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Carbon fixation and energy metabolisms of a subseafloor olivine biofilm

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Received: 6 August 2018 / Revised: 15 February 2019 / Accepted: 28 February 2019 / Published online: 12 March 2019
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Abstract

Earth's largest aquifer ecosystem resides in igneous oceanic crust, where chemosynthesis and water-rock reactions provide the carbon and energy that support an active deep biosphere. The Calvin Cycle is the predominant carbon fixation pathway in cool, oxic, crust; however, the energy and carbon metabolisms in the deep thermal basaltic aquifer are poorly understood. Anaerobic carbon fixation pathways such as the Wood-Ljungdahl pathway, which uses hydrogen (H_2) and CO_2 , may be common in thermal aquifers since water-rock reactions can produce H_2 in hydrothermal environments and bicarbonate is abundant in seawater. To test this, we reconstructed the metabolisms of eleven bacterial and archaeal metagenome-assembled genomes from an olivine biofilm obtained from a Juan de Fuca Ridge basaltic aquifer. We found that the dominant carbon fixation pathway was the Wood-Ljungdahl pathway, which was present in seven of the eight bacterial genomes. Anaerobic respiration appears to be driven by sulfate reduction, and one bacterial genome contained a complete nitrogen fixation pathway. This study reveals the potential pathways for carbon and energy flux in the deep anoxic thermal aquifer ecosystem, and suggests that ancient H_2 -based chemolithoautotrophy, which once dominated Earth's early biosphere, may thus remain one of the dominant metabolisms in the suboceanic aquifer today.

Introduction

The igneous suboceanic aquifer contains a globally-distributed chemosynthetic ecosystem supported largely by water-rock reactions [1–4]. The Calvin Cycle is the dominant carbon fixation pathway in the upper oceanic crust where cool, oxygenated seawater circulates through exposed crustal rocks [5]. However, the aquifer

extends deeper into a thermal anoxic zone that remains habitable but is more conducive to anaerobic carbon fixation pathways like the Wood-Ljungdahl pathway. The Juan de Fuca Ridge (JdFR) crustal aquifer is one location where this pathway is likely to occur since it underlies thick sediment deposits which limit thermal exchange with the overlying water column [6–8]. This is a deep thermal aquifer (>300 meters below the seafloor; ~64 °C) contained within young (~3.5 mya) basaltic oceanic crust, a prime habitat for a chemosynthetic microbial ecosystem.

The thermodynamic disequilibrium between warm aquifer fluid and reduced, Fe-bearing minerals in the crust (e.g., olivine) can be exploited by microbes for energy under the thermal conditions in which H_2 is produced [9, 10]. Molecular hydrogen is an energy-rich electron donor, and may be a driver for microbial chemosynthesis in deep aquifer ecosystems such as the JdFR where oxygen and organic matter are limited and H_2 is present [11]. The JdFR aquifer community contains distinct planktonic and sessile communities [12–17], and minerals appear to shape the structure and function of the attached

Supplementary information The online version of this article (<https://doi.org/10.1038/s41396-019-0385-0>) contains supplementary material, which is available to authorized users.

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community, favoring lineages that contain the Wood-Ljungdahl pathway [1, 12, 18, 19]. This suggests that chemosynthetic H_2 -based metabolisms are supported by water-rock reactions on Fe-bearing mineral surfaces in thermal anoxic aquifers like the JdFR.

The Wood-Ljungdahl, or reductive acetyl-CoA, pathway is an ancient carbon fixation pathway employed primarily by acetogenic bacteria, archaeal sulfate reducers, or methanogens [20–23], which commonly occupy niches close to the thermodynamic limits of life [24]. In acetogenesis, molecular hydrogen (H_2) and inorganic carbon (CO_2 or HCO_3^-) are metabolized by the two branches of the Wood-Ljungdahl pathway (the methyl and the carbonyl branches) to synthesize acetyl-CoA [20, 25], a key biomolecular precursor that links multiple carbon and energy metabolic pathways in the cell (e.g., lipid, protein, and carbohydrate metabolism). Methanogens use a different suite of enzymes in the methyl branch, eventually producing methane. The carbonyl branch, also called the acetyl-CoA pathway, is common to both methanogenesis and acetogenesis, and can be reversed with electrons from acetate being donated to sulfate as a terminal acceptor [24].

To explore the role of the Wood-Ljungdahl pathway in the carbon and energy metabolisms of microbes that live on olivine in the deep JdFR suboceanic aquifer, we produced eleven metagenome-assembled genomes (MAGs) from an olivine microbial biofilm that colonized the mineral over 4 years *in situ* (Fig. 1) [12]. The carbon fixation pathways and energy metabolisms encoded in the MAGs were identified through metabolic pathway reconstruction. We found that the primary carbon and energy pathways were acetogenesis via the Wood-Ljungdahl pathway and sulfate reduction.

Materials and methods

Overview of experimental design and study site

Details of oceanic crust mineral incubation and retrieval were previously reported ([12, 26]; Supplementary Methods). Briefly, olivine forsterite 100 (Fe_{100}) olivine (Mg_2SiO_4), forsterite 90 (Fe_{90}) olivine [$(Mg_{0.9}Fe_{0.1})_2SiO_4$], and other minerals were placed sequentially within chambers of a flow cell that was connected to an osmotic pump. The flow cell was suspended in 3.5-mya basaltic crust at IODP Hole 1301A ($47^\circ 45.210' N$, $127^\circ 45.833' W$; 2,667 m water depth) from 2004–2008 between 275 and 287 meters below seafloor (mbsf; Fig. 1a, b) [6, 26]. Seawater entrainment occurred in the borehole during the first 3 years of incubation; [27] however, in the fourth year the aquifer fluid chemistry and temperature changed to that which is more representative of the natural thermal aquifer: $\sim 64^\circ C$ aquifer fluid rich in sulfate ($17.6 \text{ mmol kg}^{-1}$), iron ($0.8 \text{ } \mu\text{mol kg}^{-1}$), and ammonium ($111 \text{ } \mu\text{mol kg}^{-1}$), with some nitrate ($0.8 \text{ } \mu\text{mol kg}^{-1}$) and a source of hydrogen that could support hydrogenotrophy ($\sim 2 \text{ } \mu\text{M } H_2$) [11, 27, 28]. Organic carbon concentrations in the aquifer are depleted relative seawater [29]. Fluid flow increased over time (along with temperature) to a final flow rate of 200 mL/year that persisted for the last year of incubation [27]. Upon recovery of the assembly aboard the ship, the olivine was frozen at $-40^\circ C$ where it remained until extraction.

DNA extraction and sequencing

All steps for sequencing and metagenome analysis are briefly summarized here; for complete details, see Supplementary Methods. Genomic DNA was extracted from

Fig. 1 Summary of subsurface olivine colonization study.

a) Simplified schematic of IODP Borehole 1301A on the eastern flank of the Juan de Fuca Ridge (well depth 367 mbsf) Modified from [6, 7, 26]. The microbial flow cell containing olivine was suspended in the basaltic basement and exposed to lateral aquifer fluid flow from the basalt. **b)** Location of Hole 1301A on the eastern flank of the Juan de Fuca Ridge at a depth of 2,667 m

Fig. 2 Clustering of the olivine metagenome contigs using tetranucleotide frequency. Eleven genomic bins were produced from the olivine metagenome; one additional bin was determined to be a contaminant (Supplementary Methods). MAGs 1–8 are Bacteria: orange, red, and yellow-toned circles denoting Firmicutes from the class Clostridia (1, 2, and 5–8), blues (3 and 4) are only classified as in the domain Bacteria, and black (not numbered) is a Proteobacterial

laboratory contaminant (genus *Ralstonia*). White circles could not be determined as belonging to any particular bin and were not analyzed further. MAGs 9–11 (pink and purple tones) are Archaea of the family Archaeoglobaceae (see Supplementary Table 3 for marker gene summary and Supplementary Table 1 for complete bin phylogeny summary). Each circle represents a contig from the metagenome assembly

~500 mg olivine sand using a modified protocol for the FastDNA Spin Kit for Soil (MP Biomedicals Catalog #116560200) and amplified by PCR as described previously [12, 30]. Genomic DNA (50–70 ng) was pooled from two olivine phases and sequenced at the Marine Biological Laboratory (MBL) in Woods Hole, MA (for more details, see ref. 31). We used an Illumina Hi-Seq1000 for 2x100 bp paired-end sequencing with dedicated-read indexing. Consensus Assessment of Sequence and Variance (CASAVA) 1.8.2 (Illumina) was used to demultiplex sequences at MBL.

Metagenome assembly and binning

Raw sequence files were concatenated into Read 1 and Read 2 files and then sent through the String Graph Assembler (SGA)'s pre-processing pipeline [32]. Reads with quality scores less than 10 or those less than 50 base pairs were eliminated. Ambiguous bases were base-called and the final read set was output into a quality-controlled, interleaved sequence pair file.

We used the Iterative deBruijn Graph Assembler–Uneven Depth (IDBA-UD) metagenome assembler v0.20 [33] and VizBin [34] to assemble and bin sequences, respectively (Supplementary Tables 1 & 2). We used PhyloPythiaS v1.0 [35] to assign taxonomy to contigs in VizBin for manual curation, CheckM v1.0.6 [36] to assess bin completeness and contamination, and PhyloSift v1.0.1 [37] with marker database v4 to verify bin taxonomy (Supplementary Methods, Supplementary Figure 1 & Supplementary Tables 2 & 3). Bins were manually curated based on PhyloPythia

taxonomy and contigs that could not be binned with confidence were marked “undetermined” (Fig. 2). These contigs were manually searched to confirm the absence of genes relevant to the carbon fixation and energy metabolisms described here. We finally named each bin according to the location and mineral it was produced from (JdFRolivine) and the bin number. Details of contamination removal (black circles; Fig. 2) are provided in Supplementary Methods.

Comparative genome analysis

We used programs in The U.S. Department of Energy Systems Biology Knowledgebase (KBbase) to determine relatedness of MAGs to genomes from ref. [15] and other publicly available genomes (Supplementary Table 4). We used Fast Whole-Genome Similarity (FastANI) KBbase v0.0.12 to determine the similarity between genomes, Rapid Annotations using Subsystems Technology (RAST) v0.0.12 to annotate proteins, and Species TreeBuilder v2.1.10 to construct phylogenetic trees based on highly conserved concatenated protein sequences (Fig. 3). See Supplementary Methods for more detail.

Protein prediction and metabolic reconstruction

Prodigal v2.6.0 [38] was used to predict proteins and determine open reading frames in the kmer 65 assembly of the olivine metagenome obtained using IDBA-UD. Predicted genes from Prodigal were input into DIAMOND v0.6.13.48 using blastp against the refseq database [39] to

Fig. 3 Maximum likelihood phylogeny of olivine MAGs using 49 highly conserved protein domains. **a)** Archaeoglobaceae. **b)** Bacteria. **c)** Clostridia. Select closely-related fluid-derived MAGs from JdFR are

represented with blue text [52]. Red text are MAGs JdFRolivine-1–11 from Fig. 2

assign function to proteins. Clusters of Orthologous Proteins (COGs) were produced from the protein file using Reverse PSI-Blast (rpsblast; National Center for Biotechnology Information, or NCBI) and were used to search for gene candidates not identified by KEGG (i.e., formate dehydrogenase). We used BlastKOALA v.2.1 (Basic Local Alignment Search Tool KEGG Orthology and Links Annotation) from the Kyoto Encyclopedia of Genes and Genomes (KEGG) for metabolic reconstruction [40].

Identification of specific genes

Putative formate dehydrogenase genes were identified for those genomes missing an annotated *fdhA* gene to complete the Wood-Ljungdahl pathway (Supplementary Table 5), and the Iterative Threading ASSEmblY Refinement v.5.1 (I-TASSER; <https://zhanglab.ccmb.med.umich.edu/I-TASSER/>) was used to determine structural relatedness of identified protein sequences (Supplementary

Fig. 4 Summary of carbon fixation, methane, nitrogen, and sulfur cycling pathways present in olivine MAGs as determined from the KEGG Pathway Module. Colored boxes indicate the presence of genes for each listed pathway. Any pathways not listed were absent from genome bins, including the Calvin cycle and other carbon fixation

pathways, and other nitrogen cycling pathways. Carbon and energy metabolisms and related pathways are separated by color and each metabolism listed on the right. Completeness for each genome is listed at the bottom of the figure

Table 6). Each putative *fdhA* protein sequence was also aligned with the acetogen *Moorella thermoacetica*'s *fdhA* protein sequence using NCBI's BLASTp. Hydrogenases, ferredoxins, and cytochromes were pulled from KEGG annotations to compare the potential for redox and electron transfer mechanisms across genomes (gene descriptions are provided in Supplementary Tables 7–9).

Results

Metagenome assembly and genome binning

Eleven high-quality (>90%, $n = 9$), or medium-quality (>81.19%, $n = 2$) draft MAGs were obtained in this study (Figs 2–4; Supplementary Figure 1) [41]. The metagenome contained 84 million reads with an average length of 108 bases post-quality filtering. Total assembly size was 30,811,948 bases (Supplementary Table 2), with an N50 of 19,191. Minimal contamination or strain heterogeneity was observed in each genomic bin (Supplementary Figure 1), likely a result of clear separation between the twelve discrete clusters of contigs based on tetranucleotide frequency (Fig. 2).

Carbon fixation pathways

We found that the majority of organisms from this deep thermal olivine biofilm community possessed complete or near-complete carbon fixation pathways (Fig. 4) based on the current KEGG Carbon Fixation Pathway Modules. At least two Clostridia (JdFRolivine-2 and 5) from the community

possess the full suite of enzymes in both branches of the Wood–Ljungdahl pathway. Another five bacterial MAGs (JdFRolivine-1, 3, 4, 6, and 8) contain near-complete Wood–Ljungdahl pathways (Figs 3, 4a, b). The phosphate acetyltransferase-acetate kinase pathway, which allows the conversion of acetyl-CoA to acetate, is a critical energy conservation step in the Wood–Ljungdahl pathway. It was only present in two Bacterial genomes, only one of which contains the complete Wood–Ljungdahl pathway (Fig. 4). This suggests that the Wood–Ljungdahl pathway may be used primarily for carbon fixation rather than energy generation in this community. Notably, all Archaeal MAGs (JdFRolivine-9, 10, and 11) also contained the complete pathway for carbon fixation via the acetyl-CoA pathway (Fig. 4), which allows for the conversion of CO₂ into acetyl-CoA. Two of these MAGs have genes coding for acetate reduction coupled to sulfate reduction (Fig. 4) [42]. Some genes that code for enzymes involved in other carbon fixation pathways were present; however, none of the MAGs contained complete pathways, supporting the notion that they do not play a role in carbon fixation in this system (Fig. 4).

Although nearly all of the bacterial MAGs encoded significant portions of the Wood–Ljungdahl pathway, most of these (five of seven MAGs) were missing annotations for one of the two genes commonly found in genomes of terrestrial acetogens (Fig. 5a, b). JdFRolivine-3 and 4 ('Bacteria') are missing the enzyme methylene-tetrahydrofolate reductase (*metVF*), and JdFRolivine-1, 6, and 8 are missing the typical formate dehydrogenase (*fdhAB*). However, we identified putative *fdhA* genes in these genomes whose predicted function includes *fdhA* (Supplementary Tables 5

Fig. 5 Wood–Ljungdahl pathway summary for olivine MAGs. **a** Wood–Ljungdahl pathway with enzymes (*a–j*) linked to **b** summary of WL pathway enzymes in each MAG. **c** Presence of potential

alternatives to formate dehydrogenase in JdFRolivine genomes (enzyme commission numbers are in brackets). Formate C-acetyltransferase is also known as pyruvate formate lyase

& 6). These MAGs also may potentially use alternate routes of formate production, whether by producing it outside of the Wood–Ljungdahl pathway or through use of analogous subunits from other formate dehydrogenases (Fig. 5c). We

also identified acetyl-CoA synthase (*acs*) gene clusters in each of the MAGs containing the Wood–Ljungdahl pathway, suggesting these organisms are true acetogens and use the Wood–Ljungdahl pathway to generate acetyl-CoA by

reducing CO₂, CO, and/or formate (Supplementary Figure 2).

Nitrogen, sulfur, and iron cycling

The JdFRolivine-8 MAG, identified as a Firmicute from the Peptococcaceae family, has both the complete pathway for nitrogen fixation and dissimilatory sulfate reduction to H₂S (Fig. 4). Three other Archaeoglobaceae and Firmicute MAGs also contained the dissimilatory sulfate reduction pathway (Supplementary Table 1; Fig. 4); however, no complete sulfate importers were identified. Although there were no complete pathways or enzymes for iron reduction, JdFRolivine-10 is a close relative of the iron reducer *Geoglobus* (Fig. 3) [43]. This MAG has genes to produce some of the proteins in the iron reduction complex of *Geoglobus acetivorans*, but not *omc* genes which are required for dissimilatory iron reduction. No *cyc2* genes used in iron oxidation were found [44], and no complete pathways for iron-dependent dissimilatory nitrate reduction were present. No other pathways were present for nitrogen, sulfur, or iron cycling, suggesting nitrogen fixation, sulfate reduction, and iron reduction are the key energy respiratory pathways occurring in this community.

Hydrogenases and electron transfer agents

The presence and abundance of different hydrogenases and other Electron Transfer Agents (ETAs) can be an indicator of modes of energy metabolism, such as hydrogenotrophy. All characterized MAGs contained hydrogenase genes (Fig. 6a; Supplementary Table 4), and most contained the *hypABCDEF* and *hyaABD* genes that encode for [NiFe] hydrogenases. JdFRolivine-3 and 4 had identical hydrogenase genes. Two Clostridia (JdFRolivine-5 and 8) were more similar in terms of presence and copies of hydrogenase genes, but JdFRolivine-8 had more gene copies of the *hya* hydrogenase, and JdFRolivine-5 contained a unique gene that was not present in any other genome, *hyaC*, the [NiFe] hydrogenase 1B-type cytochrome subunit. These two MAGs are also the only two that contained an *ech*, or ‘energy conserving’, hydrogenase gene, again showing the similarity between these two MAGs in terms of hydrogenase genes. MAGs that belong to different phylogenetic groups such as JdFRolivine-7 (Clostridia), JdFRolivine-9 (Archaeoglobaceae), and JdFRolivine-2 (Clostridiaceae) had distinct varieties of hydrogenases not found in other MAGs. Archaeal genomes also had different hydrogenase genes than bacterial genomes, except for *hypABCDEF* and *hyaABD*. Archaeal genomes were the only ones that contained the F₄₂₀-reducing hydrogenase *hnnCD* genes.

Ferredoxin genes were variable across the metagenome (Fig. 6b; Supplementary Table 5); however, most MAGs

Fig. 6 Hydrogenases and Electron Transfer Agents (ETAs) present in KEGG-annotated olivine MAGs. The number of copies of each gene in a genome is indicated on the x-axis. MAG ID numbers are listed on the y-axis. **a** Hydrogenases and related genes that play key roles in hydrogenase function. **b** Ferredoxin-related genes found in olivine MAGs. **c** Cytochromes and other ETAs. See Supplementary Tables 7–9 for gene descriptions

had several ferredoxin genes in common, with some ferredoxins unique to either individual genomes or particular lineages. All MAGs contained the *aor* and *korAB* ferredoxins, and most contained *porABDG*, the pyruvate:ferredoxin oxidoreductase, which catalyzes the reversible conversion of pyruvate to acetyl-CoA. Only bacterial MAGs (JdFRolivine-2 through 9) contained the *fpr* ferredoxin, which is generally used to reduce NADP⁺ for anaerobism. JdFRolivine-6 contained the greatest number of copies of ferredoxin genes, mainly due to extra copies of the *por* ferredoxin. JdFRolivine-1, 5, and 11 which all belong to different lineages did not contain the *fer* gene for ferredoxin.

Cytochromes and other ETAs (Fig. 6c; Supplementary Table 6) were highly variable most containing at least *sdhA*.

JdFRolivine-6 contained a unique ETA, *nrfHA*, which reduces nitrite to ammonium during respiration [45]. JdFRolivine-7 contained no cytochromes or other ETAs, and JdFRolivine-3, 4, and 8 only contained one cytochrome gene, *sdhA*.

Heterotrophy in JdFRolivine MAGs

Other than the potential for some MAGs to utilize simple carbohydrates and a few specific types of amino acids, there is little evidence for the import and degradation of organic compounds for carbon and energy in this community. Most MAGs contained just one or two amino acid transporters, only three MAGs (JdFRolivine-3, 4, and 7) contained carbohydrate importers, and three had complete glycolysis pathways (JdFRolivine-2, 7, and 8). No lipid importers were identified in this study.

Discussion

Phylogeny of lineages from the olivine biofilm and the deep JdFR aquifer

We obtained eleven draft genomes that represent undefined Bacteria, Clostridia, and Archaeoglobaceae (Supplementary Table 1), many of which are present only in attached communities of the JdFR and whose function was previously unknown. Five of the eleven MAGs had no similarity to other JdFR fluid MAG genomes (JdFRolivine-1, 2, 5, 6, 7; Fig. 3) [15], and the other six were between 76 and 99% similar to other MAGs from this system (JdFRolivine-3, 4, 8–11; Supplementary Table 4). The olivine biofilm MAGs presented herein were previously identified as biofilm organisms colonizing substrates in the JdFR aquifer. These substrates encompass a variety of Fe-bearing igneous rocks and minerals [1, 12] and the surface of a nearby steel well head exposed to venting aquifer fluid [17, 46]. This further supports the notion that these organisms are associated with the sessile, rather than the planktonic, community of the JdFR, and may particularly be associated with Fe-bearing substrates capable of producing H₂.

The microbial communities of the eastern flank aquifer of the JdFR are dominated by Firmicutes, Archaea, and other uncultured Bacteria with no close relatives [1, 12, 14, 47]. Clostridia have been found primarily in attached communities of colonized surfaces [1, 12, 17], as have members of the candidate phyla Acetothermia (formerly OP1) and Amenicenantes (formerly OP8) [12, 14, 48, 49]. Some Firmicutes and Acetothermia are capable of using the Wood-Ljungdahl pathway [18, 50, 51]. JdFRolivine-8 from this community belongs to the Family Peptococcaceae, a relative of *Candidatus Desulforudis audaxviator* [50] and

Ca. Desulfopertinax cowenii (Fig. 3; Supplementary Table 2) [52]. These organisms are common deep subsurface inhabitants of both the JdFR aquifer and continental aquifers [12, 14, 15, 50, 52], and though uncultivated, possess a suite of energy and carbon metabolisms, including the potential to fix carbon via the Wood-Ljungdahl pathway [50, 52]. The most abundant archaeal lineage in the JdFR aquifer community is Archaeoglobaceae [1, 12, 14, 16, 47]. Members of this lineage can use the acetyl-CoA, or carbonyl branch of the Wood-Ljungdahl pathway [25] and their corresponding enzymes are domain-specific. Methanogenic archaea have been detected in the JdFR aquifer through activity measurements and 16S rRNA gene sequencing [1, 11, 13, 17], but do not appear to be dominant members of this olivine biofilm community.

The co-dominance of Clostridia and Archaeoglobaceae lineages has been described in terrestrial deep subsurface environments such as continental mines and fractures as well as marine aquifers [1, 15, 16, 50, 52–54], and we are just beginning to understand their role in cycling carbon and producing energy in the deep crust. Their presence here in this JdFR community indicates there may be a shared hydrogen-based functional role for these microorganisms in deep thermal aquifers. This study also significantly expands the number of marine genomes containing the Wood-Ljungdahl pathway and suggests that this pathway is more common in these environments than previously known. Since deep thermal aquifers are isolated from the surface where photosynthetic productivity occurs, primary production in these systems is likely dependent on chemosynthesis using the available energy sources from water-rock reactions. The bifunctional Wood-Ljungdahl pathway may be favored over heterotrophy in these types of systems where oxygen and organic matter are low but hydrogen is replete [20].

Wood-Ljungdahl pathway enzymes

The Wood-Ljungdahl pathway featured prominently in the MAGs from this young, thermal basaltic aquifer, and was present in all but one of the bacterial genomes (Fig. 4). Three of the five bacterial MAGs lacking one step to complete this pathway belong to the order Clostridiales (JdFRolivine-1, 6, and 8), and the other two are classified only as Bacteria (JdFRolivine-3 and 4). The Clostridiales MAGs are missing the carbon-reducing first step in the methyl branch of the Wood-Ljungdahl pathway, which results in the production of formate and is carried out by a formate dehydrogenase enzyme [55]. Fluid MAG JdFR-65 is also missing this enzyme. JdFRolivine-3 and 4 are also missing a step in this pathway (as are the fluid MAGs JdFR-47 and 48; JdFR-46 has no Wood-Ljungdahl pathway), which requires methylene-tetrahydrofolate (methylene-

THF) reductase; however, this enzyme was previously determined to be ‘dispensable’ in an analysis of core and pan genomes of acetogens using the Wood–Ljungdahl pathway [56]. It is possible that these organisms contain enzymes that can perform these functions, but they have yet to be identified (Fig. 5c; see Supplementary Materials for details). These organisms are all predicted to be able to use the Wood–Ljungdahl pathway (Fig. 5a) and fix carbon, with putative *fdhA* genes or alternate pathways outlined here. In addition, we found *acs* gene clusters, which are markers for the Wood–Ljungdahl pathway in bacteria [18], in all bacterial MAGs (Supplementary Figure 2). These gene clusters included genes coding for enzymes in the Wood–Ljungdahl pathway and those that relate to energy conservation or generation, including ferredoxins, cytochromes, and hydrogenases.

The acetyl-CoA pathway

The acetyl-CoA pathway was complete in all Archaeoglobaceae MAGs (Fig. 4). The genes in this pathway code for the Archaeal-type carbon-fixing CODH/acetyl-CoA enzymes used in the carbonyl branch of the methanogenic Wood–Ljungdahl pathway [57]. These are analogous to the bacterial CODH/acs system of the acetogenic Wood–Ljungdahl pathway present in seven bacterial MAGs. Thus, ten of the eleven MAGs obtained from this study have complete acetyl-CoA pathways or the analogous carbonyl branch of the Wood–Ljungdahl pathway that would enable them to fix carbon.

Nitrogen, sulfur, and iron metabolism

Only JdFRolivine-8 contained a complete pathway for nitrogen fixation (Fig. 4). This MAG is phylogenetically similar to the *Ca. Desulforudis* and *Ca. Desulfopertinax* lineages (77–80%, respectively; Fig. 3). The genome of *Ca. Desulforudis audaxviator* also contains a nitrogen fixation pathway, but *Ca. Desulfopertinax cowenii*’s genome does not [50, 52]. Although nitrogen does not appear to be a limiting nutrient based on bulk measures of JdFR aquifer fluids ($[\text{NH}_4^+] = 111 \mu\text{mol kg}^{-1}$ $[\text{NO}_3^-] = 0.8 \mu\text{mol kg}^{-1}$) [28], nitrogen may become limited in the biofilm micro-niches investigated here.

Sulfate reduction may play a prominent role in energy metabolism in this community. At least four out of the eleven MAGs recovered from the olivine biofilm had complete pathways for dissimilatory sulfate reduction (Fig. 4). This is consistent with the presence of sulfate in the anoxic aquifer fluid at the time of recovery ($17.6 \text{ mmol kg}^{-1}$) [27, 28], and the prediction that the use of sulfate as a terminal electron acceptor is thermodynamically favorable in the JdFR aquifer [58]. Sulfate-reducing lineages have previously

been reported from multiple subseafloor microbial observatory studies on the eastern flank of the JdFR, including the location of this study, IODP Hole 1301A [1, 12–14, 59]. Species of *Archaeoglobus* are known to reduce sulfate either using organic carbon or H_2 as initial electron sources [19, 46]. Two of the three archaeal genomes identified from the olivine biofilm in this study (an *Archaeoglobus* member and another Archaeoglobaceae with 78% similarity to the JdFR sulfate-reducing isolate *A. sulfaticallidus*) also contain this pathway [46], indicating a metabolic commonality across the microbial community in this environment.

The only iron metabolism predicted to occur in this community is iron reduction by JdFRolivine-10. Iron oxidation could occur in this habitat as olivine contains abundant reduced iron and nitrate is present in the fluids; however, this community of organisms appears to benefit from the oxidation of iron by water, using hydrogen instead to power carbon fixation.

Hydrogenases and other ETAs

Hydrogenases have been suggested to be important components of early metabolisms on Earth [60], and may have been a precursor to respiratory Complex I and the use of chemiosmosis for energy conservation [61]. They play a key role in hydrogenotrophic metabolisms like the Wood–Ljungdahl pathway [62], and can facilitate the uptake and oxidation of H_2 to 2 H^+ using oxidants such as O_2 , CO_2 , NO_3^- , SO_4^{2-} , and fumarate [62]. The Wood–Ljungdahl pathway utilizes hydrogenases to provide reducing equivalents for CO_2 reduction in both branches, and ETAs such as ferredoxins and cytochromes, couple this pathway to energy conservation [42]. Hydrogenases are also more abundant in H_2 -rich environments such as serpentinizing systems [63], and the types of hydrogenases present are likely linked to their role in particular redox reactions that depend on specific substrates, the partial pressure of H_2 , and whether or not the reactions are coupled to the Wood–Ljungdahl pathway [64].

The distribution of hydrogenases and related genes across the olivine-hosted MAGs was variable (Fig. 6). JdFRolivine-7, a likely heterotroph and the only bacterial MAG that did not possess the Wood–Ljungdahl pathway, had few hydrogenase genes, and these were different than in the other MAGs. JdFRolivine-5 and 8 had similar complements of hydrogenases genes, including the *ech* hydrogenase, which is used by some acetogens for energy conservation by acting as a proton pump [64]. This provides a mechanism for additional ATP generation via proton pumping across the plasma membranes of these cells, allowing for an increased energy yield from acetogenesis; another valuable strategy in low energy environments like deep oceanic crust. All MAGs contained ATPases (data not

shown), suggesting that energy conservation via the generation of a proton motive force is possible in these organisms. *Moorella thermoacetica*, an acetogen and close relative of the Clostridial MAGs from this study, also uses the *ech* complex to conserve energy [64], although the *hyd* or *nfn* electron-bifurcating hydrogenases that this organism uses were not present in the olivine MAGs. The *hnnCD* hydrogenase genes found in the Archaeal genomes code for the F_{420} -reducing hydrogenase (Supplementary Table 4), which is used in the methanogenic Wood–Ljungdahl pathway [61, 62]. The two ‘Bacteria’ MAGs had identical sets of hydrogenases, ferredoxins, and cytochrome genes. Both encoded a set of hydrogenase genes, *hoxHYU*, whose product is a ‘hydrogen sensor’ that regulates the transcription of other hydrogenases. Both of these ‘Bacteria’ are evolutionarily distinct from the Clostridia in this study, which may explain why their complement of hydrogenases is unique.

Although JdFRolivine-5 and 8 shared similar hydrogenases, they did not contain similar ferredoxin or cytochrome genes. The JdFRolivine-5 genome was unusual in that it had the greatest number of ferredoxin genes and its cytochromes were most similar to the archaeal gene set. These groups shared both the *ccm* and the *sdh* genes, respiratory membrane proteins responsible for cytochrome C maturation [65] and the succinate dehydrogenase respiratory complex. Cytochrome C and succinate dehydrogenase have a role in generating energy using electron transport coupled to proton pumping. Only six MAGs contained the complete succinate dehydrogenase gene set and are able to make all four subunits, and only three of those genomes are able to produce a fully functional cytochrome C.

Similarity of JdFRolivine MAGs to other MAGs from this system

About half of the olivine biofilm MAGs are dissimilar (<75% similarity) to fluid MAGs (Supplementary Table 4). These divergent MAGs are dominant in the olivine community [12], but are less abundant or absent from the fluid community based on previous community-based studies at these sites [12, 14–16, 47, 59]. Of those JdFRolivine MAGs that are close (>75%) relatives of JdFR aquifer fluid MAGs from boreholes U1362A and U1362B [15] (Fig. 3; Supplementary Table 4), two have very high similarity (99%) with fluid MAGs (JdFRolivine-3 and 9). Of the seven JdFRolivine MAGs identified in this study that contain the Wood–Ljungdahl pathway, six of them group with other JdFR fluid MAGs and five contain the Wood–Ljungdahl pathway (JdFRolivine-1, 2, 5, and 6). All of these group with known or proposed acetogens such as *M. thermoacetica* and *Ca. D. audaxviator* (Fig. 3), which suggests

there is a potentially diverse group of acetogens in the deep biosphere at the Juan de Fuca Ridge.

Olivine community conceptual model

This olivine community has the genomic potential to grow autotrophically and heterotrophically using energy and carbon substrates originating either from aquifer fluid, water-rock reactions, or fixed carbon of the biofilm. Phosphate, organic and inorganic carbon, dinitrogen, and sulfate are all present in the JdFR aquifer fluid [28] and are potentially utilized by the community (Fig. 7) as evidenced by the presence of complete metabolic pathways for these substrates. Formate and acetate may be produced in the biofilm (Figs. 3 and 4); however, neither of these have been detected in aquifer fluids (<0.1 μM ; Tina Lin, personal communication) [28]. Key organisms in the community may be using the acetate to power sulfate or iron reduction or for fermentation [19, 43]. Molecular hydrogen is also

Fig. 7 Olivine community model depicting nutrient cycling and proposed routes of metabolites and substrates. Each oval represents a bacterial (1–8) or archaeal (9–11) cell from the olivine biofilm community. The large green structure is olivine and it contains white cloud shapes that indicate biofilms produced by the community. Colors of MAG ovals correspond to those in Fig. 2 and broad taxonomic groups are indicated below. Water reacting with reduced iron in olivine produces molecular hydrogen and iron oxides, which can be deposited on the surface of olivine as secondary minerals (orange structures on olivine). The molecular hydrogen from this reaction is presumed to help fuel the microbial biofilm through the Wood–Ljungdahl pathway. Nutrients originating from aquifer fluid (blue background) are bicarbonate or CO_2 , sulfate, nitrogen, and organic carbon. Nutrients produced or consumed by individual cells are drawn with arrows directly to or from the cell. Nutrients going into or out of multiple cells are indicated by arrows to or from the biofilm. Dashed lines are putative routes of production and consumption of nutrients. Solid lines indicate the genomic potential for metabolism of particular substrates. Blue text indicates inorganic nutrients, purple text are iron species, brown text is organic matter, and molecular hydrogen is red

present in aquifer fluid, and although its concentration is high enough to support hydrogenotrophy ($\sim 2 \mu\text{M}$) [11], additional H_2 produced on mineral surfaces could be a major source of energy fueling this microbial community (Fig. 7). The H_2 , formate, and acetate may be rapidly consumed in a biofilm community and concentrations would, therefore, be low or undetectable in the fluids.

All organisms except for the heterotroph JdFRolivine-7 have hydrogenotrophic carbon fixation pathways. The JdFRolivine-7 genome contains transporters and pathways for degrading a wide variety of organic substrates, but no genomic evidence for carbon fixation. It is, however, genetically capable of rapidly producing large amounts of formate (Fig. 5c), which may be utilized by other microbes whose genomes lack formate dehydrogenase, allowing them another energetic advantage in this energy-limited environment [66]. JdFRolivine-5 is likely the second most abundant organism in this community based on a previous study [12] and appears to rely solely on H_2 from water-rock reactions as an energy source. This organism is able to make acetate, which could supplement heterotrophy or be used for iron or sulfate reduction (Fig. 7). The JdFRolivine-3 and 4 MAGs can import simple sugars that may be produced in the biofilm, and also use the Wood–Ljungdahl pathway.

Conclusion

This study provides genomic evidence of a marine subsurface chemosynthetic community that is dependent on H_2 , presumably released as a result of water reacting with Fe-bearing minerals, and sulfate from the fluids. The Wood–Ljungdahl pathway is the predominant metabolic strategy in this community, which is at odds with the paradigm that the Calvin cycle is the dominant carbon fixation pathway in oceanic crust. This study broadens our understanding of deeper anoxic aquifer communities, which comprise the bulk of oceanic crust, and their interactions with mineral surfaces. Olivine is ubiquitous in oceanic crust and thus is likely to impact microbial community function in both basalts and ultramafic rocks, perhaps even more so in ultramafic crust where olivine comprises up to 50% of the crust. The Wood–Ljungdahl pathway is an ancient H_2 -based biosynthetic pathway used by early life on Earth, and having a better understanding of the organisms that utilize this pathway and their strategies for energy conservation may help elucidate its role in the origin and evolution of life.

Data availability

Raw sequence files and draft genomes are publicly available under the Bioproject Number PRJNA264811 (accession #s

SDWF00000000, SESU00000000-SESZ00000000, SETA00000000-SETD00000000) on the NCBI website at <https://www.ncbi.nlm.nih.gov> and at https://figshare.com/articles/olivine_contig-65_fa/7252121.

Acknowledgements Metagenome sequencing was made possible by the Deep Carbon Observatory Census of Deep Life supported by the Alfred P. Sloan Foundation and was performed at the Marine Biological Laboratory (Woods Hole, MA, USA). We are grateful for the assistance of Mitch Sogin, Susan Huse, Joseph Vineis, Andrew Voorhis, Sharon Grim, and Hilary Morrison at MBL. Andrew Fisher, C. Geoffrey Wheat, Hans Jannasch, Stefan Sievert, Keir Becker, Mark Nielsen, and the crews of submersible DSRV *Alvin* and the R/V *Atlantis* and D/V *JOIDES Resolution* assisted with flow cell development, deployment, and retrieval. William Rugh contributed to the design of the flow cells. This is a Center for Dark Energy Biosphere Investigations (C-DEBI) contribution.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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