Symbiosis of Thioautotrophic Bacteria with *Riftia pachyptila*

Frank J. Stewart, Colleen M. Cavanaugh

1 Introduction

The symbiosis between the giant vestimentiferan tubeworm *Riftia pachyptila* and an intracellular sulfur-oxidizing bacterium still fascinates researchers over 20 years after its discovery. This association, the first between a marine invertebrate and a chemoautotroph to be described, remains the best studied of the symbioses found at sulfide-rich hydrothermal vents. In the decade following the initial description of this symbiosis in 1981 (Cavanaugh et al. 1981; Felbeck 1981), many important studies have helped to characterize the physiological, biochemical, and anatomical adaptations that sustain this association (for other reviews see Fisher 1995; Nelson and Fisher 1995; van Dover 2000; Minic and Herve 2004; van Dover and Lutz 2004; Cavanaugh et al. 2005). Stable carbon isotope data and the absence of a mouth and gut strongly suggest that the adult *R. pachyptila* relies entirely on its bacterial symbionts for nutrition (see Fisher 1995; Nelson and Fisher 1995). These bacteria, which belong to the gamma subdivision of the Proteobacteria (Distel et al. 1988), oxidize reduced inorganic sulfur compounds to obtain energy and reducing power for autotrophic carbon fixation. Given their ability to synthesize C_3 compounds from a C_1 compound using chemical energy, *Riftia* symbionts are referred to as "chemosynthetic" (Cavanaugh et al. 2005).

Over the past 15 years, increasingly sophisticated experimental techniques (e.g., pressure chambers, vascular catheters) and new molecular technologies have dramatically increased our understanding of chemosynthetic symbioses. Specifically, for the *R. pachyptila* symbiosis, researchers provided new insights into the processes by which metabolites (e.g., carbon, sulfide, nitrogen) and waste products (e.g., protons) cycle among host, symbiont, and environment and identified some of the genes and corresponding enzymes involved in both host and symbiont metabolism. In addition, questions of host-

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symbiont co-evolution and symbiont transmission were addressed in a number of studies, including recent work that successfully detected the free-living tubeworm protosymbiont using 16S rRNA probes for in situ hybridization (Harmer et al. 2005).

But many questions remain unanswered, particularly regarding the mechanisms of symbiont acquisition by the tubeworm, the spatio-temporal dynamics and processes of symbiont growth and metabolism, and the genetic structure and dispersal of symbiont populations. Our pursuit of answers to these questions has been hindered by the inability to culture the *Riftia* symbiont apart from its host. Fortunately, given the recent advances in genetic techniques and the substantial progress towards sequencing the *Riftia* symbiont genome (R. Feldman and R. Felbeck, pers. comm.), researchers are poised to reveal many of the genes and genetic interactions that guide the physiological, ecological, and evolutionary processes involved in this important, ecosystem-structuring symbiosis. This chapter presents an overview of the physiological ecology and evolution of the *Riftia* symbiosis, with mention of the genes thus far described for this association and the underlying questions that may guide future research.

1.1 Discovery of the *Riftia pachyptila* Symbiosis

Scientific understanding of chemosynthetic symbioses derives in large part from studies of the unique fauna associated with deep-sea hydrothermal vents. Early explorations revealed that, in contrast to common perceptions, the deep benthos was not a cold, food-limited habitat but instead contained flourishing ecosystems localized at hot springs emanating from mid-ocean spreading centers. First characterized along the Galapagos Rift and the East Pacific Rise in the eastern Pacific Ocean, hydrothermal vents were shown to support high concentrations of free-living microorganisms and dense aggregations of invertebrates, including the vestimentiferan tubeworm *Riftia pachyptila* (Fig. 1; Lonsdale 1977; Grassle 1985; van Dover 2000). Scientists first argued that suspended particulate organic matter and free-living chemoautotrophic bacteria were being filtered from the water column to support the abundant invertebrate populations (Lonsdale 1977; Corliss et al. 1979). But studies soon revealed that the adult *R. pachyptila* lacked a mouth and gut (Jones 1981) and was therefore incapable of suspension feeding. It appeared that tubeworm nutrition, and therefore the flux of energy through the vent food web, instead depended substantially on endosymbiotic chemosynthetic bacteria.

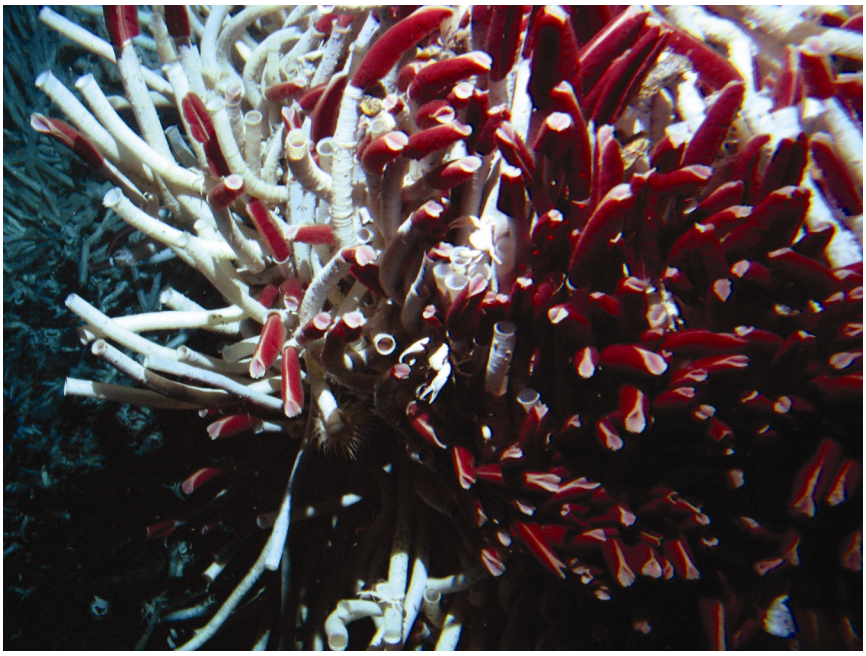

Fig. 1. *Riftia pachyptila* tubeworms on the East Pacific Rise. The branchial plume, a gill-like organ used for gas and metabolite exchange, protrudes from the white chitinous tube that protects the body of each worm

Initial evidence for a chemoautotrophic symbiosis in *R. pachyptila* came from microscopic and biochemical analyses showing Gram negative bacteria packed within the trophosome, a highly vascularized organ in the tubeworm trunk (Cavanaugh et al. 1981). Additional analyses involving stable isotope (Rau 1981), enzymatic (Felbeck 1981; Renosto et al. 1991), and physiological (Fisher et al. 1988) characterizations strongly suggested that the endosymbionts of *R. pachyptila* oxidize reduced sulfur compounds (e.g., hydrogen sulfide) to synthesize ATP for use in autotrophic carbon fixation via the Calvin cycle (i.e., "thioautotrophy"). The host tubeworm enables the uptake and transport of the substrates required for thioautotrophy (HS^-, O_2, and CO_2) and, in return, receives a portion of the organic matter synthesized by the symbiont population (Fig. 2). The bacterial population is the primary means of carbon acquisition for the symbiosis, and the adult tubeworm, given its inability to feed on particulate matter, is entirely dependent on its symbionts for nutrition.

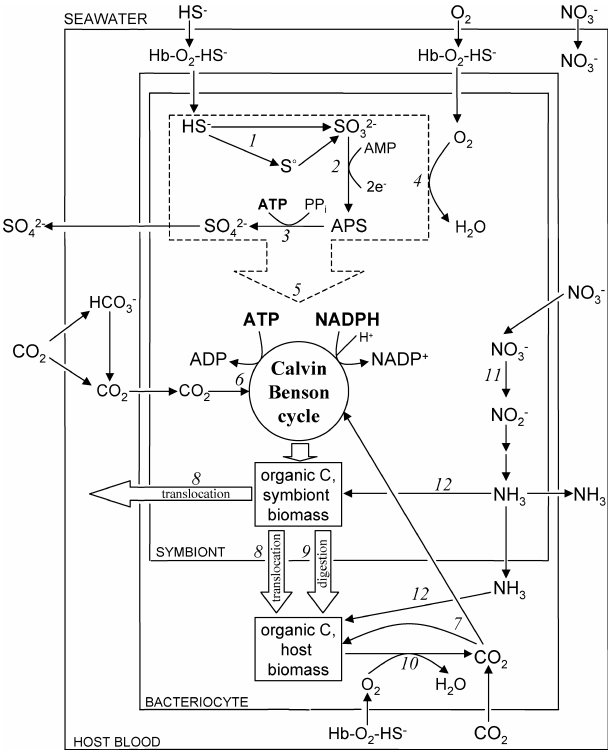

Fig. 2. Proposed model of metabolism in the symbiosis between *Riftia pachyptila* and a chemosynthetic sulfur-oxidizing bacterium. Reduced sulfur (primarily HS^-) and NO_3^- enter the tubeworm blood from the environment through unidentified transport mechanisms. CO_2 and O_2 enter by diffusion. In the blood, HS^- and O_2 simultaneously and reversibly bind hemoglobin ($Hb\text{-}O_2\text{-}HS^-$) for transport to the trophosome, where these substrates are used in symbiont sulfide oxidation (*dashed box*). HS^- is oxidized first to elemental sulfur (S^0) or directly to sulfite (SO_3^{2-}; *1*). SO_3^{2-} oxidation to sulfate (SO_4^{2-}) then proceeds through the APS pathway via the enzymes APS reductase (*2*) and ATP sulfurylase (*3*), yielding one ATP by substrate level phosphorylation. Electrons liberated during sulfur oxidation pass through an electron transport system, driving oxygen consumption (*4*) and the production of ATP and NADPH (*5*). Fixation of CO_2 occurs primarily via ribulose-1,5-bisphosphate oxygenase (RubisCO) in the Calvin Benson cycle (*6*), using ATP and NADPH generated from sulfur oxidation. Anaplerotic pathways in both host and symbiont (*7*) fix lesser amounts of 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 (*8*) and direct digestion of symbiont cells (*9*). Host oxygen consumption (*10*) occurs in typical catabolic and anabolic pathways. Nitrate (NO_3^-), the dominant nitrogen source for the symbiosis, enters via an undescribed transport mechanism and is reduced to nitrite (NO_2^-) by the symbionts via an assimilatory nitrate reductase (*11*). NO_2^- is reduced via an uncharacterized pathway to yield ammonia (NH_3), which is used for biosynthesis by both symbiont and host (*12*). Abbreviations: APS, adenosine 5′-phosphosulfate. Reprinted with permission from TRENDS Microbiol (Stewart et al. 2005)

Discovery of this obligate mutualism prompted investigators to search for similar symbioses at vents and in other marine habitats (e.g., reducing sediments, hydrocarbon seeps). To date, chemosynthetic bacteria have been found in symbiosis with invertebrate hosts from six phyla as well as with ciliate protists (see review in Cavanaugh et al. 2005). Indeed, the presence of symbiotic bacteria is a defining characteristic of some taxa. For example, all members of the tubeworm family Siboglinidae examined to date, including the vestimentiferan (e.g., *R. pachyptila*) and the smaller pogonophoran tubeworms, contain intracellular symbionts. Members of this family occur not only at deep-sea hydrothermal vents (e.g., *Oasisia*, *Ridgeia*, *Riftia*, *Tevnia* sp.; McMullin et al. 2003) but also at hydrocarbon cold seeps (e.g., *Escarpia*, *Lamellibrachia* sp.; Sibuet and Olu 1998) and mud volcanoes (e.g., *Oligobrachia*; Pimenov et al. 2000; Gebruk et al. 2003). The symbionts of most of these worms are chemosynthetic sulfur oxidizers, but methane-oxidizing bacteria (methanotrophs) have been found in one host species, the pogonophoran tubeworm *Siboglinum poseidoni* (Schmaljohann and Flügel 1987; see following chapter).

Based on analyses comparing 16S rRNA gene sequences, symbionts of the vent tubeworms fall within the gamma Proteobacteria, a broad bacterial division that includes chemosynthetic symbionts from a wide diversity of host taxa (Fig. 3, adapted from McKiness 2004; Cavanaugh et al. 2005). When compared to other chemosynthetic symbionts of the gamma Proteobacteria and to epsilon Proteobacteria episymbionts of shrimp and alvinellid worms, the 16S rRNA gene sequence from the symbiont of *R. pachyptila* clusters with sequences from symbionts of other tubeworms, including another East Pacific Rise vent tubeworm, *Ridgeia piscesae*, and two species of seep tubeworms, *Escarpia spicata* and *Lamellibrachia columna* (Fig. 3). This result supports prior studies showing that vestimentiferan tubeworms from hydrothermal vents share a single, or very similar, symbiont phylotype (Feldman et al. 1997; Laue and Nelson 1997; di Meo et al. 2000; Nelson and Fisher 2000; McMullin et al. 2003). Outside of this "tubeworm group," the *Riftia* symbiont appears most closely related to chemosynthetic symbionts of tropical lucinid clams (Fig. 3). Interestingly, both lucinid clams and vestimentiferan vent tubeworms appear to acquire their symbionts from a pool of free-living bacteria (i.e., environmental transmission, see Gros et al. 1998, 2003 and Section 14.4 below). However, the extent to which a free-living symbiont stage facilitates the invasion of multiple hosts (e.g., tubeworms and clams) over evolutionary time remains equivocal.

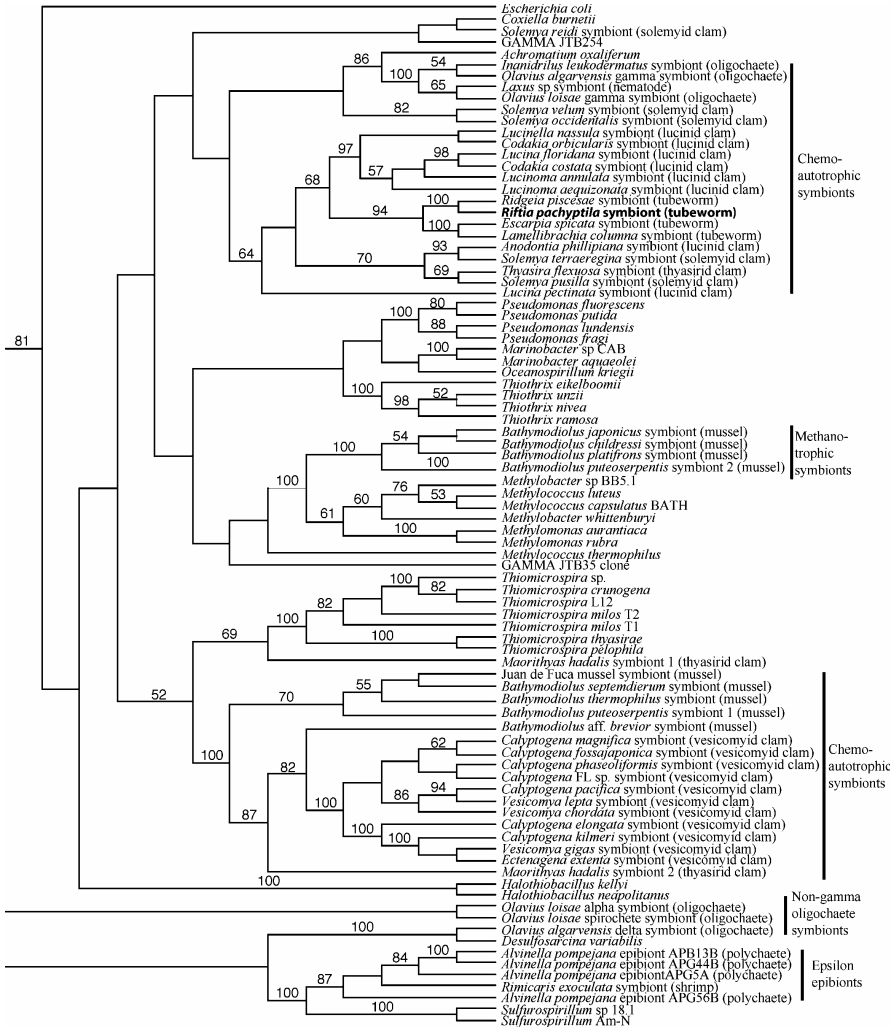

Fig. 3. Phylogeny showing the placement of the *Riftia* symbiont (*in bold*) relative to other chemosynthetic symbionts within the gamma Proteobacteria, episymbionts within the epsilon Proteobacteria, and free-living bacteria. The tree is a strict consensus of 46 trees obtained via parsimony analyses of 16S rRNA gene sequences (1,456 bp). Results greater than 50% from a 500 replicate bootstrap analysis are reported above respective branches. Symbionts are identified by host, with the common name of the host taxonomic group listed in parentheses. Adapted from McKiness (2004) and Cavanaugh et al. (2005)

For *R. pachyptila*, as for other marine chemosynthetic symbioses, much current research focuses on clarifying the mechanisms that mediate the environmental acquisition of symbionts as well as the transfer of carbon, nutrients, and sulfide within the symbiosis. These processes are vital to understanding the ecology and evolution of both the host and the symbiont. But elucidation of these mechanisms first requires knowledge of the physiochemical environment that determines the distribution and physiology of *R. pachyptila*.

1.2 Vent Habitat

Riftia pachyptila inhabits hydrothermal vent sites along the East Pacific Rise and the Galapagos Rift in the Eastern Pacific. The distribution of the tubeworm is intimately tied to the unique physiochemical characteristics of hydrothermal vents. Vent sites are typified by steep gradients between cold (~1.8°C), oxygen-rich (110 μM) bottom water and hot (up to 400°C), acidic (pH ~3 to 6) vent fluid. Hydrothermal fluids are usually laden with volcanic gases (e.g., methane and carbon dioxide) and reduced chemicals, including heavy metals and hydrogen sulfide (H_2S, HS^-, S^{2-}). Sulfide in vent fluids, produced by the geothermal reduction of seawater sulfate and the interaction of geothermally heated water with sulfur-containing rocks (e.g., basalt; Alt 1995; Elderfield and Schultz 1996; Rouxel et al. 2004), usually occurs at concentrations (3–12 mmol/kg) orders of magnitude higher than those in ambient seawater. It is at the interface (the chemocline) between the anoxic, reduced vent effluent and oxic bottom water that chemosynthetic vent symbioses thrive. Here, chemosynthetic symbionts access both the reduced compounds (e.g., sulfide) used as an energy source and the oxygen to which electrons are shuttled in aerobic energy metabolism.

While some vent symbioses, including those involving alvinellid polychaete worms and episymbiotic bacteria, congregate around sites where effluent (up to 400°C, pH ~3) directly exits the seafloor, *R. pachyptila* is typically clustered around diffuse or low flow vents. These vents, formed by ambient seawater mixing in the shallow subsurface with vent fluid, generally have a higher pH (~6), lower temperatures (1.8 to ~40°C), and, consequently, lower concentrations of reduced chemicals (e.g., sulfide up to 300 mM; Fisher 1995; van Dover 2000). But the physiochemical environment of diffuse vents is rarely stable; flow rate, temperature, and sulfide concentration may vary over timescales measured in seconds (Johnson et al. 1988a, 1994). To exploit this stochastic environment *R. pachyptila* relies on anatomical and physiological adaptations for sequestering sulfide, oxygen, inorganic carbon, and nitrogen from the

chemocline and on the ability of its endosymbionts to use these substrates for energy metabolism and biomass synthesis.

2
Anatomy and Ultrastructure

Riftia pachyptila occurs in dense clumps attached to the seafloor substrate (e.g., basalt) at low flow vents. A narrow, elongate tube composed of chitin and scleroproteins and up to three meters in length protects the soft body of the worm, which is divided into four major regions. The branchial plume lies at the anterior of the worm in direct contact with the surrounding seawater. Infused with blood vessels, this gill-like organ allows an efficient exchange of metabolites (e.g., sulfide, oxygen, carbon dioxide, inorganic nitrogen) and waste products (e.g., ammonia, protons) between the worm and the surrounding seawater. Below the plume is the vestimentum, a circular muscle that houses the heart and brain of the worm as well as glands involved in tube secretion. The vestimentum also mediates the worm's position in its tube, enabling the animal to withdraw from predation or to extend its plume to access both sulfide-rich vent fluids and oxic bottom water.

The tubeworm trunk lies between the vestimentum and the segmented opisthosome that anchors the posterior of the worm to the tube. Encapsulated within the trunk wall in *R. pachyptila*, as in other vestimentiferan and pogonophoran tubeworm species, is a unique morphological adaptation designed specifically to house bacterial symbionts: the trophosome (Cavanaugh et al. 1981; Felbeck 1981; Jones 1981). The trophosome, which in *R. pachyptila* appears to develop from mesodermal tissue (Bright and Sorgo 2003) and replaces the transient gut present in larval and young juvenile tubeworms (Jones and Gardiner 1988), is a lobular organ consisting primarily of blood vessels, coelomic fluid, and specialized host cells called bacteriocytes. Bacteriocytes are packed with chemoautotrophic sulfide-oxidizing endosymbionts that are further encapsulated within a host-derived membrane bound vacuole (Fig. 4; Cavanaugh 1983, 1994; Fisher 1990). Bacterial abundance within this tissue is high, with cell density averaging 10^9 per gram of fresh trophosome (Hand 1987) and bacterial volume estimated to occupy between 15 and 35% of total trophosome volume (Powell and Somero 1986; Bright and Sorgo 2003).

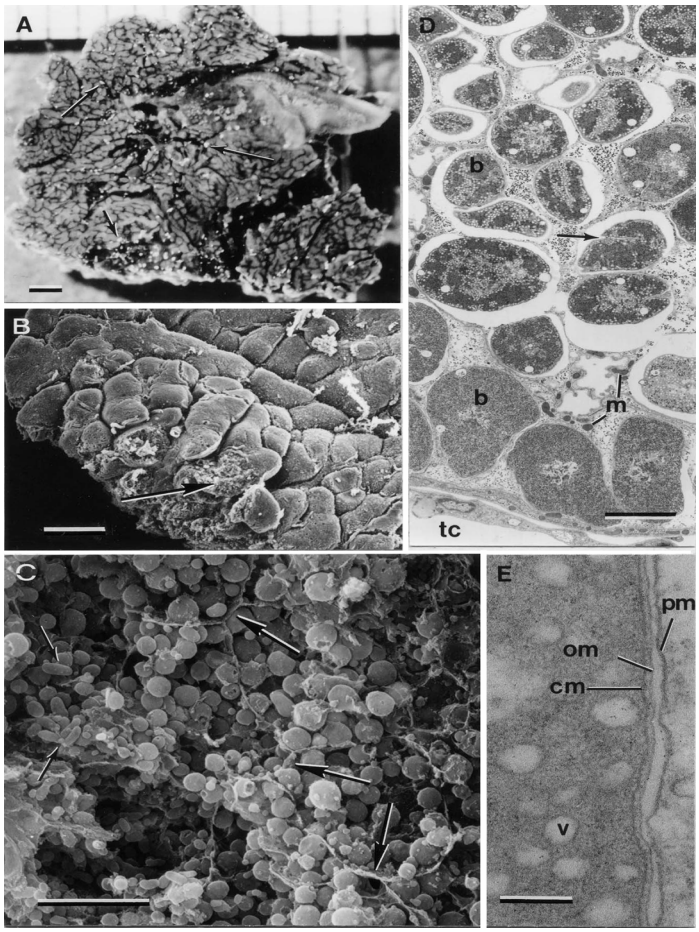

Fig. 4. *Riftia pachyptila* Jones (A-C: Galapagos Rift; D, E: 21°N, East Pacific Rise). **A** Photograph showing elemental sulfur crystals (*arrows*) scattered throughout trophosome; courtesy of M. L. Jones. **B** Scanning electron micrograph, showing lobules of trophosome; *arrow* indicates area of C (below) where surface epithelium was removed to reveal symbionts within trophosome. **C** Same, higher magnification, showing symbionts within trophosome; note spherical cells as well as rod-shaped cells (*small arrows*); *large arrows* indicate likely host cell membranes. **D** Cross section of portion of trophosome lobule, showing variable fine structure of symbionts, including membrane-bound vesicles in many cells; all symbionts contained within membrane-bound vacuoles, either singly or in groups or two or more; *arrow*, dividing bacterium; *b* bacteria; *m* mitochondria; *tc* trunk coelomic cavity. **E** Same, higher magnification, showing cell envelope of symbiont (resembling that of Gram-negative bacteria), intracytoplasmic vesicles, and peribacterial membrane; *v* vesicle; *cm* symbiont cytoplasmic membrane; *om* symbiont outer membrane; *pm* peribacterial membrane. *Scale bars*: A, 1 mm; B, 250 µm; C, 10 µm; D, 3 µm; E, 0.2 µm. Reprinted with permission from *Biol Soc Wash Bull* (Cavanaugh 1985)

In *R. pachyptila*, symbiont morphotype varies depending on location within the trophosome lobule (Fig. 5; Bosch and Grassé 1984a,b; Gardiner and Jones 1993; Bright et al. 2000). Bacteriocytes in the innermost (central) zone of the lobule primarily contain small, rod-shaped symbionts, while bacteriocytes nearer the periphery of the trophosome generally contain small and large cocci (1.6 to 10.7 µm diameter; Bright et al. 2000). Such pleomorphism may be caused either by intra-lobule biochemical gradients that impact symbiont metabolism, morphology, and growth or by differences in life cycle stage among symbiont cells (see below, Bosch and Grassé 1984a,b; Hand 1987; Bright et al. 2000).

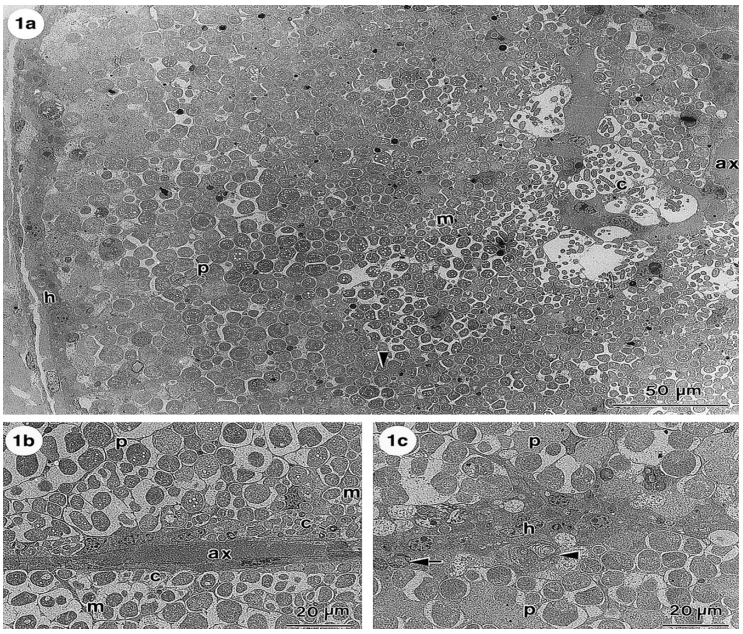

Fig. 5. Juvenile *Riftia pachyptila* transmission electron micrograph. Each trophosome lobule consists of non-symbiotic host tissue (*h*) surrounding the bacteriocytes of the peripheral zone (*p*) with large coccoid symbionts, the median zone (*m*) with small cocci, the central zone (*c*) with rods, and the non-symbiotic central axial blood vessel (*ax*). **a** Low magnification of lobule in cross section; note intracellular blood sinuses between bacteriocytes, one marked with an *arrowhead*. **b** Higher magnification of lobule center. **c** Higher magnification of periphery with degrading bacteria (*arrow*) and degrading bacteriocytes (*arrowhead*) in peripheral zone. Reprinted with permission from *Mar Biol* (Bright et al. 2000)

3
Nutritional Basis of the Symbiosis

3.1
Thioautotrophy

Nutrition in *Riftia pachyptila* depends on bacterial symbionts that fix carbon dioxide into organic matter using energy derived from the oxidation of reduced sulfur compounds. Figure 2 presents an overview of symbiont sulfur oxidation, as inferred from studies of free-living sulfur bacteria (see Kelly 1982; Nelson and Hagen 1995; Friedrich et al. 2001 for reviews of sulfur-based chemoautotrophy). The pathways by which the first half of this process, the oxidation of sulfide (H_2S, HS^-, S^{2-}) or thiosulfate ($S_2O_3^{2-}$) to sulfite (SO_3^{2-}), have not been clearly identified for the *Riftia* symbiosis and vary among different free-living thioautotrophs (Nelson and Hagen 1995). In contrast, considerable enzymatic and biochemical evidence (Felbeck 1981; Fisher et al. 1988; Renosto et al. 1991; Laue and Nelson 1994) suggests that tubeworm symbionts mediate sulfite oxidation via the energy-conserving adenosine 5′-phosphosulfate (APS) pathway (Fig. 2). In this pathway the enzyme APS reductase generates APS from adenosine monophosphate (AMP) and sulfite. In the presence of pyrophosphate, a second enzyme, ATP sulfurylase, then catalyzes the conversion of APS to ATP and sulfate (i.e., substrate level phosphorylation).

Activities of both APS reductase and ATP sulfurylase have been detected in the *R. pachyptila* trophosome (Felbeck 1981). Indeed, *Riftia* symbiont ATP sulfurylase was the first putative autotrophic ATP sulfurylase to be purified (Renosto et al. 1991). Unfortunately, neither enzyme is specifically diagnostic of thioautotrophy. ADP reductase and ATP sulfurylase operate in the reverse direction to catalyze the first steps of dissimilatory sulfate reduction by sulfate-reducing bacteria (see Peck and LeGall 1982). Also, ATP sulfurylase is ubiquitous in heterotrophic bacteria, fungi, and yeast, in which it functions in assimilatory biosynthetic pathways to incorporate sulfate into amino acids and other biomolecules (Segel et al. 1987). However, a probe specific to the gene encoding the *Riftia* symbiont ATP sulfurylase (*sopT*) was shown to specifically identify chemoautotrophic bacteria that use the APS pathway for sulfite oxidation (Laue and Nelson 1994). These results, along with the unique biochemical properties and notably high activity of *Riftia* symbiont ATP sulfurylase relative to ATP sulfurylase in photosynthetic and heterotrophic organisms (Renosto et al. 1991; Laue and Nelson 1994; Fisher 1995), strongly suggest that the *Riftia* symbiont uses this enzyme for sulfide-based chemoautotrophy. Further support for this hypothesis comes from recent crystallographic evidence showing that a key distinction between the *Riftia* symbiont ATP sulfurylase and the assimilatory enzymes from other organisms

(e.g., fungi) lies in the orientation of the mobile loop that occupies the enzyme's active site (Beynon et al. 2001). The unique "open" loop position observed in the *Riftia* symbiont potentially lowers the affinity of the enzyme for sulfate and thereby drives the reaction in the direction of ATP synthesis (Beynon et al. 2001).

Ultimately, the electrons liberated during the entire oxidation of sulfide to sulfate are shuttled through an electron transport system, yielding a proton gradient that drives ATP production via oxidative phosphorylation. In most instances the terminal oxidant in electron transport is molecular oxygen. However, both nitrate and elemental sulfur have been shown to function in *R. pachyptila* as an electron acceptor during periods of anoxia (Hentschel and Felbeck 1993; Arndt et al. 2001). ATP from both oxidative phosphorylation and substrate level phosphorylation via the APS pathway is then available for CO_2 fixation in the Calvin cycle. Autotrophy in *R. pachyptila* is evidenced by the presence and activity of diagnostic Calvin cycle enzymes, namely, phosphoribulose kinase (PRK) and the CO_2-fixing enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO; Felbeck 1981; Robinson et al. 1998), as well as by the uptake of radiolabeled CO_2 by live tubeworms (in pressure vessels), trophosome tissue homogenates, or isolated symbiont preparations (see Robinson and Cavanaugh 1995; Felbeck and Jarchow 1998; Bright et al. 2000). Indeed, the levels of carbon fixation (as RubisCO activity) in *R. pachyptila* are among the highest recorded for a chemosynthetic symbiosis (Felbeck 1981; Fisher 1995, and references therein). This is not surprising given the remarkably high growth rates observed for *R. pachyptila* – an increase of up to 1.4% of the tubeworm's total organic carbon per day (Childress et al. 1991; Lutz et al. 1994).

Thus, chemosynthesis by *Riftia* symbionts represents a significant input of fixed carbon into the vent ecosystem. To support this metabolism, the symbiosis must acquire all of the substrates necessary for both sulfide oxidation and carbon fixation: reduced sulfur, oxygen, dissolved inorganic carbon (DIC, as CO_2), and other nutrients (e.g., nitrogen and phosphorus) for use in biosynthesis. This requires that *R. pachyptila* access both oxic and anoxic environments, a task for which the tubeworm-symbiont association relies on specialized biochemistry and physiology.

3.2 Sulfide Acquisition

Thioautotrophy requires simultaneous access to both sulfide and oxygen. This dual requirement poses a unique challenge given that sulfide and oxygen occur in distinct habitat zones; in contrast to ambient seawater, the reduced vent fluid from which vent thioautotrophs obtain sulfide is typically anoxic or contains oxygen only at very low levels. In addition, sulfide spontaneously reacts with oxygen to form less-reduced sulfur compounds (S^0, $S_2O_3^{2-}$, or SO_4^{2-}; Zhang and Millero 1993). Such abiotic oxidation is typically several

orders of magnitude slower than microbially-mediated sulfide oxidation (Millero et al. 1987; Johnson et al. 1988b) but nonetheless decreases the availability of these substrates, forcing thioautotrophs to compete with oxygen for free sulfide and reside at the interface between oxic-anoxic zones. Chemosynthetic symbionts have adapted to bridge this interface via association with a eukaryotic host (Cavanaugh 1985; Cavanaugh et al. 2005). Similar to free-living sulfur bacteria, hosts of thioautotrophic symbionts use specialized behavioral, anatomical, or physiological mechanisms to spatially or temporally span the oxic-anoxic interface and sequester both sulfide and oxygen (Cavanaugh 1994; Fisher 1996; Polz et al. 2000).

In *Riftia pachyptila* simultaneous acquisition of sulfide and oxygen occurs via a remarkable biochemical adaptation. In contrast to most invertebrate and vertebrate hemoglobins, with which sulfide interacts to inhibit oxygen-binding (Weber and Vinogradov 2001 and references therein), extracellular hemoglobins (two vascular and one coelomic) synthesized by *R. pachyptila* bind sulfide reversibly and independently of oxygen (Arp et al. 1985, 1987, Childress et al. 1991, Zal et al. 1996) and are then transported via the worm's circulatory system to the trophosome for use by the symbionts. Several studies have focused on the mechanism by which hydrogen sulfide (primarily as HS-; Goffredi et al. 1997a) binds to *Riftia* hemoglobin. Initially, two free cysteine residues, each located on a distinct globin subunit (Zal et al. 1997, Zal et al. 1998, Bailly et al. 2002), were proposed as sulfide binding sites. Indeed, Bailly et al. (2003) argue that sulfide-binding cysteine residues, which are well conserved in both symbiont-containing and symbiont-free annelids from sulfidic environments but are absent in annelids from sulfide-free habitats, are ancient features and that the annelid ancestor arose within a sulfur-rich environment. When annelid ancestors colonized sulfide-free environments, these cysteine residues, in the absence of their natural ligand (sulfide), may have reacted deleteriously with other blood components, leading eventually to the loss of the sulfide-binding function during annelid evolution (Bailly et al. 2003). But in *R. pachyptila*, which inhabits sulfide-rich vents, this specialized function of annelid hemoglobins presumably has been retained. Recently, however, Flores et al. (2005) have questioned the role of cysteine residues as the sole mechanism for sulfide binding. Using crystallographic data, these authors showed that in the coelomic hemoglobin free cysteine residues are located beneath the surface of the molecule and are therefore unlikely to serve as an efficient binding site. Rather, crystallographic data and ion chelation experiments suggest that >50% of bound sulfide may be explained by chelation to 12 Zn^{+} ions near the poles of the coelomic hemoglobin molecule (Flores et al 2005). However, Zn^{+} chelation accounted for considerably less (18%) of bound sulfide in experiments using the larger of the two vascular hemoglobins (Flores et al 2005). Clearly, sulfide binding may occur through multiple mechanisms in *Riftia* hemoglobins, and additional work is needed to fully understand the role of these molecules in providing symbionts access to energy substrates and circumventing the accumulation of sulfide in the blood.

3.3 Inorganic Carbon Acquisition

The *Riftia* symbiont uses the Calvin cycle for autotrophic carbon fixation and therefore requires access to dissolved organic carbon (DIC) in the form of CO_2, the DIC species that diffuses most readily through biological membranes (Fig. 2). While the majority of DIC in seawater (pH ~8.0;) is bicarbonate (HCO_3^-; pK_a of 6.1 at in situ temperature and pressure of ~10°C and 101.3 kPa; Dickson and Millero 1987), the lower pH associated with diffuse flow vents (pH ~6.0 around *R. pachyptila* plumes) generates higher concentrations of CO_2 and gives organisms that use the Calvin cycle a distinct advantage.

DIC uptake by *R. pachyptila* depends largely on gradients of pH and CO_2. To offset internal reactions that generate protons (e.g., symbiotic sulfide oxidation of HS^- to SO_4^{2-} and H^+), the tubeworm relies heavily on proton-equivalent ion export by energy-requiring H^+-ATPases (Goffredi et al. 1999; Goffredi and Childress 2001; Girguis et al. 2002). Indeed, proton elimination, which represents the worm's largest mass-specific metabolite flux, may be the single greatest energy cost incurred by *R. pachyptila* (Girguis et al. 2002). This important process helps maintain the extracellular vascular fluid at an alkaline pH of ~7.5 (Goffredi et al. 1997b). In contrast to the surrounding vent water in which a lower pH results in an elevated CO_2 concentration, the alkalinity of tubeworm blood favors the conversion of CO_2 to HCO_3^-, thereby establishing a CO_2 gradient across the tubeworm plume (Childress et al. 1993; Goffredi et al. 1997b; Scott 2003). This gradient, from higher external [CO_2] to lower internal [CO_2], drives the diffusion of CO_2 into the blood. DIC (as CO_2 and HCO_3^-) is then transported by the vascular system to the trophosome for use in symbiont CO_2 fixation via the Calvin cycle. The method by which HCO_3^- is converted back to CO_2 at the blood-bacteriocyte interface prior to incorporation by the symbionts is unclear; however, host-derived carbonic anhydrases, enzymes that catalyze the hydration of CO_2 into HCO_3^- in both prokaryotes and eukaryotes, may play a role in this process (Kochevar and Childress 1996; de Cian et al. 2003a,b).

The mechanism of DIC incorporation impacts the stable carbon isotope signature of the *Riftia*-chemoautotroph symbiosis. In particular, relative ^{13}C enrichment in *R. pachyptila* may be due in part to the form of symbiont RubisCO used for CO_2 fixation. The $\delta^{13}C$ values (expressed as ‰; $\delta^{13}C = [(R_{sample} - R_{std})/ R_{std})] * 10^3$, where $R = {}^{13}C / {}^{12}C$) reported for thioautotrophic vent symbioses typically cluster into two distinct groups: an isotopically light "–30‰ group" composed of vent bivalves with $\delta^{13}C$ values between –27 to –35‰ and a heavier "–11‰ group" including shrimp episymbionts, free-living vent bacterial mats, and vestimentiferan tubeworms with $\delta^{13}C$ values between –9 to –16‰ (Childress and Fisher 1992; van Dover and Fry 1994; Robinson and Cavanaugh 1995; Cavanaugh and Robinson 1996; Robinson et al. 2003). In general, these two groups correspond to the RubisCO form used by a given symbiont population, with form I RubisCO

occurring in most members of the isotopically lighter group and form II in all members of the isotopically heavier group including the tubeworms (Robinson and Cavanaugh 1995; Cavanaugh and Robinson 1996). Robinson and Cavanaugh (1995) hypothesized that the difference in $\delta^{13}C$ values between these groups was due to variation in ^{13}C discrimination by the two structurally and kinetically distinct enzymes, as shown previously for form I from spinach (*Spinacia oleracea*) and form II from the proteobacterium *Rhodospirillum rubrum* (Roeske and O'Leary 1984). These authors therefore predicted that form II RubisCO of the *Riftia* symbiont discriminates less against ^{13}C than do typical form I enzymes. Indeed, Robinson et al. (2003) showed that the kinetic isotope effect (ε value), a measure of the extent to which $^{12}CO_2$ fixation is favored over $^{13}CO_2$ fixation (i.e., the degree of discrimination against ^{13}C), of purified form II RubisCO from *R. pachyptila* symbionts (ε = 19.5‰) is significantly lower than that of the form I enzyme (ε = 22 to 30‰; Guy et al. 1993). These data suggest that low ^{13}C discrimination by RubisCO may significantly impact the $\delta^{13}C$ values of tubeworm biomass.

Scott (2003) showed that rapid CO_2 fixation and steep gradients in CO_2 concentration may also contribute to ^{13}C enrichment in *R. pachyptila* biomass. RubisCO activity, by preferentially fixing $^{12}CO_2$ and leaving $^{13}CO_2$ behind, can temporarily enrich the cytoplasmic CO_2 pool. If carbon fixation is rapid, as would be expected given the high tubeworm growth rates and RubisCO activities recorded for this symbiosis, CO_2 equilibration between the isotopically heavier symbiont cytoplasm and the isotopically lighter bacteriocyte cytoplasm may not occur. The maintenance of this gradient forces RubisCO to draw from a more enriched $^{13}CO_2$ pool and therefore contributes to the relative abundance of ^{13}C in tubeworm biomass (Scott 2003).

3.4 Nitrogen Acquisition

Riftia pachyptila must also obtain all of the other macro- and micro-nutrients used in biosynthesis by both host and symbiont. While the pathways mediating assimilation and conversion of most metabolites are unknown for this symbiosis, researchers have identified some of the mechanisms of nitrogen metabolism in *R. pachyptila*. Laboratory incubations measuring the uptake of ^{15}N-nitrate ($^{15}NO_3^-$) showed that nitrate, which is abundant in situ (~40 μM; Johnson et al. 1988b), is the predominant inorganic nitrogen source for the symbiosis (Lee and Childress 1994). Indeed, activity of nitrate reductase, the bacterial enzyme mediating the reduction of nitrate to nitrite (NO_2^-), has been detected in *R. pachyptila* (Lee et al. 1999), indicating a role for nitrate reduction in either respiration or in the assimilatory pathway by which nitrate is ultimately converted to ammonia (NH_4^+) for use in biomass synthesis. Girguis et al. (2000), by measuring fluxes of inorganic nitrogen species into and out of *R. pachyptila* kept in pressurized chambers, ultimately

showed that the symbiont population reduces nitrate to ammonia not for respiratory purposes but for incorporation into both symbiont and host biomass. In addition, glutamine synthetase (GS) and glutamate dehydrogenase (GDH), the primary enzymes mediating assimilation of ammonia into amino acids, have also been detected in *R. pachyptila* (Lee et al. 1999). Though both host and symbiont synthesize these enzymes, biochemical and molecular tests (e.g., protein characterization, Southern hybridization) showed that GS measured in the trophosome was of bacterial origin (Lee et al. 1999), implicating the symbiont population in inorganic nitrogen acquisition. However, subsequent detection of high GS activity in symbiont-free branchial plume tissue indicates that the tubeworm may also assimilate ammonia directly from the surrounding seawater (Minic et al. 2001).

But further enzymatic characterization of *R. pachyptila* tissues reinforces the hypothesis that the trophosome is a primary site for nitrogen assimilation and metabolism. Minic et al. (2001) demonstrated that only the bacterial symbiont has all of the enzymes required for the de novo synthesis of pyrimidines, implying that the tubeworm is entirely dependent on the bacterium for these nucleotides. Interestingly, while absent in the symbiont, the activities of at least three of the enzymes that mediate pyrimidine catabolism were detected in the tubeworm host, suggesting that pyrimidine degradation may represent an internal source of CO_2 and NH_3 for use in host biosynthesis (Minic et al. 2001). Similarly, the catabolic enzymes involved in the synthesis of polyamines from arginine appear to be present only in the symbiont (Minic and Herve 2003). Polyamines, which play important physiological roles in processes of growth, membrane structure, and nucleic acid synthesis and which represent a potential source of carbon and nitrogen upon degradation (Tabor and Tabor 1985), may therefore be available to *R. pachyptila* only via the metabolism of the bacterial symbiont (Minic and Herve 2003). These data further emphasize the extent to which the metabolism of the tubeworm host is intertwined with that of the symbiont. Undoubtedly, genomic characterization, in conjunction with enzymatic or gene expression analyses, will reveal additional interactions that sustain this symbiosis at the molecular level.

3.5 Organic Compound Transfer and Symbiont Growth

In chemosynthetic endosymbioses the transfer of fixed carbon from the symbiont to the host for use in biomass synthesis or energy metabolism can occur via two mechanisms: 1) the symbiont excretes autotrophically-fixed carbon in the form of soluble organic molecules that are then translocated to host cells, or 2) the host directly digests bacterial cells (Fig. 2).

Both translocation and digestion appear to contribute to host nutrition in *Riftia pachyptila*. Felbeck and Jarchow (1998), using radiotracer experiments with purified *Riftia* symbionts incubated in the presence ^{14}C-bicarbonate, showed that labeled sugars, organic acids, and amino acids (primarily succinate and glutamate) were excreted into the surrounding medium by the symbionts. These simple organic compounds, some of which had been previously identified in isolated trophosome during similar radiotracer experiments (Felbeck 1985), might be important intermediates in the transfer of fixed carbon from symbionts to host (Felbeck and Jarchow 1998). Recently, pulse-chase labeling analysis and autoradiography showed directly that a considerable fraction of the organic carbon fixed by the *Riftia* symbionts is released immediately (within 15 min) after fixation and assimilated into metabolically active tubeworm tissue (Bright et al. 2000). However, the appearance of radiolabel in host tissue and the concomitant loss of label from the trophosome during the chase period suggested that symbiont digestion also contributes to host nutrition (Bright et al. 2000). Indeed, these authors consistently observed symbionts being degraded near the periphery of the trophosome lobule (Bright et al. 2000); similar ultrastructural patterns had been reported previously (Bosch and Grassé 1984a; Hand 1987; Gardiner and Jones 1993). In addition, digestion of *Riftia* symbionts has been inferred from the relatively high lysozyme activity within the *R. pachyptila* trophosome (Boetius and Felbeck 1995).

The relative contribution of translocation and digestion to tubeworm nutrition is tied to the growth dynamics of the symbiont population. Several studies show that symbiont morphology varies predictably within a trophosome lobule, with the symbiont population dominated by small, actively dividing rods at the center of the lobule and large cocci in various stages of autolysis and digestion at the periphery (Fig. 5; Bosch and Grassé 1984a; Gardiner and Jones 1993; Bright and Sorgo 2003). Two hypotheses have been put forth to explain this variation in symbiont morphology and growth (reviewed in Bright and Sorgo 2003). First, blood flow from the periphery to the center of the trophosome forms biochemical gradients that influence the metabolism, and therefore the morphology and growth, of the symbionts (Hand 1987). Second, symbiont cells and host bacteriocytes both progress through a complex cell cycle involving cell proliferation at the lobule center, followed by migration toward the periphery, and subsequent lysis and degradation (Bosch and Grassé 1984a,b). Neither hypothesis is necessarily exclusive of the other. However, symbiont carbon fixation rates were shown to be roughly equivalent at the center and periphery of the lobule (Bright et al. 2000), implying that the substrates used in chemosynthesis may not occur over a gradient in the trophosome.

Support for a cell cycle hypothesis comes from quantitative ultrastructural analyses showing that the division by rods is in balance with the lysis of cocci and that intermediate stages between rods and cocci occur within the

trophosome (Bright and Sorgo 2003). It therefore appears that in the central zone carbon fixation supports symbiont division whereas in the peripheral zone fixation supports an increase in per cell biomass, with excess carbon stored as glycogen (Sorgo et al. 2002). Recent evidence suggests that the symbiont-containing bacteriocytes undergo a similar cell cycle, with proliferation in the lobule center, terminal differentiation, and degradation in the periphery (Bright and Sorgo 2003). The life cycle hypothesis that appears to explain the gradation in symbiont size and shape within the trophosome implies a constraint on symbiont division outside the lobule center as well as a complex coordination of host and symbiont cell replication. The molecular mechanisms that regulate symbiont division, metabolism, and lysis likely involve complex symbiont-host signaling. Elucidation of these mechanisms, as well as the spatio-temporal dynamics of symbiont growth within the trophosome, remain as important goals in the study of the *Riftia* symbiosis.

4
Symbiont Transmission and Evolution

The evolutionary dynamics between symbiont and host depend largely on the symbiont transmission strategy. Symbiont transmission between successive host generations can occur environmentally (acquisition from a free-living population of symbiotic bacteria), horizontally (transfer between hosts sharing the same habitat), or vertically (transfer from parent to offspring via the egg). Vertically transmitted endosymbionts experience a unique selective regime within the host. These symbionts are effectively disconnected from their free-living counterparts and undergo a population bottleneck upon host colonization and another upon transmission (Mira and Moran 2002). These processes severely reduce the symbiont effective population size and thereby elevate the rate of fixation of slightly deleterious alleles via genetic drift (Ohta 1973; Wernegreen 2002). In addition, the asexuality and lack of recombination in endosymbionts exacerbates these genetic problems through what is known as Muller's rachet (Muller 1964; Moran 1996). In Muller's ratchet wild type recombinants cannot be introduced into the endosymbiont population (Moran and Baumann 1994; Dale et al. 2003); genetic drift therefore occurs quickly, and the population cannot recover after fixation of deleterious alleles. Because the selective regime experienced by vertically transmitted endosymbionts is inextricably linked to the reproduction and dispersal of the host, co-speciation of symbiont and host is common and is used as a marker for vertical transmission (e.g., Chen et al. 1999; Thao et al. 2000; Degnan et al. 2004).

In contrast, environmentally transmitted symbiont populations have both a free-living and a symbiotic component. Exchange between these two pools

presumably lessens the effects of Muller's ratchet by facilitating recombination between individual bacteria and leads to a larger effective population size, and therefore a potentially slower rate of nucleotide substitution, than for vertically transmitted symbionts (Moran 1996; Peek et al. 1998). Consequently, the link between symbiont and host evolution is weakened and co-speciation is not anticipated.

For the *Riftia* symbiosis, in which the adult host is completely dependent on its symbiont for sustenance, environmental transmission would seem an uncertain strategy, particularly given the erratic nature of the hydrothermal vent environment. Nonetheless, several lines of evidence strongly suggest that *R. pachyptila* acquires its symbiont de novo each generation from a pool of free-living bacteria. For example, tubeworm symbionts do not demonstrate co-speciation with their hosts, i.e., host-symbiont specificity and phylogenetic host-symbiont congruence are not evident in tubeworm symbioses. While repetitive-extragenic-palindrome PCR (REP-PCR) fingerprinting revealed that *Riftia* symbionts exhibit strain-level genetic variation that correlates with geographic location (di Meo et al. 2000), analysis of 16S rRNA gene sequences showed that vestimentiferan tubeworms belonging to the genera *Riftia, Tevnia,* and *Oasisia* appear to share a single, or very similar, symbiont phylotype (Feldman et al. 1997; Laue and Nelson 1997; di Meo et al. 2000; Nelson and Fisher 2000; McMullin et al. 2003). The distribution of a single phylotype among three vestimentiferan genera is strong evidence that vent tubeworms acquire their symbionts from a free-living pool of symbiont cells.

Other lines of evidence similarly suggest that *R. pachyptila* obtains its symbionts from the environment. PCR probing using universal eubacteria and *Riftia* symbiont-specific primers failed to detect bacterial 16 rRNA genes in DNA extracts from *R. pachyptila* eggs (Cary et al. 1993). These results suggest, but do not confirm, that the *Riftia* symbiont is not maternally transmitted via gonadal tissue. The apparent absence of symbiont cells in *R. pachyptila* eggs is consistent with the fact that tubeworm larvae and juveniles, while possessing a mouth and gut, lack symbiont-containing tissue. In contrast, adult tubeworms lack a mouth and gut and are therefore incapable of feeding autonomously. Rather, adults worms possess the bacteria-containing trophosome, the primary site for CO_2 fixation in this symbiosis.

The detection of specific functional genes in the *Riftia* symbiont also suggests environmental transmission. Millikan et al. (1999) used PCR to detect and amplify a *Riftia* symbiont gene with high sequence similarity to the flagellin gene, *fliC*, which encodes the primary subunits of the bacterial flagellum. This analysis, while showing that the *Riftia* symbiont has at least one of the genes required for flagellar synthesis, does not attest to the functional role of the flagellum in situ. Flagellar motility presumably would be unnecessary if the symbiont is always associated with the host (i.e., if the symbiont is vertically transmitted). Alternatively, if the symbiont is environmentally transmitted, a flagellum might mediate adhesion to and

invasion of the tubeworm. Indeed, several other bacterial symbionts and pathogens have been shown to use flagellum-associated structures to colonize eukaryotic hosts (e.g., Chua et al. 2003; Gavin et al. 2003; Kirov 2003; Dons et al. 2004). But, while *Riftia* symbiont *fliC* induced the formation of flagella when the gene was cloned and expressed in *Escherichia coli* (Millikan et al. 1999), characterization of in situ flagellum synthesis is necessary to demonstrate that a flagellum mediates colonization of *R. pachyptila*. Similarly, symbiont genes whose products are homologous to known signal transduction proteins have been detected in the *Riftia* symbiont (Hughes et al. 1997). These genes, *rssS* and *rssB*, encode a histidine protein kinase (RssA) and a response regulator protein (RssB) and may facilitate communication of the *Riftia* symbiont with its external environment. Alternatively, *rssA* and *rssB* may regulate survival of the symbiont while within the tubeworm host and therefore be of no direct relevance to an environmental mode of transmission. As for *fliC*, in situ expression studies will be necessary to determine the functional significance of these signal transduction genes. Fortunately, expression analyses, such as those involving mRNA hybridization to genomic or proteomic arrays and in situ hybridization to mRNA (e.g., Pernthaler and Amann 2004), while previously daunting due to the inability to culture the symbiont apart from its host, are now possible given increasingly sensitive and specific molecular approaches and advances in maintaining vent organisms under in situ conditions (i.e., in pressure vessels; e.g., Girguis et al. 2000; Felbeck et al. 2004).

Finally, if the *Riftia* symbiont is obtained anew each generation, the symbiont must exist in a free-living form in the vent habitat. Indeed, the 16S rRNA phylotype of the *Riftia* symbiont has been detected in vent environments. Harmer et al. (2005), using both PCR and in situ hybridization with symbiont-specific probes, detected the free-living form of the *Riftia* symbiont on basalt blocks and settlement traps deployed in and around clumps of tubeworms at vent sites on the East Pacific Rise. These results provide the most encouraging evidence to date of environmental transmission in the *Riftia* symbiosis. This work may guide future ecological studies examining the impact of the tubeworm symbiosis on the free-living bacterial community at vents as well as complement ongoing efforts to determine the molecular mechanisms that mediate host-symbiont recognition and entry of the symbiont into the developing tubeworm.

5
Future Directions

In this review, we have attempted specifically to include those studies that use molecular or biochemical data to describe the physiology and evolution of the

Riftia pachyptila symbiosis. While this unique chemosynthetic association has been a primary focus of hydrothermal vent research (for additional reviews see Fisher 1995; Nelson and Fisher 1995; van Dover 2000; Cavanaugh et al. 2005; Minic and Herve 2004; van Dover and Lutz 2004), studies of the molecular biology of the *Riftia* endosymbiont have been hindered greatly by the inability to grow the bacterium in pure culture. However, with the *Riftia* symbiont genome sequence in process (R. Feldman and H. Felbeck, pers. comm.), we will soon have detailed knowledge of the genes that mediate symbiont growth, metabolism, and behavior both prior to and following invasion of the tubeworm host. Several important questions remain unanswered. For instance, through what mechanisms does symbiont sulfide oxidation proceed? How are biologically important elements (e.g., nitrogen, phosphorus, sulfur, iron) obtained by and cycled within the symbiont? Do these bacterial pathways provide important intermediates required for host metabolism? What transport processes and signal pathways are responsible for host-symbiont specificity and symbiont invasion of host cells? What factors (e.g., substrate gradients, cell cycle controls) regulate symbiont growth and division, and how are these factors coordinated with the regulation of the host cell cycle? How is the genetic structure of symbiont populations affected by environmental symbiont transmission, and how does population-level genetic diversity vary over spatial, habitat, and temporal gradients and between symbiotic and free-living populations? To answer these questions it will be particularly prudent to use genomic data not only to understand symbiont evolution but also to design in situ expression studies that can accurately assess the dynamics of both gene-gene and symbiont-host interactions. Doing so presents a challenge, but one for which researchers will be rewarded with even more fascinating details about this remarkable symbiosis.

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