

# Chemosynthetic endosymbioses: adaptations to oxic–anoxic interfaces

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**Chemosynthetic endosymbioses occur ubiquitously at oxic–anoxic interfaces in marine environments. In these mutualisms, bacteria living directly within the cell of a eukaryotic host oxidize reduced chemicals (sulfur or methane), fueling their own energetic and biosynthetic needs, in addition to those of their host. In habitats such as deep-sea hydrothermal vents, chemosynthetic symbioses dominate the biomass, contributing substantially to primary production. Although these symbionts have yet to be cultured, physiological, biochemical and molecular approaches have provided insights into symbiont genetics and metabolism, as well as into symbiont–host interactions, adaptations and ecology. Recent studies of endosymbiont biology are reviewed, with emphasis on a conceptual model of thioautotrophic metabolism and studies linking symbiont physiology with the geochemical environment. We also discuss current and future research directions, focusing on the use of genome analyses to reveal mechanisms that initiate and sustain the symbiont–host interaction.**

## Introduction

Symbioses between bacteria and eukaryotes impact the physiology, ecology and evolution of all organisms. Indeed, there is growing consensus that the mitochondria and chloroplasts of eukaryotes arose from bacteria that became established intracellularly in primitive single-celled organisms 1–2 billion years ago [1]. These endosymbiotic partnerships effectively created ‘new’ organisms that were capable of invading novel metabolic and ecological niches. Similarly, contemporary associations involving intracellular autotrophic Proteobacteria and invertebrates are ubiquitous in marine-reducing environments and are particularly well-characterized in the unique ecosystems structured around deep-sea hydrothermal vents [2].

Unlike all other major ecosystems on Earth, which are driven by photosynthesis, hydrothermal vent ecosystems rely on chemosynthesis, a process by which prokaryotic organisms synthesize C3 compounds (e.g. phosphoglycerate) from C1 compounds (e.g. CO<sub>2</sub>, CH<sub>4</sub>) using chemical energy. In this review, ‘chemosynthetic’ describes microorganisms that: (i) oxidize reduced inorganic compounds (e.g. sulfide) for energy and fix CO<sub>2</sub> for biomass synthesis (i.e. chemoautotrophy), or (ii) use single carbon compounds as both an energy and carbon source (e.g. methanotrophy) [2–4]. At

hydrothermal vents, where reduced chemicals in anoxic vent effluent mix with oxygenated seawater, chemosynthetic bacteria flourish.

Although free-living chemosynthetic prokaryotes serve as the base of the food chain for some vent organisms, reliance on symbiotic chemosynthetic bacteria is the primary nutritional strategy for many vent invertebrates [5]. Both partners are inferred to benefit nutritionally from these symbioses [6,7]. The invertebrate host facilitates access to the substrates (e.g. sulfide or methane, oxygen, CO<sub>2</sub>) that are necessary for chemosynthetic metabolism (Figure 1). In exchange, the bacterial symbionts fix carbon that supports the growth and maintenance of host biomass. Given the reciprocal benefits to the partners of these associations, it is not surprising that chemosynthetic symbioses occur widely in nature. Indeed, following the discovery of symbiont-dominated vent communities, a variety of other environments characterized by the mixing of oxic and anoxic zones (e.g. hydrocarbon cold seeps, coastal sediments, mud volcanoes, whale falls) were shown to support chemosynthetic symbioses. In all of these environments, symbiosis can be viewed as a bacterial adaptation to simultaneously sequester reduced energy substrates (sulfur or methane) and oxygen by living inside a eukaryotic host. Primary production by chemosynthetic symbionts in these habitats provides a direct link between symbiosis and biogeochemical cycling of carbon and energy substrates (e.g. sulfur).

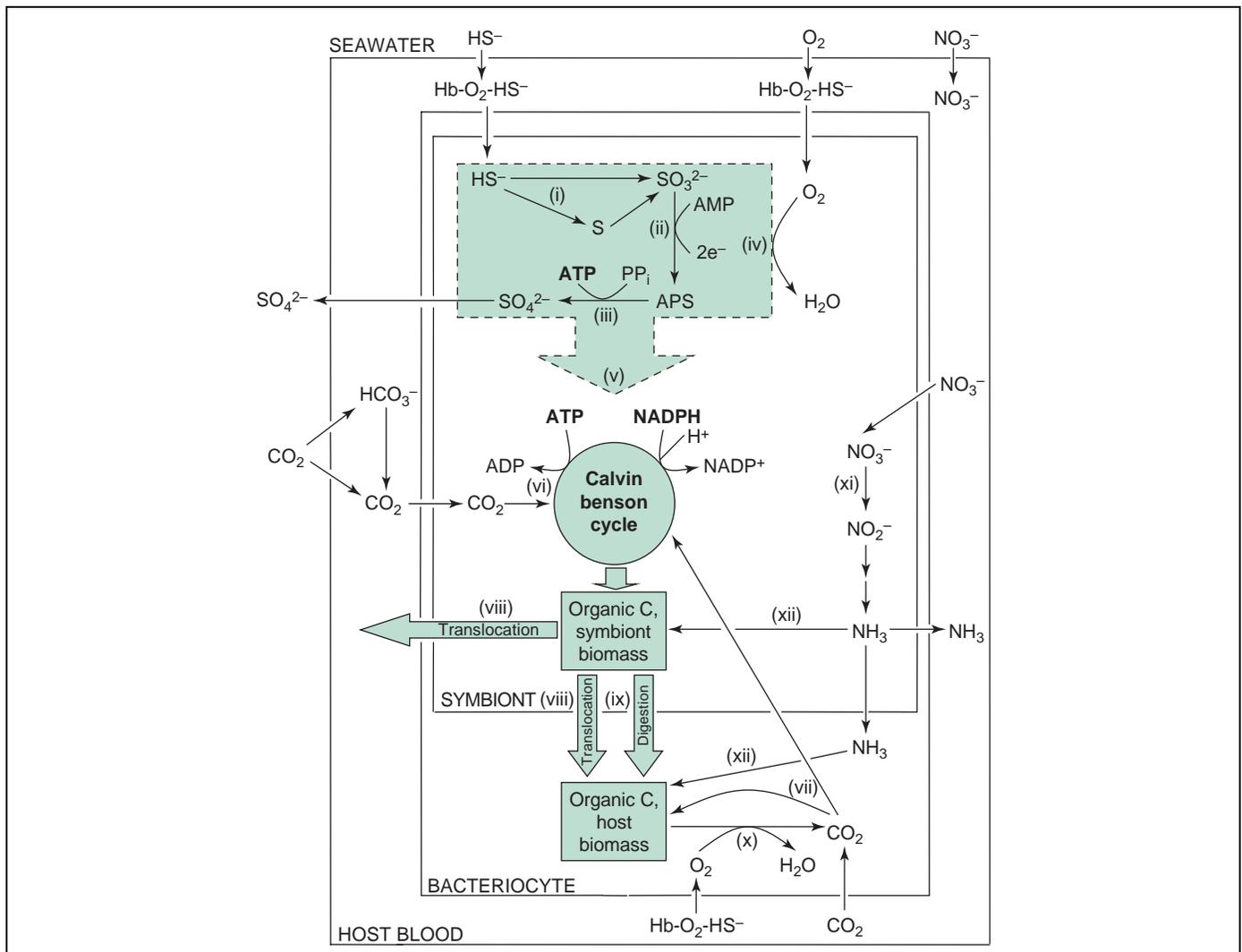
Here we review recent studies of endosymbiotic associations in which chemosynthetic (sulfur- or methane-oxidizing) bacteria live directly within the cells of a eukaryotic host. We focus on endosymbioses found at deep-sea hydrothermal vents and present a conceptual model of thioautotrophic (sulfur-oxidizing) metabolism for the vestimentiferan tubeworm *Riftia pachyptila*, the best studied of all chemosynthetic symbioses (see Ref. [8]). A detailed discussion of episymbiotic associations, which involve bacteria living on the exterior of the host and which constitute an important component of the microbial community in reducing environments, is beyond the scope of this review. However, we refer to these associations to present important contrasts to endosymbioses (for detailed information about episymbioses, see reviews by Polz *et al.* [9] and Ott *et al.* [10,11]).

## Chemosynthetic symbioses

The diversity of symbiont-bearing hosts is striking – chemosynthetic bacteria are known to associate with

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**Figure 1.** Proposed model of metabolism in the symbiosis between *Riftia pachyptila* and a chemosynthetic sulfur-oxidizing bacterium. Reduced sulfur (primarily  $\text{HS}^-$ ) and  $\text{NO}_3^-$  enter the tubeworm blood from the environment through unidentified transport mechanisms.  $\text{CO}_2$  and  $\text{O}_2$  enter by diffusion. In the blood,  $\text{HS}^-$  and  $\text{O}_2$  simultaneously and reversibly bind hemoglobin ( $\text{Hb-O}_2\text{-HS}^-$ ) for transport to the trophosome, where these substrates are used in symbiont sulfide oxidation (dashed box).  $\text{HS}^-$  is oxidized first to elemental sulfur ( $\text{S}^0$ ) or directly to sulfite ( $\text{SO}_3^{2-}$ ) (i).  $\text{SO}_3^{2-}$  oxidation to sulfate ( $\text{SO}_4^{2-}$ ) then proceeds through the APS pathway via the enzymes APS reductase (ii) and ATP sulfurylase (iii), yielding one ATP by substrate level phosphorylation. Electrons liberated during sulfur oxidation pass through an electron transport system, driving oxygen consumption (iv) and the production of ATP and NADPH (v). Fixation of  $\text{CO}_2$  occurs primarily via ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) in the Calvin Benson cycle (vi), using ATP and NADPH generated from sulfur oxidation. Anapleurotic pathways in both host and symbiont (vii) fix lesser amounts of  $\text{CO}_2$ . Transfer of organic matter from symbionts to host occurs via both translocation of simple nutritive compounds (e.g. amino acids) released by the bacteria (viii) and direct digestion of symbiont cells (ix). Host oxygen consumption (x) occurs in typical catabolic and anabolic pathways. Nitrate ( $\text{NO}_3^-$ ), the dominant nitrogen source for the symbiosis, enters via an undescribed transport mechanism and is reduced to nitrite ( $\text{NO}_2^-$ ) by the symbionts via an assimilatory nitrate reductase (xi).  $\text{NO}_2^-$  is reduced via an uncharacterized pathway to yield ammonia ( $\text{NH}_3$ ), which is used for biosynthesis by both symbiont and host (xii). Abbreviations: APS, adenosine 5'-phosphosulfate. Figure modified, with permission, from Ref. [39].

invertebrates from six metazoan phyla, as well as with ciliate protists (Table 1). These organisms host chemosynthetic bacteria either epibiotically on the exterior of the host (e.g. alvinellid tubeworms and rimecarid shrimp) or endosymbiotically within cytoplasmic vacuoles of specialized host cells called bacteriocytes (e.g. vestimentiferan tubeworms, solemyid clams; see Figure 2). Interestingly, two metabolically diverse endosymbionts can co-occur in some invertebrate hosts, such as certain species of *Bathymodiulus* mussel, which support both chemoautotrophs and methanotrophs [12]. Recent work also suggests that at least one organism, the 'scaly snail' found at vents on the Central Indian Ridge, associates simultaneously with both epibiotic and endobiotic bacteria [13]. Epibiotic bacteria of this snail form a dense layer over unique mineralized (iron sulfide) plates covering the foot of the

snail. Snail endobiotic bacteria, in contrast to all characterized chemosynthetic endosymbionts, reside within cells of the host esophageal gland [13]. Although additional molecular and enzymatic analyses are necessary to determine the metabolism of these endobacteria, the reduced digestive system of the snail suggests a reliance on symbiont chemosynthesis for host nutrition. This level of adaptation occurs in most other endosymbiont-bearing invertebrates, in which a reduction or absence of a gut coincides with a complete, or nearly complete, dependence on internal symbionts for food. Similarly, chemosynthetic endosymbionts seem to be highly adapted to life in symbiosis because all attempts to culture these bacteria apart from the host have failed. The inability to culture symbionts is undoubtedly due, in part, to our lack of understanding of the bacteriocyte microenvironment.

**Table 1. List of invertebrate taxa hosting chemosynthetic bacterial symbionts<sup>a</sup>**

Group	Common name	Symbiont-supporting tissue	Location	Habitat	Symbiont type
<b>Protozoa</b>					
Class Ciliata	Ciliate	Host cell surface	Epibiotic	Sold seeps, mangrove swamp	Chemoautotroph
<b>Porifera</b>					
Class Demospongiae					
Family Cladorhizidae	Sponge	Skeletal matrix	Extracellular	Cold seeps	Methanotroph
<b>Nemata</b>					
Subfamily Stilbonematinae	Nematode	Cuticle	Epibiotic	Reducing sediments	Chemoautotroph
<b>Mollusca</b>					
Class Bivalvia					
Subclass Protobranchia, Family Solemyidae	Clam	Gills	Intracellular	Reducing sediments, hydrothermal vents, cold seeps	Chemoautotroph
Subclass Heterodonta					
Family Lucinidae	Clam	Gills	Intracellular	Reducing sediments, cold seeps	Chemoautotroph
Family Thyasiridae		Gills	Intracellular	Reducing sediments, cold seeps	Chemoautotroph
Family Vesicomysidae		Gills	Intracellular	Hydrothermal vents, cold seeps	Chemoautotroph
Subclass Pteriomorphia					
Family Mytilidae	Mussel	Gills	Extracellular, intracellular	Hydrothermal vents, cold seeps	Chemoautotroph and/or methanotroph
Class Gastropoda					
Family Provannidae	Snail	Gills	Intracellular	Hydrothermal vents	Chemoautotroph
Family Lepetodrilidae	Limpet	Gills	Epibiotic	Hydrothermal vents	Chemoautotroph
<b>Annelida</b>					
Class Polychaeta					
Family Alvinellidae	Worm	Dorsal surface	Epibiotic	Hydrothermal vents	Chemoautotroph
Family Siboglinidae (Vestimentifera, Pogonophora)	Tubeworm	Trophosome	Intracellular	Hydrothermal vents, cold seeps, reducing sediments, fjords	Chemoautotroph
Class Clitellata					
Subfamily Phallo-drilinae	Oligochaete	Subcuticular	Extracellular	Coralline sands	Chemoautotroph
<b>Arthropoda</b>					
Class Crustacea					
Family Alvinocarididae	Shrimp	Exoskeleton	Epibiotic	Hydrothermal vents	Chemoautotroph
<b>Echinodermata</b>					
Class Echinoidea	Sea urchin	Gut	Extracellular	Reducing sediments	Chemoautotroph

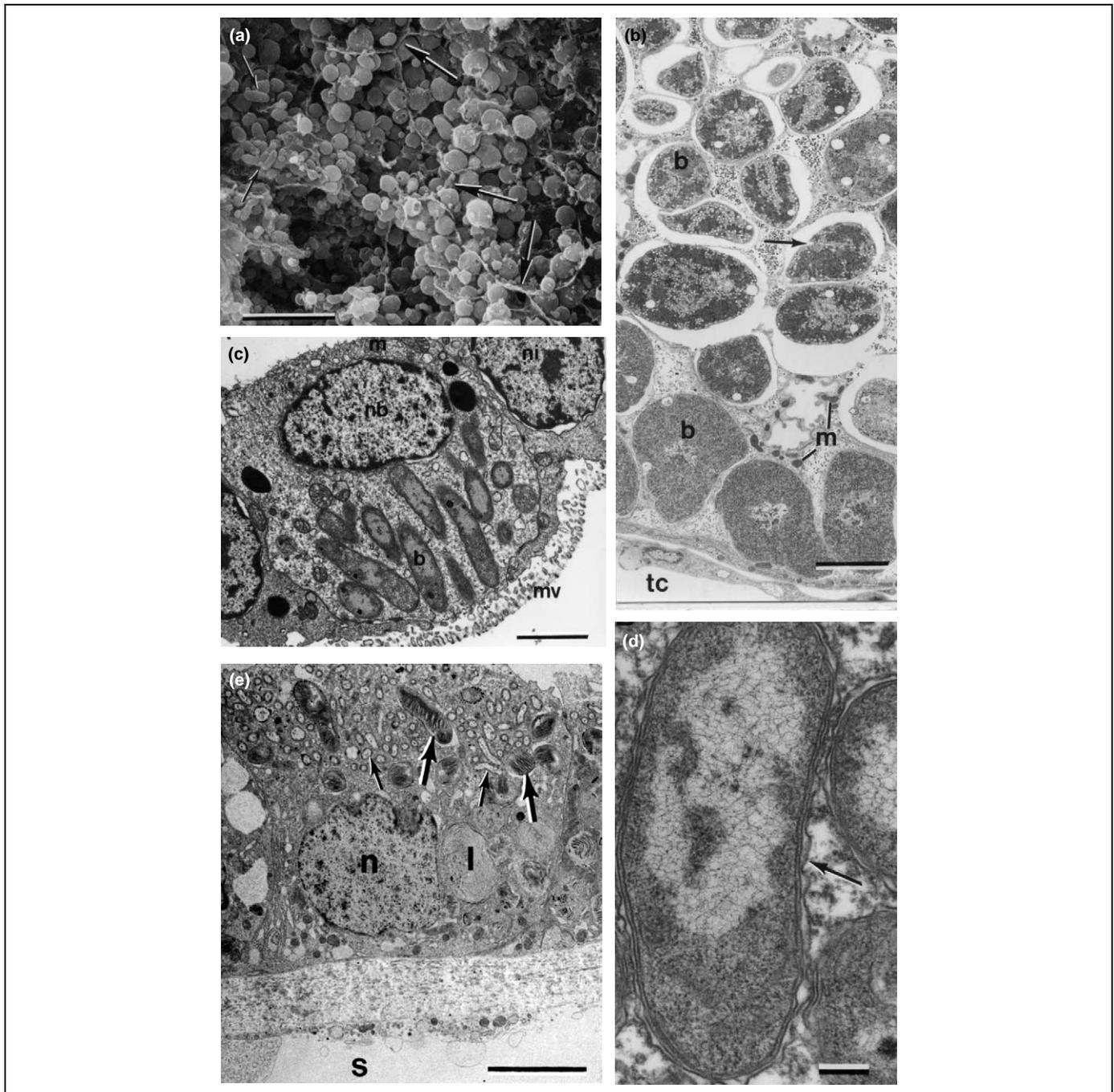
<sup>a</sup>Chemosynthetic status of symbionts inferred from ultrastructural, physiological, enzymatic, and molecular data. Table modified from and references listed in Ref. [6].

In contrast to the wide diversity of host taxa, chemosynthetic symbionts exhibit a relatively narrow phylogenetic range. Based on 16S rRNA sequences, epibiotic bacteria fall primarily within the  $\epsilon$  Proteobacteria, and the majority of endosymbiotic bacteria cluster within the  $\gamma$  Proteobacteria [5,14] (Figure 3). However, recent studies have presented interesting exceptions to this trend. For example, on the basis of 16S rRNA sequence, endosymbionts of an unnamed species of *Alviniconcha* vent gastropod cluster phylogenetically with free-living sulfur-oxidizing  $\epsilon$  Proteobacteria [15].  $\epsilon$  endosymbionts have also been reported in vestimentiferan tubeworms of the genus *Lamellibrachia* [16]. In subsequent studies, however, these authors reported the co-occurrence of  $\alpha$ ,  $\beta$  and  $\gamma$  Proteobacteria within *Lamellibrachia* sp. [17,18], suggesting that the bacteria-tubeworm relationship might not be stable over time. Several studies also indicate that multiple symbiont phylotypes co-occur within gutless oligochaetes of the genera *Inanidrilus* and *Olavius*, with one species, *O. crassitunicatus*, hosting up to six bacterial phylotypes in the extracellular space below the cuticle of the worm [19]. These bacteria include a spirochete and representatives from both the  $\gamma$  and  $\delta$  Proteobacteria. In

a previous study of a related host species, *O. algarvensis*,  $\gamma$  and  $\delta$  Proteobacterial symbionts were characterized as sulfide-oxidizing and sulfate-reducing bacteria, respectively, suggesting the potential for an internal sulfur cycle within the worm [20]. Similar work is needed to determine whether chemosynthetic metabolism underlies bacteria-bacteria and bacteria-host interactions in *O. crassitunicatus*, as well as in lamellibrachian tubeworms. Clearly, however, these associations do not fit the 'one host, one symbiont' model that applies to many chemosynthetic endosymbioses.

### Physiochemistry

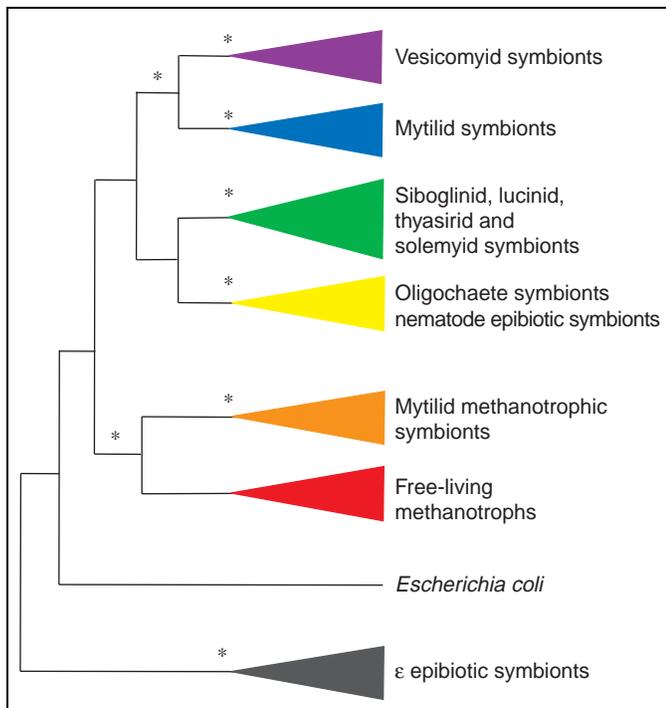
Chemosynthetic endosymbioses are inextricably linked to the physical and chemical environment. These associations predominate along chemoclines separating oxygenated zones from waters rich in CO<sub>2</sub> and reduced chemicals (e.g. sulfide, methane, ferrous iron). In non-hydrothermal environments, such as coastal marine sediments and hydrocarbon seeps, the majority of the sulfide used by thioautotrophic endosymbionts originates from microbial sulfate reduction in which SO<sub>4</sub><sup>2-</sup> serves as an electron acceptor in the anaerobic oxidation of organic



**Figure 2.** Chemosynthetic endosymbioses: scanning and transmission electron micrographs of representative symbioses. **(a,b)** *Riftia pachyptila* (vent vestimentiferan tubeworm) intracellular chemoautotrophs. **(a)** Symbionts within trophosome; symbionts occur as both spherical and rod-shaped cells (small arrows); large arrows indicate likely host cell membranes; Scale bar = 10  $\mu\text{m}$ . **(b)** Cross-section of portion of trophosome lobule, showing variable fine structure of symbionts; all symbionts are contained within membrane-bound vacuoles, either singly or in groups of two or more. Abbreviations: arrow = dividing bacterium; b = bacteria; m = mitochondria; tc = trunk coelomic cavity; Scale bar = 3  $\mu\text{m}$ . Figure reprinted, with permission, from Ref. [78]. **(c,d)** *Solemya velum* (coastal bivalve), intracellular chemoautotrophs. **(c)** Transverse section of gill filament showing rod-shaped bacteria (b) within gill bacteriocyte and intercalary cells lacking symbionts. Abbreviations: mv = microvilli; nb = nucleus of bacteriocyte; ni = nucleus of intercalary cell; Scale bar = 3  $\mu\text{m}$ . **(d)** Higher magnification of symbionts showing cell ultrastructure typical of Gram-negative bacteria and peribacterial membrane (arrow); Scale bar = 0.2  $\mu\text{m}$ . Figure reprinted, with permission, from Ref. [78]. **(e)** *Bathymodiolus puteoserpentis* (vent bathymodioline mussel) dual symbiosis. TEM showing symbiotic methanotrophs and chemoautotrophs (large and small arrows respectively), located in the apical region of bacteriocytes; nuclei (n) and lysosomal residual bodies (l) occupy the region closest to the blood sinus (s). Note centrally stacked intracytoplasmic membranes in methanotrophs; Scale bar = 5  $\mu\text{m}$ . Figure reprinted, with permission, from Ref. [79].

matter [21,22]. At these sites methane originates from either microbial methanogenesis or exposure of buried organic matter to high temperature and pressure over time (thermogenic methane [23]). By contrast, at hydrothermal vents sulfide results from the reduction of seawater sulfate during exposure to hot rock below the ocean floor and the reaction of sulfur-containing basalt with hot vent

fluids [24]. Methane at vents derives primarily from seawater–rock interactions or from degassing of magma [2]. In non-hydrothermal environments and at vents, increasing gradients of sulfide and methane generally co-occur with a decreasing gradient of oxygen. Symbioses thrive at these oxic–anoxic interfaces, accessing both the reduced chemicals and the oxygen required for chemosynthesis.



**Figure 3.** Schematic phylogenetic tree depicting the major clades of chemosynthetic endosymbionts within the  $\gamma$  Proteobacteria (unless otherwise indicated, sequences are from chemoautotrophic symbionts). This schematic is based on trees generated using a maximum parsimony algorithm on full 16S rRNA sequences. The association of these bacteria with at least six metazoan phyla suggests that chemosynthetic symbioses have evolved more than once. Episymbionts that fall within the  $\epsilon$  Proteobacteria, such as those of rimirid shrimp and alvinellid polychaetes, are included (see Table 1 for descriptions of host organisms). Clades marked by an asterisk (\*) are consistently recovered in analyses (maximum parsimony bootstrap values  $>70\%$ ). Although the host-symbiont interaction is generally species-specific, the siboglinid tubeworms share the same (or a similar) symbiont, and the lucinid clams can be co-infected by the same symbiont [72,73,80]. Certain symbionts of solemyid, thyasirid and lucinid clam species do not show consistent placement within phylogenetic trees. Schematic based, with permission, on analyses by Z.P. McKiness (PhD Thesis, Harvard University, 2004), McKiness *et al.*, unpublished data, and Ref. [5].

Such gradients are dramatic in hydrothermal vent environments, particularly those associated with 'black smokers' where hot (up to  $400^{\circ}\text{C}$ ), acidic (pH 3–6) vent effluent laden with sulfide ( $3\text{--}12\text{ mmol kg}^{-1}$ ) and methane ( $25\text{--}100\text{ }\mu\text{mol kg}^{-1}$ ) exits the seafloor and mixes with ambient bottom water ( $\sim 1.8^{\circ}\text{C}$ , pH  $\sim 8$ ) [25]. Whereas some episymbiont-hosting invertebrates (e.g. alvinellid tubeworms) withstand exposure to the high temperatures at the immediate periphery of black smokers, most endosymbiont-hosting organisms cluster around more diffuse or low flow vents, with characteristically milder temperatures ( $1.8\text{--}40^{\circ}\text{C}$ ) and pH ( $\sim 6$ ) [2]. Variation in the physiochemical characteristics of these vent waters critically affects the concentrations of energy and carbon substrates available for chemosynthesis. For example, low pH ensures that dissolved inorganic carbon (DIC) at vents is primarily in the form of  $\text{CO}_2$ , the DIC species that diffuses freely across biological membranes and is fixed by thioautotrophic symbionts via the Calvin cycle [26,27]. Similarly, studies have shown that acidity and temperature interact to determine both sulfide speciation and sulfide-metal (e.g. iron) interactions, thereby influencing the availability of free sulfide for thioautotrophy [28,29]. Vent researchers are challenged to explain how

physiochemical conditions (e.g. temperature, pH), which can vary substantially over short temporal (seconds) and spatial (centimeters) gradients, impact the physiology and ecology of symbioses. Doing so requires applying specialized sampling technology (e.g. voltametric probes and submersible chemical analyzers) to studies that aim to clarify the link between environmental substrate (e.g. sulfur) concentrations, symbiont metabolism and symbiont-host interactions.

### Metabolism – substrate acquisition

Current understanding of endosymbiont metabolism is based largely on studies of organisms from sulfide-rich deep-sea hydrothermal vents. Although the potential remains that some of these associations might use non-sulfur compounds (e.g. hydrogen) for energy, it is generally inferred that most vent endosymbionts fix carbon dioxide into organic matter using energy from the oxidation of reduced sulfur compounds (thioautotrophy). A simultaneous requirement for both sulfide and oxygen poses a unique challenge to thioautotrophs given that these substrates occur in distinct zones and that sulfide reacts abiotically with oxygen to form less reduced sulfur species (e.g. thiosulfate, sulfite, sulfate [30]), thereby limiting the availability of sulfide for biotic oxidation. To overcome these problems, host organisms have evolved specialized behavioral, morphological or biochemical mechanisms to provide their symbionts access to both sulfide and oxygen. Such mechanisms often involve physically spanning the oxic-anoxic interface, either by positioning distinct body parts in oxygenated and reduced zones (e.g. cold seep vestimentiferan tubeworms of the genus *Lamellibrachia* [31]) or by building burrows (e.g. bivalves [5]) or following migratory routes (e.g. shrimp and nematodes hosting episymbionts [32]) that bridge the interface. However, perhaps the best studied of these adaptations is the multi-hemoglobin system of the vent tubeworm *Riftia pachyptila*.

*R. pachyptila* synthesizes three extracellular hemoglobins (Hb), two in the blood and one in the coelomic fluid, which reversibly bind sulfide and oxygen with high affinity at independent sites on the protein (see [33,34]). It has been proposed that sulfide binds to these Hbs at two free cysteine residues, each located on a distinct globin type [35]. A lack of free cysteine residues in Hbs from sulfide-free environments suggests that the capacity for sulfide binding was lost during evolution as annelids that originated in anoxic sulfide-rich habitats radiated out to colonize sulfide-free environments [33]. Recently, however, Flores *et al.* [36] determined the crystal structure of *R. pachyptila* coelomic Hb, showing that free cysteine residues are located beneath the surface of the molecule and that sulfide binding might instead involve 12  $\text{Zn}^{2+}$  ions ligated to histidine and glutamate residues. Ion chelation experiments further suggested a role for  $\text{Zn}^{2+}$  in sulfide sequestration [36]. Interestingly, a similar mechanism might operate in vesicomimid clams of the genus *Calypptogena*, in which sulfide uptake and transport to symbionts are mediated via  $\text{Zn}^{2+}$ -based, non-heme sulfide-binding proteins [37,38]. If sulfide binding is similarly mediated via  $\text{Zn}^{2+}$  ions in *R. pachyptila*, it

would be of interest to determine whether this mechanism is present in annelid worms from low-sulfide regions.

### Metabolism – the *Riftia pachyptila* symbiont model

Figure 1 presents an overview of symbiotic thioautotrophy within the vent tubeworm *Riftia pachyptila*, the best studied of all chemosynthetic symbioses. The *R. pachyptila* model highlights key aspects of symbiont metabolism and symbiont–host interaction and provides a basis for comparison to future models of other endosymbioses, many of which are not yet well-characterized. Enzymatic data suggests that *Riftia* endosymbionts mediate sulfur oxidation in part via the adenosine 5'-phosphosulfate (APS) pathway, an energy-conserving mechanism that yields ATP via the oxidation of sulfite to sulfate by the enzymes APS reductase and ATP sulfurylase [5,39]. However, both enzymes also function in the reverse direction during sulfate reduction. Therefore, detection of the genes coding for these proteins does not provide conclusive evidence of thioautotrophy. However, in *R. pachyptila* biochemical (protein structure) and enzymatic evidence strongly suggests that these enzymes are used by the symbionts for sulfur oxidation [40,41].

The electrons released in sulfur oxidation, either via the APS pathway or an unidentified mechanism, are putatively channeled through a membrane-bound transport system, yielding the proton gradient that drives ATP production (Figure 1). Ultimately, the ATP produced during electron transport fuels CO<sub>2</sub> fixation via the Calvin cycle. Indeed, repeated detection of the CO<sub>2</sub>-fixing enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) in symbiont-containing tissue from diverse hosts attests to the ubiquity of the Calvin cycle in chemosynthetic endosymbionts. A fraction of the organic material generated in this cycle is eventually transferred to the host via either digestion of symbiont cells or excretion of soluble organic compounds by the bacteria [7,42] (Figure 1). In the *R. pachyptila* symbiosis, symbiont autotrophy appears to support 100% of host nutrition.

### Metabolism – extending the model

A primary challenge in symbiosis research is to determine how this basic model of symbiont metabolism and physiology (Figure 1) varies among diverse host–symbiont relationships. This model clearly does not apply to episympiotic interactions. Indeed, for some episympioses the benefit of symbiosis has yet to be defined clearly and might not be nutritional. For example, the episympionts of the newly-discovered scaly snail [13] quite possibly contribute little to the sustenance of the host. Rather, these filamentous bacteria, which morphologically resemble those of the vent polychaete *Alvinella pompejana* and the vent shrimp *Rimicaris exoculata*, are hypothesized to contribute to the mineralization of the unique iron sulfide plates that cover the foot of the snail [13]. Also, there is evidence that some episympiotic bacteria, such as those of *A. pompejana*, rely not on the Calvin cycle for carbon fixation but on the reverse tricarboxylic acid cycle [43]. Similarly, the *Riftia* model must be broadened to encompass methane-oxidizing endosymbionts (e.g. methanotrophic endosymbionts of certain *Bathymodiolus* mussels),

which not only use a distinct energy source (methane) but also fix carbon via the ribulose monophosphate pathway [44]. Although beyond the scope of this review, an in-depth understanding of the metabolic interactions driving episympiotic and methanotrophic symbioses should be a primary goal of chemosynthetic symbiosis research.

The *R. pachyptila* model also quite possibly varies among thioautotrophic endosymbionts of distinct hosts and in response to environmental conditions, particularly substrate (e.g. sulfide, methane, oxygen) concentrations. For example, several studies have focused on the use of alternate electron acceptors by thioautotrophic endosymbionts. Although most symbionts use molecular oxygen as the terminal electron acceptor during sulfur oxidation, symbionts of some host species, such as the tropical lucinid clam *Lucinoma aequizonata*, might use elemental sulfur or nitrate as a terminal oxidant during periods of anoxia [45,46]. Recently, using measurements of nitrate reductase activity and oxygen respiration, Duplessis *et al.* [47] demonstrated that the symbiont of the lucinid clam *Codakia orbicularis*, despite its high phylogenetic relatedness to the *L. aequizonata* symbiont, does not respire nitrate. Therefore, the capacity for nitrate respiration might vary, not with phylogeny but in response to environmental differences, such as varying abundances of oxygen and nitrate in clam-hosting sediments.

These types of physiological analyses will greatly complement studies that link symbiont metabolism with biogeochemical cycles and free-living microbial populations. Promising work on this front has been conducted in a study modeling the interaction between environmental sulfate reduction and sulfide oxidation by symbionts of the tubeworm *Lamellibrachia luymesii* [48]. Like *R. pachyptila*, *L. luymesii* lacks a digestive system and instead acquires energy from sulfur-oxidizing bacteria within its symbiont-containing organ (trophosome). However, in contrast to *R. pachyptila*, which occurs at hydrothermal vents, *L. luymesii* inhabits cold seeps where non-hydrothermal fluids rich in sulfide, methane and hydrocarbons bubble up through buried sediment. In these habitats, *L. luymesii* acquires sulfide not through an anterior gill-like organ, as in *R. pachyptila*, but by extending the posterior ('root') segment of its body through the sediment to access interstitial pools of sulfide [31]. Using extensive data from prior studies, including growth and mortality estimates [49] and detailed characterizations of cold seep biogeochemistry (e.g. sediment sulfide and sulfate concentrations; [50,51]), Cordes *et al.* [48] developed a tubeworm growth and sulfide-uptake model demonstrating that the persistence of tubeworm aggregations depends on the release of sulfate generated by the symbiont population of the worm. In this model, sulfate, the end-product of symbiont thioautotrophy, is released into the sediment via the 'root' of the tubeworm, providing an energetically favorable oxidant for free-living bacteria or archaea that oxidize methane or hydrocarbons present in the sediment. Sulfate reduction by these sediment microorganisms regenerates sulfide, which is taken up by the worm to fuel symbiont metabolism. In this model, the cycling of sulfur between the tubeworms and the sediment microbial consortia augments the supply of exogenous

sulfide, enabling tubeworm aggregations to persist at *in situ* population densities for over 250 years [48]. Although fully testing this model will require additional measurement (e.g. of tubeworm sulfate release), this study provides a valuable example of how to integrate physiological and environmental data for a holistic understanding of the link between endosymbioses and the surrounding ecosystem.

Attempts to link substrate (e.g. sulfide, methane) concentration with the physiology of a symbiosis have also proven informative for studies of endosymbiont-bearing mussels of the genus *Bathymodiolus*. For example, comparisons of symbiont populations in the vent mussel *B. thermophilus* from sites of low and high sulfide flux and following transplant experiments showed a loss of symbionts upon removal from a sulfide source, suggesting enhanced rates of symbiont degradation or host digestion, or a reduced rate of symbiont cell division [52]. Similarly, for *Bathymodiolus* mussels hosting dual symbionts, the ratio of methane to sulfide concentration appears to impact directly the relative abundances of methanotrophic and thioautotrophic endosymbionts. Salerno *et al.* [53], using transmission electron microscopy, examined symbiont ultrastructure across host developmental stages in dual symbiont-hosting mussels, revealing a dominance of thioautotrophs in mussels (*B. azoricus*) from a sulfide-rich vent site and a dominance of methanotrophs in mussels (*B. heckeriae*) from a methane-hydrate cold seep. Interestingly, the relative abundances of these two symbiont types seem not to vary across a range of methane concentrations (50-fold) in a recently discovered *Bathymodiolus* species from a seep in the southeast Atlantic [12]. Additional quantitative analyses will be necessary to accurately describe symbiont population dynamics and metabolism in the context of substrate (sulfide, methane, oxygen) chemistry.

Similar analyses should ultimately be applied to newly-discovered habitats supporting chemosynthetic endosymbioses. An interesting example is the Lost City, a mid-water (750–900 m) hydrothermal vent field located ~15 km off the spreading axis of the Mid-Atlantic Ridge. Unlike on-axis vent sites dominated by sulfide-rich basalt and black smokers, the Lost City vent site is characterized by carbonate deposits and cool (~100°C), alkaline (pH 10–11) effluent depleted in CO<sub>2</sub> and sulfide and enriched in H<sub>2</sub> and methane [54,55]. Although large endosymbiosis-dominated assemblages typical of hydrocarbon seeps and on-axis hydrothermal vents are notably absent from the Lost City, at least one endosymbiont-hosting species of *Bathymodiolus* mussel has been described from this site (E.G. DeChaine *et al.*, unpublished data). Preliminary investigations suggest that this mussel hosts both methanotrophic and thioautotrophic symbionts. This result is curious given the relative absence of sulfide available for thioautotrophy. Further analysis across a range of habitat conditions will help determine how the relationship between symbiont metabolism and physiochemical environment impacts the biogeographical distribution of this endosymbiosis.

## Symbiont genomics and future directions

Developing and testing models of endosymbiont metabolism (e.g. Figure 1) will benefit substantially from genetic characterization, which to date has been applied to only a small number of symbiont genes. For example, molecular analyses have been useful in describing mechanisms of nitrogen acquisition and metabolism by *Riftia pachyptila*, in which assimilatory nitrate reduction, ammonia assimilation and pyrimidine and polyamine biosynthesis seem to be dependent on the symbiont population [56,57]. Similar characterization has focused on symbiont carbon fixation enzymes, particularly the CO<sub>2</sub>-fixing enzyme RubisCO. Despite being used routinely to diagnose autotrophy, RubisCO has been described both molecularly and kinetically for only three symbionts, those of *R. pachyptila* [58], the vent gastropod *Alviniconcha hessleri* [59] and the protobranch clam *Solemya velum* [60,61]. Surprisingly, it was discovered that the symbionts encode different forms of the RubisCO enzyme, a finding that impacts interpretations of the ecology and physiology of the symbioses, as well as the stable carbon isotope ratios ( $\delta^{13}\text{C}$ ; Box 1). Recent work on *S. velum* has both characterized the structure of the RubisCO gene cluster and measured RubisCO kinetics in the context of environmental substrate (CO<sub>2</sub>) concentrations and host stable isotope ratios [60,61] (see Box 1). Such studies advance our

### Box 1. Form I and II Rubisco and $\delta^{13}\text{C}$ values in chemosynthetic symbioses

Stable carbon isotope compositions ( $\delta^{13}\text{C}$  values) have been used extensively to identify chemoautotrophy in marine endosymbioses. Whereas the  $\delta^{13}\text{C}$  of marine phytoplankton varies between –18‰ and –28‰,  $\delta^{13}\text{C}$  values of sulfur-oxidizing chemosynthetic symbioses typically cluster into two primary groups: a ‘light’ group (depleted in  $^{13}\text{C}$  relative to  $^{12}\text{C}$ ) consisting of bivalves with  $\delta^{13}\text{C}$  between –27‰ and –35‰ and a ‘heavy’ ( $^{13}\text{C}$ -enriched) group including shrimp epibionts and vestimentiferan tubeworms with  $\delta^{13}\text{C}$  values between –9‰ and –16‰. These isotopic differences relate directly to the form of ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) used to fix CO<sub>2</sub> via the Calvin cycle. Although two structurally distinct forms of RubisCO are known to occur in autotrophs, with form I consisting of four subtypes (A–D) [63], most members of the isotopically light bivalve group, including symbionts of deep-sea bathymodioline mussels and coastal solemyid clams, possess a Form IA RubisCO. By contrast, Form II occurs in members of the  $^{13}\text{C}$ -enriched group, such as the vestimentiferan tubeworm symbionts. Recent work has helped to characterize the kinetic properties underlying isotopic variation in two endosymbioses. Robinson *et al.* [64] showed that the kinetic isotope effect ( $\epsilon$  value), which reflects the discrimination against  $^{13}\text{C}$  by the purified enzyme, is significantly lower for form II RubisCO from symbionts of the vent tubeworm *Riftia pachyptila* ( $\epsilon = 19.5\%$ ) than for the form I enzyme of phototrophs ( $\epsilon = 22\text{--}30\%$ ). Similarly, the  $\epsilon$  value of form IA RubisCO from symbionts of the coastal clam *Solemya velum* has been determined ( $\epsilon = 24.5\%$ ) and, when the  $\delta^{13}\text{C}$  of the source dissolved inorganic carbon (DIC) pool is taken into account, helps to explain the placement of this symbiosis ( $\delta^{13}\text{C}$  values of –30‰ to –35‰) within the isotopically light bivalve group [61]. Clearly, additional enzymatic analysis is necessary to adequately describe isotopic discrimination by CO<sub>2</sub>-fixing enzymes among diverse symbioses, including those involving methanotrophic bacteria that fix CO<sub>2</sub> via the ribulose monophosphate pathway. Such analyses, along with monitoring of other factors that contribute to isotopic signature (e.g. the  $\delta^{13}\text{C}$  of source carbon), will aid the interpretation of  $\delta^{13}\text{C}$  values and the study of carbon cycling in symbiosis research.

### Box 2. Symbiont transmission strategy

Symbiont transmission between successive host generations can occur environmentally through a free-living population of symbiotic bacteria (e.g. rhizobia acquired by plant roots from the soil), horizontally between contemporary organisms sharing the same habitat (e.g. termite gut microflora passed via coprophagy), or vertically from parent to offspring (e.g. aphid symbionts passed from mother to embryo). Transmission strategy is generally inferred via PCR-based or visual (e.g. fluorescent *in situ* hybridization) detection of bacteria in host reproductive tissues or gametes, or via comparisons of symbiont and host phylogenies. For instance, using symbiont-specific primers for the CO<sub>2</sub> fixation enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase, Krueger *et al.* [65] detected symbionts in the ovaries of the coastal clam *Solemya velum*, suggesting that symbionts are transmitted during fertilization. Similarly, direct detection of symbionts via *in situ* hybridization with a symbiont-specific 16S rRNA probe suggests vertical transmission in the deep-sea vesicomid clam *Calyptogena magnifica* [66]. Such maternally transmitted symbionts, if involved in an association that is stable over evolutionary time, are anticipated to cospeciate with their hosts [62,67]. Topological congruence between symbiont and host phylogenies can therefore be measured to support a hypothesis of maternal transmission, as has been demonstrated in insect symbioses [68,69]. Interestingly, the vesicomid clams are the only chemosynthetic symbioses for which host-symbiont phylogenetic congruence has been demonstrated [70,71].

By contrast, environmental transmission is generally inferred from a lack of PCR-based detection in reproductive tissue, incongruence of symbiont–host phylogenies, or direct detection of a symbiont in the environment. For instance, vestimentiferan tubeworms of the genera *Riftia*, *Oasisia* and *Tevnia* share a single, or highly similar, symbiont 16S rRNA phylotype (<0.5% 16S rRNA sequence divergence [72,73]). Indeed, preliminary evidence using both PCR and *in situ* hybridization suggests that the free-living form of the tubeworm symbiont is ubiquitous in the vent environment (T.L. Harmer *et al.*, unpublished data). Similarly broad symbiont–host specificity occurs among certain species of tropical lucinid clams, for which the bacterial symbiont has not only been detected in the environment but has also been shown experimentally to co-infect multiple host species [74,75]. Although originally inferred to undergo vertical transmission, symbionts of bathymodiolid mussels might also pass through a free-living stage. Recent evidence based on population genetic and ultrastructural data for *Bathymodiolus azoricus*, a Mid-Atlantic Ridge mussel hosting both sulfur-oxidizing (thioautotrophic) and methanotrophic bacteria, indicates environmental acquisition of the thioautotroph [76] (E.G. DeChaine *et al.*, unpublished data). A primary challenge will be to assess how these variations in transmission strategy impact symbiont genome evolution.

understanding of the evolutionary relationships among symbiont functional genes, as well as the link between symbiont autotrophy and host nutrition.

Such molecular analyses must be extended to address other components of symbiont metabolism and physiology. For example, although we might have a basic understanding of energy and carbon metabolism in a few well-described symbioses (e.g. Figure 1), we have yet to fully explore the use of alternate energy sources (e.g. hydrogen oxidation) by these associations. Further, we know little of how endosymbionts and their hosts acquire, synthesize and process all of the other micronutrients (e.g. phosphorus, iron) and biomolecules that sustain the symbiosis. Exploring these topics has been hindered in part by the inability to culture endosymbionts and by the dearth of genomic data available for these species. Although laboratory culture still represents a formidable challenge in symbiosis research, projects are now underway to

### Box 3. Questions driving ongoing and future research

- To what extent is symbiont metabolism coupled to the cycling of sulfur, methane and carbon in reducing environments? How do these processes vary in response to physiochemical environmental conditions?
- What are the potential metabolic and physiological capabilities of chemosynthetic symbionts? How do these vary among diverse hosts? Is symbiont and host metabolism interdependent, as has been demonstrated for amino-acid metabolism in heterotrophic aphid endosymbionts [77]?
- Are chemosynthetic symbionts obligate autotrophs, in other words, do they lack genes encoding key enzymes in metabolic pathways necessary for utilizing exogenous organic carbon?
- How does symbiont transmission strategy affect the rate of DNA sequence evolution and the size and structure of the genome in symbiont populations?
- What do studies of extant symbioses tell us about the evolution from a free-living bacterium to a eukaryotic organelle?

sequence the genomes of the sulfur-oxidizing endosymbionts of the vent invertebrates *R. pachyptila* and *Calyptogena magnifica* and the coastal clams *S. velum* and *Codakia orbicularis*. Annotation of these genomes will reveal the molecular potential of key symbiont processes, including mechanisms by which the symbiont enters the host cell, processes chemical signals and coordinates cell division within the bacteriocyte, and metabolizes energy and carbon to maintain bacterial and host biomass. It will be interesting to examine these mechanisms in relation to the mode by which symbionts are transmitted between successive host generations (Box 2), especially given the strong impact that transmission strategy has been shown to have on genome evolution in non-chemosynthetic endosymbioses of insects [62]. Finally, genomic analyses that measure the expression of symbiont metabolism genes will be particularly useful in clarifying the link between chemosynthesis and biogeochemical cycling at oxic–anoxic interfaces (Box 3).

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