Thermodynamic models predict that H₂ is energetically favorable for seafloor microbial life, but how H₂ affects anabolic processes in seafloor-associated communities is poorly understood. Here, we used quantitative ¹³C DNA stable isotope probing (qSIP) to quantify the effect of H₂ on carbon assimilation by microbial taxa synthesizing ¹³C-labeled DNA that are associated with partially serpentinized peridotite rocks from the equatorial Mid-Atlantic Ridge. The rock-hosted seafloor community was an order of magnitude more diverse compared to the seawater community directly above the rocks. With added H₂, peridotite-associated taxa increased assimilation of ¹³C-bicarbonate and ¹³C-acetate into 16S rRNA genes of operational taxonomic units by 146% (±29%) and 55% (±34%), respectively, which correlated with enrichment of H₂-oxidizing NiFe-hydrogenases encoded in peridotite-associated metagenomes. The effect of H₂ on anabolism was phylogenetically organized, with taxa affiliated with Atribacteria, Nitrospira, and Thaumarchaeota exhibiting the most significant increases in ¹³C-substrate assimilation in the presence of H₂. In SIP incubations with added H₂, an order of magnitude higher number of peridotite rock-associated taxa assimilated ¹³C-bicarbonate, ¹³C-acetate, and ¹³C-formate compared to taxa that were not associated with peridotites. Collectively, these findings indicate that the unique geochemical nature of the peridotite-hosted ecosystem has selected for H₂-metabolizing, rock-associated taxa that can increase anabolism under high H₂ concentrations. Because ultramafic rocks are widespread in slow- and ultraslow-spreading oceanic lithosphere, continental margins, and subduction zones where H₂ is formed in copious amounts, the link between H₂ and carbon assimilation demonstrated here may be widespread within these geological settings.

The ISME Journal (2022) 16:257–271; https://doi.org/10.1038/s41396-021-01066-x

INTRODUCTION

The oxidation of molecular hydrogen (H₂) is an important source of bioavailable energy in anoxic environments, and H₂ represents a key metabolic intermediate in anaerobic syntrophy [1, 2]. Recently, aerobic H₂ oxidation was discovered to be widespread amongst microbial “dark matter” [3–5], with many aerobic microbial groups being capable of scavenging trace concentrations of atmospheric H₂ as an energy source [6]. However, the effects of H₂ on carbon utilization rate in marine microbial communities under low-oxygen conditions are poorly understood. The oxidation of H₂ with O₂ is predicted to be a thermodynamically favorable energy source for peridotite-associated microbial communities over a wide range of temperatures in ultramafic-hosted systems [7] and thus has the potential to provide important catabolic energy for seafloor-associated communities that live in the vicinity of a geological H₂ source [7, 8]. Indeed, a linkage between H₂ and microbial activity has been demonstrated in several high-temperature hydrothermal settings [8–12].

To better understand the effects of H₂ on carbon anabolism in seafloor microbial communities associated with ultramafic rocks, we used ¹³C quantitative DNA stable isotope probing (qSIP) [13, 14] with ¹³C-labeled bicarbonate, acetate, and formate in H₂ incubation experiments. The ¹³C DNA-qSIP approach identifies microbial taxa that are synthesizing new ¹³C-labeled DNA from the added ¹³C substrates, which occurs during genome replication [15]. We applied this method to microbial communities associated with partially serpentinized peridotite mylonite from Saint Peter and Saint Paul Archipelago (Arquipélago de São Pedro e São Paulo, Brasil –SPSPA) at the equatorial Mid-Atlantic Ridge.

The SPSPA is mainly composed of strongly deformed, partially serpentinitized Mg- and Fe-rich (i.e. ultramafic) rocks [16]. Serpentinization of ultramafic rocks involves the oxidation of ferrous iron in primary minerals to ferric iron in secondary minerals by water which generates abundant H₂ that can be used to conserve energy by H₂-oxidizing microbes [17]. During a recent expedition to SPSPA (AL170602) onboard the MV ALUCIA in 2017, geochemical evidence for H₂ formation was found to be recorded in serpentinized rocks and H₂ is likely generated today at SPSPA through low-temperature aqueous alteration of peridotite, mechanoradical H₂ formation, or radiolysis [17], albeit at slow
rates. Thus, we incubated partially serpentinized peridotite from SPSPA with $^{13}$C-bicarbonate, $^{13}$C-acetate, and $^{13}$C-formate in incubations with and without H$_2$ and then applied qSIP [13, 14] to quantify the effects of H$_2$ on $^{13}$C-substrate assimilation by specific operational taxonomic units (OTUs) that were associated with the ultramafic rocks.

**MATERIAL AND METHODS**

**Sampling**

Partially serpentinized peridotite (DR541-R3; 00°55'S/66°N; 29°10.70'W) and bottom seawater samples were collected from a yellowish-brown outcrop using the Deep Rover submersible in July 2017 at 327 m water depth from the northern slope east of the SPSPA, Brazil (M/V Alucia Expedition AL170602, 00°55'S; 29°21'W), a remote group of islets in the equatorial Atlantic Ocean, on the Mid-Atlantic ridge (Fig. 1A). The SPSPA belongs to the Brazilian Exclusive Economic Zone and is located within the Fernando de Noronha Environmental Protection Area. Conductivity, temperature, and density profiles were taken from several stations around the SPSPA to explore ongoing hydrothermal activity which could not be detected. The alteration mineralogy of serpentinized peridotite mylonite and fluid inclusion contents in primary minerals were determined in thin sections using a petrographic microscope and a confocal Raman spectrometer (Horiba LabRAM HR) equipped with a 20 mW 473 nm laser, astigmatic flat field spectrophotograph with a focal length of 800 mm, and a multichannel air-cooled (−70 °C) CCD detector.

Seawater was collected from directly above the peridotite rocks using an isobaric gas-tight (IGT) fluid sampler [18]. In addition, seawater was collected with a Niskin rosette from three nearby sites (Fig. 1A), two from 300 to 330 m water depth to serve as a background seawater microbial community comparison to the IGT fluids and peridotite rocks collected from dive DR541. Niskin (4–8 L) and IGT (18–75 mL) seawater for the $t_0$ comparisons were filtered onto 0.2 µm polycarbonate filters using a peristaltic pump and frozen immediately at −20 °C.

Sediments were collected from two nearby sites (Fig. 1A) using the slurp suction sampler onboard the Deep Rover submersible. Sediments were stored in 50 mL falcon tubes at −20 °C until DNA extraction.

**DNA extraction**

DNA was extracted from the seawater samples (frozen filters) using a protocol described previously [19]. In order to avoid cross-sample contamination with the rock samples, DNA was extracted from 10 to 12 g of rock samples on a separate day (Fig. 2A), in a laminar flow clean bench with pipettors that were autoclaved immediately before use (to remove contaminating DNA on the pipettors). DNA was extracted from three separate peridotite rock samples (subsamples of the same rock). These $t_0$ rock samples were collected from the same dive (DR541-R3; Fig. 1B) where peridotite rocks were sampled for the qSIP incubations (Fig. 1A). In addition, two separate carbonate rock replicates (subsamples of the same rock) were collected from dive DR540 (DR540-R3 and R4) (Fig. 1A). All $t_0$ rock samples were stored in 50 mL DNA/DNA clean falcon tubes (Fig. 2A), and DNA was extracted according to a previously published protocol [20].

The only deviation from the previous protocol was that silica glass beads from three Lysing Matrix E tubes (MP Biomedicals) were directly added to the 50 mL falcon tubes containing the rocks (Fig. 2A), which were homogenized with 10 mL C1 extraction buffer [20].

The $t_0$ peridotite rocks (DR541-R3) serve as a reference for the in situ peridotite-associated microbial community and allow comparison against the “rock-associated” community from the seawater samples. Moreover, because the $t_0$ peridotite rocks (DR541-R3) were collected from the same location as the peridotites that were used for setting up the $^{13}$C-SIP incubations, we could identify “rock-associated” OTUs (detected in the peridotite $t_0$ samples) that became labeled in the qSIP incubations that were, or were not, detected in seawater. For DNA extraction from sediments, we used the same protocol that we applied for the carbonate and peridotite rock samples, with the main difference that only 0.5 g of sediment was extracted in 2 mL lysing matrix E tubes with 1 mL of C1 extraction buffer [20]. Such a relatively small volume was required given the orders of magnitude higher microbial abundance in the sediment samples compared to the rocks (Fig. 2B).

**Experimental setup for SIP incubations**

For incubation experiments, the outermost ~3 cm of rock sample DR541-R3 was carefully removed with a sterile hammer to retrieve the rock interior. The rock interior was subsequently crushed into mm-sized fragments on a sterile surface (ethanol washed) in a fume hood for incubations. For the inocula in the SIP incubations, 2 g of crushed rock fragments from the interior of the peridotites were placed into 20 mL gas-tight glass vials which had been heated to 450 °C for 10 h prior to use.

In addition, 10 mL sterile-filtered seawater (using 0.2 µm polycarbonate filters) was added to each vial. DNA was extracted from the rocks. Therefore, the living cells in the SIP incubations should be primarily rock-associated and derived from the interior of the peridotites, as sterile filtration should have removed cells >0.2 µm in size from the added seawater. However, it is possible that some ultra-small seawater cells <0.2 µm could have passed through and made it into the SIP incubations.

Each vial was amended with either 10 mM sodium-$^{13}$C-bicarbonate (99% $^{13}$C-content, Sigma-Aldrich, Darmstadt, Germany), 2 mM sodium-$^{13}$C-acetate or 10 mM sodium-$^{13}$C-formate (99% $^{13}$C-content, Cambridge Isotope Laboratories, Andover, MA, USA) and crimp-sealed using KOH-washed butyl stoppers [21]. We acknowledge that the high concentrations of $^{13}$C-acetate and $^{13}$C-formate that were added are orders of magnitude higher compared to the measured concentrations of acetate and formate in fluids venting from the Lost City Hydrothermal field [22]. All glass vials were crimp-sealed with a butyl rubber stopper creating gas-tight conditions and the atmosphere was replaced with nitrogen gas (N$_2$) for 10 min to create low oxygen conditions. Afterward, one set of flasks was amended with H$_2$ added to the headspace (1.5 bar), with a second set as a control that did not receive H$_2$. We acknowledge that these concentrations are higher than those at most hydrothermal systems [7].

Although the O$_2$ was not measured in the incubations, we assume dissolved oxygen concentration was reduced down ca. 10-fold compared to the ambient concentration of ca. 120 µM by purging the incubation medium for a minimum of 10 min with N$_2$ [23]. The resulting low O$_2$ conditions of assumed 10–15 µM were likely further drawn down by aerobic respiration during the course of the experiment [24].

As is common practice for all DNA-SIP studies, control vials were also prepared with the same unlabeled carbon sources (referred to as “unlabeled control”) to compare the extent of $^{13}$C-labeling from the labeled incubations. The glass vials containing unlabeled control and $^{13}$C-substrates were incubated at room temperature (ca. 25 °C) terminated after 35 h and stored at −60 °C for onshore analysis. DNA was extracted from 1 g of slurry in triplicate using the same method as described above [20] and quantified fluorometrically using Qubit 3.0 fluorometer (Invitrogen, Eugene, OR, USA).

**Density gradient centrifugation and gradient fraction**

DNA samples were prepared for density gradient centrifugation according to previously defined protocols for qSIP [13, 14]. DNA of density fractions was resuspended with 30 µl molecular-grade (DEPC-treated) water and quantified fluorometrically using a Qubit fluorometer.

**Quantitative PCR (qPCR)**

Universal primers targeting the V4 hypervariable region of 16S ribosomal RNA (rRNA) genes were used to qPCR to determine density shifts in the peak DNA of buoyant density (BD) for each incubation. We used a version of the 515F primer with a single-base change (in bold) to increase the coverage of archaeal groups (515F-Y, 5′-GTYCAGCMGGCGGTAA [25]). qPCR was carried out as described previously [26]. 16S rRNA gene quantities of the density fractions were plotted against their corresponding densities and 10 fractions (on average) from each replicate set were selected for sequencing (Fig. S1; gray shaded area). Two 16S rRNA gene PCR amplicons from each density fraction (technical replicates to reduce PCR bias) were pooled and subjected to dual-indexed barcoded sequencing of 16S rRNA gene amplicons on the MiniSeq (Illumina) as described previously [27].

**Bioinformatic and qSIP analysis**

The MiniSeq reads were quality trimmed and assembled using USEARCH version 10.1.667 with the default parameters [28] resulting in 6.8 million quality checked V4 reads. Reads were then de novo clustered at 97% identity using UPARSE; OTUs represented by a single sequence were discarded [29]. Taxonomic assignments were generated by QIIME 1.9.1 [30] using the implemented BLAST method against the SILVA rRNA gene database release 132 [31]. The raw OTU table consisted of 10,654 OTUs which were further quality-filtered. The level of contamination in each density fraction for qSIP analysis was determined using previously sequenced DNA sequencing libraries from three different laboratories in our building where the samples are processed [27].
comprising 2.34% of the whole 16S rRNA gene dataset (188,273 sequence reads out of 8,046,165). Only OTUs having >12 sequences in total in each replicate (summed across all density fractions) were selected for further study since low abundance taxa cause artificial variations in qSIP calculations [33]. Intermediate files in data removal of contaminants, quality filtering, and detailed explanations of the intermediate files can be found in the following data repository: https://doi.org/10.6084/m9.figshare.13341443.v1.

Excess atomic fraction $^{13}$C (EAF) values were calculated for the 16S rRNA genes corresponding to OTUs according to a previously described study [13] using a qSIP workflow embedded in the HTS-SIP R package [34]. To calculate the bootstrap confidence intervals (CI) for significant isotope incorporation, 10,000 bootstrap replicates were run within the HTS-SIP R package; an OTU was considered as a $^{13}$C-assimilator if the lower boundary of CI was above the 0% EAF cutoff [13]. Statistical analyses and plots were performed using RStudio Version 3.3.0 [35].

qSIP measurements of OTU-specific $^{13}$C-substrate assimilation with and without H$_2$ allowed us to test whether the activity of microbial communities in the presence of H$_2$ was significantly restricted to specific phylogenetic clades (e.g., “phylogenetic signal” [33]). Page’s $\lambda$ and Blomberg’s $K$ were calculated as two independent indices of the phylogenetic signal [36, 37]; shared traits (e.g., $^{13}$C-assimilation patterns) in the context of evolutionary history (e.g., 16S rRNA gene phylogenetic relation).

**Metagenomic analysis of rock, seawater, and SIP samples**

Given the shifts in buoyant density of 16S rRNA genes in the $^{13}$C-SIP incubations (Fig. S1), we produced metagenomes from “heavy” fractions of the density gradients that indicated $^{13}$C labeling (Fig. S1). The DNA content within each of these “heavy” metagenomes was chosen for metagenomic shotgun sequencing based on the region of the CsCl gradient that exhibited a peak in the $^{13}$C-substrate incubation that had a higher CsCl density compared to the unlabeled control experiment (Fig. S1). Metagenomic libraries were prepared using Nextera XT DNA Library Prep Kit (Illumina) and following the manual provided by the manufacturer with minor modifications. The starting concentration of genomic DNA could not be set to 0.2 ng as suggested by the manufacturer’s manual due to low DNA content in the labeled SIP fractions. Instead, the PCR program in the amplification step of the fragmented DNA was increased from 12 to 15 cycles. Metagenomic libraries from the rock and seawater $\tau_0$ samples were prepared from the extracted DNA (see above) with unique barcodes using the same Nextera XT kit, were diluted to 1 nM, and pooled together for sequencing on the MiSeq (Illumina) platform.

Paired-end reads were trimmed and assembled into contigs using CLC Genomics Workbench 9.5.4 (Qiagen, Hilden, Germany), using a word size of 20, bubble size of 50, and a minimum contig length of 300 nucleotides. Reads were then mapped to the contigs using the following parameters (match/mismatch penalty, 3; insertion penalty, 3; deletion penalty, 3; minimum alignment length, 50% of reading length; minimum percent identity, 95%). Coverage values were obtained from the number of reads mapped to a contig divided by its length (i.e., average coverage). This protocol does not assemble rRNA genes [38]; thus, results are only discussed in terms of protein-encoding genes.

For annotating putative functions of ORFs in metagenomes from particular “higher-level” taxonomic groups of microorganisms, we applied a previously published bioinformatics pipeline [38]. This pipeline extracts protein-encoding ORFs from de novo-assembled contigs using FragGeneScan v. 1.30 [39], and functionally annotates ORFs against a large aggregated database (“MetaProt”) [38] using DIAMOND version 0.9.24 [40]. The MetaProt database contained predicted proteins from all protist, fungal, bacterial, and archaeal genomes (and MAGs) in the JGI and NCBI databases as of January 2021. The MetaProt database [38] also contains ORFs from all of the transcriptomes of microbial eukaryotes from the MMETS project [41]. The MetaProt database is available as a single 32 GB amino acid fasta file on the LMU Open Data website (https://data.ub.uni-muenchen.de/183/). Cutoff values for assigning hits to specific taxa were performed at a minimum bit score of 50, the minimum amino acid similarity of 60, and an alignment length of 50 residues. All scripts and code used to produce the analysis have been posted on GitHub (https://github.com/williamorsi/MetaProt-database). This approach assigns ORFs to higher-level taxonomic groups [38]. As is the case in all metagenomic studies, the incomplete nature of genomes in databases, together with the lower representation of sequenced genomes from candidate clades than from cultured ones, makes it likely that our pipeline misses annotation of ORFs that are derived from as-yet-unsequenced genomes.

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**Fig. 1** Bathymetry and petrology of SPSPA. A Bathymetry of SPSPA and sampling locations for Niskin, IGT fluids, sediments, and rocks. The location for the qSIP experiment (DS41) is shown with a yellow star. B Photo of partially serpentinized peridotite collected from St. Pauls Rocks, the interior of the rock was used for the qSIP incubations. C Dissolved oxygen vertical profiles from two sites (Niskin2, Niskin3) in close proximity to the location for qSIP (DS41). The horizontal dashed line represents the depth where samples were taken for qSIP (327 meters). The other labels indicate the water depths at which those samples were taken (see map in panel A). D Thin section photomicrographs of sheared peridotite from SPSPA. The presence of serpentine and magnetite (top two panels) is indicative of H$_2$ generation. Hyperspectral Raman map (bottom panel) showing a CH$_4$-rich inclusion in amphibole sampled from the close vicinity of the studied area.
The 16S rRNA gene amplicon sequences and metagenomic sequence data were entered in the NCBI Sequence Read Archive under BioProject ID PRJNA679196. The CTD data, metagenomic dataset, and intermediate files to produce qSIP results were deposited under https://figshare.com/authors/_mer_Coskun/9725927.

Assessing biases in metagenomes from density fractions containing 13C-enriched DNA

Sequencing metagenomic DNA from only a single 13C-enriched fraction (Fig. S1) may be biased due to [1] low GC genomes that might not be detected in 13C-enriched fraction even though they highly incorporate the labeled source, and [2] abundant organisms can sometimes be found in all fractions irrespectively of labeling [42, 43]. However, many abundant 13C-labeled OTUs were determined with statistical significance via qSIP related to Marinobacter, Alteromonas, Thaumarchaeota, 'Ca. Rokubacteria', or Nitrospirae, were represented in the 13C-metagenomes. The overlapping taxa labeled in qSIP and metagenomes from 13C-enriched SIP fractions indicate that the metagenomic sequences obtained from the selected SIP fractions are derived to a large extent from taxa that were 13C-labeled.

Phylogenetic analyses

For phylogenetic analyses of 13C-labeled 16S rRNA genes, OTUs which were at least occurring in one of the experiments were selected for alignment with MUSCLE [44] using SeaView [45]. The resulting fasta file was imported into W-IQ-TREE [46] with an option to select the best phylogenetic model using Bayesian criterion, which resulted in TIM3e+R10 algorithm using ModelFinder [47]. The phylogenetic tree was visualized and edited using iTOL [48]. Statistical analyses and plots were performed using RStudio Version 3.3.0 [35], Pagel’s λ [37] and Blomberg’s K [36] tests for significantly non-random phylogenetic distributions of 13C-utilizers from qSIP were calculated using the phylosignal R package [49].

For phylogenetic analyses of ORFs from metagenomes with similarity to HypE, niiS, and coxl based on BLASTp searches against the MetaProt database [38], ORFs were aligned against their top BLASTp hits using MUSCLE [44]. Phylogenetic analysis of the resulting amino acid alignments of the predicted proteins was conducted in SeaView using RAxML [50] with BLOSSUM62 as the evolutionary model and 100 bootstrap replicates. The resulting phylogenetic trees were displayed as unrooted cladograms using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

Rock description

Thin section petrography and Raman analysis revealed that rock sample DR541-R3 (used as the inoculum for the qSIP incubations) is a partially serpentinitized peridotite mylonite that is chiefly composed of olivine and orthopyroxene, minor amounts of clinopyroxene, and traces of Cr-spinel (Fig. 1D). Primary minerals are partially altered to serpentine, magnetite, tremolite, calcite, and aragonite which chiefly occur in veins cutting across the mylonite matrix. We observed methane-rich fluid inclusions at other locations at SPSPA but not at the sampling location (Fig. 1D). For comparison, we obtained thin sections of two additional samples from the same dive (samples DR541-R1 and DR541-R4) which revealed structural and alteration patterns similar to those of DR541-R3 suggesting all three samples experienced extensive ductile and brittle deformation followed by serpentinization.

Microbial abundance and diversity of the ultramafic-rock associated community

The concentration of 16S rRNA genes from the peridotite rock samples was 2.6 (±2)×10^6 copies per g of rock, compared to 7 (±1)×10^6 and 0.5 (±0.06)×10^6 16S rRNA gene copies per mL seawater collected with the IGT fluid samplers and Niskin rosette, respectively (Fig. 2B). qPCR quantification of 16S rRNA gene copies from the frozen peridotite rock samples showed cycle threshold (Ct) values ranging between 25 and 30 cycles, which strongly indicates that our amplified 16S rRNA genes are derived from in situ microbes associated with the rocks as opposed to contamination because all contamination controls (extraction blanks and qPCR no template controls) consistently had Ct values >35. The Chao diversity index based on the 16S rRNA gene data shows that the rock-associated community is significantly more diverse (two-sided T-test: P < 0.0001) compared to the seawater communities (Fig. 3B). 16S rRNA gene data also shows that the microbial community composition between the peridotite-associated and the seawater communities was significantly different (Analysis of Similarity [ANOSIM] R: 0.87, P < 0.0001), with approximately two-thirds of all detected OTUs being found on the peridotite rock samples and not in any seawater samples (Fig. 3C). The peridotite-associated communities included taxa that are common to hydrothermal [51] and ultramafic rock habitats [52, 53] including Nitrospirae, Rokubacteria, Entotheonellaeota, Gemmatimonadetes, and Alphaproteobacteria (Fig. 3A). These comparisons reveal a unique microbial community inhabiting the peridotite rocks compared to the seawater that was collected directly above these rocks with the IGT fluid samplers.

Fig. 2 Microbial abundance in sea floor and seawater samples. A Photograph of peridotite rock samples (dive DS41) prior to DNA extraction. B Concentration of 16S rRNA genes from sediments, rocks, IGT fluids, and Niskin collected seawater. Concentrations are normalized to per gram for sediment and rock samples, and per mL for seawater samples (IGT fluids and Niskins).
Identifying $^{13}$C-labeling for qSIP. $^{13}$C-labeling of 16S rRNA genes (defined by a shift in peak DNA buoyant density) was observed in all incubations, with average shifts in the peak $^{13}$C-DNA buoyant densities compared to control-incubations between $0.0018 \pm 0.0037$ and $0.0279 \pm 0.0076$ g ml$^{-1}$ (Fig. S1). This highlights biological variability in $^{13}$C-assimilation in our replicate treatments, but despite this variability, the average density shift in the replicate treatments shows a clear trend of $^{13}$C-enriched DNA from 16S rRNA genes compared to the unlabeled controls (Fig. S1). Therefore, $^{13}$C-labeling of microbes synthesizing new DNA occurred in all incubations. The use of replicates in qSIP allows for statistically constrained estimations of $^{13}$C-assimilation in all detectable OTUs [13]. Namely, $^{13}$C-qSIP allows for the calculation of the $^{13}$C-excess atomic fraction (EAF) for all detectable OTUs within a microbial community, together with a confidence interval (CI) that provides to test statistical significance for OTU-specific $^{13}$C-assimilation [13]. We thus applied the qSIP protocol to estimate the $^{13}$C-EAF (with CI) for all detected OTUs within each experimental treatment.

Significance of H$_2$ on the phylogenetic organization of $^{13}$C-utilizing taxa. Phylogenetic signal analyses showed that H$_2$ had a statistically significant (non-random) effect on the phylogenetic organization of the $^{13}$C-assimilating taxa in each of the three substrates tested (Table 1). Thus, the effect of H$_2$ on increased $^{13}$C-labeling of clades with $^{13}$C-bicarbonate and $^{13}$C-acetate, and to a lesser extent $^{13}$C-formate, was statistically significant in terms of the non-random distribution of specific taxa that increased their anabolism with added H$_2$.

With added H$_2$, OTUs increased assimilation of $^{13}$C-bicarbonate and $^{13}$C-acetate on average by 146% ($\pm 29\%$) and 55% ($\pm 34\%$), respectively (Fig. 4). Moreover, within the peridotite rock-associated OTUs, 54 genus-level taxonomic groups were $^{13}$C-labeled in qSIP incubations containing added H$_2$ (Fig. 5). This was more than an order of magnitude higher compared to seawater-specific $^{13}$C-labeled taxa, as well as seafloor-associated $^{13}$C-labeled taxa not detected on peridotite rocks (only detected on carbonate rocks and sediments) (Fig. 5).

The effects of H$_2$ on bicarbonate assimilation. Compared to controls ($^{13}$C-bicarbonate incubations that did not receive H$_2$) H$_2$ addition was correlated with an overall increase in $^{13}$C-assimilation by OTUs with EAF values increasing on average from $0.13 \pm 0.009$ to $0.32 \pm 0.051$ (146 $\pm 29\%$ increase) (Fig. 4). Most of these OTUs were rock-associated (Fig. 5). H$_2$ addition coincided with a two-fold drop in $^{13}$C-labeled Gammaproteobacterial OTUs (Fig. 4). In
contrast, the abundance and $^{13}$C-bicarbonate assimilation (EAF) of Thaumarchaeal OTUs in the presence of $H_2$ increased to 7.79% and 0.38 ± 0.34, respectively (Fig. 4), which were associated with the peridotite rocks (Fig. 5). $H_2$ addition coincided with a $^{13}$C-bicarbonate assimilating peridotite-associated OTU affiliated with the Atribacteria (JS1 clade) having the highest EAF of all OTUs in the $^{13}$C-bicarbonate incubations amended with $H_2$ (EAF: 0.87) (Fig. 4). In total, 85% of the $^{13}$C-bicarbonate assimilating taxa were associated with the peridotite rocks (Fig. 5).

Effects of $H_2$ on acetate assimilation. In $^{13}$C-acetate incubations with added $H_2$, the EAF value per OTU increased from 0.27 ± 0.010 to 0.42 ± 0.011 (55 ± 34% increase) relative to control incubations that did not receive $H_2$ (Fig. 4). Gammaproteobacteria and Thaumarchaeota had the highest number of OTUs that increased $^{13}$C-acetate assimilation in the presence of $H_2$ (Fig. 4). The genera showing the highest $^{13}$C-acetate assimilation in the presence of $H_2$ were Arcobacter (0.91 EAF), Marinobacterium (0.54 EAF), Nitrosomonas (0.49 EAF), Alteromonas (0.42 EAF), Nitrosporaceae (0.4 EAF), and Nitrospira (0.14 EAF) (Fig. 4). All of the acetate assimilating Nitrosporaceae (Thaumarchaea) and Nitrospira taxa were peridotite-associated (Fig. 5).

Effects of $H_2$ on formate assimilation. In contrast to acetate and bicarbonate incubations, OTUs assimilating $^{13}$C-formate in the presence of $H_2$ had on average lower EAF values compared to the control $^{13}$C-formate qSIP incubations that did not receive $H_2$ (Fig. 4 and 5) (average 0.32 ± 0.01-0.14 ± 0.02 EAF; 139 ± 11% decrease). This shows that the utilization of formate by most microbial groups was reduced in the presence of $H_2$. The composition of the community in the $H_2$-supplemented formate incubation changed substantially, with OTUs belonging to the Planctomycetes (Family Pirellulaceae) and Enthotheonellaeota exhibiting the highest EAF values from $^{13}$C-formate (up to 0.89 EAF) (Figs. 4 and 5). All of the Planctomycetes and Enthotheonellaeota formate assimilating taxa were associated with the peridotite rocks (Fig. 5).

Functional gene diversity in peridotite-associated clades assimilating $^{13}$C-substrates. In the heavy metagenomes, approximately one-third of ORFs were associated with peridotite rocks and not with seawater (Fig. 6A). This is evidence of a rock-associated community with unique protein-encoding gene content that was assimilating $^{13}$C. Within the heavy metagenomes, diversity of carbon monoxide dehydrogenase (coxL: Fig. 6B), $H_2$-oxidizing NiFe-hydrogenase assembly proteins (HypE: Fig. 7A), and dissimilatory nitrite reductase (nirS: Fig. 7B) all show bootstrap supported clades that contained ORFs from the heavy metagenomes and peridotite metagenomes. The majority (>75%) of these clades did not include any ORFs from seawater metagenomes (Figs. 6 and 7). This indicates that most of the coxL, HypE and nirS encoding organisms that assimilated the $^{13}$C-substrates in the SIP incubations were associated primarily with the peridotites.

In the peridotite rock metagenomes, there was an increased relative abundance of ORFs encoding nirS and NiFe-hydrogenase assembly proteins involved in $H_2$ oxidation (HypABCDEF) [54] compared to Niskins and IGT collected water, which was consistent across biological replicates (Fig. 7C and D). Relative abundance of HypABCDEF and nirS encoding ORFs in heavy metagenomes from SI-P incubations were also higher compared to seawater metagenomes (Fig. 7C and D).

Phylogenetic analysis of the carbon monoxide dehydrogenase large subunit (coxl) encoding ORFs reveals nine major bootstrap-supported coxl clades that include ORFs from heavy metagenomes and peridotite rock metagenomes but did not contain any coxl ORFs from seawater metagenomes (Fig. 6B). The peridotite-associated coxl clades contain a four-fold higher number (6 compared to 25) of heavy ORFs compared to seawater-associated coxl clades, and were affiliated with Labrenzia, Pelomicrobium, Denitrobaculum, Nitrolicum, Litorilinea, uncultivated Actinobacteria, SAR116 clade, and ‘Ca. Rokubacteria’ (Fig. 6B).

### DISCUSSION

The increased diversity of the peridotite-associated community compared to seawater microbial communities (Fig. 3B) is consistent with prior studies of seafloor communities associated with basaltic rocks [55]. The majority of taxa that assimilated $^{13}$C in the presence of $H_2$ were derived from this diverse peridotite-associated community as opposed to being specific to seawater (Fig. 5). Therefore, the peridotite-associated communities are enriched with the capability to utilize $H_2$ to increase their carbon assimilation from CO$_2$, acetate, and formate. Since $H_2$, acetate, and formate are formed during serpentinization [56], our results highlight the importance of $H_2$ in influencing carbon cycling in rock-hosted microbial communities.

The presence of magnetite in the serpentinized rock matrix suggests that temperatures exceeded 200 °C when rocks underwent serpentinization [57]. Because its formation requires the oxidation of ferrous iron originally contained in primary minerals to ferric iron in magnetite with water as the oxidizing agent, $H_2$ was generated during serpentinization of mylonite at St. Paul’s Rocks. Because the mylonite was only partially serpentinized, it— which would be a source of $H_2$ for the peridotite-hosted communities. Unlike peridotite-hosted alkaline hydrothermal vents with a focused flow such as the Atlantis Massif [58], we found no firm evidence of $H_2$ anomalies in the water column at SPSPA indicating that any $H_2$ had already been oxidized in the water column that has ca. 100 $\mu$M O$_2$ (Fig. 1C). However, $H_2$ in diffuse fluids emanating from the subseafloor of SPSPA could be oxidized by the rock-associated seafloor microbes (Figs. 3C, 5) before diffusing into the oxidized water column, as indicated previously by geochemical evidence in diffuse vents from the Juan de Fuca Ridge [59].
Carbon assimilation in an ultramafic rock-associated community

The observation that most $^{13}$C-assimilating taxa were peridotite-associated (Fig. 5), indicates that many taxa within this relatively diverse community (Fig. 3B) have a high affinity for the added substrates compared to the seawater microbes living directly above the rocks. Gammaproteobacteria dominate the $^{13}$C-labeled taxa that were detected in seawater, but the $^{13}$C-assimilating taxa detected only in the seafloor samples were by comparison more diverse and instead consist of Thaumarchaeota, Rokubacteria,
Peridotite-associated groups 13C-labeled in qSIP

Detected only in qSIP incubations

Not detected in seawater

Detected in seawater

Detected only in qSIP and seawater

Detected in qSIP, seawater, seafloor, not peridotites

13C-labeled in qSIP with H2 amendment

Histogram legend

Acidobacteria
Actinobacteria
Alphaproteobacteria
Bacteroidetes
Chloroflexi
Deltaproteobacteria
Epsilonbacteraeota
Euryarchaeota
Firmicutes
Gammaproteobacteria
Planctomycetes
Nitrospira
Rokubacteria
Thaumarcheota
Woeseia

**Group average 13C-EAF across all qSIP Incubations**

| Sediments | Rocks | Seafloor samples | Seawater samples | qSIP incubations |
|-----------|-------|------------------|------------------|------------------|
The distribution of carbon assimilating groups within the rock, sediment, and seawater samples. The heatmap shows the relative abundance of 16S rRNA gene sequences per group per sample (purple = more, light blue = less). Within the qSIP incubations (right-hand side), groups that had OTUs with statistically significant $^{13}$C-assimilation in qSIP are surrounded by a yellow box. The histograms on the right side of the plot show the average $^{13}$C-EAF across all qSIP incubations per group. An asterisk indicates that this group had a significant $^{13}$C-EAF within a qSIP incubation with added H$_2$ (no asterisk means labeling occurred only in qSIP incubations without H$_2$). The histograms at the top of the heatmap show the average $^{13}$C-EAF across all OTUs within each qSIP incubation, error bars represent standard deviations. The analysis shows that the majority of $^{13}$C-assimilating groups were rock-associated and that more rock-associated groups became labeled in the presence of H$_2$ (middle of the heatmap), compared to the seawater-associated groups (at the bottom of the heatmap).

Planctomycetes, Acidobacteria, Entotheonellaota, Deltaoteobacteria, and Gemmatimonadaceae (Fig. 5). The qSIP results show that the effect of H$_2$ on increased carbon assimilation is phylogenetically organized in this unique rock-associated community, with specific peridotite-associated taxa assimilating more carbon in the presence of H$_2$ compared to seawater-associated taxa (Fig. 5).

The phylogenetic signal analysis based on Blomberg’s K [36] reveals H$_2$ utilization as a shared trait in $^{13}$C-assimilating taxa, compared to the incubations that did not receive H$_2$ (Table 1, Fig. S2). This significant phylogenetic signal within $^{13}$C-assimilating taxa in the presence of added H$_2$ coincides with an order of magnitude higher number of peridotite rock-associated taxa having an increased anabolism in the presence of added H$_2$, compared to taxa not detected on the ultramafic rocks (Fig. 5). These relations point to ongoing H$_2$ production via low-temperature aqueous alteration of peridotite (Fig. 1D) which supports a unique peridotite-associated community with higher diversity (Fig. 3B) that is relatively enriched with the ability to metabolize H$_2$ compared to the overlying seawater communities (Fig. 7C).

Differential effects of H$_2$ on the assimilation of acetate, bicarbonate, and formate. The increased $^{13}$C-assimilation with added H$_2$ (Figs. 4 and 5) is supported by a higher relative abundance of the HypABCDEF [$\text{NiFe}$]-hydrogenase locus in heavy metagenomes from H$_2$-amended incubations (Fig. 7C). The HypABCDEF locus is responsible for the maturation of $\text{NiFe}$-hydrogenase in bacteria and aerobic H$_2$ oxidation [54]. The HypE ORFs within clades exhibiting $^{13}$C assimilation were affiliated with uncultured Nitrosopumilaceae, Nitrospinae bacteria, ‘Ca. Entotheonella’, and Alteromonas (Fig. 7A), which were all groups identified in qSIP as being rock-associated with increased carbon assimilation in the presence of H$_2$ (Fig. 5). The higher carbon assimilation in the presence of added H$_2$ in several rock-associated groups (Fig. 5) are consistent with H$_2$ oxidation via HypABCDEF, and could be related to higher H$_2$ concentrations in the peridotite ecosystem.

The correlation of added H$_2$ with a decreased formate assimilation by most OTUs (Fig. 4) is possibly due to inhibition of the hydrogenase unit of formate hydrogen lyase that is caused by high H$_2$ concentrations [60]. This has been observed in previous SIP studies, where H$_2$ had an inhibitory effect on formate assimilation in hot springs [10]. However, microcosm experiments with H$_2$ and formate in terrestrial alkaline fluids in the Samail Ophiolite showed that under high H$_2$ concentrations (20%, excess atmospheric pressure) certain methanogens increase methane production from formate, presumably via formate dehydrogenase [61]. Therefore, in serpentinization settings the inhibition or stimulation of formate metabolism by increased H$_2$ concentrations is likely to be dependent on the redox potential of the environment.

**Atribacteria exhibit H$_2$-dependent carbon fixation**

The higher relative abundance of $\text{nirS}$ encoding ORFs in peridotite rock and IGT metagenomes compared to the Niskin seawater metagenomes (Fig. 7D) could be explained by low-oxygen levels
Fig. 6  Metagenome ORF distribution and coxl phylogeny. A Heatmap displaying all ORFs detected from heavy SIP metagenomes and the distribution of these heavy ORFs in the rock and seawater metagenomes. Heatmap rows represent predicted proteins from the MetaProt database [38] having best BLASTp similarity to peptides encoded in ORFs from the metagenomes (columns). Colors represent length normalized read coverage from metagenomes to the ORFs. B Phylogenetic analysis (RAxML) of all detected coxl ORFs based on an alignment length of 968 amino acids. Note that most of the coxl clades were rock-specific and that these also contain the majority of heavy coxl ORFs.
Fig. 7 Distribution of HypE and nirS in metagenomes. Phylogenetic analyses (RAxML) of all detected HypE (A) and nirS (B) ORFs based on alignment lengths of 442 and 638 amino acids, respectively. Note that the separation of seawater (pink highlighted) and rock-associated (light blue highlighted) clades. Panels (C) and (D) show the relative abundance (% reads mapping, length normalized) within the different metagenomes to ORFs with best BLASTp similarity to the NiFe-hydrogenase assembly locus HypABCDEF (C) and nirS (D), respectively. Circles on nodes represent bootstrap values (black > 90%, gray > 70%, white > 50%).
commonly experienced at the benthic-seawater interface selecting for anaerobic, nitrite respiring bacteria [74]. The relative abundance of heavy nirS ORFs was higher in the presence of added H₂ (Fig. 7D), which indicates that the H₂ was selected for nitrite respiring bacteria. Most of the heavy nirS ORFs were affiliated with Marinobacter and Amphiphila (Fig. 7B), which rock-associated and exhibited some of the highest levels of 13C-assimilation with added H₂ (Figs. 4 and 5). Taken together, these results indicate that the addition of H₂ promoted increased assimilation of 13C via anaerobic nitrate-reducing bacteria, including peridotite-associated Marinobacter and Amphiphila.

The addition of H₂ affected increased carbon assimilation of peridotite-associated ammonia and nitrite-oxidizing consortia (Fig. 5) that are responsible for nitrification. Together with the activity of rock-associated nitrite reducing bacteria (Fig. 7), our results show the potential for H₂ to effect coupled nitrification and denitrification in the rock-associated community. Coupled nitrification and denitrification influence the loss of fixed nitrogen from benthic ecosystems [75]. Our findings raise the possibility that serpentinization derived H₂ may influence fixed nitrogen loss (as N₂ gas) from the peridotite-rock associated ecosystem, by stimulating the activity of nitrifiers and denitrifiers. Nitrogen cycling consortia have been found in terrestrial serpentinization systems as well, such as the Somali Ophiolite system in Oman [76]. The possibility for abiotically produced H₂ from serpentinization reactions to influence fixed nitrogen loss via coupled nitrification and denitrification in ultramafic rock ecosystems is a topic worthy of future study.

Carbon assimilation by CO oxidizers in the rock-associated community
Carbon monoxide can serve as the sole source of carbon and energy for life in environments that are low in an organic matter [77], including terrestrial serpentinization settings [78, 79]. The coxMSL enzyme (carbon monoxide dehydrogenase) catalyzes the oxidation of CO (carbon monoxide) to CO₂ in bacteria [80], and is a widespread mechanism supporting microbial survival [81, 82], particularly in extreme habitats experiencing low levels of productivity [4]. The bootstrap-supported separation of seawater and rock-associated coxL clades indicate a unique rock-associated community capable of using CO as an energy source. The substrate utilization within these peridotite-rock-associated clades of coxL encoding organisms appears to have been affected by H₂ amendments. For example, heavy coxL ORFs from SIP incubations amended with H₂ were detected within bootstrap-supported peridotite-associated clades affiliated with the ‘Ca. Rokubacteria’, Gemmatimonadetes, and SAR202 clad (Fig. 6B). This raises the possibility that CO oxidation might be related to anabolism at relatively high H₂ concentrations by some taxa within these groups.

The carbon assimilation by CO-oxidizing bacteria associated with ultramafic rocks seen here could be explained by the water–gas shift reaction (CO₂ + H₂ = CO + H₂O) [83]. Our data shows that in seafloor ultramafic rock settings where O₂ is above detection and H₂ is likely produced via serpentinization or related low-temperature alteration processes, aerobic CO-oxidizing organisms become stimulated by CO that is produced via the abiotic reduction of CO₂ with H₂. All of the CO-dehydrogenases that we detected were the molybdenum–copper-containing form which functions in aerobic CO-oxidation to CO₂, as opposed to the NiFe CO-dehydrogenases that function in anaerobic CO₂ reduction to CO [83]. Our results from peridotite-associated communities at SPSPA are similar to terrestrial serpentinization settings, where aerobic CO oxidation supports life that survives under alkaline conditions [78, 79].

Taken together, the phylogenetic analysis of coxL, HypE, and nirS show a similar pattern: the majority of heavy ORFs cluster together in bootstrap supported clades of peridotite rock-associated taxa. This trend suggests that the oxidation of H₂ and carbon monoxide, as well as dissimilatory nitrite reduction, are widespread and important physiological features for taxa that were assimilating 13C within the peridotite-associated microbial community.

Assessing effects of the qSIP incubation conditions
A comparison of effects of the substrates (acetate, formate, bicarbonate) is problematic due to the extraordinarily high concentrations of acetate and formate added relative to the in situ conditions, and potential substrate inhibition, toxicity, and pH alterations. Therefore, we do not compare the effects of the substrates to one another (e.g., we do not claim that bicarbonate is a more important carbon source compared to formate, despite the clear differences in qSIP results) but rather compare assimilation of a particular substrate with, and without, added H₂. While the added concentrations in our incubations are higher than the in situ abundances (no H₂ above background was detected in the water column), our experimental approach provides initial boundary conditions on the stimulation of carbon assimilation by H₂ for specific substrates, by specific peridotite-associated taxa in an ultramafic seafloor setting undergoing low-temperature aqueous alteration.

An increased temperature of the incubations (room temperature) relative to the in situ temperature (10–12°C), probably led to elevated microbial activity and rates of 13C substrate assimilation as shown previously for benthic microbes [84]. Moreover, purging of the incubation flasks with N₂ created dissolved O₂ concentrations at low oxygen levels (see the “Methods” section), and the labeling of known strictly anaerobic taxa indicates that anoxic regions were established during the incubation. Because O₂ was available at low concentrations in the flasks (ca. 10 µM), this explains why many of the most highly 13C-enriched taxa in the qSIP are known aerobic or facultatively anaerobic taxa (Figs. 4 and 5). However, at the flask bottom below the 3 cm column of crushed peridotite rock, anoxic conditions likely established due to a vertical O₂ gradient in the flask that commonly occurs in this experimental setup due to increased rates of aerobic respiration at the benthic–water interface [24]. Anoxic conditions in the crushed rocks at the bottom of the flasks likely promoted carbon assimilation by rock-associated strict anaerobes that also were using H₂ to increase their anabolism from the added 13C-labeled substrates. For example, this is seen in the Atribacteria that are strict anaerobes [85] that exhibited the highest 13C-bicarbonate assimilation in the presence of H₂ (Fig. 4). Moreover, the 13C-labeling of a peridotite-associated taxon affiliated with the Firmicute Paramoeditivibacter (Fig. 5) indicates anoxic conditions, as this is a strictly anaerobic organism originally isolated from a deep-sea hydrothermal vent from the Mid-Atlantic Ridge [86].

Assessing cross-feeding of 13C-labeled substrates
Cross feeding is an issue inherent to all SIP studies. It is possible that some of the 13C was fixed from bicarbonate into organic molecules and subsequently assimilated by heterotrophs. The labeling of heterotrophic taxa with 13C-bicarbonate indicates that some of the 13C-bicarbonate was taken up by autotrophs and assimilated by heterotrophs as DOM or POM. Alternatively, heterotrophic carbon fixation occurs through anaerobic carbon fixation reactions in heterotrophs that can account for 2–8% of cell carbon [87, 88] and could explain a portion of the 13C labeling in heterotrophs seen here. It is not possible that the 13C from the organic substrates would be remineralized and be taken up as bicarbonate because the natural bicarbonate concentration in seawater is ~2.3 mM and will dilute the remineralized 13C label to undetectable levels [19]. Therefore, the results in bicarbonate incubations not only show the primary utilizers but underpin complex ecological interactions in the microbial food web. We also note that formate can rapidly interconvert with CO₂ and therefore some of the formate may have been taken up as 13C-bicarbonate [89]. These results support the hypothesis that anaerobic carbon fixation reactions in heterotrophs represent
CONCLUSIONS

Our findings demonstrate that H2 has a quantitatively significant impact on microbial carbon assimilation in seafloor ultramafic rock microbial ecosystems (Fig. 8). The qSIP results show that this effect of H2 on increased carbon assimilation is phylogenetically organized, and the distribution of the carbon assimilating taxa shows a higher diversity of peridotite-associated taxa assimilating carbon in the presence of H2 compared to taxa that were not detected on the ultramafic rocks. The data support thermodynamic predictions that oxidation of H2 is energetically favorable for seafloor-associated microbial life in settings where H2-containing aequous solutions mix with seawater [7], including those where H2 is formed via high-temperature or low-temperature aqueous alteration [91, 92], rock comminution in fault zones, and radiolysis.

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270

271
AUTHOR CONTRIBUTIONS

WDO, WE, FK, and FS conceived the idea for the study. OC, WDO, FK, and FS wrote the paper. OC, AV, FK, and FS produced data. OC, WDO, FK, and FS analyzed data. All authors participated in editing the paper and interpreting the results.

FUNDING

This work was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation)—Project-ID 364653263—TRR 235 to WDO and WE, and under Germany’s Excellence Strategy—EXC 2077-390741603. The work was also supported by the Dalio Explore Fund and LMU Mentoring Program. Open Access funding enabled and organized by Projekt DEAL.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41396-021-01066-x.

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ACKNOWLEDGEMENTS

We thank the Captain and crew of the MV Alucia and the Brazilian Authorities, Brazilian Navy, and ICMBio for sampling permits (No. 61074.002427/2017-20 granted by Estado-Maior Da Armada/Marinha Do Brasil on 10 April 2017) and access to the Saint Paul’s and Peter’s Rocks Archipelago.

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