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One sentence summary:

Functional gene, geochemical, and incubation evidence reveal active, methane- and sulfur-cycling microbial communities in deep basaltic ocean crust.
Sediment-covered basalt on ridge flanks constitutes most of Earth’s oceanic crust, but the composition and metabolic function of its microbial ecosystem is largely unknown. Here, we demonstrate the presence of methane- and sulfur (S)-cycling microbes in subseafloor basalt of the eastern flank of the Juan de Fuca Ridge. Depth horizons with functional genes indicative of methane-cycling and sulfate-reducing microorganisms are enriched in solid-phase S and total organic carbon (TOC), host δ^{13}C- and δ^{34}S-isotopic values with a biological imprint, and show clear signs of microbial activity when incubated in the laboratory. Downcore changes in C and S cycling show discrete geochemical intervals with chemoautotrophic δ^{13}C-TOC signatures potentially attenuated by heterotrophic metabolism.

Subseafloor basaltic crust represents the largest habitable zone by volume on Earth (1). Chemical reactions of basalt with seawater flowing through fractures (veins) release energy that may support chemosynthetic communities. Microbes exploiting these reactions are known from basalt exposed at the seafloor, where the oxidation of reduced S and iron (Fe) from basalt with dissolved oxygen and nitrate from seawater supports high microbial biomass and diversity (2, 3). Multiple lines of evidence that include textural alterations (4), depletions in δ^{34}S-pyrite (FeS₂) (5) and δ^{13}C-dissolved inorganic carbon (DIC) (6), and DNA sequences from borehole observatories (7, 8) suggest active microbial communities in subseafloor basalt. Yet, direct evidence of these communities is still missing. In this study we combine sequencing of genes diagnostic of microbial methane- and S-cycling with geochemical and isotopic analyses of C- and S-pools and laboratory-based incubations to identify microbial ecosystem components in deep subseafloor basalt.

The 3.5 million-year-old basement at Hole U1301B was sampled during Integrated Ocean Drilling Program (IODP) Expedition 301 in 2004 (Fig. S1) (9). Site U1301 is covered by a 265-m thick sediment layer and lies ~2 km south of ODP Site
1026, which it resembles in temperature profile, lithology, and sediment chemistry (9). Given anticipated poor recovery due to brecciation of the upper basement (265-350 meters below seafloor; mbsf), coring was restricted to an interval of pillow basalts and massive lavas (351-583 mbsf). Sulfate concentrations (~16mM) and vein carbonates indicate basalt fluids are derived from seawater, which enters ~80 km south at Grizzly Bare outcrop and discharges near U1301B, at Baby Bare and Mama Bare outcrops (9, 10; Fig. S1B). Yet, the basement at U1301 differs from seafloor-exposed basalt in its uniformly high temperature (~64°C) (9) and lack of fresh photosynthesis-derived organic matter, dissolved oxygen and nitrate (7, 11). These conditions preclude chemosynthetic S- and Fe-oxidation reactions that require oxygen or nitrate as electron acceptors (12), but may enable growth of strict anaerobes, such as many methanogens, methane-oxidizing archaea and sulfate reducers.

By sequencing genes encoding the α subunit of methyl coenzyme M reductase (mcrA), a gene unique to methanogens and anaerobic methane-oxidizers (13), and the β subunit of dissimilatory sulfite reductase (dsrB), a gene found in sulfate- and sulfite-reducing microbes (14), we demonstrate the presence of key genes of methane-cycling and sulfate-reducing microbes (Methods in Supporting Online Material). We detect mcrA in 5 of the 10 samples and dsrB in 4 of the 6 samples tested (Table S1).

The phylogenetic diversity of mcrA genes is restricted to two groups: the Juan de Fuca Methanogen Group (JdFMG), which falls into an uncultivated cluster within the Methanosarcinales, and anaerobic methane-oxidizing archaea (ANME-1; Fig. 1A). Close relatives of the JdFMG were first reported as ‘Unidentified Rice Field Soil mcrA’ from paddy soil (15), and have since also been found in marine habitats, including JdF Ridge hydrothermal vent chimneys and seafloor-exposed basalt ~100 km west of U1301B (Fig. S2) (16-17). Recently, a close relative of JdFMG, which uses hydrogen (H₂), acetate, methanol and trimethylamine as energy substrates for
methanogenesis, was enriched in wetland soil (18). Given that substrate use follows phylogenetic divisions in mcrA (19), the JdFMG are likely to be methanogens with similar substrate requirements. ANME-1 occur widely in marine sediments and methane seeps, and are believed to gain energy from the anaerobic oxidation of methane (AOM) (20). Two distinct ANME phylotypes occur at U1301, one closely related to ANME-1 from methane seeps, and another clustering with only one other sequence, from subseafloor sediment (Fig. S3). We detect JdFMG in four, and ANME-1 in three out of ten basalt samples. Two samples contain both groups (Table S1).

The phylogenetic diversity of dsrB is limited to one group, the Juan de Fuca Sulfate Reducing Group (JdFSRG), which falls into Cluster IV, a deeply-branching dsrB cluster without cultured members, first reported from hydrothermal sediment (Fig. 1B and S4, Table S1) (21). Remarkably, the only other dsr sequences reported so far from the subseafloor, i.e. sediment of the Peru Margin (22), fall into this cluster, which is widespread in shallow marine sediment and terrestrial aquifers.

The study of solid-phase S-pools through analyses of acid-volatile sulfide (AVS), chromium-reducible S (CRS), and sulfate-S (SO₄-S) provides insights to redox processes (5, 23), and shows a relationship with dsrB distributions at U1301: dsrB was only found in a relatively reduced ‘intermediate depth interval’ (~430-520 mbsf, 14R-26R), in samples with AVS as the main S pool in alteration halos (14R-1-11) or host rock (17R-170, 20R-1-57, 23R-2-21; Fig. S5, Table S1). Samples from this interval have higher AVS, CRS, and total S (Fig. S5, Table S2), contain large pyrite fronts (14R-1-65P, 15R-4-142P; Fig. S5), and have lower δ³⁴S-AVS, -CRS, and -SO₄-S, compared to the more oxidized upper (1R-12R) and lower coring intervals (30R-36R; Fig. S6, Table S1). Consistent with higher Fe³⁺/Fe⁰Total ratios, which indicate halos to be more oxidized than host rock (Table S1), pyrite is generally
absent from halos or veins. Outside the intermediate depth interval, the near absence of pyrite from host rock, and mixed clay-Fe-oxyhydroxide-dominated halos and veins are further evidence of pervasive oxidative alteration.

To examine micro- and macroscale variations in microbial S-cycling, the $\delta^{34}$S of pyrite grains were analyzed (Tables S1 and S3, Fig. 2). Though variable, the $\delta^{34}$S-pyrite grains (-72.4 to 1.2‰; Table S3) are typically lower than those of AVS (-9.3 to -0.2‰), CRS (-13.7 to 0‰), SO$_4$-S (-6.5 to 0‰), mantle S (0‰) (5), dissolved sulfate in bottom sediments at ODP Site 1026 (+30‰) (24), or seawater (+21‰; Fig. 2).

Locally, the $\delta^{34}$S of pyrite grains reach very negative values (-72‰), consistent with the addition of highly $^{34}$S-depleted secondary sulfide to basement rock (23). These low $\delta^{34}$S-pyrite values indicate single-step sulfate reduction (25) or repeated cycles of sulfate reduction and S oxidation (26). The co-occurrence of low $\delta^{34}$S-pyrite, dsrB, and mcrA of ANME-1 in two samples (14R-1-11, 17R-1-70) suggests local coupling between methane and S-cycling by sulfate-dependent AOM.

Depth profiles of TOC content, $\delta^{13}$C-TOC, and $\delta^{13}$C-carbonate at U1301B are consistent with functional gene- and $^{34}$S-data (Fig. 3). The TOC content is highest in the intermediate depth interval in cores with mcrA, dsrB, and low $\delta^{34}$S-pyrite (Fig. 3A, Table S4). The $\delta^{13}$C-TOC is in the range of dissolved organic C (DOC) in fluids from nearby 1026B and Baby Bare Springs (BBS; Fig. 3B, Table S4) and lower than seawater DOC (-21.1‰; 6). The $\delta^{13}$C-carbonate is higher than $\delta^{13}$C-DIC at 1026B or BBS (Fig. 3C, Table S5) and overlaps with $\delta^{13}$C-DIC of bottom seawater (-1.4‰; 10).

$\delta^{13}$C-TOC values in the upper coring interval (4R-5R) and near the bottom (23R-26R; -34.6 to -32.0‰) are close to $\delta^{13}$C-DOC from nearby BBS (-34.6‰; Fig. 3B). The absence of O$_2$ and the high $^{13}$C-TOC depletion relative to carbonate (~ -30 to -35‰) suggest C fixation by the reductive acetyl CoA pathway – an anaerobic
pathway found in all methanogens and acetogens, and certain sulfate and iron
reducers (Fig. S7, Tables S6 and S7; 27). The presence of dsrB but not mcrA in these
samples suggests that sulfate reducers or other groups, but not methanogens, produce
this low $\delta^{13}$C-TOC.

$\delta^{13}$C-TOC at the top (2R) and in the intermediate depth interval (-28.4 to -
21.6‰) are close to $\delta^{13}$C-DOC from borehole 1026B (-26.1‰, Fig. 3B; 6). The $^{13}$C-
depletion relative to inorganic C is lower than in the other layers (~ -20 to -26‰), but
also falls in the range of the reductive acetyl CoA pathway (Table S7), and, consistent
with mcrA detection, could be impacted by autotrophic methanogenesis. In addition,
elevated heterotrophic activity is possible, since degradation of chemoautotrophy-
derived OC, e.g. by AOM, methanogenesis or fermentation, would lower the $\delta^{13}$C-
carbon and potentially raise the $\delta^{13}$C-TOC. The alternative explanation, enhanced
breakdown of photosynthesis-derived OC in the intermediate depth interval, is
unlikely given that sediment inclusions are absent (9). Similarly, influx of labile DOC
or unaltered DIC from seawater is incompatible with the 7-11 kyr greater DOC age
compared to bottom seawater and the 4-8‰ decrease in $\delta^{13}$C-DIC along the flowpath
from Grizzly Bare outcrop to 1026B and BBS, respectively (6, 10).

To rule out a fossil origin of functional genes, C and S compounds in subseafloor
basalt, we incubated pieces from the interior of three rocks used for functional gene
analyses (1R-1-79, 14R-1-11, 23R-2-21) at 65°C in anoxic, sulfate-rich media
containing H$_2$, acetate, methanol, and dimethyl sulfide as energy substrates (Table
S8). After two years, aliquots were transferred to fresh media, and incubated for
another five years using triple-autoclaved basalt pieces as substrata. By then, low
concentrations of $^{13}$C-depleted methane (-54 to -65‰) had formed indicating the
presence of active methanogenic microorganisms (Table S9).
The variability in $\delta^{34}\text{S}$-pyrite, $\delta^{13}\text{C}$-TOC and $\delta^{13}\text{C}$-carbonate indicates that micro- and macro-scale geochemical changes related to mineralogy, fracturing and/or fluid flow strongly influence microbial activity. These chemical microniches may explain the coexistence of sulfate reducers and methanogens at U1301 and in other igneous habitats, despite higher energy yields of sulfate reduction compared to methanogenesis (28). In addition, some methanogens can survive in the presence of sulfate reducers by consuming non-competitive methyl substrates (19); this explanation is consistent with the ability of a close relative of JdFMG to use methanol (18), and the production of biogenic methane in basalt incubations containing sulfate and methanol (Table S9, Fig. S8). The sources of energy substrates to sulfate reducers and methanogens at U1301B remain unknown. A potential source of $\text{H}_2$ is the oxidative alteration of Fe or S minerals, while short-chain organic acids and alcohols might be produced by breakdown of TOC (19, 28-29) or Fischer-Tropsch-type synthesis (30).

Samples from the eastern flank of the Juan de Fuca Ridge provide the first integrated view of microbial community structure and activity in subseafloor basalt. Our analyses of functional gene, geochemical, and isotopic data reveal strong micro- and macroscale heterogeneity of microbial C- and S-cycling, and indicate a key role of chemoautotrophy in supporting Earth’s oceanic crustal biosphere.
References


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documented and available in the Supplementary Tables. The functional gene sequence
data are available from Genbank database.
Figure 1. Phylogenetic trees of functional genes. (A) McrA sequences from U1301B are in bold magenta type face. Close relatives based on microarray analyses of JdF Ridge hydrothermal vent chimneys and seafloor basalt are in green (16) and cyan (17), respectively. (B) DsrB sequences from U1301B are in bold magenta type face, and sequences from subseafloor sediment off Peru in cyan (22). Bootstrap support (in %, 1,000 replications) is indicated at each branching point.
Figure 2. Macro-and micro-scale distribution of S-isotopic data. On the left, $\delta^{34}$S-depth profile of pyrite granules, analyzed by laser ablation and secondary ion mass spectrometry (SIMS), and bulk S pools (AVS, CRS). On the right, thin section micrograph showing individual pyrite granules and their $\delta^{34}$S. The dashed magenta line indicates the sampling depth of the thin section. The dashed black lines mark the intermediate depth interval. Pyrite grains with a sufficient diameter for $\delta^{34}$S-determination (10 µm) were limited to this interval. The scale bar is 200 µm.
Figure 3. Depth-related trends in (A) TOC content, (B) δ¹³C-TOC, and (C) δ¹³C-carbonate. Cores with functional gene detection are indicated in A and B. Dashed vertical lines indicate δ¹³C-DOC (B) and –DIC (C) values from 1026B and BBS. Because the carbonate content of rock samples used in (A) and (B) was too low for analyses, δ¹³C from carbonate veins are shown in (C). The reduced intermediate depth interval falls between the dashed horizontal lines. All δ¹³C are in ‰ vs. VPDB.