

ORIGINAL ARTICLE

Methanogenic archaea are globally ubiquitous in aerated soils and become active under wet anoxic conditions

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The prototypical representatives of the *Euryarchaeota*—the methanogens—are oxygen sensitive and are thought to occur only in highly reduced, anoxic environments. However, we found methanogens of the genera *Methanosarcina* and *Methanocella* to be present in many types of upland soils (including dryland soils) sampled globally. These methanogens could be readily activated by incubating the soils as slurry under anoxic conditions, as seen by rapid methane production within a few weeks, without any additional carbon source. Analysis of the archaeal *16S ribosomal RNA* gene community profile in the incubated samples through terminal restriction fragment length polymorphism and quantification through quantitative PCR indicated dominance of *Methanosarcina*, whose gene copy numbers also correlated with methane production rates. Analysis of the $\delta^{13}\text{C}$ of the methane further supported this, as the dominant methanogenic pathway was in most cases acetoclastic, which *Methanocella* cannot perform. Sequences of the key methanogenic enzyme methyl coenzyme M reductase retrieved from the soil samples before incubation confirmed that *Methanosarcina* and *Methanocella* are the dominant methanogens, though some sequences of *Methanobrevibacter* and *Methanobacterium* were also detected. The global occurrence of only two active methanogenic archaea supports the hypothesis that these are autochthonous members of the upland soil biome and are well adapted to their environment.

The ISME Journal (2012) 6, 847–862; doi:10.1038/ismej.2011.141; published online 10 November 2011

Subject Category: microbial ecology and functional diversity of natural habitats

Keywords: upland soils; biological soil crust; methanogens; *16S rRNA*; *mcrA*; $\delta^{13}\text{C}$

Introduction

The methanogenic *Archaea* (methanogens) produce nearly all biogenic methane on Earth. All known methanogens fall into the phylum *Euryarchaeota*, where they form six distinct orders: *Methanomicrobiales*, *Methanocellales*, *Methanosarcinales*, *Methanobacteriales*, *Methanococcales* and *Methanopyrales*. The pathways for generating CH_4 in methanogens vary and include methanol and CO_2 reduction, acetate cleavage, as well as CH_4 production from a variety of methylated compounds. With the exception of marine sediments and hypersaline mats (Oremland, 1988), methanogenesis in natural systems is dominated by CO_2 reduction (hydrogenotrophic methanogenesis) and acetate cleavage (acetoclastic methanogenesis; Conrad, 2005).

Although the biochemical machinery in methanogens varies with the pathway used, few functional genes, which encode for key enzymes in the

production of methane, are common to all known methanogens (Hedderich and Whitman, 2006). Of those, the methyl coenzyme M reductase (*mcrA*) that is responsible for the last step in all methanogenic pathways—the reduction of the methyl group bound to coenzyme M—is typically used as functional gene marker for analysis of methanogenic communities along with the *16S ribosomal RNA* (*rRNA*) gene (Luton *et al.*, 2002).

Methanogens are strict anaerobes, and methanogenesis was shown to be fully suppressed upon exposure to oxygen in both pure culture and in soil (Fetzer *et al.*, 1993; Fetzer and Conrad, 1993; Yuan *et al.*, 2009). Nevertheless, the ability of methanogens in pure culture and soil to survive oxygen and desiccation stress conditions for several hours to days has also been shown (Kiener and Leisinger, 1983; Fetzer *et al.*, 1993; Ueki *et al.*, 1997; Liu *et al.*, 2008; Ma and Lu, 2011). Although numbers of viable cells decreased with time, in many types of methanogens a core population was able to survive the stress and proliferate again.

In addition, the typical energetic yields for hydrogenotrophic and acetoclastic methanogenesis ($\Delta G^0 = -131$ and -36 kJ, respectively) are lower than those obtained by iron, nitrate and sulphate reduction ($\Delta G^0 = -228.3$, -163.2 and -152.2 kJ,

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Received 17 February 2011; revised 27 June 2011; accepted 31 August 2011; published online 10 November 2011

respectively), and organisms performing these respirations pathways successfully compete with methanogens over hydrogen (Thauer *et al.*, 1977, 1989). As a result, methanogenesis typically occurs only in highly reduced anoxic environments, where oxygen is absent and alternative electron acceptors, such as nitrate and sulphate, are depleted (Whitman *et al.*, 2006).

In contrast to the notions presented above, Peters and Conrad (1995) demonstrated that several types of aerated soils could become methanogenic when incubated under anoxic conditions as slurry. Among these soils was a South African arid soil, which was stored in dry state at room temperature for nearly 10 years before incubation. Few other researchers have reported the occurrence of methanogenesis in aerated soils and/or the presence of methanogens. For example, West and Schmidt (2002) were able to induce methanogenesis in alpine soils when incubated under an H₂/CO₂-enriched atmosphere (but not under pure N₂ atmosphere), and Teh *et al.* (2005) found methanogenesis occurring in tropical forest soils containing up to 19% O₂. In three other cases, researchers measured methanogenic activity in pasture soil and were able to retrieve sequences affiliated to methanogens (Nicol *et al.*, 2003; Gattinger *et al.*, 2007; Radl *et al.*, 2007). However, these authors have attributed most of this activity to the effect that livestock had on the soil by enriching it with nutrients from urine and manure and by introducing rumen microflora to the soil. Last, Poplawski *et al.* (2007) retrieved sequences of *Methanosarcina* and *Methanocella* in a Swedish barley field but did not attempt to measure potential methanogenic activity.

The discovery of potential methanogenesis in arid soils is of particular interest, as it is least expected in a soil that is dry and oxic throughout most of the year and where input of organic carbon is particularly low and grazing ruminants are scarce. Particularly for these reasons, if active methanogens are to be found in arid soils, they can demonstrate their autochthonous origin as soil microorganisms (rather than as allochthones being introduced by ruminants) and ability to survive in anoxic microniches of upland soils around the world.

Arid soils differ from soils in humid areas by their unique profile. Unlike most soils, soils in arid parts do not display a profile of layers representing different stages of bedrock erosion and a gradient of nutrient concentrations. Rather, the bulk soil is undifferentiated, eroded bedrock poor in nutrients, which is in turn covered by a densely populated mat of microorganisms, termed biological soil crust (BSC). The BSC is an association of microorganisms, which is formed practically in any soil whose water budget restricts the development of higher plants. The primary members and first colonizers of BSCs are filamentous cyanobacteria, which aggregate the soil using exopolysaccharides. Fungi, microalgae

and lichens are often also associated with mature BSCs and sometimes bryophytes as well (Belnap *et al.*, 2003). An extensive literature exists on the abovementioned members of BSCs but studies (particularly molecular) on the prokaryotic inhabitants of this mat (other than cyanobacteria) are limited (Garcia-Pichel, 2002).

Our goals in this study were to compare the methanogenic potential of different aerated soils collected globally and, more importantly, to uncover the identity of the active methanogens in these soils and the potential methanogenic pathways. We focused primarily on dryland environments and collected samples not only from Israel, Australia and Utah, but also from a forest and a meadow in Germany for comparison. We screened the soil samples for methanogenic potential, and attempted to decipher the methanogenic pathways through stable isotopes analysis and by the use of an inhibitor for aceticlastic methanogenesis. We then analyzed the identity of the archaeal populations in these soils and quantified their sizes through specific quantitative PCR (qPCR) assays.

Materials and methods

Soil samples and soil characterization

Soil samples were collected from several sites around the world as listed in Table 1. The sampling done in the Negev Desert, Israel, comprises our most comprehensive sampling. The sites were previously described by Angel and Conrad (2009). Samples were taken from the BSC where it existed, from 0 to 10 cm and from 10 to 20 cm depth. Apart from the Negev Desert samples, samples from Utah and Australia were also from dryland environments, whereas the samples obtained in Germany were from temperate forest and grassland. The gravimetric water content of the samples was determined before all further analyses.

Incubation conditions and gas measurements

For determining the methanogenic potential of the soils, the samples were incubated under wet anoxic conditions, as supposed to occur after flooding: 5 g of soil sample was placed in a 26-ml glass tube, supplemented with 5 ml of sterile distilled deionized water and the tube was capped with a butyl rubber stopper (cleaned by boiling in water before use). The headspace was repeatedly evacuated and flushed with N₂ for 10 min and left with a 0.5 bar overpressure. Each sample was set up in triplicate and incubated at 25 °C in the dark for 48 days. Samples that demonstrated methanogenic activity were later incubated again using fresh soil, but supplemented with 2% methyl fluoride (CH₃F) to inhibit aceticlastic methanogenesis (Janssen and Frenzel, 1997). Gas samples were taken from the tube headspaces every 6 days using a gas-tight

Table 1 Characteristics of the study sites and samples and methanogenic potentials

Location	Site	Soil type (USDA) ^a	Sampling layer	pH	C_{org}, N_{tot} (%)	Methane production rate ($nmol\ gdw^{-1}\ d^{-1}$)	Lag ^b (days)	R ²	Inhibited methane production rate ($nmol\ gdw^{-1}\ d^{-1}$) ^c	Lag (days)	R ²
Avdat, Negev Plateau, Israel	Natural field—intershrub 30°47'N 34°45'E	Silt loam	BSC	8.3	0.46, 0.30	3081.6	20	0.99	629.02	24	0.96
			0–10 cm		0.38, 0.01	1.03	24	0.90			
			10–20 cm		0.33, 0.01	0.46	23	0.94			
-	Shrub 30°47'N 34°45'E	Silt loam	BSC	8.4	0.66, 0.05	3710.73	16	0.96	1793.76	21	0.96
			0–10 cm		0.34, 0.01	1.54	22	0.93			
			10–20 cm		0.26, BLD	1.23	23	0.82			
Limnan (constructed mini-catchment)	30°48'N 34°45'E	Silt loam	BSC	7.9	2.00, 0.15	5657.59	11	0.99	3471.14	15	1.00
			0–10 cm		1.27, 0.09	540.95	16	1.00			
			10–20 cm		0.96, 0.05	224.6	16	1.00			
Agricultural field	30°52'N 34°48'E	Loam	0–10 cm	8.5	0.28, BLD	0.62	3	0.71			
			10–20 cm		0.25, 0.03	BLD					
Arava Valley South, Israel	Wadi (dry riverbed) 29°58'N 35°05'E	Sand	BSC	8.4	0.98, BLD	4.39	22	0.99			
			0–10 cm		0.06, BLD	BLD					
			10–20 cm		0.08, BLD	BLD					
Agricultural field	30°04'N 35°08'E	Sand	0–10 cm	8.5	0.38, 0.14	BLD					
			10–20 cm		0.18, BLD	BLD					
North Utah, USA	Natural field	Silt loam	0–10 cm	6.5	4.71, 0.37	222.98	52	0.95	23.3	28	1.00
			0–10 cm		2.03, 0.11	138.35	13	0.93	49.53	26	0.99
			Sandy loam		2.61, 0.14	3.49	19	0.99			
			Silt loam		2.63, 0.16	8.98	62	0.94			
Niflold Plain, Australia	Savannah	Silt loam	0–10 cm	4.8	0.72, 0.10	11.61	59	0.91			
			0–10 cm		0.35, 0.43	374.5	38	0.93			
			Loamy sand		1.21, 0.10	0.63	38	0.97			
Giessen, Germany	Meadow	Silt loam	0–7.5 cm	5.9	4.24, 0.40	1860.07	33	0.92	305.48	27	0.97
			7.5–15 cm		3.48, 0.35	1428.4	42	0.85	171.91	37	0.98
Marburg, Germany	Forest	Sandy loam	0–10 cm	3.5	20.83, 0.92	1.2	55	0.99			
			0–10 cm		20.07, 0.90	1.58	62	0.98			

Abbreviations: BLD, below limits of detection; BSC, biological soil crust.

^a<http://soils.usda.gov/technical/aids/investigations/texture/>^bInitiation of linear methane production (extrapolated from linear regressions).^cInhibition of acetoclastic methanogenesis using 2% CH₃F (see Materials and methods).

pressure-lok syringe (Vici, Baton Rouge, LA, USA) and analyzed immediately. Methane and CO₂ concentrations were analyzed using a gas chromatograph equipped with a methanizer (Ni-catalyst at 350 °C, Chrompack, Varian Deutschland GmbH, Darmstadt, Germany) and a flame ionization detector (Shimadzu Deutschland, Duisburg, Germany).

Analysis of stable carbon isotopes

The carbon isotope ratios ¹³C:¹²C in the CH₄ and CO₂ were determined using a gas chromatograph combustion isotope ratio mass spectrometer against the Vienna Pee Dee Belemnite (V-PDB) standard, as described previously (Conrad *et al.*, 2009). Isotopic calculations and estimation of the approximate fraction of hydrogenotrophic methanogenesis of the total methanogenesis were done after Conrad (2005). Briefly, the fraction of the newly formed CH₄ between two time points is given by:

$$\delta_2 = f_n \delta_n + (1 - f_n) \delta_1 \quad (1)$$

where δ_1 , δ_2 and δ_n are the isotopic signatures of the CH₄ at times 1 and 2 and of the newly formed, respectively, whereas f_n is the fraction of the newly formed CH₄ between times 1 and 2.

The apparent fractionation factor for the conversion of CO₂ to CH₄ is given by:

$$\alpha_{\text{app}} = (\delta_{\text{CO}_2} + 10^3) / (\delta_{\text{CH}_4} + 10^3) \quad (2)$$

where δ_{CO_2} and δ_{CH_4} are the isotopic signatures of the carbon in CO₂ and CH₄, respectively.

For convenience, ε is often used instead of α . The two can be easily converted through:

$$\varepsilon = 10^3(\alpha - 1) \quad (3)$$

The relative fraction of H₂/CO₂-derived CH₄ in the total generated CH₄ was determined by:

$$f_{\text{H}_2} = (\delta_{\text{CH}_4} - \delta_{\text{ma}}) / (\delta_{\text{mc}} - \delta_{\text{ma}}) \quad (4)$$

where δ_{ma} and δ_{mc} are the specific isotopic signatures of the carbon in CH₄ produced solely from acetate and H₂/CO₂, respectively. The $\delta^{13}\text{C}$ in the organic matter was analyzed at the Institute for Soil Science and Forest Nutrition (IBW) at the University of Göttingen, Göttingen, Germany, using an elemental analyzer coupled to a mass spectrometer. Measurements were done before and after acidification, the difference being due to carbonate (Nüsslein *et al.*, 2003).

Slurry sampling

After incubations were completed, the tubes were opened in an anoxic glove box, and ~1 g of slurry was sampled for nucleic acid extraction, immediately frozen in liquid nitrogen and stored at -80 °C until analysis.

Nucleic acid extraction, RNA purification and complementary DNA synthesis

Total nucleic acids were extracted by disrupting 0.5 g of soil or slurry sample in a Lysing Matrix E tube (MP Biomedicals) in the presence of phosphate

buffer, 10% SDS solution and phenol using a FastPrep-24 bead beater (MP Biomedicals, Heidelberg, Germany). The process was repeated three times using fresh buffers and phenol, and the supernatant was collected. The extractant was then purified using standard phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol purification, and the supernatant was transferred to Non-Stick RNase-free Microfuge Tubes (Ambion, Applied Biosystems, Darmstadt, Germany). The nucleic acids were precipitated using 20% polyethylene glycol and 5 µg of glycogen (Ambion), washed once with ice-cold 75% EtOH and resuspended in low TE buffer.

In cases in which the extractant had colour, it was further purified using MicroSpin S-200 HR (GE Healthcare, Freiburg, Germany). Ten microliters of the extractant was diluted in 90 µl of low TE buffer to be used as DNA template. For complementary DNA synthesis from all RNA templates, 50 µl of nucleic acid extractant was digested with TURBO DNase (Ambion) and later purified using RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany). Complete DNA removal was verified by failure to obtain PCR amplification product with the purified RNA template, using the conditions described below. Complementary DNA was synthesized using ImProm-II Reverse Transcriptase (Promega, Mannheim, Germany) using 100 ng purified RNA and 0.5 µg µl⁻¹ of random hexamer primers, as described by the manufacturer. The resulting complementary DNA product was used directly as a template for consecutive PCR reactions. PCR amplifications were performed identically for both DNA and complementary DNA templates. For a detailed description of the procedure, see the Supplementary Information.

Primers and probes used in this study

Table 2 summarizes the primers and probes used in this study to monitor and quantify the archaeal populations. For sequence affiliation, oligonucleotide design and evaluation, and reconstruction of phylogenetic trees, the ARB software package was used (Ludwig *et al.*, 2004). Analysis of 16S *rRNA* gene sequences and oligonucleotide design was done using the Silva database (Pruesse *et al.*, 2007), whereas for analyses of *mcrA* sequences a specific ARB database was built. The *mcrA* database comprised 5200 translated nucleic acid sequences, which were obtained from EMBL (<http://www.ebi.ac.uk/embl/>), aligned using Promals (Pei and Grishin, 2007) and manually corrected. The *mcrA* ARB database is available at: <http://www.mpi-marburg.mpg.de/downloads/conrad/>. The qPCR primers and probe targeting the 16S *rRNA* gene of *Methanocella* were designed using Primrose (Ashelford *et al.*, 2002).

PCR amplification

PCR reactions were conducted in triplicates of 25 µl, which were pooled to minimize reaction variability. Each PCR reaction contained 5 µl GoTaq Flexi 5 ×

Table 2 Primers and probes used in this study

Oligo. name ^a	Target	Oligo. sequence (5'-3')	Position ^b	GC (%)	Tm ^c	Amplicon size	Essay	Reference
ARCH109 - F	Archaea	AHDGCTCAGTAAACAGRT	109–125	42.2	56		TRFLP/Sequencing	Miyashita et al. (2009)
ARCH934 - R	16S rRNA gene	GTGCTCCCGCCGCAATTCCT	915–934	65	68	826		Grosskopf et al. (1998)
U1406 - R		AGGGGGCTGCTGTRC	1392–1406	70	63	1298	qPCR std.	Amann et al. (1995)
ARCH364 - F		CGGGGYGCASCAGGGGGGAA	364–383	75–80	75	553	qPCR	Burggraf et al. (1997)
MSL812 - F	<i>Methanosarcinales</i>	GTAAACGATRYTCGCTAGGT	812–831	40–50	62			
MSL860 - P	16S rRNA gene	AGGGAAGCCGTGAAAGCGARCC	860–880	62–67	70		qPCR	Yu et al. (2005)
MSL1159 - R		GGTCCCCACAGWGTACC	1143–1159	65	63	354		
Mcl282 - F	<i>Methanocella</i> 16S	ATCMGTACGGGTTGTGGG	282–299	56–61	65			
Mcl609 - P	rRNA gene	ATCCAGCGGCTTAACCGTTGGKCK	609–632	54–63	72		qPCR	This study
Mcl832 - R		CACCTAGCGRGCATCGTTTAC	813–832	52–57	64	510		
771F	<i>Crenarchaeota</i> 16S	ACGGTGAGGGATGAAAAGCT	753–771	53	63			Ochsenreiter et al. (2003)
957R	rRNA gene	CGGGCTTCACTCCAAATTG	957–974	56	62	203	qPCR	
mlas-mod - F	Universal <i>mcrA</i> gene	GGYGGTGTMGDDTTCACMCARTA	976–998a	43–65	68			Modified from: Steinberg and Regan (2008)
mcrA-rev - R		CGTTTCATBGGCGTAGTTVGGRTAGT	1421–1444a	42–54	66	469	Sequencing/qPCR	Steinberg and Regan (2008)

^aThe following primer name suffixes are used: - F – forward primer, - R – reverse primer, - P – dual labelled probe.

^bPositions are based on the following: primers targeting the 16S rRNA gene - *E.coli*; primers targeting the *mcrA* gene - *M. thermautotrophicus mcrA* gene accession number: U10036 (following Steinberg and Regan, 2008);

^cCalculated using Nearest Neighbour method with OligoAnalyzer 3.1 (<http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/>).

Green Buffer (Promega), 1.5 mM MgCl₂, 20 mg BSA (Roche Diagnostics, Mannheim, Germany), 0.75 U of GoTaq DNA polymerase (Promega) and 1 µl of template. For amplification of the *16S rRNA* gene, 0.5 µM of each primer was used, whereas for amplification of *mcrA* 0.8 µM was used. The following program was used for the amplification of the *16S rRNA* gene: 94 °C for 4 min, followed by 28 cycles of 94 °C for 1 min, 52 °C for 30 s and 72 °C for 45 s, and a single step of final elongation at 72 °C for 10 min. For the amplification of *mcrA*, the following program was used: 94 °C for 4 min, followed by five touchdown cycles of 94 °C for 30 s, 60–1 °C for 45 s and 72 °C for 30 s, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a single step of final elongation at 72 °C for 10 min. Following amplification, the PCR products were purified using GeneE-lute PCR purification Kit (Sigma-Aldrich, Munich, Germany).

TRFLP analysis

For terminal restriction fragment length polymorphism (TRFLP) analysis, the forward primer was labeled with the fluorescent dye 6-carboxyfluorescein. Approximately 200 ng of purified PCR products was digested overnight at 37 °C with 20 U of the restriction enzyme *TaqI* and 2 µl of 10 × Buffer *TaqI* (Fermentas, St. Leon-Rot, Germany). Following digestion, samples were desalted using SigmaSpin Post-Reaction Clean-up Columns (Sigma); aliquots of 3 µl were mixed with 10 µl of HiDi Formamide (Applied Biosystems, Darmstadt, Germany) and 0.3 µl of the internal DNA standard MapMarker1000 (BioVentures, Murfreesboro, TN, USA). The samples were denatured at 94 °C for 2 min and loaded into an ABI 3100 automated gene sequencer (Applied Biosystems) for separation of the TRFs. TRFLP data were retrieved by comparison with the internal standard using GeneScan 3.71 software (Applied Biosystems).

Cloning and sequencing and phylogenetic analysis

Samples with high methanogenic potential from the following locations were used for cloning of both *16S rRNA* and *mcrA* genes: Avdat natural field and Liman: BSC; Giessen: 0–7.5 cm; and Nifold Plain 2 0–10 cm. Libraries were constructed using purified PCR products and pGEM-T Easy cloning kit (Promega). Double-coverage sequencing services were provided by ADIS (Cologne, Germany) or GATC (Konstanz, Germany) using the primers T7f and M13r targeting flanking regions of the insert. A total of 250 clones containing *16S rRNA* gene insert and 78 clones containing *mcrA* gene inserts were analyzed. All sequences were deposited into the GenBank (<http://www.ncbi.nlm.nih.gov>) and received the following accession numbers: HQ268968–HQ269217 and HQ269218–HQ269295 (*16S rRNA* and *mcrA*, respectively). Raw sequence data were analyzed using SeqMan 4.05 software (DNASar, Madison, WI, USA), where the primers

and vector data were trimmed. The *16S rRNA* gene sequences were aligned using the SINA Webaligner (<http://www.arb-silva.de/aligner/>) against the Silva 102 database. *mcrA* sequences were converted to amino-acid sequences and aligned against the ARB database described above. Reconstruction of a maximum likelihood phylogenetic trees was conducted with RAxML 7.04 using rapid hill climbing algorithm and with GTRMIX and PROT-MIX-JTT evolutionary models for *16S rRNA* and *mcrA* genes, respectively (<http://sco.h-its.org/exelixis/software.html>; Stamatakis, 2006).

qPCR Assays

qPCR assays were used to quantify the *16S rRNA* gene copies of the general archaeal, crenarchaeal, *Methanosarcina* and *Methanocella* populations. All qPCR reactions were performed on an iCycler thermocycler equipped with a MyiQ detection system (Bio-Rad, Munich, Germany) and the data were analyzed using iQ5 Optical System software (Bio-Rad). For all assays, standards containing known numbers of DNA copies of the target gene were used. Standards were serially diluted and used for construction of calibration curves in each reaction. The standard for the general archaeal and the *Methanosarcina* assays was prepared from a pure culture of *Methanosarcina thermophila* according to Lueders and colleagues (2004). The standards for *Crenarchaeota* and *Methanocella* assays were prepared from clones containing *16S rRNA* genes affiliated with either *Crenarchaeota* or *Methanocella* as a plasmid insert. Total *Archaea* and *Crenarchaeota* assays were based on SYBR Green (Ambion). Each reaction was 25 µl in volume and contained the following mixture: 12.5 µl SYBR Green JumpStart Taq ReadyMix, 3 or 2 mM MgCl₂ (total *Archaea* and *Crenarchaeota* assays, respectively) and 0.8 ng µl⁻¹ BSA, 0.66 or 0.44 µM of each primer (total *Archaea* and *Crenarchaeota* assays, respectively), and 5 µl of template. For the two assays, the program used was: 94 °C for 4 min, followed by 40 cycles of 94 °C for 30 s, 66 or 63 °C for 30 s (total *Archaea* and *Crenarchaeota* assays, respectively), 72 °C for 30 s and 85 °C for 10 s for signal reading. The assays targeting the *16S rRNA* gene of *Methanosarcina* and *Methanocella* were based on dual-labeled probes. Each reaction was 25 µl in volume and contained the following mixture: 12.5 µl JumpStart Taq ReadyMix, 4 mM MgCl₂, 0.8 ng µl⁻¹ BSA (Ambion), 0.5 µM of each primer, 0.2 µM of the dual-labeled probe and 5 µl of template. For the two assays, the program used was: 94 °C for 4 min, followed by 40 cycles of 94 °C for 30 s and 60 °C for 60 s for annealing, extension and signal reading.

Data transformation and statistical analysis

Affiliation of TRFs to their respective archaeal taxa was done using *in silico* prediction of the restriction

sites in the sequence data. For principal component analysis, TRFLP data were transformed and standardized as described previously (Angel *et al.*, 2010). Principal component analysis plots were computed using Canoco 4.53 and plotted using CanoDraw 4.12 (<http://www.canoco.com>).

Results

Methanogenic potential in upland soils

The methanogenic potential in upland soils was tested by incubating the samples under flooded conditions with anoxic gas headspace. Of the 27 soil samples tested, 22 produced at least some CH₄ during the incubation (Table 1). We observed strong differences in the extent of the methanogenic potential of the soils, which were expressed in up to four orders of magnitude difference in daily production rates. Of the 22 samples, we classified 10 as having a strong methanogenic potential (highly methanogenic soils), meaning they had CH₄ production rates in the hundreds or thousands nmolgdw⁻¹ per day. In most incubations, CH₄ was detected within 7–14 days, but a linear CH₄ production began, on average, at day 21. Interestingly, CH₄ production rates and lag times did not correlate with parameters such as precipitation, soil water content and organic matter, which would seem to be most important for supporting methanogenesis. For example, desert crust samples from the Negev Desert, Israel, had more than double the rate of potential CH₄ production than the meadow samples. With respect to the desert samples, we observed that the BSC layer was the only truly active layer in the soil, whereas layers below it had a very low or no methanogenic potential. The only exception are the samples from the Liman (constructed desert minicatchment), whose soil periodically behaves like pond sediments when the Liman is flooded (after heavy rain), and also builds up much quicker than the native soil due to alluviation processes (as it is built to drain its surrounding area).

Stable isotope analysis of CH₄ and CO₂

In parallel to measuring potential CH₄ production rates of the soils, we have also determined the isotopic signature ($\delta^{13}\text{C}$) of the carbon in the CH₄ and CO₂ being produced. This was done to estimate the proportion of each of the two main methanogenic pathways—hydrogenotrophic and acetate—our experiments. In addition, those samples that we classified as highly methanogenic were also incubated with CH₃F to inhibit acetate methanogenesis. This allowed us to specifically determine the isotopic signature of the CH₄ produced through hydrogenotrophic methanogenesis (δ_{mc}).

The BSC samples from Israel showed a temporal trend during the incubation by which CH₄ began with a light isotopic signature of the carbon

($\delta^{13}\text{C}$ = approximately -57 on average) and became heavier with time (up to approximately -35 on average), whereas the isotopic signature of the carbon in the CO₂ became slightly lighter over time starting at $\delta^{13}\text{C}$ = approximately -9 and shifting toward approximately -15 , on average (Table 3). A similar phenomenon with respect to CH₄ but not to CO₂ was observed for incubations of the samples from Utah and Germany, whereas the incubations from Australia showed a relatively stable isotopic signature in both CH₄ and CO₂ over time. Fractionation factors (ϵ) in all non-inhibited incubations were smaller than those in the inhibited incubations, but higher than those obtained for purely acetate methanogenesis that typically range between ~ 21 and 27 (Krzycki *et al.*, 1987; Gelwicks *et al.*, 1994), indicating a mixture of both pathways. To precisely determine the contribution of hydrogenotrophic and acetate methanogenesis, knowledge of the exact isotopic signatures of the carbon in the CH₄ produced in each pathway is required. One can, however, estimate the relative contribution of each pathway if the isotopic signature of the carbon in the CH₄ ($\delta^{13}\text{C}\text{-CH}_4$) produced in the inhibited incubations is used as the specific signature for CH₄ produced from H₂/CO₂ alone (δ_{mc}) and the isotopic signature of the organic carbon in the soil is used to estimate the signature of CH₄ produced from acetate. The measured $\delta^{13}\text{C}$ of the organic carbon in the soil samples (which is the substrate for acetate) differed somewhat between sites but ranged from approximately -20.2 to -27.9 . The fractionation factor for acetate methanogenesis in pure cultures varies with respect to substrate concentration and can reach up to $\epsilon = \sim 25.6$ when substrate is not limiting (Goevert and Conrad, 2009). As acetate concentrations were low in our incubations (relative to those used by Goevert and Conrad, 2009), one can expect a relatively high substrate turnover and assume a low fractionation factor (~ 10). Using Equation 4 and these assumptions, the fractions of hydrogenotrophic methanogenesis in the system were estimated (Table 3). In all cases, except the samples from Australia, methanogenesis began with a dominance of hydrogenotrophic methanogenesis but later on dropped to about 10–25% of the CH₄ being produced.

Molecular characterization of the methanogenic community

We characterized the archaeal community in the soil samples and incubated slurries through cloning and TRFLP profiling of the 16S rRNA and compared the preincubated with the post-incubated state of both inhibited and non-inhibited incubations. Surprisingly, the diversity of the methanogenic community in our samples was very low and nearly identical, although samples were obtained from different parts around the world. In all of the post-incubated samples, we identified only *Methanosarcina*- and

Table 3 Stable carbon isotope analysis of the methane and CO₂

	$\delta^{13}\text{C-C}_{\text{org}}$ (‰)		$\delta^{13}\text{C}$ (‰)				Apparent fractionation factor (ϵ_{app})		δ_{mca} (‰)	Fraction of H ₂ /CO ₂ methanogenesis	
	Non-acidified	Acidified	CH ₄ begin ^b	CH ₄ end	CO ₂ begin	CO ₂ end	ϵ begin	ϵ end	δ_{mca} (‰)	f_{H_2} begin	f_{H_2} end
Israel (Natural field)	-5.4	-21.1	-57.3 ± 4.9	-34.9 ± 2.5	-9.1 ± 2.1	-15.3 ± 1.2	53 ± 6	25 ± 4	-31.1	0.74	0.1
Israel + CH ₃ F			-66.3 ± 3.2	-68.7 ± 0.6	-7.5 ± 1.67	-12.2 ± 1.1	61 ± 3				
Utah (sample 1)	-26.4	-26.6	-74.4 ± 6.8	-44.4 ± 3.4	-24.8 ± 1.8	-24.9 ± 0.6	54 ± 10	27 ± 4	-36.6	1.00	0.25
Utah + CH ₃ F				-70.4 ± 3.6		-25.2 ± 0.4	49 ± 3				
Australia (sample 2)	-20.6	-20.4	-69.6 ± 4.4	-65.4 ± 2.7	-20.0 ± 0.1	-16.8 ± 0.7	34 ± 7	46 ± 6	-30.4	0.72	0.67
Australia + CH ₃ F			-85.3 ± 6.0	-82.6 ± 0.9	-17.1 ± 0.6	-15.2 ± -6.2	76 ± 3				
Germany (0-7.5 cm)	-28.3	-28.2	-80.1 ± 2.9	-51.9 ± 11.0	-22.2 ± 0.8	-22.7 ± 0.8	63 ± 4	30 ± 11	-38.2	0.95	0.27
Germany + CH ₃ F			-82.1 ± 3.16	-89.4 ± 1.2	-22.8 ± 0.1	-21.3 ± 0.4	69 ± 3				

^aδ_{mca} was calculated by assuming a $\epsilon = -10\text{‰}$ fractionation from organic carbon (see text).

^b'Begin' and 'end' refer to the first third and last two thirds of the incubation period, respectively.

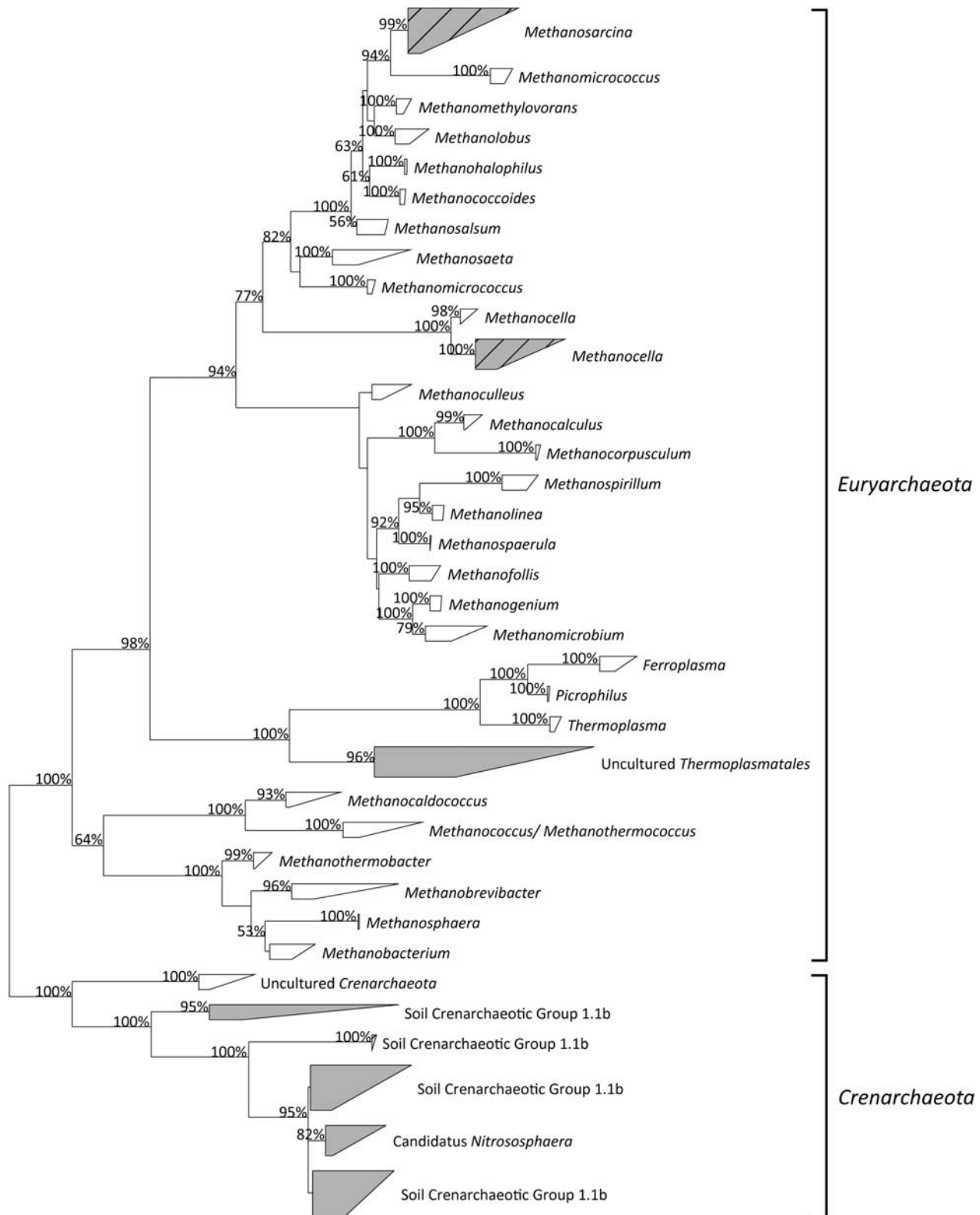
Methanocella-related sequences along with a variety of crenarchaeal sequences, typically associated with aerated soils such as soil crenarchaeotic group 1.1b and occasionally sequences related to *Thermoplasmatales* (Figure 1). The latter two groups were detected as sole members of the archaeal communities in the samples before incubation, whereas none of the methanogens could be detected in our 16S rRNA clone libraries or TRFLP profiles. Inhibition with CH₃F resulted in lower CH₄ production rates but did not change the identity of the methanogenic community. Not only did we detect nothing but *Methanosarcina* and *Methanocella* as methanogenic members in our post-incubated samples, their within-group sequence diversity was itself very low. For example, the average sequence difference between *Methanosarcina* from Avdat BSC (Israel) and Giessen meadow soil (Germany) was only 1.8% ± 0.025 and that between the *Methanocella* members only 3.3% ± 0.1.

TRFLP analysis

We used TRFLP profiling to decipher relative changes in the archaeal community from *in situ* to post-incubated state and with respect to the effect of inhibition with CH₃F. A total of 67 replicated TRFLP profiles, representing the soil samples before incubation and the inhibited and uninhibited slurry incubations, are summarized in Figure 2. As expected, all preincubated samples (green circles) had similar 16S rRNA profiles on both DNA and RNA levels and clustered very tightly with respect to the primary principal component, where methanogens showed the highest loadings. Most post-incubated samples (red and blue circles) were found on the right to the ordinate, indicating methanogenic activity, and their approximate CH₄ production rates can be deduced from the fitted isolines (LOESS fit). Those samples that did not demonstrate potential methanogenic activity remained close to the preincubated samples in these plots. *Methanosarcina* was more abundant than *Methanocella* in most methanogenic samples on the DNA level and seemed to have been overwhelmingly dominant on the RNA level. It seems that the inhibition with CH₃F, while reducing methanogenic activity, did not have an effect on the composition of the methanogenic community.

Molecular characterization using the mcrA marker gene

Our inability to detect methanogens in the 16S rRNA gene TRFLP profiles and clone libraries of the preincubated soil samples is most likely the result of their low relative abundance compared with the populations of *Crenarchaeota* and *Thermoplasmatales*. Any community member that comprises 1% or less of the community would be undetected in TRFLP profiles (due to the method's threshold and



0.10

Figure 1 Maximum likelihood phylogenetic tree based on aligned partial archaeal 16S rRNA gene sequences. Sequences were aligned against the SILVA 102 database using the SINA aligner, and the tree was calculated with RAxML 7.04 using rapid hill climbing algorithm and GTRMIX evolutionary model. Bootstrap values above 50% (out of 100 trials) are displayed next to the nodes. Shaded clusters contain sequences that were only detected in the preincubated soil samples, whereas sequences from the post-incubated slurries cluster both into the shaded clusters and into the shaded clusters with diagonal lines.

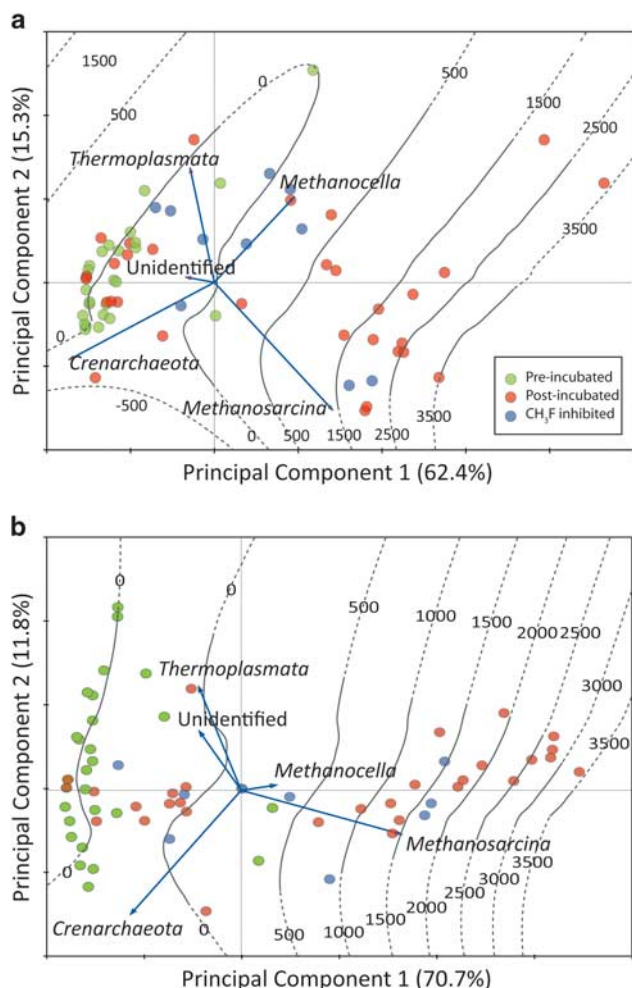


Figure 2 Principal component analysis plots of the archaeal community as deciphered from the *16S rRNA* gene TRFLP profiles. Circles indicate individual samples. The relative abundance of each of the archaeal types can be estimated from the perpendicular projection of each sample to the individual vectors, whereas the length of each vector indicates the variance (range of relative abundance values) of its respective archaeal type. CH_3F was used to inhibit acetoclastic methanogenesis. Fitted methane production rates are shown as isolines; (a) DNA TRFLP profiles and (b) rRNA TRFLP profiles.

standardization procedures) and also in the clone libraries (considering our sequencing efforts). We therefore performed an analysis of the methanogenic community in both the preincubated soil samples and the post-incubated slurries using the *mcrA* functional gene. Although we were unable to amplify the gene in all our samples (most likely due to the low abundance of methanogens in the samples before incubation), we did obtain PCR products from at least one sample at each site. The phylogenetic affiliation of these sequences is presented in Figure 3. The majority of the *mcrA* sequences belonged to either *Methanosarcina* or *Methanocella*, confirming our observation from the analysis of the *16S rRNA* genes and transcripts of the post-incubated slurries. We did, however, detect

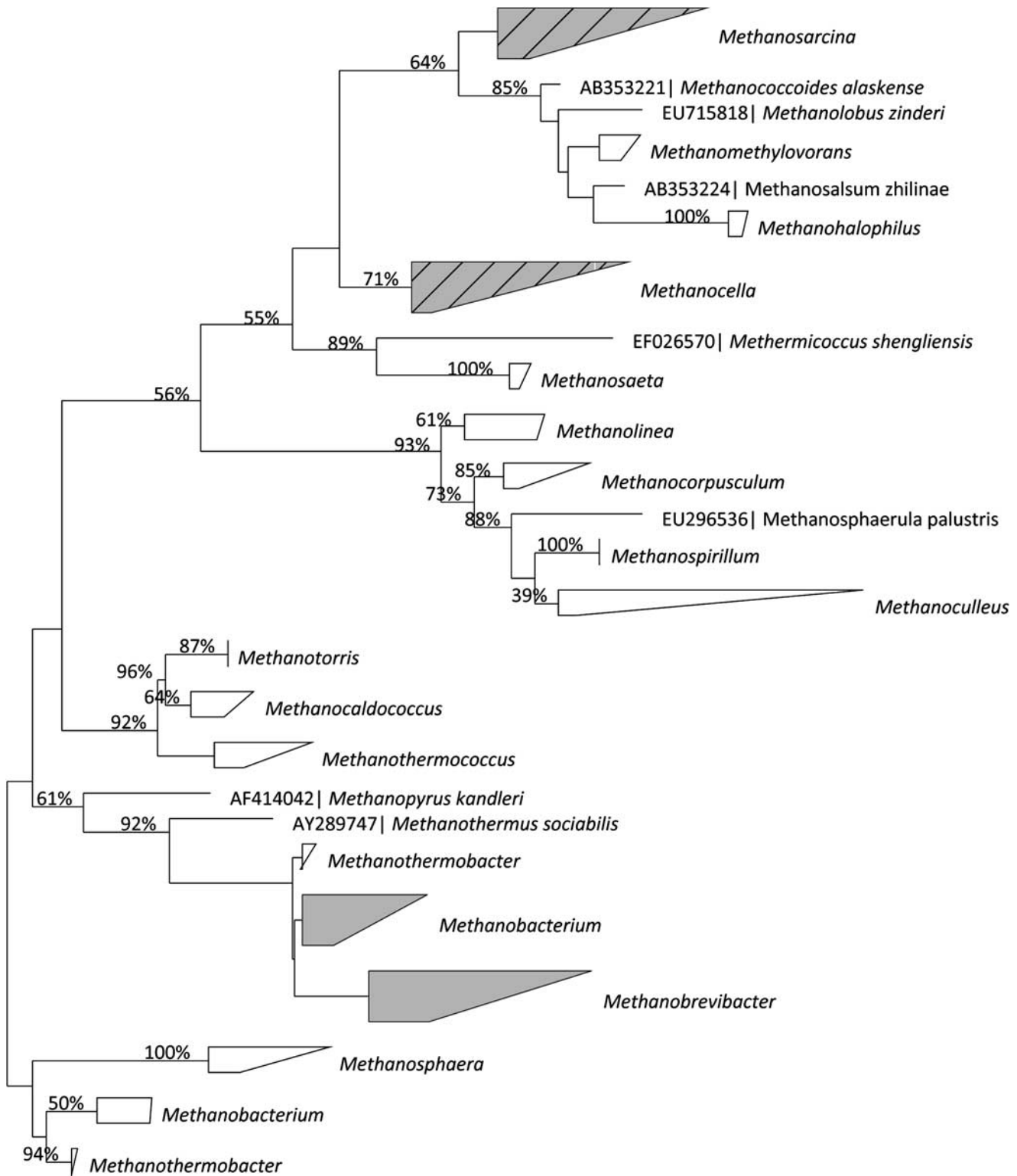
several sequences belonging to *Methanobrevibacter* and *Methanobacterium*, which were not detected in the post-incubated samples using the *16S rRNA* gene analysis.

Quantification of the methanogenic community

To determine the quantitative effect of our incubations on the different members of the archaeal community, we used qPCR and quantified the total archaeal, the crenarchaeal communities and the two methanogens that were identified in the phylogenetic analysis. Quantification was performed on the soil samples before incubation and the post-incubated slurries. Copy numbers of the archaeal *16S rRNA* gene in the highly methanogenic samples rose between 0.5 and 2 orders of magnitude in the post-incubated slurries compared with the preincubated soils and were moderately correlated to CH_4 production rates (Figure 4a). Both methanogen types were undetected in the preincubated soil samples and non-methanogenic samples (that is, they were below 10^3 copies) but reached up to 10^9 copies gdw^{-1} after the incubation. Copy numbers of *Methanosarcina* and *Methanocella* *16S rRNA* gene were within one order of magnitude from each other. As both methanogen types contain three copies of the *16S rRNA* gene in their genomes, the actual number of methanogenic cells is a third of the gene copy numbers displayed in the figure. Copy numbers of both methanogens were positively correlated to CH_4 production rates, but *Methanosarcina* was better correlated (Figures 4c and d). In contrast, gene copy numbers of *Crenarchaeota* did not change in most samples between the pre- and post-incubated states and showed no correlation to CH_4 production rates (Figure 4b).

Discussion

When the domain *Archaea* was first described by Woese and Fox (1977), it was thought to be an ancient lineage of prokaryotes (hence the name), which evolved early in the evolutionary history in a world which was hotter and whose atmosphere and hydrosphere were much more reduced. This notion emerged from the fact that at that time the known archaea were predominantly extremophiles or anaerobes (Woese *et al.*, 1990; Takai and Horikoshi, 1999). In the past two decades though, environmental surveys using molecular screening tools have detected numerous archaeal sequences in virtually all mesothermic environments, including aerated soils, ocean water and freshwater (DeLong, 1992; Bintrim *et al.*, 1997; Schleper *et al.*, 1997). It is mainly based on these studies that *Archaea* are now considered as cosmopolitan as *Bacteria*, adapted to their ecological niches and perform a variety of metabolic activities (DeLong, 1998; Schleper *et al.*, 2005; Leininger *et al.*, 2006; Auguet *et al.*, 2010).



0.10

Figure 3 Maximum likelihood phylogenetic tree based on aligned partial amino-acid sequences of the *mcrA*. Amino-acid composition was deduced from DNA sequences, and the tree was calculated with RAxML 7.04 using rapid hill climbing algorithm and PROTMIX-JTT evolutionary model. Bootstrap values above 50% (out of 100 trials) are displayed next to the nodes. Shaded clusters contain sequences that were only detected in the preincubated soil samples, whereas sequences from the post-incubated slurries cluster both into the shaded clusters and into the shaded clusters with diagonal lines clusters.

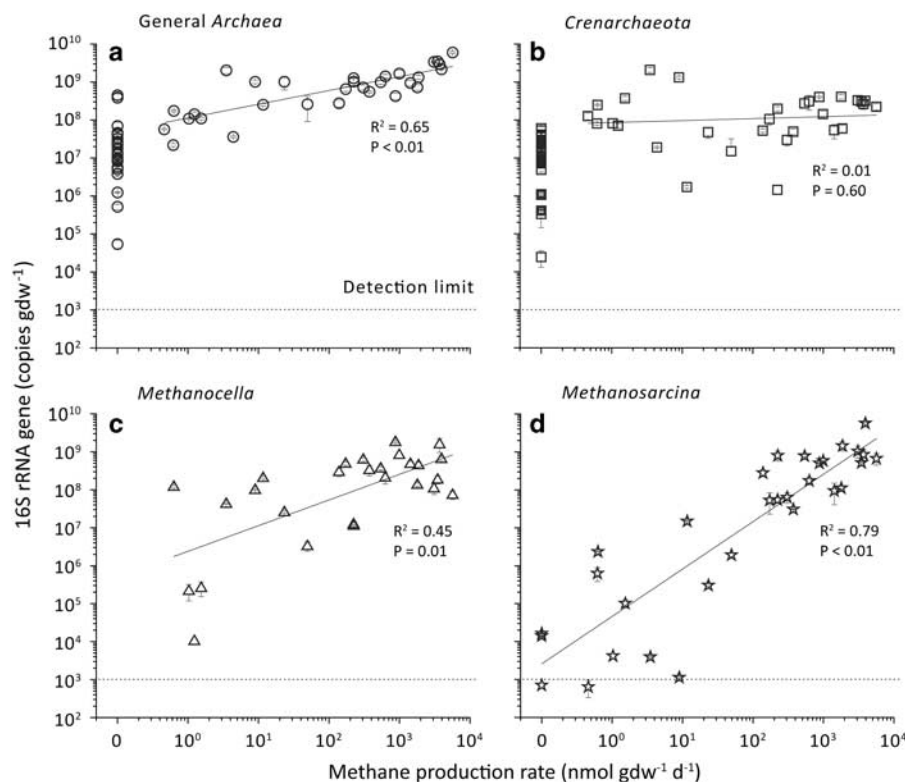


Figure 4 Archaeal 16S rRNA gene copy numbers are quantified using qPCR plotted against potential methane production rates. Both preincubated soil samples as well as post-incubated slurries are shown in these plots. The preincubated samples as well as the slurries that showed no methanogenic potential are positioned at 0 on the x axis.

Here, we have shown that methanogens are also ubiquitous in mesophilic, aerated soils around the world. Through simple anoxic incubations with only water but no additional substrate added, nearly all the soil samples produced CH_4 from degradation of soil organic matter. Many of our samples had very low methanogenic potential and long lag times, but some samples reacted very quickly and produced ample amounts of CH_4 within a few days. Particularly intriguing is the fact that in the arid samples from Israel it was the topmost part of the soil—the BSC—which held nearly all of the methanogenic potential of the soil. Although it is true that BSCs contain most of the microbial biomass and activity in desert soils (Garcia-Pichel and Belnap, 2003), it is also the layer most exposed to oxygen. In fact, as BSCs are mostly formed by photosynthetic microorganisms, they can turn hyperoxic at parts during daytime when moist (Garcia-Pichel and Belnap, 1996). The existence of soils with such high methanogenic potential and fast response indicates that they are also active, at least at certain times, under natural conditions.

It is difficult to suggest from the data what determines the difference in methanogenic potential in the different soil samples. Important factors that might reduce the methanogenic potential are low quantity and quality of available organic matter (Wagner *et al.*, 2005; Guérin *et al.*, 2008), low soil pH

(Phelps and Zeikus, 1984) and the availability of alternative electron acceptors (Peters and Conrad, 1996; Yao *et al.*, 1999). Desert soils are typically neutral/alkaline, well buffered and are poor in nitrogen, iron and manganese, which could serve as alternative electron acceptors (Knight, 1991). Sulfate may be available in desert soils containing gypsum, but we found no evidence for substantial sulfate reduction in our incubations (data not shown). It is unclear to which extent the abiological characteristics of desert soil can explain the relatively high methanogenic potentials of some of our desert samples. However, biological activity in the BSCs that typically form on the surface of most desert soils may support methanogenesis through the supply of fresh organic matter from photosynthesis by cyanobacteria during daytime and the depletion of oxygen through respiration activity during nighttime (Garcia-Pichel and Belnap, 1996; Lange, 2003; Angel *et al.*, 2011).

Similar to many natural environments, we found a mixture of both acetoclastic and hydrogenotrophic methanogenesis in our incubations (Conrad, 1999), but the specific proportion of each pathway varied between samples and even within certain samples as incubation proceeded. All incubations began with CH_4 being produced mostly or entirely out of hydrogen and CO_2 , but in nearly all cases shifted to being predominantly acetoclastic as the incubation

proceeded. This pattern is known from studies on methanogenesis in rice field soils (Roy *et al.*, 1997), and is explained by the temporal change in thermodynamic conditions and differential expression of microbial activity upon flooding (Glissmann and Conrad, 2002; Conrad, 2002). The ratio of acetoclastic methanogenesis in these samples stabilized around 60–70%, which is very close to the ratio found in many natural environments in general (Conrad, 1999) and anoxically incubated upland soils in particular (Peters and Conrad, 1996). Methanogenesis in the samples from Australia, on the other hand, remained predominantly hydrogenotrophic throughout the incubation.

Although methanogenic potential thus seems to be a global trait of upland soils (albeit of different characteristics), we have recovered a remarkably low diversity of methanogens in our samples. Of the 30 known methanogenic genera, we found only 2 closely related genera in our post-incubated samples belonging to *Methanosarcina* and *Methanocella*, both types are of high ecological importance. *Methanosarcina* are, along with *Methanosaeta*, the only methanogens capable of performing acetoclastic methanogenesis and are therefore predominant methanogens in most natural environments (Liu and Whitman, 2008). *Methanocella* is a newly discovered genus, also globally distributed but colonizes rice roots in particular (Grosskopf *et al.*, 1998). *Methanocella* was also shown to be of high importance as one of the primary CH₄ producers in rice fields, probably feeding on fermentation products of root exudates (Lu and Conrad, 2005). Although phylogenetically closely related to *Methanosarcina* and similar in their cellular structure, *Methanocella* species can only perform hydrogenotrophic methanogenesis (Sakai *et al.*, 2010).

In our samples, it appears that *Methanosarcina* was the dominant methanogens. Certainly, this stems from our analysis of methanogenic pathways, where in the latter part of the incubations acetoclastic methanogenesis accounted for roughly two-thirds of the newly formed CH₄, but also generally speaking *Methanosarcina* had a higher relative abundance in the TRFLP profiles and their absolute abundance correlated better with CH₄ production rates. Similarly, *Methanosarcina* and *Methanocella* were also the methanogenic types that were found by Nicol *et al.* (2003) in a pasture soil in Scotland and by Poplawski *et al.* (2007) in a barley field in Sweden, thus reinforcing our notion that these types are universal upland soil methanogens. In cases where heavy grazing was involved, the authors have also found *Methanosarcina* as dominant methanogens, but along with it other types that could be directly associated to rumen microflora (Gattinger *et al.*, 2007; Radl *et al.*, 2007). To our knowledge, there is only one molecular study explicitly targeting archaea in desert soils (Soule *et al.*, 2009), detecting only Crenarchaeota of the group 1.1b in the native soil.

The phylogenetic analysis of the preincubated soils using *mcrA* revealed also the presence of a few other methanogens that are closely affiliated with *Methanobrevibacter* and *Methanobacterium*. None of these methanogens could be enriched in our incubations and it remains unclear whether these were viable cells. Nevertheless, their presence does imply that the diversity of the methanogenic community in these aerated soils is potentially wider.

The formation of biogenic CH₄ when aerated soils are incubated anoxically testifies not only for the presence of active methanogens but also for the presence of many other anaerobic microorganisms. As CH₄ is only an end product in a cascade of anaerobic degradation processes, other viable anaerobic microorganisms from fermenters to acetogens and syntrophs need to be present in the soil for it to be formed (Garcia *et al.*, 2000; Stams and Plugge, 2009). The presence and potential activity of such anaerobes in upland soils have indeed been shown in the past (Kusel and Drake, 1994; Degelmann *et al.*, 2009). It thus indicates that upland soils host not just methanogens but entire consortia of anaerobes capable, at least potentially, to carry a full anaerobic degradation pathway.

Methanogens of the type *Methanosarcina* and *Methanocella* appear to be autochthonous and universal members of the biome in aerated soils. Despite being exposed to constant oxygen flux when the soil is dry, a core population of these methanogens is able to survive and become rapidly active when incubated anoxically with water. The existence of a unique and viable upland soil methanogenic population is an indication for a natural niche for these microorganisms, yet their activity in field conditions still remains to be shown.

Acknowledgements

We thank Dr Claudia Kammann of Giessen FACE Experiment for providing the samples from Giessen (Germany); Professor Andreas Brune for sampling in Australia and Professor JC Ensign for sampling in Utah. The Zuckerberg Institute for Water Research of Ben-Gurion University of the Negev, Israel, for allowing the use of their facilities; the Arava Institute for Environmental Studies, Israel, for providing boarding services and administrative assistance; The Institute for Soil Science and Forest Nutrition (IBW) at the University of Göttingen, Germany, for determining the isotopic signature of the soil organic carbon; The Analytical Chemical Laboratory of the Philipps University, Marburg, for the CN analyses; and the Chair of the Physical Geography, Aachen, for analysis of the soil texture. RA was supported by a fellowship of the Max Planck Society.

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