Thermophilic Lithotrophy and Phototrophy in an Intertidal, Iron-rich, Geothermal Spring

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Abstract

Hydrothermal systems, including terrestrial hot springs, contain diverse and systematic arrays of geochemical conditions that vary over short spatial scales due to progressive interaction between the reducing hydrothermal fluids, the oxygenated atmosphere, and in some cases seawater. At Jinata Onsen, on Shikinejima Island, Japan, an intertidal, anoxic, iron- and hydrogen-rich hot spring mixes with the oxygenated atmosphere and sulfate-rich seawater over short spatial scales, creating an enormous range of redox environments over a distance ~10 m. We characterized the geochemical conditions along the outflow of Jinata Onsen as well as the microbial communities present in biofilms, mats, and mineral crusts along its traverse via 16S amplicon and shotgun metagenomic sequencing. The microbial community changed significantly downstream as temperatures and dissolved iron concentrations dropped and dissolved oxygen rose. Near the spring source, primary productivity appears limited, and is fueled primarily by oxidation of ferrous iron and molecular hydrogen by members of the Zetaproteobacteria and Aquificae, while downstream the microbial community is dominated by oxygenic Cyanobacteria. At Jinata Onsen, Cyanobacteria are abundant and productive even at ferrous iron concentrations of ~150 μM, which challenges the idea that iron toxicity limited cyanobacterial expansion in the Precambrian oceans. Several novel lineages of Bacteria are also present at Jinata Onsen, including previously uncharacterized members of the Chloroflexi and Caldithrichaeota phyla, positioning Jinata Onsen as a valuable site for future characterization of these clades.

Importance

High temperatures and reducing conditions allow hot springs to support microbial communities that are very different those elsewhere on the surface of the Earth today; in some ways, these environments and the communities they support can be similar to those that existed on the early Earth and that may exist on other planets. Here, we describe a novel hot spring system where hot, iron-rich but oxygen-poor water flows into the ocean, supporting a range of unique microbial communities. Metagenomic sequencing recovered many novel microbial lineages, including deep-branching and uniquely thermotolerant members of known groups. Comparison of the biological productivity of communities in the upstream part of the hot spring,

supported by biological iron and hydrogen oxidizing metabolisms, to downstream microbial mats, supported by oxygenic photosynthesis, provides insight into the potential productivity of life on the early Earth and other planets where oxygenic photosynthesis is not possible.

Introduction

A major theme of environmental microbiology has been the enumeration of microbial groups that are capable of exploiting diverse chemical potentials that occur in nature (e.g. Broda 1977, Strous et al. 1999, Bryant et al. 2007, Ward et al. 2018a). Hot springs, with their varied chemical compositions, provide reservoirs of novel microbial diversity, where environmental and geochemical conditions select for lineages and metabolisms distinct from other Earth-surface environments (e.g. Eloe-Fadrosh et al. 2016, Beam et al. 2016). In addition to their value as sources of microbial diversity, hot springs also provide valuable test beds for understanding microbial community processes driven by different suites of metabolisms (e.g. Inskeep et al. 2005)—this in turn provides a process analog window into biosphere function during early times in Earth history, for example when the O_2 content of surface waters was low or non-existent. In contrast to most surface ecosystems which are fueled almost entirely by oxygenic photosynthesis by plants, algae, and Cyanobacteria (Ward and Shih, in review), hot spring microbial communities are commonly supported by lithotrophic or anoxygenic phototrophic organisms that derive energy and electrons for carbon fixation by oxidizing geologically sourced electron donors such as Fe^{2+}, sulfide, and molecular hydrogen (e.g. Kawasumi et al. 1998, Spear et al. 2005, Ward et al. 2017a). These communities may therefore provide insight into the function of low-productivity communities on the early Earth, before the Great Oxygenation Event ~2.3 billion years ago as oxygenic photosynthesis came to dominate primary productivity thereafter (Kharecha et al. 2005, Canfield et al. 2006, Sleep and Bird 2007, Ward and Shih, in review).

Here, we present a geomicrobiological characterization of a novel Precambrian Earth process analog site: Jinata Onsen, on Shikinejima Island, Tokyo Prefecture, Japan. At Jinata hot spring, anoxic, iron-rich hydrothermal fluids feed a subaerial spring that flows into a small bay, and mixes with seawater over the course of a few meters. Over its course the waters transition from low-oxygen, iron-rich conditions analogous to some aspects of the early Proterozoic oceans, toward iron-poor and and oxygen-rich conditions typical of modern coastal oceans. Coupled to geochemical measurements, 16S amplicon sequencing and shotgun metagenomic sequencing provide an overview of the microbial community composition along the hot spring transect as well as metagenome-assembled genomes of diverse novel microbial lineages that inhabit these springs.

Materials and Methods:

Geological context and sedimentology of Jinata:

Jinata Onsen is located at 34.318 N, 139.216 E on the island of Shikinejima, Tokyo Prefecture, Japan. Shikinejima is part of the Izu Islands, a chain of volcanic islands that formed in the last few million years along the northern edge of the Izu-Bonin-Mariana Arc (Kaneoka et al. 1970). Shikinejima is formed of Late Paleopleistocene- to-Holocene non-alkaline felsic volcanics and Late-Miocene to Pleistocene non-alkaline pyroclastic volcanic flows (Figure 1).

The source water of Jinata Onsen emerges anoxic, iron-rich, and gently bubbling from the spring source (Figure 1, Figure 2). Temperatures at the source are ~62°C. Water emerges into the Source Pool, which has no visible microbial mats or biofilms (Figure 2D). Surfaces are instead coated with a fluffy red precipitate, likely a poorly ordered or short range-ordered ferric

iron oxide phase such as ferrihydrite. Flow from the Source appears to be—at least in part—tidally charged, with the highest water levels and flow rates occurring at high tide. At low tide, flow rates drop and the water level of the source pool can drop by decimeters. Downstream, the spring water collects into a series of pools (Pool 1-3) (Figure 2C,E-F), which cool sequentially. Pool 1 contains iron oxides like the Source Pool, but also develops macroscopic microbial streamers that are in iron oxides. Streamers are very fine (mm-scale) and delicate (break apart on contact with forceps) but can reach several centimeters in length. Downstream pools (Pools 2 and 3) also mix with seawater during high tide due to wave action, but this seawater influence does not appear to influence the Source or Pool 1. Samples were collected and temperatures were measured at high tide, reflecting the lowest temperatures experienced by microbes in the pools—at low tide, hot spring input is dominant and temperatures rise (observed range at each site in Supplemental Table 1). Subaqueous surfaces in Pools 2 and 3 are covered in thick microbial mats. In Pool 2, the mat is coated in a layer of fluffy iron oxide similar to that in the source pool, with dense microbial mat below (Figure 2E). Pool 3 contains only patchy iron oxides, with mostly exposed microbial mats displaying a finger-like morphology. These "fingers" were 0.5-1 cm in diameter and up to ~5 cm long and were closely packed and carpeting surfaces of Pool 3 below the high tide line. potentially related to turbulent mixing from wave action during high tide (Figure 2F). The Outflow is the outlet of a channel connecting Pool 2 to the bay. Its hydrology is dominantly marine with small admixtures of inflowing spring water (Figure 2G).

Jinata hot spring was visited twice for observation and community DNA sampling in 2016 (January and September), and again for observation and gas sampling in October 2017 and April 2018. These visits corresponded to a range of tidal conditions, including a spring low and high tide in September 2016. General features of the spring were consistent across this period (including abundance and distribution of iron minerals and microbial mats), differing primarily in an apparent tidal dependence in flow rate and water level of the spring and the amount of seawater influence on Pool 3. These differences in flow and mixing led to variation in water temperatures of 3-10 °C (Supplemental Table 1). At high tide, flow rate of the spring increases, as does seawater influx to Pool 3. During the spring low tide, the spring flow stagnated and the water level of Source Pool and Pool 1 dropped by decimeters. During less extreme low tides observed on other dates, the spring flow was low but nonzero and the water level of the Source Pool did not drop significantly.

Sample collections:

Five sites were sampled at Jinata Onsen: the Source Pool, Pool 1, Pool 2, Pool 3, and the Outflow (Figure 1, Figure 2). During the first sampling trip in January 2016, two whole community DNA samples were collected from each site for 16S amplicon sequencing. During the second sampling trip, additional DNA was collected from the Source Pool and Pool 2 for shotgun metagenomic sequencing.

Samples were collected as mineral scrapings of loosely attached, fluffy iron oxide coating from surfaces and clasts upstream (Source Pool and Pool 1) and as samples of microbial mat downstream (Pools 2 and 3, and Outflow) using sterile forceps and spatulas (~0.25 cm^3 of material). Immediately after sampling, cells were lysed and DNA preserved with a Zymo Terralyzer BashingBead Matrix and Xpedition Lysis Buffer. Lysis was achieved by attaching tubes to the blade of a Makita JR101DZ cordless reciprocating saw and operating for 1 minute. Aqueous geochemistry samples consisted of water collected with sterile syringes and filtered through a 0.2 μm filter. Gas samples were collected near sites of ebullition emerging from the

bottom of the source pool; collection was done into serum vials by water substitution, and then sealed underwater to prevent contamination by air.

Geochemical analysis:

Dissolved oxygen (DO), pH, and temperature measurements were performed *in situ* using an Extech DO700 8-in-1 Portable Dissolved Oxygen Meter (FLIR Commercial Systems, Inc., Nashua, NH). Iron concentrations were measured using the ferrozine assay (Stookey 1970) following acidification with 40 mM sulfamic acid to inhibit iron oxidation by O_2 or oxidized nitrogen species (Klueglein and Kappler 2013). Ammonia/ammonium concentrations were measured using a TetraTest NH_3/NH_4^+ Kit (TetraPond, Blacksburg, VA) following manufacturers instructions but with colorimetry of samples and NH_4Cl standards quantified with a Thermo Scientific Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA) at 700 nm to improve sensitivity and accuracy. Anion concentrations were measured via ion chromatography on a Shimadzu Ion Chromatograph (Shimadzu Corp., Kyoto, JP) equipped with a Shodex SI-90 4E anion column (Showa Denko, Tokyo, JP).

Presence of H_2 and CH_4 in gas samples was qualitatively determined with a Shimadzu GC-14A gas chromatograph within 12 hours of collection to minimize oxidation of reduced gases. Quantitative gas composition was measured following methods described in Suda et al. 2017. In brief, gas samples were analyzed using a gas chromatograph (GC-4000, GL Sciences) equipped with both a pulsed discharge detector (PDD) and a thermal conductivity detector (TCD). The GC was equipped with a ShinCarbon ST packed column (2 m × 2.2 mm ID, 50/80 mesh) connected to a HayeSepo Q packed column (2 m × 2.2 mm ID, 60/80 mesh) to separate O_2, N_2, CO_2, and light hydrocarbons. Temperature was held at 40°C for 6 minutes before ramping up to 200°C at 20°C/min. This temperature was held for 6 minutes before ramping up to 250˚C at 50˚C/min before a final hold for 15 minutes. The value of standard errors (SE) were determined by replicate measurement of samples. The detection limit was on the order of 1nmol/cc for H_2 and CH_4.

16S sequencing and analysis:

Following return to the lab, microbial DNA was extracted and purified with a Zymo Soil/Fecal DNA extraction kit. The V4-V5 region of the 16S rRNA gene was amplified from each extract using archaeal and bacterial primers 515F (GTGCCAGCMGCCGCGGTAA) and 926R (CCGYCAATTYMTTTRAGTTT) (Caporaso et al., 2012). DNA was quantified with a Qubit 3.0 fluorimeter (Life Technologies, Carlsbad, CA) according to manufacturer's instructions following DNA extraction and PCR steps. All samples yielded PCR amplicons when viewed on a gel after initial pre-barcoding PCR (30 cycles). Duplicate PCR reactions were pooled and reconditioned for five cycles with barcoded primers. Samples for sequencing were submitted to Laragen (Culver City, CA) for analysis on an Illumnia MiSeq platform. Sequence data were processed using QIIME version 1.8.0 (Caporaso et al., 2010). Raw sequence pairs were joined and quality-trimmed using the default parameters in QIIME. Sequences were clustered into de novo operational taxonomic units (OTUs) with 99% similarity using UCLUST open reference clustering protocol (Edgar, 2010). Then, the most abundant sequence was chosen as representative for each *de novo* OTU (Wang et al., 2007). Taxonomic identification for each representative sequence was assigned using the Silva-115 database (Quast et al., 2013) clustered at separately at 99% and at 97% similarity. Singletons and contaminants (OTUs appearing in the negative control datasets) were removed. 16S sequences were aligned using MAFFT (Katoh et al. 2002) and a phylogeny constructed using FastTree (Price et al. 2010). Alpha diversity was estimated using the Shannon Index (Shannon 1948) and Inverse Simpson metric (1/D) (Simpson

1949; Hill 1973). All statistics were calculated using scripts in QIIME and are reported at the 99% and 97% OTU similarity levels. Multidimensional scaling (MDS) analyses and plots to evaluate the similarity between different samples and OHK environments were produced in R using the vegan and ggplot2 packages (R Core Team 2014, Oksanen et al. 2016, Wickham 2009).

Metagenomic sequencing and analysis:

Following initial characterization via 16S sequencing, four samples were selected for shotgun metagenomic sequencing: JP1-A and JP3-A from the first sampling trip, and JP1L-1 and JP2-1 from the second sampling trip. Purified DNA was submitted to SeqMatic LLC (Fremont, CA) for library preparation and 2x100bp paired-end sequencing via Illumina HiSeq 4000 technology. Samples JP1-A and JP3-A shared a single lane with two samples from another project (Ward 2017, Ward et al. 2018a), while JP1L-1 and JP2-1 shared a lane with one sample from another project.

Raw sequence reads from all four samples were co-assembled with MegaHit v. 1.02 (Li et al. 2016) and genome bins constructed based on nucleotide composition and differential coverage using MetaBAT (Kang et al. 2015), MaxBin (Wu et al. 2014), and CONCOCT (Alneberg et al. 2013) prior to dereplication and refinement with DAS Tool (Sieber et al. 2018) to produce the final bin set. Genome bins were assessed for completeness and contamination using CheckM (Parks et al. 2014), tRNA sequences found with Aragorn (Laslett and Canback 2004), and presence of metabolic pathways of interest predicted with MetaPOAP (Ward et al. 2018b). Genes of interest (e.g. coding for ribosomal, photosynthesis, and electron transport proteins) were screened against outlier (e.g. likely contaminant) contigs as determined by CheckM using tetranucleotide, GC, and coding density content. Coverage was extracted using bbmap (Bushnell 2016) and samtools (Li et al. 2009). Genes of interest (e.g. coding for ribosomal, photosynthesis, iron oxidation, and electron transport proteins) were identified from assembled metagenomic data locally with BLAST+ (Camacho et al. 2008), aligned with MUSCLE (Edgar 2004), and alignments manually curated in Jalview (Waterhouse et al. 2009). Phylogenetic trees were calculated using RAxML (Stamakis 2014) on the Cipres science gateway (Miller et al. 2010). Node support for phylogenies was calculated with transfer bootstraps by BOOSTER (Lemoine et al. 2018). Trees were visualized with SeaView (Gouy et al. 2010) and the Interactive Tree of Life viewer (Letunic and Bork 2016). Because sequencing depth of each sample in the full metagenome was uneven, relative abundance of genes of interest between metagenomic datasets was normalized to the coverage of *rpoB* genes in each raw dataset as mapped onto the coassembly. Like the 16S gene, *rpoB* is a highly conserved, vertically-inherited gene useful for taxonomic identification of organisms, but has the added advantage that it is only known to occur as a single copy per genome (Case et al. 2007) and is more readily assembled in metagenomic datasets (e.g. Ward et al. 2018a).

Results

Geochemistry

Geochemical measurements along the flow path of Jinata Onsen revealed a significant shift from hot, low-oxygen, high-iron source water to cooler, more oxygen-rich water with less dissolved iron downstream. Geochemistry measurements of Jinata source water are summarized in Table 1 and Supplemental Table 1, while geochemical gradients along the stream outflow are summarized in Figure 3 and Supplemental Table 2. Source waters were slightly enriched in chloride relative to seawater (~23.2 g/L), depleted in sulfate (~1.63 g/L) but approached seawater

concentrations downstream as mixing increased. Water emerging from the source was 62°C, very low in dissolved oxygen (~0.15 mg/l), at pH 5.4, and contained substantial concentrations of dissolved iron (~250 μM Fe^{2+}). After emerging from the source pool, the spring water exchanges gases with the air due to mixing associated with water flow and gas ebullition, and DO rose to 1.24 mg/L at the surface of the source pool. As water flows downstream from the source pool, it cools slightly, exchanges gases with the atmosphere, and intermittently mixes with seawater below Pool 1.

While there is significant variability in the flow rate from the spring based on tides (and resulting shifts in water level and temperature), the overall geochemistry of the source water and the microbial community appeared largely similar between the January and September 2016.

Both H_2 and CH_4 were qualitatively detected in bubbles from the source pool following initial sampling in September 2016. However, during subsequent analyses to quantify the gas composition in October 2017 and April 2018 the gas was determined to contain CO_2, CH_4, N_2 (Supplemental Table 2). This subsequent non-detection of H_2 may be related to temporal variability in the gas composition at Jinata (e.g. following tidal influence; significant variability was observed in the CO_2:N_2 ratio between two sampling dates, Supplemental Table 2) or may reflect oxidation of H_2 between sampling and analysis; however, the detection limit of H_2 for these later measurements was ~1 nmol/cc, well above the energetic and ecological limits for hydrogenotrophic metabolisms (e.g. Ji et al. 2017) leaving open the possibility of biologically significant H_2 fluxes at Jinata around the time of sampling. This possibility is supported by observations of high relative abundances of microbes with the capacity for hydrogenotrophy, discussed more below.

Sequencing

16S and metagenomic sequencing of microbial communities at Jinata Onsen revealed a highly diverse community. In total, 16S amplicon sequencing recovered 456,737 sequences from the 10 samples at Jinata (Supplemental Table 3, Supplemental Table 4). Reads per sample ranged from 26,057 Source Pool Sample A to 97,445 for Pool 1 Sample A (median 43,331, mean 45,673, and standard deviation 19,568). Assessment of sampling depth was estimated using Good's Coverage (Good 1953). On average, 74% of the microbial community was recovered from Jinata samples at the 99% OTU level based on the Good's Coverage statistic (ranging from 54% coverage in the Outflow Sample A to 85% in the Pool 1 Sample A) and 87% at the 97% OTU level (74% for the Outflow Sample A to 94.5% for the Pool 1 Sample B). The incomplete sampling—despite sequencing to relatively high depth (>18000 reads per sample)—probably reflects uneven diversity. Greater than 50% of the reads observed at most sites map to the 10 most abundant taxa (Supplemental Table 4). MDS analysis (Supplemental Figure 1) demonstrates that samples from the same site are highly similar, and adjacent sites (e.g. Source and Pool 1, Outflow and Pool 3) show significant similarity. However, there is a significant transition in microbial community diversity between the most distant samples (e.g. Source and Outflow).

Shotgun metagenomic sequencing of four samples from Jinata Onsen recovered 121 GB of data, forming a 1.48 Gb coassembly consisting of 1531443 contigs with an N50 of 1494 bp. Nucleotide composition and differential coverage-based binning of the coassembly via multiple methods followed by dereplication and refinement resulted in a final set of 161 medium- or high-quality metagenome-assembled genomes (MAGs) following current standards (i.e. completeness >50% and contamination <10%) (Bowers et al. 2017). These MAGs are from diverse phyla of Bacteria and Archaea (Figure 4); metagenome and MAG statistics with tentative taxonomic

assignments for recovered MAGs are available in the Supplementary Information (Supplemental Table 5), while MAGs of particular interest are discussed in depth below and shown in phylogenetic trees alongside reference strains in Figures 5-7.

Discussion

The primary trends at Jinata are the transition from hot, low-oxygen, high-iron source waters to cooler, iron-depleted, oxygen-rich water in downstream regions (Figure 3). Following this geochemical transition is a major shift in the composition and productivity of the microbial community, from a high-temperature, lithotrophic community apparently fueled by iron- and hydrogen-oxidation which produces little biomass (at least in net) upstream, to a lower temperature, oxygenic photosynthesis-fueled community with well-developed, thick microbial mats downstream. This shift in community composition is summarized in Figure 3, with complete diversity data in the Supplemental Information (including OTU counts per samples in Supplemental Table 4). Below, we discuss the overall physiological and taxonomic trends across the spring sites as inferred from diversity and genomic analysis.

Iron and hydrogen oxidation

The hot spring water emerging at the Source Pool at Jinata contains abundant bioavailable electron donors including dissolved Fe^{2+} and likely H_2 (though measurements of gas content varied, as discussed in Results above) (Table 1). These electron donors appear to fuel productivity and determine the microbial community upstream at the Source Pool and Pool 1, where microbial mats are not well developed. The low accumulation of biomass in upstream regions of Jinata are similar to other microbial ecosystems fueled by iron oxidation (e.g. Oku-Okuhachikurou Onsen, Ward et al. 2017a, Fuschna Spring, Helger et al. 2012, and Jackson Creek, Roden et al 2012), in which lithotrophic communities appear much less productive and capable of accumulating less biomass than communities fueled by oxygenic photosynthesis (such as those in downstream regions at Jinata).

The most abundant organisms in the Source Pool are members of the Aquificae family Hydrogenothermaceae. Members of this family of marine thermophilic lithotrophs are capable of both iron and hydrogen oxidation (Takai and Nakagawa 2014) and may be utilizing either Fe^{2+} or H_2 at Jinata. The seventh most abundant OTU in the Source Pool samples is a novel sequence 89% similar to a strain of *Persephonella* found in an alkaline hot spring in Papua New Guinea. *Persephonella* is a genus of thermophilic, microaerophilic hydrogen oxidizing bacteria within the Hydrogenothermaceae (Götz et al. 2002); the potential difference in bioenergetics between closely related alkaliphiles in Papua New Guinea and strains living at pH 5.5 at Jinata Onsen may be an interesting target for future research. Despite their abundance as assessed by 16S sequencing (Figure 3), only four partial Aquificae MAGs were recovered from Jinata of which only one (J026) was reasonably complete (~94%). Two Aquificae MAGs recovered Group 1 NiFe hydrogenase genes, which may be used in hydrogenotrophy; the absence of hydrogenases from the other MAGs may be related to their low completeness, or could reflect a utilization of iron or other electron donors and not H_2 in these organisms.

The other most abundant organisms near the source are members of the Zetaproteobacteria—a group typified by the neutrophilic, aerobic iron-oxidizing genus *Mariprofundus* common in marine systems (Emerson et al. 2007). Zetaproteobacteria and Hydrogenothermaceae together made up ~30-65% of 16S sequences in the Source Pool and Pool 1, and so appear to drive the base of ecosystem productivity in these upstream pools.

The relative abundance of Hydrogenothermaceae drops off to less than 1% of sequences where microbial mats become well developed downstream of Pool 1, but Zetaproteobacteria continue to make up a few percent of reads in Pool 2 and Pool 3 where dissolved iron concentrations are still significant (Figure 3). This suggests that shifts in the relative abundance of may be due more to the increase in abundance of other organisms, rather than a drop in the number of Zetaproteobacteria or their ability to make a living oxidizing iron. In contrast, the absence of Hydrogenothermaceae downstream may be a real signal driven by the rapid disappearance of H_2 as an electron donor. However, in both cases, a drop in relative abundance is likely related to the increasing total biomass (i.e. number of cells) downstream as Cyanobacteria become more productive, leading to sequences from Hydrogenothermaceae and Zetaproteobacteria being swamped out by increases numbers of Cyanobacteria, Chloroflexi, and other sequences. This provides an indirect proxy for the relative productivity of lithotrophs versus oxygenic phototrophs in this environment.

Members of the Mariprofundaceae have been observed to have an upper temperature limit for growth of 30°C (Emerson et al. 2010). The Zetaproteobacteria found at Jinata thrive at temperatures up to 63 degrees. This currently represents a unique high-temperature environment for these organisms. In particular, the third most abundant out in the Source Pool and Pool 1 sample A is an unknown sequence that is 92% identical to a sequence from an uncultured zetaproteobacterium from a shallow hydrothermal vent in Papua New Guinea (Meyer-Dombard et al. 2013). This sequence likely marks a novel lineage of high-temperature iron-oxidizing Zetaproteobacteria. Four MAGs affiliated with the Zetaproteobacteria were recovered from Jinata with completeness estimates by CheckM ranging from ~80 to ~97% (J005, J009, J030, and J098). While these MAGs did not recover 16S genes, RpoB- and concatenated ribosomal protein-based phylogenies illustrated that members of this group at Jinata Onsen do not belong to the characterized genera *Mariprofundus* or *Ghiorsea*, but instead form separate basal lineages within the Zetaproteobacteria (Figure 5). Despite their phylogenetic distinctness, these MAGs largely recovered genes associated with aerobic iron oxidation, including a terminal O_2 reductase from the C-family of Heme Copper Oxidoreductases for respiration at low O_2 concentrations and Cyc2 cytochrome genes implicated in ferrous iron oxidation in Zetaproteobacteria and other taxa (e.g. Chlorobi) (Han et al. 2011, Kato et al. 2015, He et al. 20170). Hydrogenase catalytic subunit genes were not recovered in zetaproteobacterial MAGs even at high completeness, suggesting that these organisms are not hydrogenotrophic, though the possibility of uncharacterized hydrogenases cannot be discarded. J098 did not recover a Cyc2 cytochrome gene; based on phylogenetic position this MAG captures a member of the most basal Zetaproteobacteria lineage recovered to date, which if correct may have diverged prior to the evolution of iron oxidation in this group. However, this MAG is also only 80% complete and so there is a significant probability of failure to recover this gene even if it were present in the source genome (MetaPOAP False Negative estimate 0.205). J005 and J030 did not recover genes for carbon fixation via the Calvin cycle such as the large and small subunits of rubisco, phosphoribulose kinase, or carboxysome proteins; the high completeness of these MAGs (~94-97%) makes it incredibly unlikely that these genes would all fail to be recovered (MetaPOAP False Negative estimates 10^{-5}-10^{-7}), suggesting that these strains may be heterotrophic or rely upon an alternative carbon fixation pathway. Over all, the genetic and apparent physiological novelty of Jinata Zetaproteobacteria, along with the site's ease of access relative to typical deep marine settings, makes this a promising target for future isolation and detailed characterization of these taxa.

Seven MAGs were recovered from the enigmatic phylum Calditrichaeota (J004, J008, J042, and J075) (Figure 6). Calditrichaeota is a phylum of bacteria with few isolated or sequenced members. The best known of these is *Caldithrix abyssi* (Miroshnichenko et al. 2003); this taxon was characterized as an anaerobic thermophile capable of lithoheterotrophy H_2 oxidation coupled to denitrification and organoheterotrophic fermentation (Alauzet and Jumas-Bilak 2014, Marshall et al. 2017). The Calditrichaeota MAGs reported here are up to 97% complete (J004) and contain members with variable metabolic capabilities. Aerobic respiration via A-family Heme Copper Oxidoreductases could potentially be coupled to autotrophic hydrogen oxidation (via the Group 1d NiFe hydrogenase in J042) or iron oxidation (via the *pioA* gene in J075); however, *Caldithrix abyssi* appears incapable of aerobic respiration despite encoding an A-family Heme Copper Oxidoreductase (Kublanov et al. 2017). Unlike previously described Calditrichaeota which are all heterotrophic (Marshall et al. 2017), most of the Calditrichaeota MAGs reported here possess the capacity for carbon fixation via the Calvin cycle. J004 is closely related to *Caldithrix abyssi*, while the other MAGs form two distinct but related clades (Figure 6). These MAGs significantly expand the known genetic and metabolic diversity of this under characterized phylum, and Jinata Onsen may serve as a valuable resource for further research on the physiology and ecology of the Calditrichaeota phylum.

The abundance at Jinata of microbes with the genetic capacity for hydrogenotrophy suggests that H_2 may be contributing to lithoautotrophy near the hot spring source, despite H_2 concentrations being low (below the detection of ~1 nmol/cc in the gas phase of our quantitative gas analyses, or ~1 nM in the aqueous phase, Amend and Shock 2001). However, this is unsurprising, as the oxidation of H_2 coupled to O_2 reduction is an incredibly thermodynamically favorable process even at vanishingly low substrate concentrations (e.g. $\Delta_rG' < -340$ kJ/mol under conditions at Jinata with substrate concentrations at our limit of detection, Flamholtz et al. 2012). Consistent with this thermodynamic favorability, biology has been shown to make use of this metabolism in environments such as hot springs with H_2 concentrations near our detection limits (D'Imperio et al. 2008) and in Antarctic soils where microbes rely on uptake of trace atmospheric H_2 at concentrations around 190 ppbv (Ji et al. 2017). Improved quantification of H_2 concentrations and measurement of hydrogenase activity and the productivity of hydrogenotrophic microbes will be needed in future to determine the relative contribution of hydrogen oxidation to productivity at Jinata.

Oxygenic photosynthesis

Cyanobacteria are nearly absent from near the source pool, but begin to appear around Pool 1 and become dominant starting in Pool 2. The most abundant Cyanobacteria present are predominantly members of Subsection III, Family I. This group includes *Leptolyngbya*, a genus of filamentous non–heterocystous Cyanobacteria that has appeared in other hot springs of similar temperatures (e.g. Ward et al. 2017a, Roeselers et al. 2007, Bosak et al. 2012). Diverse cyanobacterial MAGs were recovered, including members of the orders Pleurocapsales (J083), Chroococcales (J003 and J149), and Oscillatoriales (J007, J055, and J069).

Cyanobacteria performing oxygenic photosynthesis appear to dominate primary productivity in downstream regions of the hot spring, and the filamentous morphology of the strains present here allow them to contribute to the cohesive fabric of the microbial mat.

In the outflow samples, chloroplast sequences become abundant, most closely related to the diatom *Melosira.* Algae are at very low abundance upstream of the Out Flow, potentially inhibited by high temperatures, high iron concentrations, ecological competition, or other

characteristics of the hot spring water, but the higher seawater influence at the Out Flow appears to create a more permissive environment.

Cyanobacteria are sometimes underrepresented in iTag datasets as a result of poor DNA yield or amplification biases (e.g. Parada et al. 2015, Trembath-Reichert et al. 2016), but the low abundance of Cyanobacteria near the Source Pool was confirmed by fluorescent microscopy, in which cells displaying cyanobacterial autofluorescence were observed abundantly in samples from the downstream samples but not in the Source Pool (Supplemental Figure 2).

Thick microbial mats, and large accumulations of organic carbon, first appear in Pool 2 when Cyanobacteria become abundant. This appears to be related to the high productivity of oxygenic photosynthesis relative to lithotrophic metabolisms (e.g. Ward et al. 2017a, Ward et al. 2018c). Consistent with expectations of the nitrogen demand of highly productive oxygenic phototrophic ecosystems relative to poorly productive lithotrophic systems, the abundance of genes for biological nitrogen fixation via nitrogenase was 2.5 times higher in Pool 2 and Pool 3 than near the source (*nifD*/*rpoB* of 0.075 versus 0.03).

Previously, it has been suggested that high ferrous iron concentrations are toxic to Cyanobacteria, and that this would have greatly reduced their productivity under ferruginous ocean conditions such as those that may have persisted through much of the Archean era (Swanner et al. 2015). The high cyanobacterial productivity observed at Jinata under high iron concentrations suggest that Cyanobacteria can adapt to ferruginous conditions, and therefore iron toxicity might not inhibit Cyanobacteria over geological timescales. Indeed, the soluble iron concentrations observed at Jinata are higher (150-250 μM) than predicted for the Archean oceans (<120 μM, Holland 1984) or observed at other iron-rich hot springs (~100-200 μM, Pierson et al. 1999, Ward et al. 2017a), making Jinata an excellent test case for determining the ability of Cyanobacteria to adapt to high iron concentrations. Culture-based physiological experiments may be useful to determine whether Jinata Cyanobacteria utilize similar strategies to other iron-tolerant strains (e.g. the *Leptolyngbya*-relative *Marsacia ferruginose*, Brown et al. 2010) or whether Jinata strains possess unique adaptations that allow them to grow at higher iron concentrations than known for other environmental Cyanobacteria strains. This will in turn provide insight into whether iron tolerance is due to evolutionarily conserved strategies or whether this is a trait that has evolved convergently multiple times.

Diverse novel Chloroflexi from Jinata Onsen

In addition to the primary phototrophic and lithotrophic carbon fixers at Jinata, 16S and metagenomic data sets revealed diverse novel lineages within the Chloroflexi phylum. A total of 23 Chloroflexi MAGs were recovered, introducing substantial genetic and metabolic diversity that expands our understanding of this group. While the best known members of this phylum are Type 2 Reaction Center-containing lineages such as *Chloroflexus* and *Roseiflexus* within the class Chloroflexia (e.g. Thiel et al. 2018), phototrophy is not a synapomorphy of the Chloroflexi phylum or even the Chloroflexia class (e.g. Ward et al. 2015a) and most of the diversity of the phylum belongs to several other classes made up primarily of nonphototrophic lineages (Ward et al. 2018a). The bulk of Chloroflexi diversity recovered from Jinata belongs to “Subphlyum I”, a broad group of predominantly nonphototrophic lineages that was originally described based on the classes Anaerolineae and Caldilineae (Yamada and Sekiguchi 2009), but also encompasses the related classes Ardenticatenia, Thermoflexia, and *Candidatus* Thermofonsia (Kawaichi et al. 2013, Dodsworth et al. 2014, Ward et al. 2018a).

16S analysis indicates that members of the Chloroflexi class Anaerolineae are common throughout Jinata with the exception of the Outflow (average 3.5% relative abundance). The Anaerolineae have generally been isolated as obligately anaerobic heterotrophs (e.g. Sekiguchi et al. 2003, Yamada et al. 2006), but genome sequencing of isolates and MAG data from a range of environments has revealed the capacity for aerobic respiration across members of this clade (e.g. Hemp et al. 2015ab, Pace et al. 2015, Ward et al. 2015b, Ward et al. 2018f). It is also likely that a large fraction of 16S sequences annotated as Anaerolineae at Jinata Onsen belong to the sister class *Candidatus* Thermofonsia (Ward et al. 2018a). Three Anaerolineae MAGs were recovered from Jinata (J082, J097, and J130), as compared to seven associated with *Ca.* Thermofonsia (J027, J033, J036, J038, J039, J064, and J076). MAG J036 is an improved version of the genome previously reported as JP3_7 (Ward et al. 2018a), a close relative of *Ca.* Roseilinea gracile (Klatt et al. 2011, Tank et al. 2017, Thiel et al. 2017). J036 contains a 16S gene that is 96% similar to that of *Ca*. Roseilinea gracile, and thus these strains are probably best classified as distinct species within the same genus. Unlike other phototrophs in the Chloroflexi phylum that are capable of photoautotrophy via the 3-hydroxypropionate bicycle or the Calvin Cycle (Klatt et al. 2007, Shih et al. 2017), J036 and *Ca.* Roseilinea gracile do not encode carbon fixation and are likely photoheterotrophic. Previous analyses suggested that the Roseilinea lineage belongs to the Anaerolineae (Klatt et al. 2011) or Thermofonsia (Ward et al. 2018a) classes; however, our updated phylogeny presented here places J036 and Roseilinea in a separate lineage along with J033 and J162, diverging just outside of the Anaerolineae+Thermofonsia clade, suggesting that these strains may instead be yet another class-level lineage within the broader "Subphylum I" of Chloroflexi (Figure 7).

Members of the Chloroflexi class Caldilineae were present at up to ~1% abundance at Jinata in the 16S dataset. Members of the Caldilineae have previously been isolated from intertidal hot springs in Iceland (Kale et al. 2013) and Japanese hot springs (Sekiguchi et al. 2003). Characterized organisms in this class are filamentous, anaerobic, or facultatively aerobic heterotrophs (Sekiguchi et al. 2003, Grégoire et al. 2011, Kale et al. 2013); and therefore these taxa may play a role in degrading biomass within low-oxygen regions of microbial mats. Several MAGs from within the Caldilineae and related lineages were recovered in the metagenome, potentially reflecting novel class-level diversity within the Chloroflexi. Three MAGs were recovered that form a clade sister to the previously characterized members of the Caldilineae class *Caldilinea* and *Litorilinea* (J095, J111, and J123), forming a deeply branching lineage within this class. Like other members of the Caldilineae, these strains encode aerobic respiration via A family Heme Copper Oxidoreductases and both a *bc* complex III and an Alternative Complex III, and are therefore likely at least facultatively aerobic. J095 also encodes carbon fixation via the Calvin cycle as well as a Group 1f NiFe hydrogenase, suggesting a potential capability for lithoautotrophy by hydrogen oxidation, expanding the known metabolic diversity of this class and the Chloroflexi phylum as a whole.

The Chloroflexi class Ardenticatenia was first described from an isolate from an iron-rich Japanese hydrothermal field (Kawaichi et al. 2013) and has since been recovered from sulfidic hot springs as well (Ward et al. 2018e). A MAG closely related to *Ardenticatena maritima* was recovered from Jinata Onsen, J129. While *Ardenticatena maritima 110S* contains a complete denitrification pathway (Hemp et al. 2015), MAG J129 did not recover any denitrification genes. This could be related to the relatively low completeness of this MAG (~70%), but False Negative estimates by MetaPOAP (Ward et al. 2018c) indicates that the probability that all four steps in the canonical denitrication pathway would fail to be recovered in J129 given their presence in the

source genome is less than 0.8%, suggesting that most if not all denitrification genes are truly absent and that the capacity for denitrification is not universal within members of *Ardenticatena*. This would be consistent with broad trends in the apparently frequent modular horizontal gene transfer of partial denitrification pathways between disparate microbial lineages to drive rapid adaption and metabolic flexibility of aerobic organisms in microoxic and anoxic environments, for reasons that are still not well established (Chen and Strous 2013, Stein and Klotz 2016).

MAG J114 branches at the base of Subphylum I of the Chloroflexi, potentially the first member of a novel class-level lineage. The divergence between Anaerolineae and Caldilineae has been estimated to have occurred on the order of 1.7 billion years ago (Shih et al. 2017). The phylogenetic placement of J114 suggests that it diverged from other members of Subphylum I even earlier, and it may be a good target for future investigation to assess aspects of the early evolution of the Chloroflexi phylum. J114 encodes aerobic respiration via an A family Heme Copper Oxidoreductase and an Alternative Complex III like many other nonphototrophic Chloroflexi lineages (e.g. Ward et al. 2015a, Ward et al. 2018a) as well as a Group 1f NiFe hydrogenase and carbon fixation via the Calvin Cycle, suggesting the capacity for aerobic hydrogen-oxidizing autotrophy—a lifestyle not previously described for members of the Chloroflexi. The Alternative Complex III encoded by J114 branches basally to a clade of ACIII sequences from other Subphylum I Chloroflexi, potentially reflecting vertical inheritance of ACIII from the last common ancestor of this clade; however, the A-family Heme Copper Oxidoreductase encoded by J114 is in a more derived position closely related to sequences from members of the Caldilineae, and may have been acquired via horizontal gene transfer from a member of this group.

Conclusions

Jinata Onsen is a environment supporting especially strong geochemical gradients over short spatial scales. The transition from low-oxygen, iron- and hydrogen-rich hot spring source water to oxygen-rich ocean water takes place over just a few meters, and results in an almost complete change in microbial community. We have recovered substantial genetic and metabolic novelty from metagenomic data from Jinata Onsen, highlighting how hot spring microbial communities (particularly those of understudied iron-rich systems) are hotbeds of poorly characterized microbial clades. In addition, due to its utility as an environment to investigate the diversity and ecology of microbes, including thermal tolerant iron-oxidizing Zetaproteobacteria and iron-tolerant Cyanobacteria, this system is significant for its relevance as a process analog for environments through Earth history and potentially habitable environments in Mars' past.

The diversity of iron oxidizing bacteria at Jinata is very different than in other Fe^{2+} -rich springs and environments. For example, in freshwater systems such as Oku-Okuhachikurou Onsen in Akita Prefecture, Japan (Ward et al. 2017), and Budo Pond in Hiroshima, Japan (Kato et al. 2012), iron oxidation is driven primarily by the activity of chemoautotrophs such as members of the Gallionellaceae (Ward et al. 2017). In contrast, at Chocolate Pots hot spring in Yellowstone National Park, USA, iron oxidation is primarily abiotic, driven by O2 produced by Cyanobacteria, with only a small contribution from iron oxidizing bacteria (Trouwborst et al. 2007, Fortney et al. 2018).The distinct iron-oxidizing community at Jinata Onsen may be related to the salinity of the spring water, or biogeographically by access to the ocean, as Zetaproteobacteria are typically found in marine settings, particularly in deep ocean basins associated with hydrothermal iron sources (Emerson et al. 2010). Despite the taxonomically distinct iron oxidizer communities between Jinata and Oku-Okuhachikurou Onsen, both

communities support only limited biomass in regions dominated by iron oxidizers (Ward et al. 2017a), perhaps reflecting the shared biochemical and bioenergetic challenges iron oxidation incurred by diverse iron oxidizing bacteria including Gallionellaceae and Zetaproteobacteria (Emerson et al. 2010, Bird et al. 2011, Ward et al. 2017a).

Throughout Earth history, the metabolic opportunities available to life, and the resulting organisms and metabolisms responsible for driving primary productivity, have been shaped by the geochemical conditions of the atmosphere and oceans. Over the course of Earth's four-and-a-half billion-year history, the redox state and overall geochemical conditions of the oceans have varied systematically. The modern, sulfate-rich, well-oxygenated oceans we see today reflects a relatively recent state—one typical of only the last few hundred million years (e.g. Lyons et al. 2014). For the first half of Earth history, until ~2.3 billion years ago (Ga), the atmosphere and oceans were anoxic (Johnson et al. 2014), and the oceans were largely rich in dissolved iron but poor in sulfur (Walker and Brimblecombe 1985). At this time, productivity was low and fueled by metabolisms such as methanogenesis and anoxygenic photosynthesis (Khareacha et al. 2005, Canfield et al. 2006, Ward et al. 2018c). Following the expansion of oxygenic photosynthesis by Cyanobacteria and higher primary productivity around the Great Oxygenation Event ~2.3 Ga (Fischer et al. 2016, Ward et al. 2016, Crockford et al. 2018, Ward et al. 2018c), the atmosphere and surface ocean accumulated some oxygen, and the ocean transitioned into a state with oxygenated surface waters but often anoxic deeper waters, rich in either dissolved iron or sulfide (Canfield 1998, Poulton et al. 2010, Johnston et al. 2009, Johnston et al. 2010). Many individual environments have been characterized that are interpreted to be analogous to a particular period in Earth history; these include Lake Matano, in Indonesia, interpreted as being analogous to the ferruginous ocean (Crowe et al. 2008), Oku-Okuhachikurou Onsen in Akita Prefecture, Japan, similar to conditions just following the GOE (Takashima et al. 2011, Ward et al. 2017a), and Lake Cadagno in Switzerland and the Black Sea, analogous to conditions hypothesized for euxinic Proterozoic oceans (Canfield et al. 2010, Scott et al. 2008). These analogs are each valuable in their own right, but the major differences in context at each site makes it difficult to isolate individual variables that lead to shifts in microbial community and productivity.

At Jinata Onsen, this range of geochemical conditions is recapitulated over just a few meters, providing a useful test case for probing the shifts of microbial productivity over the course of Earth history as conditions vary over short spatial scales. In particular, the concomitant increase in net primary production at Jinata as the community shifts from lithotrophy toward water-oxidizing phototrophy (i.e. oxygenic photosynthesis) is consistent with estimates for greatly increased primary production following the evolution of Cyanobacteria around the GOE (Sleep and Bird 2007, Ward et al. 2016, Ward et al. 2017a, Crockford et al. 2018, Ward et al. 2018c, Ward and Shih 2018).

The dynamic abundances of redox-active compounds including oxygen, iron, and hydrogen at Jinata may not only be analogous to conditions on the early Earth, but may have relevance for potentially habitable environments on Mars as well. Early Mars is thought to have supported environments with metabolic opportunities provided by the redox gradient between the oxidizing atmosphere and abundant electron donors such as ferrous iron and molecular hydrogen sourced from water/rock interactions (e.g. Hurowitz et al. 2010), and production of these substrates may continue today (Stamenkovic et al. 2018, Dzaugis et al. 2018). Understanding the potential productivity of microbial communities fueled by lithotrophic metabolisms is critical for setting expectations of the presence and size of potential biospheres on other worlds and early in Earth history (e.g. Ward et al. 2017a, Ward 2017, Ward et al. 2018d).

Uncovering the range of microbial metabolisms present under the environmental conditions at Jinata, and their relative contributions to primary productivity, may therefore find application to predicting environments on Mars most able to support productive microbial communities.

Data availability:

Raw 16S and metagenomic sequence data have been uploaded to the Sequence Read Archive (Submission #SUB4558398) and MAGs have been uploaded to Genbank (Submission #SUB4557661). All data will be made publicly available immediately following processing.

Figure 1:

Location of Jinata Onsen on Shikinejima Island, Japan, and inset overview sketch of field site with sampling localities marked.

Figure 2:

Representative photos of Jinata. A) Panorama of field site, with source pool on left (Pool 1 below), Pool 2 and 3 in center, and Out Flow to bay on right. B) Undistorted view north up the canyon. C) Undistorted view south toward bay, overlooking Pool 2. D) Source pool, coated in floc-y iron oxides and bubbling with gas mixture containing H2, CO2, and CH4. E) Pool 2, with mixture of red iron oxides and green from Cyanobacteria-rich microbial mats. F) Close up of textured microbial mats in Pool 3. G) Close up of Out Flow, where hot spring water mixes with ocean water.

T	63°C
pH	5.4
DO	4.7 μM
Fe^{2+}	261 μM
NH_3/NH_4^+	87 μM
Cl^-	654 mM
SO_4^{--}	17 mM
NO_3^-	<1.6 μM
NO_2^-	<2.2 μM
HPO_4^-	<1 μM

Table 1: Geochemistry of source water at Jinata Onsen.

Figure 3:

Summary of geochemical and microbiological trends along the flow path of Jinata Onsen. Top: panoramic view of Jinata Onsen, with source pool at left and flow of spring water toward the bay at right, with sampling locations indicated. Middle: geochemical transect across the spring, showing temperature (°C) and dissolved Fe(II) and O_2 (μM). Bottom: stacked bar chart of relative community abundance of dominant microbial phyla as determined by 16S amplicon sequencing. Sequence data were binned at the phylum level averaged at each sample site. Reads that could not be assigned to a phylum were discarded; all phyla that do not make up more than 2% of the community at any one site have been collapsed to "Other". Near the source, the community is predominantly made up of iron- and/or hydrogen-oxidizing organisms in the Proteobacteria and Aquificae phyla. As the hot spring water flows downstream, it equilibrates with the atmosphere and eventually mixes with seawater, resulting in downstream cooling,

accumulation of oxygen, and loss of dissolved iron due to biological and abiotic processes. Oxygenic Cyanobacteria become progressively more abundant downstream Hydrogen- and iron-oxidizing lithotrophs dominate near the source, but phototrophic Cyanobacteria come to dominate downstream. Additional community diversity is found Supplemental Table 4.

Figure 4:
Phylogeny of Bacteria and Archaea based on concatenated ribosomal proteins. Numbers in parentheses next to phylum labels refer to number of MAGs recovered from Jinata Onsen. Labels for phyla with two or fewer MAGs recovered from Jinata omitted for clarity. Reference alignment modified from Hug et al. 2016. Full list of MAGs recovered available in Supplemental Table 5.

Figure 5: Phylogeny of the Zetaproteobacteria, rooted with Alphaproteobacteria, built with concatenated ribosomal protein sequences. Data from Singer et al. 2011, Mori et al. 2017, Makita et al. 2017, and other draft genomes available on Genbank. All nodes recovered TBE support values greater than 0.7. In cases where reference genomes have a unique strain name or identifier, this is included; otherwise Genbank WGS genome prefixes are used.

Figure 6: Phylogeny of the Calditrichaeota, rooted with Bacteroidetes, built with concatenated ribosomal protein sequences. Data from Kublanov et al. 2017 and other draft genomes available on genomes have a unique strain name or identifier, this is included; otherwise Genbank WGS genome prefixes are used.

Figure 7: Detailed phylogeny of the Chloroflexi phylum, with class-level clades highlighted in gray, built with concatenated ribosomal protein sequences. The large basal class Dehalococcoidia, which was not observed in 16S or metagenome data from Jinata, is omitted for clarity. Contains MAGs reported here, members of the Chloroflexi phylum previously described (Chang et al. 2011, Kuznetsov et al. 2011, Sorokin et al. 2012, Kawaichi et al. 2015, Dodsworth et al. 2014, Hedlund et al. 2015, Ward et al. 2015a, Ward et al. 2015b, Hemp et al. 2015a, Hemp et al. 2015b, Hemp et al. 2015c, Pace et al. 2015, Ward 2017, Ward et al. 2018a, Ward et al. 2018e, Ward et al. 2018f), and members of the closely related phylum Armatimonadetes as an outgroup (Dunfield et al. 2012, Ward et al. 2017). MAGs described here highlighted in green, MAGs previously reported from Jinata Onsen highlighted in pink. All nodes recovered TBE support values greater than 0.7. In cases where reference genomes have a unique strain name or identifier, this is included; otherwise Genbank WGS genome prefixes are used.

Supplemental Figure 1:
Multidimensional scaling plot of Jinata samples. Each point represents the recovered microbial community from a given sample, with sites identified by color and sample type by shape. Samples plotting close to each other are relatively more similar in community composition. Abundance data are transformed by the 4^{th} root to down-weight the effect of abundant taxa. Stress value is 0.0658.

Supplemental Figure 2:

672 Microscopy images of sediment (Source and Pool 1) or mat (Pool 2, Pool 3, and Out Flow). Left
673 are light microscopy images. Center and right are fluorescence images. At center, blue signal is
674 DAPI-stained (Excitation: 365nm, Emission: BP445~50nm). At right, red is autofluorescence
675 signal of Cyanobacteria (BP395~440nm, LP470nm). Scale bars 50 μm.

	pH	T (°C)	Fe(II) (µM)	DO (µM)	Descriptions
Source	**5.4**	**60-63**	**260**	**4.7 (source) 39 (surface)**	**Fluffy red iron oxide precipitate**
Pool 1	**5.8**	**59-59.5**	**265**	**58**	**Reddish precipitate and streamers in shallower regions, more yellowish deeper**
Pool 2	**6.5**	**44.5-54**	**151**	**134**	**Iron oxide-coated microbial mats. Orange to orange-green.**
Pool 3	**6.7**	**37.3-46**	**100**	**175**	**Green or mottled orange-green microbial mats, commonly with 1-5cm finger-like morphology.**
Outflow	**6.5**	**27-32**	**45**	**234**	**Ocean water within mixing zone at high tide, with constant flow of spring water from Pool 2. Thin green microbial mats.**

676 **Supplemental Table 1:** Geochemistry and brief description at sampling sites along the flow path
677 of Jinata Onsen as discussed in the text.

	Average of gas compositions (percent composition)								
Sampling dates (mm/dd/yyyy)	Measurement number	N_2	SE	O_2	SE	CH_4	SE	CO_2	SE
10/03/2017	2	30.5	4.6	0.10	0.01	0.04	0.01	69.3	4.6
04/13/2018	4	55.5	5.5	0.07	0.04	0.05	0.01	44.4	5.0

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679
680 **Supplemental Table 2:** Gas composition of bubbles collected from the Source Pool at Jinata
681 Onsen.
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Sample:	Reads:	OTUs (99%):	Good Coverage (99%):	Shannon Index (99%):	Inverse Simpson (99%):	OTUs (97%):	Goods Coverage (97%):	Shannon Index (97%):	Inverse Simpson (97%):
Source A	26057	9558	0.724	10.594	83.020	4632	0.884	8.196	23.035
Source B	49340	1439	0.790	10.275	44.714	5530	0.932	7.229	12.835

		2							
Pool 1 A	97445	2116 6	0.848	10.128	56.287	10160	0.935	8.080	24.682
Pool 1 B	57250	1055 9	0.872	8.794	33.323	4766	0.945	6.414	12.005
Pool 2 A	41515	1311 4	0.759	9.754	24.340	7710	0.873	8.118	14.702
Pool 2 B	45171	1721 1	0.697	10.708	50.836	10525	0.832	8.980	25.783
Pool 3 A	45148	1598 8	0.722	10.287	33.295	9302	0.853	8.351	16.880
Pool 3 B	29778	1202 3	0.682	10.894	84.725	6625	0.837	8.553	31.520
Outflow A	32382	1774 1	0.542	11.931	57.572	11290	0.738	10.262	28.674
Outflow B	32651	8881	0.797	9.237	28.728	4210	0.909	6.373	9.850

Supplemental Table 3:
Diversity metrics of Jinata sequencing. Diversity metrics calculated for both 99% and 97% sequence identity cutoffs for assigning OTUs.

Supplemental Table 4:
16S data as OTU table with sequences.

Supplemental Table 5:
High- and medium-quality metagenome-assembled genomes (MAGs) (>50% completeness and <10% contamination) recovered from Jinata Onsen.

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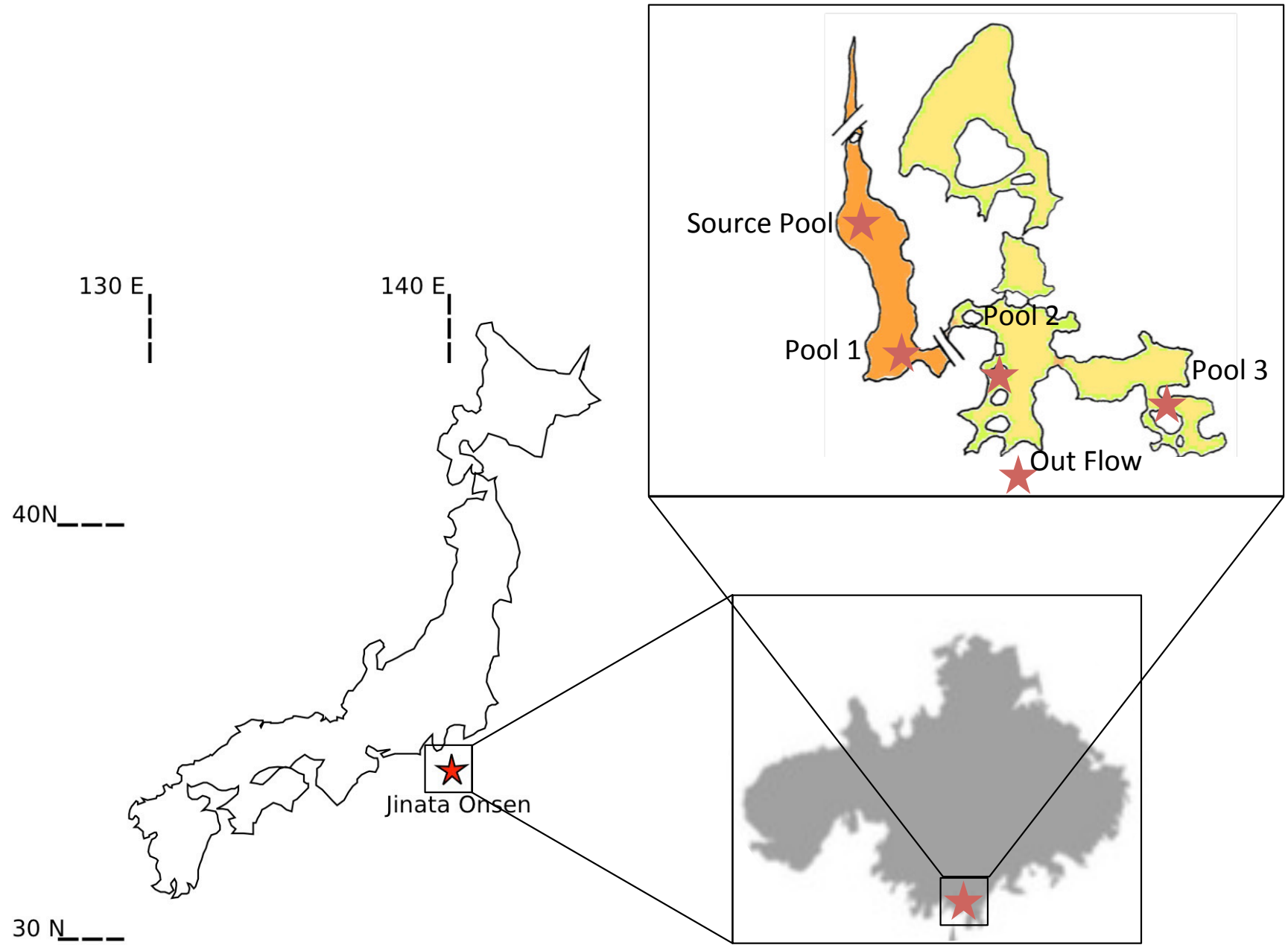
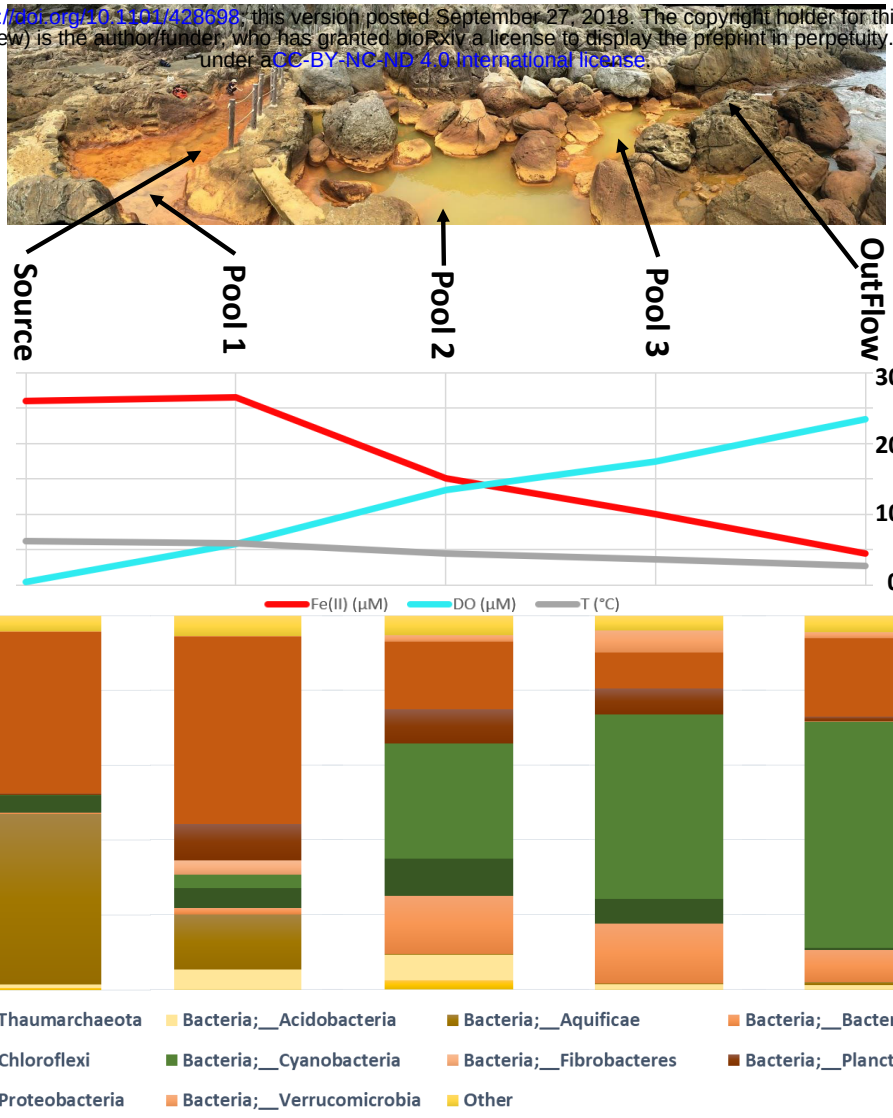
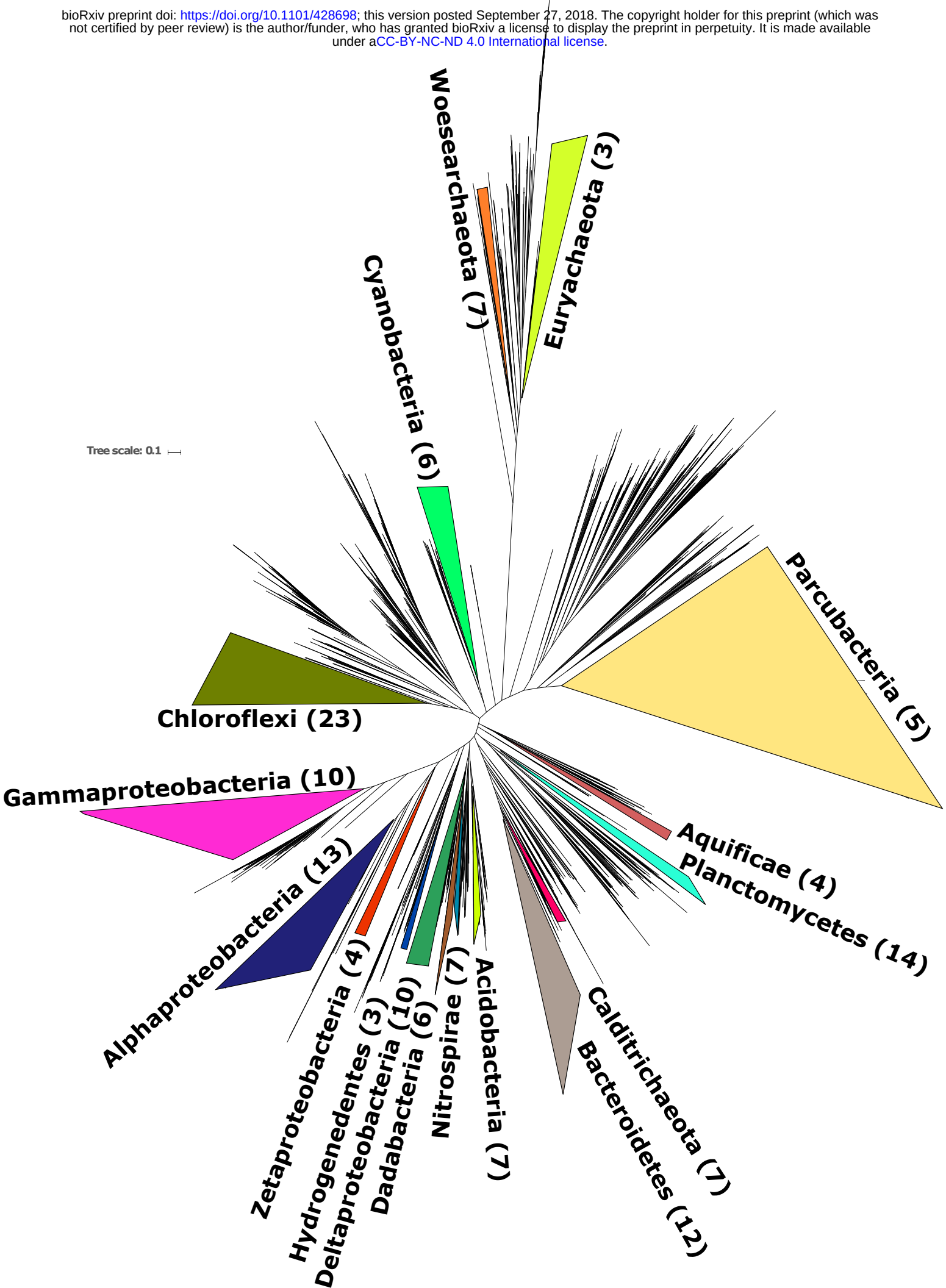
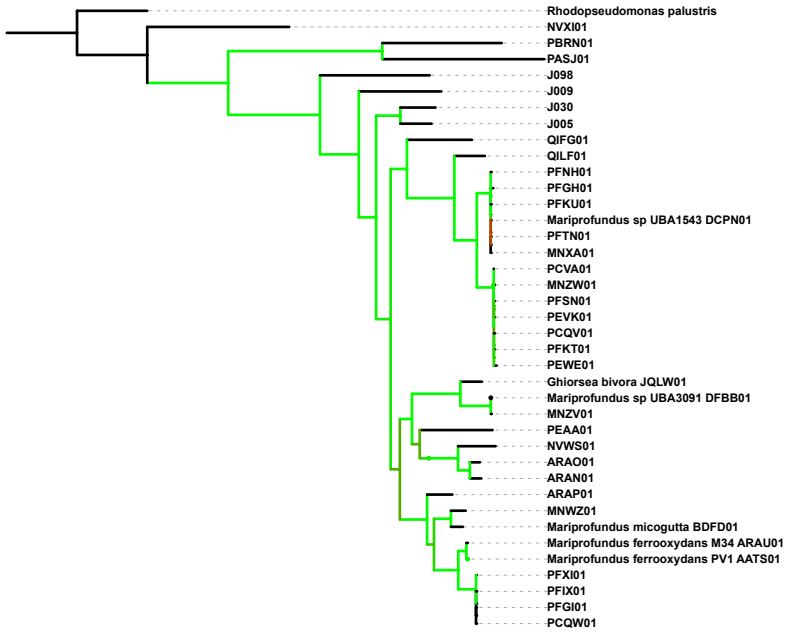

130 E
140 E
40N
30 N
Jinata Onsen
Source Pool
Pool 1
Pool 2
Pool 3
Out Flow

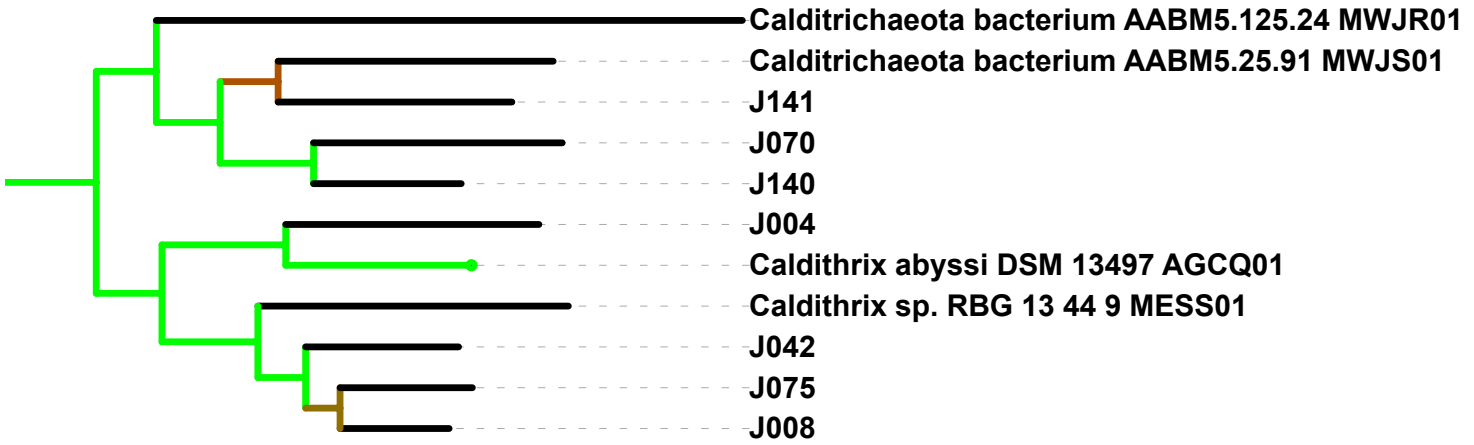

A)
B)
C)
D)
E)
F)
G)





Tree scale: 0.1




Calditrichaeota bacterium AABM5.125.24 MWJR01
Calditrichaeota bacterium AABM5.25.91 MWJS01
J141
J070
J140
J004
Caldithrix abyssi DSM 13497 AGCQ01
Caldithrix sp. RBG 13 44 9 MESS01
J042
J075
J008