

Metagenomic analysis of permafrost microbial community response to thaw

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Permafrost contains an estimated 1672 Pg carbon (C), an amount roughly equivalent to the total currently contained within land plants and the atmosphere¹. This reservoir of C is vulnerable to decomposition as rising global temperatures cause the permafrost to thaw². During thaw, trapped organic matter may become more accessible for microbial degradation and result in greenhouse gas emissions⁴. Despite recent advances in the use of molecular tools to study permafrost microbial communities⁶⁻⁹, their response to thaw remains unclear. Here, we employed deep metagenomic sequencing to determine the impact of thaw on microbial phylogenetic and functional genes and related this data to measurements of methane emissions. Metagenomics, the direct sequencing of DNA from the environment, allows for the examination of whole biochemical pathways and associated processes, as opposed to individual pieces of the metabolic puzzle. Our

metagenome analyses revealed that during transition from a frozen to a thawed state there were rapid shifts in many microbial, phylogenetic and functional gene abundances and pathways. After one week of incubation at 5°C, permafrost metagenomes converged to be more similar to each other than while they were frozen. We found that multiple genes involved in cycling of C and nitrogen shifted rapidly during thaw. We also constructed the first draft genome from a complex soil metagenome, which corresponded to a novel methanogen. Methane previously accumulated in permafrost was released during thaw and subsequently consumed by methanotrophic bacteria. Together these data point towards the importance of rapid cycling of methane and nitrogen in thawing permafrost.

We collected three intact frozen permafrost soil cores with their overlying seasonally thawed active layers from Hess Creek (HC), Alaska. This is a black spruce forest site containing many meters of frozen peat and the C was dated to 1200 ybp¹⁰. Other soil properties and microbial respiration rates were previously characterized¹⁰. Frozen active layer and permafrost layer samples from each core were thawed and incubated for 7 days at 5°C under a He headspace. During the incubations, CH₄ (Fig. 1a) and CO₂ (Supplementary Fig 1) concentrations were monitored in the headspace and DNA was extracted for 16S ribosomal RNA (rRNA) and metagenome sequencing.

There was a burst of CH₄ from the permafrost within 48 h of thaw, followed by a significant ($P = 0.05$) decrease in concentration from day 2 to day 7 (Fig. 1a). To determine (a) if methane release was due to post-thaw production or from trapped gas, (b) whether the methane was consumed by methanotrophs or anaerobic methane oxidizers, and (c) the CH₄ oxidation potential over time, we treated additional samples with 1500

ppm CH₄ and 2-bromoethane sulphonic acid (BES) and measured CH₄ levels daily. BES is an inhibitor of archaeal methanogenesis and methyl-coenzyme M reductase (MCR)-dependent anaerobic methane oxidation. Rapid release of CH₄ from samples treated with BES suggested that the CH₄ primarily originated from gas present in the permafrost prior to thaw (Supplementary Fig. 2), as previously reported¹¹. Subsequent CH₄ consumption in both BES and non-BES-treated samples was indicative of CH₄ oxidation by methanotrophic bacteria (Fig. 1b). The oxygen utilized for methane oxidation presumably originated from permafrost water or aerobic microsites in the samples¹². Together these data indicate CH₄ levels are dynamic in thawing permafrost.

To determine the phylogenetic and functional gene repertoire before and after thaw, we performed deep metagenome sequencing of samples from two of the three replicate cores (cores 1 and 2). DNA was extracted from frozen active layer and permafrost samples and from samples thawed at 5°C for 2 and 7 days. This resulted in 12 samples for metagenome sequencing.

Due to low DNA yield we used emulsion PCR (emPCR) to generate random shotgun short insert libraries with minimum amplification bias¹³. Sequencing yielded a total of 176 million reads and 39.8 Gb of raw sequence. The individual metagenome reads were annotated by comparison to protein-coding and small subunit (SSU) rRNA genes (Supplementary Table 1). We achieved relatively good assembly of the metagenome data, despite the high microbial diversity of soil. In total we obtained 9.7 Mb of sequence in 3758 contigs greater than 1 kb in length. The longest contig was 67.4 kb in length and 98 contigs were greater than 10 kb.

A draft genome of a novel methanogen was constructed from the metagenome data (Fig. 2), due to the relatively high abundance of methanogens in the samples (0.2 – 4% of total 16S rRNA gene sequences) and its low population heterogeneity. To generate the draft genome, contigs were binned by tetranucleotide frequency and read coverage¹⁴. The draft genome was comprised of 1.9 Mb of sequence in 174 contigs. To confirm that the clustered contigs represented a draft genome, we validated the assembly through single copy gene analysis (Supplementary Table 2) and by alignment of the draft genome to a related reference genome (Supplementary Fig. 3). This is the first example of successful assembly of a draft genome from a highly complex soil metagenome. The nearest sequenced relative was the recently described *Methanocellales*¹⁵ order at a nucleotide identity level of ~65% (Fig. 2). Single copy gene analysis demonstrated it was related to members of *Methanomicrobia* (Supplementary Fig. 4). The abundance of this novel methanogen correlates with the observed CH₄ in the samples and suggests that it may be an important player in CH₄ production under frozen conditions. It has previously been reported that trapped CH₄ in permafrost is biological in origin and that methanogenesis can occur at sub-zero temperatures¹¹. The draft genome also included genes for nitrogen fixation. Although nitrogen-fixing methanogens have been previously described¹⁶, this draft genome is the first indication that they are present in permafrost.

The metagenome data revealed core specific shifts in some community members (Fig. 3a), including *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* orders. We found that *Actinobacteria* increased in both cores during thaw (Supplementary Fig 5). *Actinobacteria* have previously been found at high abundance in permafrost⁹, which is thought to be due to their maintenance of metabolic activity and DNA repair mechanisms

at low temperatures¹⁷. Most archaeal sequences identified in the metagenomic data were methanogens in the phylum *Euryarchaeota* (62%-95%), including the *Methanomicrobia* that was represented in our draft genome. In total, four orders of methanogens (*Methanosarcinales*, *Methanomicrobiales*, *Methanomicrobia*, and *Methanobacterales*) were detected. As the permafrost thawed, the methanogens (including *Methanomicrobia*) increased in relative abundance (Supplementary Fig. 6). These orders are known to be metabolically versatile and can utilize a variety of substrates¹⁸.

18S rRNA gene sequences from land plants (*Streptophyta*) were the most abundant Eukaryotic reads in the metagenome data, likely originating from undecomposed detritus. 18S rRNA gene sequences also originated from fungi, protists, amoebae, algae, and other eukaryotic phyla (Supplementary figure 7.) Few consistent changes in the Eukarya were observed after thaw, although the *Streptophyta* decreased in core 2, presumably due to microbial degradation of plant material (Supplementary Fig. 7)

A greater phylogenetic distance was observed between frozen and day 2 samples than between day 2 and day 7 samples (Supplementary Fig. 8), based on 454 pyrotag sequencing of 16S rRNA genes, suggesting that the community composition shifted rapidly upon thaw. The difference was more pronounced in the permafrost than in the active layer. OTUs changing significantly ($P < 0.05$) in abundance during thaw were largely from uncultivated taxa (Supplementary Fig. 9 and Supplementary Table 3).

We used quantitative PCR (qPCR) to measure the absolute abundances of specific phyla before and after thaw. The qPCR results confirmed that there was a significant increase in *Actinobacteria* in both cores after thaw, *Bacteroidetes* changed in a core

dependent manner, and no significant changes were observed in *Chloroflexi* (Supplementary Figure 10).

Our observation that methane was consumed after thaw (Fig. 1) was correlated to detection of sequences representative of bacterial methanotrophs in relatively high amounts (~0.25% to 0.65% relative abundance). Two forms of methane monooxygenases were detected: particulate methane monooxygenase (*pmoA*) represented the majority (~80%) and the rest were soluble methane monooxygenase (*mmoX*). The metagenomic results were confirmed by qPCR of *pmoA*, *mcrA* (encoding the methyl coenzyme-M reductase alpha subunit), and 16S rRNA genes from Type I and Type II methanotrophs. Both the *pmoA* gene and Type II methanotrophs significantly increased in abundance after thaw ($P < 0.01$). Although Type I Methanotrophs were detectable at low levels (<100 copies per ng), they did not differ in abundance between the frozen and thawed samples. *McrA* sequences from methanogenic archaea were detected but did not change significantly during thaw (Supplementary Fig. 11). *McrA* and 16S sequences originating from anaerobic methane oxidizers were not detected. Collectively, these data and our BES incubation experiments (Fig. 1b), suggest that bacterial Type II methanotrophs and the particulate methane monooxygenase enzyme were involved in consumption of methane released during permafrost thaw.

We tracked simultaneous shifts in the total gene complement from the metagenome data to obtain a global view of functional response to thaw. The active layer samples were relatively similar before and after thaw. By contrast, the two frozen permafrost metagenomes differed dramatically prior to thaw (Fig. 3b). In addition, functional genes in frozen active layer and permafrost samples were distinct from each

other, including differences in several key metabolic pathways such as energy metabolism, nitrogen fixation, amino acid transport, oxidative phosphorylation, and anaerobic respiration (Supplementary Fig. 12). During thaw, the permafrost metagenomes rapidly converged and neared those in the active layer samples (Fig. 3b). The convergence of function was not matched by a convergence of phylogenetic composition during this short-term incubation (Fig. 3a), suggesting that disparate community responses to thaw can have similar functional consequences.

The high sequencing depth used provided the sensitivity necessary to characterize subtle changes in the abundance of thousands of genes that had a cumulative significant affect, as confirmed by multiple statistical analyses (Supplementary Figs 13-15 and Supplementary Table 4). We specifically targeted genes involved in C and N cycling by grouping KEGG genes into subsets (Fig. 3c-f). Several genes involved in the N cycle shifted in abundance during thaw (Fig. 3c). For example, nitrate reductase I genes significantly increased, suggesting nitrate was available as a terminal electron acceptor, which was confirmed by its presence in the chemical data¹⁰. Ammonification and denitrification genes increased during thaw in both cores. However, core 1 did not show an increase in the *nosZ* gene, responsible for converting N₂O to N₂, suggesting that it had potential for N₂O greenhouse gas emission during thaw. By contrast, several genes in the nitrogen fixation pathway decreased during thaw in both cores. Changes in the N cycle observed in the metagenomic data were confirmed by qPCR of specific genes for nitrate reduction (*narG*, encoding nitrate reductase I, alpha subunit) and nitrogen fixation (*nifH*, encoding the nitrogenase iron protein), that increased and decreased in abundance after thaw, respectively (Supplementary Fig. 16).

The metagenomic strategy also allowed us to identify changes in a diverse set of C cycle genes not easily accessed by other approaches such as qPCR or analysis of enzymatic activities. These included genes involved in central metabolism that increased during thaw (Fig. 3d). Genes involved in the transport and metabolism of specific carbohydrates were also enriched after thaw, but these changes were mainly core-specific. For example, in core 2, we observed significant increases ($P < 0.01$) in the cellobiose transport system and a corresponding increase in cellulose degradation and hemicellulose degradation (Fig. 3f). Chitin degradation genes and some sugar transport systems were specifically enriched in core 1 (Fig. 3g) (see Supplementary Table 5 for a complete list). It is important to keep in mind that decreases in relative abundance may also be due to growth of other community members lacking those particular genes. These data reflect a rapid response of specific members of the microbial community in permafrost to thaw, likely due to the availability of particular substrates that become accessible. Although the cores were taken from the same site within meters of each other and would presumably have similar chemical and physical properties, there were subtle differences between the genetic responses to thaw in the replicate cores. For example, we observed a higher level of dissolved organic carbon (DOC) and higher C density in core 2 (Supplementary Table 6) that is correlated with the core 2 specific enrichment of C processing genes during thaw. These findings emphasize the importance of replication, even for complex soil metagenomes.

In summary, these detailed analyses reveal for the first time the rapid and dynamic response of permafrost microbial communities to thaw. The thaw-induced shifts that we detected directly support conceptual models of C and N cycling in arctic soils

(Fig. 4), in which microbes play a central role in greenhouse gas emissions and destabilization of stored permafrost C.

Methods Summary

Triplicate frozen permafrost cores were collected from Hess Creek, Alaska to a depth of 1 m. Active layer (~35cm depth) and permafrost (~85 cm depth) samples were cut from the frozen cores using a band saw in a -20°C cold room. Approximately 10 g of frozen soil were incubated at 5°C for one week under a He headspace. CH₄ and CO₂ concentrations were measured on days 2 and 7 of incubation. In separate experiments, CH₄ oxidation was measured in permafrost samples incubated with 2-bromoethane sulphonic acid and CH₄ (1500 ppm). CH₄ was subsequently measured in the headspace every 24 h for 7 days.

Shotgun metagenomic sequencing was conducted on DNA extracted from two replicate cores (active layer and permafrost) while frozen and after 2 and 7 days of incubation. Since the DNA yield was too low to prepare standard sequencing libraries, we used emPCR^{13,19}, for amplification prior to sequencing. This method escapes biases common in other amplification methods. The DNA was sequenced using the Illumina GAII platform with 2 x 113 bp cycles. Metagenomic reads were annotated by comparison to the KEGG genes database²⁰ using BLASTX²¹ ($e \leq 1 \times 10^{-5}$) and to the Greengenes²² and SILVA²³ databases using BLASTN²¹ ($e \leq 1 \times 10^{-20}$). For assembly, raw reads from all samples were combined. After quality filtering and dereplication, k-mers (32-mers) with a depth less than two were removed from the dataset¹⁴. The filtered reads were then assembled using Velvet²⁴. Approximately 10 assemblies were

generated, and the one generating the longest contigs was chosen for further analysis. Contigs were annotated through the Integrated Microbial Genomes & Metagenomics (IMG/M) system²⁵. To group contigs into a draft genome, large contigs (>15 Kb in length) were clustered based on tetranucleotide frequency and coverage. The largest bin was grown by the recruitment of smaller contigs to the large cluster¹⁴.

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Author Contributions J.J., M.W., and K.D. conceived the incubation experiments. M.W. collected the samples. M.W. and S.B. conducted the incubation experiments. R.M., K.C., and K.D. performed the DNA extractions. R.M. created the shotgun sequencing libraries and conducted bioinformatics analyses. R.M. and K.D. performed statistical analyses. R.M. and M.D. performed qPCR experiments. R.M. and J.J. wrote the paper. All authors discussed the results and commented on the manuscript. E.R. M.W. and J.J. obtained funding for the study.

Author Information All annotated assembled sequences were incorporated into the Integrated Microbial Genomes & Metagenomes (IMG/M) system and are available at http://img.jgi.doe.gov/cgi-bin/m/main.cgi?section=TaxonDetail&page=taxonDetail&taxon_oid=2067725009. Raw Illumina and 454 pyrotag sequence reads and a list containing the subset of contigs belonging to the draft methanogen genome are available at https://www.jgi.doe.gov/downloads/Permafrost_metagenome. Reprints and permissions will be available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to J.K.J. (jrjansson@lbl.gov).

Figure legends

Figure 1. Bacterial oxidation of trapped CH₄ released from HC permafrost after thaw.

a, CH₄ headspace concentration during incubation of permafrost and active layer samples at 5°C for 2 and 7 days. Values are means ± standard error. **b**, CH₄ headspace concentrations during 7 days incubation at 5°C from permafrost samples without BES and with BES. Values are normalized to CH₄ concentrations after the initial release of CH₄ during thaw (day 1).

* $P = 0.05$ ($n = 3$).

Figure 2. Draft methanogen genome assembly.

Draft methanogen genome. Features correspond to concentric circles, starting with the outermost circle: (1) Illumina sequence coverage averaging 73 X, (2) 174 contigs making up the draft genome. Contigs shown are scaled according to size and are roughly ordered by mapping to the reference genome (65% identity at the nucleotide level). (3) GC content heat map (dark blue to light green = low to high values). (4) Methanogenesis genes (orange) and nitrogen fixation genes (blue). The true size of the genome is not known due to gaps between the contigs.

Figure 3. Thaw induced shifts of phylogenetic and functional genes in metagenomes.

a, nMDS analysis of the relative abundance of 16S rRNA genes from the metagenomes. **b**, Principle component analysis of relative abundance of KEGG genes in metagenomes. The percent of variation explained by the principle components is indicated on the axes. Arrows illustrate rapid shift in functional gene composition upon thaw in two disparate permafrost samples. **c-f**, Heat maps indicating differences in relative abundances of specific genes between frozen (Day 0) and thawed (Day 7) permafrost metagenomes (HC

cores 1 and 2). **c**, Nitrogen cycle **d**, central metabolism **e**, cellulose degradation **f**, chitin degradation, sugar metabolism and transport.

Figure 4. Conceptual model of C and N cycling in arctic soils.

Slow rates of methanogenesis by cold adapted methanogens occur in permafrost and active layer soils. Over time, methane accumulates in the permafrost and is initially consumed by bacterial methanotrophs upon thaw. In the permafrost, N₂ fixation genes are abundant. As the permafrost thaws, microbial degradation of organic C occurs rapidly and other nitrogen sources become available with an increase in dissimilatory and assimilatory nitrate reduction processes.

Methods

Soil sampling

We obtained soil cores from a site in Interior Alaska that represents a C-rich form of permafrost. This site is a lowland soil near Hess Creek (HC), AK, north of Fairbanks, AK and south of the Yukon River off the Dawson Highway (65°40'12.84" N, 149°04'36.24" W). The HC soil contains large quantities of organic matter in both the active layer and permafrost depths ¹⁰. The ambient temperature of the soil was approximately -2 °C. Both sites have an overstory of black spruce (*Picea mariana*).

Soil cores were collected in March 2007 to a depth of 1 m while the soils were still frozen to the surface. The permafrost table began at 63 cm depth. Observed variability of active layer thickness is generally low in Central Alaska. Taking into account that the present-day conditions are among the warmest during the last 2-3 thousand years, it is unlikely that thawing reached the sampled permafrost depths (V.

Romanovsky, personal communication). We collected three replicate cores within a ~100 m² area. In the field, cores were scraped free of surface organic matter contaminants that froze to the core during the drilling process, wrapped in aluminum foil, placed into 4 inch-diameter PVC tubes and capped. Cores were taken to the Cold Regions Research and Engineering Laboratory (CRREL) at Fort Wainwright Army Base in Fairbanks, AK. In a large -20 °C cold room, cores were again scraped of surface organic matter with sterile blades and cut into segments using a band saw. We selected sub-samples for sequencing according to depth: active layer (Core 1: 25-35cm; Core 2: 20-30cm; Core 3: 20-30cm) and permafrost horizon (Core 1: 75-85cm; Core 2: 75-100cm; Core 3: 85cm-95cm). Metadata collection and physical and chemical data for these samples are described elsewhere ¹⁰. Briefly, these data showed that nutrients and labile C fractions were available to support microbial growth.

Incubation experiments

Frozen soil cores were cut using a band saw to excise experimental soil aliquots. Ice lens features and historical measurements at the sites indicate that the permafrost surface was at approximately 50-60 cm. Approximately 10 g of intact frozen soil were added to autoclaved glass jars (237.5 mL). The jars were sealed with septa fitted tops and were flushed with high purity He at 15 psi for 45 s and were then incubated at 5° C in the dark. 10 ml of headspace was taken from the jars immediately after the soil was added and after 2 and 7 days incubation during which the soil thawed. The gas samples were injected into He-cleared gas vials for methane measurements via GC-FID. CO₂ concentrations in the headspace gas were measured at time zero, immediately after being placed in the cold room, and on days 2 and 7 using 2ml syringe injections of headspace

gas into an infrared gas analyzer. All measurements were performed in triplicate.

For the CH₄ oxidation experiments, 5 g of soil was added to autoclaved glass jars (120 mL). Bromoethanesulfonate (BES) (190 mM) was added to a subset of the samples to a final concentration of 10 mM. An equivalent volume of sterile H₂O was added to non-BES incubations. The jars were sealed with septa fitted tops and flushed with He at for 45 s. Methane was added to all samples with a final average concentration of 1500 ppm and they were incubated at 5°C in the dark. Headspace CH₄ was measured every 24 h for 7 days via GC-FID. All treatments were incubated in triplicate.

DNA extraction

Three DNA extractions per sample were conducted according to a modified bead-beating protocol²⁶⁻²⁸. Approximately 0.5 g of soil was added to 2.0 ml tubes containing 1.4 mm ceramic spheres, 0.1 mm silica spheres, and one 4 mm glass bead (MP Biomedicals). Hexadecyltrimethylammonium bromide (CTAB) extraction buffer containing 10% CTAB in 1M NaCl, 0.1M ammonium sulfate, and 0.5ml phenol:chloroform:isoamylalcohol (25:24:1) were added and shaken in a FastPrep Instrument (MP Biomedicals) at 5.5 m s⁻¹ for 30 s. Following bead beating, the samples were extracted with chloroform and precipitated in a PEG 6000/1.6M NaCl solution. Pellets were washed with 70% ethanol and resuspended in molecular biology grade water. The three extractions were combined at this step and purified using an AllPrep DNA/RNA kit (Qiagen). Extracts were quantified using Quant-iT dsDNA HS assay kit (Invitrogen) according to the manufacturer's manual.

16S rRNA gene sequencing and analysis

Each sample was amplified with the primer pair 926f/1392r as described in Kunin et al ²⁹. The reverse primer included a 5 bp barcode for sample multiplexing during sequencing. After PCR, samples were purified using a MiniElute PCR purification kit (Qiagen) and quantified using the Agilent Labchip System. Samples were then pooled at equal concentrations. Sequencing of the amplicons was performed using Roche 454 GS FLX as per the manufacturer's instructions.

Sequences were analyzed using the software tool PyroTagger ³⁰. Sequences that were less than 220 bp in length and those with greater than 3% low quality bases (quality score < 27) were removed. Unique sequences were clustered at 97% identity using the Uclust option ³¹. Cluster representatives were classified using Greengenes ²². The phylogeny of cluster representatives was inferred using an approximate maximum likelihood method designed for large alignments as implemented by the software FastTree ³². The weighted UniFrac metric ³³ was used to quantify differences in community composition. Statistical tests for differentially abundant operational taxonomic units (OTUs) were made using the Metastats methodology ³⁴ with 1000 permutations to compute nonparametric *P*-values.

Emulsion PCR paired-end library generation

Starting material for library generation ranged from 1 ng to 10 ng depending on the total amount of DNA obtained from the extractions. The DNA was sheared to 300-500 bp in 100 µl round-bottom glass tubes on a Covaris-S instrument (Covaris). Ends were repaired using the End-It DNA Repair Kit (EPICENTRE Biotechnologies) according to the manufacturer's instructions. End-repaired DNA was purified using

phenol:chloroform:isoamylalcohol (25:24:1), precipitated in ethanol, and resuspended in molecular biology grade water.

Illumina paired-end (PE) linkers were ligated to the end-repaired DNA by adding 1 μ l PEG, 1 μ l 10X ligation buffer, 1 μ l T4 DNA ligase (5U/ μ l) (Fermentas), 20 μ M each of the Illumina PE adapters, and incubated at 22°C for 1 h. DNA was purified using a MiniElute Reaction Cleanup kit (Qiagen).

Standard library creation protocols were not used due to low DNA yield (only a few nanograms per sample). Therefore, Illumina PE libraries were made via linker-mediated emulsion PCR (emPCR) amplification as described previously^{13, 19}. This method theoretically escapes bias common in other methods, such as multiple displacement amplification (MDA), by isolating DNA molecules in aqueous droplets (15). Briefly, a PCR mix was created using the linker-ligated DNA, 10X Pfu PCR buffer, BSA, dNTPs, primers, water, and Pfu Turbo DNA polymerase (Stratagene). 50 μ l was reserved as a non-emulsion control and the rest was added dropwise to 400 μ l of an oil-surfactant mixture. The resulting emulsion was subjected to 40 PCR cycles. The emulsion was broken by addition of diethyl ether and DNA was recovered using chloroform and subsequently by using a MiniElute PCR Purification kit. Products were run on a 2% low melt agarose gel and 250-500 bp sized products were cut from the gel and purified using a MiniElute Gel Extraction Kit (Qiagen). Libraries were quantified on a Bioanalyzer DNA 1000 chip (Agilent). Sequencing was performed according to the manufacturer's instructions (Illumina). The flow cell was sequenced using Illumina GAII technology generating 2x113 bp paired end reads with an average insert size of approximately 300 bp.

Annotation of metagenomic sequence reads

Metagenomic reads were annotated through comparison to the Kyoto Encyclopedia of Gene and Genomes (KEGG) database²⁰ using BLASTX²¹ at an e-value cutoff of 1×10^{-5} . Relative abundance of each gene was determined by taking the number of hits to that gene in a given sample and dividing by the total number of hits to the KEGG gene databases. Small subunit ribosomal RNA gene sequences in the metagenome were identified through BLASTN²¹ by comparison to the Greengenes database²² (16S) and the Silva database²³ (18S) at an e-value cutoff of 1×10^{-20} . Methane monooxygenase (*mmo*) genes were identified through additional comparisons to all known *mmo* genes in the NCBI nt and nr nonredundant databases (BLASTX, e-value cutoff: 1×10^{-5}). Each putative *mmo* read was checked against the entire KEGG genes database and was discarded if it matched another gene with a higher bit score.

Statistical analyses

Ordination based on the relative abundance of KEGG genes was performed using principle component analysis (PCA). Identification of individual genes differing in relative abundance between the frozen active and permafrost layer samples was conducted using a binomial test implemented in the R package³⁵ ShotgunFunctionalizerR³⁶. Genes were considered enriched in a given layer if the relative abundance was significantly different between layers from both cores. *P*-values were corrected for multiple tests using the Benjamini-Hochberg correction factor³⁷.

For comparisons between the pyrotag and metagenome data, phylogenetic information was extracted from the metagenomes using MEGAN³⁸. Hits corresponding to specific taxa were retained if their bit scores were within 10% of the best bit score, and

if the minimum score was 35 as suggested for short reads. Additionally, singleton hits were excluded from the analysis. For analysis of pyrotag data in comparison to the metagenomic data, a subset of the data was made of only the samples whose metagenome were also sequenced ($n=12$). Based on this subset, any taxa (cluster) was excluded that was only represented once, as was the case for the metagenome dataset. These analyses were carried out in R³⁵. Ordination of taxonomic community data was carried out using the ordination method nonmetric multidimensional scaling with a Bray-Curtis distance measure. Where appropriate, *P*-values were corrected for multiple tests using the Benjamini-Hochberg correction factor. Non-parametric analysis of variance based on dissimilarities was carried out using adonis function in the vegan package of R³⁹. Hierarchical clustering was performed using the R package pvclust⁴⁰, with 10,000 bootstrap replications.

Response of C and N cycle genes to thaw

To determine the change in genes relevant to C and N after thaw, we selected a subset of KEGG genes based on C and N cycle pathways. Difference in abundance was determined by comparing the relative abundance at the frozen and Day 7 time points and calculating the log₂ fold change.

Metagenome assembly and annotation

All sequence data was combined, screened, and trimmed using a k-mer based filtering approach. K-mers with a depth less than two were removed as described previously¹⁴ because they are not expected to contribute to the assembly and because the reduction reduces memory requirements for subsequent assembly steps. Filtered sequences were assembled by Velvet²⁴ from all reads using a k-mer length of 51,

insertion length of 300 bp, and an expected coverage of 100. Coverage was calculated by mapping all reads back to the contigs using BWA at default parameters⁴¹. The assembly used approximately 5% of de-replicated reads. All contigs were submitted to the US Department of Energy Joint Genome Institute Integrated Microbial Genomes & Metagenomes (IMG/M) system (<http://img.jgi.doe.gov/cgi-bin/m/main.cgi>)²⁵ for gene calling and functional annotation.

BLASTN searches of all contigs were performed against the NCBI-nt nonredundant database. Assignment to phylogenetic groups at the level of class by was done using MEGAN³⁸ at default parameters. By this measure, ~80% of the large contigs (>10 Kb in length) originated from methanogenic taxa. Therefore, we focused our further assembly efforts on methanogens. Larger contigs were clustered by genome based on tetranucleotide frequencies (TNF) and coverage, two properties expected to be present in contigs derived from the same genome¹⁴. Briefly, Hierarchical Agglomerative Clustering using Euclidean Distance (maximum distance: 0.13) was performed on all contigs greater than 15 Kb in length. The largest cluster was manually inspected and contigs with a coverage that differed from the mean by more than one standard deviation were removed. The largest bin was grown by recruiting the remaining contigs (<15 Kb) where the TNF distance was < 0.18. Coverage cutoff was set at one standard deviation from the mean.

For the single copy gene analysis, we selected 19 protein encoding genes (*rpl1p*, *rpl2p*, *rpl3p*, *rpl4lp*, *rpl5p*, *rpl6p*, *rpl11p*, *rpl13*, *rpl14p*, *rpl19e*, *rps9p*, *rps10p*, *rps11p*, *rps13p*, *dnaG*, *pyrG*, *nusA*) that were identified previously by Wu and Eisen⁴² to be universally distributed in bacteria, exist as single copy genes, and are recalcitrant to

lateral gene transfer. Because the Wu and Eisen study focused on bacteria, we further confirmed that the selected genes exist unambiguously as single copy genes in all methanogens with a sequenced genome. The occurrence of each gene in the draft genome was determined from the IMG/M functional annotations.

For phylogenetic analysis, we selected a subset of the 19 single copy genes (*rpl1p*, *rpl2p*, *rpl3p*, *rpl5p*, *rpl6p*, *rpl19e*, *rps11p*). These genes were extracted from the draft genome and downloaded from 15 methanogen genomes (NCBI accession numbers: NC_009712, NC_003551, NC_000916, NC_013790, NC_007681, NC_014658, NC_000909, NC_014002, NC_007955, NC_009464, NC_014570, NC_009051, NC_008942, NC_007796), selected to represent the diversity of known methanogens. DNA sequences were concatenated and then aligned using MUSCLE³¹. Low confidence parts of the alignments were trimmed using gblocks at default settings⁴³. A phylogenetic tree comparing the draft genome with other methanogen genomes was inferred using FastTree³².

For further validation of the draft genome, we selected a related reference genome determined by phylogenetic analysis of the single copy genes. The draft genome was compared to its sequenced relative (NC_009464) using BLASTN with an e-value cutoff of 1×10^{-5} and the penalty for a nucleotide mismatch set at -1. A given base in the reference genome was considered covered if it was part of the alignment. The number of times each base in the reference genome was covered by draft genome was counted. To determine the number of times any given base is expected to be covered when comparing two finished genomes, the same analysis was conducted comparing NC_009464 with two

genomes (NC_007955 and NC_014002) that were found to be approximately as distant to the reference genome as the draft genome.

Quantitative PCR

We used quantitative PCR (qPCR) to quantify the abundances of *mcrA*, *nifH*, *narG*, *pmoA* functional genes, and the 16S rRNA genes of Type I and Type II methanotrophs. Experiments were performed on unamplified DNA samples in 25 μ l volumes. The PCR conditions and primers are given in Supplementary Table 7. We used the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA). All PCRs were run on an iCycler machine (Bio-Rad Laboratories, Hercules, CA, USA). Reactions consisted of 2 μ l of template DNA (diluted to 2 ng/ μ l) and 1 μ l each of the forward and reverse primers (10 μ M). Standards were created by amplifying the gene fragment of interest from a reference genome (in the case of *mcrA*, *nifH*, *narG*, and the 16S rRNA gene sequences) or by synthesis of the positive control (in the case of *pmoA*) as described previously⁴⁴. PCR products resulting from the amplification were cloned using a Zero Blunt TOPO TA cloning kit and One Shot TOP 10 chemically competent cells (Invitrogen, Carlsbad, CA, USA).

Methods References

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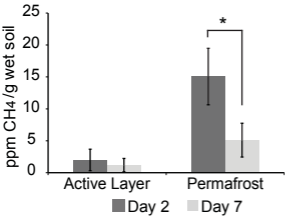
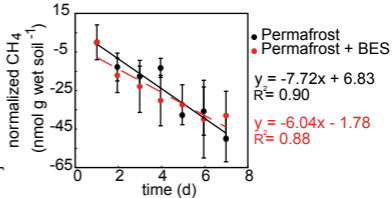
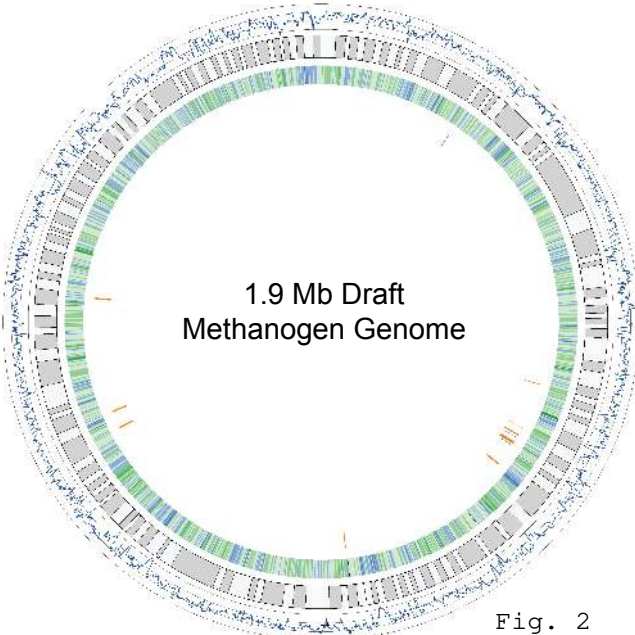
a**b**

Fig. 1



1.9 Mb Draft
Methanogen Genome

Fig. 2

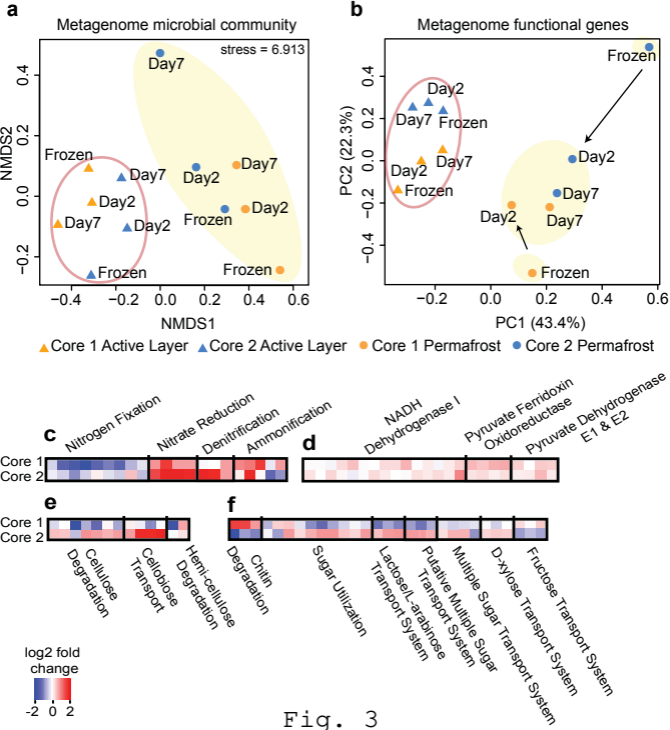


Fig. 3

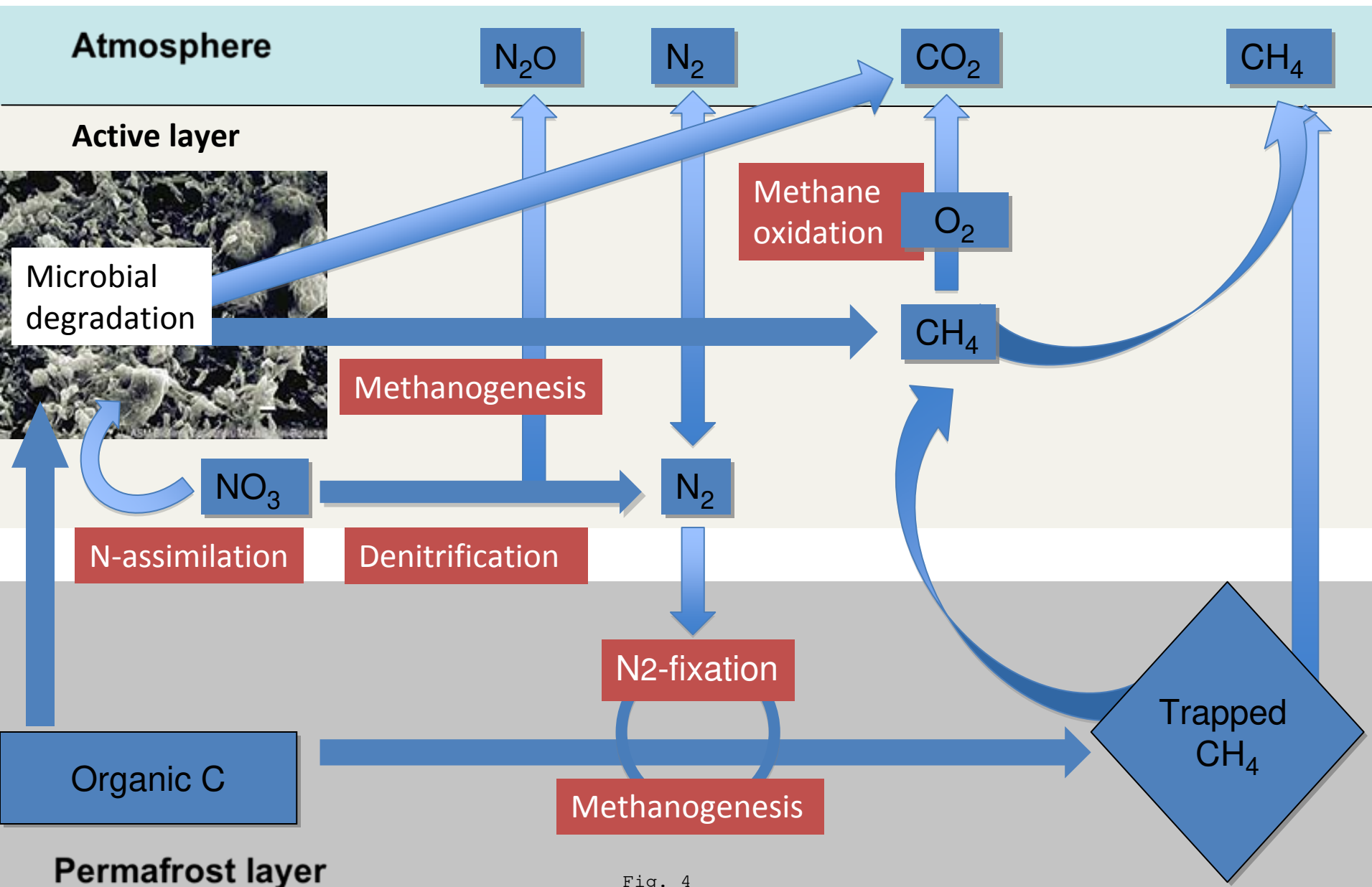


Fig. 4

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