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Research Article

RESEARCH ARTICLE

Methanogens in the Antarctic Dry Valley permafrost

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One sentence summary: The genome of methanogen Ant1 of the genus Methanosarcina reported for the first time from the 15 000 years old permafrost of the Antarctic Dry Valleys contains proofs of cold-adapted enzymes and pathways.

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ABSTRACT

Polar permafrost is at the forefront of climate change, yet only a few studies have enriched the native methane-producing microbes that might provide positive feedbacks to climate change. Samples Ant1 and Ant2, collected in Antarctic Miers Valley from permafrost sediments, with and without biogenic methane, respectively, were evaluated for methanogenic activity and presence of methanogens. After a one-year incubation of both samples under anaerobic conditions, methane production was observed only at room temperature in microcosm Ant1 with CO_2/H_2 (20/80) as carbon and energy sources and was monitored during the subsequent 10 years. The concentration of methane in the headspace of microcosm Ant1 changed from 0.8% to a maximum of 45%. Archaeal 16S rRNA genes from microcosm Ant1 were related to psychrotolerant *Methanosarcina lacustris*. Repeated efforts at achieving a pure culture of this organism were unsuccessful. Metagenomic reads obtained for the methane-producing microcosm Ant1 were assembled and resulted in a 99.84% complete genome affiliated with the genus *Methanosarcina*. The metagenome assembled genome contained cold-adapted enzymes and pathways suggesting that the novel uncultured *Methanosarcina* sp. Ant1 is adapted to sub-freezing conditions in permafrost. This is the first methanogen genome reported from the 15 000 years old permafrost of the Antarctic Dry Valleys.

Keywords: Antarctic; permafrost; metagenome; methane; methanogen; *Methanosarcina*

INTRODUCTION

Recent years have been marked by particular interest in the problem of increased greenhouse gas (GHG) emissions in permafrost regions (Graham *et al.* 2012), with carbon dioxide and methane as the main components. Methane, the most potent GHG, is emitted to Earth's atmosphere from a variety of natural and human-influenced sources, including biogenic methane produced by methanogenic archaea (Bapteste *et al.* 2005). While the production of methane by methanogenic archaea has been recorded across many sub-surface environments, there are no data reporting the existence of viable methanogens in Antarctic permafrost.

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Antarctic permafrost has little organic carbon, ranging from 0.01% in the Dry Valleys up to 1.5% in the Antarctic coastal oases (Gilichinsky *et al.* 2007). Nevertheless, an estimated 21 × 10^{18} g of organic carbon is buried beneath the Antarctic Ice Sheet (Wadham *et al.* 2012). Therefore, as snow- and ice-free areas and thickness of permafrost active layers expand with climate change the liberated organic matter may stimulate microbial methanogenesis, adding to the release of methane trapped in permafrost. Previous studies have demonstrated the presence of methane in bottom sediments of Antarctic lakes (Franzmann *et al.* 1992; Smith, Miller and Howes 1993; Karr *et al.* 2006) and the potential for methane hydrate accumulation in sedimentary basins beneath the Antarctic Ice Sheet (Wadham *et al.* 2012). Methane was found in permanently frozen sedimentary rocks in ice-free Antarctic lake and marine sediment oases, as well as in sediments of temporary melt water streams. Biogenic methane (330 μmol kg^{-1}) was discovered in marine deposits on Bellingshausen Station, King George Island (Karaevskaya *et al.* 2014) and in lake sediments of Larsemann Hills (230 μmol kg^{-1}) (Demidov *et al.* 2013). Biogenic methane was recorded in epicryogenic middle Pleistocene lake sediments of Dry Valleys (Wilson *et al.* 2002; Gilichinsky *et al.* 2007). Gilichinsky *et al.* (2007) also showed the presence of abiogenic methane and its homologs (ethane and propane) and ethylene in quartzfeldspathic gravels, sands and sediments of the Sirius Group.

Despite a wide distribution of methanogenic archaea in Arctic permafrost affected soils (Ganzert *et al.* 2007; Wagner and Liebner 2010), only a few strains (*Methanobacterium arcticum* $M2^T$, *Methanobacterium veterum* $MK4^T$ and *Methanosarcina* sp. JL01) have been isolated from Arctic permafrost (Rivkina *et al.* 2007; Krivushin *et al.* 2010; Shcherbakova *et al.* 2011). In Antarctica, psychrophilic *Methanogenium frigidum* and mesophilic *Methanococcoides burtonii* were both isolated from the anoxic methane-saturated hypolimnion of Ace Lake (Franzmann *et al.* 1992, 1997). Studies of contemporary environments in Antarctica such as soil (Berlemont *et al.* 2011), surface snow (Lopatina *et al.* 2016), microbial mats (Varin *et al.* 2012) and surface marine waters (Dickinson *et al.* 2016) using metagenomic approaches were devoted to characterizing the microbial community structure. No metagenome analyses of Antarctic deep subsurface permafrost have been reported. A recent study of the Antarctic permafrost soils up to 0.5 m deep using the 16S rRNA pyrosequencing approach detected archaeal sequences affiliated with *Halobacteria* and *Methanomicrobia* only in surface soils (Goordial *et al.* 2016). In Antarctic deep subsurface permafrost, methanogens were only detected via 16S rRNA gene analysis from the permanently frozen marine deposits of the King George Island (Bellingshausen Station) and sedimentary lacustrine deposits of Bunger Hills Oasis (Oazis II Station) (Karaevskaya *et al.* 2014; Manucharova *et al.* 2016). Viable methanogens in low numbers (2–22 cells g^{-1}) were detected in samples of the Dry Valleys permafrost (Gilichinsky *et al.* 2007), although no isolates were attained. Methane in concentrations from 20 to 330 μmol kg^{-1} was detected in permafrost with temperatures of −0.5 to −1.0°C from the vicinity of Bellingshausen Station (Karaevskaya *et al.* 2014). The anoxic microcosms of these samples showed incorporation of ^{14}C into CH_4 from both bicarbonate ($H^{14}CO_3^-$) and acetate ($^{14}CH_3COO^-$). While these microcosms produced detectable amount of methane, all attempts to isolate methanogenic archaea failed.

Though signatures of biogenic methane and methane production have been shown in some of Antarctic permafrost samples (Gilichinsky *et al.* 2007), there is no documented record of isolation of cultivable methanogens from Antarctic permafrost. In this paper, we show that methanogens can be enriched from ancient Antarctic permafrost that had biogenic methane, suggesting the methanogens can survive extremely harsh freezing conditions and be resurrected. We describe the microbial community in these microcosms using a metagenomic approach that produced a genome of the methanogen *Methanosarcina*.

MATERIALS AND METHODS

Site location, permafrost coring and physicochemical characteristics of the permafrost

The study site (Fig. 1) was located on the delta prograding into the western edge of Lake Miers (78°05′49.4″S 163°48′44.8″E, elevation 190 m) in the Miers Valley, Antarctica (Wilson *et al.* 1996; Gilichinsky *et al.* 2007). In the austral summer of 1995, the permafrost core was aseptically extracted with a fluid-free drilling machine that prevented down-hole contamination (Khlebnikova *et al.* 1990; Shi *et al.* 1997). The DH4-95 core extracted from a depth of 4.98 m was comprised of permafrost with well-sorted coarse to medium-coarse ice-cemented quartz-feldspathic gravel and coarse sands mostly of eolian and fluvial origin. Below 4.98 m to the base of the drill hole only clear sediment-free ice was recovered (Gilichinsky *et al.* 2007). The temperature inside the borehole was −17°C. Permafrost core samples were kept frozen after coring and during transportation; in the laboratory the permafrost samples were stored in a freezer at −20°C until analyzed. The physicochemical composition of the DH4-95 core was reported (Gilichinsky *et al.* 2007). Briefly, pH 7.8–8.05; dry residue 0.45–0.55g L^{-1}; anions (mmol per 100 g): CO_3^{2-} below detection level; HCO 0.3; Cl- 0.2-0.3; NO^{3-} below detection limit; SO_4 0.22–0.5; Ca^{2+} 0.12–0.2; Mg^{2+}/K^+ 0.06–0.08; Na^+ 0.33–0.67.

Sample age and methane concentration

For this study, two samples were selected from the DH4-95 core: sample Ant1 subsampled from 2.04 m and sample Ant2 subsampled from 3.75 m. Ice content was 75% in sample Ant1 and 50% in sample Ant2. Sediment age was determined by thermoluminescence. At 2.9 m depth an age of 20 500 ± 2500 years was measured suggesting an average sedimentation rate of 0.14 m per thousand years (Wilson *et al.* 1996). Ages for samples Ant1 and Ant2 were estimated as ~15 000 and ~30 000 year old, respectively. Sediments recovered from 4.9 m depth (immediately above the basal ice in the DH4-95 drill hole) were deposited during the last interglacial cycle and are older than 30 000 years (Wilson *et al.* 1996).

Permafrost DH4-95 core samples were degassed and the concentration of CH_4 in the pore gas was measured as described previously (Gilichinsky *et al.* 2007) using a headspace method (Alperin and Reeburgh 1985) and a Shimadzu GC-mini-2 (Tokyo, Japan) gas chromatograph with a hydrogen-flame ionization detector and argon as a carrier gas. The $\delta^{13}C$ of methane was measured in the same samples using a gas chromatograph isotope ratio mass spectrometer (GC-IRMS) operating in continuous flow mode (GC, Hewlett Packard 5890 Series II; and IRMS, Finnigan Delta S). Isotope ratios (‰) were given as δ values versus the Vienna Pee Dee Belemnite standard (Coplen 1994). The experimental error of $\delta^{13}C$ was ±0.2‰.

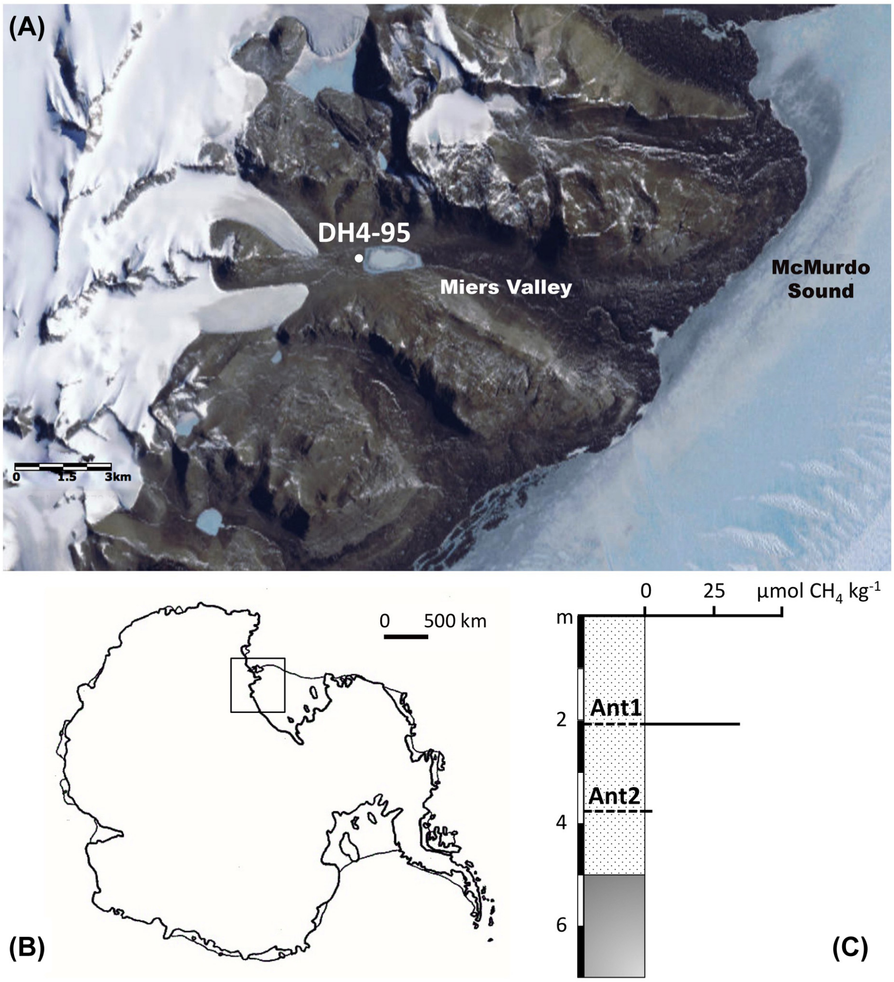

Figure 1. View of Miers Valley obtained using the USGS LandsatLook Viewer, and location of borehole DH4-95 (**A**). Miers Valley located within Victoria Land west of McMurdo Sound (**B**). Position of permafrost samples (Ant1 and Ant2) in the DH4-95 core and *in situ* concentration of methane detected in the pore gas (**C**). Permafrost core: ░ sand, ▓ ice.

Growth medium and microcosm cultivation

Anaerobic microcosms were set up in August 2005 using 1 g of either Ant1 or Ant2 samples and 100 ml of modified anaerobic medium DSMZ 141. During this work, we followed an approach for enrichment and isolation of permafrost methanogenic archaea described previously (Rivkina *et al.* 2007). A total of four microcosms were designed for each sample using either acetate (10 mM) or 80% H_2 and 20% CO_2 gas mixture as substrate, and growth temperatures of 6° or 20°C. The medium DSMZ 141 was modified as follows (per liter of distilled water): 0.34 g KCl, 5.0 g NaCl, 0.4 g $MgCl_2x6H_2O$, 0.25 g NH_4Cl, 1.4 g $CaCl_2x2H_2O$, 0.15 g K_2HPO_4, 0.1 g yeast extract, 0.1 g tryptone, 10 ml vitamin solution (medium DSMZ 141), 10 ml trace element solution (medium DSMZ 320), 0.5 g L-cysteine-HClxH_2O, 0.5 g Na_2Sx9H_2O, 0.50 ml Na-resazurin solution (0.1% w/v), pH was adjusted to 7.0 and the headspace was overpressurized by 1 atm with sterile H_2/CO_2 (80/20).

Trace element solution SL-10 contained in 1 L of distilled water: 10.00 ml HCl (25%; 7.7 M); 1.50 g $FeCl_2x4H_2O$; 70.00 mg $ZnCl_2$; 100.00 mg $MnCl_2x4H_2O$; 6.00 mg H_3BO_3; 190.00 mg $CoCl_2x6H_2O$; 2.00 mg $CuCl_2x2H_2O$; 24.00 mg $NiCl_2x6H_2O$; 36.00 mg $Na_2MoO_4x2H_2O$. First $FeCl_2$ was dissolved in the HCl and diluted in water, after the other salts were added and dissolved.

The vitamin solution contained, in 1 L of distilled water: 2.00 mg biotin; 2.00 mg folic acid; 10.00 mg pyridoxine-HCl; 5.00 mg thiamine-HCl × 2 H_2O; 5.00 mg riboflavin; 5.00 mg nicotinic acid; 5.00 mg D-Ca-pantothenate; 0.10 mg vitamin B12; 5.00 mg p-aminobenzoic acid; 5.00 mg lipoic acid. Vitamins were prepared under 100% N_2 gas atmosphere and sterilized by filtration at 0.2 μm pore size.

All ingredients (except bicarbonate, vitamins, cysteine and sulfide) were dissolved under 80% H_2 and 20% CO_2 gas mixture for 30–45min to make the medium anoxic. Then, bicarbonate was added and dissolved, and the pH of the medium was adjusted to 6.8. The medium (100 ml) was dispensed into 250 ml anaerobic Wheaton^{TM} glass serum bottles under 80% H_2 and 20% CO_2 gas atmosphere and autoclaved. Appropriate volumes of the stock solutions were injected into the sterile medium with hypodermic syringes.

Growth was estimated by measuring the concentration of methane in the gas phase . The measurements were performed from July 2005 through February 2016 as indicated in Fig. 2. The microcosms were subsampled nine times with attempt to obtain methanogen into a pure culture (Fig. 2; green arrows). Every time

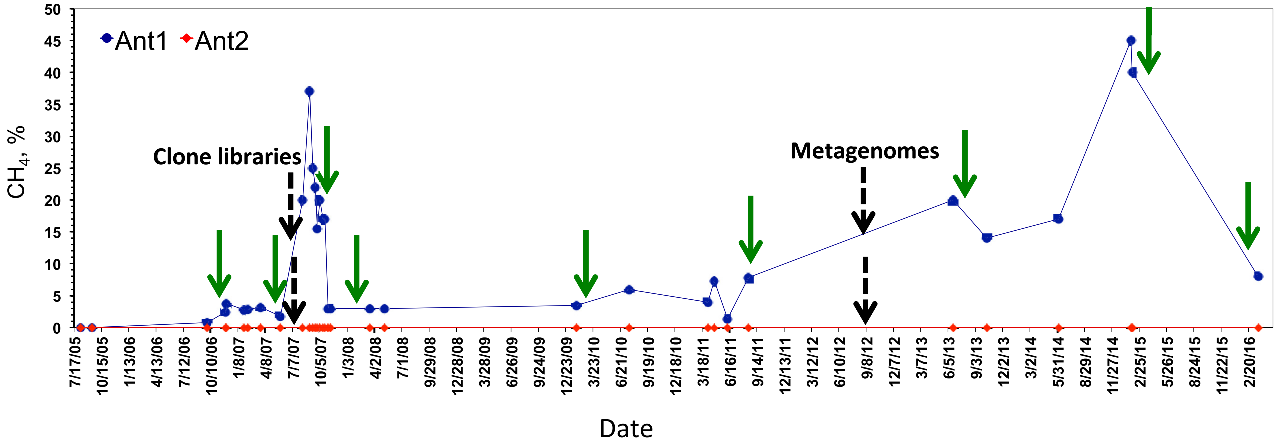

Figure 2. Monitoring of methane oscillation in permafrost microcosms Ant1 and Ant2. Green arrows indicate addition of phosphate-buffered basal medium (DSMZ 141) and gas mixture of H_2/CO_2 (80/20). Black arrows indicate time points when microcosms were sampled for extraction of the genomic DNA.

1 ml of microcosm was transferred in duplicate to a new anaerobic bottle with 5 ml of fresh DSMZ 141 medium under 80% H_2 and 20% CO_2 gas atmosphere and incubated for one year at room temperature. Then, 2 ml of fresh DSMZ 141 medium was added back to microcosms and the headspace was overpressurized by 1 atm with sterile H_2/CO_2 gas mixture. Then, microorganisms from microcosms were tested for ability to grow on the R2A and tryptic soy agar media.

Microscopy

Phase-contrast micrographs were made with a LUMAM Microscope (LOMO, Russia) with 90 × 15 magnification during repetitive examinations of the microcosms. Cell suspensions for microscopic counts were fixed in glutaraldehyde (2.5%), and counts were made in triplicate in a hemocytometer.

Isolation of genomic DNA

Total community genomic DNA (gDNA) was extracted from 1 ml of microcosms (H_2/CO_2, 20°C) for samples subsampled in July 2007 and in quadruplicate for samples subsampled in September 2012 using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). The gDNA from five bacterial isolates was obtained using the UltraClean® Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). The concentration of gDNA was measured using a NanoDrop 2000 (Thermo Scientific Inc.).

Cloning and Sanger sequencing

The 16S rRNA genes were amplified from the total community gDNA using high-fidelity AccuPrime Pfx DNA polymerase (Invitrogen, Carlsbad, CA) and Bacteria- or Archaea-specific primers. The Bacteria-specific primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT TTA CGA CTT-3') were used (Weisburg *et al.* 1991). For amplification of the archaeal 16S rRNA gene the Archaea-specific primers Arch21F (5'-TTC CGG TTG ATC C(C/T)G CCG GA-3') and Arch958R (5'-(C/T)CC GGC GTT GA(A/C) TCC AAT T-3') were used (DeLong 1992; Cytryn *et al.* 2000). The 16S rRNA amplification consisted of initial denaturation for 5 min at 94°C; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min (Archaea) or 2 min (Bacteria); and a final extension at 72°C for 7 min. The genomic DNA from *Shewanella* or *Methanococcus* served as positive controls for amplification of 16S rRNA gene from bacteria or archaea, respectively.

PCR products were purified from UltraPureTM Agarose (Invitrogen) using QIAquick Gel Extraction kit (QUAGEN Inc, Valencia, CA) and ligated into pCR 2.1-TOPO vectors (Invitrogen, Carlsbad, CA), followed by transformation into One Shot Mach1TM-T1R chemically competent *E. coli*. Transformants were plated onto LB agar containing 50 μg ml^{-1} kanamycin and X-gal, and incubated overnight at 37°C. A total of 96 white colonies were selected and regrown in LB broth containing 50 μg ml^{-1} kanamycin overnight at 37°C with aeration. Clones were then sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit and a primer T7 targeting position from plasmid (Invitrogen). Sequences were determined by resolving the sequence reactions on an Applied Biosystems 3730 automated sequencer.

Analysis of Sanger sequences

The 16S rRNA gene sequences for archaeal clones were assign to operational taxonomic units (OTUs) with the furthest clustering algorithm using the mothur v.1.39.1 program (Schloss *et al.* 2009). A total of 16 low quality and chimeric sequences were excluded from analyses. All positions containing gaps and missing data were eliminated. The Neighbor-Joining tree (Saitou and Nei 1987) was constructed in MEGA 7 (Kumar, Stecher and Tamura 2016). Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei and Kumar 2004) and presented in units of the number of base substitutions per site.

Illumina HiSeq sequencing and metagenome analysis

A total community gDNA library was prepared using TruSeq DNA sample prep kit v.2 without whole genome amplification. The Illumina HiSeq 2000 platform was used to acquire paired-end 150 bp metagenomics reads. Demultiplexing of the sequencing data, and generating fastq files were carried out on the Illumina's BaseSpace Sequence Hub. The demultiplexed sequencing reads were uploaded to the MG-RAST automated processing pipeline (Meyer *et al.* 2008). For Ant1, 4.0 Gb of non-assembled reads, representing 23.4 million sequences with an average length of 172 bp and 13.9 Gb of non-assembled reads representing 122.1 million sequences with an average length of 113 bp for

Ant2, were processed in MG-RAST for gene calling and annotation. Comparison of Ant1 and Ant2 metagenomes was done in MG-RAST using the 'best hit classification' function with RefSeq database (Pruitt *et al*. 2012) as the annotation source, a maximum e-value cutoff of 10^{-5}, a minimum percent identity of 60%, and a minimum alignment length of 15 bp. The phenotypic composition of the microbial community in each microcosm was analyzed using METAGENassist (Arndt *et al*. 2012) and the MG-RAST generated taxonomy file.

Genome reconstruction from metagenomic dataset Ant1

Adaptors and low-quality reads were trimmed with the Trimmomatic software (Bolger, Lohse and Usadel 2014) and metagenomic reads were assembled using the metaSPAdes assembler v.3.7 with k-mer size set to 21, 33, 55 and 77 (Nurk *et al*. 2013). Contigs below 1000 bp were culled after assembly. Quality of the assembly was assessed using the QUAST (Gurevich *et al*. 2013). The VizBin application (Laczny *et al*. 2015) was used for visualization and subsequent binning of metagenomic sequences based on similar coverage and k-mer frequency. Gene calling and annotation of protein coding sequences was conducted with PROKKA (Seemann 2014) using combined curated versions of the Uniprot databases for archaea, bacteria and viruses as a reference for the BLAST alignment-based annotations (Seemann 2014). The quality of the binned genome was assessed using CheckM (Parks *et al*. 2015). The 16S rRNA sequences were identified using RNAmmer (Lagesen *et al*. 2007). The average nucleotide identity (ANI) and correlation indexes of tetranucleotide signatures were determined based on BLAST+ by using *in silico* calculator of the JSpecies Web Server (Richter *et al*. 2016).

Protein structure reconstruction

To construct ribbon depictions of the elongation factor 2 (EF2), DeepViewer (Kaplan and Littlejohn 2001) was used. The structural model was generated with SwissModeller and ProMod3 Version 1.0.0 (Guex and Peitsch 1997) using automated homology modeling. Homology modeling was conducted by aligning the target EF2 sequences to the amino acid sequence of a *Sacchromyces cerevisiae* EF2 template from the SWISS-MODEL Template Library (Arnold *et al*. 2006; Kiefer *et al*. 2009). All target EF2 sequences had at least 30% sequence identity and 98% coverage to the template.

Nucleotide sequences

The partial 16S rRNA gene sequences were submitted to the GenBank under accession numbers JX876650–JX876740. The metagenome raw data were uploaded to the MG-RAST as project entitled Antarctic Permafrost Microcosms, ID mgp10234. The assembled metagenome Ant1 was uploaded to the Integrated Microbial Genomes and Microbiomes (IMG/M) under GOLD Project ID: Gp0137128. The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession MCHG00000000 for the entire metagenome and MDTP00000000 for the reconstructed genome (Buongiorno *et al*. 2016). The latest versions are versions MCHG01000000 and MDTP02000000.

RESULTS

Microcosms experiment and methane production

Permafrost sample Ant1 contained 29.0 μmol CH_4 kg^{-1} of sediment, with $\delta^{13}C$ at −54.8‰ indicating a biogenic origin of the CH_4, and 0.06 μl ethylene kg^{-1} of sediment, while sample Ant2 contained 0.3 μmol CH_4 kg^{-1} of sediment, with no biogenic CH_4 detected, and 0.04 μl ethylene kg^{-1} of sediment. These permafrost samples, Ant1 with biogenic CH_4 and Ant2 without any biogenic CH_4 (Fig. 1B), were used in microcosm experiments designed to provoke growth of different groups of methanogens in response to temperature (6°C and 20°C) and type of carbon source (acetate vs. H_2/CO_2). After anoxic incubation for one year, methane production was detected in microcosm Ant1 on H_2/CO_2 (80/20) gas mixture at 20°C, but not in any Ant2 microcosms. There was no methane production detected in Ant1 at 6°C or on acetate. Microcosm growth and methane production in Ant1 were monitored for a decade. The methane concentration in the headspace changed from 0.8% to maximum of 45% (Fig. 2). There was no evident increase in methane production after the addition of either fresh medium or H_2/CO_2 substrate. Total cell numbers were ~3 fold higher in microcosm Ant1 (1.08×10^8 cells ml^{-1}) over microcosm Ant2 (3.68×10^7 cells ml^{-1}). Archaea and bacteria were detected by PCR in Ant1, whereas only bacteria were detected in microcosm Ant2 (Fig. S1, Supporting Information). The archaeal cell number in Ant1 was 5.46×10^7 cells ml^{-1} and these cells produced up to 37% of CH_4 in August 2007 (Fig. 2). The average rate of CH_4 production was estimated during the period from January 29, 2007 to August 31, 2007, to be 0.019 fmol CH_4 $cell^{-1}$ day^{-1}.

Approaches to the isolation of archaeal and bacterial cultures

The phase contrast image of methane-producing microcosm Ant1 shows cells grouped in multicellular aggregates similar to characterized *Methanosarcina* (Fig. 3). An empty shell of a diatom of the genus *Navicula* was also observed under the microscope along with *Methanosarcina*-like cells (Fig. 3). The microcosms were kept in the dark and no visual algae growth was observed suggesting that diatom shell originated from the permafrost sediment. All efforts to isolate methanogenic Achaea into a pure culture were unsuccessful. To determine whether the permafrost microcosms contained bacteria capable of growth on other nutrient media, anoxic Petri dishes were inoculated with 100 μl of both microcosms and bacterial growth was detected on the tryptic soy agar at 20°C only from microcosm Ant2. A total of five bacterial strains were isolated from microcosm Ant2. Based on 16S rRNA phylogenetic analyses, four strains were related to *Microbacterium* (phylum Actinobacteria) and one to *Paracoccus* (phylum Proteobacteria, class Alphaproteobacteria), and these strains did not show any growth during following transfer. Therefore, the microcosms were studied using a culture-independent approach. Samples for the preparation of clone libraries and metagenomes were collected during active methane production (Fig. 2). Yields of the total genomic DNA were 0.43 and 0.15 μg per ml of Ant1 and Ant2 microcosms, respectively.

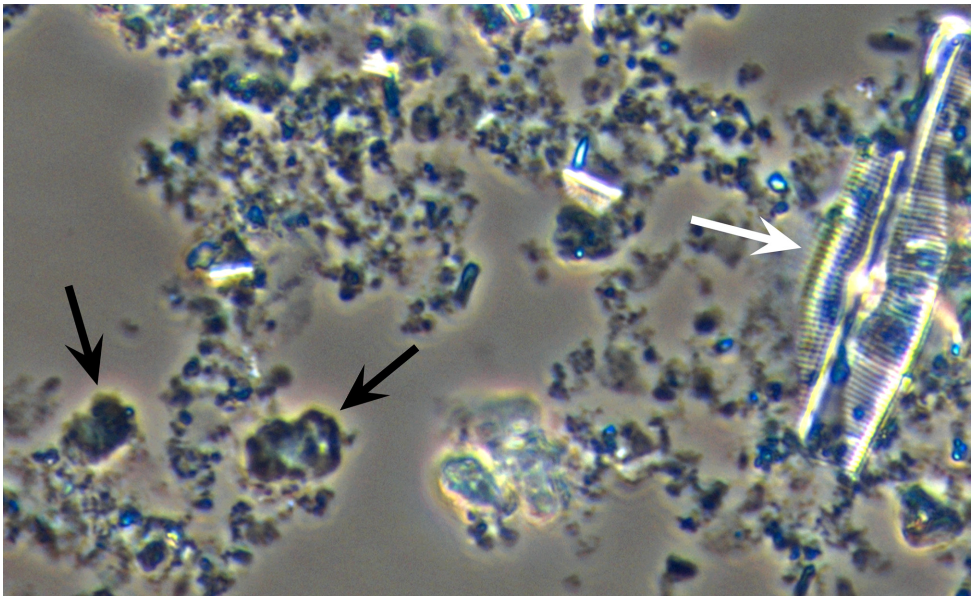

Figure 3. Phase contrast image of methane producing microcosm Ant1, black arrows show *Methanosarcina* cells, which formed multicellular aggregates, white arrow shows shell of diatom of the genus *Navicula*. The diatom shell was preserved in the permafrost and corresponds to the age of permafrost. Bar is 10 μm.

Microcosm community structure based on non-assembled metagenomes

Non-assembled metagenome sequences analyzed using the MG-RAST pipeline showed that metagenomes Ant1 and Ant2 had 0.49% and 0.81% ribosomal RNA genes, 85.54% and 83.97% predicted proteins with known functions, and 13.98% and 15.22% predicted proteins with unknown function. Taxonomic identification was assigned using RefSeq database (Pruitt *et al.* 2012). Microbial community structure based on the most abundant classes (>0.2%) is shown in Fig. 4.

The metagenome from methane-producing microcosm Ant1 contained 48.58% of predicted genes identified as archaeal genus *Methanosarcina*. The microbial community in non-methane-producing microcosm Ant2 contained a low abundance (0.19%) of methanogens from the class Methanomicrobia, and was dominated (82.08%) by Alphaproteobacteria in comparison to 10.42% in Ant1. The Alphaproteobacteria type II methanotrophs (0.85% vs. 0.23%) were more abundant in Ant2 with the dominant genus *Methylobacterium*, followed by *Methylocystis, Methylosinus* and *Methylocella*. On the contrary, the Gammaproteobacteria type I methanotrophs were less abundant in Ant2 (0.04% vs. 0.09%), and were represented by genera *Methylobacter* and *Methylococcus*. Bacteria from the nitrogen cycle were detected in both metagenomes, whereas ammonia-oxidizing bacteria were 10 times more abundant in Ant1 (0.28% vs. 0.02%) while nitrite-oxidizing bacteria were twice more abundant in Ant2 (0.46% vs. 0.28%). Sequences affiliated with *Microbacterium* and *Paracoccus*, bacteria that showed growth in tryptic soy agar, were detected in the metagenome Ant2 at 0.03% and 0.21% abundance, respectively. The abundance of these sequences in the metagenome Ant1 was lower, with < 0.01% *Microbacterium* and 0.06% *Paracoccus*. Sequences related to the diatom of the genera *Phaeodactylum* (the order Naviculales) and *Thalassiosira* (the class Coscinodiscophyceae) were detected in metagenome Ant1 at a low abundance below 0.01%. The METAGENassist analysis (Arndt *et al.* 2012) showed that microcosm Ant1 contained 62.9% anaerobic microorganisms, 14.5% known facultative anaerobes and 21.9% unknown. On the contrary, microcosm Ant2 contained only 0.6% known anaerobic microorganisms, 65.8% of facultative anaerobic microorganisms, and 33.2% unknown. The indicator resazurin remained reduced throughout the experiment and confirmed that conditions stayed anoxic in both microcosms. Other partial genomes binned from the metagenome Ant1 were related to anaerobic bacteria *Alkalibacter* sp., *Lutispora* sp. and *Brevundimonas* sp.

Metagenome assembled genome of *Methanosarcina* Ant1

A draft genome of a novel uncultured methanogen was constructed from the metagenome data, owing to the relatively high abundance of *Methanosarcina* sequences in the microcosm Ant1 (47.68% of all metagenomic reads mapped to the reconstructed genome with an average coverage of 570x). Metagenomic binning (Fig. S2, Supporting Information) resulted in the recovery of a nearly complete genome of a methanogenic archaeon, identified by the presence of the alpha subunit of the methyl coenzyme-M reductase (*mcrA*) gene. The Metagenome assembled genome (MAG) Ant1 was determined to be 99.84% complete by using the Euryarchaeota-specific marker set of housekeeping genes (Parks *et al.* 2015), and had low contamination (1.41%) and 0% strain heterogeneity. The MAG Ant1 comprised 4.3 Mb of sequence in 342 contigs over 1000 bp in length with largest contig of 107 490bp; N50 = 22,152; and L50 = 59 (Buongiorno *et al.* 2016). The draft MAG Ant1 had 38% GC content and a total of 3593 genes, which encoded 3244 putative proteins with 932 proteins referred as hypothetical. It contained 53 tRNAs, 11 predicted CRISPR regions, 29 transposases and 11 cytochromes and number of these genes differed from their number in genomes of related *Methanosarcina* strains (Table S1, Suppoting Information). Two miscellaneous RNAs (misc-RNA) were detected in MAG Ant1, which were fewer than the 5–18 misc-RNA genes in other *Methanosarcina* species (Table S1, Supporting Information). Only one rRNA operon was detected in the MAG Ant1, yet each of 26 finished genomes of the *Methanosarcina* species available in the Integrated Microbial Genomes (IMG), including

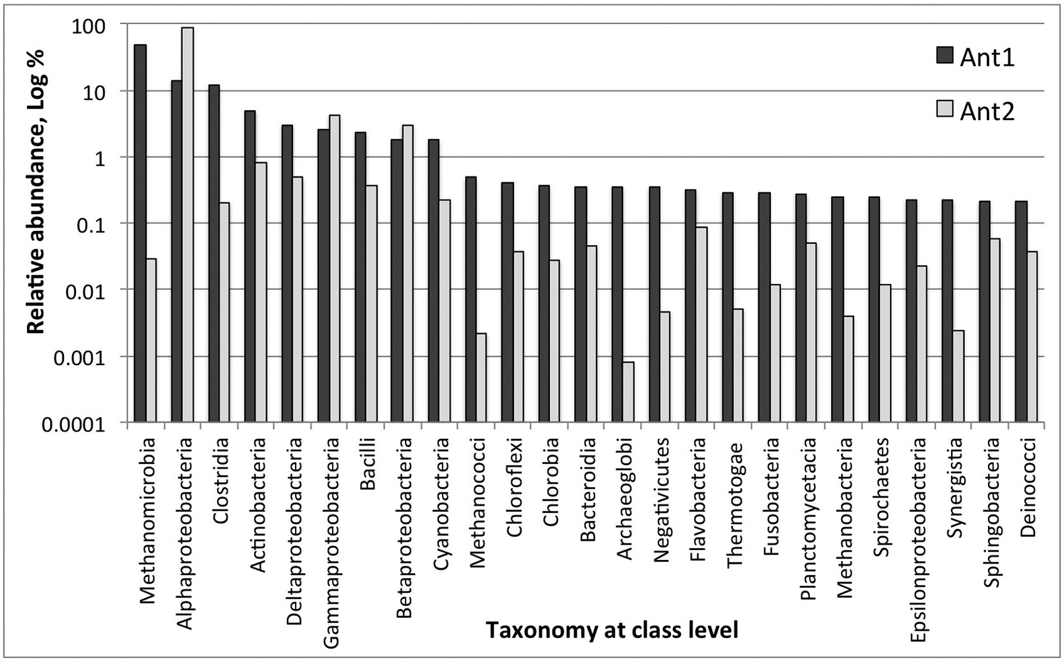

Figure 4. Diversity of conservative genes taxonomically assigned to microbial classes identified in Ant1 and Ant2 metagenomes. Microorganisms detected in abundance >0.2% are shown. Plot constructed at the Log10 scale.

Methanosarcina lacustris Z-7289, has three copies of the rRNA operon.

The average nucleotide identity between the MAG Ant1 and whole-genome sequences of known species of *Methanosarcina* determined by using the *in silico* DNA-DNA hybridization method (Table S2, Supporting Information) is lower than the recommended cutoff point of 94% for species delineation (Konstantinidis and Tiedje 2005; Goris *et al.* 2007; Richter *et al.* 2016) supporting the claim that methanogen Ant1 belongs to novel species of the genus *Methanosarcina*. The tetra-nucleotide distribution regression coefficient (Table S3, Supporting Information) is below the cutoff (<0.989), further demonstrating that the methanogen Ant1 differs greatly and belongs to a novel uncultured species.

Phylogenetic analysis of 16S rRNA genes obtained from clones and MAG Ant1

A total of 78 archaeal 16S rRNA gene clones derived from microcosm Ant1 clustered into three OTUs (Fig. 5). The 16S rRNA gene sequence from the MAG Ant1 and one clone from the Bunger Hills Oasis permafrost clustered with these 16S rRNA clones (Fig. 5). These sequences fell into three OTUs at 97% identity threshold (Fig. 5) and were related to *Methanosarcina lacustris*. A relatively low value (73%) in the bootstrap test (1000 replicates) between MAG Ant1 and closest cultivable strains of the species *Methanosarcina lacustris* suggests that the methanogen identified as MAG Ant1 belongs to a new species of the genus *Methanosarcina* and is most closely related to several strains of *M. lacustris*, including strain Z-7289, a psychrotolerant methanogen isolated from a fen in Moscow (Zhilina and Zavarzin 1991) and strains MM and MS from anoxic lake sediments in Switzerland (Simankova *et al.* 2003). High similarity of 16S rRNA genes from MAG Ant1 and clone from permafrost of the Bunger Hills Oasis (Fig. 5) suggests that the methane-producing *Methanosarcina* may be common in Antarctic permafrost.

Methane metabolism

Methane metabolism genes from Ant1 MAG were most closely related to those of its closest relatives, identified based on 16S rRNA phylogeny (Fig. 6). The most conspicuous features of methane metabolism of the Ant1 MAG include the presence of several subunits of formylmethanofuran dehydrogenase and the presence of the entire operon encoding methyl coenzyme M reductase (Mcr) and genes for hydrogenotrophic methanogenesis (*fmd, ftr, mch, mtd, mer, mtrABCDEFGH* and *hdrABCDE*). These suites of genes indicate that methanogen Ant1 can grow with H_2/CO_2 like *M. lacustris* and *M. soligelidi*. Additionally, acetoclastic genes encoding carbon monoxide dehydrogenase, acetate kinase, acetyl-coenzyme A synthetase, phosphate acetyltransferase and the acetyl-CoA decarbonylase/synthase complex provide evidence that this organism is capable of acetoclastic methanogenesis (Fig. 6). Methanol metabolism genes encoding the three subunits of methanol-corrinoid protein co-methyltransferase show potential for growth with methanol. The genome contains monomethylamine methyltransferase and dimethylamine corrinoid protein genes, suggesting growth with methylamines. An incomplete formate dehydrogenase operon suggests that growth with formate is not likely. These findings suggest that this MAG was from the key methanogen producing methane in microcosm Ant1.

Cold adaptation strategies detected in MAG Ant1

Studies on EF2 from the model archaeal psychrophile *Methanococcoides burtonii* showed the enzyme activity dependence on temperature (Thomas and Cavicchioli 1998, 2002). The EF2 protein coding sequence was found in MAG Ant1. A three-dimensional ribbon model of EF2 enzyme (GTPase) from the MAG Ant1 (Fig. 7) was compared to EF2 (GTPase) models from *Methanosarcina lacustris*, the closest relative to Ant1 by 16S rRNA, a mesophilic relative *Methanosarcina acetivorans*, and a psychrophilic isolate *Methanolobus psychrophilus*. The EF2

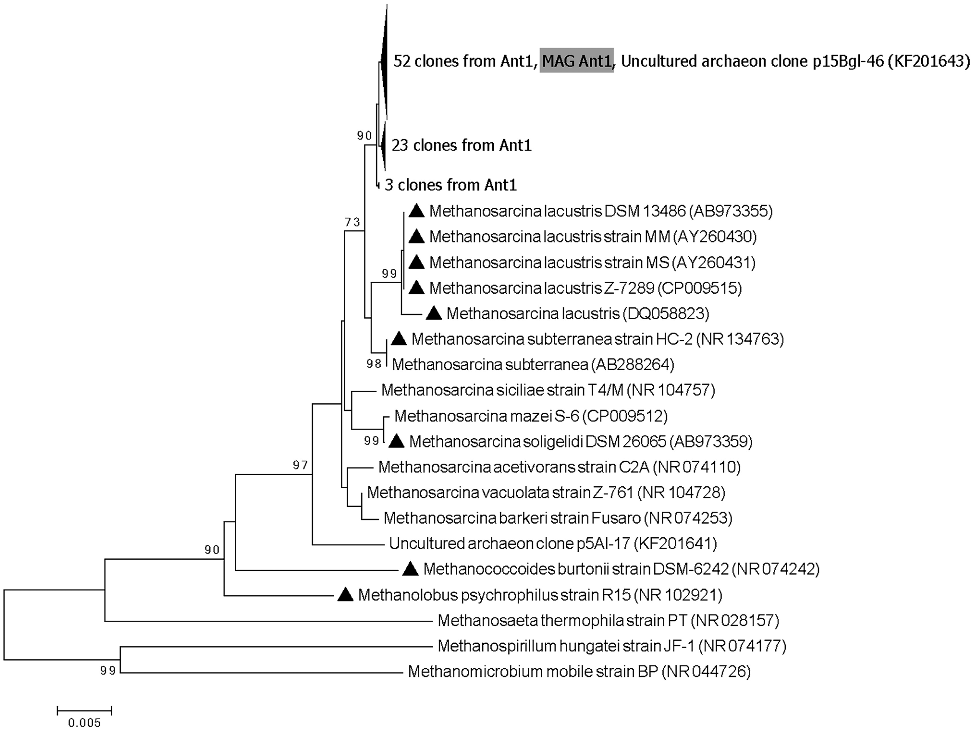

Figure 5. Phylogenetic tree for 16S rRNA gene sequences showing the relationship between the clones derived from microcosm Ant1, MAG Ant1 (highlighted), clones (p15Bgl-46 and p5AI-17) from Bunger Hills Oasis and King George Island permafrost samples, respectively, and close *Methanosarcina* relatives. Relationships were inferred by using the Neighbor-Joining method with 1000 bootstrap replicates. The sequences from *Methanospirillium hungatei* strain JF-1 and *Methanomicrobium mobile* strain BP were used as outgroups. Black triangles indicate psychrophilic or psychrotolerant relatives.

model of the MAG Ant1 had a higher alpha helical content compared to its mesophilic relative, *M. acetivorans*, but was similar to other psychrophilic or psychrotolerant relatives (Table 1). Modifications, such as increased alpha helical content, increased substrate affinity, stronger polar and weakened hydrophobic interactions, allow for greater flexibility in cold environments (Thomas and Cavicchioli 2002; Madigan et al. 2014). A previous study showed that the psychrophile *M. burtonii* generates unsaturated lipids by selective saturation (Cavicchioli 2006). The MAG Ant1 contains evidence for *de novo* synthesis of unsaturated diether lipids through a functional mevalonate pathway (Smit and Mushegian 2000). Specifically, present in the genome are the enzymes acetyl-CoA acyltransferase (EC 2.3.1.9), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (EC 1.1.1.34) and Mevalonate kinase (EC 2.7.1.36). A protein BLAST search initiated by a mevalonate kinase sequence from MAG Ant1 revealed <87% identity to mevalonate kinases from other *Methanosarcina*. Genes encoding the DNA DSB repair Rad50 ATPase, 15 heat shock proteins and 2 cold-shock DEAD-box proteins were detected in the MAG Ant1, indicating that several defense strategies against environmental stresses are available to the *Methanosarcina* sp. strain Ant1. In comparison, the mesophilic *Methanosarcina barkeri* DSM 804 contained 10 heat shock proteins, though no cold-shock proteins were detected. In addition, adaptation to low water activity can be achieved through accumulation of compatible solutes (Russell *et al.* 1995). The MAG Ant1 contains several different transporters for common compatible solutes on the same contig, including glycine betaine/carnitine/choline transport ATP-binding protein *opu*CA, glycine betaine/carnitine/choline transport system permease protein *opu*CB and choline-binding protein precursor. Motility was not observed in *Methanosarcina*-like cells Ant1, but the presence of several flagellar proteins, including flagellar proteins H and J, and flagellar accessory protein *flaH* were found in MAG Ant1.

DISCUSSION

Although methanogenic archaea are often successfully isolated from cold biotopes (Kendall *et al.* 2007; Rivkina *et al.* 2007; Wagner and Liebner 2010), there is currently no methanogen isolate from Antarctic deep subsurface permafrost. Studies concerning the distribution of Antarctic permafrost methanogens in general are scarce, leaving gaps in our knowledge about biochemical or genomics capabilities of these microorganisms. In this study, growth and activity of a methanogen was observed from 15 000-year-old permafrost (sample Ant1) where biogenic methane (concentration 29.0 μmol CH_4 kg^{-1} of sediment) was present *in situ*.

The discovery of methane and evidence of methanogenic archaea in 15 000-year-old sediments and absence in 30 000-year-old sediments contributes to our understanding of the paleo-history of Lake Miers. Currently, Lake Miers is a small freshwater lake filled by meltwater from the Miers and Adams glaciers. However, the Miers Valley was occupied by Glacial Lake Trowbridge between 10 000 and 23 000 years ago. This lake was at least 80 m deep and extended to more than 156 m above present sea level at its maximum extent 18 000–19 000 years ago. By 13 000–14 000 years ago evaporation had considerably reduced the size of the lake and resulted in the precipitation of calcite and gypsum evaporates (Clayton-Greene, Hendy and Hogg 1988). Our findings are consistent with this. The sample Ant2 from 3.75 m deep was about 30 000 years old (Gilichinsky *et al.* 2007) and this horizon developed before Glacial Lake Trowbridge appeared in

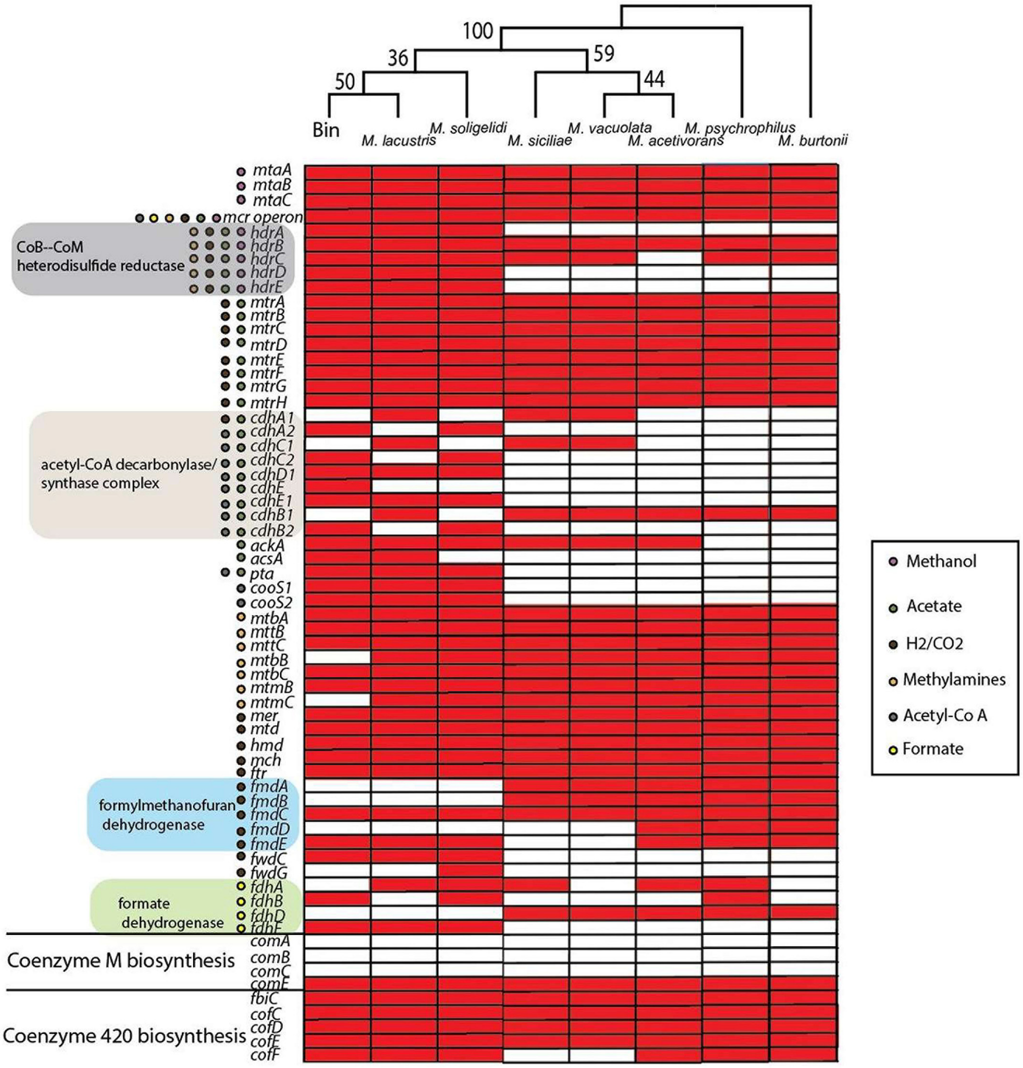

Figure 6. Manual inspection of the genome alongside mesophilic and psychrophilic relatives (by 16S rRNA gene) shows that the entire operon encoding methyl coemzyme M reductase and genes for hydrogenotrophic methanogenesis were present in binned genome Ant1. Bootstraps represent results after 1000 replicate trees. All trees are drawn to scale, with scale bar representing substitutions per site. Red color indicates that gene is present in the genome.

Table 1. Number of predicted EF-2 alpha helical domains.

Organism	Relation to temperature	Number of alpha helices
Methanogen Ant1 MAG (this study)	Antarctic permafrost at −18°C	23
Methanolobus psychrophilus	Psychrophilic	21
Methanosarcina lacustris	Psychrotolerant	22
Methanosarcina acetivorans	Mesophilic	18

the Miers Valley (Clayton-Greene, Hendy and Hogg 1988). However, the sample Ant1 from 2.04 m, estimated to be ~15 000 years old, was deposited under lake water. An observation of diatom shells in microcosm Ant1 supports our conclusion about the lake origin of these sediments. Diatoms, rotifers, cyanobacteria and plankton were found to be present in Lake Miers (Baker 1967). The bottom waters and lake bed of Lake Miers is described as a reducing environment based on low <1.5ppm oxygen, presence of NH_4^+, absence of NO_3^- and NO_2^- (Bell 1967). Methane (2 μg l^{-1}) was detected immediately above the sediment/water interface (Andersen, McKay and Wharton 1998). The presence of methanogenic archaea and discovery of methane in the sample from 2.04 m (Ant1) supports our assumption that these sediments were of lake origin, anoxic and contained methane and methanogens before they become frozen. These data suggest that cells of the methanogen Ant1 have remained viable in permafrost sediments for ~15 000 years.

Methanogen cells preserved in Antarctic permafrost sample from Lake Miers did not respond immediately to nutrient addition, producing a detectable amount of methane only after a year. The long recovery phase may reflect the low number of methanogens in Antarctic permafrost, extended time

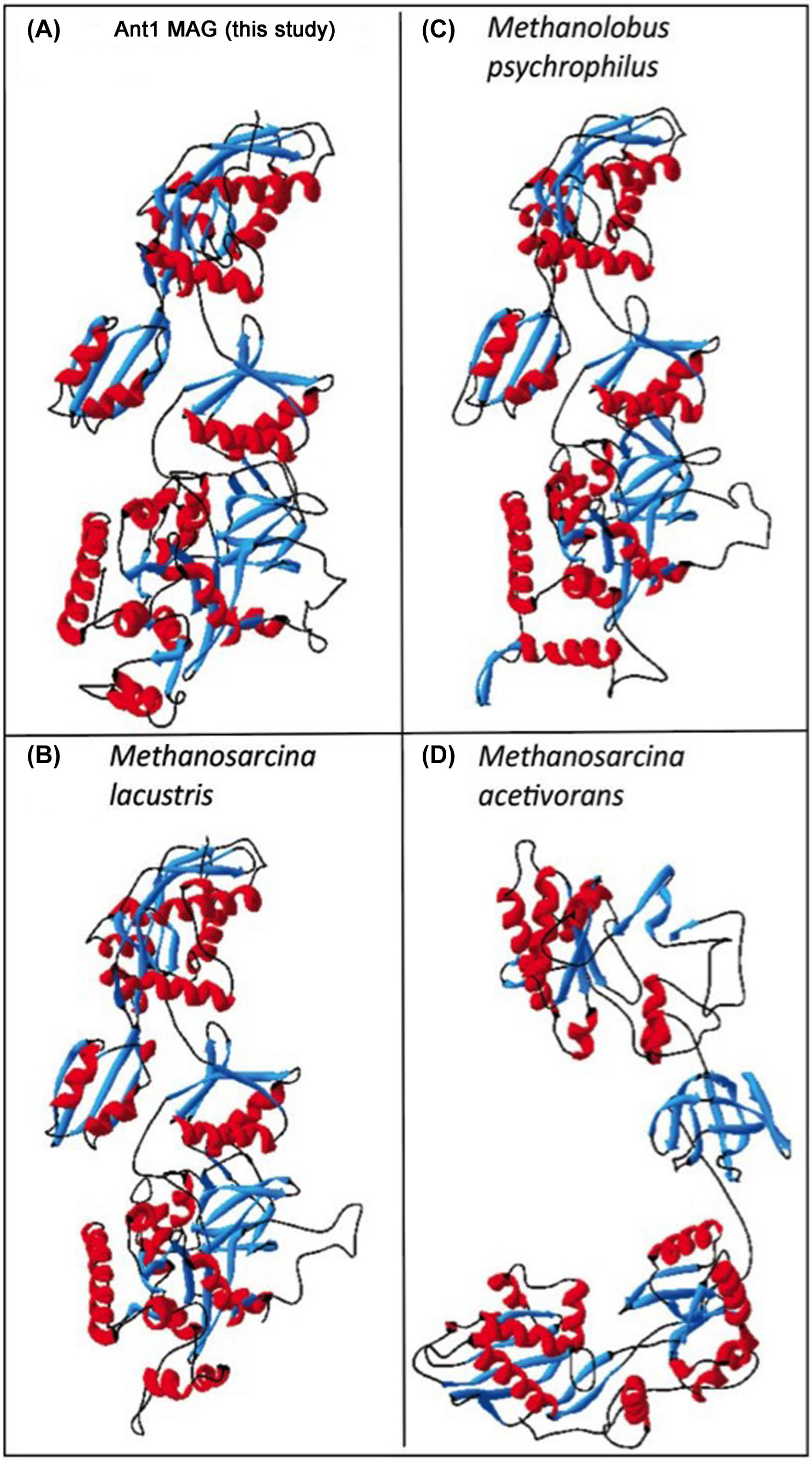

Figure 7. Comparison of three-dimensional ribbon models of EF-2 (GTPase) from the binned genome Ant1 (**A**), *Methanosarcina lacustris* (**B**), *Methanolobus psychrophilus* (**C**), and *Methanosarcina acetivorans* (**D**). Alpha helices are in red, beta sheets are in blue, and loops are in black.

needed for recovery from prolonged period of inactivity inside the frozen sediments, and/or slow growth rate. The estimated doubling time of 150 different methanogenic archaea showed that methanogens from cold biotopes tended to be slow-growing microorganisms (Jablonski, Rodowicz and Lukaszewicz 2015). Specifically, psychrophilic methanogens, such as *Methanosaeta pelagica* isolated from marine tidal flat sediment (Mori *et al.* 2012) and *Methanogenium frigidum* isolated from sediments of Ace Lake Antarctica (Franzmann *et al.* 1997; Mori *et al.* 2012), showed doubling times of 298 and 70 h, correspondingly. Mesophilic *Methanosarcina barkeri* detected in low-temperature anaerobic bioreactor displayed a doubling time of 581 h when grown on H_2/CO_2 at 15°C (Gunnigle *et al.* 2013). The increase of headspace methane in microcosm Ant1 proves that cultivable methanogens were present and could be detected in deep subsurface permafrost samples from the Dry Valleys. The CH_4 production rate in the microcosm Ant1 was compatible with the mean rate of methanogenesis estimated at 0.014–0.015 fmol CH_4 $cell^{-1}$ day^{-1} at 5°C for acetate enrichments of Arctic permafrost samples (Rivkina *et al.* 2007); and at 0.017 fmol CH_4 $cell^{-1}$ day^{-1} at 21°C with 50-kPa hydrogen in the headspace as the energy source for *Methanoculleus submarinus* (Colwell *et al.* 2008).

The fact that the methane concentration in the headspace increased and decreased constantly may suggest a possibility of existence of both methane production and anaerobic methane oxidation processes in the same microcosm. Previously studied methanogens, including *Methanosarcina barkeri*, were able to anaerobically oxidize small quantities of methane simultaneously with methane production and produced CO_2, methanol, and acetate (Zehnder and Brock 1979, 1980). A keyword search of 98 *Methanosarcina* strains, deposited to the IMG server, revealed that all of them contain protein-coding genes annotated as probable Quinol monooxygenase YgiN (COG1359) and a similar gene was identified in MAG Ant1. Previous studies showed that methanogens might form syntrophic connections with sulfate reducing bacteria (SRB) during anaerobic methane oxidation (Zehnder and Brock 1979; Shawn 2017; Skennerton *et al.* 2017). The metagenome analysis indicated the presence of SRB from the orders Desulfobacterales and Desulfovibrionales at 1.02% in microcosm Ant1. Genes involved in sulfate uptake and reductions were identified in microcosm Ant1. Sulfate was not added to microcosms as component of growth medium, but was present in original permafrost samples (Gilichinsky *et al.* 2007). Other possible scenarios for methane decrease are that CH_4 leaked out of the headspace, pushed out by over pressure; CH_4 was oxidized by aerobic methanotrophs, which were present at 0.2% and subsisted on a small amount of oxygen that leaked in; or co-existence of these processes.

The metagenomic analysis of both Ant1 and Ant2 microcosms revealed a high percentage of predicted proteins with known functions. Given the high level of known proteins in the metagenomic data, and the fact that the vast majority of the reads in metagenome Ant1 belonged to our methanogen, it may be suggested that genetic studies using organisms like Ant1 as model for targeting questions about the response to permafrost thawing would yield reasonably translatable results. Therefore, using a metagenome-binning technique, we were successful in reconstructing a nearly complete *Methanosarcina* genome from permafrost methane-producing enrichment Ant1. The current microcosm experiment led to the enrichment of actively methane-producing microorganisms, and methanogenic pathways described through genomic approaches and predictions suggested that methanogen Ant1 is capable of using diverse substrates for growth and methane production, including H_2/CO_2, methanol, methylamines and fermentation byproducts, such as acetate. However, methanogenic activity was not detected when acetate was added to the microcosm as the sole carbon source, indicating that acetate is not actually used for growth or it is not the preferred substrate when others are available. Similarly to the current study, active methanogenic enrichments from Arctic Holocene permafrost were only obtained on H_2/CO_2 (80/20), but not acetate, as substrate, even though the methanogen *Methanosarcina mazei* strain JL01, which was isolated from that permafrost H_2/CO_2 enrichment, uses acetate, methanol, monomethylamine, dimethylamine and trimethylamine as sole carbon sources (Rivkina *et al.* 2007). H_2/CO_2 turned out to be a favorable substrate for methanogenic enrichments from both Arctic and Antarctic permafrost. Nevertheless, numerous attempts to obtain a pure culture of the methanogen Ant1 on H_2/CO_2, acetate, methanol and methylamines were unsuccessful. It is not clear why methanogen Ant1 does not grow in pure culture, however, efforts to isolate microorganisms from permafrost environments are commonly met with mixed results. For example, it was observed that permafrost microorganisms exhibited high rates of cell proliferation and biochemical processes immediately after permafrost thawing, and yet not all bacterial isolates recovered from ancient permafrost showed growth after repetitive transfers (Vorobyova *et al.* 1997). Inability to grow in the laboratory is usually bound to either undetermined nutrient requirements, demands for co-culture with other microorganisms, and/or changed environmental characteristics (Stewart 2012). Unraveling these unknowns will improve growth and maintenance of rare species. Nevertheless, there are examples of isolating pure cultures of methanogenic archaea from Siberian permafrost, for example *Methanosarcina mazei, Methanobacterium veterum* and *Methanobacterium arcticum* (Rivkina *et al.* 2007; Krivushin *et al.* 2010; Shcherbakova *et al.* 2011). Characterization of these species has shown the ability of the methanogenic archaea to withstand extreme permafrost conditions including long-term freezing.

The close phylogenetic relationship of methanogen Ant1 assembled in the present study to psychrotolerant *M. lacustris*, as well as adaptations to low temperature, including higher EF2 alpha helical content, and the presence of cold-shock and heat-shock proteins, suggest that methanogen Ant1 stayed viable after thousands of years at −17°C as was measured inside the borehole after core extraction (Gilichinsky *et al.* 2007) or below as extrapolated during temperature reconstruction for 40 000 years before present (Cuffey *et al.* 2016). The latter study estimated the surface temperature in West Antarctica for ages greater than 11 000 years ago to be colder by −5.8°C and this allows us to suggest that methanogen Ant1 was active at cold temperatures before being frozen inside permafrost. Previously, it was shown that the properties of archaeal EF2 proteins reflect the growth temperature of the archaea (Thomas and Cavicchioli 1998; Thomas and Cavicchioli 2000). The similarity of EF2 from MAG Ant1 to EF2 from psychrophilic relatives supports the cold adaptive nature of methanogen Ant1. Additional evidence of cold adaptation is the presence of a *de novo* pathway for unsaturated diether lipid synthesis. The importance of unsaturation in lipids to low-temperature adaptations has been demonstrated for *Methanococcoides burtonii* showing that the proportion of unsaturated ether lipids was significantly higher in cells grown at 4°C than in cells grown at 23°C (Nichols *et al.* 2004). Hostile habitats, e.g. permafrost, impact the microbial inhabitants at different organismal levels resulting in genome instability, and cell death. DNA double-strand breaks (DSBs) are lethal for microorganisms and must be repaired to preserve chromosomal integrity and cell viability. For the initial recognition and repair of DNA DSBs, the presence of the conserved Mre11-Rad50 double-stranded-break repair system is essential. Genes that are predicted to code for endonuclease Mre11 and ATPase Rad50 were detected in the MAG Ant1. A similar Mre11-Rad50 DSB repair system was found in the genome of mesophilic hydrogenotrophic methanogen *Methanococcus maripaludis* (Hendrickson *et al.* 2004), suggesting that such a mechanism for DNA repair is essential across the archaeal tree of life. An osmoregulatory response to low water activity associated with the permafrost environment was suggested due to presence of several transporters for known compatible solutes, such as carnitine and glycine betaine. Additional work is required to isolate the methanogen Ant1 into pure culture and to conduct growth studies showing its temperature requirements.

In conclusion, the presented data prove the existence of viable methanogens in 15 000-year-old permafrost from Miers Valley. This is the first report showing a preservation of methanogens in extremely harsh ancient permafrost of the Antarctica Dry Valleys. These data provide additional proof that metabolic versatility makes *Methanosarcina* spp. widespread and allows it to inhabit a diverse array of environments such as dumps, sewage heaps, deep sea vents, deep subsurface groundwater, ruminant's gut and Siberian and Antarctic permafrost (Galagan *et al.* 2002; Rivkina *et al.* 2007; Galand *et al.* 2008; Liu and Whitman 2008). The existence and preservation of *Methanosarcina* sp. in colder Antarctic permafrost could be explained by the wide metabolic plasticity of the genus and its evolutionary advancement among methanogenic archaea.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

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