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Coupling Metabolite Flux to Transcriptomics: Insights Into the Molecular Mechanisms Underlying Primary Productivity by the Hydrothermal Vent Tubeworm *Ridgeia piscesae*

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Abstract. Deep-sea hydrothermal vents host highly productive ecosystems. Many of these communities are dominated by vestimentiferan tubeworms that house endosymbiotic chemoautotrophic bacteria that provide the hosts with their primary nutritional needs. Rates of carbon fixation by these symbioses are also among the highest recorded. Despite the breadth of physiological and biochemical research on these associations, the underlying molecular mechanisms that regulate host and symbiont metabolite flux and carbon fixation are largely unknown. Here we present metabolite flux and transcriptomics data from shipboard high-pressure respirometry experiments in which we maintained *Ridgeia piscesae* tubeworms at conditions comparable to those *in situ*. Host trophosome was used for cDNA library construction and sequencing. Of the 19,132 clones sequenced, 10,684 represented unique expressed sequence tags (ESTs). The highest proportions of genes are involved with translation, ribosomal structure and biogenesis, cellular processing, and signal transduction. There was moderate representation of genes involved in metabolite exchange and acid-base regulation. These data represent the first concomitant surveys of metabolite flux rates and gene expression for a chemoautotrophic symbiosis during net autotrophy, and they suggest that—in the case of *Ridgeia piscesae*—host-symbiont interactions such as cell cycle regulation may play a significant role in maintaining physiological poise during high productivity.

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Abbreviations: EST, expressed sequence tag; HPRS, high-pressure respirometry system.

Introduction

Thirty years ago, scientists discovered active hydrothermal vents in the ocean off the Galapagos Islands (Corliss *et al.,* 1979). Like the findings of Darwin 140 years earlier, the discovery of hydrothermal vents on the Galapagos rift fundamentally altered our notions about life on Earth. These underwater hot springs discharge chemically altered seawater that is low in pH and enriched in hydrogen sulfide, iron, and other metals and gasses (Von Damm, 1990). Although temperatures of vent effluents can exceed 380 °C (Urabe *et al.,* 1995; Schmidt *et al.,* 2007), mixing with cold seawater results in biologically habitable regimes. Indeed, hydrothermal vents support extensive biological communities, comparable in biomass to rainforests and coral reefs (Gaill *et al.,* 1997; Sarrazin and Juniper, 1999; Govenar *et al.,* 2005). Although a majority of vent endemic species are heterotrophs, animals that have nutritional symbioses with chemoautotrophic bacteria constitute as much as 96% of the community biomass (Sarrazin and Juniper, 1999; Govenar *et al.,* 2004). These chemoautotrophic symbionts harness energy *via* the oxidation of reduced chemical species and fix inorganic compounds for growth and biosynthesis. The co-availability of chemically reduced and oxidized compounds within vent mixing zones (called diffuse flows) is ideal for chemoautotrophic activity.

Hydrothermal vent tubeworms were the first chemoautotrophic symbioses to be described (Jones, 1981). The best known of these symbioses is the giant vent tubeworm *Riftia*

pachyptila Jones, 1981 (hereafter referred to as *Riftia*), a monospecific genus within the family Siboglinidae (Rouse, 2001). Tubeworms are the dominant megafaunal species at many vent sites, growing in enormous aggregations and hosting numerous other species such as mussels, polychaete worms, limpets, and crabs (Hessler and Smithey, 1983; Shank *et al.,* 1998; Govenar *et al.,* 2005). Tubeworms are devoid of a mouth or digestive tract, and host their intracellular chemoautotrophic bacteria within a vascularized organ called the trophosome (Cavanaugh *et al.,* 1981; Felbeck, 1981). These worms cannot ingest particulate organic matter; therefore they are entirely reliant on their symbionts for nutrition and must provide all the substrates necessary for symbiont chemoautotrophy. As a result, vent vestimentiferans thrive in diffuse flows, positioning their plumelike gill at the interface of vent flow and bottom-water mixing (Childress *et al.,* 1991). Because these diffuse flows are spatially and temporally heterogeneous, with large fluctuations in chemistry and temperature (Johnson *et al.,* 1986, 1988; Chevaldonne *et al.,* 1991; Shank *et al.,* 1998; Luther *et al.,* 2001; Mullineaux *et al.,* 2003; Le Bris *et al.,* 2005), vent vestimentiferans have evolved to rapidly acquire and store substrates from the environment to dampen the variations in substrate availability. For example, they can bind both oxygen and sulfide in their blood (Arp and Childress, 1983; Flores *et al.,* 2005), and *Riftia* has been shown to store enough oxygen and sulfide to sustain chemoautotrophy for hours without environmental input (Arndt *et al.,* 1998; Girguis and Childress, 2006).

The unique nature of vestimentiferan symbioses has encouraged many physiological and biochemical studies of host and symbionts (for review, see Stewart and Cavanaugh, 2006). Until recently, however, little was known about the metabolic rates of these symbioses. Vent tubeworms usually die within several hours at one atmosphere, making it difficult to study whole-animal processes at atmospheric pressure. To address this problem, investigators developed a shipboard flow-through high-pressure respirometry system (HPRS) that maintains chemoautotrophic symbioses at *in situ* pressures, simultaneously delivers dissolved sulfide and oxygen at rates sufficient to support the high metabolic rates of the organisms, and concurrently measures changes in metabolite flux *via* a membrane inlet mass spectrometer (Girguis *et al.,* 2000, 2002; Girguis and Childress, 2006; Fig. 1B). This technique allows for well-controlled respirometric experiments and is very effective at keeping deep-sea organisms alive both on board ship and in the laboratory. Using this system, they found that *Riftia* actively takes up nitrate—a first for any animal—that is reduced by the symbionts to provide ammonia for growth and biosynthesis (Girguis *et al.,* 2000), and that it eliminates hydrogen ions—the primary waste product of symbiont metabolism—at unprecedented rates *via* numerous H^+-ATPases (Girguis *et al.,* 2002).

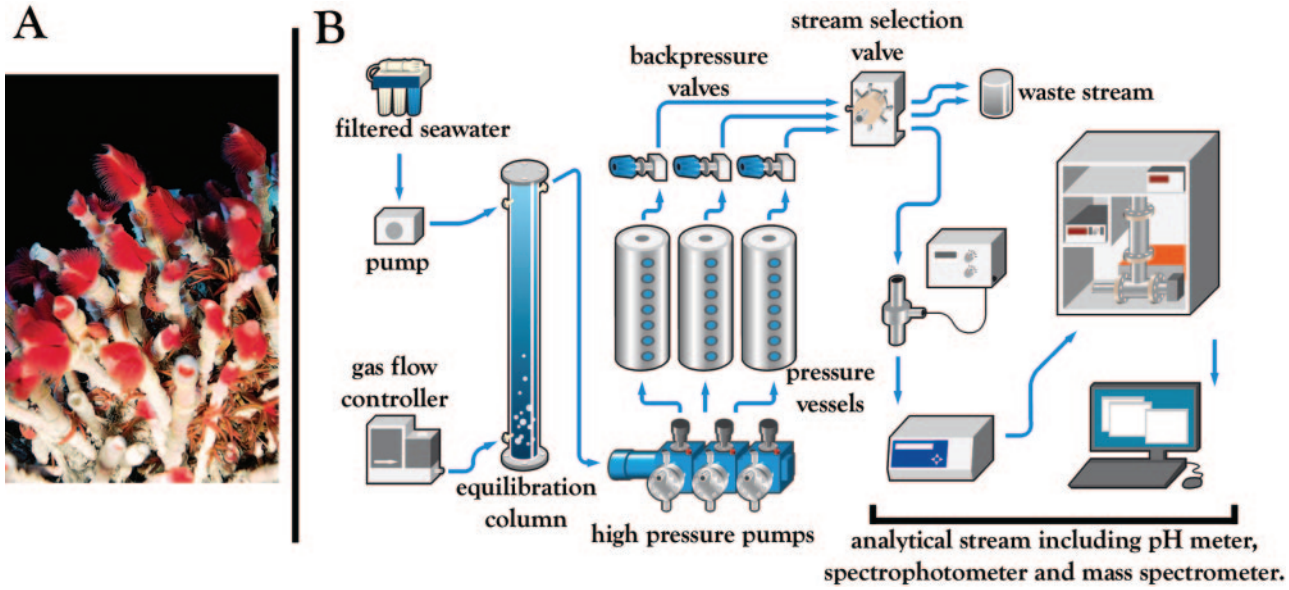

Figure 1. (A) Photograph of a *Ridgeia piscesae* aggregation *in situ*. Photograph courtesy of V. Tunnicliffe, University of Victoria. (B) Schematic of the high-pressure respirometry system (HPRS). Sterilized (0.2-μm filter) seawater is pumped into the equilibration column, where it is mixed with gasses and compounds to mimic diffuse vent effluent. This solution is delivered to three high-pressure metering pumps, which generate 27.5 MPa pressures in the temperature-controlled pressure aquaria located between the pumps and the backpressure valves. Aquarium effluents are directed toward a stream-selection system that sends each stream to analysis and sampling.

The most recent, and arguably most exciting, work has demonstrated that *Riftia* tubeworms are among the most productive organisms on Earth (Girguis and Childress, 2006). Using the shipboard HPRS, the authors measured astonishingly high net primary productivity, up to 25 micromoles of dissolved inorganic carbon per gram of wet weight per hour. Conservative extrapolations of the mass specific carbon fixation rates into community productivity (based upon published community data including tubeworm density, biomass, and *in situ* chemical measurements; Govenar *et al.,* 2005) estimate that at least 6 to 9 kilograms of inorganic carbon per square meter per year are fixed into organic carbon (Girguis and Childress, 2006). These rates are comparable to the high primary productivity found in kelp forests and Chilean costal upwelling zones (carbon fixation of *ca.* 7 $kg \cdot m^2 \cdot y^1$; Daneri *et al.,* 2000). In addition, Girguis and Childress (2006) observed that the host (or symbionts) actively modulates inorganic carbon uptake and fixation in response to lower environmental substrate concentrations, maintaining modest carbon fixation until conditions are sufficient to support elevated primary productivity. Recently, Markert and colleagues (2007) showed that *Riftia* symbionts might possess two carbon fixation pathways, the reductive tricarboxylic acid cycle and the Calvin Benson Bassham cycle, which is possibly an adaptation to the dynamic vent environment. A recent study by Sanchez and colleagues (2007) examined differential gene expression between tissues of *Riftia* (comparing gill, body wall, and trophosome), and found that cathepsin and chitinase precursor transcripts, carbonic anhydrase (RpCAbr), and other genes were highly represented in the gill tissue, whereas another carbonic anhydrase (RpCAtr), myohemerythrin, and other genes were highly specific to the trophosome tissue. These differences in gene expression may reflect the role of different tissues in supporting chemoautotrophic function.

Collectively, however, these studies focus largely on the metabolic responses of vestimentiferans to the vent environment. In some respects, all the prior physiological or biochemical studies of *Riftia* have been "black box" approaches, studying biochemical activities *in vitro*; whole-animal metabolic activity with little insight into the underlying molecular regulation; or gene and protein expression with little environmental context. Although the recent genomic, proteomic, and transcriptomics studies in this and other hydrothermal vent organisms have provided valuable insight into gene expression and biochemical potential, these studies used freshly collected specimens as their source material, many of which had spent several hours or more in conditions that were markedly different from their *in situ* environment (*e.g.,* high oxygen concentrations and low pressures, Table 1). In short, such data cannot be allied to organismal activity at environmentally relevant conditions.

An effective approach to studying gene and protein expression in deep-sea organisms (especially in light of the difficulties in conducting physiological experiments *in situ*) is to conduct shipboard experiments that reproduce *in situ* conditions, and to rapidly collect materials for analyses post-experimentation. To that end, we have adapted the HPRS to enable rapid depressurization and preservation of biological samples for subsequent molecular analyses (see Materials and Methods). This system allows us to rapidly depressurize the organisms and preserve them in reagents or liquid nitrogen in 4 to 7 min.

In light of the paucity of data on the molecular mechanisms underlying vestimentiferan symbiotic interactions, in particular during high metabolic activity, we conducted a

Table 1

Hydrothermal vent metazoan, symbiotic prokaryotes, and select free-living prokaryotic genomes, transcriptomes and proteomes examined to date

Organism	Genome	Transcriptome	Proteome	References
METAZOANS				
Riftia pachyptila		X		Joint Genome Institute in progress; Sanchez *et al.,* 2007
Ridgeia piscesae		X		Joint Genome Institute in progress; this study
Alvinella pompejana		X		Joint Genome Institute in progress
MICROORGANISMS (partial list)				
Symbionts				
Endoriftia persephone	X		X	Robidart *et al.,* 2008; Markert *et al.,* 2007
Ruthia magnifica	X			Newton *et al.,* 2007
Nautilia sp.	X			Campbell *et al.,* 2001
Alvinella pompejana epibionts	X*			Campbell *et al.,* 2003
Free-living				
Thiomicrospira crunogena	X			Scott *et al.,* 2006
Methanococcus jannaschii	X		X	Bult *et al.,* 1996; Zhu *et al.,* 2004
Beggiatoa sp.	X			Teske and Nelson, 2004

* Metagenome.

series of experiments that employ the HPRS to ally metabolic rates with host gene expression during high net productivity in the vestimentiferan tubeworm *Ridgeia piscesae* Jones, 1985. *Ridgeia piscesae* is a tubeworm that flourishes at vents in the Pacific Northwest (Jones, 1985). It has widely variable morphologies that appear related to its local geochemical milieu. The "short and fat" form of *Ridgeia* (Fig. 1A), used in this study, is physiologically and morphologically very similar to *Riftia* (Jones, 1985; Flores *et al.,* 2005; Carney *et al.,* 2006; Urcuyo *et al.,* 2003, 2007), but is found in more vigorous diffuse flows and can exhibit higher activity and growth (DeBovoise and Taghon, 1988). We chose to work with *Ridgeia* because of its amenability to shipboard manipulations (individuals are modest in size and very active), and its genetic and biochemical similarity to *Riftia* (the two species are phylogenetically allied, have similar hemoglobins, and likely host the same symbionts; Rouse, 2001; Flores *et al.,* 2005; DiMeo *et al.,* 2001). Also, *in situ* observations suggested that *Ridgeia* aggregations are capable of extremely rapid growth rates (J. Delaney, University of Washington, pers. obs.). The experiments and data presented here examine gene expression in the *Ridgeia* host trophosome (in particular the bacteriocytes) during periods of high primary productivity, in order to identify the mechanisms employed by these hosts to support high symbiont chemoautotrophic productivity.

Materials and Methods

Animal collection

Ridgeia piscesae tubeworms were collected during a research expedition from August to September 2006 to the Mothra (47°55.4′N by 129°06.4′W) and Main Endeavour vent fields (47°57.0′N by 129°5.82′W) along the Juan de Fuca ridge. Tubeworms were collected *via* the DSV *Alvin* from a depth of about 2300 m, brought to the surface in a thermally insulated container, and promptly moved to ice-cold seawater. We specifically collected the "short and fat" *Ridgeia* morphotype from active sulfide chimneys. Tubeworms that weighed between 5 and 20 g and were responsive to touch were immediately placed into flow-through, high-pressure respirometry aquaria for experimentation.

Experimental apparatus and design

During all respirometry experiments, tubeworms were maintained in the high-pressure respirometry system (HPRS; as described in Girguis and Childress, 2006). The aquaria were maintained at 27.5 MPa (4000 psi) by high-pressure pumps (American Lewa, Inc.) and backpressure valves. All experiments were maintained at 15 °C (a temperature measured near the base of *Ridgeia* aggregations *in situ*; Urcuyo *et al.,* 2007). To simulate the seawater chemistry found *in situ*, 0.2-m filter-sterilized seawater was pumped into an acrylic gas equilibration column and bubbled with carbon dioxide, hydrogen sulfide, oxygen, and nitrogen to achieve the desired concentrations of dissolved gas. For the experiment outlined here, we maintained *R. piscesae* at conditions previously shown to support high primary productivity by *Riftia* (5.5 mmol l^{-1} dissolved inorganic carbon; 215–230 μmol l^{-1} H_2S; 150 μmol l^{-1} O_2; 40 μmol l^{-1} NO_3^-; pH 5.9; 15 °C; 27.5 MPa; Girguis and Childress, 2006). In the absence of data on *Ridgeia* physiology, we chose to replicate the chemical conditions that stimulated net carbon fixation in *Riftia*, which we believe is a reasonable representation of conditions found on the Juan de Fuca chimneys where these specimens were collected. At the end of the experiments, the worms were quickly removed from the pressure vessels; dissected into gill, vestimentum, body wall, and trophosome sections (taking care to selectively harvest the lobules containing the bacteriocytes); and flash-frozen in liquid nitrogen for subsequent RNA extraction (see below). In general, samples could be flash-frozen within 5 min of depressurization.

Determination of metabolite uptake rates

To determine substrate uptake rates, seawater was collected pre- and post-aquarium for chemical analyses. For total CO_2 (ΣCO_2) and O_2 concentrations, 30 ml of effluent was added to N_2-purged 50-ml serum vials containing 3 ml of concentrated NaOH. ΣCO_2 concentrations were measured by adjusting the pH to 3 using degassed HCl, and measuring P_{CO_2} in the headspace using a blood gas analyzer (Capni-Con II, Cameron Instruments). O_2 concentrations were measured using a gas chromatograph with a thermal conductivity detector (Agilent 6890N, as in Childress *et al.,* 1984). For ΣH_2S concentrations, 1.5 ml of effluent was preserved with 0.227 mol l^{-1} zinc acetate and kept frozen and in the dark for later analysis. Subsequently, ΣH_2S concentrations were determined using the method of Cline (1969). Cline's "B" reagent consisted of 0.6% FeCl and 0.4% *N,N*-dimethyl-*p*-phenylenediamine in 50% HCl. A standard curve was generated using sodium sulfide concentrations varying between 1 μmol l^{-1} and 1 mmol l^{-1}. All samples were measured on a Smart spectrophotometer (Bio-Rad, Hercules, CA) at 670 nm. pH was determined on board ship using a double-junction pH electrode and high-precision pH meter (Radiometer, Inc.).

RNA extractions

Flash-frozen trophosome tissues were quickly cut into 1-cm pieces on a dry-ice-cooled stainless steel dissecting tray and placed immediately into pre-chilled (−20 °C) RNAice (Ambion Inc.) and allowed to equilibrate overnight at −20 °C. One gram each of both trophosome and plume from experimental and control specimens of *R. piscesae* was used to extract total RNA according to manufacturer's

guidelines (MaxiPrep total RNA extraction kit, Qiagen, Inc.). After extraction, total RNA was quantified on a microscale UV spectrophotometer (Nanodrop, Inc.) and quality checked by gel electrophoresis on an RNA microchip (Agilent, Inc.). Subsequently, Poly- $(A)^+$ RNA was isolated using oligo-(dT)-chromatography, and eluted into Tris-EDTA buffer, pH 7 (Sigma, Inc.).

cDNA synthesis and EST library construction

A 500-μg sample of total RNA from pooled trophosome tissues was sent to the Joint Genome Institute (DOE, Walnut Creek, CA) for cDNA synthesis, library construction, and sequencing. Briefly, polyadenylated RNA was reverse transcribed with superscript reverse transcriptase using dT primer (5′-GACTAGTTCTAGATCGCGAGCGGCCGC-CCTTTTTTTTTTTTTTTVN-3′). cDNA was synthesized with *E. coli* DNA ligase, *E. coli* DNA polymerase I, and *E. coli* RNaseH. Ends were repaired with T4 DNA polymerase. An SalI adapter (5′TCGACCCACGCGTCCG-3′ and 5′-P04-CGGACGCGTGGG-3') was ligated to cDNA, and the product was digested with NotI. Digested cDNA was gel-purified, directionally ligated into SalI- and NotI-digested pCMVsport6, then transformed into ElectroMAX T1 DH10B cells. The transformation stock was plated onto 254 × 254-mm dry bioassay agar plates using glass beads. The plates were incubated for 18 h at 37 °C, then colonies were picked using a QPix2 XT colony robot picker and placed into 384-well microtiter dishes containing LB medium and 7.5% glycerol. Sequence template was prepared by rolling-circle amplification. Di-deoxy terminator sequencing was performed using BigDye terminator chemistry and an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA) on 11,520 unique clones in both the 5′ and 3′ directions.

Sequence analysis

Sequence reads were called *via* ABI and PHRED version 0.020425.c sequence analysis software and were manually verified. Sequences were trimmed and edited in Sequencher ver. 4.1 (GeneCodes), and identified using the BLAST-based analysis program BLASTX (National Center for Bioinformatics Information; NCBI; Altschul *et al.,* 1997), which compared our sequence to six non-redundant peptide sequence databases (GenBank CDS translations, RefSeq Proteins, PDB, SwissProt, PIR, and PRF). Sequences were then manually annotated on the basis of significant hits to annotated proteins (E value $< 10^{-10}$). BLAST was run between the fasta file containing all of the sequences that passed quality standards, and the KOG database (Tatusov *et al.,* 2003). Sequences with KOG E values of $< 10^{-10}$ were categorized and quantified according to the functional category of the homologous gene.

Results

Metabolite flux

Specimens of *Ridgeia piscesae* were successfully maintained in our high-pressure respirometry system (HPRS) for 6 days under *in situ* environmental conditions (6 days was the duration of the expedition, though tubeworms have been maintained for up to 4 months in previous incubations). Worms exhibited little or no evidence of net carbon uptake during the first 6 h of incubation (similar to *Riftia*, Girguis *et al.,* 2000). Subsequently ΣH_2S, ΣCO_2, O_2, and NO_3^- uptake increased, as well as proton equivalent elimination, indicating that the tubeworms were engaged in active chemoautotrophic sulfide oxidation and carbon fixation (Table 2). ΣH_2S uptake rates averaged 5.47 ± 0.37 μmol·g^{-1}·h^{-1}, also comparable to those of *Riftia* (Girguis and Childress, 2006). ΣCO_2, O_2, and NO_3^- uptake rates averaged 3.51 ± 1.76, 13.7 ± 1.9, and 1.42 ± 0.714 μmol·g^{-1}·h^{-1} respectively. Proton equivalent elimination, previously shown to be a good indicator of chemoautotrophic function (Girguis *et al.,* 2002), was also high, averaging 16.1 ± 2.6 μeq·g^{-1}·h^{-1}, with a peak of 47.5 μmol·g^{-1}·h^{-1}. The worms also deposited new tube near their gills at a rate of about 1 cm·day^{-1} during these experiments (n = 11). These uptake rates persisted for greater than 66 h, and were maintained through

Table 2

Metabolite uptake and proton-equivalent elimination by Ridgeia piscesae *during net chemoautotrophy*

Measurement (n = 5)	ΣH_2S	Oxygen	ΣCO_2	NO_3^-	H^+ equivalent elimination
Concentration (μmol l^{-1})*	218.8 ± 11.4	224 ± 9.21	5637 ± 262.5	25.3 ± 6.81	16.1 ± 2.6
Uptake (μmol·g^{-1}·h^{-1})	5.47 ± 0.37	13.7 ± 1.9	3.51 ± 1.76	1.42 ± 0.714	(μequiv.·g^{-1}·h^{-1}) NA

Mean ± standard deviation of sulfide (ΣH_2S), oxygen, total inorganic carbon (ΣCO_2) and nitrate concentrations in seawater, as well as uptake rates and proton elimination rates, by *Ridgeia piscesae* during net chemoautotrophy. Seven freshly-collected specimens of *Ridgeia* were placed into high-pressure aquaria and adjusted to produce diffuse flow-like conditions These conditions were maintained for 72 hours prior to tissue sampling and flash-freezing.

* All concentrations are in micromoles per liter, with the exception of H^+ equivalent elimination, which is given in microequivalents per gram per hour.

the end of the experiment (at which point the worms were rapidly depressurized and flash-frozen). These metabolite uptake rates and tube growth rates indicate that *R. piscesae* thrived under our experimental conditions.

Gene expression

The 11,520 unique clones obtained generated a total of 23,040 unique expressed sequence tags (ESTs) of which 19,132 contained insert and were of sufficient quality to use for BLAST analyses. Of these ESTs, 20.22% (3868) and 5.45% (1042) yielded BLAST hits with E values $< 10^{-10}$ when run against Genbank databases (see Materials and Methods) and the KOG database (Tatusov *et al.,* 2003) respectively. ESTs with KOG homologies were categorized accordingly (Fig. 2). The majority of these genes were involved with translation, biogenesis, transcription, metabolism, cell processing, and signaling mechanisms. Select transcripts representing genes from these various categories expressed in the trophosome under HPRS conditions were identified and binned to depict those transcripts with the highest abundance (Table 3). These expressed genes fall under a range of categories involved with eukaryotic cell cycle, signaling, regulation, metabolism, and stress response. Specific transcripts belonging to well-described vestimentiferan tubeworm hemoglobins involved with binding of sulfide and oxygen were also abundant in the trophosome. Key enzymes involved with metabolism were present in the EST libraries, including a previously described vestimentiferan carbonic anhydrase (DeCian *et al.,* 2003). Genes involved with dealing with cell stress were identified, including a homolog to a heat-shock protein (HSP70). We also recovered a hypothetical protein, glycoprotein, and RNA helicase that have been expressed in organisms challenged with infection or inflammation (Table 3). Transcription factors were identified along with 10 genes involved with cell signaling and regulation, including ubiquitin, a putative growth hormone, and a voltage-dependent anion channel. Genes involved with chitin interactions, a major component of the body wall, were also

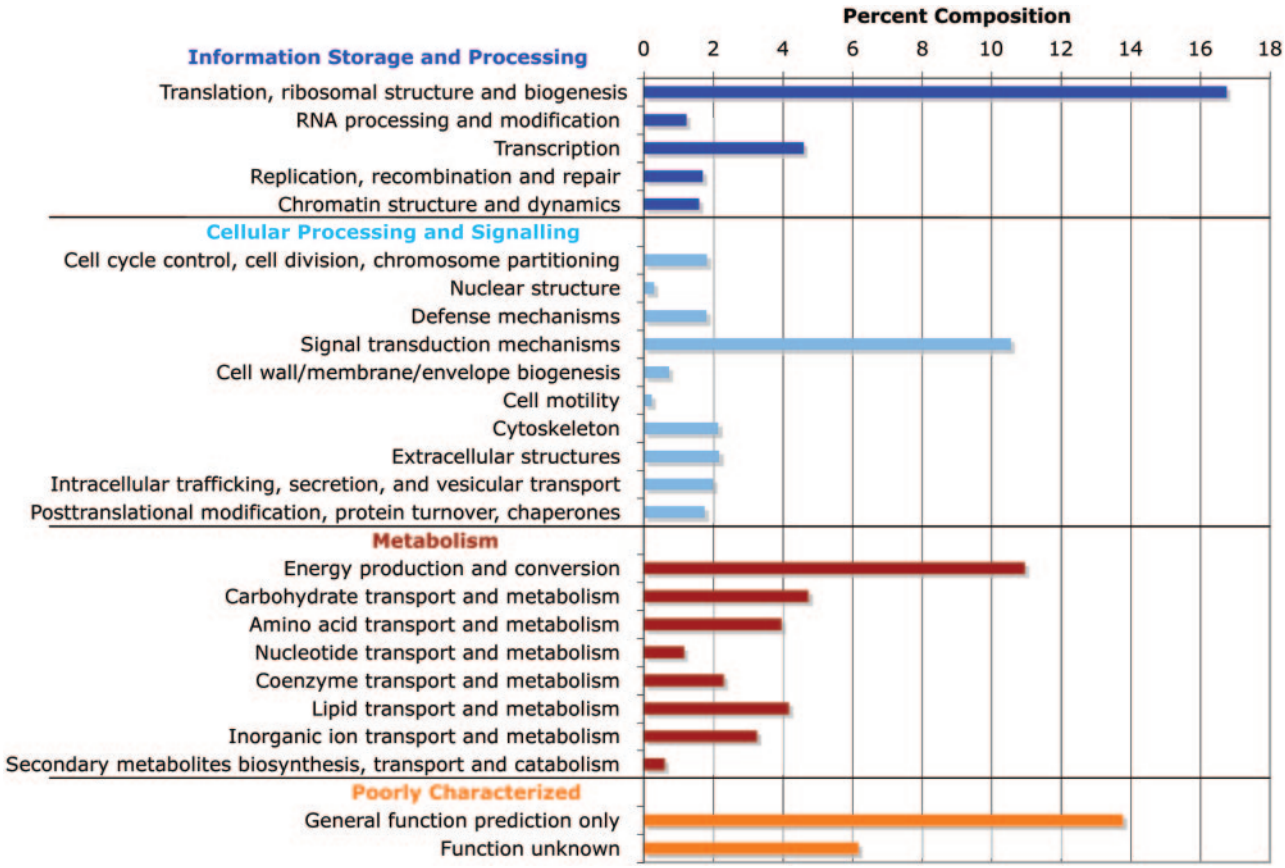

Figure 2. KOG analysis depicting all hits to the eukaryotic KOG database (Tatusov *et al.,* 2003) with E values of greater than 10^{-10}. KOG hits were categorized according to their homologs in the database. *Ridgeia* expressed sequence tags were highly diverse in function, but expression of protein biosynthesis genes, metabolic genes (in particular energy production and acid-base regulation) and signal transduction genes was very high.

Table 3

List of representative sequences from Ridgeia piscesae *expressed sequence tag libraries with known homologies and* E *values less than* E^{-10} *(* * *= 14 genes with highest representation in library)*

Putative identification	*E* value	Organism	GENBANK Accession number
Mitochondrial sequence			
16S ribosomal DNA	0	*Ridgeia piscesae*	AF315054.1
Cytochrome *c* oxidase	$1.00E^{-150}$	*Lamellibrachia satsuma*	AF342671
Oxoglutarate receptor	$1.00E^{-112}$	*Homo sapiens*	Q02978
Body wall/tube			
β-chitin-specific protein	$4.00E^{-21}$	*Riftia pachyptila*	AF266752.1
Chitinase*	$8.00E^{-91}$	*Rattus norvegicus*	Q6RY07
Cell cycling/regulation			
Ubiquitin	$4.00E^{-19}$	*Caenorhabditis elegans*	P14792
skpA (apoptosis)	$2.00E^{-29}$	*Drosophila melanogaster*	NM_166861
RF-C/Activator 1	$4.00E^{-12}$	*Homo sapiens*	AF085736.1
Leishmanolysin-like peptidase*	$2.00E^{-92}$	*Mus musculus*	Q8BMN4
Cell stress mediators			
BAT1 ATP-dependent helicase (inflammation)	$5.00E^{-29}$	*Crassostrea gigas*	AF075691.1
Heat-shock protein 70	$1.00E^{-138}$	*Caenorhabditis elegans*	P27420
Amphiphysin-like lipid raft protein (endocytosis)	$1.00E^{-34}$	*Candida dubliniensis*	AJ634665.1
Glycoprotein RsRG3-13 (*Ralstonia* infection)	$3.00E^{-10}$	*Nicotiana tabacum*	AB293950.1
Metallothionein	$1.00E^{-16}$	*Danio rerio*	BC152694
Hypothetical Protein (immune-related)	$6.00E^{-14}$	*Eristalis tenax*	AM706411.1
Mucin-5AC precursor *	$3.00E^{-10}$	*Homo sapiens*	P98088
Barrier-to-autointegration factor*	$7.00E^{-20}$	*Drosophila melanogaster*	Q9VLU0
Cytoskeleton			
β-actin*	0	*Lumbricus terrestris*	X96514.1
α-tubulin	0	*Pectinaria gouldii*	AY855263.1
β-tubulin	$2.00E^{-35}$	*Aplysia californica*	AY256661.1
Metabolism			
Carbonic anhydrase	0	*Riftia pachyptila*	AJ439711
Cytosolic malate dehydrogenase	$1.00E^{-110}$	*Gallus gallus*	Q5ZME2
NADH dehydrogenase	$1.00E^{-119}$	*Lumbricus terrestris*	Q34947
Glutamate dehydrogenase	$2.00E^{-09}$	*Salmo salar*	AJ532827.1
Glyceraldehyde 3-phosphate dehydrogenase	$8.00E^{-05}$	*Mus musculus*	BC023196.1
Oxygen/sulfide binding			
Hemoglobin (α subunit)*	$1.00E^{-175}$	*Ridgeia piscesae*	DQ414407.1
Hemoglobin (β subunit)*	$1.00E^{-135}$	*Ridgeia piscesae*	AY250083.1
Hemerythrin	$1.00E^{-11}$	*Periserrula leucophryna*	AY312845.1
Myohemerythrin*	$1.00E^{-35}$	*Theromyzon tessulatum*	Q9GYZ9
Signaling			
Allatotropin (regulation of growth hormones)	$2.00E^{-07}$	*Spodoptera frugiperda*	CAD98809
Guanine nucleotide binding factor	$4.00E^{-52}$	*Brachiostoma lancolatum*	AY130392.1
Protein kinase C1 receptor	$5.00E^{-76}$	*Dicentrarchus labrax*	DQ836932.1
KDEL endoplasmic reticulum protein retention receptor 1	$2.00E^{-47}$	*Mus musculus*	BC007146.1
Serine/Threonine kinase	$2.00E^{-17}$	*Nicotiana tabacum*	DQ459385.1
Calmodulin*	$1.00E^{-17}$	*Patinopecten* sp.	P02595
S-adenosylhomocystein hydrolase/AHCY	$2.00E^{-16}$	*Mus musculus*	BC015304.1
Neurogenic locus notch homolog protein 1*	$5.00E^{-15}$	*Danio rerio*	P46530
Transcription factors			
MADS-box	$3.00E^{-13}$	*Triticum aestivum*	DQ512346.1
Stat 4-like	$1.00E^{-16}$	*Gallus gallus*	AB275897.1
Transcription factor DBF1 (salt stress)	$6.00E^{-11}$	*Physcomitrella patens*	DQ202211.2
Unknown function			
Histidine-rich glycoprotein*	$6.00E^{-30}$	*Plasmodium lophurae*	P04929
Other transcription and translation			
Serine/arginine repetitive matrix protein 2*	$4.00E^{-22}$	*Mus musculus*	Q8BTI8
Viral			
Glycoprotein X precursor*	$1.00E^{-13}$	*Equine herpesvirus type 1 (strain AB4P)*	Q6S6WO
Immediate-early protein*	$6.00E^{-10}$	*Herpesvirus saimiri (strain 11)*	Q01042

recovered. Mitochondrial rRNA genes were highly expressed, making up 7.44% of all sequenced ESTs (Table 3).

Discussion

The data presented here offer a first glimpse into the relationship between metabolite flux, chemoautotrophic activity, and host bacteriocyte gene expression in a chemoautotrophic symbiosis. As previously mentioned, studying organisms maintained in experimental systems affords a unique opportunity to relate gene expression to experimental conditions. In this case, *Ridgeia piscesae* metabolite uptake rates, in particular ΣH_2S and ΣCO_2, were among the highest measured to date, comparable to those of extremely productive *R. pachyptila* and exceeding rates measured in free-living associations (*e.g.,* Nelson and Hagen, 1995). In addition, *R. piscesae* sustained these carbon uptake rates continuously for more than 66 h, underscoring the capacity of the species for rapid and continuous net carbon acquisition and fixation. These data are the first measurements of *R. piscesae* metabolite flux, and support previous suppositions that hydrothermal vent vestimentiferan tubeworms are tremendous primary producers, comparable in body growth and mass specific rates to productive photoautotrophs (Lutz *et al.,* 1994; Girguis and Childress, 2006).

These metabolite flux rates also provide significant context for our gene expression libraries. Because of the ability to rapidly preserve tissues post-experimentation, the ESTs recovered in our libraries represent genes expressed by *R. piscesae* bacteriocytes (and other trophosomal tissues) during high chemoautotrophic activity (Fig. 2; Table 3). These ESTs provide a glimpse into host molecular processes underlying tremendous primary productivity. With respect to metabolism and homeostasis, we have recovered genes that encode for enzymes involved in acid-base regulation and inorganic carbon acquisition (in particular carbonic anhydrase, or CA). This finding corroborates previous studies—from biochemical assays to gene sequencing—that emphasize the importance of CA in enabling vestimentiferans to cope with the highly variable changes in environmental pH, as well as with the rapid production of protons resulting from symbiont sulfide oxidation (Kochevar and Childress, 1995; Goffredi *et al.,* 1999a, b; De Cian *et al.,* 2003; Sanchez *et al.,* 2007). In particular, sulfate and proton equivalents are the primary end products of symbiont sulfide oxidation (Girguis *et al.,* 2002), and active vestimentiferan symbionts generate protons at unprecedented rates (up to 119 $\mu eq \cdot g \cdot h^{-1}$; Girguis *et al.,* 2002). Additionally, vestimentiferans must acquire inorganic carbon and transport it to the trophosome, while concurrently eliminating protons at rates sufficient to prevent metabolic acidosis. CA plays a significant role in transporting inorganic carbon (Goffredi *et al.,* 1999a, b), while also buffering and indirectly transporting protons from the bacteriocytes and trophosome into the vascular blood, where gill H^+ ATPases eventually eliminate them into the environment (Girguis *et al.,* 2002). We also found high representation of genes involved with oxygen and sulfide binding, namely hemoglobin alpha and beta subunits, as well as a hemerythrin (an iron-containing non-heme protein that binds oxygen). Blood- and tissue-borne binding proteins have been shown to increase the transport of oxygen (and in this case sulfide; Arp and Childress, 1983), as well as the rate of diffusion into tissues (Sidell, 1998). All together, expression of these genes suggests that *Ridgeia* supports high productivity by further facilitating the diffusion of inorganic carbon, oxygen, and sulfide into the bacteriocytes, and the elimination of protons out of the bacteriocytes.

The majority of the genes recovered from the library, however, are implicated in mediating cell stress and governing cell regulation (Fig. 2, Table 3). Heat-shock proteins (such as the HSP70 that was recovered in our libraries) have been shown to be expressed during host-cell signaling cascades that occur during bacterial infection (Davies *et al.,* 2006). We also identified a Bat1 homolog belonging to the DEAD-box family of RNA-binding proteins, previously demonstrated to be an important mediator of inflammation (Alcock *et al.,* 2001). The identification of genes potentially encoding for viral precursors is very intriguing, as little is known about the role that viruses play in hydrothermal vent systems. A recent study showed that viruses are found in high densities at hydrothermal vents and likely cause significant microbial mortality (Ortmann and Suttle, 2005). However, the role of viruses in chemoautotrophic symbioses remains to be examined, though they could be a major factor in tubeworm mortality.

In addition to cell stress response and viral protein precursors, many genes were found that are known to be involved with regulating cell cycle, apoptosis, and cell growth; these genes include ubiquitin and homologs for skpA and RF-C activator 1, known mediators of cell cycle and replication in *Drosophila* (Murphy, 2003; Tsuchiya *et al.,* 2007). Development and cell patterning genes such as a brachyenteron-like homolog, important in insect development and a member of the MADS-box family of transcription factors, implicated in development and differentiation in *Rhizobium* infections of root nodules, were also recovered (Heard and Dunn, 1995; Messengy and Dubois, 2003). Although the regulation of bacteriocyte development has not been described for any vestimentiferan, morphological studies suggest that a complicated bacteriocyte and symbiont cell cycle occurs in these associations (Bright and Sorgo, 2003). In *Riftia*, the endosymbionts appear to change from a rod-like morphotype near the axial blood vessel to larger, rotund cocci at the periphery of the trophosome lobule. The host bacteriocytes also change along with the symbionts, having the morphological appearance of undergoing apoptosis near the periphery of the lobules. It has

been suggested that a significant fraction of the symbiont population may also be digested at any given time by the host (Bright and Sorgo, 2003).

These morphological observations suggest that the expressed gene repertoire in the trophosome may be responsible for far more than simply enhanced substrate delivery. Many of these described cell cycle and stress response pathways require well-established signaling cascades, and this study finds expression of such genes (such as a homolog to the neuropeptide growth hormone allatotropin as well as several involved with phosphorylation-based cellular signaling, see Table 3; Elekonich and Horodyski, 2003; Cooper *et al.,* 2006). In such a closed and intimate association as that between hydrothermal vent tubeworms and their bacterial symbionts (*e.g.,* bacterial symbionts in the adult host are never in contact with the external milieu), regulation of bacteriocyte and symbiont cell density and division is likely paramount to the association's survival. The host must promote chemoautotrophic activity by its symbionts while preventing symbiont overgrowth to its detriment. Genes involved with regulating cell stress, cycle, and signaling are likely to be important in mediating these processes. Ongoing experiments are employing quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and mRNA capture to better quantify the changes in host bacteriocyte gene expression between individuals of *Ridgeia* exhibiting differences in net chemoautotrophy.

The data presented here focus on the suite of genes expressed by the host bacteriocytes and associated tissues during elevated chemoautotrophic activity by the intracellular symbionts. Although this illustrates how the host might sustain substrate availability while potentially regulating host transcriptome or symbiont cell cycle, it provides an incomplete story in the absence of data on symbiont gene expression. We are currently developing approaches to recover and sequence symbiont mRNA from these same experimental organisms, and to ally them to host gene expression. The genes recovered from genomes of two chemoautotrophic symbionts (*Riftia pachyptila* and *Ruthia magnifica*; Newton *et al.,* 2007; Robidart *et al.,* 2008) can help us identify their mode of action. Microbial-mediated apoptosis has been shown to be an important function in both symbiotic (Foster and McFall-Ngai, 1998; Heddi *et al.,* 2005) and pathogenic associations (Hofman *et al.,* 2004; Guiney, 2005; Zhang and Bliska, 2005) and may play an important role in these vestimentiferan symbioses as well. Other enhanced COG (clusters of orthologous groups) categories in the *Riftia* symbiont genome corroborate the indications of high metabolic rates and cell turnover in this study. As with the host bacteriocyte transcriptome, quantitative and higher resolution analyses will enable us to better understand the mode of communication between host and symbiont and the methods of regulating chemoautotrophic function and host and symbiont growth.

Although model systems like the insect-bacteriocyte, squid light organ, and *Rhizobium*/root nodule associations have provided insight into the types of genes and proteins that are involved in regulating host-symbiont cell cycle and cell-cell signaling (Gage, 2004; Heddi *et al.,* 2005; Moran *et al.,* 2005; Visick and Ruby, 2006), no model system has yet been identified to study metazoan-chemoautotrophic symbiont interactions. The nature of vestimentiferan associations—namely their efficacy as primary producers and their amenability to shipboard experimentation—make them an effective model system for studying these interactions. Despite the added challenges of working with complex experimental apparatus, the benefits of studying these associations in the carefully controlled conditions on board ship cannot be undervalued. Resulting from the first study of a chemoautotrophic symbiosis (or any deep-sea organism) that couples metabolite flux and transcriptomics, the approaches and results presented here have challenged our preconceptions about these associations, but have illustrated the potential range of experiments and wealth of information that can be gathered in such studies. The expressed genes recovered so far represent a fraction of a larger pool whose members are currently being sequenced, but already offer insight into the types of mechanisms that may be regulating these tubeworm symbioses. We believe that coupling experimental physiology with genomics and proteomics offers the best means by which we can further our understanding of these extraordinary organisms, and promises to be an exciting avenue of present and future research.

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Literature Cited

Alcock, R. J., J. H. Williams, and P. Price. 2001. The central MHC gene, BAT1, may encode a protein that down-regulates cytokine production. *Genes Cells* **6:** 487–494.

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25:** 3389–3402.

Arndt, C., D. Schiedek, and H. Felbeck. 1998. Anaerobiosis in the hydrothermal vent tube-worm *Riftia pachyptila*. In *Proceedings of the First International Symposium on Deep-Sea Hydrothermal Vent Biology: Funchal, Madeira, Portugal, 20–24 October 1997. Cah Biol. Mar.* **39:** 271–273.

Arp, A. J., and J. J. Childress. 1983. Sulfide binding by the blood of the hydrothermal vent tube worm *Riftia pachyptila*. *Science* **219:** 295–297.

Bright, M., and A. Sorgo. 2003. Ultrastructural reinvestigation of the trophosome in adults of *Riftia pachyptila* (Annelida, Siboglinidae). *Invertebr. Biol*. **122:** 347–368.

Bult, C. J., O. White, G. J. Olsen, L. Zhou, R. D. Fleischmann, G. G. Sutton, J. A. Blake, L. M. FitzGerald, R. A. Clayton, J. D. Gocayne, *et al.* 1996. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. *Science* **273:** 1058–1073.

Campbell, B. J., C. Jeanthon, J. E. Kostka, G. W. Luther III, and S. C. Cary. 2001. Growth and phylogenetic properties of novel bacteria belonging to the epsilon subdivision of the Proteobacteria enriched from *Alvinella pompejana* and deep-sea hydrothermal vents. *Appl. Environ. Microbiol.* **67:** 4566–4572.

Campbell, B. J., J. L. Stein, and S. C. Cary. 2003. Evidence of chemolithoautotropy in the bacterial community associated with *Alvinella pompejana,* a hydrothermal vent polychaete. *Appl. Environ. Microbiol*. **69:** 5070–5078.

Carney, S. L., J. F. Flores, K. M. Orobona, D. A. Butterfield, C. R. Fisher, and S. W. Schaeffer. 2006. Environmental differences in hemoglobic gene expression in the hydrothermal vent tubeworm, *Ridgeia piscesae*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **146:** 326–337.

Cavanaugh, C. M., S. L. Gardiner, M. L. Jones, H. W. Jannasch, and J. B. Waterbury. 1981. Prokaryotic cells in the hydrothermal vent tube worm *Riftia pachyptila*: possible chemoautotrophic symbionts. *Science* **213:** 340–342.

Chevaldonne, P., D. Desbruyeres, and M. Le Haitre. 1991. Time-series of temperature from three deep-sea hydrothermal vent sites. *Deep-Sea Res. A* **38:** 1417–1430.

Childress, J. J., A. J. Arp, and C. R. Fisher, Jr. 1984. Metabolic and blood characteristics of the hydrothermal vent tubeworm *Riftia pachyptila*. *Mar. Biol*. **83:** 109–124.

Childress, J. J., C. R. Fisher, J. A. Favuzzi, R. E. Kochevar, N. K. Sanders, and A. M. Alayse. 1991. Sulfide-driven autotrophic balance in the bacterial symbiont-containing hydrothermal vent tubeworm, *Riftia pachyptila* Jones. *Biol. Bull.* **180:** 135–153.

Cline, J. D. 1969. Spectrophotometric determination of hydrogen sulfide in natural seawaters. *Limnol. Oceanogr.* **3:** 454–458.

Cooper, B. J., B. Key, A. Carter, N. Z. Angel, D. N. J. Hart, and M. Kato. 2006. Suppression and overexpression of adenosylhomocysteine hydrolase-like protein 1 (AHCYL1) influences zebrafish embryo development: a possible role for AHCYL1 in inositol phospholipid signaling. *J. Biol. Chem.* **281:** 22471–22484.

Corliss, J. B., J. Dymond, L. I. Gordon, J. M. Edmond, R. P. V. Herzen, R. D. Ballard, K. Green, D. Williams, A. Bainbridge, K. Crane, and T. H. van Andel. 1979. Submarine thermal springs on the Galapagos Rift. *Science* **203:** 1073–1083.

Daneri, G., V. Dellarossa, R. Quiñones, B. Jacob, P. Montero, and O. Ulloa. 2000. Primary production and community respiration in the Humboldt Current System off Chile and associated oceanic areas. *Mar. Ecol. Prog. Ser.* **197:** 41–49.

Davies, E. L., M. M. Bacelar, M. J. Marshall, E. Johnson, T. D. Wardle, S. M. Andrew, and J. H. Williams. 2006. Heat shock proteins form part of a danger signal cascade in response to lipopolysaccharide and GroEL. *Clin. Exp. Immunol.* **145:** 183–189.

DeBevoise, A. E., and G. L. Taghon. 1988. RNA:DNA ratios of the hydrothermal-vent vestimentiferans *Ridgeia piscesae* and *R. phaeophiale* indicate variations in growth rates over small spatial scales. *Mar. Biol.* **97:** 421–426.

De Cian, M. C., A. C. Andersen, X. Bailly, and F. H. Lallier. 2003. Expression and localization of carbonic anhydrase and ATPases in the symbiotic tubeworm *Riftia pachyptila*. *J. Exp. Biol*. **206:** 399–409.

Elekonich, M. M., and F. M. Horodyski. 2003. Insect allatotropins belong to a family of structurally-related myoactive peptides present in several invertebrate phyla. *Peptides* **24:** 1623–1632.

Felbeck, H. 1981. Chemoautotrophic potential of the hydrothermal vent tube worm, *Riftia pachyptila* Jones (Vestimentifera). *Science* **213:** 336–338.

Flores, J. F., C. R. Fisher, S. L. Carney, B. N. Green, J. K. Freytag, S. W. Schaeffer, and W. E. Royer, Jr. 2005. Sulfide binding is mediated by zinc ions discovered in the crystal structure of a hydrothermal vent tubeworm hemoglobin. *Proc. Natl. Acad. Sci. USA* **102:** 2713–2718.

Foster, J. S., and M. J. McFall-Ngai. 1998. Induction of apoptosis by cooperative bacteria in the morphogenesis of host epithelial tissues. *Dev. Genes Evol.* **208:** 295–303.

Gage, D. J. 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol. Mol. Biol. Rev.* **68:** 280–300.

Gaill, F., B. Shillito, F. Menard, G. Goffinet, and J. J. Childress. 1997. Rate and process of tube production by the deep-sea hydrothermal vent tubeworm *Riftia pachyptila. Mar. Ecol. Prog. Ser.* **148:** 135–143.

Girguis, P. R., and J. J. Childress. 2006. Metabolite stoichiometry and chemoautotrophic function of the hydrothermal vent tubeworm *Riftia pachyptila*: responses to environmental variations in substrate concentrations and temperature. *J. Exp. Biol*. **209:** 3516–3528.

Girguis, P. R., J. J. Childress, M. Pospesel, N. T. Desaulniers, F. Zal, and H. Felbeck. 2000. Fate of nitrate acquired by the hydrothermal vent tubeworm *Riftia pachyptila*. *Appl. Environ. Microbiol.* **66:** 2783–2790.

Girguis, P. R., J. J. Childress, J. A. Freytag, K. A. Klose, and R. Stuber. 2002. Effects of metabolite uptake on proton-equivalent elimination by two species of deep-sea vestimentiferan tubeworm, *Riftia pachyptila* and *Lamellibrachia* cf *luymesi*. *J. Exp. Biol.* **205:** 3055–3066.

Goffredi, S. K., P. R. Girguis, J. J. Childress, and N. T. Desaulniers. 1999a. Physiological functioning of carbonic anhydrase in the hydrothermal vent tubeworm *Riftia pachyptila*. *Biol. Bull.* **196:** 257–264.

Goffredi, S. K., J. J. Childress, F. H. Lallier, and N.T. Desaulniers. 1999b. The ionic composition of the hydrothermal vent tube worm *Riftia pachyptila*: evidence for the elimination of SO_4^{2-} and H^+ and for a Cl^-/HCO^{3-} Shift. *Physiol. Biochem Zool.* **72:** 296–306.

Govenar, B., M. Freeman, D. C. Bergquist, G. A. Johnson, and C. R. Fisher. 2004. Composition of a one-year-old *Riftia pachyptila* community following a clearance experiment: insight to succession patterns at deep-sea hydrothermal vents. *Biol. Bull*. **207:** 177–182.

Govenar, B., N. Le Bris, S. Gollner, J. Glanville, A. B. Aperghis, S. Hourdez, and C. R. Fisher. 2005. Epifaunal community structure associated with *Riftia pachyptila* in chemically different hydrothermal vent habitats. *Mar. Ecol. Prog. Ser.* **305:** 67–77.

Guiney, D. G. 2005. The role of host cell death in *Salmonella* infections. *Curr. Top. Microbiol. Immunol.* **289:** 2835–2840.

Heard, J., and K. Dunn. 1995. Symbiotic induction of MADS-box gene during development of alfalfa root nodules. *Proc. Natl. Acad. Sci. USA* **92:** 5273–5277.

Heddi, A., A. Vallier, C. Anselme, H. Xin, Y. Rahbe, and F. Wackers. 2005. Molecular and cellular profiles of insect bacteriocytes: mutualism and harm at the initial evolutionary step of symbiogenesis. *Cell Microbiol*. **7:** 293–305.

Hessler, R. R., and W. M. Smithey, Jr. 1983. The distribution and community structure of megafauna at the Galapagos Rift hydrothermal

vents. Pp. 735–770 in *Hydrothermal Processes at Seafloor Spreading Centers,* P. A. Rona, K. Bostrom, L. Laubier, and J. K. L. Smith, eds. Plenum Press, New York,.

Hofman, P., B. Waidner, V. Hofman, S. Bereswill, P. Brest, and M. Kist. 2004. Pathogenesis of *Helicobacter pylori* infection. *Helicobacter* **9 Suppl. 1:** 15–22.

Johnson, K. S., C. L. Beehler, C. M. Sakamoto-Arnold, and J. J. Childress. 1986. In situ measurements of chemical distributions in a deep-sea hydrothermal vent field. *Science* **231:** 1139–1141.

Johnson, K. S., J. J. Childress, and C. L. Beehler. 1988. Short-term temperature variability in the Rose Garden [eastern Pacific Ocean] hydrothermal vent field: an unstable deep-sea environment. *Deep-Sea Res. A* **35:** 1711–1722.

Jones, M. L. 1981. *Riftia pachyptila*, new genus, new species, the vestimentiferan tubeworm from the Galapagos Rift geothermal vents. *Proc. Biol. Soc. Wash.* **93:** 1295–1313.

Jones, M. L. 1985. On the Vestimentifera, new phylum: six new species, and other taxa, from the hydrothermal vents and elsewhere. In *The Hydrothermal Vents of the Eastern Pacific: an Overview,* M. L Jones, ed. *Bull. Biol. Soc. Wash.* **6:** 117–158.

Kochevar, R. E., and J. J. Childress. 1995. Carbonic anhydrase in deep-sea chemoautotrophic symbioses. *Mar. Biol.* **125:** 375–383.

Le Bris, N., B. Govenar, C. Le Gall, and C. R. Fisher. 2005. Variability of physico-chemical conditions in 9°50′N EPR diffuse flow vent habitats. *Mar. Chem.* **98:** 167–182.

Luther, G.W. III, T. F. Rozan, M. Talliefert, D. B. Nuzzio, C. A. Di Meo, T. M. Shank, R. A. Lutz, and S. C. Cary. 2001. Chemical speciation drives hydrothermal vent ecology. *Nature* **410:** 813–816.

Lutz, R., T. Shank, D. Fornari, M. Lilley, K. Von Damm, R. Haymon, and D. Desbruyeres. 1994. Rapid rates of colonization and growth of vestimentiferan tube worms at newly-formed hydrothermal vents. *Nature* **371:** 663–664.

Markert, S., C. Arndt, H. Felbeck, D. Becher, S. M. Sievert, M. Hugler, D. Albrecht, D. J. Robidart, S. Bench, S. Feldman, *et al.* 2007. Physiological proteomics of the uncultured endosymbiont of *Riftia pachyptila*. *Science* **315:** 247–250.

Messenguy, F., and E. Dubois. 2003. Role of MADS box proteins and their cofactors in combinatorial control of gene expression and cell development. *Gene* **316:** 1–21.

Moran, N. A., P. H. Degnan, S. R. Santos, H. E. Dunbar, and H. Ochman. 2005. The players in a mutualistic symbiosis: insects, bacteria, viruses, and virulence genes. *Proc. Natl. Acad. Sci. USA* **102:** 16919–16926.

Mullineaux, L. S., C. H. Peterson, F. Micheli, and S. W. Mills. 2003. Successional mechanism varies along a gradient in hydrothermal fluid flux at deep-sea vents. *Ecol. Monogr.* **73**: 523–542.

Murphy, T. D. 2003. *Drosophila skpA,* a component of SCF ubiquitin ligases, regulates centrosome duplication independently of cyclin E accumulation. *J. Cell Sci.* **116:** 2321–2332.

Nelson, D. C., and K. D. Hagen. 1995. Physiology and biochemistry of symbiotic and free-living chemoautotrophic sulfur bacteria. *Am. Zool.* **35:** 91–101.

Newton, I. L., T. Woyke, T. A. Auchtung, G. F. Dilly, R. J. Dutton, M. C. Fisher, K. M. Fontanez, E. Lau, F. J. Stewart, P. M. Richardson, *et al.* 2007. The *Calyptogena magnifica* chemoautotrophic symbiont genome. *Science* **315:** 998–1000.

Ortmann, A. C., and C. A. Suttle. 2005. High abundances of viruses in a deep-sea hydrothermal vent system indicates viral mediated microbial mortality. *Deep-Sea Res. I* **52:** 1515–1527.

Robidart, J. C., S. R. Bench, R. Feldman, S. Podell, A. Novoradovsky, T. Gaasterland, and H. Felbeck. 2008. Metabolic versatility of the *Riftia pachyptila* endosymbiont revealed through metagenomics. *Environ. Microbiol.* **10:** 727–737.

Rouse, G. W. 2001. A cladistic analysis of *Siboglinidae caullery*, 1914 (Polychaeta, Annelida): formerly the phyla Pogonophora and Vestimentifera. *Zool. J. Linn.. Soc.* **132:** 55–80.

Sanchez, S., S. Hourdez, and F. Lallier. 2007. Identification of proteins involved in the functioning of *Riftia pachyptila* symbiosis by Subtractive Suppression Hybridization. *BMC Genomics* **8:** 337.

Sarrazin, J., and S. K. Juniper. 1999. Biological characteristics of a hydrothermal edifice mosaic community. *Mar. Ecol. Prog. Ser.* **185:** 1–19.

Schmidt, K., A. Koschinsky, D. Garbe-Schönberg, L. M. de Carvalho, and R. Seifert. 2007. Geochemistry of hydrothermal fluids from the ultramafic-hosted Logatchev hydrothermal field, 15°N on the Mid-Atlantic Ridge. *Chem. Geol.* **242:** 1–21.

Scott, K. M., S. M. Sievert, F. N. Abril, L. A. Ball, C. J. Barrett, R. A. Blake, A. J. Boller, P. S. Chain, J. A. Clark, C. R. Davis, *et al.* 2006. The genome of deep-sea vent chemolithoautotroph *Thiomicrospira crunogena* XCL-2. *Plos Biol.* **4:** 383.

Shank, T. M., D. Fornari, K. L. Von Damm, M. D. Lilley, R. M. Haymon, and R. A. Lutz. 1998. Temporal and spatial patterns of biological community development at nascent deep-sea hydrothermal vents (9°50′ N, East Pacific Rise). *Deep-Sea Res*. II **45:** 465–515.

Sidell, B. D. 1998. Intracellular oxygen diffusion: the roles of myoglobin and lipid at cold body temperature. *J. Exp. Biol.* **201:** 1119–1128.

Stewart, F. J., and C. M. Cavanaugh. 2006. Symbiosis of thioautotrophic bacteria with *Riftia pachyptila. Prog. Mol. Subcell. Biol*. **41:** 197–225.

Tatusov, R. L., N. D. Fedorova, J. D. Jackson, A. R. Jacobs, B. Kiryutin, E. V. Koonin, D. M. Krylov, R. Mazurnder, S. L. Mekhedov, A. N. Nikolskaya, *et al.* 2003. The COG database: an updated version includes eukaryotes. *BMC Bioinformatics* **4:** 41–55.

Teske, A., and D. C. Nelson. 2004. The genera *Beggiatoa* and *Thioploca.* In *The Prokaryotes: an Evolving Electronic Resource for The Microbiological Community,* 3rd ed., release 3.17. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt, eds., Springer, New York.

Tsuchiya, A., Y. H. Inoue, H. Ida, Y. Kawase, K. Okudaira, K. Ohno, H. Yoshida, and M. Yamaguchi. 2007. Transcriptional regulation of the *Drosophila rfc1* gene by the DRE-DREF pathway. *FEBS J.* **274:** 1818–1832.

Urabe, T., E. T. Baker, J. Ishibashi, R. A. Feely, K. Marumo, G. J. Massoth, A. Maruyama, K. Shitashima, K. Okamura, J. E. Lupton, *et al.* 1995. The effect of magmatic activity on hydrothermal venting along the superfast-spreading East Pacific Rise. *Science* **269:** 1092–1095.

Urcuyo, I. A., G. J. Massoth, D. Julian, and C. R. Fisher. 2003. Habitat, growth and physiological ecology of a basaltic community of *Ridgeia piscesae* from the Juan de Fuca Ridge. *Deep-Sea Res. I* **50:** 763–780.

Urcuyo, I. A., D. C. Bergquist, I. R. MacDonald, M. VanHorn, and C. R. Fisher. 2007. Growth and longevity of the tubeworm *Ridgeia piscesae* in the variable diffuse flow habitats of the Juan de Fuca Ridge. *Mar. Ecol. Prog. Ser.* **344:** 143–157.

Visick, K. L., and E. G. Ruby. 2006. *Vibrio fischeri* and its host: it takes two to tango. *Curr. Opin. Microbiol.* **9:** 632–638.

Von Damm, K. L. 1990. Seafloor hydrothermal activity: black smoker chemistry and chimneys. *Annu. Rev. Earth Planet. Sci.* **18:** 173–204.

Zhang, Y., and J. B. Bliska. 2005. Role of macrophage apoptosis in the pathogenesis of *Yersinia*. *Curr. Top. Microbiol. Immunol*. **289:** 151–173.

Zhu, W., C. I. Reich, G. J. Olsen, C.S. Giometti, and J.R. Yates. 2004. Shotgun proteomics of *Methanococcus jannaschii* and insights into methanogenesis. *J. Proteome Res.* **3:** 538–548.