Effect of Temperature on Anaerobic Ethanol Oxidation and Methanogenesis in Acidic Peat from a Northern Wetland

Martina Metje and Peter Frenzel*

Max Planck Institute for Terrestrial Microbiology, D-35043 Marburg, Germany

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The effects of temperature on rates and pathways of CH_4 production and on the abundance and structure of the archaeal community were investigated in acidic peat from a mire in northern Scandinavia (68°N). We monitored the production of CH_4 and CO_2 over time and measured the turnover of Fe(II), ethanol, and organic acids. All experiments were performed with and without specific inhibitors (2-bromoethanesulfonate [BES] for methanogenesis and CH_3F for acetoclastic methanogenesis). The optimum temperature for methanogenesis was 25°C ($2.3 \mu\text{mol CH}_4 \cdot \text{g} [\text{dry weight}]^{-1} \cdot \text{day}^{-1}$), but the activity was relatively high even at 4°C ($0.25 \mu\text{mol CH}_4 \cdot \text{g} [\text{dry weight}]^{-1} \cdot \text{day}^{-1}$). The theoretical lower limit for methanogenesis was calculated to be at –5°C. The optimum temperature for growth as revealed by real-time PCR was 25°C for both archaea and bacteria. The population structure of archaea was studied by terminal restriction fragment length polymorphism analysis and remained constant over a wide temperature range. Hydrogenotrophic methanogenesis accounted for about 80% of the total methanogenesis. Most 16S rRNA gene sequences that were affiliated with methanogens and all *McrA* sequences clustered with the exclusively hydrogenotrophic order *Methanobacteriales*, correlating with the prevalence of hydrogenotrophic methanogenesis. Fe reduction occurred parallel to methanogenesis and was inhibited by BES, suggesting that methanogens were involved in Fe reduction. Based upon the observed balance of substrates and thermodynamic calculations, we concluded that the ethanol pool was oxidized to acetate by the following two processes: syntrophic oxidation with methanogenesis (i) as an H_2 sink and (ii) as a reductant for Fe(III). Acetate accumulated, but a considerable fraction was converted to butyrate, making volatile fatty acids important end products of anaerobic metabolism.

Peatlands cover $400 \times 10^6 \text{ km}^2$ worldwide, and their distribution is centered in the northern hemisphere (22). In spite of low rates of primary production, northern peatlands have accumulated 200 to 455 Pg carbon since the last glaciation, corresponding to 20 to 30% of the global soil carbon pool (18, 22, 55). Hence, northern peatlands have been a substantial sink for atmospheric CO_2 , but they are also sources of atmospheric methane (42, 49). When the “global warming potential” of CH_4 is factored in, carbon sequestration and CH_4 emission are balanced (48). The ongoing temperature increase in the north (25) is expected to affect this balance. Higher temperatures may transform permafrost soils to wetlands and accelerate mineralization in extant peatlands. Temperature may also affect biogeochemical cycles and pathways of carbon flow, as was shown in vitro for other cold ecosystems (19, 53).

Although countless CH_4 fluxes have been measured in northern peatlands (42, 49), little is known about methanogenic substrates and the dominating biochemical pathways. In general, H_2 - CO_2 and acetate are the preferred substrates for methanogenesis. With carbohydrates as the main substrate, about two-thirds of the CH_4 produced in nature should originate from acetate (8). In a northern peatland (Alaska, 60°N), acetate was found to be an end product that was not used at all by methanogens (12, 26). In contrast, acetoclastic methanogenesis accounted for up to 85% of the methanogenesis in a

temperate peatland (42°N, Michigan), but there were large seasonal fluctuations (2).

Significant microbiological and physiological work has been done for temperate bogs (27), but we are not aware of a study of northern peatlands examining the methanogenic community, substrate usage, and the effect of temperature. In minerotrophic mires, Fe reduction may compete with methanogenesis for substrates. Hence, we focused on (i) methanogenesis and Fe(III) reduction, (ii) the carbon substrates and biochemical pathways involved, and (iii) the structure of the methanogenic community. Our experiments were conducted with acidic peat from a mire in northern Finland (68°N) and covered the temperature range from 4°C to 60°C in 2°C steps. We combined culture-independent approaches with process measurements. In a pilot study we became aware that ethanol was a major intermediate in the samples, and the concentrations were up to 10.5 mM. Methanogens are virtually unable to use primary alcohols (62), but they may take advantage of the H_2 released during anaerobic oxidation of ethanol (32, 51, 59). To determine the most probable pathways involved in anaerobic ethanol oxidation, we combined inhibitor experiments with mass balance and thermodynamic calculations.

MATERIALS AND METHODS

Sampling site and peat. Peat samples were obtained from a mire in northern Finland (68°14.32'N, 27°11.44'E) in September 2002. The mean annual temperature in this region is –1.1°C. The mean daily temperatures are above 0°C during 5 or 6 months of the year, and the maximum mean daily temperature is 13.4°C in July (data for Ivalo at 68°36'N, 27°24'W) (35). The water table was close to the surface. Bubble-free peat samples were obtained from below the water table with sterile polyethylene terephthalate bottles (Nalgene). Samples were stored at 4°C until they were analyzed. The peat originated from fragments of sedges and

* Corresponding author. Mailing address: Max Planck Institute for Terrestrial Microbiology, Karl von Frisch Str., D-35043 Marburg, Germany. Phone: (49) (0)6421-178820. Fax: (49) (0)6421-178809. E-mail: frenzel@staff.uni-marburg.de.

mosses. It also contained some fine roots that were presumably alive when the samples were taken. The dry weight was 4.8%, and the loss on ignition was 77%. The pH was 4.1.

Incubation. The peat samples were diluted 1:1 (vol/vol) with O₂-free autoclaved distilled water and blended. The resulting slurries (8 ml) were placed into sterile test tubes (16 ml), which were closed with butyl rubber stoppers and capped. All handling was done in an anaerobic box under an N₂ atmosphere. The slurries were incubated in a custom-made temperature gradient block that was heated at one end and cooled at the other (28, 54). The gradient covered the range from 4°C to 60°C in 2°C steps. Two replicates per temperature were incubated for 4 weeks. The headspace concentrations of CH₄ and CO₂ were measured up to three times per week after shaking to equilibrate the gas and liquid phases. The concentration of H₂ in the headspace was measured at the end of the experiment. Pore water samples were taken at the beginning and at the end of the experiment and were analyzed for fatty acids and alcohols. Similarly, slurry samples were taken to measure Fe(II) concentrations.

Acetoclastic methanogenesis was determined by inhibition with methyl fluoride (CH₃F) (final mixing ratio, 1%), a specific inhibitor of acetoclastic methanogenesis (16, 29). An oxygen-free solution of sodium 2-bromoethanesulfonate (BES) (final concentration, 40 mM) was used to completely inhibit methanogenesis. In a pilot experiment with acidic peat a BES concentration of 40 mM was found to be necessary to inhibit methanogenesis completely. Two replicates were incubated for 4 weeks at 4, 10, 15, 25, 30, 37, and 45°C.

At temperatures between 15 and 30°C, the CH₄ concentration increased linearly until day 15 to 20. After this, the rate of CH₄ production decreased. At lower and higher incubation temperatures, the increase was linear with time during the whole incubation period (1 month). Hence, CH₄ production was calculated (i) from the linear part of the curves by regression analysis (termed "rate") and (ii) from the amount of CH₄ that accumulated until the end of the experiment after 1 month (termed "accumulation").

Analytical techniques. CH₄ and CO₂ were analyzed with a gas chromatograph with a flame ionization detector (SRI-9300; SRI Instruments, Torrance, Calif.) with H₂ as the carrier gas; the instrument was equipped with a custom-made methanizer with an Ni-based catalyst. Calibration was done with certified standards (Messer-Griesheim, Krefeld, Germany). H₂ was analyzed with a gas chromatograph equipped with a temperature-conductivity detector (SRI-9300; SRI Instruments, Torrance, Calif.) with N₂ as the carrier gas.

Liquid samples were filtered through 0.2-μm membrane filters (Schleicher & Schuell, Dassel, Germany) and stored at -20°C until analysis. Organic acids (lactate, formate, acetate, propionate, butyrate, and caproate) were measured by high-performance liquid chromatography on an Aminex HPX-87H ion exclusion column (Bio-Rad Laboratories, Hercules, Calif.) with a refractive index detector (RI2000; Sykam, Gilching, Germany) and a UV detector (UVIS 205; Linear Instruments, Reno, Nev.). Acetone, methanol, propanol, 2-propanol, butanol, 2-butanol, and ethanol were measured by using a gas chromatograph equipped with a flame ionization detector (Carlo Erba 8000) and a BP 20 column (inside diameter, 0.32 mm; length, 25 m; 0.5 μm; SGE, Austin, Tex.) with 1-pentanol as an internal standard (final concentration, 10 mM). Chromatograms were analyzed with the Peak Simple software (SRI Instruments, Torrance, Calif.).

Samples used for Fe(II) analysis were taken from a peat slurry at the beginning and end of incubation at 4, 10, 15, 25, 30, 37, and 45°C (with and without BES). Fe(II) was extracted with 0.5 M HCl and measured as described by Phillips and Lovley (44), as modified by Ratering and Schnell (45).

DNA extraction and PCR amplification. Slurries samples were obtained at the beginning of the experiment and after 4 weeks of incubation at 4, 10, 15, 25, 30, 37, and 45°C. The samples were homogenized with a pestle and mortar to break up macroscopic peat structures. DNA was extracted with a FastDNA SPIN kit for soil used according to the manufacturer's instructions (Qbiogene, Carlsbad, Calif.). To remove PCR-inhibiting compounds (mainly humic acids) from the extract, two further washing steps with guanidine thiocyanate (5.5 mM; Sigma) were necessary.

Archaeal 16S rRNA genes were amplified using primers Ar109f and Ar915r or Ar915r labeled at the 5' end with 6-carboxyfluorescein (56) (MWG Biotech, Ebersberg, Germany). The PCR was performed as follows: 30 s at 94°C, 45 s at 53°C, and 1.5 min at 72°C for 32 cycles, a primary denaturation step consisting of 3 min at 94°C, and final DNA synthesis for 5 min at 72°C. The gene encoding the α-subunit of the methyl-coenzyme M reductase was amplified using primers ME1 and ME2 (24) (MWG Biotech, Ebersberg, Germany). The PCR was performed as follows: 45 s at 94°C, 45 s at 50°C, and 1.5 min at 72°C, a primary denaturation step consisting of 3 min at 94°C, and final DNA synthesis for 5 min at 72°C. PCR products were purified with a QIAquick PCR purification kit (QIAGEN, Hilden, Germany).

For real-time PCR, DNA was extracted and purified as described above and was quantified using the PicoGreen assay (40). Archaeal small-subunit rRNA genes were quantified by real-time PCR by using an iCycler iQ real-time PCR system (Bio-Rad, Munich, Germany) and primers Ar109f and Ar915r (23).

T-RFLP analysis. Terminal restriction fragment length polymorphism (T-RFLP) analysis was performed as described previously (6). In short, purified 16S rRNA gene fragments were quantified by UV photometry (Biophotometer; Eppendorf, Hamburg, Germany). The fluorescently labeled PCR products (70 ng) were digested with TaqI (Promega, Mannheim, Germany) and analyzed with an ABI PRISM 373 DNA sequencer (Applied Biosystems, Weiterstadt, Germany). The electropherograms were analyzed with GeneScan, version 2.1 (Applied Biosystems). Relative amplicon frequencies were determined by determining relative signal intensities of terminal restriction fragments (T-RFs) from peak heights (40). Signals with a peak height that was less than 100 relative fluorescence units were regarded as background noise and excluded from the analysis. The percentages of fluorescence intensity represented by single T-RFs were calculated relative to the total fluorescence intensity of all T-RFs.

Cloning, sequencing, and phylogenetic analysis. Gene libraries for archaeal 16S rRNA and *mcrA* sequences were constructed using DNA extracts from the original peat sample. PCR products were ligated into pGEM-T vector plasmids (Promega, Mannheim, Germany) and transformed into *Escherichia coli* JM109 competent cells (Promega, Mannheim, Germany) according to the manufacturer's instructions. 16S rRNA genes were directly amplified with the archaeon-specific primers Ar109f and Ar915r. The resulting amplicons were restricted with TaqI. Plasmid DNA was sequenced with an automated ABI Prism BigDye terminator cycle Ready Reaction kit with AmpliTaq polymerase FS (Applied Biosystems) according to the manufacturer's instructions using primers M13 rev-29 (5'-CAGGAAACAGCTATGACC-3') and T7 (5'-TAATACGACTC ACTATAGGG-3'). 16S rRNA gene and *mcrA* sequences were assembled with SeqMan II (DNASTAR) and compared with the sequences available in the GenBank database using the BLAST network service to determine the approximate phylogenetic affiliations. Chimeric sequences of 16S rRNA genes were identified by Chimera Check of Ribosomal Database Project II (release 8.1) (7). Alignment and phylogenetic analysis of 16S rRNA gene sequences were done with ARB (38). Additional sequences that were potentially related to the retrieved clones were added to the existing tree using the ARB parsimony tool. 16S rRNA gene sequences (>790 bases) were selected to construct an archaeal base frequency filter (50 to 100% similarity), which was subsequently used to generate an initial maximum-likelihood tree with the TreePuzzle tool (10,000 puzzling steps; Schöniger-von Haeseler substitution model [52]; parameter estimation uses, neighbor-joining tree). In addition, the tree topology was evaluated using neighbor joining (Felsenstein distance correction), Phylip DNAPARS, and AxML as implemented in ARB. *Aquifex pyrophilus* was used as the outgroup.

An *mcrA* sequence database was constructed with 505 sequences which are publicly available from NCBI (<http://www.ncbi.nlm.nih.gov/>). The partial *mcrA* sequences obtained were assembled and checked with the LASERGENE software package (DNASTAR). After translation and alignment of the resulting amino acid sequences, an initial tree was constructed by neighbor joining with the PAM correction. Our sequences were added by quick add parsimony as implemented in ARB. For treeing, 85 *McrA* sequences were selected to construct a base frequency filter (25 to 100% similarity; 134 valid columns) (39), which was subsequently used to generate a maximum-likelihood tree with the TreePuzzle tool (1,000 puzzling steps; WAG substitution model [61]; parameter estimation by neighbor-joining tree). In addition, the tree topology was verified by PROTPARS (maximum parsimony) and PROTDIST with FITCH as the distance matrix, both from the PHYLIP package (version 3.573c; J. Felsenstein, University of Washington; <http://evolution.genetics.washington.edu/phylib.html>), and by neighbor joining with the PAM correction (ARB). *Methanopyrus kandleri* was used as the outgroup (accession no. AF414042).

Thermodynamic calculations. Thermodynamic calculations were done for all of the reactions shown in Table 1 except ethanol oxidation with Fe(III) as the e⁻ acceptor. Because the concentration and speciation of Fe(III) were not known, no calculation was possible. Standard Gibbs free energies (ΔG^0) were calculated from the standard Gibbs free energies of formation (G_f^0) of the reactants and products (58) (Table 1). The standard reaction enthalpies (ΔH^0) were calculated from the enthalpies of formation (H_f^0) of the reactants and products (10, 11, 34). ΔG^0 values were corrected for temperature by using the Van't Hoff equation (10). The actual Gibbs free energy (ΔG) under nonstandard conditions was calculated by using the Nernst equation (10). H₂, CH₄, and CO₂ were assumed to be gases. All other compounds were assumed to be dissolved. The concentrations and partial pressures that were actually measured were used to calculate ΔG at the beginning and end of the experiment. For H₂ we had only endpoint measurements. We assumed a steady-state situation with constant partial pres-

TABLE 1. Stoichiometries and Gibbs free energies for processes relevant for ethanol, acetate, and CH₄ turnover

Process	Stoichiometry	ΔG^0	Reference(s)
Direct ethanol oxidation to acetate by a methanogen	$2\text{CH}_3\text{CH}_2\text{OH} + \text{CO}_2 \rightarrow 2\text{CH}_3\text{COO}^- + \text{CH}_4 + 2\text{H}^+$	-31.7	62, 63
Syntrophic ethanol oxidation to acetate and H ₂	$\text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + 2\text{H}_2 + \text{H}^+$	49.5	13
Hydrogenotrophic methanogenesis	$4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$	-130.7	
Acetoclastic methanogenesis	$\text{CH}_3\text{COO}^- + \text{H}^+ \rightarrow \text{CO}_2 + \text{CH}_4$	-75.7	
Ethanol oxidation with Fe(III)	$\text{CH}_3\text{CH}_2\text{OH} + 4\text{Fe(III)} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + 4\text{Fe(II)} + 5\text{H}^+$		36
Butyrate formation	$2\text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2 \rightarrow \text{CH}_3(\text{CH}_2)_2\text{COO}^- + 2\text{H}_2\text{O}$	-107.6	58

tures throughout the experiment and combined all measurements into one graph. For calculations we used values interpolated by a kernel-weighted regression of H₂ against temperature (SYSTAT, version 11) (see Fig. 1).

Nucleotide sequence accession numbers. All sequences generated in this study have been deposited in the GenBank and EMBL databases under the following accession numbers: 16S rRNA sequences, DQ089002 to DQ089010; and *mcrA* sequences, DQ099684 to DQ099723, DQ090725, and DQ099726.

RESULTS

Reactants and processes. Even at temperatures between 4°C and 10°C the rates of CH₄ production were high (0.25 to 0.5 $\mu\text{mol} \cdot \text{g} [\text{dry weight}]^{-1} \cdot \text{day}^{-1}$). At the optimum temperature (25°C) the rate was 2.3 $\mu\text{mol} \text{CH}_4 \cdot \text{g} (\text{dry weight})^{-1} \cdot \text{day}^{-1}$. Similarly, the optimum temperature for CH₄ accumulation was 25°C (Fig. 1). Inhibition with CH₃F reduced the rates of CH₄ production only slightly, suggesting that >80% of the CH₄ was produced from H₂-CO₂ at the optimum temperature (25°C) (Fig. 1). However, at temperatures between 37°C and 45°C acetoclastic methanogenesis dominated, even at a very low rate of CH₄ production. BES completely inhibited methanogenesis at all temperatures.

The lowest H₂ partial pressures (4 Pa; 0.06 $\mu\text{mol} \text{g} [\text{dry weight}]^{-1} \cdot \text{day}^{-1}$) were observed at temperatures around the optimum temperature for methanogenesis (Fig. 1). At the lowest and highest temperatures, the H₂ partial pressures were much higher (200 and 2,000 Pa at 4°C and 45°C, respectively). CO₂ accumulated over the whole temperature gradient and particularly under nonmethanogenic conditions at temperature up to 60°C (Fig. 2). The increase was nonlinear and slowed with time.

FIG. 1. Accumulation of CH₄ and fraction of CH₄ produced from H₂-CO₂. (Left panel) Fraction of CH₄ calculated from the initial rates with and without CH₃F. (Right panel) H₂ partial pressures after 1 month of incubation at different temperatures. The line indicates the overall trend calculated with a kernel-weighted regression. gDW, grams (dry weight).

Acetate and butyrate accumulated with optimum temperatures of 32°C and 30°C, respectively. The maximum concentrations were 6 mM for acetate and 5 mM for butyrate (data not shown). The net accumulation or consumption was calculated by determining the difference between the final and initial concentrations (Fig. 3). Butyrate accumulation was reduced only slightly by CH₃F (data not shown) but was inhibited completely by BES (Fig. 3). Acetate accumulation was decreased by about 50% at 25°C in the presence of BES and even more at higher temperatures (Fig. 3). Propionate accumulated both with and without inhibitors, but it accumulated only at temperatures around the optimum temperature for methanogenesis and at a relatively low rate (10 $\mu\text{mol} \cdot \text{g} [\text{dry weight}]^{-1} \cdot \text{month}^{-1}$) (data not shown). The formate concentration was at the detection limit ($\leq 1.5 \mu\text{M}$) at temperatures between 4 and 50°C, but it increased at higher temperatures at a rate of up to 40 $\mu\text{mol} \cdot \text{g} (\text{dry weight})^{-1} \cdot \text{month}^{-1}$ in the control experiment (data not shown). In the physiological temperature range, however, acetate and butyrate were the most important volatile fatty acids, and they accumulated at rates of 38 and 50 $\mu\text{mol} \cdot \text{g} (\text{dry weight})^{-1} \cdot \text{month}^{-1}$, respectively, at the optimum temperatures (Fig. 3).

Ethanol exhibited the strongest net turnover of all the alcohol species detected. At the optimum temperature for methanogenesis its concentration decreased from 10.5 mM to 0.01 mM (detection limit). A net accumulation of ethanol was observed only at temperatures higher than 50°C. The

FIG. 2. CO₂ accumulation after 1, 6, 12, 20, and 27 days. gDW, grams (dry weight).

FIG. 3. Net turnover of Fe(II), ethanol, acetate, and butyrate ($\mu\text{mol} \cdot \text{g} [\text{dry weight}]^{-1} \cdot \text{month}^{-1}$) with and without BES. A negative value indicates net consumption, and a positive value indicates accumulation. The slight ethanol accumulation at temperatures above 50°C is obscured by the symbols. CH_4 concentrations (Fig. 1) are included for comparison. gDW, grams (dry weight).

concentrations of methanol, propanol, and 2-butanol were much lower, and the maximum concentrations were between 0.01 and 0.5 mM (data not shown). The optimum temperature for Fe(II) accumulation was the same as that for methanogenesis (25°C) (Fig. 3). At 4°C and 45°C about 50 $\mu\text{mol Fe(II)} \cdot \text{g} (\text{dry weight})^{-1} \cdot \text{month}^{-1}$ accumulated in the controls, but up to 350 $\mu\text{mol Fe(II)} \cdot \text{g} (\text{dry weight})^{-1} \cdot \text{month}^{-1}$ accumulated at 25°C. No Fe(II) accumulation was observed with BES.

FIG. 4. Gibbs free energies under in situ conditions calculated with the initial and final concentrations. A, homoacetogenesis; B, syntrophic ethanol oxidation; C, butyrate synthesis; D, acetoclastic methanogenesis; E, hydrogenotrophic methanogenesis; F, ethanol oxidation to acetate and CH_4 . See Table 1 for stoichiometries.

FIG. 5. Compositions and percentages of terminal restriction fragments in the field sample (A) (from two extractions) and after 1 month of incubation at different temperatures (B).

The actual Gibbs free energies of CH_4 formation from both $\text{H}_2\text{-CO}_2$ and acetate were exergonic over the whole temperature range (Fig. 4). The ΔG for the oxidation of ethanol was negative at all temperatures. The ΔG values for homoacetogenesis were negative only at low and high temperatures.

In summary, our results showed that temperature influenced CH_4 production rates together with the net turnover of fatty acids, ethanol, and Fe(II). Ethanol exhibited the highest net turnover of all compounds measured. Both the direct oxidation of ethanol by methanogens and the syntrophic oxidation of ethanol with H_2 as an intermediate were thermodynamically feasible under in situ conditions. CH_4 production was paralleled by the net accumulation of acetate, butyrate, and Fe(II) and by the net consumption of ethanol.

Structure and quantification of the archaeal community. The T-RF frequencies were almost constant at temperatures between 4 and 30°C. At 37 and 45°C, the archaeal population became less diverse. When the experiment began, 60% of all T-RFs exhibited a 92-bp fragment, and 20% exhibited a 184-bp fragment. After 1 month of incubation the 92-bp fragment was still the most abundant T-RF at all temperatures (60 to 80%). The 184-bp fragment occurred only at temperatures between 4 and 30°C (frequency, 10 to 20%) (Fig. 5).

In total, 24 archaeon-specific 16S rRNA gene clones were analyzed. All tree calculation methods confirmed that 14 of the 24 clones clustered with uncultured *Crenarchaeota*; 10 clones clustered with sequences from a boreal forest soil in Finland (accession no. AJ428031 and AJ428025) (65), two clones clustered with a sequence from New Zealand (accession no. AF402987), and one clone each clustered with sequences retrieved from a freshwater lake (accession no. AF402987) and a groundwater well (accession no. AJ583398). Nine clones (FL-01, FL-04, FL-10, FL-11, FL-18, FL-27, FL-50, FL-52, and FL-56) clustered in the

FIG. 6. Maximum-likelihood tree for the 16S rRNA gene. The tree was constructed using the Treepuzzle tool as implemented in ARB. Sequences obtained in this study are indicated by boldface type. Because the corresponding clone sequences formed a coherent cluster, they were merged into one branch. Scale bar = 10% sequence divergence. The values at the nodes are Treepuzzle support values.

order *Methanobacteriales* next to clones from an acidic mire in Bavaria (accession no. AJ459876, AJ459881, and AJ459882) (27) (Fig. 6). Clone FinSSU_65 and its closest relatives from a shallow marine sediment (accession no. AB107831) and a rice root sample (accession no. AY125683) clustered in a new deep lineage of archaeal sequences from deep-sea hydrothermal vents (DHVE6; euryarchaeotic group II) (57). All 44 McrA sequences were affiliated with the *Methanobacteriales* and formed a tight cluster with *Methanobacterium formicum* and *Methanobacterium bryantii* (Fig. 7). The latter species was first isolated from an ethanol-oxidizing syntrophic coculture of “*Methanobacterium omelianskii*” (5).

An analysis of the clone sequences in silico showed that the 92-bp T-RF could be affiliated with the *Methanobacteriales* and the 184-bp T-RF could be affiliated with the *Crenarchaeota*. Hence, the major archaeal groups detected by T-RFLP anal-

ysis were identical to the most abundant lineages found in the clone library. While the T-RFLP pattern stayed remarkably constant with temperature (Fig. 5), the target numbers of both archaea and bacteria showed that the optimum temperature for net population growth was 25°C (Fig. 8).

DISCUSSION

Methanogenesis and ethanol consumption were highly dependent on temperature. The substrate dynamics reacted similarly with temperature, indicating that there was tight coupling between the underlying processes. The optimum temperature, 25°C, is too high for psychrophilic organisms (41), but the rate at 4°C, which was about 1/10 that at the optimum temperature, indicated that a psychrotolerant population was present. A Ratkowsky plot of the square root of methane accumulation

FIG. 7. Maximum-likelihood tree for McrA. Sequences obtained in this study are indicated by boldface type. Scale bar = 10% sequence divergence. The values at the nodes are TreePuzzle support values. *Methanopyrus kandleri* was used as the outgroup (not shown).

versus temperature (46) gave a theoretical temperature limit for methanogenesis close to -5°C . Ethanol has rarely been considered an important intermediate in peat, but it has been found in other different anoxic environments, where it may account for up to 14% of the carbon flux to CH_4 (9, 51). Syntrophic ethanol oxidation has been demonstrated in peat amended with ethanol (21, 27). However, ethanol has never previously been found to play as an important role in natural peat samples as we observed in the northern Finland bog.

Reactants and processes. Part of the ethanol available at the beginning of our experiment may have been released from the fine roots collected together with the peat. However, a wide range of eukaryotic and prokaryotic microorganisms are able to produce ethanol under anoxic conditions (47) and may have provided the larger part of the ethanol. Ethanol fermentation is the physiological response of plants to hypoxia. Anaerobiosis becomes even more demanding in the dark, when enhanced ethanol release from submersed roots may

FIG. 8. Quantitative PCR with primers for the archaeal and bacterial 16S rRNA genes. The numbers of targets at the start of the experiment (horizontal lines) are compared to the numbers of targets recovered after 1 month of incubation at different temperatures (means $\pm$ standard errors; $n = 9$ to 28). gDW, grams (dry weight).

occur (60). Hence, a supply of ethanol to a wetland soil or peat may be considered a natural process.

The molar ratio for ethanol consumption and CH_4 accumulation was 4:1 at 25°C (Fig. 3). Direct utilization of ethanol by methanogens is quite unusual (66). Only the thermophilic marine isolate *Methanogenium organophilum* is known to grow on primary alcohols other than methanol, but the growth is less efficient than that on secondary alcohols (62, 63). More common is the syntrophic ethanol oxidation to acetate (Table 1) that may be coupled to different hydrogen-scavenging partners (32, 50, 59).

Direct oxidation of ethanol and hydrogenotrophic methanogenesis were exergonic for both the initial and final conditions (after 4 weeks of incubation) (Fig. 4). The syntrophic oxidation of ethanol to acetate and H_2 became less favorable at the end of incubation, mainly at temperatures around the optimum temperature (Fig. 4). This agrees with a decrease in the CH_4 production rate with time, as observed in the temperature range between 15 and 30°C. In addition, the high concentrations of acetate (6 mM) and butyrate (5 mM) that accumulated at the optimum temperature may have inhibited CH_4 production due to the formation of undissociated acids, as found in other acidic peats (27, 64). When preparations are treated with BES, the methanogenic precursors should accumulate with time, but the net accumulation of acetate was even lower with BES (Fig. 3). This observation is consistent with syntrophic ethanol oxidation to acetate that is suppressed when H_2 is no longer consumed by methanogens.

The theoretical molar ratio of ethanol to CH_4 is 2:1 for both direct and syntrophic ethanol oxidation (Table 1), but the observed ratio was 4:1 at 25°C (Fig. 3). Assuming that CH_4 originated completely from H_2 derived from ethanol, at least 50% of the ethanol consumed must have entered another metabolic pathway.

Fe(III) may be formed even below the water table in an

FIG. 9. Pathways proposed from the balance between the initial and final concentrations of substrates and products at 25°C and 4°C. The concentrations for pools (rectangles) are expressed in $\mu\text{mol} \cdot \text{g}$ (dry weight) $^{-1}$, and the flow rates are expressed in $\mu\text{mol} \cdot \text{g}$ (dry weight) $^{-1} \cdot \text{month}^{-1}$. The negative and positive numbers in the rectangles indicate net consumption and accumulation, respectively. The rhombuses represent the reactions, and the lowercase letters in the rhombuses indicate the following reactions: a, $\text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + 2\text{H}_2 + \text{H}^+$; b, $\text{CH}_3\text{CH}_2\text{OH} + 4\text{Fe}^{3+} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + 4\text{Fe}^{2+} + 5\text{H}^+$; c, $\text{CH}_3\text{COO}^- + 8\text{Fe}^{3+} + 2\text{H}_2\text{O} \rightarrow 2\text{CO}_2 + 8\text{Fe}^{2+} + 7\text{H}^+$; d, $2\text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2 \rightarrow \text{CH}_3(\text{CH}_2)_2\text{COO}^- + 2\text{H}_2\text{O}$; e, $\text{CH}_3\text{COO}^- + \text{H}^+ \rightarrow \text{CH}_4 + \text{CO}_2$; f, $4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$. The negative and positive numbers associated with the arrows indicate the amount of substrate leaving a pool and entering a reaction and the amount of product entering the subsequent pool, respectively. The question marks indicate an unknown source or sink.

otherwise anoxic environment, if O_2 is released from plant roots (14). Fe(III) may accumulate at levels high enough to suppress methanogenesis for a prolonged time (17). However, CH_4 production started at the beginning without a lag. Fe(II) accumulated at all temperatures and accumulated optimally at 25°C, the optimum temperature for methanogenesis (Fig. 4). The accumulation of up to 350 $\mu\text{mol Fe(II)} \cdot \text{g}$ (dry weight) $^{-1} \cdot \text{month}^{-1}$ makes iron reduction a candidate for the missing ethanol sink. Theoretically, 4 mol Fe(III) is reduced per mol ethanol consumed (Table 1) (36). Fe(III)-reducing microorganisms may suppress methanogenesis competing for H_2 (1, 8) or acetate (17), and methanogens themselves may divert electrons via extracellular quinones to Fe(III) (4). By reducing Fe(III), methanogens may metabolize H_2 to levels that make CH_4 production thermodynamically unfavorable (37). However, methanogenesis and Fe(III) reduction proceeded in parallel (Fig. 3), and conditions were permissive for hydrogenotro-

phic and acetoclastic methanogenesis at the beginning and end of the experiment (Fig. 4). The BES concentration used in the inhibition experiment was high, but it was necessary to inhibit methanogenesis completely, as found in a previous dose-response experiment (data not shown) and in pure cultures (67). Provided that BES had no nonspecific effect on Fe(III)-reducing microorganisms, Fig. 3 suggests that CH_4 production and Fe(III) reduction were linked.

Corresponding to the consumption of ethanol, equimolar accumulation of acetate was expected (Table 1), but this was not observed (Fig. 3). Inhibition with CH_3F showed that acetoclastic methanogenesis accounted for 20% of the total methanogenesis and hence consumed some of the missing acetate. However, the concurrent accumulation of butyrate (Fig. 3) led us to suggest that most acetate was converted to butyrate (Table 1). This suggestion was supported by thermodynamic (Fig. 4) and mass balance calculations (Fig. 9) (see below). Interestingly, no butyrate accumulated with BES (Fig. 3), while it was expected to accumulate if it was used as a syntrophic substrate. Similarly, no butyrate accumulated in a rice field soil amended with 20 mM BES (20). However, fermentative butyrate formation seems not to be inhibited by BES, at least when the carbon supply is high (20).

It is widely accepted that at low temperatures CH_4 is produced by acetate cleavage, whereas H_2 - CO_2 is basically utilized by homoacetogens (15, 19, 31, 53). However, different authors have described the prevalence of hydrogenotrophic methanogenesis in acidic peat at low temperatures (2, 3, 12, 26, 33). Thermodynamic calculations showed that homoacetogenesis from H_2 - CO_2 was permissive only at low and very high temperatures, whereas acetoclastic methanogenesis and hydrogenotrophic methanogenesis were exergonic at all temperatures (Fig. 4). Homoacetogenesis cannot be ruled out, but methanogenesis was based mainly on H_2 - CO_2 even at 4°C. This conclusion was further supported by the population analysis discussed below.

CO_2 accumulation and hence overall mineralization even increased at temperatures above 35°C, when methanogenesis and net population growth of archaea decreased (Fig. 1, 2, and 8). A similar uncoupling of microbial growth and activity at nonphysiologically high temperatures has been found in upland soils (44a). Abiotic CO_2 production linked to Fe reduction has been found in vitro at a very low pH (44b), but whether this occurs under in situ conditions is not known. In our experiment, Fe could be ruled out as an e^- acceptor, because Fe(II) accumulation decreased to nearly zero at temperatures above 35°C (Fig. 3). In addition, H_2 (Fig. 1) accumulated in the same temperature range together with formate (data not shown). The peat never experiences temperatures as high as 50°C in situ, and organisms that might be involved in this activity are totally unknown.

Structure and quantification of the archaeal community. Altogether, the diversity of archaeal 16S rRNA gene clones was low. More than one-half of the 16S rRNA gene clones clustered with uncultured *Crenarchaeota* (data not shown). *Crenarchaeota* are a diverse class of microorganisms which also occur in peatlands (30, 43). Because their physiology is mostly unknown, a detailed discussion of the phylogenetic affiliation would not contribute to our understanding of metabolic pathways.

Most of the other sequences were related to the *Methanobacteriales*, which use only H_2 or formate as an e^- donor. They were closely affiliated with the type strain of *Methanobacterium bryantii* (Fig. 6), which was isolated from a syntrophic ethanol-oxidizing coculture of "*Methanobacterium omelianskii*" (5). The ability to utilize ethanol directly for CH_4 production is known for *Methanogenium organophilum*, a member of the *Methanomicrobiales*. However, none of the clones could be affiliated with the *Methanomicrobiales*. These results indicate that syntrophic instead of direct ethanol oxidation was the prevailing process. Confirming this further, the McrA sequences clustered in the *Methanobacteriaceae* (Fig. 7). The phylogenetic affiliations of the clones were the same with all treeing methods applied.

The 16S rRNA gene-based T-RFLP patterns shown in Fig. 8 did not indicate any temperature-dependent change in the population structure. The 92-bp T-RF, which exhibited the highest relative abundance, could be assigned to the 16S rRNA gene clone sequences clustering with the order *Methanobacteriales* (Fig. 6), corresponding to the high proportion of hydrogenotrophic methanogenesis. Real-time PCR showed that there was conspicuous temperature-dependent net population growth. The levels of both archaeal and bacterial targets were highest at 25°C, in accordance with the optimum temperature for CH_4 , ethanol, and Fe turnover.

Balance calculations. We suggest that at 25°C the ethanol pool was split into the following two main branches: (i) syntrophic oxidation of ethanol in cooperation with methanogens, which was confirmed by thermodynamic calculations and was supported by phylogenetic analysis, and (ii) oxidation of ethanol by Fe(III) reduction (Fig. 9). Hydrogenotrophic methanogenesis contributed 80% of the total methanogenesis. Hence, a minor fraction of acetate was consumed by acetoclastic methanogenesis, while butyrate synthesis accounted for the larger fraction. The in situ conditions were thermodynamically favorable for all the processes mentioned above (Fig. 4).

We are aware of the limitations of this approach, which does not account for the ongoing production of fermentation products (e.g., ethanol and acetate). However, because of the large amount of ethanol that was available at the beginning of the experiment, this may be a minor problem. However, we cannot decide if the ethanol was consumed directly by Fe-reducing bacteria (36) or indirectly via an interspecies H_2 transfer to Fe-reducing bacteria or by a drain of reducing equivalents from methanogens via extracellular electron shuttles to Fe(III) (4). In summary, the proposed split of the ethanol pool at 25°C into two main branches was strongly supported by the balance between ethanol and the end products (Fig. 9). Some H_2 needed for butyrate synthesis lacks in the balance, but the flow of carbon is fairly well constrained.

At 4°C the substrate flow was different (Fig. 9). The net production of Fe(II) was negligible (Fig. 3). The balance of ethanol and acetate agreed well with the proposed syntrophic ethanol oxidation. This was further supported by thermodynamic calculations (Fig. 4). However, the balance cannot account for the H_2 produced (Fig. 9). One may speculate that organic matter (humins) acted as an electron acceptor, but we have no evidence for this yet. Similarly, humins may be the unknown electron donor at 25°C (Fig. 9).

In summary, the microbial populations were well adapted

to low temperatures, as evident from the high activity and the theoretical lower temperature limit for methanogenesis, -5°C . Ethanol played a major role in the flow of carbon and reductants. Syntrophic oxidation to acetate was the key process leading to CO_2 reduction at all temperatures. At low temperatures, most H_2 resulting from ethanol oxidation ended up in CH_4 , while at the optimum temperature an equal amount was used to reduce Fe(III) . According to the T-RFLP analysis and clone libraries, the peat methanogens were affiliated with the *Methanobacteriales*. Some lines of evidence led us to speculate that methanogens may be involved in Fe reduction. The archaeal diversity was not affected by temperature even when population growth occurred, suggesting that the archaeal population was remarkably resistant to perturbations. The close correspondence between structure and function is exciting, but the following challenge for future work remains: to go beyond analysis of structure and function and to explain why a population exists at a particular site but not at other sites.

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