diverse genetic and functional characteristics of chemosynthetic symbiosis in bivalves, which reflect the evolutionary ecology of both hosts and symbionts within this important invertebrate group.

## Host morphology

The unusual nutritional strategy used by these symbiotic bivalves is reflected in their atypical morphology. Symbiotic bivalves are characterized by rudimentary or absent digestive

systems. The principal anatomical adaptations are reduction of labial palps, reduction in length and volume of intestine, reduction in stomach volume, disappearance of crystalline style and style sac, reduction in digestive tubule volume, disappearance of digestive gland secretory cells, and, in some cases, total disappearance of the digestive system (Boss and Turner 1980). Bivalves typically harbor their symbionts in typically large and conspicuous gills, often accounting for more than one third of the animal's total soft tissue weight (Distel 1998). However, chemosynthetic symbioses within Bivalvia are excellent model systems for studying the evolution of bacteria–eukaryote interactions, as they display a range of intimacies with some symbionts being housed intracellularly within specialized gill cells called bacteriocytes (as in the bathymodiolin mussels and the vesicomid and lucinid clams) or extracellularly within the subfilamentar tissue of the host gill (as in the thyasirid clams) (Dubilier et al. 2008). In some *Idas* and *Adipicola* species, the symbionts are epibiotic and are attached to the surface of the gill tissue (Southward 2008). The relevance of this intimacy to host function and metabolism is not currently known.

## Host diversity

### Solemyidae

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*. The solemyid range in size from less than 30 to over 200 mm (*Acharax bartschii*) and form symbioses with thioautotrophic bacterial symbionts within their gill epithelial cells.

Solemyids live in “Y”-shaped burrows (Stewart and Cavanaugh 2006) (Fig. 1) and are capable of swimming by jet propulsion through flexure of the shell margins with a pulsing expulsion of water through the posterior aperture (Reid 1980). Several key studies of solemyid symbioses have focused on the Atlantic species *Solemya velum*, which inhabits soft coastal sediments from Florida to Nova Scotia (Vokes 1955) and its Pacific congener *Solemya reidi*. Each of these hosts associates with a single, unique symbiont phylotype, whose carbon fixation supports most if not all of the nutritional requirements of the adult host (Krueger et al. 1992). Symbiont DNA has been extracted from the host ovarian tissue (Krueger et al. 1996b), raising the hypothesis that the symbiont is transmitted maternally (vertically) between successive host generations via the egg (Krueger et al. 1996b). To our knowledge, all attempts to culture symbionts of *S. velum* (or other hosts) have failed. Nonetheless, the existence of a free-living form of the symbionts could explain the rare signals of

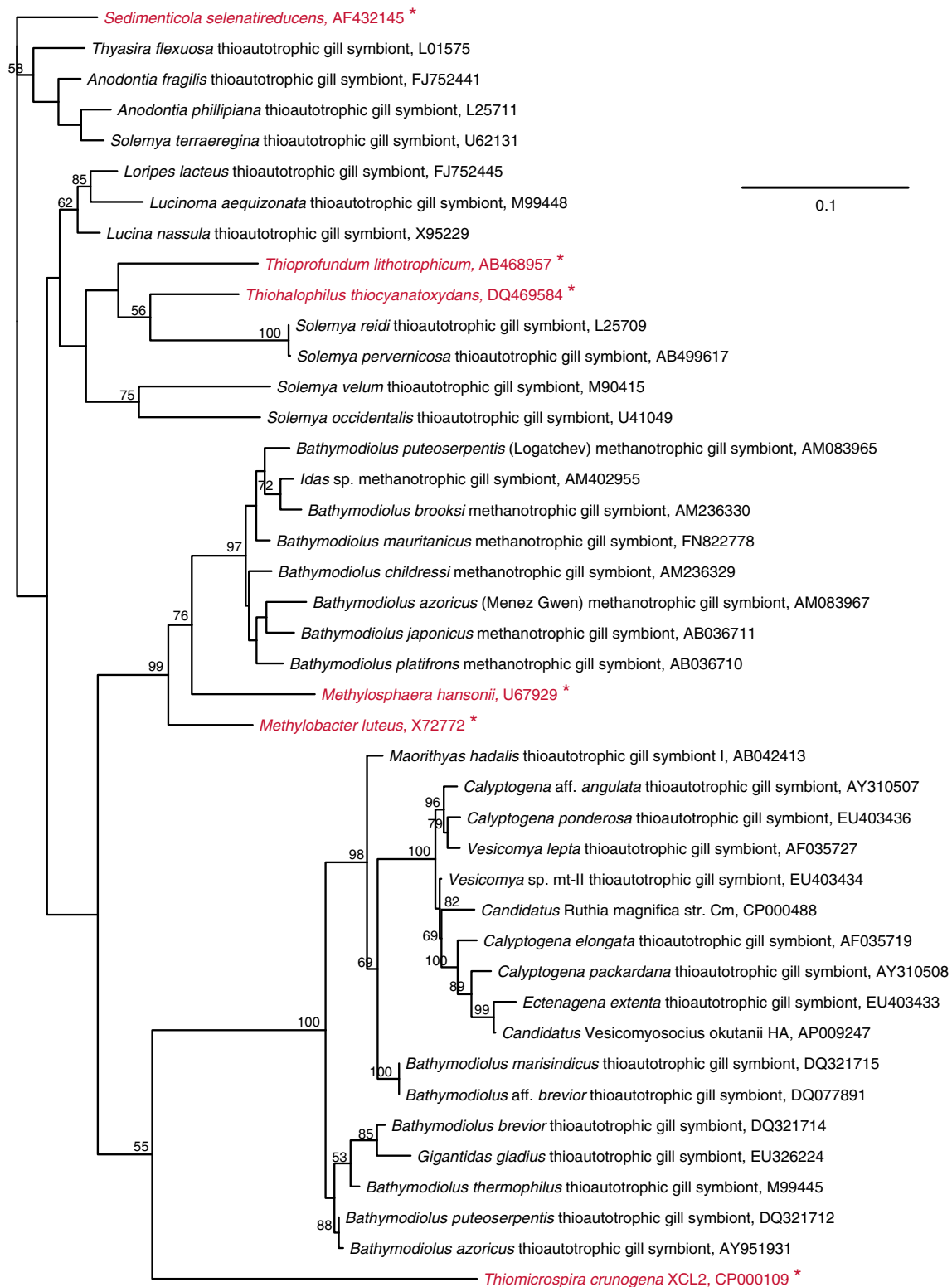


**Fig. 1** Sediment cross section exposing the characteristic Y-shaped burrow dug by *S. velum*. Positioning itself at the triple junction of the Y, the bivalve alternates between actively pumping oxygenated water from the upper arms of the burrow through the mantle cavity and across the gills and accessing reduced sulfur compounds diffusing up from the anoxic zones below and pumped through a ventral incurrent opening in the mantle. Scale bar equals 2.5 cm

horizontal transfers between hosts (Stewart et al. 2009a, Stewart and Cavanaugh 2009).

### Lucinidae

The family Lucinidae is by far the most disparate and species-rich of the chemosynthetic hosts and occupies the greatest variety of habitats over a broad geographical range. Habitats occupied by the over 400 living Lucinidae species range from intertidal zones and offshore sediments, seagrass beds (Barnes and Hickman 1999; Johnson et al. 2002), mangrove muds (Schweimanns and Felbeck 1985; Lebata 2001), and sewage effluent sites (Herry et al. 1989). Some species are associated with cold seeps and mud volcanoes (Brissac et al. 2010; Callender and Powell 1997; Carney 1994), oxygen minimum zones (Cary et al. 1989; Oliver and Holmes 2006), and a single species (*Bathyaustriella thionipta*) from a hydrothermal vent site (Glover et al. 2004). The lucinid range from a few millimeters to 150 mm in size and harbor thioautotrophic symbionts within their gill epithelial cells (Frenkiel and Moueza 1995; Taylor and Glover 2000). The lucinid symbionts are thought to be laterally transmitted each generation, leading to the increased genetic diversity seen within hosts (Williams et al. 2004). Phylogenetic analyses places the Lucinidae symbionts in a  $\gamma$ -proteobacterial clade including *Solemya* and vestimentiferan tube worm symbionts (Cavanaugh et al. 2006) (Fig. 2).



**Fig. 2** Maximum likelihood tree illustrating the phylogenetic relationships among several thioautotrophic and methanoautotrophic symbionts of different bivalve hosts based on full-length 16S rRNA gene sequences selected from the ARB-SILVA SSU-Ref-108 database

(<http://www.arb-silva.de>). Free-living bacteria are indicated with an asterisk. *Agrobacterium tumefaciens* [D14500], serving as an outgroup, was pruned from the ML tree. Bootstrap values  $\geq 50\%$  (1,000 replicates) are placed at the branch nodes

## Vesicomylidae

The Vesicomylidae comprises at least 50 species occurring ubiquitously at hydrothermal vents, hydrocarbon seeps, and whale falls throughout the world's oceans (Baco et al. 1999; Goffredi et al. 2003). They range in size from less than 5 mm to the extremely large species from vents and seeps that reach shell lengths up to 280 mm. Vesicomylidae retain only a rudimentary gut and rely instead on thioautotrophic gill endosymbionts for nutrition (Cavanaugh 1983).

Vesicomylid endosymbionts are found in host ovaries (Cary and Giovannoni 1993), and their genomes are highly reduced (Newton et al. 2007; Kuwahara et al. 2007) supporting the hypothesis that symbiont acquisition occurs via maternal inheritance in this group. Indeed, Vesicomylidae show a strong coupling of symbiont–host genetic variation (Peek et al. 1998). These symbioses are therefore considered useful models for studying bacteria–eukaryote coevolution. However, Stewart et al. (2009a, b) observed instances of decoupled symbiont and host mitochondrial phylogenies suggesting a mixed mode of symbiont transmission characterized by predominantly maternal transmission with periodical events of lateral symbiont acquisition.

Molecular analyses of symbionts from various species of Vesicomylidae from widely separated habitats show that they belong to one clade of  $\gamma$ -proteobacteria (Cavanaugh et al. 2006; Dubilier et al. 2008). These form a sister clade to the thioautotrophic symbionts from *Bathymodiolus* species, distinct from the symbiont group associated with shallow water families Lucinidae, Thyasiridae, and Solemyidae.

## Mytilidae

Bathymodiolin mussels (family Mytilidae) are one of the most ecologically successful metazoans in the deep-sea; they are ubiquitous within these habitats, colonizing hydrothermal vents and cold seeps to wood falls, whale carcasses, and oil impregnated muds from oil-drilling platforms (Taylor and Glover 2010; Dubilier et al. 2008). The occurrence of bathymodiolin mussels in this broad range of environments may be attributed to their nutritional flexibility; they are capable of obtaining nutrition from filter feeding (Page et al. 1991) in addition to receiving nutrition from chemosynthetic symbionts (Dubilier et al. 1998; Fujiwara et al. 2010). The mussels can be very large; for example, *Bathymodiolus boomerang* reaches shell lengths of 370 mm and *Gigantidas gladius* to 316 mm (von Cosel and Marshall 2003). Sulfur and/or methane-oxidizing bacteria housed in their gill bacteriocytes cells have been reported for all studied species of bathymodiolins. All work done to date suggests that the *bathymodiolin* symbionts are acquired from the environment (Won et al. 2003)

## Thyasiridae

Among the families of chemosymbiotic bivalves, the Thyasiridae stand out in several ways. Thyasiridae vary the most in their anatomical characters and in the extent of their nutritional reliance upon symbionts (Dufour 2005). Although Thyasiridae range in size from less than 1 to around 110 mm (*Conchocele bisecta*), most are less than 10 mm. They are globally distributed from polar to equatorial latitudes and live at all depths ranging from the intertidal to deepest trenches in the ocean (Taylor and Glover 2010).

Unlike the other chemosynthetic Bivalve families, only a fraction of Thyasiridae species harbor symbionts (Dufour 2005) which in most species are observed to be extracellular (Taylor and Glover 2010; Dufour 2005). This group may therefore be representative of early stages in the evolution of chemosynthetic bacterium–bivalve symbioses.

## Symbiont transmission strategies

In order to continue to propagate these symbioses, each generation the host animals must be inoculated with the bacterial symbiont at some point during the life cycle, referred to as the transmission strategy for that organism. The transmission strategy utilized by a symbiosis has generally been inferred from phylogenetic evidence (lack or presence of codiversification) or the presence of bacterial DNA in the gametes of the host (Krueger et al. 1996b). Only for one bivalve group, bathymodiolin mussels, has the process of host colonization been experimentally tested. These experiments suggest that in these hosts, the symbionts are reacquired from the surrounding seawater after induced loss (Kadar et al. 2005; Won et al. 2003). The implications for host niche differentiation are quite clear: A host able to take up a genetically diverse pool of symbionts adapted to localized habitats may be at an advantage compared to those with static relationships. Similar mechanisms of niche adaptation have been suggested in other invertebrate symbioses, for instance, corals (Iglesias-Prieto et al. 2004).

Symbionts can be transmitted to the next host generation along a spectrum of transmission strategies: from strict vertical to strict lateral transmission. Vertically transmitted symbionts are passed down via the gametes (often intracellular). This transmission strategy imposes both a population bottleneck on the symbionts (reducing the symbiont genetic diversity available to the host and increasing the mutational load for the symbiont through genetic drift (Allen et al. 2009; Moran 1996)) as well as imposing codiversification (evidence often seen in phylogenetic analyses of host and symbiont). A vertical transmission strategy has been suggested for the Solemyidae and Vesicomylidae, based on phylogenetic and microscopic evidence. However, recent



analyses suggest that vesicomid bivalves are not strictly maternally transmitted (Stewart et al. 2009b) and that the symbiont of *S. velum* has a genome that contrast with those seen in vertically transmitted symbionts (Stewart et al. 2009a).

At the other end of the transmission strategy spectrum are those symbionts that are strictly laterally transmitted. These hosts must reacquire their symbionts each generation from a presumed free-living pool of bacteria. In contrast to the vertically transmitted symbioses, symbionts that are laterally transmitted are likely to be more genetically diverse within and between hosts, leading to the possibility of evolutionary conflicts. The bathymodiolin mussels and lucinid clams are thought to gain their symbiont through lateral transmission, culminating in the great diversity of symbionts we see in these hosts (Caro et al. 2007; Duperron et al. 2009). However, it is possible that the lateral transmission strategy comes with a substantial benefit; it would allow a host, during colonization of a new habitat, to sample bacteria from that environment, thereby selecting the organisms best suited for the symbiosis given the ecological parameters present.

## Habitat

There are numerous environments in the biosphere where the biogeochemistry favors the colonization and emergence of chemosynthetic metabolisms. These sites are unified by the simultaneous availability of reduced compounds and molecular oxygen. Deep-sea hydrothermal vents were the first habitats in which chemosynthesis-driven primary production was shown to fuel large animal communities (Cavanaugh et al. 1981; Cavanaugh 1983). Hydrothermal vents result from geothermally driven circulation of seawater in the crust linked to magmatic activity at oceanic ridges. A few years after the discovery of the hydrothermal vent fauna, similar communities were discovered at cold seeps (Paull et al. 1984) where hydrocarbons and/or gas hydrates escape from the subsurface at continental margins. Later chemosymbiotic bivalves were found in a range of environments where sulfuric compounds originate from decaying organic matter as, such as in sea-grass beds (Cavanaugh 1983) and whale-falls (Bennett et al. 1994). Relevant to our discussions below, although each of these environments provides the biogeochemistry necessary for

chemosynthetic metabolism, they differ in regards to the concentrations of particular reduced compounds and sources of nutrients available to the symbioses (Table 1). These geochemical differences may select for associations best specialized for a particular niche.

## Symbiont diversity

It is striking that chemosynthesis has evolved in such phylogenetically diverse bivalve families ranging from the ancient Solemyidae, the bathymodiolin mussels to the heterodont families Thyasiridae, Lucinidae, and Vesicomidae. In contrast to the remarkable phylogenetic divergence of the hosts involved in these symbioses, their chemosynthetic symbionts cluster all within a single bacterial class, the  $\gamma$ -proteobacteria, on the basis of 16S rRNA gene sequences (Distel and Cavanaugh 1994) (Fig. 2). Recent phylogenetic analyses of these symbionts revealed at least nine phylogenetically distinct clades within this bacterial class, most of which were interspersed with sequences from free-living bacteria (Dubilier et al. 2008). This evidence suggests that these symbioses have evolved multiple times, from different free-living bacterial ancestors.

The genetic and genomic diversity of within-host symbiont populations has been explored in a number of different chemosynthetic bivalves. The diversity of the population found within the host has the potential to affect host physiology, evolutionary adaptability, and define host niche. For example, in symbioses where a single, primary bacterial symbiont is known, polymorphisms can be observed with regards to symbiont genome number and bacterial size (as in the clam *Codakia orbicularis*) (Caro et al. 2007), and these differences may influence symbiont transcriptional response. A large amount of genetic diversity is expected in symbionts that are laterally transmitted (Stewart et al. 2009a, b; Dechaine et al. 2006), but even within primarily vertically transmitted symbioses, cryptic genetic diversity has been discovered within these presumed homogenous populations (Stewart and Cavanaugh 2009; Stewart et al. 2009b). The significance of this population level diversity is not yet known, but the implications for the evolution of the symbioses are large. Strict vertical transmission of symbionts is known to dramatically reduce genome content (Moran 2003). Indeed, the vertically transmitted vesicomid

**Table 1** Typical pore water concentrations of reduced compounds in different habitats

Habitat	Sulfur	Methane	References
Hydrothermal vents	3–40 mmol/kg	0.1–3.4 mmol/kg	Kelley et al. (2005); Cowen et al. (2002)
Cold seeps	0.57–19.43 mmol/kg	0.06–0.8 mmol/kg	Kelley et al. (2001); Barry et al. (1997)
Seagrass beds	5–35 $\mu$ mol/kg	2–20 $\mu$ mol/kg	Deborde et al. (2010); Calleja et al. (2007)

symbionts have undergone dramatic genomic reductions, with the loss of many physiological capabilities (Kuwahara et al. 2007; Newton et al. 2007; Roeselers et al. 2010). If a symbiont population loses the ability to synthesize a particular nutrient, this can be particularly disadvantageous for the host. However, the existence of population level diversity within a host suggests avenues through which the bacterium might recuperate some function relevant to the symbiosis, perhaps through homologous recombination.

Within the bivalves, the bathymodiolin mussels are conspicuous for the huge diversity of symbionts found within an individual host's tissues. Up to six bacterial symbionts have been identified in *Idas* species, with all symbionts capable of chemosynthesis (Duperron et al. 2008). More commonly, however, a dual symbiosis is seen in the mussels with a thioautotrophic symbiont co-occurring within the same cells as a methanoautotrophic symbiont (Duperron et al. 2006; Dechaine and Cavanaugh 2005). As detailed below, the levels of methane and sulfur in a particular environment may control the numbers of these distinct symbionts found within the host cells. This kind of symbiotic plasticity in combination with high dispersal ability, based on their larval shell morphology and small egg size (Le Pennec and Beninger 2000), may have allowed the bathymodiolin mussels to reach current cosmopolitan biogeographic distribution (Kyuno et al. 2009).

### Functional genomics and ecology of chemosynthetic symbioses

The functionality of the chemosynthetic symbioses relies on both the availability of substrates for chemosynthetic metabolism and the existence of particular metabolic pathways in the symbiont to utilize those substrates. There is a link, therefore, between geochemistry and the type of symbiosis that can colonize a particular environment (Cordes et al. 2010). Hosts have adapted in a variety of ways to provide their symbionts with these essential substrates (for example, the behavioral extension of the foot to reach sulfur in thyasirid bivalves (Dufour and Felbeck 2003), but environmental concentrations are likely to dominate any ecological framework determining the localization and abundance of these symbioses.

#### Sulfur

For bivalves harboring thioautotrophic symbionts, the availability of sulfur (as well as a terminal electron acceptor such as oxygen) in their geochemical environment is an important variable in determining the presence of these symbioses (Van Dover 2000). Indeed, sulfur availability may define niche differentiation at cold seeps in *Monterey Bay*, where *Calyptogena kilmeri* inhabits areas of high porewater sulfide

concentrations and *Calyptogena pacifica* beds surround the periphery of the *C. kilmeri* zone where sulfide concentrations are lower (Barry et al. 1997). Increased sulfide consumption rates, sulfide binding ability, and internal sulfide levels, as well as symbiont energy turnover, as measured by sulfide oxidation potential, sulfur metabolism enzymes, and symbiont densities suggest that *C. pacifica*, is physiologically better equipped for the uptake and transport of sulfide (Goffredi and Barry 2002).

The loss of genes encoding the synthesis of dissimilatory nitrate reductase in the *C. pacifica* symbiont *Candidatus Ruthia magnifica* and retention of the pathway in the *C. R. magnifica* symbiont *Candidatus Vesicomysocius okutanii* may reflect differences in host geochemical ecology. The *C. V. okutanii* specimen for the *Candidatus V. okutanii* genome project was collected off Hatsushima Island, in the Sagami Bay seep sites (Kuwahara et al. 2007). The vesicomysid hosts found at Sagami Bay protrude their feet deep within the sediment to access sulfide and may spend the majority of their time in anoxic conditions, where nitrate may be the more abundant oxidant (Masuzawa et al. 1992). As eukaryotic hosts must use oxygen as a terminal electron acceptor, the retention of the nitrate reductase in the seep clam symbionts may reduce competition for oxygen between host and symbiont (Newton et al. 2008).

Thioautotrophic symbionts are the most common type in Bivalvia and are found in bathymodiolin mussels as well as solemyid, thyasirid, lucinid, and vesicomysid clams (Cavanaugh et al. 2006). In laboratory-controlled experiments, reducing the availability of sulfur to the lucinid host induced physiological and morphological changes in the symbiont. The symbiont populations are greatly reduced, their shape and size altered, and the gill of the host was restructured (Caro et al. 2009). These changes can be interpreted as the presumed digestion of the symbiont, a process that is likely normal and ongoing at a lower rate when sulfur is readily available but accelerated when geochemical conditions are not favorable. There are bivalves that seem to be better equipped to handle periods of low sulfur availability. Among them are thyasirid and lucinid clams that can turn to particulate feeding to supplement their diet under sulfur starvation (Dufour and Felbeck 2006). Another adaptation to periods of sulfur starvation on the part of the symbioses is the reacquisition of symbionts after the populations are diminished. After experimentally induced symbiont loss, the reacquisition of symbionts was seen in *Bathymodiolus azoricus* (Kadar et al. 2005). However, it remains unclear whether or not the symbionts were repopulated from the standing intracellular pool of organisms or reacquired from the environment.

#### Methane

Mussels of the *Bathymodiolus* genus are the only known bivalve symbioses to harbor either or both sulfur and

methane-oxidizing symbionts within their cells. These symbioses were first described in the late 1980s from organisms found at cold seeps (Cavanaugh et al. 1987), but subsequently, *Bathymodiolus* mussels have been found to have a wide biogeographic distribution, being found globally in environments such as cold seeps and hydrothermal vents (DeChaine and Cavanaugh 2005). Mussels that harbor methanoautotrophic symbionts exclusively include *Bathymodiolus japonicus*, *Bathymodiolus platifrons* and *Bathymodiolus childressi* (Childress et al. 1986; Fujiwara et al. 2000). In these hosts, the symbionts have been identified through 16S rRNA gene sequence analysis, morphological features typical of methanotrophs (such as stacked intracellular membranes), the incorporation of radiolabeled <sup>14</sup>C methane, and diagnostic enzymes.

The *Bathymodiolus* mussels harbor their symbionts, whether sulfur or methane oxidizing, primarily within epithelial cells in the gill tissue (Fiala-Médioni et al. 2002; Fisher et al. 1993), although these bacteria have been detected in other organs of the host as well (Streams et al. 1997). The location of the symbionts has led to speculation about how these hosts acquire their symbionts; it is hypothesized that the symbionts are endocytosed by the epithelial gill cells from the surrounding seawater (Won et al. 2003). The cytological observations support a lateral transmission strategy for these mussels. Additional evidence comes from the lack of congruence between host and symbiont phylogenies (DeChaine et al. 2005) and the fact that multiple host species can harbor the same symbiont phylotype and diverse phylotypes exist within a single host (Duperron et al. 2006; Won et al. 2003; DeChaine and Cavanaugh 2005).

It has long been observed that environmental concentrations of methane and sulfur predict the presence of the chemosynthetic symbioses (Barry et al. 1997; Goffredi and Barry 2002; Sibuet and Olu 1998); this is not surprising, as these symbioses require access to these reduced chemicals. Interestingly, however, environmental levels of these geochemicals may also control the relative numbers of methanoautotrophic and thioautotrophic symbionts in *B. azoricus* and *Bathymodiolus heckeriae* (Salerno et al. 2005; Riou et al. 2008). The ability of the *Bathymodiolus* mussels to harbor both kinds of symbionts and utilize either reduced substrate as an energy source may be a significant advantage in colonizing vent, seep, and woodfall environments.

#### Electron acceptors and anoxic conditions

In order for the chemosynthetic symbioses to function, the symbionts need access to a terminal electron acceptor. In the process of sulfur or methane oxidation, electrons from these reduced substrates can be donated to oxygen (oxic conditions) or a molecule like nitrate (anoxic conditions). Some

chemosynthetic bivalves (such as *S. reidi* and *Lucinoma aequizonata*) have shown evidence of nitrate respiration when under anoxic and/or oxic conditions (Hentschel and Felbeck 1995; Wilmot and Vetter 1992). The ability to utilize an alternative electron acceptor is a potentially significant trait for these symbioses as the environments in which they reside are often anoxic; indeed, the animals often bridge the oxic/anoxic interface to be able to acquire both reduced substrate and electron acceptor. The host animal, unable to utilize nitrate as a terminal electron acceptor, is likely to consume available oxygen for its own respiration under hypoxic conditions. More evidence for the ability of symbionts to respire nitrate comes from genomic sequencing of the vesicomid clam symbionts. As mentioned earlier, the *C. kilmeri* symbiont, has a functional nitrate reductase while the closely related *C. R. magnifica* symbiont does not (Kuwahara et al. 2007; Newton et al. 2007, 2008). Evidence from comparative genomic analyses suggests that *C. R. magnifica*'s symbiont lost the ability to utilize nitrate—the nitrate respiration genes are now pseudogenes in this organism (Newton et al. 2008). Clearly the pressure to maintain functionality of the nitrate reductase in *C. R. magnifica*'s symbiont could not overcome the effects of genomic reduction and pseudogenization in this lineage. The observation that the primarily vertically transmitted vesicomid symbionts are occasionally laterally transmitted (Stewart et al. 2009a, b) opens up an interesting evolutionary scenario for their hosts—the loss nitrate respiration might be recouped through homologous recombination between divergent symbionts.

#### Conclusions and perspectives

Much remains to be learned about the biology of chemosymbiosis in bivalves. Continued genetic, physiological, and evolutionary explorations promise to further increase our understanding of these remarkable symbiotic associations.

Advances in single-cell methods that combine imaging techniques with metabolic analyses, such as nanometer-scale secondary ion mass spectrometry and Raman microscopy, permit the visualization of uptake and distribution of substrates from the environment as well as measuring the rates at which substrates are acquired (Lechene et al. 2007). Single-cell genomics in combination with methods that allow isolation of specific cells of interest from microscopic regions of tissue/cells/organisms such as laser capture microdissection will be particularly valuable for the characterization of novel chemosynthetic symbiosis and to identify the roles of the different bacteria within hosts harboring multiple symbionts.

The recent introduction of next generation sequencing methods in microbial ecology has offered a powerful alternative to traditional clone library-based and microarray

assessments of microbial diversity. Using barcoded amplicon pyrosequencing, a single run can generate >1 Gb of sequence data, one can increase by orders of magnitude the ability to detect rare but potentially functionally important genetic variants in a given microbial ecosystem. This technology has recently been used to probe the rare genetic diversity in symbiont populations (Stewart and Cavanaugh 2009).

Similarly, advances in next-generation sequencing methods have revolutionized the application of metatranscriptome analyses, which until recently were limited to microarray experiments or analysis of mRNA-derived cDNA clone libraries. High-throughput sequencing of cDNA pools derived directly from microbial communities allows analysis of the (meta) transcription profiles and may provide a molecular framework for interpreting physiochemical measurements of metabolism in chemosynthetic symbionts (Stewart et al. 2011).

Chemosynthetic symbiotic bacteria have never been cultured and their hosts, especially those adapted to extreme deep-sea environments, are difficult to maintain in the laboratory. Nevertheless, despite the advantages of culture-independent detection methods, through the enrichment and isolation of bacterial symbiont species in pure culture, one would be able to fully characterize the physiology of these remarkable organisms. Surprisingly, alternative isolation efforts using, e.g., diffusion chambers with minimal amounts of host tissue or co-occurring microorganisms, or long-term incubation experiments have not yet been documented. It is possible that certain co-adapted intracellular symbionts with highly reduced genomes can never be cultivated in pure culture in vitro. However, novel culturing strategies targeting those chemosynthetic symbionts that show signs of temporal environmental stages deserve adequate investigation.

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