

Microbial survival mechanisms within serpentizing Mariana forearc sediments

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Editor: Lee Kerkhof

Abstract

Marine deep subsurface sediment is often a microbial environment under energy-limited conditions. However, microbial life has been found to persist and even thrive in deep subsurface environments. The Mariana forearc represents an ideal location for determining how microbial life can withstand extreme conditions including pH 10–12.5 and depleted nutrients. The International Ocean Discovery Program Expedition 366 to the Mariana Convergent Margin sampled three serpentizing seamounts located along the Mariana forearc chain with elevated concentrations of methane, hydrogen, and sulfide. Across all three seamount summits, the most abundant transcripts were for cellular maintenance such as cell wall and membrane repair, and the most abundant metabolic pathways were the Entner–Doudoroff pathway and tricarboxylic acid cycle. At flank samples, sulfur cycling involving taurine assimilation dominated the metatranscriptomes. The *in situ* activity of these pathways was supported by the detection of their metabolic intermediates. All samples had transcripts from all three domains of Bacteria, Archaea, and Eukarya, dominated by *Burkholderiales*, *Deinococcales*, and *Pseudomonales*, as well as the fungal group Opisthokonta. All samples contained transcripts for aerobic methane oxidation (*pmoABC*) and denitrification (*nirKS*). The Mariana forearc microbial communities show activity not only consistent with basic survival mechanisms, but also coupled metabolic reactions.

Keywords: IODP, mariana forearc, metatranscriptome, microbial survival, serpentine mud volcano, subsurface sediment

Introduction

The marine deep subsurface covers a vast area of approximately 70% of the Earth's surface. Within the marine deep subsurface there are various environments including abyssal plains, hydrothermal vents, gas hydrates, and tectonic plate interactions, which serve as microbial habitats (Orcutt et al. 2011). Many studies have focused on which microorganisms colonize these types of environments and the potential for their activity (Biddle et al. 2008, Biddle et al. 2011, Brazelton et al. 2012, Reese et al. 2012, Baker and Dick 2013, Orcutt et al. 2013, Labonté et al. 2015, Meyer et al. 2016, Marshall et al. 2018, Tully et al. 2018, Farag et al. 2020). The current estimate for microbial diversity in the marine subsurface is approximately 7.9×10^3 to 6.1×10^5 bacterial amplicon sequence variants (ASV) and 3.3×10^4 to 2.5×10^6 archaeal ASVs (Hoshino et al. 2020), yet there are many microbial groups and metabolic processes awaiting discovery (Hug et al. 2016, Hoshino et al. 2020, Nayfach et al. 2020). A few studies have further elucidated putative activities of *in situ* microbial communities including the Baltic Sea Basin (Zinke et al. 2017) and Peru Margin (Orsi et al. 2013). This dynamic environment offers a unique Earth analog to extraterrestrial exploration due to serpentization reactions occurring within the Mariana forearc mud volcanoes. Previous studies on accretionary prisms that form mud volcanoes have found microbial activity to use surrounding geochemical re-

sources such as sulfide, sulfate, nitrogen, and methane for energy (Jones et al. 2015). Serpentization at the Mariana forearc releases methane and hydrogen gas which can be moved upward through these mud volcanoes and be used for metabolic processes by microbial communities. This provides an opportunity to understand how geophysical processes impact the survival of the microbial community.

The Mariana forearc is a nonaccretionary subduction zone that lies less than 100 km west of the Mariana Trench (Uyeda 1982, Curtis et al. 2013, Fryer et al. 2017). The Mariana system consists of an active subduction zone where the Pacific plate is consistently subducting beneath the Philippine plate. A chain of serpentinite mud volcanoes have formed in this forearc along the intersection of faults in the Philippine plate. Modern serpentine mud volcanoes on Earth have only been found along the Mariana forearc thus far (Fryer et al. 2020); however, Earth's geologic record indicates that serpentinite volcanism occurred as early as 3.8 billion years ago (Pons et al. 2011, Fryer et al. 2020). The origination of the serpentinite minerals found within the Mariana forearc sediments occurs from the subducted overlying seawater on the Pacific Plate interaction with the ultramafic, olivine-rich minerals contained on the overlying Philippine Plate (Fryer et al. 2020). Faults within the Philippine and Pacific plates permit fluids, mud, and rocks to be transported up through the resulting conduits, cre-

Received: February 23, 2022. Revised: December 7, 2022. Accepted: January 10, 2023

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ating seamounts. This transport throughout the seamount sediments can provide resources for microbial life such as sulfur, carbon, and hydrogen cycling.

Many microorganisms can use resources produced from serpentinization reactions for their unique chemosynthetic metabolisms, thus allowing for life to thrive (Schulte et al. 2006, McCollom and Seewald 2013) resulting in a 'serpentinite biosphere' (Fujioka et al. 2002). As serpentinization reactions occur, hydrated materials from the interaction may eventually reach the seafloor thus forming mud volcanoes, or seamounts (Schrenk et al. 2013). The mud volcanoes formed from serpentinization allow for the release of fluids that are typically extremely alkaline (reaching pH 12.5) and contain greater concentrations of methane, hydrogen, acetate, and formate (Fryer et al. 2003).

The Mariana forearc was sampled in December 2016–February 2017 during the International Ocean Discovery Program (IODP) Expedition 366. This expedition focused on understanding mass transport, geochemical cycling, spatial and temporal variability of slab-related fluids and metamorphic and tectonic processes, physical properties of the subduction zone, and biological activity (Fryer et al. 2017). Conical and South Chamorro seamounts located along the chain were previously sampled on Leg 125 and Leg 195, respectively (D'Antonio and Kristensen 2004, Savov et al. 2005, Albers et al. 2020, Fryer et al. 2020). However, neither of these expeditions focused on microbial life. Microorganisms have been investigated previously in the South Chamorro seamounts by lipids and 16S rRNA gene analyses (Mottl et al. 2003, Curtis et al. 2013).

Previous studies of the Mariana forearc have focused on geochemistry from pore fluids, petrology, and sedimentology. There have been no studies to date examining the microbial communities within the Mariana forearc and if they are actively withstanding the extremely alkaline conditions, especially in Asút Tesoru. Examining the microbial community diversity and functionality through metatranscriptomics and metabolomics provides insight into the microbial communities surviving and possibly active without the limitation of cultivation alkaline systems.

Here we report the first metatranscriptome and metabolome results for microbial communities within subsurface marine sediment of three Mariana forearc serpentine mud volcanoes. The flanks and summits of Yinazao (IODP Site U1492), Asút Tesoru (IODP Sites U1493, U1494, U1496), and Fantangisña (IODP Site U1497) mud volcanoes were sampled during the International Ocean Discovery Program Expedition 366 to the Mariana Convergent Margin in December 2016–February 2017. We extracted total RNA (e.g. tRNA, mRNA, rRNA) as a proxy for microbial activity to determine which microorganisms were active and which of their genes were being transcribed. We also extracted and identified small metabolites to show which of these functions are supported by the presence of small intermediate molecules.

Materials and methods

Site description

The Mariana forearc, which lies less than 100 km west of the Mariana Trench, was sampled onboard the D/V JOIDES Resolution during IODP Expedition 366 (Fig. 1) (Fryer et al. 2017, Eickenbusch et al. 2019). Yinazao seamount sits 55 km west of the trench and the summit (borehole U1492A) is approximately 13 km above the subducting slab. Fantangisña seamount is 62 km from the Mariana trench and the summit (boreholes U1497B and U1497B) lies approximately 14 km above the subducting slab. Asút Tesoru

seamount is the furthest west of the trench at 72 km and the summit (boreholes U1496A and U1496B) lies approximately 18 km above the subducting slab. Two flank samples were also taken from Asút Tesoru, boreholes U1493B and U1494A. The subducting slab under each seamount has experienced a wide range of temperatures from 80°C beneath Yinazao up to 250°C under Asút Tesoru (Fig. 1) (Fryer et al. 2017, Eickenbusch et al. 2019).

The pH increased with depth in Yinazao seamount from 7.7 to 11.1, while the pH increased in Asút Tesoru from 11.8 to 12.4 in the first few meters of sediments (Fryer et al. 2017). Fantangisña seamount increased in pH from 9.0 to 11 in the first 10 meters of sediment. Asút Tesoru had the highest methane concentrations ranging from 1.0 to 6.9 mM in the summit samples, while in the flanks, methane concentrations ranged from 0.9 to 52 µM, indicating greater serpentinization activity in the summit (Fryer et al. 2020). Yinazao summit had methane concentrations ranging from below detection to 30 µM. Fantangisña summit methane concentrations ranged from below detection to 6.6 µM (Fryer et al. 2017).

Sample collection

Subsections of the whole round cores from depths of 1–20 meters below seafloor were collected using sterile cut syringes, stored in 50 ml cryotubes, and frozen at -80 °C. Drilling fluid was also collected in 50 ml conical vials and frozen at -80°C. Sediment cores were screened on board for perfluoromethyldecaline (PFMD) and perfluoromethylcyclohexane (PMCH) using gas chromatography to evaluate potential contamination from drill fluid penetration. Sediment collected for Single-Amplified Genome (SAG) analysis was preserved in a 5% glycerol solution and stored at -80°C. Sediment and drill fluid samples were shipped on dry ice and stored at -80°C at Texas A&M University-Corpus Christi for RNA analysis and to University of Tennessee-Knoxville for metabolomic analysis. Samples chosen for DNA and RNA extractions were selected based on the corresponding geochemical profile, cellular abundance, and those without potential contamination from drilling fluid and total cell abundance (Eickenbusch et al. 2019). For full methodology of potential tracer contamination and cellular abundance please see Eickenbusch et al. 2019. Seven sediment samples had cell counts above detection limit with below detection for perfluorocarbon detection (Table 1) (Eickenbusch et al. 2019). One summit sample was extracted from Yinazao seamount (U1492A-1H1), two flank samples (U1493B-4F2 and U1494A-1F3) and two summit samples (U1496A-1F1 and U1496B1F1) were extracted from Asút Tesoru Seamount, and two summit samples were extracted from Fantangisña Seamount (U1497B-1F1 and U1497B-6F2) (Table 1).

RNA extraction

A clean hood was used for all extractions and was sterilized with 70% ethanol, RNaseZap (Ambion, Foster City, CA, USA), and UV light. All consumables were RNase-free and glassware was baked at 450°C for four hours prior to use. Individuals extracting samples wore a hairnet, face mask, gloves, cuffed lab coat, and shoe covers to prevent contamination. Sediments collected for metatranscriptomic analyses were extracted in 10 gram replicates per sample using a modified hot alkaline lysis method (Morono et al. 2014). A second chloroform: isoamyl alcohol step was included instead of a phenol: chloroform: isoamyl alcohol separation. The final step of the alkaline lysis method using a spin column filled with polyvinylpyrrolidone was not used. Instead, the pellet of RNA was resuspended in 100 µl of 10 mM Tris-HCl (pH 8.0) for downstream analyses. DNA was removed from samples using the

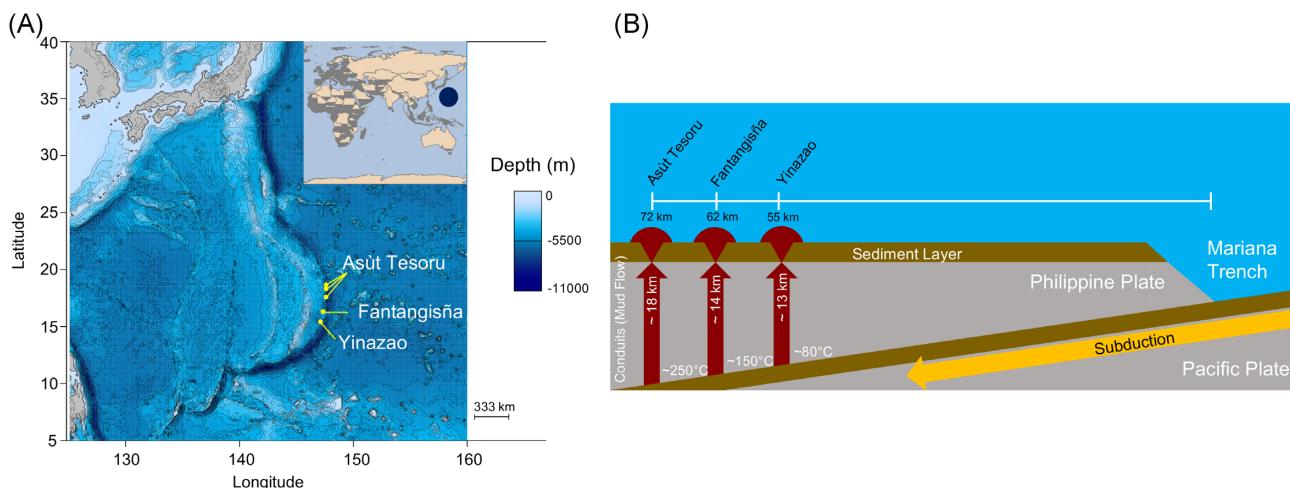


Figure 1. Geography of the Mariana forearc. (A) Bathymetric map of the Mariana forearc serpentine mud volcanoes with the yellow points indicating sampling sites. (B) Schematic of the Mariana system where the serpentine mud volcanoes are in conjunction with the Mariana Trench.

TURBOTM DNA-free kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The DNase-treated extracts were used as a template for PCR using universal primers (515F and 926R) to ensure extracts contained only RNA (Parada et al. 2016, Zinke et al. 2017). DNA-free extractions were cleaned and concentrated using the Zymo RNA Clean and Concentrator kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions. Resulting cleaned RNA extractions were quantified using the Qubit HS RNA Assay on a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). A no-template control consisting of sterile water and extraction reagents (i.e. extraction blank) and drilling fluid were extracted alongside the samples to assess contamination.

Metatranscriptome sequencing

A total of seven samples, a sample of extracted drill fluid, and an extraction blank were sequenced (Table 1). Molecular Research DNA Laboratory (Shallowater, TX, USA) conducted library preparation and sequencing using an Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA) with 2×150 basepair paired-end read chemistry. Whole transcriptome amplification was done using the QuantiTect Whole Transcriptome kit (Qiagen, Germantown, MD, USA) then quantified using the Qubit RNA HS Assay kit (Life Technologies, Carlsbad, CA, USA). Libraries were prepared using Nextera DNA Flex library preparation kit (Illumina Inc., San Diego, CA, USA) following the manufacturer's instructions (Table 2) generating sequences approximately 300 bp in length. All raw sequences were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under project accession number PRJNA592129.

Sequence analysis

Sequence quality was monitored using FastQC (Andrews 2010) and forward and reverse reads were merged using Flash version 2.2.0 (Magoč and Salzberg 2011). Adapters were trimmed and quality was filtered using TrimGalore! version 0.6.2 (Krueger 2015) with a Phred score of 25 (Mullis et al. 2019). Reads were annotated using BlastX in Diamond (Buchfink et al. 2015) against the Non-Redundant database (NR, downloaded in April 2019) from NCBI. MEGaGenome ANalyzer version 6.18.0 was used to predict genes in all merged and unmerged reads from each sample (Huson and Weber 2013). Transcripts were annotated against

Clusters of Orthologous Groups (COG) (Galperin et al. 2014), SEED, and InterProGo (Huson and Weber 2013) databases. Annotations were normalized to the smallest number of reads within a sample using the program MEGAN (Huson and Weber 2013). Sequences were annotated using Prokka version 1.11 on default settings (Seemann 2014) and Kyoto Encyclopedia of Genes and Genomes (KEGG). Orthologies were assigned using KofamScan version 1.1.0 (Aramaki et al. 2019). The Carbohydrate-Active enZymes database was used to specifically annotate transcripts associated with carbon cycling (Lombard et al. 2013). To compare gene expression from the Mariana forearc to the Mariana backarc (previously sampled in 2016), gene sequences for key metabolic genes for oxygen, nitrogen, hydrogen, methane, and sulfur cycling were downloaded from UniProt (accessed July 2020) (Consortium 2018, Trembath-Reichert et al. 2019). All sequences annotated for genes of interest were downloaded and clustered using CD-Hit at 90% similarity (Fu et al. 2012). Once clustered, all genes of interest were used to create a database using BLAST v 2.9.0 (Altschul et al. 1990). All reads from each sample were compared to each database using BLASTX and the top hit was recorded for each sample. All annotations were normalized to transcripts per million (tpm) for comparison to the Mariana backarc metatranscriptomes (Trembath-Reichert et al. 2019).

Microbial community analysis was performed by isolating reads annotated as SSU 16S rRNA transcripts using phyloFlash version 3.3b3 (Gruber-Vodicka et al. 2019) and were classified using Silva 138 release for the entire SSU 16S/18S rRNA genes (Quast et al. 2012, Yilmaz et al. 2014).

Contamination removal

The drill fluid used on Expedition 366 was extracted alongside samples as well as a no-template control to serve as quality control to ensure no contamination was introduced upon sampling the Mariana forearc. Sequences were trimmed of adapters and above a phred score of 25. The trimmed, good-quality reads from the drill fluid and no-template control were concatenated to use as a reference library for removing contamination. To remove potential contamination from samples, BBSSplit was used to map sample reads to the 'contamination' reads and create two output files (Bushnell 2014). BBSSplit uses BBMap, an ultra-fast aligner to map reads to a reference library (Bushnell 2014), which in this case is the 'contamination' sequences. The output from BBSSplit

Table 1. Mariana forearc samples collected and analyzed from IODP Expedition 366 to the Mariana Convergent Margin.

Seamount	Sample	Latitude	Longitude	Water Depth (meters below seafloor)	Location on Seamount	Trench Axis (km)	Depth to the Mariana Trench Axis (km)	Subducting Slab (km)	Cell Counts (cells/cm ³)	PFMD Tracers Peak Area
Virnzaizo	U1497A-1H1	15°42'57"N	147°10'50"E	1.36	3666	Summit	55	13	1.40E+04	4.89E-02*
Astut Tasonu	U1493B-4F2	17°59'16"E	147°06'00"E	17.8	3259	Peak	72	25	6.74E+0	2.24E02
Astut Tasonu	U1494A-1F3	18°30'89"E	147°06.0003"E	3.0	2200	Peak	72	25	6.33E+03	1.12E+03*
Astut Tasonu	U1496A-1F1	18°65'99"E	147°06.0998"E	0.9	1244	Summit	72	25	4.64E+05	6.76E+02*
Fantangisia	U1496B-1F1	18°32'22"E	147°13.2606"E	0.81	2020	Summit	72	25	1.49E+06	2.81E+02*
Fantangisia	U1497B-6F2	16°32'22"E	147°13.2606"E	1.4	2020	Summit	62	19	1.04E+05	6.09E+02
				20.6					1.09E+05	9.49E+02

* Denotes PFMD tracer concentration in adjacent samples.

Table 2. Sequencing metrics from each sample from raw sequences to cleaned annotated reads.

Sample	DNA Concentration (ng/µL)	Library Concentration (ng/µL)	Average Library Size (bp)	Sequence Yield	Quality Reads	# Reads after contamination removal
U1492A-1H1	652.00	10.70	603	5.95 Gbp	23893776	14274204
U1493B-4F2	536.00	5.04	661	4.04 Gbp	15826022	5827010
U1494A-1F3	556.00	6.18	543	2.91 Gbp	10790044	3335528
U1496A-1F1	676.00	14.90	680	5.35 Gbp	22122126	17109268
U1496B-1F1	688.00	7.50	676	5.61 Gbp	19415465	18319848
U1497B-1F1	672.00	19.10	678	5.38 Gbp	23262527	19758282
U1497B-6F2	728.00	16.30	656	5.14 Gbp	21190914	15294686
Drill Fluid	644.00	10.20	654	4.71 Gbp	20672320	NA
Extraction Control	652.00	9.66	640	3.89 Gbp	13835894	NA

creates two files where the reads mapped to the reference library and another file with reads that did not successfully map (Bushnell 2014). Only sequences that did not map to the 'contamination' sequences were then used for downstream analyses.

Following the extra contamination controls recommended by Orsi et al. 2020 for low biomass samples, reference genomes for taxa commonly associated with contamination (i.e. 'kitome') (Sheik et al. 2018) were downloaded from the National Center for Biotechnology Information in July of 2021 (Orsi 2020). These taxa included *Burkholderia*, *Pseudomonas*, *Rhizobium*, *Sphingobacterium*, *Sphingomonas*, *Staphylococcus*, and *Streptococcus*. Complete genome assemblies for each taxon were downloaded (Table S1), concatenated, and used as a reference to align the metatranscriptome sequences that did not align to contamination controls using bbmap version 38.90 using 'perfectmode' (Bushnell 2014, Orsi 2020). Sequences that did not align to the reads from the drill fluids, the no-template controls, or the reference genomes of common contaminants, were used for downstream annotation. See statistical analysis section for further checks on contamination.

Metabolomics

Metabolites were extracted and measured as described previously (Bird et al. 2019) at the Biological and Small Molecule Mass Spectrometry Core (BSMMSC), University of Tennessee, Knoxville, TN (RRID: SCR_021368). Briefly, metabolites were extracted from whole sediments for the entire sediment cores by grinding triplicate 0.5 g subsamples in a mortar and pestle in liquid nitrogen, and the metabolome was measured using a Dionex Ultimate 3000 UPLC with a Thermo Scientific Exactive Plus Orbitrap MS (Lu et al. 2010). Generated data were analyzed using the MAVEN software package to visualize chromatograms and extract intensity (ion counts), retention time (min), and mass (*m/z*) data (Melamud et al. 2010). Metabolite intensities were normalized to sample mass. Metabolites were identified using a list of known retention times (± 1 min) and *m/z* (± 5 ppm) values, and ion counts for each were compiled from areas under the curve. A negative control of extraction materials was used that yielded no metabolites.

Statistical analyses

Taxonomic distribution and functional genes annotated from the metatranscriptome samples were transformed using Hellinger distance (Hellinger 1909, Legendre and Legendre 2012). Hellinger transformation standardized the number of reads to normalize sampling effort across all samples. The resulting normalized annotations were used to construct a series of Canonical Correspondence Analyses (CCA) to identify the combination of five or fewer environmental variables, which best explain the taxonomic and

functional diversity. The best model was identified using an exhaustive search of every combination of five or fewer environmental parameters and selected based on the highest adjusted r^2 value. Overall model significance and significance of individual environmental variables was determined using permutational ANOVA (1000 permutations) (Legendre and Legendre 2012).

Taxa commonly associated with contamination, also referred to as the 'kitome' (Sheik et al. 2018), were analyzed to determine if the potential contaminants were true inhabitants of the Mariana forearc. The 16S/18S rRNA transcripts identified in the 'kitome' included *Burkholderiales*, *Chloroplast*, *Cyanobacteriales*, *Enterobacterales*, *Lactobacterales*, *Pseudomonadales*, *Rhizobiales*, *Sphingobacteriales*, *Sphingomonadales*, *Staphylococcales*, *Streptomycetales*, and *Opisthokonta* and were used to construct a non-metric multidimensional scaling ordination was created using Bray-Curtis distance (Bray and Curtis 1957) and visualized using ggplot2 (Supplemental Figure 1) (Wickham 2016). To determine if the potential 'kitome' contaminants were true contaminants, the samples should cluster closely to one another with little to no significant geochemical or physical parameters as they are ultimately derived from the same source and not the sample environments.

Metabolites identified from this study were assessed in a similar fashion to taxonomic and functional annotations from the metatranscriptomes. The metabolites identified were normalized using Hellinger distance (Hellinger 1909). Because of the larger sample size, a full CCA model was fit using all measured environmental parameters to explain differences in observed metabolites. The full model was assessed for significance using a permutational ANOVA (1000 permutations) and then the most parsimonious set of environmental parameters were selected using a combination of both forward and reverse stepwise regression to maximize adjusted R^2 values (Blanchet et al. 2008). The significance of the reduced model and individual retained environmental parameters was determined using permutational ANOVA (1000 permutations). All statistical analyses were performed using the vegan R package (Oksanen et al. 2013) in R (Team 2013).

Results

Site description

The pH at Yinazao seamount increased from 7.7 at 0 mbsf to 11 at 10 mbsf (Fryer et al. 2017). Fantangisña seamount increased from a pH of 9.0 at 0 mbsf to 11 at 11 mbsf but decreased back to 8.2 at 21 mbsf (Fryer et al. 2017). The pH at Asút Tesoru increased from 11 to 12 pH in the first mbsf (Fryer et al. 2017). Hydrogen concentrations ranged from 0.1 to 88 µM in Yinazao seamount, with the maximum concentration at 20 mbsf (Fryer et al. 2017).

Fantangisña summit hydrogen concentrations ranged from 2.6 to 390 μM . Hydrogen concentrations in Asùt Tesoru flank sites hydrogen concentrations ranged from 1.7 to 310 μM , whereas the summit sites ranged from 8 to 5.2 mM. Methane concentrations were minimal in Yinazao and Fantangisña summits, ranging from 0.1 to 30 μM and 0.1 to 6.5 μM , respectively. Methane concentrations were much greater in Asùt Tesoru (0.1 to 53 μM) summit samples than the flanks (1.0 to 6.9 mM) (Fryer et al. 2017).

Bioinformatic analyses

Sample, no-template control, and drilling fluid sequence libraries ranged from 2.9 to 6.0 billion base pairs (Table 2). Reads that passed quality control ranged from 11 to 24 million base pairs. After contamination was removed based on no-template controls and reference genomes, sample reads ranged from 3.3 to 20 million base pairs. Sample reads that were annotated ranged from 0.8 to 8.5 million, unclassified reads ranged from 0.1 to 0.3 million, and unassigned reads ranged from 0.8 to 7.1 million. Unassigned reads, or reads that did not match to any references in the annotation databases, included transcripts that were putatively annotated but not within the confidence intervals within the algorithm. The annotations from MEGAN against InterPro2Go were split into four overall categories: Biological Process (12%–48%), Cellular Components (9%–37%), Molecular Function (14%–44%), and Unassigned (5.1%–44%) across the entire metatranscriptome. Whole transcriptome amplification can be limited due to GC content of transcripts amplified which could result in skewed amplification which in turn could affect the reads passing quality control and annotation (Martin and Wang 2011).

Microbial community diversity

The Mariana forearc seamounts exhibited a large diversity of microbial taxa based on the 16S rRNA transcripts including Bacteria, Archaea, and Eukarya across all summit and flank sites. Archaea contributed between 0% and 0.7% with an average of 0.3% of the microbial community. Bacteria were the dominant microorganisms within the seamounts ranging from 32% to 94% with an average of 64%. Eukaryota contributed an average of 35% with a range of 5.9%–69% among the Mariana forearc sites. Sequences that could not be assigned classifications at the order level ranged from 0.4% to 13%, with an average of 5.4% across the samples. Of those 16S rRNA transcripts that could not be identified to an order level, 2.2%–19% were Bacteria and 0%–10% Eukaryota. All transcripts that were identified as Archaea could be classified to the order level. Sequences that were unclassified at the genus level ranged from 36% to 77% with an average of 48% (Fig. 2A and B).

Taxa identified as Archaea were in low abundance across all samples. Asùt Tesoru seamount flank site (sample U1496B-1F1) had the greatest relative abundance of Archaea, specifically Nanoarchaeales of 2.3%. Other Archaea orders identified in other seamounts include A10, Halobacteriales, Nitrosphaerales, Thermoproteales, and Woesearchaeales. Archaea were not identified in the Asùt Tesoru summit (sample U1496A-1F1), or either summit sites on Fantangisña seamount (samples U1497B-1F1 and U1497B-6F2).

Bacteria had the highest relative abundance in all samples except for Asùt Tesoru flank U1493B-4F2. Sequences most closely related to Betaproteobacteria, specifically Burkholderiales, were identified in all sites with the greatest relative abundance in Yinazao summit (sample U1492A-1H1) at 52%, Asùt Tesoru summit (sample U1496A-1F1) at 53%, and Fantangisña summit (sample U1497B-1F1) at 36%. Sequences most closely related to Acidobacteriae Subgroup 2 contributed 6.8% to the overall microbial

community within Fantangisña summit sample U1497B-6F2. Sequences closely relating to Alphaproteobacteria, including Rhizobiales were identified in all sites except Yinazao summit (sample U1492A-1H1) ranging from 0.1% to 4%. Sequences identified as Psuedomonadales were present in all samples except for Yinazao summit (sample U1492A-1H1) ranging from 0.2% to 13%. All sites contained sequences identified as Lactobacillales ranging in relative abundance from 0.1% to 6.4%. All sites had sequences identified as Bacillales identified ranging from 0.2% to 1.5% of the microbial communities. Only Asùt Tesoru summit sample U1496B-1F1 had sequences closely related to Deinococcales, which comprised a majority of bacterial taxa at 44%. Yinazao seamount summit sample U1492A-1H1 had sequences identified as Candidatus Obscuribacteriales, which contributed a minor portion of the microbial community at 2.2%.

All samples were dominated by sequences identified as Opisthokonta and Charophyta. Opisthokonta comprised between 32% and 65% among the flank samples (U1493B-4F2 and U1494A-1F3) and two of the summit samples (U1492A-1H1 and U1496B-1F1). Opisthokonta was less than 5% for the other two summit samples U1496A-1F1 and U1497B-1F1. Sequences identified as Opisthokonta in the flank samples of Asùt Tesoru (U1493B-4F2 and U1494A-1F3) were greater in relative abundance compared to summit samples. Charophyta, composed of green algae typically found in freshwater, was the dominant eukaryote identified in Asùt Tesoru summit sample U1496A-1F1.

Taxonomic diversity within the Mariana forearc seamounts was different between the flank and summit sites based on Canonical Correspondence Analysis (CCA). The flank samples of Asùt Tesoru (U1493B-4F2 and U1494A-1F3) were different from each other as well as all other summit sites for all three seamounts (Fig. S3). The overall model for the taxonomic diversity was significant ($P = 0.02$), and the geochemical and abiotic parameters that were significant in determining the microbial diversity differences among each sample included depth ($P = 0.02$), phosphate ($P = 0.02$), iron ($P = 0.02$), and strontium ($P = 0.04$) (Fig. S3). In contrast, a Nonmetric Multidimensional Scaling (NMDS) analysis of taxa that were removed as contaminants showed no distinction between the summits and flanks (Fig. S1). The fact that these geochemical features could be distinguished in the CCA plots suggests that the microbial populations were true inhabitants, rather than contaminants resulting from the drilling operations.

Cellular maintenance

Sequences annotated for cellular maintenance were between 3.5% and 10% of the metatranscriptomes (Fig. 3). The flank sites on Asùt Tesoru seamount exhibited some key differences in expression when compared to the summit sites. Sample U1493B-4F2, Asùt Tesoru seamount, had relatively greater expression for outer membrane porin biosynthesis specific to Bacteria. Sample U1493B-4F2 had relatively greater gene expression for phosphate transporter and flagellar regulator (Flk) (Supplemental File 1). Site U1494A-1F3 was the only sample that exhibited gene expression for resistance, nodulation, and cell division efflux pump. All samples shared similar levels of gene expression for P-type ATPases for energy consumption. All samples also exhibited expression for membrane insertases including yidC for Bacteria, oxa1 for mitochondrial membranes, and cytochrome c oxidase assembly protein cox18 for Eukarya. All sites also had greater gene expression for major facilitator superfamily and cytochrome c oxidase subunit I compared to other transcripts (Supplemental File 1).

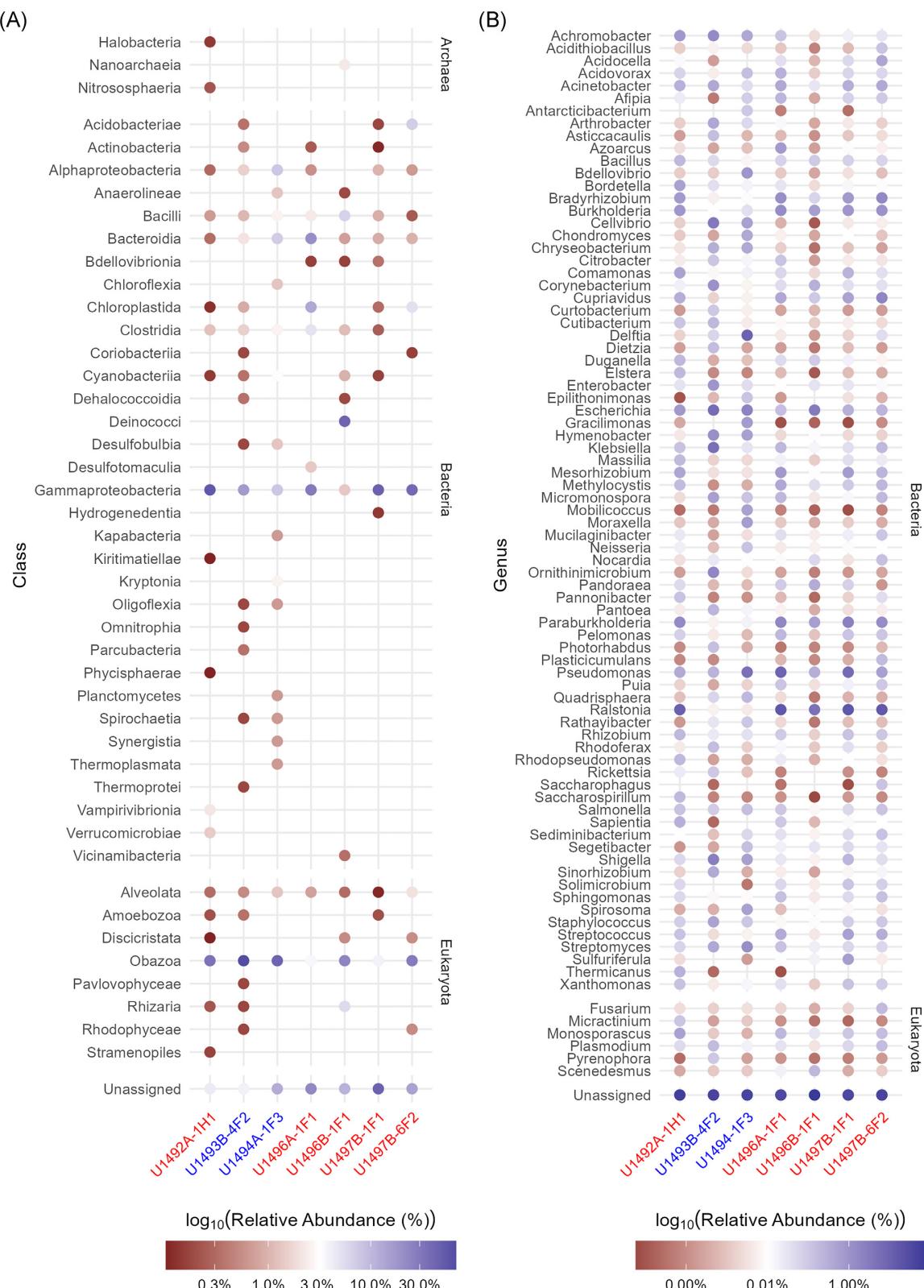


Figure 2. Taxa breakdown of microbial community classification from all samples. The 16S SSU ribosomal RNA gene was used for classifying Bacteria and Archaea and the 18S SSU ribosomal RNA gene was used for classifying Eukaryota. The ‘other’ group is comprised of taxa that makes up less than 0.1% of the individual sample. Sample names in red are summit samples and sample names in blue are flank samples. (A) Class level breakdown of microbial diversity. (B) Genus level breakdown of microbial diversity.

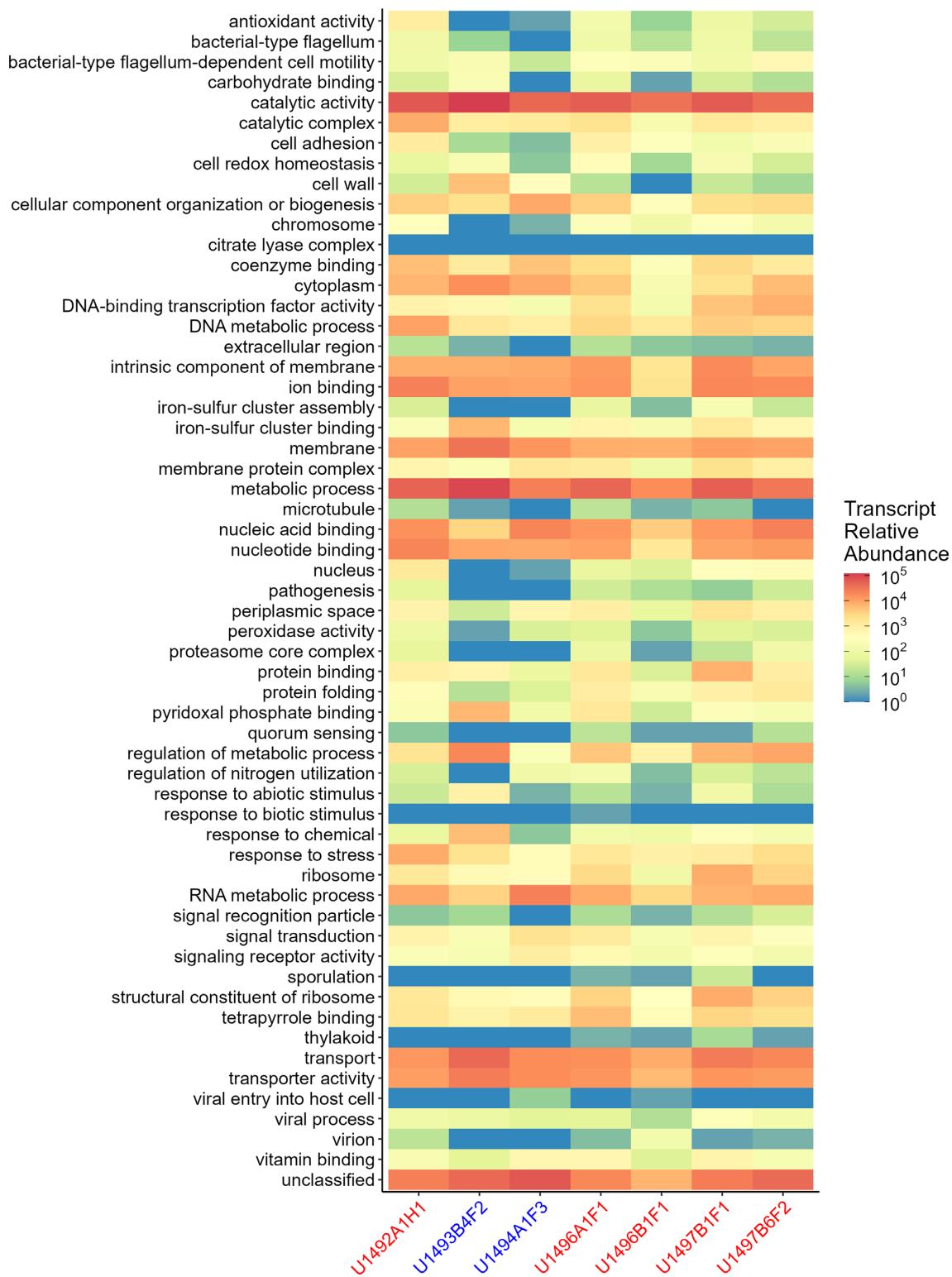


Figure 3. Categorical breakdown of normalized metatranscriptome annotations from InterPro2Go. Sample labels in red font indicate summit samples and labels in blue indicate flank samples.

Metabolic processes

The Mariana forearc microbial communities exhibited various metabolic capabilities including carbon and sulfur cycling. Most of the gene expression was for catalytic activity, metabolic activity, and transport (Fig. 3). Microbial communities also exhibited stress response and secondary metabolism activities that can be used

for survival mechanisms. Biological processes annotated with InterPro2Go included cellular processes such as flagellum biosynthesis, adhesion, homeostasis, DNA replication and repair, and metabolic processes. The cellular component category included formation of all cellular components (e.g. cytoplasm, membrane, ribosomes). Molecular function included genes required for cat-

alytic activities and binding (e.g. protein binding, transporter activity, coenzyme binding). Sequences annotated as unclassified are sequences that aligned to existing protein sequences but are currently unclassified. Sequences that were not assigned did not align to anything.

The relative abundance of assigned transcripts here are relative to the entire metatranscriptome of each sample and should not be considered equivalent to genes expressed *in situ* in the Mariana forearc. On average, approximately 79% transcript reads were not able to be assigned using InterPro2Go, COG, and SEED databases. Most sequences that could be assigned were annotated as biological processes (1%–16%), followed by molecular function (1%–14%), and cellular components (0.4%–5.7%). Sequences that were unclassified ranged from 0.2% to 6.4%. Geochemical parameters were used to determine if any metatranscriptome samples were significantly impacted. The overall model for functional diversity was significant ($P = 0.02$). The significant geochemical values correlating with mRNA composition were bromide ($P = 0.01$), phosphate ($P = 0.01$), silicon ($P = 0.02$), Total Inorganic Carbon (TIC) ($P = 0.04$), and Total Organic Carbon (TOC) ($P = 0.01$) (Fig. S3).

Carbohydrate utilization

Transcripts for carbohydrate utilization ranged from 3% to 11% within these samples and differed between the flank and summit samples. The flank samples (U1493B-4F2 and U1494A-1F3) along Asùt Tesoru seamount had relatively greater gene expression for various fermentation pathways and methylglyoxal metabolism. Both flank sites had gene expression for mixed acid and lactate fermentation, while the summit sites did not. Both flank sites had relatively greater gene expression for ethylmalonyl-CoA pathway and ethanolamine utilization than the other sites (Supplemental File 1).

There were some similarities found across all sites including gene expression for the Entner-Doudoroff pathway, citrate metabolism, Calvin-Benson cycling, acetyl-CoA biosynthesis to butyrate, the serine-glyoxylate cycle, and tricarboxylic acid cycle (TCA). The Entner-Doudoroff pathway was expressed in all samples especially from Asùt Tesoru flank (U1493B-4F2) and Fantangisña summit (U1497B-1F1). Citrate metabolic transcripts were found in all samples, especially in Asùt Tesoru flank (U1493B-4F2). The Calvin-Benson cycle, serine-glyoxylate cycle, TCA cycle, and biosynthesis of butyrate was found in all samples to be greatly expressed across all sites. The TCA cycle was greatly expressed in all summit holes except for Asùt Tesoru (U1496B-1F1).

Redox cycling

All samples contained transcripts for denitrification, including nitrate reductase (*narGH*), nitrite reductase (*nirKS*), periplasmic nitrate reductase (*napAB*), nitric oxide reductase (*norBC*), and nitrous oxide reductase (*nosZ*) (Fig. 4). Additionally, transcripts were also detected for ammonia oxidation (*amoABC*). All samples also had transcripts for ammonia monooxygenase (*amoABC*). Dissimilatory sulfur cycling transcripts were found in all samples, but in lower abundance than methane oxidation (*pmoABC*) and denitrification (*nirKS* and *nosZ*) (Fig. 4). Sulfate reduction (*sat*, *aprAB*, and *dsrAB*) was expressed in all samples. The greatest gene expression for sulfur cycling was found for sulfur oxidation (*soxB* and *sqr*). All sites had more transcripts for aerobic methane oxidation (methane monooxygenase *pmoABC*) than other dissimilatory processes. All sites contained less transcripts for methanogenesis and anaerobic methane oxidation (methyl co-enzyme M reductase *mcrABCDG*).

The major categories for carbohydrate-active enzymes identified were carbohydrate esterases, carbohydrate-binding modules, glycoside hydrolases, glycosyltransferases, and polysaccharide lyases (Fig. 5). The most relatively abundant category was the glycoside hydrolases which included enzymes for breaking down hemicellulose, peptidoglycan, polysaccharides, and starch. The second most relatively abundant category of annotations was the glycosyltransferase categories, with most enzymes annotated as cellulose and lipopolysaccharide degradation. All samples had high relative abundance for breaking down peptidoglycan, polysaccharides, starches, and cellulose. The flank sites (U1493B-4F2 and U1494A-1F3) were found to have distinct capabilities including breaking down peptidoglycan, hemicellulose, and murein.

Metabolites

A total of 33 metabolites were identified across all the seamounts of the Mariana forearc (Kevorkian 2019). The annotated transcripts that were identified for metabolites are shown in Fig. 6. The geochemical parameters with significant correlation for the identified small organic molecules were determined through principal component analyses and Canonical correspondence analyses (Fig. 7), and the Asùt Tesoru summit formed a distinct cluster from Yinazao and Fantangisña summits. The overall model for metabolite diversity was significant ($P = 0.001$) and the significant geochemical parameters include strontium ($P = 0.06$), magnesium ($P = 0.001$), sulfate ($P = 0.001$), chloride ($P = 0.001$), manganese ($P = 0.04$), and sodium ($P = 0.04$).

Discussion

Microbial diversity

Gammaproteobacteria was identified in the highest relative abundance across all samples, especially *Burkholderiales*. Previous studies have found *Burkholderiales* to be abundant within serpentizing systems because of their ability to oxidize hydrogen and assimilate carbon using the Calvin-Benson-Bassham pathway (Brazelton et al. 2012, Schrenk et al. 2013, Purkamo et al. 2015). *Burkholderiales* were the most abundant taxa at the Outokumpu Drill Hole in Finland (Purkamo et al. 2015) and were in high relative abundance in Lost City hydrothermal field and the Tablelands Ophiolite, Newfoundland (Brazelton et al. 2012). The presence of [NiFe]-hydrogenase sequences in all samples from the Mariana forearc are indicative of hydrogen oxidation, which is a metabolic pathway present in *Burkholderiales* (Brazelton et al. 2012). The availability of hydrogen in the Mariana forearc sediments as well as the hydrogen oxidation transcripts support that the taxa identified as *Burkholderiales* could be native to this environment.

Burkholderiales and *Deinococcales* can assimilate formaldehyde formed from aerobic methane oxidation using the serine pathways (Seyler et al. 2020). The tetrahydrofolate pathway could also be used to oxidize formate found within the environment (Seyler et al. 2020). Due to the greater methane concentrations within Asùt Tesoru seamount, *Deinococcales* could have a large resource of formaldehyde and formate to use for assimilation. Small molecules containing carbon, such as methane and formate, can become the sustaining carbon sources due to the serpentizing reaction occurring within the Mariana forearc seamounts since dissolved inorganic carbon is limited by the hyperalkaline conditions. Asùt Tesoru seamount had the highest pH, approaching 12.5 in the summit, so the microbial community, especially dominant

Pathway	Gene Name	Gene	U1492A-1H1	U1493B-4F2	U1494A-1F3	U1496A-1F1	U1496B-1F1	U1497B-1F1	U1497B-6F2	Scale
Calvin Benson Bassham	ribulose-biphosphate carboxylase	rbcLS	197	84	57	224	387	1372	251	1
Reductive TCA	ATP-citrate lyase	aciAB	624	130	56	449	182	1028	97	100
Hydroxypropionate-hydroxybutyrate	4-hydroxybutyryl-CoA dehydratase	abfD	116	240	79	225	139	290	258	1000
Methane Oxidation	methane monooxygenase	pmoABC	400370	569544	534051	168758	442719	332742	283385	10000
Methanogenesis	methyl co-enzyme M reductase	mcrABCDG	167	806	3749	354	348	363	179	1
Hydrogen Oxidation	Ni-Fe hydrogenase, group 1	hyaAB	626	354	946	1010	3365	957	629	100
	quinone-reactive Ni/Fe-hydrogese	hydAB3	440	948	877	452	740	1140	243	1000
Oxygen Respiration	cytochrome c oxidase, aa3-type	coxABC	3782	232	657	8264	1064	5616	4549	1
	cytochrome bd ubiquinol oxidase	cydAB	22933	101873	55822	21038	2587	12619	56741	1000
	cytochrome c oxidase, cbb3-type	ccoNOP	47066	13937	11446	73249	35117	40665	391003	10000
Denitrification	nitrate reductase	narGHI	1384	504	522	4787	1054	820	423	1
	nitrite reductase	nirK	334315	239318	204803	426431	107589	338248	180706	100
	periplasmic nitrate reductase	napAB	782	992	57	2697	1936	1534	518	1000
	nitrite reductase	nirS	253	64	210	287	1441	500	277	10000
	nitric oxide reductase	norBC	1061	939	53	922	722	960	335	1
Ammonia Oxidation	nitrous oxide reductase	nosZ	99683	23333	81913	116090	362307	60165	33585	100
	ammonia monooxygenase	amoABC	76484	36753	100502	120766	28100	172560	37587	1000
Sulfur Reduction and Oxidation	sulfate adenylyltransferase	sat	677	3474	69	3178	493	13830	2138	10000
	adenylylsulfate transferase	aprAB	1353	209	53	12196	348	1377	1608	1
	dissimilatory sulfite reductase	dsrAB	1461	240	375	1793	696	778	555	10000
	thiosulfate reductase	phsABC	2904	1520	289	4791	5821	2537	1028	100
	S-sulfosulfanyl-L-cysteine sulfohydrolase	soxB	2665	3870	1725	29347	2407	7774	3417	10000
	sulfide:quinone oxidoreducatse	sqr	656	635	1691	2691	438	2125	488	10000

Figure 4. Normalization of transcripts for redox cycling across oxygen, nitrogen, sulfur, carbon, and hydrogen cycling and was annotated using hand-curated databases. Numerical values indicate transcripts per million.

members like Burkholderiales and Deinococcales would have an advantage using formate or formaldehyde for carbon assimilation.

The dominant Eukaryota class was Obozoa, specifically Opisthokonta, which include fungi and metazoans. This taxon could be flourishing in the flank locations on Asüt Tesoru due to the less extreme conditions than those found at the summit sites (Lopez-Fernandez et al. 2018). Opisthokonta have been found in various deep biosphere environments including continental and marine systems (Quaiser et al. 2011, Petro et al. 2017, Lopez-Fernandez et al. 2018). In marine sediments, Opisthokonta have been found to range from 1% to 50% of the microbial community (Petro et al. 2017). One major clade of opisthokonts consists of fungi which use a wide array of metabolisms including oxygen respiration, sulfate reduction, ammonia fermentation, and/or nitrate respiration (Müller et al. 2012). Common electron acceptors of the opisthokonts include succinate, hydrogen, ethanol, nitrite, and elemental sulfur (Müller et al. 2012). Based on the geochemistry and active metabolisms found within the metatranscriptomic data, the opisthokonts may be using denitrification and/or sulfate reduction for metabolic purposes. Alternatively, the fungal community could be working with the bacterial and archaeal communities in consortia for nitrogen and sulfur cycling. For example, anaerobic fungi can produce H₂ along with formate, lactate, and acetate when in consortia (Drake et al. 2017, Eickenbusch et al. 2019). These compounds were all found in relatively high concentrations in the Mariana forearc.

Significant geochemical correlation with microbial diversity

Taxonomic, functional, and metabolite diversity were significantly different between the summit and flank sites of the Mariana fore-

arc. This strong trend of differences between summit and flank samples is found in the geochemical data as well. The geochemical parameters that were significant for the models generated in this study were in some cases orders of magnitude different across samples such as strontium (range 4.3–580 µM), iron (range Below Detection to 8.4 µM), TOC (0–23 ppm), and TIC (1.5–26 ppm) (Fryer et al. 2017). Yinazao summit sample U1492A-1H1 was driven the most by TIC and strontium for functional diversity and metabolites. The CAZy annotations generated from this study support TIC driving functional diversity in Yinazao summit because there were very little transcripts annotated for organic carbon sources for metabolism (Fig. 5). The summit sites of Asüt Tesoru were driven most by TOC and phosphate, which is supported by the functional annotations for carbon cycling. Polysaccharide, starch, and hemicellulose degradation were found to be relatively high in transcripts in Asüt Tesoru summit compared to all other samples indicating microorganisms could be using more organic carbon than inorganic in this seamount summit. Bromide, as a conservative tracer, indicated a significant geochemical contribution by hydrothermal vent fluids in structuring the taxonomic and functional diversity of the microbial communities in the flank sites of Asüt Tesoru.

Cellular maintenance

As depth increases in marine sediments, cellular maintenance becomes the most important process due to the limited resources available (Zinke et al. 2017, Bradley et al. 2018, Reese et al. 2018, Mullis et al. 2019). Membrane structure and repair transcripts were greatly expressed, specifically for outer membrane porin biosynthesis and resistance, nodulation, and cell division efflux pump enzymes. Outer membrane porin biosynthesis specific to

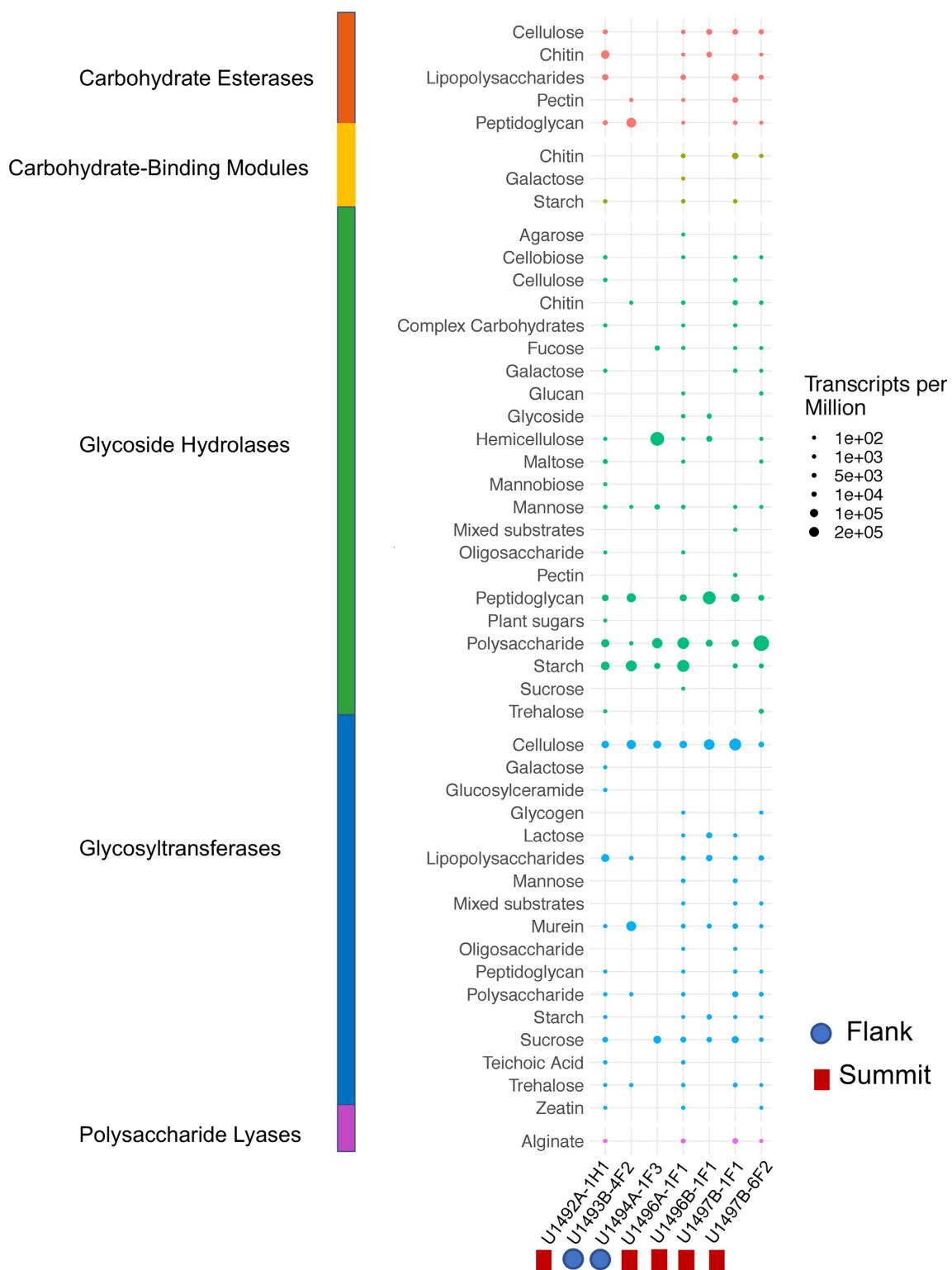


Figure 5. Normalized per million transcripts against the Carbohydrate-Active enZymes database (CAZy) (Lombard et al. 2014).

Bacteria indicates formation of pores within the cellular membrane to gain entry and release into the cell (Nikaido 1992). Resistance, nodulation, and cell division efflux pump enzymes are a part of a large family of polypeptides that are thought to connect the inner and outer membranes (Schülein et al. 1992).

All samples shared similar levels of gene expression for P-type ATPases, which are found among Bacteria and Eukarya and transport compounds such as lipids or ions, across a membrane using ATP hydrolysis as the energy source (Axelsen and Palmgren 1998). Asüt Tesoru flank site U1493B-4F2 had relatively greater gene expression for ornithine/lysine/arginine decarboxylases, which can

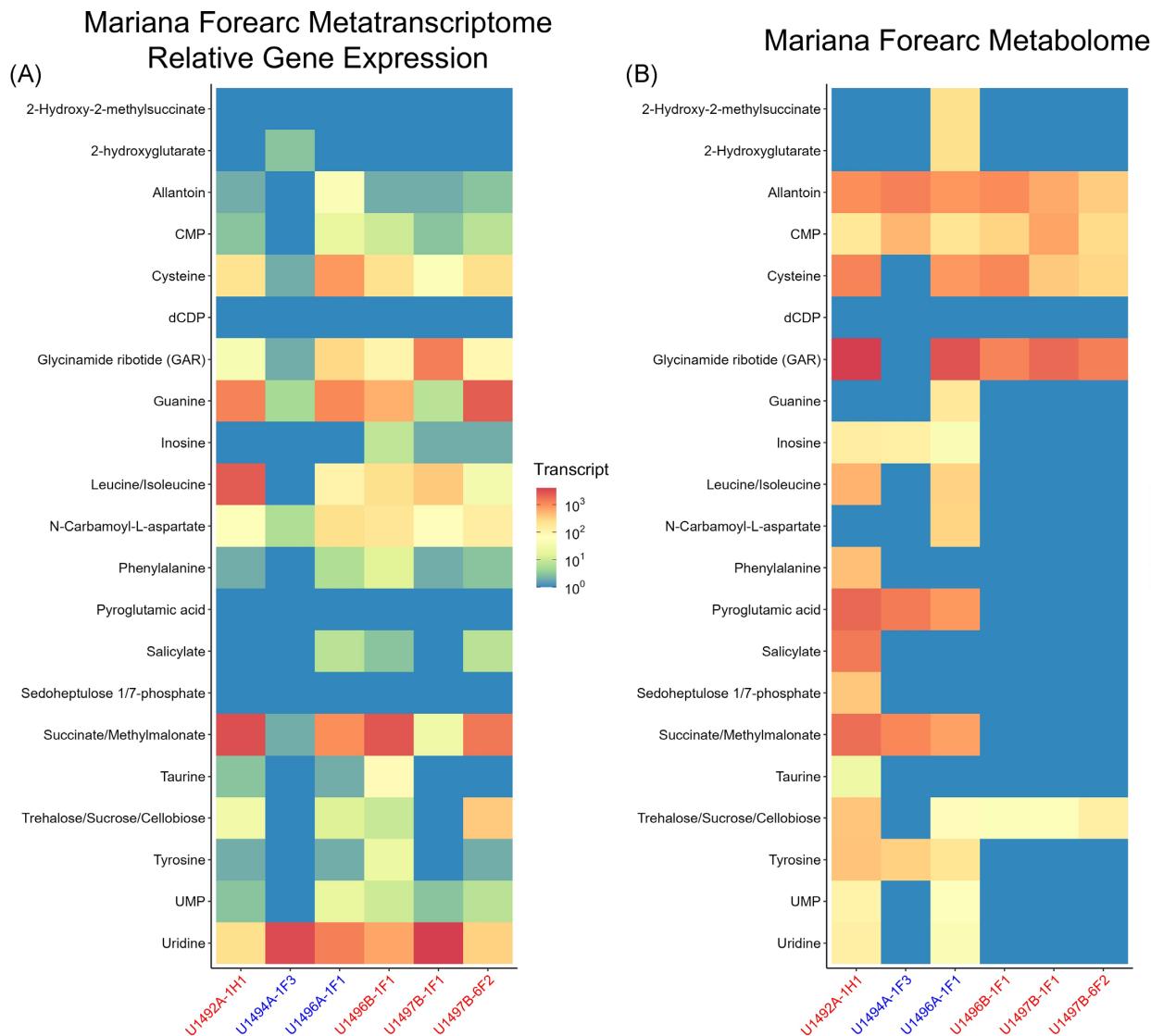


Figure 6. Metabolites identified in the Mariana forearc. All transcript relative abundances and metabolite values have been log transformed for visualization purposes. Sample labels in red labels indicate summit samples and blue labels indicate flank samples. **(A)** Reads annotated from metatranscriptomes that correspond to the metabolites identified. **(B)** Metabolites identified in the Mariana forearc that correspond to the metatranscriptome annotations.

provide a source of organic nitrogen available as substrates for growth (Klouche et al. 2007).

Fermentation

All samples collected from the summits of Yinazao, Asùt Tesoru, and Fantangisña seamounts, and the flanks of Asùt Tesoru contained transcripts for fermentative metabolic pathways. Short-chain organic acids including formate, acetate, propionate, butyrate, and lactate serve as key metabolic intermediates and can be the resulting end products of microbial fermentation pathways (Stams 1994, Wellsbury et al. 2002, Worm et al. 2010, Bird et al. 2019, Eickenbusch et al. 2019, Mullis et al. 2019). Citrate metabolism constitutes a vital role in fermentation metabolisms (Starrenburg and Hugenholtz 1991, Hugenholtz 1993). Fermentation metabolisms were found in all samples which could be associated with metabolizing citrate. Transcripts identified to be associated in citrate metabolism were found in all sites, especially the flank site U1493B-4F2 on Asùt Tesoru. Due to the high

relative abundance of transcripts for fermentation and citrate metabolisms, microbial communities could be utilizing citrate to contribute to fermentation.

Fermentative metabolism has been hypothesized to be feasible and energetically-favorable in serpentinizing environments (Postec et al. 2015, Twing et al. 2017, Trutschel et al. 2022). The organic molecules produced in serpentinization reactions include methane, which is extremely high in concentration in Asùt Tesoru seamount (Holm et al. 2015). Many serpentinizing environments across terrestrial and marine environments have been found to contain microbial communities that have fermentation pathways including Pronym Bay Hydrothermal Field (Postec et al. 2015), Coastal Range Ophiolite Microbial Observatory California, USA (Twining et al. 2017), Ney Springs California, USA (Trutschel et al. 2022), and a serpentinizing spring of Manleluag, Philippines (Wang et al. 2022). The Mariana forearc follows a similar trend of fermentation pathways expressed in relatively high abundance across Asùt Tesoru which had the highest methane and hydrogen concentrations of the dataset.

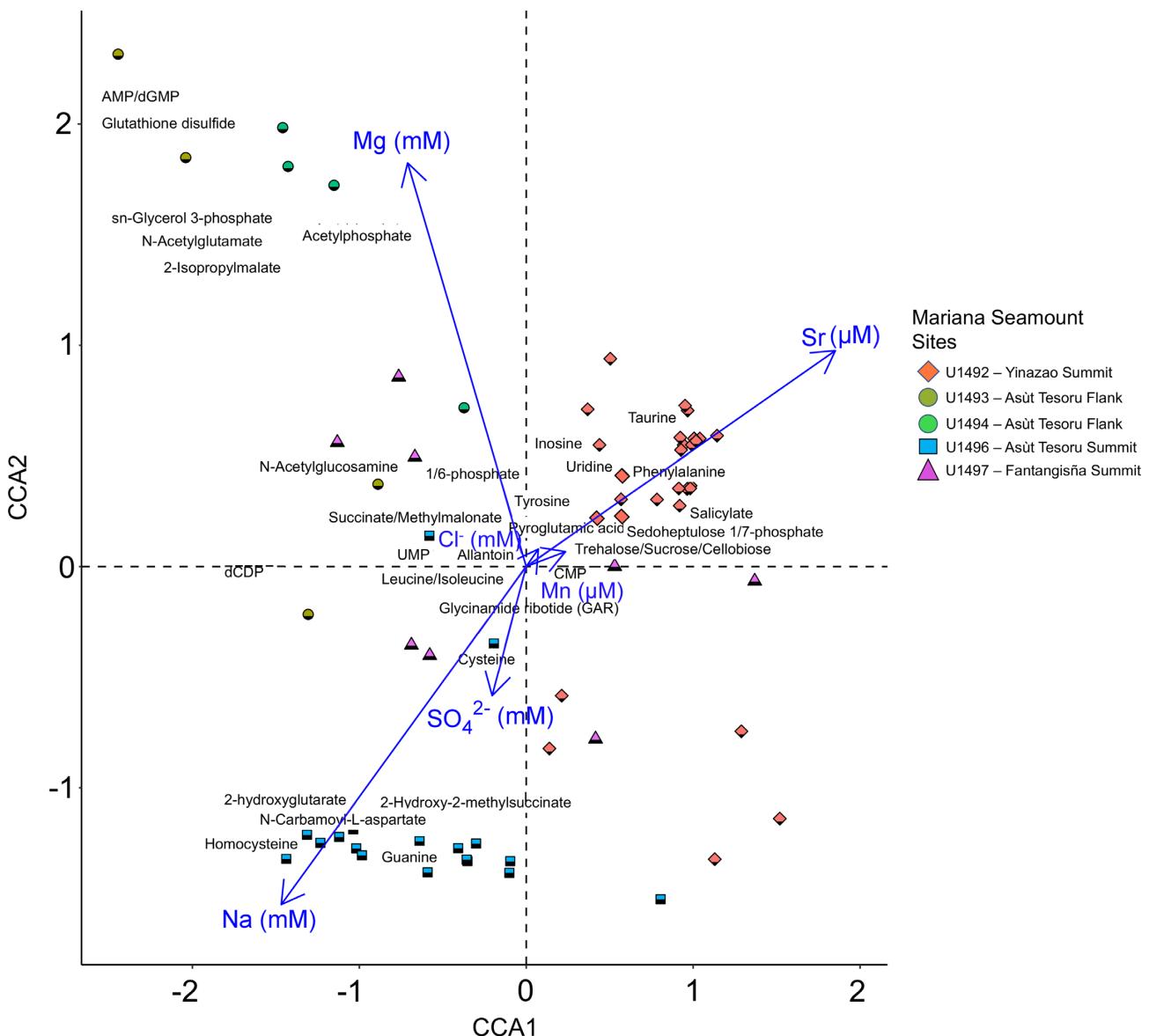
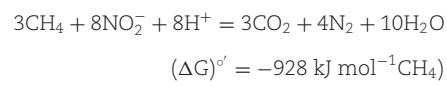


Figure 7. Canonical Correspondence Analysis (CCA) of the identified metabolites within each site along the Mariana forearc. Based on the CCA magnesium, strontium, chloride, manganese, and sodium are geochemical parameters that have significant correlation with the metabolite concentrations across each site.

Methane and nitrogen cycling

The data presented here support the previous findings that the methane production is primarily from abiotic or thermogenic processes (Eickenbusch et al. 2019, Fryer et al. 2020). Previous thermodynamic calculations of the abiotic formation of methane indicate that methane formation can occur under aqueous conditions in the subducting slab from hydrogen and carbonate without an energetic expenditure (Eickenbusch et al. 2019). This abiotic pool of methane within Asüt Tesoru can serve as a source for metabolism in microorganisms. The relatively greater gene expression for methane monooxygenase (*pmoABC*) gives evidence of microbial activity oxidizing the methane. Methane oxidation may be coupled with denitrification reactions, specifically nitrite to dinitrogen (Raghoebarsing et al. 2006, Ettwig 2010, Hu et al. 2014). Nitrite reduction (*nirKS*) had higher transcript abundance relative to other dissimilatory metabolic genes in all samples indicating partial denitrification within each sample. All steps of denitrification were transcribed in all samples; however the highest

relative abundance of transcripts for denitrification was for nitrite reductase. Due to methane being a low-reactivity molecule in the first step of methanotrophy, coupling methane oxidation to another more energy-producing reaction would offer microorganisms more energy yield (Ettwig et al. 2010, Momper et al. 2017). At standard temperature and pressure, the coupling of methane oxidation to denitrification is feasible for energy production (Ettwig et al. 2010, Zhu et al. 2016), with the actual energy yield depending on the relative concentrations of reactants and products:



The coupling of methane oxidation and denitrification occurs in both terrestrial and marine environments (Ettwig et al. 2010). Methanotrophs produce methanol and formate which can be used as carbon or energy sources for denitrifying Bacteria (Ettwig et al. 2010), such as *Pseudomonas* and *Ralstonia*, which were iden-

tified in relatively high abundance across many of the samples in this study (Householder et al. 2000, Spain and Krumholz 2011, Sharma et al. 2015). The presence of formate in the Mariana forearc is not only indicative of serpentinization reactions but can also be indicative of methanotrophy (Ettwig et al. 2010, Eickenbusch et al. 2019). The formate formed in the Mariana forearc could be used by the denitrifiers for carbon or energy due to the energy-limited conditions.

A terrestrial serpentinizing seep in the Philippines (Jakobs et al. 2013, Woycheese et al. 2015) also contained methanogens, methanotrophs, and denitrifiers thus indicating potential coupling of anaerobic methane oxidation and denitrification. However, the biochemical mechanisms of anaerobic methane oxidation and denitrification within the marine deep subsurface are still unclear (Itävaara et al. 2016). Anaerobic methanotrophy has been heavily investigated in Archaea and sulfate reducing Bacteria (Orphan et al. 2002, Joye et al. 2004, Nauhaus et al. 2005, Bhatarai et al. 2019); however, there have been few studies on the consortia of anaerobic methane oxidation and denitrification within *in situ* microbial communities in the marine subsurface. The Mariana forearc microbial communities could contain novel taxa and metabolic pathways yet to be identified for coupling anaerobic methane oxidation and denitrification.

The Mariana forearc is presumably anoxic due to the serpentinizing reaction actively occurring (Fryer et al. 2017, Fryer et al. 2020); however, there were numerous oxidases expressed in the metatranscriptomes from this study. The oxidases identified included cytochrome oxidases, pyruvate oxidases, and peroxidases, which indicate there could be aerobic microenvironments within the Mariana forearc sediments (Inskeep et al. 2007, Bhattacharya et al. 2020). Aerobic microorganisms and processes have been identified in seawater-sediment interfaces indicating oxic conditions (Tiago et al. 2004, Rowe et al. 2017). If there are aerobic microniches within the Mariana forearc sediments, this would be the first reporting of aerobic processes occurring within active serpentinization systems in sediments (Suzuki et al. 2014, Crespo-Medina et al. 2017, Fones et al. 2019). The presence of transcripts for these enzymes could also indicate that these organisms are poised to detoxify oxidized compounds they are exposed to, which could be exposure from sampling the sediments.

Sulfur cycling

Taurine utilization has been reported to transport taurine into bacterial cells (Kertesz 2001, Cook and Denger 2006, Xing et al. 2019). Bacteria and Archaea utilize taurine, also referred to as 2-aminoethanesulfonic acid, in strictly anoxic conditions (Cook and Denger 2006, Xing et al. 2019). Taurine is widespread in the environment and consists of an amino group that can be cleaved leaving the remaining taurine to be used as a nitrogen, carbon, or sulfur source (Chien et al. 1997, Ōmura et al. 2001, Cook and Denger 2006). Some microorganisms, such as *Clostridium*, have been found to assimilate sulfur under anaerobic conditions, which can explain the greater abundance of transcripts for taurine metabolism within Asút Tesoru seamount. The relatively low concentration of ammonium in U1494A-1F3 can further indicate the need for a nitrogen source for cellular maintenance and assimilation purposes (Fryer et al. 2017, Wheat et al. 2018).

Mariana backarc compared to mariana forearc

Previous metatranscriptomic analyses determined the dominant metabolisms include hydrogen oxidation, denitrification, and sulfide and/or thiosulfate oxidation along the Mariana backarc (ac-

cession bioproject number PRJNA454888) (Trembath-Reichert et al. 2019). The Mariana forearc exhibits all redox metabolisms to some degree, however there are key differences. Aerobic methane oxidation (*pmoABC*) was greatly transcribed in all samples, which was elucidated by hand-curating an annotation database as we have done here. Since methane concentrations are so high at Asút Tesoru, microbial methanotrophy may not make a noticeable change in the geochemistry. However, biological methane oxidation is likely occurring based on the presence of *pmoABC* transcripts. The abiotic formation of methane far outweighed the biological consumption of methane thus leading to methanotrophy being a hidden metabolic cycle within the microbial community. The main difference observed between these two sites is the large amount of gene expression for methane oxidation in all samples along the Mariana forearc. Based on the large amount of gene expression for denitrification and anaerobic methane oxidation, there could be coupling of these reactions.

The Mariana forearc serves as a unique serpentinization site in the marine subsurface due to the extremely high pH and wide array of metabolisms identified. Previously there have been few studies that have hypothesized methane oxidation and denitrification co-occurring and possibly being coupled to increase the energetic payoff (Woycheese et al. 2015, Trutschel et al. 2022). Previous thermodynamic calculations and predictions for a terrestrial serpentinizing environment in California were found to support coupling of anaerobic methane oxidation and denitrification and was primarily conducted through metagenomic data and bioenergetic calculations (Trutschel et al. 2022). The Mariana forearc is the first marine serpentinizing environment to document transcription of genes supporting the same metabolisms. Previous metatranscriptomes from serpentinizing systems have been constrained to terrestrial subsurface environments (Sabuda et al. 2020, Seyler et al. 2020). Identifying anaerobic methane oxidation and denitrification metabolic transcripts in the Mariana forearc further supports the potential coupling of these metabolisms to increase the energetic payoff for microbial communities in these extreme serpentinizing conditions.

Metabolome and gene expression

The identified metabolites from the Mariana forearc were compared to the annotated metatranscriptomes from the three seamounts to determine if there were similarities between gene expression and metabolic products. Allantoin was identified in the metabolome data for all samples that were also analyzed for metatranscriptomics. Transcripts for allantoin biosynthesis were annotated in one flank site and one summit sample of Asút Tesoru (U1494A-1F3 and U1496A-1F1). Allantoin is a product of purine degradation and can be used as a potential catabolic substrate in energy-limited marine sediments (Bird et al. 2019). Microorganisms have also been found to use allantoin as a nitrogen source in energy-limited conditions (Switzer et al. 2020). Ammonium concentrations in the Mariana forearc seamounts were much greater than concentrations found in seawater (0–10 µM) ranging from 34 to 110 µM in Yinazao, below detection to 250 µM in Asút Tesoru, and below detection to 74 µM in Fantangisña. Ammonium can be a by-product of allantoin degradation (Switzer et al. 2020) which could in part explain the high ammonium concentrations in the Mariana forearc sediments, especially Asút Tesoru. Asút Tesoru summit samples also had extremely high TOC concentrations when compared to the other metatranscriptomic samples (17 and 23 ppm) which could contribute to the high ammonium concentrations via decomposition.

The most common metabolites identified in the metabolome were found to be nucleotide components. Cytidine monophosphate (CMP) was found in all samples analyzed for metabolomics that also had pairing metatranscriptome data and found in the summits of Asút Tesoru and Fantangisña metatranscriptomes. Cysteine, guanine, and uridine were identified in metatranscriptomic and metabolomic samples, but were inconsistent across the same sample (i.e. only found in metabolome or metatranscriptome of a sample). Cysteine, guanine, uridine, and CMP are all components of nucleic acids (Pascal 2008); and observation of these molecules correlates well with the observed relative gene expression for processes involving each of these molecules in the microorganisms need to generate nucleic acids for cell replication and transcription of DNA to create RNA molecules. Each of these products are most likely not as abundant in the metabolite data due to not completing the translation of RNA to proteins to eventually create the metabolites.

The metabolite glycinamide ribotide (GAR) was identified in all samples except for Asút Tesoru flank site U1494A-1F3, while the metatranscriptomes had gene expression for GAR in all summit sites of each seamount. Glycinamide ribotide is an intermediate metabolite in the synthesis of purines (Warren and Buchanan 1957). The annotation and identification of GAR in both datasets indicate active anabolic purine metabolism in the microbial communities, which could be used for cell replication or transcription processes. N-Carbamoyl-L-aspartate metabolite was identified in one of the summit sites of Asút Tesoru (U1496A-1F1), while gene expression for pyrimidine biosynthesis was found for all samples except the flank site of Asút Tesoru (U494A-1F3). N-Carbamoyl-L-aspartate is an intermediate in pyrimidine biosynthesis (Robitaille et al. 2013), which indicates again microbial communities attempting to create pyrimidines based on gene expression.

Caveats and limitations

Metatranscriptomic data are very useful in the detection and quantification of putative cellular activity; however, there are remaining limitations regarding the data produced (Aguiar-Pulido et al. 2016). The first major limitation is the type of RNA that is extracted and sequenced is usually dominated by ribosomal RNA rather than messenger RNA (mRNA), which mRNA is used to annotate functionality (Peano et al. 2013, Aguiar-Pulido et al. 2016). The second limitation is the instability of RNA in nature. The single-stranded structure causes an RNA molecule's half-life to be much shorter compared to DNA molecules, even as short as minutes (Selinger et al. 2003, Steiner et al. 2019). Background sampling would provide additional insight into environmental sampling to determine if there is overlap in the microbial community from surrounding sediments. We advocate collecting background samples that can be used for comparison to determine if the results are specific to the sampling site, or if there is contamination or ubiquity across the surrounding sampling area. Overall, metatranscriptomic studies can be highly informative in deducing functional capabilities and putative activities in nature, but still have limitations. Comparing different datasets such as metatranscriptomes and metabolomes allows for a more robust analysis that offers a more in-depth perspective of microbial ecology.

Summary

The microbial communities isolated within the serpentining, hydrogenic, methanogenic sediments of the Mariana forearc are active under extremely harsh conditions. Metatran-

scriptomic analyses provided key insight into the diversity of metabolisms and survival mechanisms within the Mariana forearc. The metabolisms include hydrogen oxidation, denitrification, and anaerobic methane oxidation. We propose there to be cryptic anaerobic methane oxidation coupled with denitrification in the Mariana forearc sediments. Assimilatory processes for cellular maintenance were highly abundant in the transcriptomes of the active communities. Microorganisms from the sediment communities also exhibited gene expression for secondary metabolite biosynthesis which could include biosynthesis of toxic compounds including antimicrobials. Metabolite identified as intermediates for biosynthesis of nucleic acids, amino acids, and osmolytes indicate that cell survival may dominate cellular processes in the Mariana forearc. The microbial communities within the Mariana forearc sediments are not only surviving harsh conditions, they are scavenging what is available and coupling nutrients for metabolic activity.

Supplementary Data

Supplementary data are available at [FEMSEC](#) online.

Author Contributions

MMM, BKR, and KGL developed the research idea. MMM processed all samples and performed all molecular analyses. KGL, RK, HFC, and SRC designed the metabolomics study; EDT and RK performed the metabolomics analyses; and KGL, RK, EDT, HFC, and SRC all participated in metabolomics data analysis. MMM and BKR analyzed the metatranscriptome and metabolome data. JDS assisted in statistical analyses and metatranscriptome analyses. All authors contributed to data generation and data analyses. MMM and BKR wrote the manuscript. All authors contributed to reviewing and editing the manuscript.

Acknowledgments

We would like to thank the Science Party and Crew for IODP Expedition 366 (Mariana Convergent Margin) for sample collection. We thank Rachel Weisend, Morgan Sobol, and Christian Cunningham for assistance in laboratory procedures. We thank Dr Laura Zinke, Dr Brett Baker, Dr Benjamin Tully, and Dr Ian Rambo for bioinformatic insight. We thank Dr Blair Sterba-Boatwright for statistical insight. This project was funded by the IODP #GG009393 to KGL and RK, and NASA Exobiology #NNX16AL59G to KGL.

Conflicts of interest. None declared.

Funding

This project was funded by the IODP #GG009393 to KGL and RK, and NASA Exobiology #NNX16AL59G to KGL.

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