**Supplementary Methods**

Supplement to:

*Microbial residents of the Atlantis Massif's shallow serpentinite subsurface*

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*Sampling Location*

Below is a brief summary of the sampling process; further details are available elsewhere (1, 2). During IODP Expedition 357 to the Atlantis Massif (Mid-Atlantic Ridge 30°N), rock cores were collected from seventeen drilled holes at nine sites(**Figure 1**): two sites on the eastern end of the southern wall (Sites M0068 and M0075), three sites in the central section of the southern wall north of the Lost City hydrothermal field (Sites M0069, M0072, and M0076), and two sites on the western end (Sites M0071 and M0073. Onshore investigations of the split cores indicated that the central sites preserved in-situ basement sequences, whereas the cores from the eastern and western sites consisted of talus blocks eroded from the top of the massif during uplift but showing a similar alteration history as the in-situ sequences (2). Rock and water samples were collected for a wide range of geological, physical, chemical, and biological measurements by the scientific party, as described in (3). In this report, we describe new environmental DNA sequencing results from the rock cores and water collected during the expedition.

*Description of Rock Samples*

Two seabed drill systems were utilized during this expedition: the British Geological Survey (BGS; Edinburgh, United Kingdom) RockDrill2 (RD2) and the Center for Marine Environmental Sciences (MARUM; Bremen, Germany) Meeresboden-Bohrgerät 70 (MeBo70). Both drills are remotely operated systems that are lowered onto the seabed, with power and control maintained from the surface via an umbilical cable. Additional details on the drill systems were provided in previous reports (2, 3). More than 57 m of cores were recovered by the drill systems, with borehole penetration ranging from 1.30 to 16.44 meters below seafloor and core recoveries as high as 74.76% of total penetration (2). For microbiological investigations, a 2–20 cm section of whole round core from one end was selected for pooled analyses to be completed on shore. The piece was typically divided into two portions, one of which was flame-sterilized with a handheld butane torch inside a flame-sterilized steel box to remove surface contamination. The serpentinite rock samples included in this study that were flame-sterilized are 0AMRd010, 024, 031, 033, 048, 049, 058, and 072 (**Data set S1**). All rock core samples for microbiological analyses were stored at -80oC on the ship and then shipped on dry ice directly to the JAMSTEC Kochi Core Center in Japan for processing.

The rock core samples varied greatly in mineralogy, texture, and structure (3). Rock types included serpentinized harzburgites and dunites, intruded by gabbros and dolerite dikes, as well as minor basaltic rocks. In addition, talc-rich rocks (talc-amphibole schists) were typically recovered at contacts between the serpentinites and mafic rocks and are considered to be the product of Si mobility during alteration (3, 4). Some samples were very hard, with very few veins or fissures, and remained as intact cores until subsampling, while others were already rubbly and soft immediately after recovery from the seafloor. The exteriors of intact pieces were shaved off with a steam-sterilized band saw on a frozen stage in a microbiologically clean room at the Kochi Core Center, leaving only the pristine interior sections for downstream microbiological analyses. Shaving the exteriors of fractured and rubbly samples was not practical, therefore these samples were washed three times with ultrapure, sterilized water before additional processing.

Of the 89 rock core subsamples dedicated for microbiology studies, 35 were processed for this study. These 35 rock samples included 18 characterized as serpentinites with varying talc overprinting, and the DNA sequencing results for 15 of these serpentinite samples exceeded our data quality thresholds (see below). Each rock sample was assigned a standardized IODP sample ID upon recovery that includes the expedition number, site, hole, core number, core type, section number, piece number (for hard rock), and interval in centimeters measured from the top of the section. For example, “357-69A-3R-2, 35–40 cm” is a sample removed from the interval 35–40 cm below the top of Section 2, Core 3R, from Hole M0069A during Expedition 357. During DNA extraction and sequencing in the lab in Utah, rock core subsamples were assigned new IDs: e.g. “0AMRd005” where AMR stands for Atlantis Massif Rock. Each rock sample is labeled with both IDs in **Data set S1**.

*DNA Extraction from Rocks*

At the Kochi Core Center, after sawing or washing the exteriors of the frozen rock samples, each rock core sample was homogenized into fine grains with a sterile mortar and pestle, as described elsewhere (1). The resulting rock powders were subsectioned into sterile plastic tubes under a HEPA-filtered processing workstation and then refrozen, then shipped on dry ice to the shorebased laboratory in Utah where they were processed in a dedicated room supplied with filtered air and low levels of dust particles. Moreover, two molecular workstations with UV lamps (UVP Model UV3 HEPA PCR Cabinet, Analytik, Jena, Germany; and AirClean Model 600 PCR Workstation, AirClean Systems, Creedmore, North Carolina, USA) were used for aliquoting the rocks into microcentrifuge tubes and for sensitive stages during the DNA extraction, washing, and purification.

DNA was extracted with a custom protocol that involving neither commercial extraction kits nor phenol. All reagents were prepared in our laboratory with molecular-grade powders and ultrapure water. Development of our protocol benefitted from previous reports on the optimization of DNA extraction and purification methods (5–10). First, 0.5 g of rock powder was placed in 2 mL sterile tubes and mixed with 1000 μL of DNA Extraction Buffer (0.03M Tris-HCl, 0.01M EDTA, 0.02M EGTA, 0.1M KH2PO4, 0.8M guanidine HCl, 0.5% Triton-X 100, pH 10), 150 μL of 20% sodium pyrophosphate solution, and 150 μL of 50 mM dATP (Jena Bioscience, Jena, Germany). The DNA extraction buffer was filtered sterilized, boiled in a microwave for two minutes, and exposed to UV light for 30 minutes before adding to the rock powder. The tubes were shaken and vortexed briefly before an overnight incubation at 4 oC, which is thought to allow time for the chelation of salts as well as for the prevention of DNA adsorption to phyllosilicates and other minerals by saturating binding sites with dATP and pyrophosphate. The DNA extraction buffer was verified to contain below-detection levels of DNA with the Qubit 2.0 fluorometer with dsDNA (High-Sensitivity) Assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

The following day, tubes were frozen at -80oC and then thawed at 54oC, 600 rpm for an hour in a shaking Thermomixer. This freeze-thaw step was followed by increasing the heat to 75oC and the speed to 1500 rpm for 30 minutes. Tubes were beaten in a MiniBeadBeater-16 Model 607 (Biospec, Bartlesville, Oklahoma, USA) (40 s at 3450 oscillations/min). No beads were added for the beating step in order to avoid unnecessary contamination and because our initial tests indicated that the DNA yield did not improve by adding commercial beads to a preparation of crushed rocks. No enzymes were added to extractions in order to avoid additional contamination. Tubes were centrifuged (3 min, 6100 rcf), and the supernatant was transferred to clean tubes. For each rock sample, 3-5 replicate extractions were performed. At this point, DNA in the lysates was quantified with the Qubit 2.0 fluorometer with HS dsDNA assay kit, and the total DNA yield per gram of rock was calculated by summing the quantifications of all replicate extractions for each sample and dividing by the total grams of rock sample included in the extractions (**Table 1**). The DNA detection limit per gram of rock sample was determined for each sample by calculating the total maximum DNA yield that could have been achieved by the extraction while remaining below the detection limit of the Qubit 2.0 fluorometer (0.05 ng per μL).

*Washing and Purification of DNA from Rocks*

In order to minimize the loss of DNA, we did not perform phenol extractions or ethanol precipitations. Instead, crude lysates were washed with Vivacon2 filtration units (100,000 MWCO) (VIVACON 2-PCR Grade (ETO), Sartorius, Göttingen, Germany) by centrifugation for 35 min at 2,500 rcf and then washed with 65oC sterile TE (Tris-10mM, EDTA-1mM) and then 65oC ultra-pure water. The VivaSpin membranes are expected to retain DNA molecules larger than 600 bp, which we verified with gel electrophoresis of test samples. DNA in the washed preparations was again quantified with the Qubit 2.0 fluorometer with HS dsDNA.

SCODA (synchronous coefficient of drag attenuation) technology implemented with the Aurora purification system (Boreal Genomics, Vancouver, British Columbia, Canada) was used for purification and concentration of DNA from these extremely low-biomass samples. The procedure was conducted with a modification of the manufacturer's standard protocol. The Aurora cartridge and dams were soaked in 10% household bleach (5% sodium hypochlorite) for thirty minutes and thoroughly washed with Milli-Q water (MilliporeSigma, Burlington, Massachusetts, USA). Both cartridge and dams were placed under a UV lamp for an hour. The dams were inserted into their proper locations on the cartridge, and the molten agarose was poured into the designed gap between the dams. The cartridge with molten agarose was exposed to UV light for 15-20 min. The cartridge buffer chambers, and extraction well were filled with 0.25x TBE Buffer. The extraction well was sealed with PCR tape. A pre-run protocol was conducted prior to every run to remove any possible contaminating DNA in the Aurora cartridge.

The sample, after achieving the required level of conductivity (⪯100 μS/cm) after washing with the Vivacon2filter, was loaded into the sample chamber in the cartridge and was placed onto the cold plate in the Aurora instrument. The “106-0001-CA-D\_AURORA\_DNA\_CLEAN- UP\_PROTOCOL” was selected in the Aurora software, which is expected to recover DNA molecules 0.3-50 kb in length. The run completes in 4 hours, and the purified DNA was extracted from the concentration well. The output volume was 40-70 μl for each run, and each replicate extraction was processed on a separate Aurora run. Then, the purified outputs of all replicate extractions were pooled for a final run to concentrate and further purify a final DNA prep for that rock sample. The final result of this protocol is that tiny quantities of DNA from multiple aliquots of large volumes of crushed rock were concentrated and purified in a single, final preparation. The DNA in the final, purified preparation was again quantified with the Qubit fluorometric method.

*Extraction of DNA from Seawater*

Prior to the deployment of the drill and beginning of drilling operations at each site, a cast of the ship’s conductivity, temperature, and depth (CTD) Niskin bottle rosette was undertaken to capture a water column profile, focusing on the bottom water (2). The ship’s wireline CTD rosette with six 10 L Niskin bottles was used to collect water from as close to the seafloor as possible (generally 2–3 m above seafloor). Three of the 10 L bottles were triggered near the seafloor at each site. An additional three bottles were triggered at several shallower depths in the water column at each site. Additionally, a 4 L bucket was used to collect surface water near the ship. A total of 76 seawater samples were collected for DNA analysis. These samples include 20 that were collected from "shallow water” (depth of 200-600 meters below the sea level (mbsl)), 37 from "deep water" (depth of 700-1600 mbsf), 19 collected from "surface water" (with a bucket from the ship or with the CTD Niskin bottles from a few meters below the surface). The details related to sample names, site and hole numbers, as well as cast numbers can be found in **Data set S2**. The shallow and deep water samples were collected from CTD Niskin bottles into pre-cleaned 4 L cubic containers and stored at 4°C until further processing. All water samples (surface, shallow, and deep) were filtered through 0.22 μm mesh Sterivex filter cartridges (Millipore, Billerica, Massachusetts, USA) using a peristaltic pump (Model 07518-60, Cole-Parmer, Vernon Hills, Illinois, USA); 1–4 L of fluid was filtered in duplicate per sample. Sterivex filter cartridges were frozen at –80°C and shipped on dry ice to shore-based laboratory in Utah, where they were extracted following a previously described method for DNA extraction and purification (11).

*Extraction of DNA from Air*

Air from the general lab area and the clean room in the shore-based laboratory in Utah was filtered through 0.1 μm Puradisc 25 mm PTFE syringe filters (GEHealthcareWhatman, Pittsburgh, Pennsylvania, USA) by a dual head Air Cadet Model 420-2901-00FK (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The air was vacuumed through the filter for 9 h each time. The air was filtered in triplicate, and replicates were combined during the DNA extraction. DNA extraction and purification were performed on the three air samples following the same protocols and using the same reagents as for the rock samples. Therefore, the air samples also serve as extraction blanks for comparison with the rock samples.

*DNA Sequencing and Sequence Analyses*

Bacterial 16S rRNA gene amplicon sequencing was conducted by the Michigan State University genomics core facility on all of the samples (rock, water, and lab air). All samples were sequenced twice (i.e. sequencing replicates). The V4 region of the bacterial 16S rRNA gene was amplified with dual-indexed Illumina fusion primers (515F/806R) as described elsewhere (12). Amplicon concentrations were normalized and pooled using an Invitrogen SequalPrep DNA Normalization Plate. After library quality control (QC) and quantitation, the pool was loaded on an Illumina MiSeq v2 flow cell and sequenced using a standard 500 cycle reagent kit. Base calling was performed by Illumina Real Time Analysis (RTA) software v1.18.54. Output of RTA was demultiplexed and converted to fastq files using Illumina Bcl2fastq v1.8.4.

16S rRNA gene amplicon sequences were processed with cutadapt v. 1.15 (13) and DADA2 v. 1.10.1 (14) according to a protocol available at https://github.com/Brazelton-Lab/Atlantis-Massif-2015. This protocol includes quality trimming and filtering of reads, removal of chimeras, and inference of amplicon sequence variants (ASVs). Rock and air samples containing fewer than 500 total counts after quality filtering, and water samples containing fewer than 2000 total counts after quality filtering, were excluded from further analyses. Taxonomic classification of all ASVs was performed with DADA2 using the SILVA reference alignment (SSURefv132) and taxonomy outline (15). Rock samples included 287 ASVs (14% of the total ASVs in rocks) that were classified as Archaea. Because the focus of this study was to identify a list of likely rock-inhabiting microbes and not statistical comparisons of bacterial communities, we chose to include the archaeal ASVs in subsequent analyses. Raw counts were converted to proportions to normalize for variations in sequencing depth among samples. The proportional abundances of all 17,081 unique ASVs among all rock, water, and air samples were used to calculate the Morisita-Horn community dissimilarity between each pair of samples. Similar results were obtained with other metrics of dissimilarity (e.g. Bray-Curtis). The non-metric multi-dimensional scaling (NMDS) plot was generated using the distance, ordinate, and plot\_ordination commands in the R package phyloseq v.1.26.1 (16). The major sources of contamination and the level of contamination in each sample were estimated with SourceTracker2, version 2.0.1 (17). Serpentinite samples with >15% of sequences attributed to lab air were excluded from further analyses (0AMRd014A, 031C, 033A, 033C, 034A, 036A, 045B, 067C, 071A, and 073A). In addition, only one of each pair of sequencing replicates was included in further analyses in order to avoid pseudo-replication in later statistical tests and because this only affected three samples (0AMRd030, 057, and 072) at this stage. In each case, the replicate with lesser air contamination was included.

Differential abundance was tested with the R package edgeR v. 3.24.3 (18) as recommended elsewhere (19). We used edgeR to contrast the total read counts of ASVs in serpentinite rock samples compared to three groups of water samples (surface, shallow, deep). ASVs that were absent in all serpentinites and ASVs with low variance (<1e-6) were excluded from the comparisons. The output of the three edgeR tests (serpentinite samples compared to each of the three groups of water samples) was three lists of ASVs with significant differential abundances (false discovery rate (FDR) < 0.05) in serpentinite samples or water samples. The final list of ASVs was created by deleting ASVs with greater abundances in any of the categories of water samples (as determined by the edgeR tests) from the original list of ASVs from which all air ASVs had already been removed. Finally, rare ASVs (those that did not have >=100 counts in a single sample) were excluded from the final results, merely as a conservative abundance filter for reporting a final list of ASVs expected to be present in serpentinite rocks. No comparisons of diversity were attempted after removing rare ASVs.

**References**

1. Orcutt BN, Bergenthal M, Freudenthal T, Smith D, Lilley MD, Schnieders L, Green S, Früh-Green GL. 2017. Contamination tracer testing with seabed drills: IODP Expedition 357. Sci Drill 23:39–46.

2. Früh-Green GL, Orcutt BN, Green SL, Cotterill C, Morgan S, Akizawa N, Bayrakci G, Behrmann J-H, Boschi C, Brazleton WJ. 2017. Expedition 357 methods. Atlantis Massif: Serpentinisation and life. Proc Int Ocean Discov Program 357.

3. Früh-Green GL, Orcutt BN, Rouméjon S, Lilley MD, Morono Y, Cotterill C, Green S, Escartin J, John BE, McCaig AM, Cannat M, Ménez B, Schwarzenbach EM, Williams MJ, Morgan S, Lang SQ, Schrenk MO, Brazelton WJ, Akizawa N, Boschi C, Dunkel KG, Quéméneur M, Whattam SA, Mayhew L, Harris M, Bayrakci G, Behrmann J-H, Herrero-Bervera E, Hesse K, Liu H-Q, Ratnayake AS, Twing K, Weis D, Zhao R, Bilenker L. 2018. Magmatism, serpentinization and life: Insights through drilling the Atlantis Massif (IODP Expedition 357). Lithos 323:137–155.

4. Früh-Green GL, Orcutt BN, Green SL, Cotterill C, Morgan S, Akizawa N, Bayrakci G, Behrmann JH, Boschi C, Brazleton WJ. 2017. Expedition 357 summary. Proc Int Ocean Discov Program 357.

5. Direito SO, Marees A, Röling WF. 2012. Sensitive life detection strategies for low-biomass environments: optimizing extraction of nucleic acids adsorbing to terrestrial and Mars analogue minerals. FEMS Microbiol Ecol 81:111–123.

6. Saeki K, Sakai M. 2009. The influence of soil organic matter on DNA adsorptions on andosols. Microbes Environ 0904270086–0904270086.

7. Lever MA, Torti A, Eickenbusch P, Michaud AB, Šantl-Temkiv T, Jørgensen BB. 2015. A modular method for the extraction of DNA and RNA, and the separation of DNA pools from diverse environmental sample types. Front Microbiol 6:476.

8. Barton HA, Taylor NM, Lubbers BR, Pemberton AC. 2006. DNA extraction from low-biomass carbonate rock: An improved method with reduced contamination and the low-biomass contaminant database. J Microbiol Methods 66:21–31.

9. Schrenk MO, Kelley DS, Delaney JR, Baross JA. 2003. Incidence and diversity of microorganisms within the walls of an active deep-sea sulfide chimney. Appl Env Microbiol 69:3580–3592.

10. Tanaka T, Sakai R, Kobayashi R, Hatakeyama K, Matsunaga T. 2009. Contributions of phosphate to DNA adsorption/desorption behaviors on aminosilane-modified magnetic nanoparticles. Langmuir 25:2956–2961.

11. Brazelton WJ, Thornton CN, Hyer A, Twing KI, Longino AA, Lang SQ, Lilley MD, Früh-Green GL, Schrenk MO. 2017. Metagenomic identification of active methanogens and methanotrophs in serpentinite springs of the Voltri Massif, Italy. PeerJ 5:e2945.

12. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Env Microbiol 79:5112–5120.

13. Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17:10–12.

14. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 13:581–583.

15. Pruesse E, Peplies J, Glöckner FO. 2012. SINA: Accurate high-throughput multiple sequence alignment of ribosomal RNA genes. Bioinformatics 28:1823–1829.

16. McMurdie PJ, Holmes S. 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLOS ONE 8:e61217.

17. Knights D, Kuczynski J, Charlson ES, Zaneveld J, Mozer MC, Collman RG, Bushman FD, Knight R, Kelley ST. 2011. Bayesian community-wide culture-independent microbial source tracking. Nat Methods 8:761–763.

18. Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139–140.

19. McMurdie PJ, Holmes S. 2014. Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible. PLOS Comput Biol 10:e1003531.