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Accessibility
Metatranscriptomics reveal differences in in situ energy and nitrogen metabolism among hydrothermal vent snail symbionts

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Despite the ubiquity of chemoautotrophic symbioses at hydrothermal vents, our understanding of the influence of environmental chemistry on symbiont metabolism is limited. Transcriptomic analyses are useful for linking physiological poise to environmental conditions, but recovering samples from the deep sea is challenging, as the long recovery times can change expression profiles before preservation. Here, we present a novel, in situ RNA sampling and preservation device, which we used to compare the symbiont metatranscriptomes associated with Alviniconcha, a genus of vent snail, in which specific host–symbiont combinations are predictably distributed across a regional geochemical gradient. Metatranscriptomes of these symbionts reveal key differences in energy and nitrogen metabolism relating to both environmental chemistry (that is, the relative expression of genes) and symbiont phylogeny (that is, the specific pathways employed). Unexpectedly, dramatic differences in expression of transposases and flagellar genes suggest that different symbiont types may also have distinct life histories. These data further our understanding of these symbionts’ metabolic capabilities and their expression in situ, and suggest an important role for symbions in mediating their hosts’ interaction with regional-scale differences in geochemistry.

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Introduction

Endosymbioses among marine invertebrates and chemoautotrophic bacteria have a key role in the ecology and biogeochemistry of deep-sea hydrothermal vents and similar environments. Symbionts derive energy by oxidizing reduced compounds (for example, sulfide, methane, hydrogen) and fix inorganic carbon, providing nutrition to their hosts (Felbeck, 1981; Fisher and Childress, 1984; Childress et al., 1986, 1991; Girguis and Childress, 2006; Petersen et al., 2011). Much is known about host biochemical and morphological adaptations to both their symbionts and the environment (reviewed in Stewart et al., 2005; Childress and Girguis, 2011).

Surprisingly less, however, is known about the relationship between symbiont physiology and the environment, in particular how variations in environmental geochemistry influence symbiont metabolic activity and, in turn, how this affects the ecology of the animal–microbe association. The symbioses between the deep-sea snail Alviniconcha and its chemoautotrophic symbionts afford a unique opportunity to examine these relationships. Alviniconcha are prosannid gastropods that are indigenous to vents in the Western Pacific and Indian Ocean and harbor chemoautotrophic symbionts within host cells located in the gill (Suzuki et al., 2006). At the Eastern Lau Spreading Center in the southwestern Pacific (ELSC, Supplementary Figure S1), genetically distinct Alviniconcha ‘types’ (likely cryptic species) form associations with three lineages of chemoautotrophic Proteobacteria: two \(\gamma\)-proteobacteria (termed \(\gamma-1\) and \(\gamma\)-Lau) and an \(\varepsilon\)-proteobacterium (Beinart et al., 2012). A recent study of these host-symbiont associations (hereafter referred to as holobionts), found striking patterns of distribution along the ~300 km length of the ELSC, wherein snails hosting \(\varepsilon\)-proteobacteria dominated the northern vent fields and those hosting \(\gamma\)-proteobacteria dominated the southern fields (Beinart et al., 2012). Vent fluids also showed marked changes in geochemistry along this range,
with substantially elevated hydrogen and hydrogen sulfide concentrations in the northernmost fields (Mottl et al., 2011; Beinart et al., 2012). This unprecedented pattern of holobiont distribution across this 300 km spreading center suggests a link between symbiont physiology and the environment; namely that differences in the availability of reduced compounds may influence the realized niche of the holobionts as a function of their symbionts’ metabolic capacity.

Our understanding of these observed patterns would be facilitated by analyses that reveal the symbionts’ physiological poise in situ. Transcriptomic studies have been used to relate changes in gene expression to environmental conditions (Gracey, 2007; Gracey et al., 2008). Previous studies have also used transcriptomics to study gene expression in both host and chemosynthetic symbionts (Harada et al., 2009; Stewart et al., 2011; Wendeberg et al., 2012). However, using transcriptomics to study patterns of gene expression in the deep sea (that is, in situ) is especially challenging. Organisms are typically held in ambient seawater during sampling and recovery, so their transcriptional profiles likely change in the hours between collection and preservation (Wendeberg et al., 2012).

To better examine the relationship between Alviniconcha symbiont physiology and the observed regional-scale differences in geochemistry, we developed a novel in situ sampling and preservation system that allowed us to quickly homogenize and preserve holobionts at the seafloor, simultaneously stopping transcription and stabilizing nucleic acids for downstream analysis. Using this device, we collected individual Alviniconcha from four vent fields spanning the previously observed geochemical gradient along the ELSC. Gene expression analyses revealed key differences among symbiont types in the expression of genes relating to hydrogen and sulfur oxidation. In contrast, similarities in patterns of gene expression relating to nitrogen metabolism—which deviate from canonical models of nitrate reduction—suggest a potentially unique strategy of nitrogen utilization that is shared among these symbionts. We also observed differences in gene expression that may be relevant to the maintenance and transmission of these associations. Our results clearly illustrate differences in physiological poise among these symbiont types, underscore the likely role of symbiont physiology in structuring holobiont distribution, and provide insights into how these phylogenetically distinct symbionts have evolved to exploit resources and niches in these highly dynamic environments.

**Materials and methods**

**Instrument design**

To preserve holobiont RNA in situ, we designed a sample container/homogenizer termed the In Situ Mussel And Snail Homogenizer (ISMASH; Figures 1a and b). The ISMASH consists of a 1 l stainless steel blending cylinder, the bottom of which contains a rotating blade assembly and preservative inlet. The top is open to permit sample insertion but is sealed post-collection via a magnetically-latched lid equipped with a one-way check valve. The ISMASH can be operated at any depth attainable by the submersible. During operations, the ISMASH is deployed open and empty on the submersible’s working platform. When individual specimens are deposited in the cylinder, the operator places the magnetic lid on the cylinder, and RNALater (Ambion Inc., Grand Island, NY, USA) is pumped in from the bottom, displacing the less-dense seawater through the lid’s check valve. After pumping sufficient RNALater to ensure thorough flushing, a hydraulic motor actuates the blade assembly and the sample is homogenized. For a complete instrument description and operational procedures, see Supplementary Information.

**Sample collection**

Alviniconcha holobionts (Figure 1c) were collected along the ELSC using the ROV Jason II aboard the R/V Thomas G Thompson during expedition TM-235 in 2009. One snail was collected randomly from among large aggregations at each of the four vent fields (Kilo Moana and Tow Cam in the north, ABE and Tu’i Malila in the south; Table 1, Supplementary Figure S1), then homogenized in situ. Time limitations prohibited additional sampling. Homogenization was completed within 4–10 min of collection. Upon recovery, the ~11 homogenates were carefully transferred to sterile glass jars, incubated overnight at 4 °C, and then frozen and maintained at −20 °C for ~5 months before extraction.

**Nucleic acid extraction, library preparation, and sequencing**

Before extraction, homogenates were thawed and rehomogenized in a clean, sterile blender (Waring Inc., Torrington, CT, USA) to maximize uniformity. One 2-ml aliquot was taken from each sample and centrifuged at 14 000 x g for 10 min in a refrigerated centrifuge. RNA was extracted from the pellet using TRIzol (Invitrogen Inc., Grand Island, NY, USA) per the manufacturer’s protocol. After each extraction, RNA was assessed with an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA) to determine concentration and integrity.

To maximize mRNA representation in our metatranscriptomic libraries, we preferentially removed eukaryotic and bacterial ribosomal RNA (rRNA) using sample-specific rRNA probes as in Stewart et al. (2010). Each of the four libraries was sequenced in a separate, gasketed quadrant using Titanium chemistry, yielding a full plate on a
DNA was extracted from an additional 2 ml aliquot per sample using a DNeasy Blood & Tissue kit (Qiagen Inc., Venlo, Netherlands) per the manufacturer’s protocol. DNA samples were used to assess host and symbiont identities, as well as microbial diversity (while the gill endosymbionts are typically monocultures, our technique would also include any epibionts or other microbes associated with the snail). We confirmed host genotypes by sequencing 500 bp of the mitochondrial cytochrome oxidase I gene (as in Beinart et al., 2012; Table 1). The identity and abundances of the major symbiont types were assessed using SYBR Green qPCR assays on a Mx3005P real-time thermal cycler (Stratagene Inc., Santa Clara, CA, USA), using previously described primers and protocols (Beinart et al., 2012). Microbial diversity was assessed via pyrosequencing of the V1–V3 region of the bacterial 16S rRNA gene (Dowd et al., 2008).

Metatranscriptomic analyses

Sequences were filtered of rRNA sequences using BLASTN (cutoff bit score = 50) against a custom database of rRNA sequences derived from the SILVA LSU and SSU databases and microbial genomes (Pruesse et al., 2007). Non-rRNA reads were further filtered for potentially artifactual duplicate sequences (reads of equal length sharing 100% sequence identity) using custom scripts as in the study of Gomez-Alvarez et al. (2009). The remaining reads were annotated using BLASTX against the NCBI nr database (as of 28 April 2011; bit score cutoff = 50). BLASTX results were examined for taxonomic representation, gene content, and functional pathways in MEGAN (Huson et al., 2011).

Given the representation of both host and symbiont sequences in the libraries, we analyzed reads annotated as eukaryotic or bacterial separately. Each sample’s eukaryotic and bacterial transcriptomes were normalized to the total number of non-rRNA reads assigned to their respective taxonomic division in MEGAN. Reads not assigned a division-level taxonomic identification were excluded from further analysis.

To validate the results from MEGAN, we submitted our entire raw transcriptomic data set and, separately, the fraction of non-rRNA reads identified as bacterial in origin to MG-RAST (Meyer et al., 2011).
Table 2 Metatranscriptome sequence characteristics

|        | Kilo Moana | Tow Cam | ABE | Tu'i Malila |
|--------|------------|---------|-----|-------------|
| Total reads | 199679     | 165105  | 182311 | 171587     |
| rRNA reads | 102675     | 83436   | 67669 | 135141     |
| Non-rRNA reads | 97004     | 81669   | 114642 | 36446     |
| Taxon-assigned proteins % Eukaryotic | 71% | 60% | 58% | 37% | (MG-RAST accn.) (4492532.3) (4492531.3) (4492530.3) (4492529.3) |
| % Bacterial | 27% | 38% | 39% | 59% | (MG-RAST accn.) (4491348.3) (4491346.3) (4491347.3) (4491344.3) |
| % γ-Lau* (16S) | 0/0% | 0/0% | 0/0% | 59/51% | |
| % e (16S) | 84/100% | 98/100% | 0/2% | 0/1% |

*Proportion of symbiont 16S rRNA gene copies in ISMASH DNA as determined via 454 pyrosequencing/quantitative PCR.

2008). There was broad agreement between results derived from MEGAN and those from MG-RAST. Unless otherwise specified, all described results are derived from manual searches of the bacterial data sets with SEED/Subsystems annotations in MG-RAST (e-value = 10^-10). All eight datasets are now publicly available on MG-RAST (Table 2).

Bacterial 16S rRNA gene diversity from amplicon pyrosequencing was assessed in QIIME v1.4.0 (Caporaso et al., 2010). Raw pyrosequencing flowgrams were denoised using the QIME Deneroiser (Reeder and Knight, 2010), then sequences were filtered for chimeras using the de novo implementation of the UCHIME chimera checker (Edgar et al., 2011). Sequences were then clustered at 97% identity using UCLUST (Edgar, 2010), and the resulting operational taxonomic units were analyzed using the default QIME pipeline.

**Results and discussion**

**Transcriptome characteristics and taxonomic composition**

The ISMASH was highly effective at preserving RNA in situ. Assessments of extracted RNA showed good preservation, with clearly defined eukaryotic and prokaryotic rRNA peaks (Supplementary Figure S2). Rapid in situ homogenization likely facilitated the penetration of preservative Throughout the tissues of these large-shelled organisms. More importantly, in situ homogenization arrests metabolism and alleviates concerns about transcriptional changes that might arise if specimens are simply submerged in a preservative in situ, or are recovered in seawater and preserved on board ship.

Between 160000 and 200000 metatranscriptomic reads were recovered from each sample (Table 2). Of these, 37–79% matched the rRNA database. Between 10–27% of the total reads (17000 and 49000) matched to protein-coding genes in the NCBI nr database. In total, we recovered between 9450 and 18915 putatively bacterial and between 6434 and 28412 putatively eukaryotic protein-coding transcripts per sample. Approximately 50–60% of transcripts identified as bacterial in origin were successfully assigned a functional annotation, compared with just 12–13% of eukaryotic transcripts. Consistent with our objective of examining symbiont gene expression, subsequent analyses focused solely on genes of bacterial origin. Expression data presented hereafter are normalized to the total number of protein-coding transcripts assigned to bacteria in each sample, and scaled to represent a 10000 read library.

Host genotyping and assessment of the symbiont compositions from DNA extracts demonstrated that the distribution of sampled holobionts along the spreading center—as well as the observed specificity among host and symbiont types—was concordant with previous results (Beinart et al., 2012).

Partial *Alviniconcha* mitochondrial cytochrome oxidase I gene sequences showed that all four sampled snails had >99% identity to genotypes previously described from the ELSC (Table 1). Taxonomic assignment of bacterial 16S rRNA genes amplified from DNA extracts revealed that 84–98% of bacteria in each sample matched *Alviniconcha* symbiont reference sequences, with the most abundant operational taxonomic units in each sample matching reference sequences at 99–100% identity (Table 2). ε-proteobacterial symbionts dominated the host type II samples from the two northern fields (Kilo Moana and Tow Cam), and γ-proteobacterial symbionts dominated the host type I and type III samples from the two southern fields (ABE and Tu’i Malila, respectively). Though three of the four samples were dominated by a single symbiont phylotype, the Tu’i Malila sample appeared to simultaneously host both γ-1 and γ-Lau phylotypes (Table 2).

To help confirm that the overall taxonomic composition of the data set was reflected in key metabolic pathways, taxonomic assignments for genes involved in sulfur metabolism, hydrogen oxidation, and nitrogen metabolism were manually reviewed in MEGAN (Supplementary Table S1). Despite the uncertainties associated with taxonomy assignment to individual short reads, there was very little evidence for expression of ε-proteobacterial transcripts in γ-dominated samples, or vice versa.

**Sulfur metabolism**

Sulfur oxidation genes were well represented in all metatranscriptomes, constituting 2–3% of the total bacterial transcripts in each sample (Figure 2, Supplementary Table S1). The γ-dominated samples showed expression of sulfur oxidation genes and pathways typical of chemoautotrophic γ-proteobacteria (Figure 3, Supplementary Table S1). From both γ-dominated samples, we recovered transcripts from the core periplasmic Sox genes, soxXYZ, though not the soxCD genes. The Sox complex without SoxCD is utilized for the incomplete
oxidation of sulfide or thiosulfate, resulting in the deposition of periplasmic elemental sulfur granules (Grimm et al., 2008; Ghosh and Dam, 2009). The \(\gamma\)-dominated metatranscriptomes also contained transcripts for sulfide:quinone (oxido)reductases (Sqr) and sulfide dehydrogenases (Fcc), which

![Figure 2](image_url)

**Figure 2.** Summarized differences in expression of the most abundant categories of symbiont genes. Only gene categories with membership comprising >0.5% of the total data set are represented. Blue- and yellow-shaded bars indicate relative levels of expression in \(\gamma\)-or \(\epsilon\)-dominated metatranscriptomes, respectively, on a linear scale. Dark gray bars indicate the base-10 logarithm of the odds ratio \(((G_\gamma/T_\gamma)/(G_\epsilon/T_\epsilon))\), where \(G\) = no. reads in that category and \(T\) = total no. of reads. Positive log (odds ratios) indicate genes more likely to be expressed in \(\gamma\)-dominated metatranscriptomes (‘Protein biosynthesis’ abundance and ‘Sulfate reduction’ log ratio bars exceed the axis limits in the figure at the scale presented). Genes are summarized by the Level 2 of the SEED Subsystems ontology as annotated in MEGAN4.
oxidize sulfide to elemental sulfur in the periplasm (Frigaard and Dahl, 2008; Ghosh and Dam, 2009). Consistent with the expression of these genes and pathways, we frequently observe elemental sulfur granules in the gills of *Alviniconcha* hosting γ-proteobacteria. The further oxidation of this elemental sulfur to sulfate is thought to involve additional pathways (Ghosh and Dam, 2009), such as reverse dissimilatory sulfate reduction (rDSR). Indeed, the DSR genes *dsrABCEFJKMLOPS* were highly expressed in both γ-dominated samples, with *dsrEFHR* genes present in at least one γ-dominated sample (*dsrEFHR* abundances determined via MG-RAST IMG annotation). The presence of *dsrEFH* transcripts indicates use of this pathway for the oxidation of reduced sulfur species. Here, the reverse DSR pathway is likely coupled to sulfite oxidation via an indirect pathway involving APS reductase and sulfate adenylyltransferase, both of which were identified in the γ-dominated metatranscriptomes.

Notably, the expression of an incomplete Sox complex along with reverse DSR and indirect sulfite oxidation pathways is common among γ-proteobacterial chemosynthetic endosymbionts (Harada *et al*., 2009; Markert *et al*., 2011; Stewart *et al*., 2011). We detected transcripts from both pathways in the metatranscriptome derived from a snail dominated by the γ-1 phylotype, suggesting that both pathways are present and being expressed in this symbiont. However, as the other metatranscriptome was derived from a mixed symbiont community of two γ-proteobacterial phylotypes, we cannot say whether these pathways are expressed by one or both of the γ-proteobacterial symbionts.

In contrast to the γ-dominated metatranscriptomes, the ε-dominated metatranscriptomes from...
the northern two vent fields lacked reverse DSR transcripts, but were replete with complete Sox–multienzyme complex and Sqr transcripts (Figure 3, Supplementary Table S1). The full Sox–multienzyme complex is employed by ε-proteobacteria for the complete oxidation of sulfide, thiosulfate, and/or elemental sulfur (Yamamoto and Takai, 2011). Additionally, we detected high expression of sqr genes, despite the fact that the ε-proteobacterial symbiont, like its free-living relatives from the genus *Sulfurimonas*, has not been observed to form visible sulfur granules (Sievert et al., 2008). Many ε-proteobacterial genomes encode genes for Sqr in addition to the Sox complex, though the role of Sqr in sulfur oxidation and its relationship to the Sox pathway is still uncertain in these symbionts and other ε-proteobacteria.

**Hydrogen metabolism**

Previous work has shown that *Alviniconcha* hosting ε-proteobacterial symbionts dominate at the northern vent fields, where hydrogen concentrations are highest, suggesting that hydrogen might serve as an electron donor for these symbionts (Beinart et al., 2012). The metatranscriptomes from the two ε-dominated, northernmost samples revealed the potential for respiratory hydrogen oxidation, with both samples expressing genes allied to Group 1 NiFe-hydrogenases (~0.5% of all transcripts, Figure 3 and Supplementary Table S1). This group of enzymes, often called uptake hydrogenases, are membrane-bound, respiratory enzymes that oxidize hydrogen and donate electrons to the quinone pool (Vignais and Billoud, 2007). Hydrogen oxidation transcripts for Group 1 NiFe-hydrogenases were also recovered—though with much lower representation—from the γ-dominated metatranscriptomes (Figure 3).

The presence of hydrogenase transcripts in all four of the *Alviniconcha* metatranscriptomes indicates that hydrogen could be an electron donor in both the γ- and ε-proteobacterial symbionts of *Alviniconcha*. However, the difference in expression of hydrogenases between the ε- and the γ-dominated individuals suggests that hydrogen oxidation potentially has a larger role in the energy metabolism of the holobionts with ε-proteobacteria (Figure 3, Supplementary Table S1). Phylogenetic analysis of the *Alviniconcha* ε-proteobacterial endosymbionts from the ELSC shows that they are closely allied to members of the genus *Sulfurimonas* (Beinart et al., 2012), many of which are able to utilize both hydrogen and reduced sulfur compounds as electron donors (Nakagawa et al., 2005). A recent study employed a suite of molecular, physiological, and geochemical approaches to show that vent mussels with γ-proteobacterial symbionts can oxidize hydrogen to support carbon fixation (Petersen et al., 2011). Future studies will use similar approaches to elucidate the degree to which these *Alviniconcha* ε-proteobacterial endosymbionts rely on hydrogen for energy production.

**Carbon fixation**

Transcript representation and abundances revealed clear differences in carbon fixation pathways between γ- and ε-dominated individuals (Figure 4, Supplementary Table S1). Key genes of the Calvin-Benson-Bassham cycle, including those encoding Form II Ribulose-1,5-bisphosphate carboxylase/oxygenase and phosphoribulokinase, were enriched in γ-dominated metatranscriptomes. In contrast, the three key genes associated with the reductive tricarboxylic acid (rTCA) cycle (ATP citrate lyase, 2-oxoglutarate oxidoreductase, and fumarate reductase) were found only in snails hosting ε-proteobacteria.
These patterns are consistent with previous studies of carbon fixation in both free-living and symbiotic chemosynthetic microorganisms. In these studies, the most prominent nitrogen sources were ammonia (DNR) and nitrite reductase (Nar) activities in the periplasm of some symbionts, indicating that both assimilatory nitrate reduction and dissimilatory nitrite reduction are present in these symbionts (Potter et al., 1999). However, both γ- and ε-dominated metatranscriptomes showed expression of the narK nitrite/nitrate transporter and cytoplasmic ammonifying nitrite reductases, representing a potential mechanism for assimilation of periplasmically-reduced nitrate via the shuttling of nitrite into the cytoplasm and subsequent reduction to ammonium and assimilation by glutamine synthetase-glutamate synthase (Figure 3, Supplementary Table S1). This model represents an alternative to the typical pathway for DNR.

Nitrogen metabolism

All organisms need nitrogen for growth and biosynthesis, requiring the assimilation of an exogenous source of nitrogen. At vents, dissolved organic nitrogen (for example, free amino acids) is quite low (Johnson et al., 1986), while inorganic nitrogen compounds are typically abundant. Some vent fluids contain nM to μM concentrations of dissolved ammonium, which is easily assimilated by many organisms. However, there are no data on ammonium concentrations at the ELSC (Tivey, 2007). Nitrate, however, is typically very abundant in seawater surrounding vents (occurring at ~40 μM; Johnson et al., 1986).

Nitrate can be used both as a primary nitrogen source for biosynthesis and growth and as a respiratory terminal electron acceptor. Assimilatory nitrate reduction canonically utilizes the cytoplasmic nitrate reductase Nas. Dissimilatory nitrate reduction (DNR) frequently utilizes the membrane-bound respiratory nitrate reductase Nar, but in some bacteria may also be catalyzed via a periplasmic–enzyme complex (Nap) (Potter et al., 2001). The nitrite generated by DNR may be further reduced to ammonia via dissimilatory nitrate reduction to ammonia (DNRA), which may then be utilized for biosynthesis (though Nap has not typically been associated with nitrate assimilation, Berks, 1995). In both assimilatory nitrate reduction and DNRA, the resulting ammonium is typically assimilated by the glutamine synthetase-glutamate synthase or glutamate dehydrogenase pathways (Reitzer, 2003).

In all of the metatranscriptomes, we found the expression of some genes typically associated with both assimilatory and dissimilatory nitrate reduction, as well as for ammonium assimilation. Curiously, though, we did not find evidence for complete expression of any of the canonical pathways. Instead, periplasmic nitrate reductase (Nap) appeared to catalyze nitrate reduction as the first step for both assimilation and respiration.

Genes involved in ammonium assimilation comprised a substantial portion of all metatranscriptomes, indicating that all Alviniconcha symbionts were poised to assimilate ammonium either from the reduction of nitrate or from the environment (Figure 3, Supplementary Table S1). Both ε- and γ-proteobacterial symbionts showed substantial expression of glutamine synthetase-glutamate synthase and ammonium transporters. As mentioned above, all symbionts were poised for nitrate reduction in the periplasm via the Nap complex. We did not detect the periplasmic nitrite reductase Nrf, which is typically the next step in Nap-catalyzed DNRA (Potter et al., 1999). However, both γ- and ε-dominated metatranscriptomes showed expression of the narK nitrite/nitrate transporter and cytoplasmic ammonifying nitrite reductases, representing a potential mechanism for assimilation of periplasmically-reduced nitrate via the shuttling of nitrite into the cytoplasm and subsequent reduction to ammonium and assimilation by glutamine synthetase-glutamate synthase (Figure 3, Supplementary Table S1). This model represents an alternative to the typical pathway for DNR.

The substantial expression of genes involved in respiratory denitrification suggests another possible fate for periplasmic nitrite (Figure 3, Supplementary Table S1), raising the possibility that Alviniconcha symbionts may be utilizing nitrate as an alternative electron acceptor to oxygen, potentially reducing competition for oxygen with the host (Hentschel and Felbeck, 1993; Hentschel et al., 1996). Though we did not detect transcripts for the canonical dissimilatory nitrate reductase Nar, in other bacteria, Nap has been shown to catalyze the first step in aerobic denitrification, as the presence of oxygen inhibits activity of Nar (Potter et al., 2001). We posit that Nap has a similar role in Alviniconcha symbionts. All of our samples contained transcripts for the rest of the denitrification pathway, including periplasmic respiratory cytochrome cd1 nitrite reductase NirS, the nitric oxide–reductase complex Nor, and the nitrous oxide–reductase complex Nos. Denitrification genes were more abundantly and consistently expressed in the ε-dominated metatranscriptomes, where they constituted around 2% of all reads. These genes were less abundant (0.3% of reads) in the γ-proteobacterial metatranscriptomes. Notably, NirS, which serves the reduction of nitrite to nitric oxide, and thus may serve to commit nitrite to a dissimilatory pathway, was ~50-fold more abundant in the ε-dominated samples (Figure 3).

Functionally, this assemblage of assimilatory and dissimilatory genes may represent a strategy well suited for life around hydrothermal vents, where fluid mixing leads to ammonium concentrations that are inversely correlated with availability of oxygen as a terminal electron acceptor. The model outlined in Figure 3 would permit purely respiratory reduction of nitrate in holobionts exposed to oxygen-poor and ammonium-rich vent fluid. Conversely, holobionts in more aerobic conditions, with less access to ammonium, could decrease complete denitrification to dinitrogen in favor of assimilating...
nitrates to meet the needs of biosynthesis. Under this hypothesis, the expression differences we observed between γ- and ε-dominated metatranscriptomes would suggest that these symbionts were engaged primarily in either nitrogen assimilation or denitrification in response to variations in water chemistry in their respective habitats.

Despite differences in expression, the complement of nitrogen genes was remarkably consistent between ε- and γ-dominated metatranscriptomes. The similarities in nitrogen gene content were especially striking in light of the large differences we observed in sulfur and carbon pathways. Where the latter differences were typical of pathways found in free-living members of their respective proteobacterial classes, the combination of nitrogen-related genes we observed in both classes of Alviniconcha symbionts was fairly unusual, but could potentially catalyze functionally identical pathways in both classes of symbiont. At hydrothermal vents, the capacity to use nitrates for both biosynthesis and respiration is widespread among ε-proteobacteria (Sievert and Vetriani, 2012), including the free-living relatives of Alviniconcha ε-proteobacterial symbionts, Sulforimonas (Takai et al., 2006; Sievert et al., 2008; Sikorski et al., 2010). Nitrate respiration is less common among free-living vent γ-proteobacteria (Sievert and Vetriani, 2012), though, curiously, patterns of nitrogen gene expression similar to the Alviniconcha symbionts have been observed in other γ-proteobacterial symbionts, such as those associated with vent tubeworms (Markert et al., 2011; Robidart et al., 2011). Although this model remains to be validated, such functional convergence may reflect similar selective pressures imposed by life in this environment, as well as the symbiotic lifestyle.

Flagellar genes
Flagellar genes showed striking differences in expression between ε- and γ-proteobacterial metatranscriptomes (Figure 4, Supplementary Table S1), hinting at potential differences in host–symbiont interactions. The ε-dominated samples expressed transcripts for at least 30 flagellum-related genes, including flagellins flaAB and transcripts associated with the flagellar hook, ring, motor, basal body, and biosynthesis (Supplementary Table S1). Only 10 flagellum-related genes were recovered from γ-dominated metatranscriptomes and, in aggregate, were ~20-fold lower in abundance. While the diversity of roles that flagellar genes have in other symbiotic bacteria complicates interpretation (Anderson et al., 2010), we propose three hypotheses that might explain the observed differences in expression.

First, if flagella are used primarily for motility, their abundant expression in mature-host associations may signal differences in symbiont motility and transmission dynamics between the ε- and γ-proteobacterial symbionts. Riftia tubeworm symbionts, while possessing a large number of genes related to motility and chemotaxis, do not appear to possess flagella while inhabiting the host trophosome (Harmer et al., 2008; Robidart et al., 2008). Instead, they may utilize flagella during horizontal transmission (Harmer et al., 2008), when flagellar motility could be important in escaping from parental host tissue and/or chemotaxis towards a new host. Here, the abundant flagellar gene expression in ε-symbionts could indicate that they are actively transmitted throughout the lifetime of the host. The lower expression observed in the γ-hosting snails might in turn reflect either a different transmission strategy or, potentially, temporal differences in symbiont transmission.

Second, flagellar proteins are commonly used in host recognition and attachment. It is plausible that the expression of flagellar genes in ε-dominated metatranscriptomes relates to host recognition (that is, specificity). Flagellar proteins are critical to symbiont recognition and colonization in other systems: for example, the Euprymna-Vibrio symbioses (Nyholm et al., 2000; Millikan and Ruby, 2004), another highly specific, horizontally transmitted marine symbiosis. A previous study found that one Alviniconcha host type nearly always hosted solely ε-proteobacterial symbionts, while others hosted mixed populations of the two γ-proteobacterial symbiont lineages and, occasionally, the ε-proteobacterial symbionts (Beinart et al., 2012). The expression of flagellin in ε-proteobacterial symbionts may contribute to this specificity.

Finally, flagellar genes may also mediate nutritional export from symbiont to host. Ring- and hook-associated flagellar proteins have been shown to have an important secretory role in the intracellular symbionts of aphids (Maezawa et al., 2006; Toft and Fares, 2008), though they have lost genes for the flagellin tail proteins, which were abundantly expressed in our ε-dominated samples. In Alviniconcha, the mode of nutrient transfer in γ-hosting individuals may predominantly be via digestion of symbiont cells, as is thought to be the case for γ-proteobacterial symbionts of other vent animals (Lee et al., 1999). In contrast, translocation of small organic compounds from symbiont to host may have a bigger role in Alviniconcha hosting ε-proteobacteria. Compound-specific isotopic observations made by Suzuki et al. (2005) suggest as much, demonstrating that symbiont-associated fatty acids are detected in non-symbiotic host tissues of γ-, but not ε-hosting Alviniconcha.

Transposons
Another surprising difference between symbiont types was the increased abundance of transposases in the γ-dominated metatranscriptomes, where predicted transposases accounted for 3–7% of all bacterial transcripts via IMG in MG-RAST (SEED called around 2–5% of the transcripts transposons). Predicted transposases were present
in the ε-dominated samples, but were much lower in abundance and matched largely to different transposase families (Figure 4, Supplementary Table S1). The presence of transposons in the genomes of other chemoautotrophic symbionts is variable: they are undetected in the genomes of the vertically-transmitted endosymbiont of vent clams (Kuwahara et al., 2007; Newton et al., 2007), while those of the horizontally transmitted vent tubeworm endosymbionts contain at least one (Robidart et al., 2008; Gardebrecht et al., 2012), and nearly 20% of an endosymbiont genome from the oligochaete Olavius algarvensis is composed of transposable elements (Woyke et al., 2006).

Moran and Plague (2004) have proposed that the proliferation of mobile genetic elements occurs early during the transition to an obligate intracellular lifestyle, as genetic bottlenecks during transmission decrease the effective population size of the symbionts, leading to decreased strength of selection. Combined with the lack of a free-living life stage, this is thought to relax purifying selection on the symbiont genome. Although conflicting with the current hypothesis that both γ-proteobacterial and ε-proteobacterial symbionts are transmitted via the environment, the dramatic differences in transposase expression observed among these metatranscriptomes hints at the possibility that these two distinct symbiont-host associations either represent different stages of evolutionary development, or exhibit differing transmission modes. When taken in conjunction with the corresponding differences in flagellar gene expression, these data raise the hypothesis that ε-proteobacterial symbionts in Alviniconcha, relative to the γ-proteobacterial symbionts, experience more frequent dispersal and fewer genetic bottlenecks.

Conclusions

Our results reveal that Alviniconcha symbionts exhibited marked differences in gene expression related to energy metabolism. The predominance of both hydrogen oxidation and DNR genes in the ε-dominated metatranscriptomes would suggest that these holobionts live in more highly reduced and potentially less oxygen-rich fluids. This is consistent with the previously observed patterns of distribution across a regional gradient, wherein ε-hosting Alviniconcha were most abundant in the more sulfidic, hydrogen-rich fluids found at the northern vent fields (Beinart et al., 2012). Though these differences in expression do not necessarily imply differences in metabolic capability (this is better addressed via genomic sequencing of each symbiont type), their striking correlation with holobiont biogeography supports the hypothesis that symbiont physiology has an important role in habitat partitioning among the host types in this genus.

Unexpectedly, the observed differences in flagellar genes and transposons between ε- and γ-dominated metatranscriptomes hint at differences in symbiont life histories, potentially related to the dynamics of association between host and symbiont, including mode of transmission and nutrient exchange. Though other chemoautotrophic symbioses have been described with widely varying specificities and transmission mechanisms (reviewed in Dubilier et al., 2008), Alviniconcha is thus far unique in that close relatives within the genus host unrelated symbionts, each with apparently variable degrees of specificity (Beinart et al., 2012).

The data presented here illustrate the value of using in situ preservation and shore-based transcriptomics to examine symbiont physiological poise. This technology and methodology allowed us to test a priori hypotheses as well as to identify previously unrecognized differences among these symbionts. Future studies should employ such approaches when studying both host and symbiont gene expression within and among different geochemical habitats to better understand the complex relationships among symbionts, their hosts, and the environment.

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