

ORIGINAL ARTICLE

Coupled RNA-SIP and metatranscriptomics of active chemolithoautotrophic communities at a deep-sea hydrothermal vent

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The chemolithoautotrophic microbial community of the rocky seafloor potentially provides a large amount of organic carbon to the deep ocean, yet our understanding of the activity and metabolic complexity of seafloor organisms remains poorly described. A combination of metagenomic, metatranscriptomic, and RNA stable isotope probing (RNA-SIP) analyses were used to identify the metabolic potential, expression patterns, and active autotrophic bacteria and archaea and their pathways present in low-temperature hydrothermal fluids from Axial Seamount, an active submarine volcano. Metagenomic and metatranscriptomic results showed the presence of genes and transcripts for sulfur, hydrogen, and ammonium oxidation, oxygen respiration, denitrification, and methanogenesis, as well as multiple carbon fixation pathways. In RNA-SIP experiments across a range of temperatures under reducing conditions, the enriched ^{13}C fractions showed differences in taxonomic and functional diversity. At 30 °C and 55 °C, Epsilonproteobacteria were dominant, oxidizing hydrogen and primarily reducing nitrate. Methanogenic archaea were also present at 55 °C, and were the only autotrophs present at 80 °C. Correspondingly, the predominant CO_2 fixation pathways changed from the reductive tricarboxylic acid (rTCA) cycle to the reductive acetyl-CoA pathway with increasing temperature. By coupling RNA-SIP with meta-omics, this study demonstrates the presence and activity of distinct chemolithoautotrophic communities across a thermal gradient of a deep-sea hydrothermal vent.

The ISME Journal (2016) 10, 1925–1938; doi:10.1038/ismej.2015.258; published online 12 February 2016

Introduction

Venting fluids at deep-sea hydrothermal systems export members of the extensive microbial communities of the rocky seafloor. Within the fluids circulating through the seafloor, chemolithoautotrophs convert CO_2 to biomass using the geochemical energy created from chemical disequilibria when hydrothermal fluid mixes with seawater (Amend *et al.*, 2011). Previous studies found high rates of carbon fixation and concentrations of organic carbon and ATP in venting fluids and plume waters (Karl *et al.*, 1980; Jannasch and Wirsen, 1981; Jannasch *et al.*, 1989; Wirsen *et al.*, 1993). Energetic modeling suggests that seafloor mixing zones are likely to be the most productive of vent habitats with respect to microbial growth, potentially providing an important source of carbon to the deep ocean (McCollom, 2005).

16S ribosomal RNA (rRNA) gene surveys and culture-dependent approaches have revealed the

extensive taxonomic diversity and metabolic plasticity of chemolithoautotrophs at deep-sea hydrothermal vents (Reysenbach and Shock, 2002; Sievert and Vetriani, 2012). Further studies of diffuse hydrothermal fluids have shown that the population structure of the seafloor community can vary with time (Huber *et al.*, 2002, 2003; Perner *et al.*, 2009), space (Opatkiewicz *et al.*, 2009; Huber *et al.*, 2010), and geochemical gradients (Huber *et al.*, 2007; Perner *et al.*, 2010; Akerman *et al.*, 2013). In addition, the genomes of key autotrophs from hydrothermal vents have revealed the broad array of CO_2 fixation pathways and energy acquisition strategies used by these organisms (Takai *et al.*, 2005; Campbell *et al.*, 2009; Yamamoto and Takai, 2011; Meyer and Huber, 2014).

Recent metagenomic and metatranscriptomic studies from hydrothermal plumes and diffuse hydrothermal fluids have provided additional insights into the metabolic potential and gene expression patterns of the microbial communities in hydrothermal systems (for example, Lesniewski *et al.*, 2012; Anantharaman *et al.*, 2013; Baker *et al.*, 2013; Anderson *et al.*, 2014; Urich *et al.*, 2014). Studies of hydrothermal vent plumes, which are a mixture of deep-water and hydrothermal vent microbes (Dick *et al.*, 2013), demonstrated the metabolic versatility

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Received 5 June 2015; revised 7 December 2015; accepted 10 December 2015; published online 12 February 2016

of plume microbial communities, with genes for ammonium, sulfur, and methane oxidation highly expressed (Lesniewski *et al.*, 2012; Anantharaman *et al.*, 2013) and presence of both chemolithoautotrophic populations such as SUP05 and heterotrophic archaea (Dick *et al.*, 2013; Li *et al.*, 2015). Targeting the seafloor chemolithoautotrophic community is especially important for determining their role in the primary production of biomass by dark fixation. However, as these subsurface communities are difficult to sample, studies of their microbiology have relied on sampling vent fluids, which represent a mixture of seafloor hydrothermal fluids and deep seawater consisting of 75–99% seawater and 1–25% hot hydrothermal end-member. This results in an extremely diverse microbial community (Huber *et al.*, 2007) where the seafloor microbial signal can be dampened by the overwhelming signature of seawater mixing.

Stable isotope probing (SIP) is one powerful tool for focusing on specific active communities through incorporation of isotopically labeled substrates under near natural conditions (Dumont and Murrell, 2005). During a SIP experiment, the active community will incorporate the labeled substrate and increase in biomass relative to the non-active fraction of the community. The active community can then be identified using various molecular techniques (Dumont and Murrell, 2005; Neufeld *et al.*, 2007). Recently, SIP studies have expanded from purely taxonomic identification (for example, Lueders *et al.*, 2004; Bernard *et al.*, 2007; Glaubitz *et al.*, 2010) to using DNA-SIP to construct metagenomic libraries of the labeled populations (Dumont *et al.*, 2006; Neufeld *et al.*, 2008). SIP studies have also coupled RNA-SIP with metatranscriptomics to link actively transcribed genes to specific microbial populations (Huang *et al.*, 2009; Dumont *et al.*, 2013). The first study to attempt mRNA-SIP used reverse transcriptase-PCR and primers specific to naphthalene degrading genes to understand the specific bacterial species that were degrading this compound in groundwater (Huang *et al.*, 2009). A more recent study was able to construct a metatranscriptome from a SIP experiment of lake sediments looking at aerobic methanotrophs and showed the presence of transcripts for methane oxidation, carbon assimilation and nitrogen metabolism (Dumont *et al.*, 2013).

Although SIP presents an excellent way to link community structure and function, there are limitations that must be considered. The active microbial community must be able to take up the labeled isotope and incorporate it into nucleic acids within a given incubation time under particular experimental conditions. Too short of an incubation may bias against microbes with slow growth rates and lead to incomplete labeling of the community, while too long of a time may lead to nonspecific labeling of the community (that is, cross-feeding) (Radajewski *et al.*, 2003). It is also important to differentiate between

assimilation and metabolism of a labeled isotope. For example, the general uptake of ^{13}C -labeled bicarbonate into anaplerotic pathways of the TCA cycle may result in nonspecific labeling of the community (Yakimov *et al.*, 2014). Thus, it is important to take these limitations into account when analyzing SIP results. In this study, traditional metagenomics and metatranscriptomic analyses of diffuse vent fluids were coupled with RNA-SIP metatranscriptomics to determine which autotrophs were active under relevant seafloor conditions, what metabolisms and carbon fixation pathways were used, and how these organisms function across different temperatures representing seafloor thermal gradients.

Experiments were carried out at Axial Seamount, a submarine volcano located on the Juan de Fuca Ridge and a site of long term geological, chemical, and biological study (Chadwick *et al.*, 2010). 16S rRNA and functional gene surveys have shown that diverse Epsilonproteobacteria dominate across diffuse vents at Axial and their community structure can often be linked to the chemistry of the vent sampled (Huber *et al.*, 2007; Opatkiewicz *et al.*, 2009; Akerman *et al.*, 2013; Meyer *et al.*, 2013). Epsilonproteobacteria were first isolated from hydrothermal vents in 2001 (Campbell *et al.*, 2001) and all isolates to date are chemolithoautotrophic and either moderately thermophilic hydrogen oxidizers or mesophilic hydrogen and sulfur oxidizers (Campbell *et al.*, 2006). Recent studies of hydrothermal vent Epsilonproteobacteria also show that many are capable of nitrate reduction and denitrification (Campbell *et al.*, 2006; Vetriani *et al.*, 2014), as well as nitrogen fixation (Meyer and Huber, 2014). In addition to Epsilonproteobacteria, Gammaproteobacteria, specifically SUP05 populations, were also shown to be abundant and active within both diffuse fluids and plumes at Axial (Bourbonnais *et al.*, 2012; Akerman *et al.*, 2013; Anderson *et al.*, 2013). Although SUP05 remains uncultivated from the deep ocean, genomic evidence indicates these bacteria possess genes to carry out both sulfide and hydrogen oxidation, similar to many Epsilonproteobacteria (Takai *et al.*, 2005; Campbell *et al.*, 2006; Anantharaman *et al.*, 2013; Meyer and Huber, 2014). Hydrogen oxidation is a more energetically favorable reaction compared with sulfur oxidation and thus may be an alternate energy pathway for these chemolithoautotrophs in venting fluids (Amend *et al.* 2011).

In addition to the dominance of sulfur and hydrogen-oxidizing microbes, Axial Seamount has also been a study site of seafloor methanogenic archaea, with the detection, quantification, and isolation of many mesophilic, thermophilic, and hyperthermophilic strains from diffuse fluids (Huber *et al.*, 2002; Ver Eecke *et al.*, 2012; Meyer *et al.*, 2013; Ver Eecke *et al.*, 2013), including isolation of the first nitrogen-fixing hyperthermophilic methanogen (Mehta and Baross, 2006). Growth experiments with methanogenic archaea isolated

from diffuse fluids at Axial indicate they require concentrations of hydrogen 10 times lower than previous estimates and have the potential for syntrophic relationships with hydrogen producing heterotrophs (Ver Eecke *et al.*, 2012, 2013).

Building upon previous studies, we used metagenomics and metatranscriptomics to first establish the metabolic potential and gene expression patterns of the microbial community from a singular vent at Axial Seamount, Marker 113. We then used RNA-SIP combined with metatranscriptomics to separate out the chemolithoautotrophic signature in the mixed fluids in order to determine the key taxa and metabolisms representative of the warm, reducing seafloor habitat. Results showed the metabolic complexity of the vent microbial community and highlight the diverse metabolic pathways of the potential members of the seafloor chemolithoautotrophic community across different temperatures.

Materials and methods

Fluid collection and vent chemistry

Diffuse hydrothermal vent fluids (24 °C) were collected from Marker 113 vent (Ver Eecke *et al.*, 2012; 45.9228, -129.9881) at Axial Seamount on 27 September 2013 on board the *R/V Falkor* using the deep-sea research submarine ROV *ROPOS*. Sampling depth at Marker 113 vent was 1521 m. Fluids were collected 2 cm above the seafloor using the hydrothermal fluid and particle sampler (HFPS) (Butterfield *et al.*, 2004) and the large volume water sampler (LVWS) (Wommack *et al.*, 2004). Both instruments had integrated temperature sensors at the point of intake to allow for constant temperature monitoring while drawing fluids. For RNA-SIP experiments, 4–5 l of fluid were pumped into either an acid-washed 4-l Tedlar bag using the HFPS or into a 5-l acid-washed plastic carboy using the LVWS. For DNA and RNA collection, the HFPS was used to pump 3 l of diffuse fluid at a rate of 100–150 ml min⁻¹ through a 0.22 µm, 47 mm GWSP filter (Millipore, Billerica, MA, USA) that was then preserved *in situ* with RNALater (Ambion, Grand Island, NY, USA) as previously described in Akerman *et al.* (2013). Fluid samples were also collected for chemistry and analyzed for alkalinity, hydrogen sulfide, ammonia, methane, and hydrogen concentrations following methods described in Butterfield *et al.* (2004). The oxygen concentration was measured using a Seabird (Bellevue, WA, USA) 63 Optical oxygen sensor, and the pH was measured using an AMT (Rostock, Germany) deep-sea glass pH electrode, both plumbed to the HFPS. For total cell counts, fluids collected via the HFPS were preserved in 3–4% paraformaldehyde, stained with acridine orange, and counted via epifluorescent microscopy. For virus-like particle counts, fluids collected via the LVWS were preserved in 1% formalin, stained with

Table 1 Comparison of Marker 113 and seawater chemistry characteristics

Fluid Characteristic	Marker 113	Seawater
Temperature, °C	24.1	2.4
Percent seawater	96	100
Magnesium, mmol kg ⁻¹	49.1	51.5
Total CO ₂ , mmol kg ⁻¹	4.6	2.3
Hydrogen sulfide, mmol l ⁻¹	0.6	0.0
Ammonia, µmol l ⁻¹	3.5	<0.6
Silica, µmol l ⁻¹	636	155
pH	6.2	7.8
Hydrogen, µmol kg ⁻¹	<1.0	0.0
Methane, µmol kg ⁻¹	13.2	0.0
Oxygen, µmol l ⁻¹	7.0	28.3
Microbial cells per ml	4.4 × 10 ⁵	1.8 × 10 ⁴
Virus-like particles per ml	1.6 × 10 ⁶	ND

Abbreviation: ND, no data.

Percent seawater was calculated via the conservative tracer magnesium (Mg): $[\text{Mg}]_{\text{vent}}/[\text{Mg}]_{\text{seawater}} \times 100$. Most seawater data are reported from Akerman *et al.* (2013). Seawater magnesium and total CO₂ were measured from a 2013 sample, whereas oxygen data were reported from a 2015 CTD cast at 1520 m.

2.5x SYBR Gold (Invitrogen, Grand Island, NY, USA), and counted via epifluorescent microscopy (Table 1).

RNA-SIP experimental set-up

For HFPS samples, fluid was pumped using a peristaltic pump from the Tedlar bag to six evacuated 500 ml Pyrex bottles, each filled nearly to capacity (530 ml). For LVWS samples, fluid was pumped from the carboy into each of four evacuated 1 liter Pyrex bottles to nearly full capacity (1060 ml). Before filling, ¹³C-labeled sodium bicarbonate (Cambridge Isotope Laboratories, Tewksbury, MA, USA) or ¹²C sodium bicarbonate (Sigma, St Louis, MO, USA) was added to each bottle for a final concentration of 10 mM additional bicarbonate. During filling, steps were taken to minimize introduction of oxygen and outgassing of fluid. After filling, 1–2.5 ml of 10% HCl was added to ensure a pH similar to vent conditions (pH < 6.5). Pure H₂ was added by syringe to each bottle: 20 ml (900 µmol) to each HFPS bottle and 60 ml to each LVWS bottle. ¹³C- and ¹²C-bicarbonate amended HFPS bottles were each incubated at 30 °C, 55 °C, and 80 °C for 36 h. A pair of ¹³C- and ¹²C-bicarbonate amended LVWS bottles were each incubated at 80 °C, one set for 18 h; the other for 36 h. After incubation, the fluid in each bottle was filtered through 0.22 µm Sterivex filters (Millipore), preserved in RNALater (Ambion), and frozen at -80 °C.

Isopycnic centrifugation, fractionation and RNA-SIP library preparation

RNA from SIP experiments was extracted using the mirVana miRNA isolation kit (Ambion) with an added bead-beating step using RNA PowerSoil beads (MoBio, Carlsbad, CA, USA). A total volume of

100 μl was extracted and was then DNase treated using the Turbo-DNase kit (Ambion). Gradient preparation, isopycnic centrifugation, and gradient fractionation were performed as described in Lueders (2010). For each gradient sample, 5.1 ml of CsTFA ($\sim 2 \text{ g ml}^{-1}$, GE Healthcare Life Sciences, Piscataway, NJ, USA), 185 μl formamide, and a mixture of 750 ng RNA and gradient buffer solution (0.1 M Tris-HCl, 0.1 M KCl, 0.1 mM EDTA) up to 1 ml were first mixed in a 15 ml tube. Once mixed, the refractive index was measured for each sample to ensure a median density of $\sim 1.80 \text{ g ml}^{-1}$. Samples were then loaded into 4.9 ml OptiSeal tubes (Beckman Coulter, Brea, CA, USA), placed into a VTi 65.2 vertical rotor (Beckman Coulter) and spun at 37 000 r.p.m. at 20 $^{\circ}\text{C}$ for 64 h using an Optima L-80 XP ultracentrifuge (Beckman Coulter). Each gradient was fractionated into 12 tubes of approximately 410 μl each and the refractory index of each fraction was measured to determine density. RNA was precipitated with isopropanol and the pellet was washed with 70% ethanol as described in Lueders (2010). RNA concentration of each fraction was determined using the RiboGreen quantification kit (Invitrogen) and a Gemini XPS plate reader (Molecular Devices, Sunnyvale, CA, USA).

Figure 1 depicts the density fractions chosen for sequencing. At each temperature, four metatranscriptomic libraries were constructed, two from the ^{12}C -control, named ^{12}C -light and ^{12}C -heavy, and two from the ^{13}C -experiment, named ^{13}C -light and ^{13}C -heavy. The two density fractions with the highest RNA concentration in the ^{12}C -control incubation were combined to construct the ^{12}C -light library, which represents the natural community. Similarly, two density fractions with the highest RNA concentration in the ^{13}C -experiment incubation were combined to construct the ^{13}C -heavy library, which represents the labeled autotrophic community. The other two libraries from each temperature, ^{12}C -heavy and ^{13}C -light, were constructed to act as additional controls. To construct the ^{12}C -heavy library, density fractions were chosen that corresponded to the same density fractions used to construct the ^{13}C -heavy library. The ^{12}C -heavy library represents the heavier density unlabeled community and acts as a key comparison with the ^{13}C -heavy labeled community to confirm that the populations in the ^{13}C -heavy library have taken up the labeled isotope and are not naturally found at heavier nucleic acid densities. To construct the ^{13}C -light library, density fractions were chosen that corresponded to the same density fractions used to construct the ^{12}C -light library. The ^{13}C -light library represents the lighter density community in the ^{13}C -experiment, which includes both labeled and unlabeled populations that are found at lighter densities. For all libraries, double-stranded complementary DNA was constructed and metatranscriptomic library preparation and analysis was carried out as described in the Supplementary methods. Raw read data are available through the

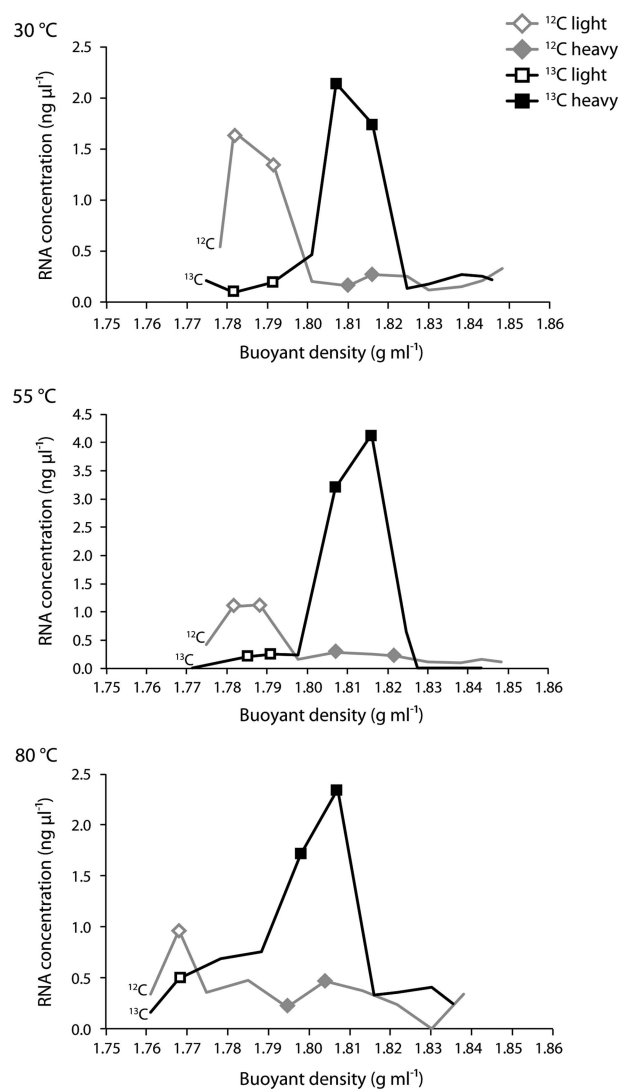


Figure 1 Fractionation plots of RNA-SIP experiments for each of the three temperatures. Plots depict the RNA concentration ($\text{ng } \mu\text{l}^{-1}$) vs buoyant density (g ml^{-1}) for each fraction. RNA from fractions denoted by a symbol was used for metatranscriptomic library preparation and sequencing.

European Nucleotide Archive for all 20 RNA-SIP metatranscriptomes, under sample accession numbers ERS743595 through ERS743614.

Metagenomic and metatranscriptomic library preparation and analysis

The 47 mm flat filters were first cut in half with a sterile razor, with half used for DNA and half used for RNA extraction. RNA and DNA were extracted and prepared for sequencing as described in the Supplementary methods. For all metagenomic and metatranscriptomic libraries, paired-end partially overlapping reads were merged and quality filtered using custom Illumina utility scripts (<https://github.com/meren/illumina-utils>). Merged reads were assembled using CLC Genomics Workbench (v 7.0) and default settings. Assembled contigs from each

library were submitted to the DOE Joint Genome Institute's Integrated Microbial Genome Expert Review (IMG/ER). A detailed description of open reading frame mapping, rRNA identification, and statistical analyses can be found in the Supplementary methods. Raw metagenomic and metatranscriptomic read data are available through the European Nucleotide Archive under sample accession numbers ERS614323 and ERS614324 for the Marker 113 metagenome and metatranscriptome, respectively.

Results

At Marker 113, the average temperature of the diffuse fluids collected at 2 cm above the seafloor was 24 °C. Gas and chemical analysis of the diffuse fluid showed H₂ concentrations were below the limit of detection (<1 μmol kg⁻¹), whereas the CH₄ concentration (13.2 μmol kg⁻¹) equaled or exceeded CH₄ concentrations measured elsewhere at Axial (Butterfield *et al.*, 2004; Ver Eecke *et al.*, 2012). The concentration of O₂ in the fluid was 7 μM (Table 1). The natural DIC concentration measured at Marker 113 was 4.6 mmol kg⁻¹. We extrapolated the percent seawater of the diffuse fluid using the conservative tracer magnesium (Mg), where percent seawater was calculated as $[Mg]_{vent}/[Mg]_{seawater} \times 100$ (Table 1). The percent seawater of the diffuse fluid at Marker 113 was ~96% seawater, meaning it contained ~4% hydrothermal fluid. From these measurements, we estimated the amount of DIC in the end-member fluid (100% hydrothermal fluid) to ~60 mmol kg⁻¹. The concentrations of prokaryotic cells and virus-like particles were 4.4×10^5 ml⁻¹ and 1.6×10^8 ml⁻¹, respectively (Table 1).

For the RNA-SIP experiments, there appeared to be uptake of the ¹³C-labeled bicarbonate at all three incubation temperatures, resulting in a higher peak density of the community RNA in the ¹³C-experiment compared with the ¹²C-control (Figure 1). On average, the peak RNA density for the controls was 1.78 (±0.009), whereas the average peak RNA density for the ¹³C experiments was 1.81 (±0.007). Of the three 80 °C SIP experiments, only the 18-h LVWS experiment was used for analysis. This is the 80 °C experiment shown in Figure 1 and throughout the article. The number of sequencing reads and percent total rRNA for each experiment is shown in Supplementary Table S1. Hierarchical clustering of the four libraries for each temperature based on the relative abundance of transcripts annotated to the KEGG Ontology (KO) database showed that ¹²C-light and ¹³C-heavy libraries clustered together, separating from the ¹²C-heavy and ¹³C-light libraries at all three temperatures. This indicates the functional similarities between the two communities (Figure 2). In addition, ¹²C-heavy libraries clustered separately from the ¹³C-heavy libraries at all temperatures, demonstrating the ¹³C-heavy libraries were functionally different from the

communities at the same densities in the ¹²C-heavy controls (Figure 2). In the 55 °C experiment, the ¹²C-light and ¹³C-heavy communities consisted of Epsilonproteobacteria and Methanococci, whereas the ¹²C-heavy and ¹³C-light libraries consisted mainly of Actinobacteria, Alphaproteobacteria, and Betaproteobacteria. At 30 °C, Epsilonproteobacteria were present in all four libraries, but in highest abundance in the ¹²C-light and ¹³C-heavy libraries at 99% and 98.6% of the community, respectively. At 80 °C, the ¹³C-light library was taxonomically very different from the other three fractions, with a high percentage of Betaproteobacteria, as well as the occurrence of Actinobacteria, and Alpha- and Gammaproteobacteria. In contrast, Methanococci were dominant in the other three 80 °C fractions. Although Methanococci occurred in both the ¹²C-light and ¹³C-heavy libraries, this group was also in the ¹²C-heavy library. However, based on annotated transcripts there was still separation between the ¹²C-heavy and ¹³C-heavy communities, which indicated that the ¹³C-heavy library was representative of the labeled autotrophic community. In addition, the presence of transcripts attributed to the class Thermococci, a known class of heterotrophic species, in both the ¹²C-light and ¹³C-heavy libraries indicated the possibility of nonspecific labeling in the experiment. Finally, Betaproteobacteria were present in annotated transcripts for SIP metatranscriptomic libraries across all three temperatures (Figure 2). These transcripts were taxonomically classified and represented by various heterotrophic genera including *Burkholderia*, *Ralstonia*, and *Curvibacter*. In all SIP metatranscriptomes, Betaproteobacteria comprised a small percentage, between 0.0003% and 0.2%, of 16S rRNA sequences yet they accounted for a high proportion of the annotated transcripts in a few RNA-SIP metatranscriptomes.

In the RNA-SIP experiments, the majority of RNA was found in only a few fractions across the density gradient, with some fractions having no RNA (Figure 1). This suggests that the majority of the community took up the ¹³C-labeled bicarbonate, which is not entirely surprising given vent systems are dominated by autotrophs and hydrogen amendment pushed these experiments toward autotrophy (Orcutt *et al.*, 2011). Owing to the presence of RNA in only a few fractions, taxonomic analysis of all density fractions was not possible and thus we were not able to completely verify separation of ¹²C and ¹³C-labeled RNA in our experiments. Consequently, the results of the RNA-SIP experiments more resemble enrichment experiments when compared with SIP studies of soil, sediment, and marine surface waters (for example, Lueders *et al.*, 2004; Neufeld *et al.*, 2008; Dumont *et al.*, 2013) and should be treated as such.

Comparison of the 16S rRNA community composition between the Marker 113 metagenome and the ¹³C-heavy metatranscriptomes showed that while the un-manipulated diffuse fluid community was highly

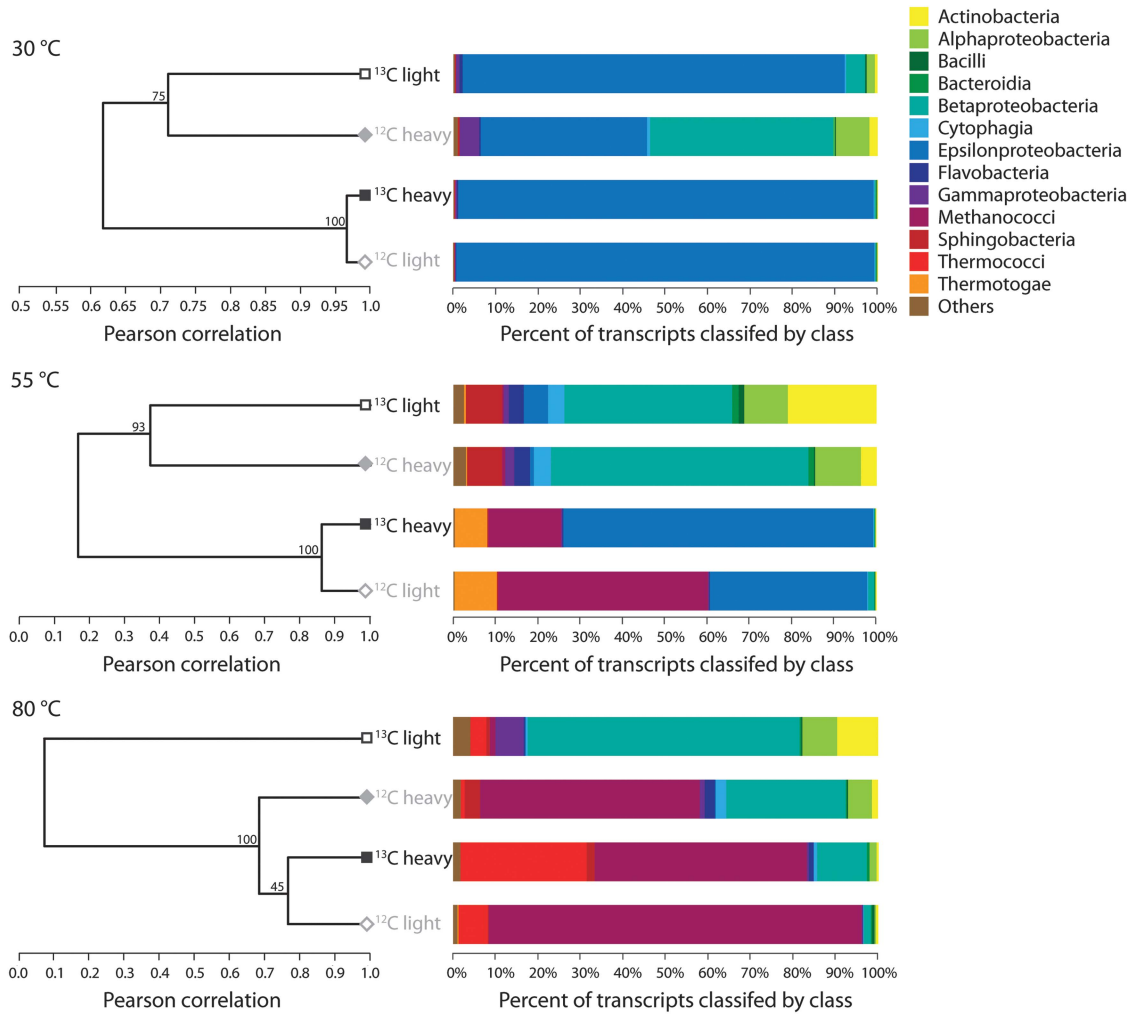


Figure 2 Hierarchical clustering of the four SIP library fractions for each temperature, based on the relative abundance of KO annotated transcripts. The bar graphs display the taxonomy of annotated transcripts at the class level.

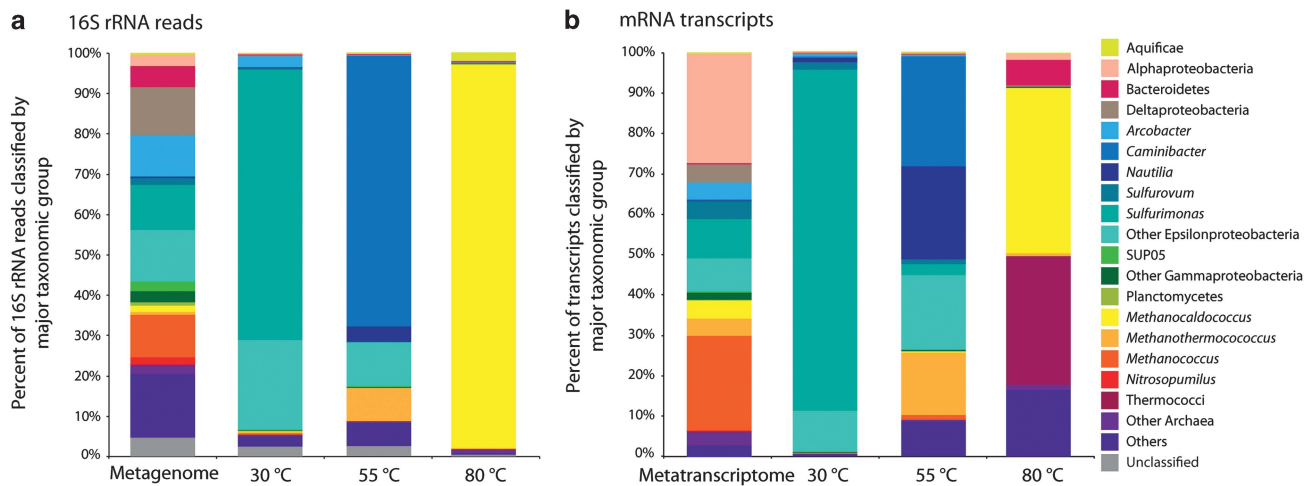


Figure 3 16S rRNA gene (a) and annotated transcript taxonomy (b), classified by major taxonomic group for the ^{13}C -heavy metatranscriptome at each of the three incubation temperatures. Included for comparison is the Marker 113 metagenome and metatranscriptome.

diverse, a different taxonomic group became dominant under hydrogen amended conditions at each incubation temperature (Figure 3a). In the Marker 113 metagenome, there was a mix of both known vent and seawater communities (Figure 3a). The most dominant bacterial groups included Delta-, Epsilon-, and Gammaproteobacteria, which made up 12%, 35%, and 6.5% of the community, respectively. Archaeal 16S rRNA gene sequences comprised 17% of all 16S rRNA gene sequences in the metagenome, with 9% of all 16S rRNA gene sequences classifying to the genus *Methanococcus* (Figure 3a). The 16S rRNA of the SIP metatranscriptomes displayed much less diversity. At 30 °C, the autotrophic community consisted mostly of *Sulfurimonas*, which are known mesophilic Epsilonproteobacteria at vent systems, and made up 66% of the 16S rRNA sequences (Figure 3a). At 55 °C, Epsilonproteobacteria were again dominant, with the thermophilic hydrogen-oxidizing genera *Caminibacter* being the most prevalent. In addition, 9% of 55 °C 16S rRNA sequences were classified to the thermophilic methanogen *Methanothermococcus*. At 80 °C, methanogens, specifically *Methanocaldococcus* species, comprised 95% of the 16S rRNA sequences (Figure 3a).

The taxonomic classification of the transcripts from the Marker 113 metatranscriptome and the three ¹³C-heavy metatranscriptomes was similar to the 16S rRNA community data, but also highlighted distinct differences. Transcripts associated with Alphaproteobacteria, Epsilonproteobacteria, and the mesophilic methanogenic genus *Methanococcus* comprised the majority of the Marker 113 metatranscriptome (Figure 3b). At 30 °C, the taxonomic composition of the transcripts was similar to the 16S rRNA, with the dominance of *Sulfurimonas* (Figure 3b). Some differences between 16S rRNA and

transcript community composition were seen at both 55 °C and 80 °C. In addition to *Caminibacter*, *Nautilia* classified transcripts were prevalent at 55 °C, as were transcripts classified as *Methanothermococcus*, a thermophilic methanogen (15% of all annotated transcripts, Figure 3b). At 80 °C, *Methanocaldococcus* transcripts accounted for 50% of annotated transcripts, with a majority of the rest classified as other archaeal groups including Thermococci (Figure 3b).

Genes were present for a number of carbon fixation pathways in the Marker 113 metagenome, metatranscriptome, and SIP metatranscriptomes. In the metagenome, genes were present for five carbon fixation pathways: the reductive TCA (rTCA) cycle, reductive acetyl-CoA pathway, 3-hydroxypropionate bicycle, 3-hydroxypropionate/4-hydroxybutyrate cycle, and the Calvin Benson Bassham (CBB) cycle. Transcripts were present for all pathways except the 3-hydroxypropionate bicycle (Figure 4). The rTCA cycle was the most abundant carbon fixation pathway in the Marker 113 metagenome, metatranscriptome, and the 30 °C and 55 °C SIP metatranscriptomes (Figure 4). Transcripts for rTCA cycle genes comprised over 4% of all annotated transcripts at 30 °C and were all taxonomically identified as Epsilonproteobacteria, specifically *Sulfurimonas*, *Sulfurovum*, and *Sulfuricurvum* species (Figures 4 and 5). Reductive TCA cycle transcripts comprised about 2% of total annotated transcripts at 55 °C and were mostly assigned to *Caminibacter* and *Nautilia* species (Figures 4 and 5). Transcripts for the reductive acetyl-CoA pathway, the pathway through which methanogens fix carbon, were observed at 80 °C, making up 1% of total annotated transcripts. In addition, transcripts for the rTCA cycle were present and comprised about 0.5% of all annotated transcripts in the 80 °C experiment (Figure 4).

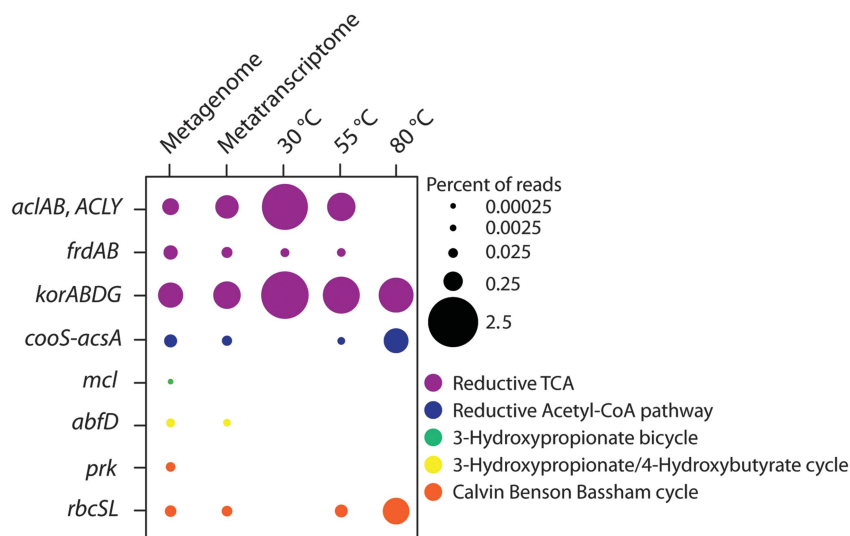


Figure 4 Percentage of reads annotated to genes for carbon fixation pathways for the Marker 113 metagenome, metatranscriptome and the ¹³C-heavy metatranscriptomes from each of the three incubation temperatures. *acl*, ATP-citrate lyase; *frd*, fumarate reductase; *kor*, 2-oxoglutarate ferredoxin oxidoreductase; *cooS-acsA*, carbon-monoxide dehydrogenase; *mcl*, malyl-CoA lyase; *abfD*, 4-hydroxybutyryl-CoA dehydratase; *prk*, phosphoribulokinase; *rbc*, ribulose-bisphosphate carboxylase.

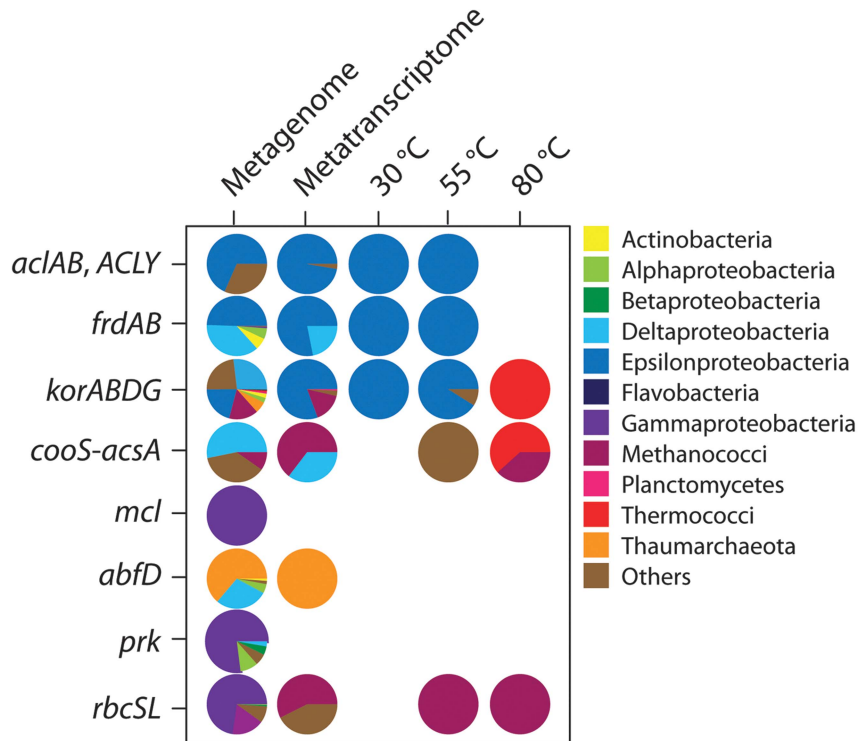


Figure 5 Taxonomy of reads annotated to genes for carbon fixation pathways, classified by class, for the Marker 113 metagenome, metatranscriptome, and the three ^{13}C -heavy metatranscriptomes. Gene names are identical to those listed in Figure 4.

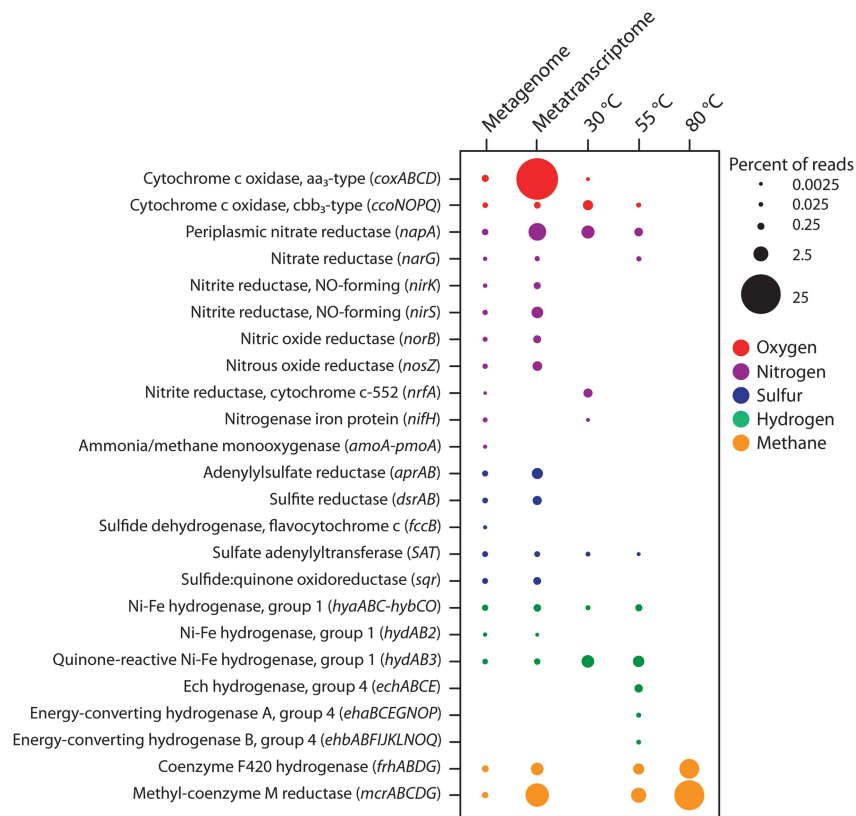


Figure 6 Percentage of reads annotated to genes for key oxygen, nitrogen, sulfur, hydrogen, and methane metabolism genes for the Marker 113 metagenome, metatranscriptome, and the ^{13}C -heavy metatranscriptomes from each of the three incubation temperatures.

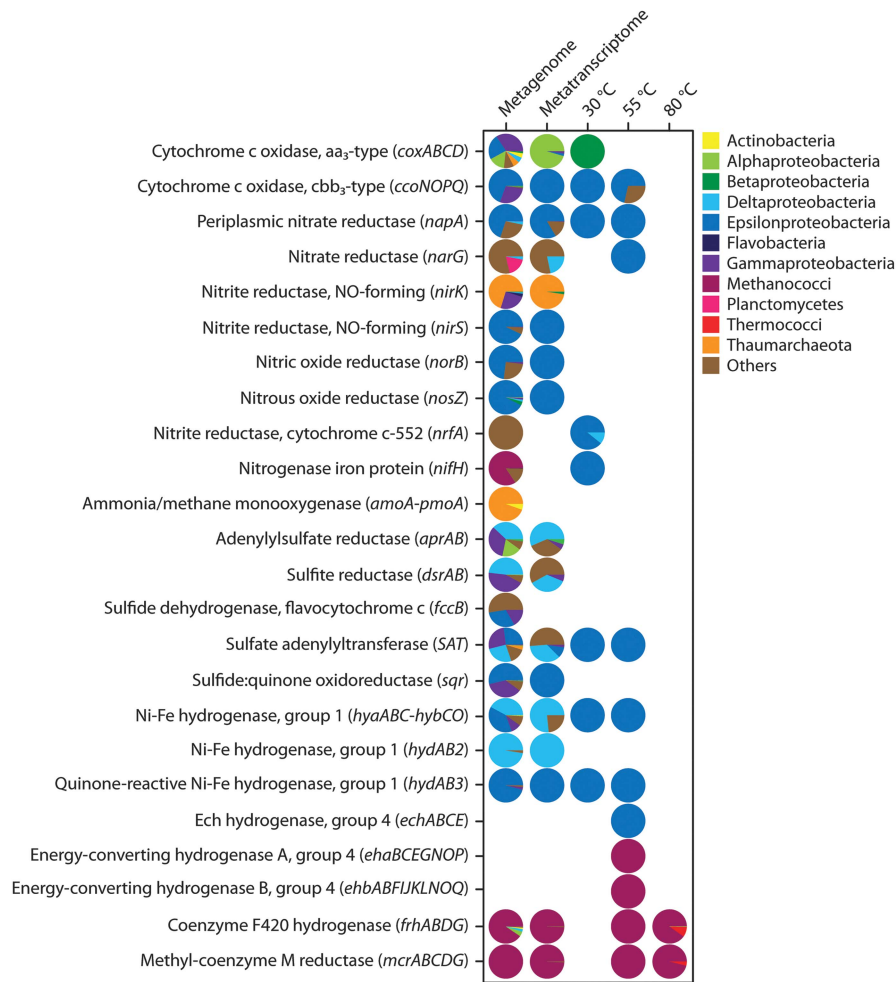


Figure 7 Taxonomy of reads annotated to genes for oxygen, nitrogen, sulfur, hydrogen, and methane metabolism genes, classified by class, for the Marker 113 metagenome, metatranscriptome, and the ¹³C-heavy metatranscriptomes from each of the three incubation temperatures.

Transcripts from this experiment were attributed to methanogens and Thermococci (Figure 5). Rubisco gene transcripts (CBB cycle) were also present at 55 °C and 80 °C and assigned to methanogen genera, specifically *Methanothermococcus* and *Methanocaldococcus* (Figures 4 and 5).

Genes were also present and expressed for many different metabolic pathways. The genes included in Figure 6 were chosen based on the inferred metabolisms of the groups seen in the 16S rRNA and metatranscriptomic sequences and focused on oxygen, nitrogen, sulfur, hydrogen, and methane metabolisms. In the metagenome, genes attributed to oxygen respiration, nitrate reduction, denitrification, ammonium oxidation, nitrogen fixation, sulfur oxidation and reduction, hydrogen oxidation, and methanogenesis were all present (Figure 6). Expression was highest for the cytochrome *c* oxidase aa₃-type gene complex (*cox*), which comprised over 25% of all annotated transcripts in the metatranscriptome (Figure 6). Transcripts for the *coxABCD* genes were classified mostly as Alpha-, Gamma-, and

Epsilonproteobacteria (Figure 7). Other genes that were highly expressed in the metatranscriptome were *napA*, a periplasmic nitrate reductase, and genes involved in methanogenesis (Figure 6). Transcripts for methanogenesis genes made up almost 10% of all annotated transcripts, with the majority (58%) attributed to the mesophilic *Methanococcus* genus (Figure 7). Along with *napA*, other denitrification genes were also expressed, including genes for nitrite, nitric oxide, and nitrous oxide reductases, with 85% of denitrification gene transcripts classified as Epsilonproteobacteria (Figures 6 and 7). For sulfur metabolism, transcripts for sulfate reduction/oxidation genes *dsrAB*, *aprAB*, and *SAT* were classified as Gamma- and Deltaproteobacteria, as well as the archaeal genera *Archaeoglobus* and *Caldivirga* (Figure 7). The sulfur metabolism transcripts classified as Epsilonproteobacteria were for the genes *SAT* and *sqr* (Figure 7).

At 30 °C, the SIP metatranscriptome revealed a transition in the community from the use of oxygen to the use of nitrate as a terminal electron acceptor.

Transcripts were present for both cytochrome *c* oxidase genes as well as for the nitrate reductase gene *napA* (Figure 6). Of the two cytochrome *c* oxidase gene complexes present, the high-affinity *cbb₃* type, *ccoNOPQ*, was more highly expressed. Hydrogen oxidation was the main autotrophic metabolism, as hydrogenase genes *hya*, *hyb*, and *hyd* were all expressed (Figure 6). Transcripts for hydrogen oxidation genes, *napA*, as well as for the *cbb₃*-type cytochrome *c* oxidase gene complex *ccoNOPQ* were all classified as Epsilonproteobacteria, specifically as *Sulfurimonas*, *Sulfurovum*, *Sulfuricurvum*, and *Arcobactor* species (Figure 7). At 55 °C, there was still some evidence of oxygen respiration, with limited expression of the *cbb₃*-type cytochrome *c* oxidase gene, but nitrate reduction transcripts for *napA* and *narG* genes were more abundant (Figure 6). Hydrogen oxidation and methanogenesis were the main autotrophic metabolisms. Hydrogenase genes, *hya*, *hyb*, *hyd*, and *ech* were expressed and mostly classified as known thermophilic hydrogen oxidizers *Caminibacter* and *Nautilia* (Figures 6 and 7). Methanogenesis genes were also expressed at 55 °C and classified as the thermophilic methanogen *Methanothermococcus* (Figures 6 and 7). Methanogenesis was the dominant metabolism at 80 °C, with 18.5% of all annotated transcripts attributed to the *mcr* and *frh* gene complexes (Figure 6). Nearly 95% of transcripts annotated to genes for methanogenesis were classified as the hyperthermophile *Methanocaldococcus* (Figure 7).

Discussion

The microbial communities in low-temperature diffuse hydrothermal fluids are metabolically and taxonomically complex because of the mixing of deep seawater and seafloor vent communities. This mixing can make it difficult to tease apart the vent, seafloor, and seafloor signatures from those of deep-water microorganisms growing in background seawater near the seafloor (Akerman *et al.*, 2013). In this work, we set out to characterize the hydrogenotrophic chemolithoautotrophic community present in vent fluids using both meta-omics and RNA-SIP. Through metagenomics and metatranscriptomics, we first determined the taxonomic diversity and range of metabolic processes in un-manipulated diffuse fluids. We then used RNA-SIP metatranscriptomics in hydrogen-enriched incubations to specifically target the seafloor chemolithoautotrophic community and to identify the key taxa and metabolisms occurring across different temperatures representative of the warm, reducing seafloor habitat. Analysis of un-manipulated fluids showed a phylogenetically and metabolically diverse group of bacteria and archaea from both background seawater and vent environments including members of the seafloor community. However, under experimental

conditions at elevated temperatures, hydrogen amendment, and reducing conditions, diversity was greatly reduced. Still, distinct genera and metabolisms dominated the seafloor autotrophic community when comparing different temperature regimes.

In the shipboard experimental treatment of vent fluids, there were a number of factors that needed to be addressed, including the introduction of oxygen, outgassing of methane and hydrogen, pH changes, limited access to samples, and timing of incubations. Hydrogen concentrations at Marker 113 are low, yet previous work has shown that seafloor hyperthermophilic methanogenic archaea are abundant there and require a minimum hydrogen concentration of ~17–23 μM for growth (Ver Eecke *et al.* 2012). Given these known constraints, hydrogen was added to the SIP experiments at this level in order to mimic likely energy-rich, reducing, and warm seafloor conditions. When hydrogen was not added, there was no label uptake at any temperature (30 °C, 55 °C and 80 °C) and little RNA was recovered (data not shown).

The length of incubations is also a challenge to SIP experiments, as it is important to minimize cross-feeding, the uptake of the labeled isotope by a non-target community. The incubation time of the experiments was estimated from previous work with cultured representatives from mesophilic, thermophilic, and hyperthermophilic seafloor communities (Mehta and Baross, 2006; Ver Eecke *et al.*, 2012; Meyer and Huber, 2014). In our 80 °C experiments, the 36-h incubations resulted in almost all transcripts assigned to heterotrophic bacteria, indicating the consumption of labeled organic matter and an incubation time that was too long to capture the autotrophic community (Supplementary Figure S1). Cross-feeding also appeared to occur, but to a lesser extent, in the 80 °C 18-h incubation, with about 30% of annotated transcripts assigned to the heterotrophic phylum Thermococci, and specifically to the genera *Thermococcus*, *Palaeococcus*, and *Pyrococcus*. A possible syntrophic relationship between hyperthermophilic heterotrophs like *Thermococcus* and methanogens has been previously observed (Ver Eecke *et al.*, 2012) and may explain the labeling of this heterotrophic community even in our shorter 18-h incubation. The length of our experiments plus amendment with hydrogen resulted in RNA being concentrated in only a few density fractions (Figure 1) and thus we were unable to taxonomically analyze all fractions to verify separation of ¹²C and ¹³C-labeled RNA. Our RNA-SIP experiments instead resemble experimental enrichments with a labeled substrate, in this case ¹³C-labeled bicarbonate. In density fractions with low RNA concentrations, mainly in the ¹²C-heavy and ¹³C-light controls, we found a high abundance of transcripts for a common laboratory contaminant. Betaproteobacteria comprised a large percentage of the annotated transcripts from many of the ¹²C-heavy

and ^{13}C -light metatranscriptomes, regardless of temperature. This group has recently been shown to be present as a common lab contaminant in extraction kit reagents and is abundant in sequence data of samples with low microbial biomass (Salter *et al.*, 2014), consistent with our findings.

Given the challenges of carrying out RNA-SIP under simulated seafloor conditions, it was important to compare our experimental treatments to the Marker 113 metagenome and metatranscriptome of un-manipulated fluids, which represent the potential and active metabolic processes occurring within mixed seawater-vent fluids as they exit the seafloor. The Marker 113 metagenome was comprised of genes for a diverse array of aerobic and anaerobic metabolic processes including sulfur oxidation and reduction, hydrogen oxidation, denitrification, and methanogenesis (Figure 6). Transcripts for all of these processes were also observed in the Marker 113 metatranscriptome. In addition, genes for many different carbon fixation pathways were observed and expressed, again showing the metabolic flexibility of vent communities (Figure 4).

Epsilonproteobacteria comprised over one-quarter of the 16S rRNA gene sequences in the metagenome and 8% of annotated transcripts in the metatranscriptome. Epsilonproteobacteria are an abundant and metabolically versatile group in many habitats at deep-sea hydrothermal vents, including diffuse vent fluids (Huber *et al.*, 2003; Takai *et al.*, 2003; Campbell *et al.*, 2006). The metabolic flexibility of the Epsilonproteobacteria was observed in both the Marker 113 metagenome and metatranscriptome, with genes and transcripts for both hydrogen and sulfur oxidation present. The measured oxygen at Marker 113 was just below $7\ \mu\text{M}$, indicating a low oxygen environment at the point of venting and the potential for use of oxygen or nitrate as terminal electron acceptors. Epsilonproteobacteria appeared to take advantage of these conditions, as both cytochrome *c* oxidase and denitrification genes that classified as Epsilonproteobacteria were present and expressed. In fact, in the Marker 113 metatranscriptome, all transcripts for the *cbb*₃-type cytochrome *c* oxidase, along with 85% of all denitrification gene transcripts, were classified as Epsilonproteobacteria, demonstrating the genomic plasticity of this key phylum and the ability of these bacteria to flourish across redox gradients at hydrothermal vents and within the underlying seafloor.

Methanogenic archaea are also important autotrophs at Marker 113. Long-term chemistry measurements at Marker 113 have shown anomalously high methane concentrations in the diffuse vent fluids, which suggest a biologically derived source and prominence of methanogenesis at this site (Ver Eecke *et al.*, 2012). Previous work using quantitative PCR and sequencing of the *mcrA* gene for methanogenesis have shown the presence of different methanogen genera at a wide range of temperatures at Marker 113 (Ver Eecke *et al.*, 2012). In this study, we observed

the expression of genes associated with mesophilic, thermophilic, and hyperthermophilic methanogens including *Methanococcus*, *Methanothermococcus*, and *Methanocaldococcus* genera, respectively. In the Marker 113 metatranscriptome, 66% of archaeal transcripts and nearly half the transcripts for the *mcr* gene complex (*mcrABG*) for methanogenesis were classified as the mesophilic group *Methanococcus*. Methanogens are thus present and active across a wide temperature range and are important contributors to deep-sea primary production at this site.

In response to the hydrogen enrichment of diffuse vent fluids, the main autotrophic metabolism in the 30 °C and 55 °C SIP experiments was hydrogen oxidation coupled with use of oxygen or nitrate as a terminal electron acceptor. Molecular hydrogen is a key energy source for hydrothermal plume, diffuse fluid, seafloor, and vent symbiont communities (Petersen *et al.*, 2011; Wankel *et al.*, 2011; Anantharaman *et al.*, 2013). Within the 55 °C experiment, hydrogenase gene transcripts were classified as *Caminibacter* and *Nautilia*, both known thermophilic, hydrogen oxidizing, nitrate (or sulfur) reducing Epsilonproteobacteria genera (Meyer and Huber, 2014). At 30 °C, hydrogenase gene transcripts were classified mostly as *Sulfurimonas* and *Sulfuricurvum*, specifically as *Sulfurimonas sp. GD1*, *Sulfurimonas denitrificans* and *Sulfuricurvum kujiense*, all of which possess the capability to oxidize hydrogen (or sulfide) using either nitrate or oxygen. Although there are many mesophilic Epsilonproteobacteria that use both oxygen and nitrate, there is currently only one vent-associated cultured representative from the *Sulfurimonas* genus that has been shown to both oxidize hydrogen and reduce nitrate, *Sulfurimonas parvalvinellae*, isolated from a vent at the Mid-Okinawa Trough (Takai *et al.*, 2006). *Sulfurimonas parvalvinellae* is a facultative anaerobe with optimal growth observed with nitrate as the sole electron acceptor (Takai *et al.*, 2006). The results seen in the 30 °C SIP metatranscriptome suggest a similar metabolism for seafloor *Sulfurimonas* species at Axial Seamount.

Likely, the little oxygen present in the fluids was consumed during the 36-h enrichment with hydrogen in all incubations. Accordingly, the transition between aerobic and anaerobic metabolisms was seen in the 30 °C, and to a lesser extent in the 55 °C SIP experiments. In both the 30 °C and 55 °C, the *cbb*₃-type cytochrome *c* oxidase gene was the cytochrome oxidase gene most highly expressed. The *cbb*₃-type cytochrome *c* oxidase is present in bacteria only and has been shown to be a high-affinity cytochrome *c* oxidase, with the highest activity under low oxygen conditions (Ekici *et al.*, 2012). The depletion of oxygen probably induced the expression of genes for nitrate reduction at 30 °C and 55 °C by Epsilonproteobacteria. Transcripts for nitrate reduction via the *napA* gene comprised about 2% of the total annotated transcripts in the 30 °C experiment and 0.5% in the 55 °C experiment.

Nitrate reduction via the *nap* operon has been shown to be highly conserved and widespread throughout the Epsilonproteobacteria (Vetriani *et al.*, 2014). NapA has been shown to be a high-affinity nitrate reductase and only expressed during low nitrate conditions (Potter *et al.*, 1999), which may explain the prevalence of this gene within the Epsilonproteobacteria, as hydrothermal fluid is depleted in nitrate compared with deep seawater (Vetriani *et al.*, 2014).

At 30 °C, other mesophilic autotrophic groups, specifically SUP05, were not detected. However, we did not add sulfide to the enrichments, which may have been limiting further growth of sulfide-oxidizing autotrophs. SUP05 is a key sulfur and hydrogen oxidizer in the deep sea, oxygen minimum zones, and hydrothermal plumes (Walsh *et al.*, 2009; Stewart *et al.*, 2012; Anantharaman *et al.*, 2013) and is present and active at Axial Seamount and specifically at Marker 113 (Akerman *et al.*, 2013; Anderson *et al.*, 2013). Sequences identified to the SUP05 group were present in low concentrations in both the metagenome and metatranscriptome but were absent in the 30 °C SIP metatranscriptome. Given their ubiquity in the deep ocean and hydrothermal plumes where temperatures rarely exceed 5 °C, SUP05 populations likely have a lower growth optima temperature than 30 °C and thus are not expected to be present in our SIP experiments. In addition, another explanation could be the possible lack of nitrate reduction genes in SUP05 populations. A recent genomic study of two SUP05 isolates from a hydrothermal plume revealed they did not possess genes for nitrate reduction (Anantharaman *et al.*, 2013). In our SIP experiment, the main metabolic process occurring at 30 °C was hydrogen oxidation using oxygen or nitrate. This lack of nitrate reduction genes could also explain why SUP05 was not present in our mesophilic SIP experiment.

In the Marker 113 metatranscriptome, most transcripts identified as methanogenic archaea were classified as the putatively mesophilic *Methanococcus* genus, but in our SIP experiments, methanogenesis at higher temperatures was observed. Methanogenesis was the only autotrophic metabolism observed at 80 °C, with nearly 20% of transcripts annotated to methanogenesis genes and all were classified as the hyperthermophilic genus, *Methanocaldococcus*. At 55 °C, along with the thermophilic Epsilonproteobacteria, *Methanothermococcus*, a thermophilic methanogen, was present, making up 16% of the community. Although carbon fixation gene transcripts for the reductive acetyl-CoA pathway were observed at 55 °C and 80 °C, transcripts for Rubisco genes were also present and were all classified as *Methanothermococcus* and *Methanocaldococcus*. These methanogens are not using Rubisco for carbon fixation as they lack other important enzymes in the CBB cycle, namely phosphoribulokinase, but they have been shown to actively synthesize the enzyme (Finn and Tabita,

2004). The function of Rubisco in methanogens is not fully understood, but it could have a role in purine recycling (Finn and Tabita, 2004). These experiments show that at Marker 113, thermophilic and hyperthermophilic subseafloor methanogens are active in subseafloor carbon cycling.

In conclusion, metagenomic and metatranscriptomic analyses revealed the high taxonomic diversity and extensive metabolic potential of microbial communities found in venting fluids at hydrothermal vents. RNA-SIP experiments under simulated subseafloor conditions revealed that different phylogenetic groups and metabolisms dominated at different temperature regimes. Future experiments at geochemically diverse sites will help to further determine the extent of taxonomic and metabolic diversity across hydrothermal vents and the key autotrophic metabolisms that occur under different redox and geothermal conditions.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

This work was funded by the Gordon and Betty Moore Foundation Grant GBMF3297 and NSF Center for Dark Energy Biosphere Investigations (C-DEBI) (OCE-0939564). The data collected in this study is based upon work supported by the Schmidt Ocean Institute during cruise FK010-2013 aboard R/V *Falkor*. We thank the captain and crew of the R/V *Falkor*, as well as the entire ROV ROPOS group. Chris Algar, Dave Butterfield, Ben Larson, Jim Holden, Giora Proskurowski, Lisa Zeigler Allen, Kevin Roe, Lucy Stewart, and Begum Topcuoglu provided critical support in sample collection and experimental design and execution. We would also like to thank the ISME J editor and reviewers for the detailed and helpful comments on the manuscript. This is C-DEBI contribution number 267.

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