

Hydrogenotrophic Methanogenesis by Moderately Acid-Tolerant Methanogens of a Methane-Emitting Acidic Peat

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The emission of methane ($1.3 \text{ mmol of CH}_4 \text{ m}^{-2} \text{ day}^{-1}$), precursors of methanogenesis, and the methanogenic microorganisms of acidic bog peat (pH 4.4) from a moderately reduced forest site were investigated by in situ measurements, microcosm incubations, and cultivation methods, respectively. Bog peat produced CH_4 (0.4 to $1.7 \mu\text{mol g} [\text{dry wt}] \text{ of soil}^{-1} \text{ day}^{-1}$) under anoxic conditions. At in situ pH, supplemental H_2 - CO_2 , ethanol, and 1-propanol all increased CH_4 production rates while formate, acetate, propionate, and butyrate inhibited the production of CH_4 ; methanol had no effect. H_2 -dependent acetogenesis occurred in H_2 - CO_2 -supplemented bog peat only after extended incubation periods. Nonsupplemented bog peat initially produced small amounts of H_2 that were subsequently consumed. The accumulation of H_2 was stimulated by ethanol and 1-propanol or by inhibiting methanogenesis with bromoethanesulfonate, and the consumption of ethanol was inhibited by large amounts of H_2 ; these results collectively indicated that ethanol- or 1-propanol-utilizing bacteria were trophically associated with H_2 -utilizing methanogens. A total of 10^9 anaerobes and 10^7 hydrogenotrophic methanogens per g (dry weight) of bog peat were enumerated by cultivation techniques. A stable methanogenic enrichment was obtained with an acidic, H_2 - CO_2 -supplemented, fatty acid-enriched defined medium. CH_4 production rates by the enrichment were similar at pH 4.5 and 6.5, and acetate inhibited methanogenesis at pH 4.5 but not at pH 6.5. A total of 27 different archaeal 16S rRNA gene sequences indicative of *Methanobacteriaceae*, *Methanomicrobiales*, and *Methanosarcinaceae* were retrieved from the highest CH_4 -positive serial dilutions of bog peat and methanogenic enrichments. A total of 10 bacterial 16S rRNA gene sequences were also retrieved from the same dilutions and enrichments and were indicative of bacteria that might be responsible for the production of H_2 that could be used by hydrogenotrophic methanogens. These results indicated that in this acidic bog peat, (i) H_2 is an important substrate for acid-tolerant methanogens, (ii) interspecies hydrogen transfer is involved in the degradation of organic carbon, (iii) the accumulation of protonated volatile fatty acids inhibits methanogenesis, and (iv) methanogenesis might be due to the activities of methanogens that are phylogenetic members of the *Methanobacteriaceae*, *Methanomicrobiales*, and *Methanosarcinaceae*.

Peat bogs are wetland ecosystems in which biomass production exceeds biodegradative activity, are often acidic, and emit approximately 3 to 7% of the global annual emission of the greenhouse gas methane (CH_4) (2, 15). Peat bogs cover less than 3% of the Earth's terrestrial surface (8) but harbor approximately 30% of the global reserves of soil carbon and soil nitrogen, and the underlying peat is often anoxic (18). In anoxic environments with negligible concentrations of inorganic electron acceptors (e.g., sulfate or nitrate), methanogenesis is often the main terminal microbiological process during the biodegradation of organic matter. H_2 - CO_2 and acetate are considered the main substrates for methanogenesis (37, 58, 62); although normally less important than H_2 - CO_2 and acetate, formate and methanol can also be significant substrates for methanogens (4).

It is estimated that about two-thirds of the methane produced in nature originates from acetoclastic methanogenesis (14). However, H_2 - CO_2 appears to be a significant precursor of the methane that is emitted from acidic peats (29, 62). In

northern peatlands, although acetoclastic methanogenesis occurs in the upper vegetated zones of the peat, methanogenesis shifts toward the reduction of CO_2 in deeper zones (7, 44, 54). Acetate and one-carbon compounds (e.g., formate) can even accumulate in acidic peat (22), indicating that these compounds are not the primary source of methane that is emitted from such habitats. Supplemental acetate can be inhibitory to methanogenesis in acidic peat samples, whereas glucose and H_2 can be stimulatory (62).

Little is known about acid-tolerant methanogens (4). Only one acid-tolerant H_2 -utilizing methanogen, identified as a member of the *Methanobacteriaceae*, has been reported in the literature (63). The methanogenic microbiota in acidic peat are unresolved. Gene sequences indicative of members of *Methanobacteriaceae*, *Methanococcaceae*, *Methanosarcinaceae*, and *Methanomicrobiales* have been detected in a blanket bog peat (20, 36); however, the methanogens present in the zone of highest methanogenic activity in this bog peat were not identified (36).

An investigation designed to resolve both the methanogenic community and the flow of carbon and reductant in bog peat has thus far not been reported. The main objectives of this study were to (i) assess precursors of methane during the flow of carbon and reductant in an anoxic acidic bog peat and (ii)

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enumerate and identify the cultured microorganisms associated with methanogenesis.

MATERIALS AND METHODS

Description of the site and collection of peat. The acidic bog is located approximately 700 m above sea level (50°08'40" N, 11°51'55" E) in the Fichtelgebirge, a forest with medium-sized mountains in east-central Germany. The soil is Cambic Podzol on granite bedrock with peat accumulation (33), and *Picea abies* and *Sphagnum* spp. dominate the vegetation of the site. During the investigation period (1998 and 1999), the bog lacked vegetation and was covered with water (5 to 30 cm depending on the amount of precipitation). The annual mean air temperature at the site approximates 6.5°C, with temperature extremes of approximately 30 and -20°C; annual precipitation averages 1,100 mm per year (33). During the sampling periods, the peat temperature at a depth of 5 to 10 cm was 9.4 ± 2.2°C ($n = 10$) and the air temperature was 12.6 ± 4.0°C ($n = 10$) (these data were obtained at approximately 11 a.m. on 10 different days). Peat was collected in sterile airtight vessels and stored on ice until processed (within 8 h); it had a dry weight of 12% ± 2% (dry weights were determined on three different dates, and each determination was done in triplicate).

Assessment of in situ gas emissions. Gases were collected in round Plexiglas (Degussa, Darmstadt, Germany) chambers (1.9 liters, 227 cm²) (34) that were placed on the water surface above the peat. Gas emissions were analyzed with samples from two chambers at each sampling date. Gas samples ($n = 14$) were withdrawn at gas outlets with syringes, injected into gastight evacuated bottles, and analyzed for CH₄ in the laboratory.

Anoxic microcosms. Microcosms with large headspaces were 500-ml screw-cap, butyl rubber-stoppered infusion flasks (Merck ABS, Dietikon, Switzerland) containing 15 g (dry wt) of peat per flask under 100% argon. Microcosms with small headspaces were 125-ml infusion flasks containing 12 g (dry wt) of peat per flask. Substrates were added from sterile anoxic stock solutions (adjusted to pH 4.5) or from bottles containing sterile gases. Microcosms were incubated horizontally in the dark at 20°C, and each microcosm experiment was performed in triplicate. Substrate/product stoichiometries were corrected with values obtained in control microcosms lacking supplemental substrates. The pH of peat during incubations was usually between 4.3 and 4.5, with extremes of 4.0 and 4.8.

Cultivation media. Anoxic media were prepared using modified Hungate techniques (24). Anoxic media were boiled, subsequently cooled under an argon gas phase, dispensed into culture tubes or serum bottles, and autoclaved. Unless otherwise indicated, gas phases were 100% argon. The sterile anoxic mineral solution used for the most-probable-number (MPN) dilution series (see below) contained (in milligrams per liter) (NH₄)₂SO₄, 25; CaCl₂ · 2H₂O, 10; MgCl₂ · 6H₂O, 10; NaCl, 200; NH₄Cl, 200; and KH₂PO₄, 200. The pH of the mineral solution was adjusted to 4.5 with HCl. Anoxic tryptic soy broth containing glucose (TSB; Difco Laboratories, Detroit, Mich.) was used at 2.75 g liter⁻¹ (yielding an initial glucose concentration of 1.5 mM); the pH was adjusted to 4.5 (unless otherwise indicated). Undefined medium (UM) was anoxic mineral solution (above) supplemented with (per liter) yeast extract, 0.5 g; tryptone, 0.5 g; vitamin solution (3), 10 ml; and trace element solution (3), 10 ml. The pH of UM was adjusted to 4.5 or 6.0. UM supplemented with antibiotics (UM_{ab}) was UM with (per liter) penicillin, 0.3 g; streptomycin, 0.2 g; and kanamycin, 0.2 g. Selective methanogen medium (SMM) contained (per liter) mineral solution, 10 ml; vitamin solution, 10 ml; trace element solution, 10 ml; acetate-free fatty acid solution (61), 1 ml; and cell-free enrichment culture fluid (see below), 100 ml. The pH of SMM was adjusted to 4.5 or (in one case) 6.5. For preparation of cell-free enrichment culture fluid, methanogenic UM enrichment cultures were centrifuged at 10,000 × g for 20 min; the supernatant fluid was freed of particulate matter by microfiltration (pore size 0.2 μm). The filtered supernatant fluid constituted the cell-free enrichment culture fluid and was stored at 4°C under 100% argon. The gas phase of SMM was H₂-CO₂ (8:2) or argon-H₂-CO₂ (78:18:4).

Enumeration of cultured microorganisms. Cultured microorganisms were estimated by the MPN technique (10). Peat was processed inside a Mecaplex (Grenchen, Switzerland) O₂-free chamber (the gas phase was 100% N₂). For extraction of microorganisms, 50 g (wet wt) of peat and 200 ml of anoxic mineral solution (see above) were placed in 500-ml infusion flasks; the flasks were then sealed with a rubber stopper and placed on an end-over-end shaker (60 cycles per min) for 10 h at 2°C. Tenfold serial dilutions of peat were prepared in sterile anoxic mineral solution by using anoxic techniques (13). Inoculated tubes of anoxic TSB that showed both a consumption of glucose and an increase in optical density at 660 nm (≥0.05) were scored positive for general fermentative anaerobes. Methanogen numbers were estimated in various media with an argon-H₂-CO₂ (78:18:4) gas phase. MPN tubes for which the stoichiometries of H₂ con-

sumed with respect to CH₄ produced were approximately 4:1 were scored positive for methanogens. TSB tubes were scored positive for methanogens if at least 0.1 μmol of CH₄ was produced per tube (methane was not detected in uninoculated controls). MPN analyses were performed in triplicate. Culture tubes were incubated horizontally at 20°C in the dark.

Enrichment of methanogens under acidic conditions. A total of 10 g (wet wt) of peat was added to 120-ml serum bottles that contained 50 ml of UM and had an argon-H₂-CO₂ (78:18:4) gas phase. Enrichments were incubated at 20°C in the dark. After the fourth transfer into the same medium, enrichment cultures that yielded stoichiometries of H₂ consumed with respect to CH₄ produced of approximately 4:1 were used to inoculate SMM. Experiments with methanogenic enrichments were performed in triplicate with SMM.

Analytical methods. Gases (CH₄, CO₂, and H₂) were analyzed by gas chromatography, and organic acids and alcohols were quantified by high performance liquid chromatography (28). Unless otherwise indicated, no distinction is made between organic acids and their salt forms. Redox potentials of peat ($n = 9$) were measured with a Schott PT-6280 platinum redox electrode (Schott Glasgeräte, Hofheim, Germany) with Ag/AgCl as a reference system and a WTW pH 90 digital pH meter (Wissenschaftlich Technische Werkstätten, Weilheim, Germany). Redox potentials were calculated by using the following equation: E_h (mV) = $E_{Ag/AgCl}$ (mV) - 0.76 [T(°C) - 25°C] + 222.67 (55). The pH of peat ($n = 10$) was measured with a U457-S7/110 combination pH electrode (Ingold, Steinbach, Germany). Oven-dried (60°C) peat was homogenized with a ball mill (MM2; Retsch, Haan, Germany) and analyzed for total carbon content ($n = 3$) with an element analyzer (CHN-O rapid; Foss-Heraeus, Hanau, Germany). The total inorganic carbon content of peat ($n = 1$) was determined after heating peat at 500°C for 12 h. The aqueous phase of bog peat was analyzed for dissolved organic carbon ($n = 1$) with a liquiTOC (Foss-Heraeus). The dry weight of peat ($n = 6$) was determined by weighing peat before and after drying at 60°C for 48 h. Ammonium ($n = 4$) and nitrate plus nitrite ($n = 4$) were determined by flow injection analysis (QuickChemAE; Lachat Instruments, Milwaukee, Wis.). Sulfate ($n = 1$) was measured by ion chromatography (DX-100 with an AS4 A column [Dionex, Sunnyvale, Calif.]). Acetate ($n = 3$) was quantified by high-performance liquid chromatography (28).

FISH and identification of methanogens. Fluorescently labeled oligonucleotide probes (Table 1) were obtained from Interactiva (Ulm, Germany). Cells were fixed in 4% formaldehyde solution, immobilized on gelatin-coated slides, and dehydrated with ethanol at increasing concentrations (50, 80, and 98%). Fluorescent in situ hybridization (FISH) and quantification of hybridized cells in comparison to total cell counts (as determined by staining with 4',6-diamidino-2-phenylindole [DAPI]) were done by published protocols (52); formamide and NaCl concentrations in the buffers are listed in Table 1. Methanogens were also microscopically identified by the fluorescence of coenzyme F₄₂₀ (11). The following reference organisms were used for FISH: *E. coli* (DSM 423), *Methanosarcina* sp. strain WH1 (DSM 4659), *Methanobacterium bryantii* M.o.H.G. (DSM 862), *Methanococcus voltae* PS (DSM 1537), and *Methanogenium cariaci* JR1 (DSM 1497). The reference organisms were grown as suggested by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany [http://www.dsmz.de]).

Phylogenetic analyses. DNA was extracted from methanogenic enrichments and highest CH₄-positive MPN dilutions by using the FastDNA SPIN Kit (Bio 101, Carlsbad, Calif.) as specified by the manufacturer. PCR was performed in a T-gradient cyler (Biometra, Göttingen, Germany) by using 33 cycles of 94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1.5 min. The final PCR extension step was at 72°C for 5 min. To construct an archaeal 16S rRNA gene library, almost full-length archaeal 16S rRNA gene fragments were amplified using primers Arch21Fa and GM4R (Table 1). PCR products were ligated into pGEM-T (Promega, Mannheim, Germany) and transformed into *E. coli* JM109 (Promega) as specified by the manufacturer. For screening of the gene library by denaturing gradient gel electrophoresis (DGGE), 570-bp 16S rRNA gene fragments were directly amplified from 1 μl of resuspended clones using the primer pair ARC344Fa-Clamp plus ARC915 (Table 1). The Wizard Plus Minipreps DNA purification system (Promega) was used for the preparation of plasmids, and DNA was sequenced commercially at MWG Biotech (Ebersberg, Germany). DGGE analysis was also directly performed on extracted DNA from methanogenic enrichments or the highest CH₄-positive MPN dilutions (10⁻⁶ to 10⁻⁷). Fragments (470 to 570 bp) of 16S rRNA genes were amplified by PCR using the primer pairs ARC344Fa-Clamp plus ARC915 for *Archaea*, ARC344Fa-Clamp and MSX860 for *Methanosarcinales*, ARC344Fa-Clamp and MS821 for *Methanosarcina*, and 341F-Clamp/907R for *Bacteria* (Table 1).

DGGE was performed by following a published protocol (39); the temperature was 60°C, the denaturant (urea and formamide) gradient was 20 to 80%, the electrophoresis time was 18 h, and the voltage was 100 V. Gels were stained with

TABLE 1. Sequences of oligonucleotides used as PCR primers and for FISH with corresponding concentrations of formamide and NaCl

Probe or primer	Sequence (5'-3')	Target organisms	% Formamide in hybridization buffer	Concn of NaCl in washing buffer (mM)	Reference
EUB338	GCTGCCTCCCGTAGGAGT	Bacteria	0	900	1
ARC915	GTGCTCCCCGCCAATTCCT	Archaea	20	225	53
MS821	CGCCATGCCTGACACCTAGCGAGC	<i>Methanosarcina</i>	30	112	45
MSMX860	GGCTCGTTTCACGGCTTCCT	<i>Methanosarcinales</i>	30	112	45
NON ^a	ACTCCTACGGGAGGCAGC	NA ^b	NA	NA	32
Arch21Fa	TCCGGTTGATCCYGSCRG ^c	Archaea	NA	NA	9
ARC344Fa-Clamp	CGCCCGCCGCGCCCGCGCCCGTCCCGCCGCCCGCCCG- ACGGGGYGCAGCAG ^c	Archaea	NA	NA	45
GM4R	TACCTTGTTACGACTT	All organisms	NA	NA	40
341F-Clamp	CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGG- CCTACGGGAGGCAGCAG	Bacteria	NA	NA	39
907R	CCGTCAATTCMTTGTGATTT	Bacteria	NA	NA	39

^a Used as negative control.

^b NA, not applicable.

^c Sequence modified from original publication.

SYBR Gold (Molecular Probes, Eugene, Oreg.). Selected DGGE bands were excised, and the DNA was extracted with water, reamplified, and sequenced commercially at MWG Biotech.

Sequences were aligned and phylogenetically analyzed using the program package ARB (Department of Microbiology, Technical University Munich, Germany [http://www.arb-home.de]). For calculation of phylogenetic trees, neighbor-joining, parsimony, and maximum-likelihood algorithms were applied to 16S rRNA gene sequences that were longer than 1.4 kb; a consensus tree was drawn using consistent branchings from all three methods, while inconsistent branches were drawn as multifurcations. Partial sequences were added to the consensus tree by maximum-parsimony analysis without changing the tree topology.

Nucleotide sequence accession numbers. All 16S rRNA gene sequences have been deposited in the EMBL nucleotide sequences database. Accession numbers AJ459876 to AJ459902 have been assigned to the following archaeal sequences: AMC 3, AEC 11, AEC 10, AEC 9, AEC 8, AMC 6, AEC 3, AEC 6, AEC 2, AMC 7, AEC 4, AMC 4, AMC 5, AEC 5, AED 4, AED 9, AED 7, AED 8, AED 5, AED 6, AED 10, AED 3, AMC 2, AMC 1, AEC 1, AED 1, and AED 2. Accession numbers for bacterial sequences are given in the dendrogram.

RESULTS

Chemical characteristics of acidic bog peat. The peat had a pH of 4.4 ± 0.2 and a mean total carbon content of 319 ± 30 g of C kg (dry wt) of soil⁻¹. The organic and inorganic carbon contents in the peat were 329 and 1.2 g of C kg (dry wt) of soil⁻¹, respectively. In the aqueous phase, the ammonium concentration was below 0.1 mM, the nitrate plus nitrite and sulfate concentrations were always below 0.01 mM, and the acetate concentration never exceeded 0.2 mM. The in situ redox potential of the bog was 550 to 650 mV in the water layer above the peat and 10 to 98 mV at a depth of 4 to 7 cm below the water/peat interface. The steepest redox gradient was observed at the water/peat interface (data not shown). The redox potential did not change significantly below a depth of 7 cm; the lowest redox potential measured was -50 mV.

In situ emission of methane at the bog. CH₄ was emitted at a rate of 1.3 mmol of CH₄ m⁻² day⁻¹ in September 1998. This rate was within the range of CH₄ emission rates (0.02 to 15 mmol of CH₄ m⁻² day⁻¹) that were obtained at the same site in 1995 (August through October) and 1996 (June through November) (C. Kuhner and H. L. Drake, unpublished data).

Methanogenesis and the production of H₂ by peat. In anoxic microcosms at in situ pH (4.3 to 4.5), CH₄ was produced at

rates that ranged from 0.36 to 1.71 μmol of CH₄ g (dry weight) of soil⁻¹ day⁻¹ (four sampling dates). Supplemental H₂-CO₂ stimulated the linear production of CH₄ (Fig. 1A). CH₄ was initially produced in stoichiometries that averaged 3.1 mol of H₂ consumed per mol of CH₄ formed (Fig. 1A, phase I), and approximately 1 mol of CH₄ was formed per mol of CO₂ consumed in H₂-supplemented microcosms (data not shown). These stoichiometries were indicative of hydrogenotrophic methanogenesis ($4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$). In addition to CH₄, acetate and small amounts of butyrate and propionate were formed in H₂-supplemented microcosms (Fig. 1B). Acetate production in H₂-supplemented microcosms exceeded that in nonsupplemented microcosms, indicating that significant amounts of H₂-derived reductant were recovered in acetate (Fig. 1B, phase II).

Supplemental formate was readily consumed, stimulated the production of H₂, CO₂, and acetate, and initially inhibited methanogenesis in the peat (Fig. 2A and B and data not shown). During the initial phase of activity when formate was utilized, the ratio of formate consumed to CO₂, H₂, and acetate produced approximated 8:6:4:1 (data not shown). In contrast, supplemental acetate and methanol persisted in anoxic microcosms, and acetate inhibited methanogenesis (data not shown). Although supplemental formate was not readily utilized for methanogenesis, the consumption of H₂ and the re-engagement of methanogenesis occurred after formate was consumed to depreciable levels (Fig. 2A and B).

Ethanol and 1-propanol were readily consumed and stimulated the production of CH₄ (Fig. 2C and data not shown). The consumption of ethanol or 1-propanol was concomitant with the stoichiometric production of acetate and propionate, respectively (data not shown), indicating that these substrates were oxidized. Ethanol- and 1-propanol-supplemented peat initially produced trace amounts of H₂ that were subsequently consumed. CH₄ production was concomitant with the apparent consumption of CO₂ (Fig. 2C and D and data not shown). When H₂ (70 μmol g [dry wt] of soil⁻¹) and ethanol (40 μmol g [dry wt] of soil⁻¹) were added simultaneously to peat microcosms, the ethanol was not consumed until the H₂ was de-

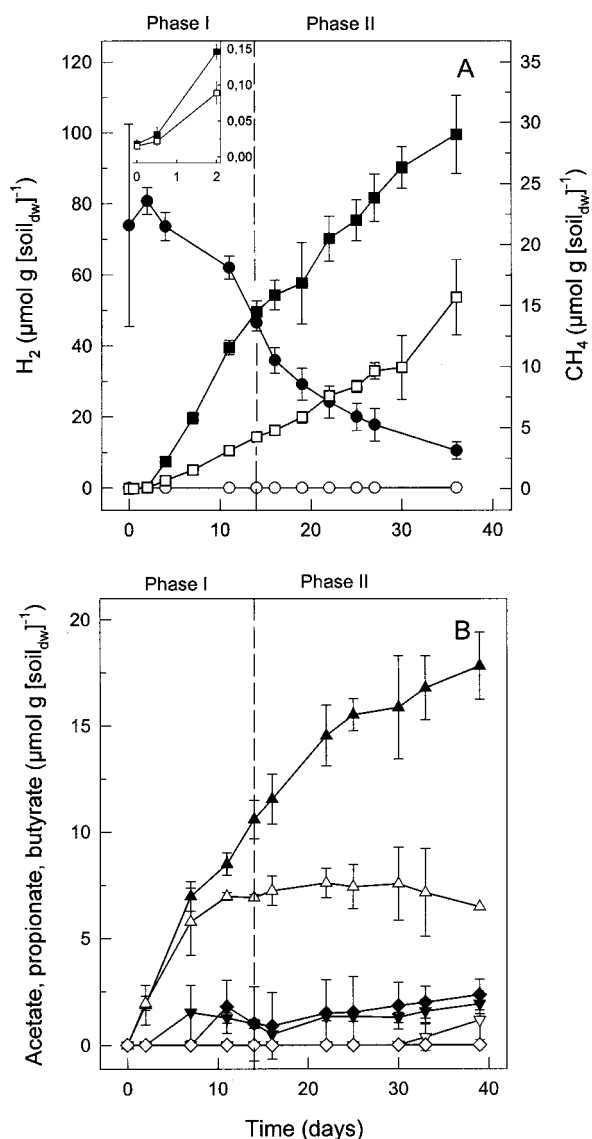


FIG. 1. Effects of supplemental H₂ on the production of CH₄ (A) and volatile fatty acids (B) in bog peat microcosms. Solid symbols indicate microcosms amended with H₂; open symbols indicate controls (unamended); ●, ○, H₂; ■, □, CH₄; ▲, △, acetate; ▼, ▽, propionate; ◆, ◇, butyrate. A detailed view of the production of CH₄ during the first 2 days is shown in the inset.

pleted (data not shown), suggesting that methanogens did not use ethanol as a substrate.

The results described above indicated that H₂ was a significant precursor of CH₄. Therefore, the capability of the peat-associated microbes to produce H₂ from endogenous substrates was evaluated. In microcosms with small headspaces (see Materials and Methods), unamended peat initially produced trace amounts of H₂ that were subsequently consumed (Fig. 3A). The amount of H₂ increased significantly when bromoethanesulfonate (20 mM), an inhibitor of methanogenesis (19), was added to the microcosms (Fig. 3A).

When peat was supplemented with TSB, large amounts of H₂ transiently accumulated and the production of methane decreased significantly (Fig. 3B); ethanol (0.5 mM) was initially

produced during the first 5 days and was consumed by day 15 (data not shown). The glucose (1.5 mM) present in TSB was depleted after 24 h. Acetate (9 mM), propionate (3 mM), butyrate (3 mM), isovalerate (1 mM), and isobutyrate (1 mM) were produced within 15 days and were not consumed within the first 31 days of incubation. However, analysis of microcosms after 80 days of incubation indicated that about half of the 9 mM acetate that initially accumulated during the first 31 days of incubation was consumed between days 31 and 80. In contrast, the acetate that accumulated in TSB-supplemented microcosms during the initial incubation period of 31 days was not subsequently consumed when such microcosms contained 20 mM bromoethanesulfonate (data not shown). The consumption of acetate that was produced in microcosms containing TSB and H₂-CO₂ occurred only after H₂ was consumed to trace levels, and acetate was totally consumed within 80 days (data not shown). Methanogenesis was inhibited by 80 to 96% in microcosms amended with acetate, propionate, or butyrate (5 mM each) (Table 2). These supplemental fatty acids were not consumed during 31 days of incubation.

Enumeration of cultured methanogens from peat and product analyses of MPN series. An MPN value of 3×10^7 methanogens g (dry wt) of soil⁻¹ was obtained for hydrogenotrophic methanogens, a value that was approximately 1% of the MPN value for general fermentative anaerobes and 30 times greater than the MPN value for unspecified methanogens obtained in TSB (Table 3). MPN values for unspecified methanogens in TSB at pH 6.0 and pH 6.8 were similar to those obtained at pH 4.5 (data not shown). Antibiotics (streptomycin, kanamycin, and penicillin) greatly decreased the MPN values obtained for hydrogenotrophic methanogens (Table 3).

Acetate and propionate were the only soluble fermentation products detected in all of the growth-positive MPN dilutions (10^{-2} to 10^{-9}) when TSB (containing glucose) was used as the growth medium (data not shown). Butyrate and isovalerate were detected in the 10^{-2} to 10^{-6} MPN dilutions. Ethanol was detected only in the 10^{-5} to 10^{-6} MPN dilutions of bog peat, indicating that ethanol producers outnumbered ethanol degraders in the peat. CH₄ was detected in TSB-MPN tubes up to dilutions of 10^{-6} (Table 3). However, the largest amounts of CH₄ were detected in the 10^{-2} TSB-MPN dilutions, in which the concentrations of acetate (and ethanol) were lower than those obtained in higher TSB-MPN dilutions (data not shown). Collectively, the MPN analyses indicated that organisms that might link the consumption of acetate or ethanol to methanogenesis (via acetoclastic methanogens, syntrophic acetate oxidizers, syntrophic ethanol oxidizers, etc.) at pH 4.5 occurred in numbers that were substantially smaller than the number of hydrogenotrophic methanogens that were viable at this pH.

Enrichment of acid-tolerant methanogens from peat. Attempts to obtain methanogenic enrichments were unsuccessful with UM (pH 4.5 and 6.0) supplemented with either 5 mM acetate or 5 mM formate. Successful methanogenic enrichments were obtained with H₂-CO₂-supplemented UM (pH 4.5) and subsequently maintained in either H₂-CO₂-supplemented SMM or H₂-CO₂-supplemented UM (pH 4.5). The ending pH approximated 5.5. After prolonged enrichment, the relative number of *Archaea* and *Bacteria* detected by FISH was 70 and 7%, respectively. Approximately 4 mol of H₂ was consumed per mol of CH₄ produced in methanogenic enrich-

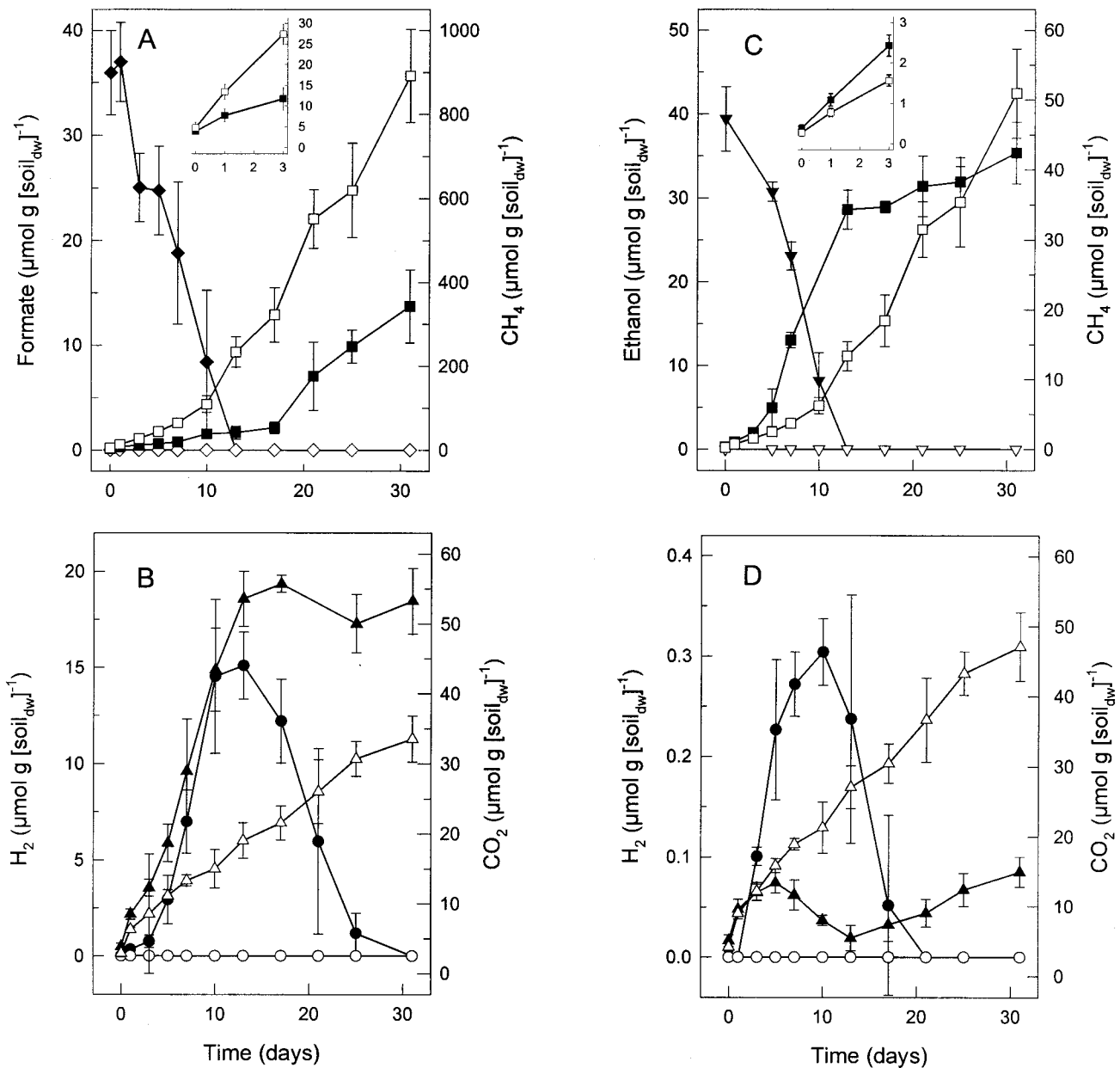


FIG. 2. Effects of supplemental formate (A and B) or ethanol (C and D) on the production of CH_4 (A and C), H_2 (B and D), and CO_2 (B and D) in bog peat microcosms. Solid symbols indicate amended microcosms; open symbols indicate controls (unamended); \blacklozenge, \diamond , formate; \blacksquare, \square , CH_4 ; $\blacktriangledown, \triangledown$, ethanol; \bullet, \circ , H_2 ; $\blacktriangle, \triangle$, CO_2 . A detailed view of the production of CH_4 during the first 3 days is shown in the insets (A and C).

ments. Methanogens identified by coenzyme F_{420} fluorescence or FISH were mainly small, straight rods, some of which were aggregated in short chains of up to four cells. Long, curved rods were also observed. Neither acetate nor ethanol was consumed by methanogenic enrichments. Methanogenesis by the enriched methanogens was similar at pH 4.5 and 6.5; however, methanogenesis was significantly inhibited by 5 mM acetate at pH 4.5 but not at pH 6.5 (Fig. 4). The production of CH_4 from H_2 - CO_2 by the methanogenic enrichments was completely inhibited by the antibiotics streptomycin, kanamycin, and penicillin (data not shown).

Phylogenetic analyses of cultured methanogens. A total of 27 different archaeal 16S rRNA gene sequences were obtained from methanogenic enrichments and methanogenic MPN dilutions. 16S rRNA gene sequences included 10 sequences obtained from excised DGGE bands and 17 clone sequences. Most of these 27 sequences were closely related to known phylogenetic groups of methanogens. A total of 21 sequences (AMC 3 to AMC 7, AEC 2 to AEC 6, AEC 8 to AEC 11, and AED 4 to AED 10) clustered with *Methanobacteriaceae*, 4 (AMC 1, AMC 2, AEC 1, and AED 3) clustered with *Methanomicrobiales*, and 2 (AMC 1 and AMC 2) clustered with

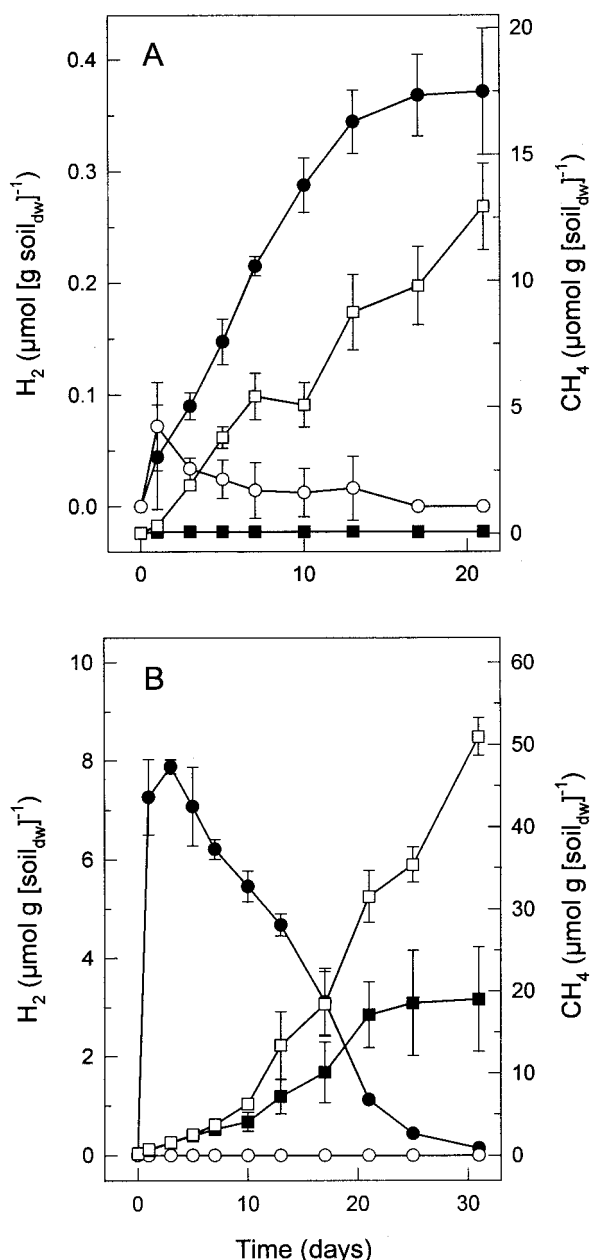


FIG. 3. Effects of 20 mM bromoethanesulfonate (A) or easily fermentable carbon (TSB containing glucose) (B) on the production of H₂ and CH₄ in bog peat microcosms. Solid symbols indicate amended microcosms; open symbols indicate controls (unamended); ●, ○, H₂; ■, □, CH₄.

Methanosarcinaceae. For each group, one or two representative sequences are displayed in Fig. 5. Five clone sequences of the *Methanobacteriaceae* cluster were from the highest CH₄-positive MPN dilutions of peat in UM (AMC 3 to AMC 7) and were closely related to the VeH52 isolate from rice paddy soil (97 to 99% sequence similarity). Two clone sequences from the highest CH₄-positive peat dilution (10⁻⁶) in TSB (AMC 1 and AMC 2) clustered with sequences of *Methanomicrobiales* and were closely related to a clone sequence retrieved from a Welsh bog peat (97% sequence similarity). Clone sequences

TABLE 2. Effects of supplemental volatile fatty acids on the production of CH₄ in acidic bog peat (pH 4.5)

Expt	Volatile fatty acid (5 mM)	pK _a	Mean amt of CH ₄ produced (nmol g [dry wt] of soil ⁻¹ day ⁻¹) ± SD ^a
A ^b	None	NA ^c	268 ± 19
	Formate	3.75	28 ± 4
	Acetate	4.76	8 ± 4
B ^d	None	NA	541 ± 88
	Propionate	4.88	96 ± 11
	Butyrate	4.82	112 ± 28

^a Rates were calculated from the initial phase of activity (7 to 11 days of incubation; a minimum of five data points were used to calculate rates) in anoxic microcosms (0.95 ≤ r² ≤ 0.98).

^b Bog peat sample was collected on 5 May 1999.

^c NA, not applicable.

^d Bog peat sample was collected on 25 June 1999.

from methanogenic enrichments were affiliated with the same methanogenic groups to those for the clone sequences that were obtained from the highest CH₄-positive MPN dilutions. Likewise, all partial 16S rRNA gene sequences that were obtained from DGGE bands were affiliated with the same methanogenic groups as the clone sequences, i.e., *Methanobacteriaceae* and *Methanomicrobiales*, except for two sequences (AED 1 and AED 2); these sequences were retrieved after (semi) specific PCR amplifications with primer pair ARC344Fa-clamp and MS821 and fell into the *Methanosarcinaceae* cluster. AED 1 was found to be very similar to an archaeal 16S rRNA gene sequence (98% sequence similarity) of an uncultured rice field soil archaeon; in contrast, AED 2 was only distantly related to this sequence.

Seven bacterial 16S rRNA gene sequences were obtained from methanogen enrichments in UM; they were phylogenetically related to *Bacteroidales* (BED 7), the *Acidobacterium* phylum (BED 5 and BED 6), and various strains and clone sequences of the *Clostridiales* (BED 1 to BED 4) (Fig. 5). The three bacterial 16S rRNA gene sequences obtained from the highest CH₄-positive MPN dilution (BMD 1 to BMD 3) were most similar to *Serratia fonticola* (BMD 3; 99% sequence similarity), *Propionispira arboris* (BMD 2; 96% sequence similarity), and an uncultured delta proteobacterium (BMD 1; 98% sequence similarity) (Fig. 5).

TABLE 3. MPN of cultured anaerobes and methanogens in acidic bog peat

Metabolic type	Medium	Substrate	MPN of organisms g (dry wt) of soil ^{-1a}
General fermentative anaerobes	TSB (pH 4.5)	Complex	1 × 10 ⁹ (2 × 10 ⁸ -5 × 10 ⁹)
Methanogens	TSB (pH 4.5)	Complex	1 × 10 ⁶ (3 × 10 ⁵ -6 × 10 ⁶)
H ₂ -CO ₂ -utilizing methanogens	UM (pH 4.5)	H ₂ -CO ₂	3 × 10 ⁷ (6 × 10 ⁶ -1 × 10 ⁸)
H ₂ -CO ₂ -utilizing methanogens	UM _{ab} (pH 4.5)	H ₂ -CO ₂	3 × 10 ² (7 × 10 ¹ -2 × 10 ³)

^a Values in parentheses represent confidence intervals with 95% probability.

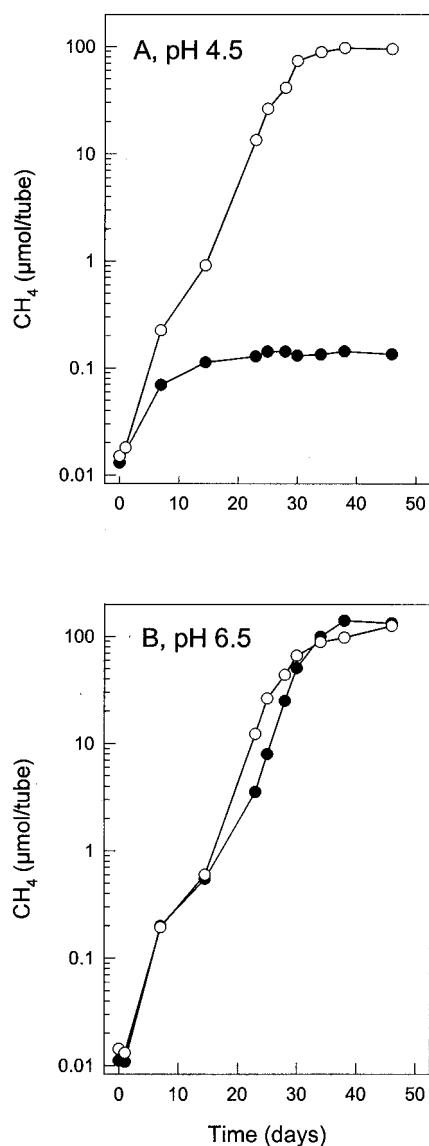


FIG. 4. Effect of 5 mM acetate on the production of CH₄ from H₂-CO₂ by the methanogenic enrichment culture at pH 4.5 ($<pK_{a \text{ acetic acid}}$) (A) and pH 6.5 ($>>pK_{a \text{ acetic acid}}$) (B). Values are the means of triplicates. Symbols: ●, amended with acetate; ○, controls (unamended).

DISCUSSION

Hydrogenotrophic methanogenesis and the interspecies transfer of H₂. In contrast to terrestrial environments and pH-neutral wetlands, in which CH₄ is produced predominantly by acetoclastic methanogens (6, 14, 30, 59), H₂ was the main substrate for methanogenesis in the acidic bog peat investigated in this study. Indeed, acetate inhibited methanogenesis, and attempts to obtain acetoclastic, methanogenic enrichments at both pH 4.5 and 6.0 were unsuccessful. These results are consistent with earlier reports (29, 62) and with recent isotope data (7, 44) obtained from acidic bog peats and northern peatlands. Acetate is a main intermediate during the anaerobic decomposition of organic matter (38) and can accumulate to high levels in the absence of acetoclastic methanogenesis in

peatlands (22, 51, 58). In the present study, in situ accumulation of acetate was not evident, presumably because under in situ conditions, acetate was oxidized to CO₂ via aerobic respiration or other oxidative microbial processes (e.g., via the dissimilation of iron or nitrate) or oxidized syntrophically to CO₂ by the concerted activity of acetate-oxidizing anaerobes and hydrogenotrophic methanogens. The latter possibility is indirectly supported by the results from TSB-supplemented anoxic microcosms (Fig. 3B). In microcosms, about 50% of the acetate produced from glucose was consumed slowly after an extensive incubation period. Bromoethanesulfonate inhibited both the consumption of acetate and the production of methane, indicating that acetate degradation was indeed linked to methanogenesis. In contrast, supplemental H₂ stimulated the consumption of acetate after H₂ was consumed, indicating that the initial stimulation of hydrogenotrophic methanogens enhanced the usually slow syntrophic degradation of acetate. The syntrophic degradation of acetate under anoxic conditions can occur in methanogenic bioreactors (50) and lake sediments (42).

Syntrophic interactions of certain anaerobic microorganisms are based on the transfer of H₂ to a hydrogenotrophic partner (37, 48). The acidic bog peat evaluated in the present study produced small, transient amounts of H₂ from endogenous sources (Fig. 3A), and the amount of this transiently produced H₂ increased significantly in TSB-supplemented bog peat (Fig. 3B). The production of H₂ became more evident when methanogenesis was inhibited or when the peat was amended with primary alcohols. Since H₂ was transiently produced concomitant with the consumption of alcohols and the production of CH₄, H₂ appears to be an important substrate for the production of CH₄ in alcohol-supplemented microcosms (Fig. 2B and C). This conclusion was corroborated by the observation that ethanol was not consumed in the presence of high partial pressures of H₂. The MPN values for hydrogenotrophic methanogens were approximately 30-fold greater than the MPN values for the unspecified methanogens that were obtained in TSB lacking supplemental H₂ (Table 3). The absence of CH₄ in 10⁻⁷ TSB-MPN dilutions indicated that no suitable precursor for methanogenesis (i.e., H₂) was produced beyond TSB-MPN dilutions of 10⁻⁶. These collective observations indicate that H₂ is an intermediate in the overall decomposition of organic matter in this acidic bog and that hydrogenotrophic methanogens outnumber H₂-producing microorganisms in the bog peat.

Primary alcohols are produced by fermentation and are intermediates in the anoxic decomposition of organic matter to CH₄ and CO₂ (38). Alcohols were quickly consumed in anoxic peat microcosms, demonstrating that the anaerobic microbiota of the peat had a substantial capacity to consume alcohols. Anaerobic bacteria that degrade primary alcohols in syntrophic association with H₂-scavenging anaerobes have been isolated from pH-neutral environments and have pH optima that approximate pH 7 (47, 48, 60, 65). The syntrophic methanogenic consortia of the bog peat characterized in the present study is moderately acid tolerant and is probably composed of microorganisms that have not been described yet. The moderately acid-tolerant hydrogenotrophic methanogens appear to be syntrophically associated with H₂-producing anaerobes and

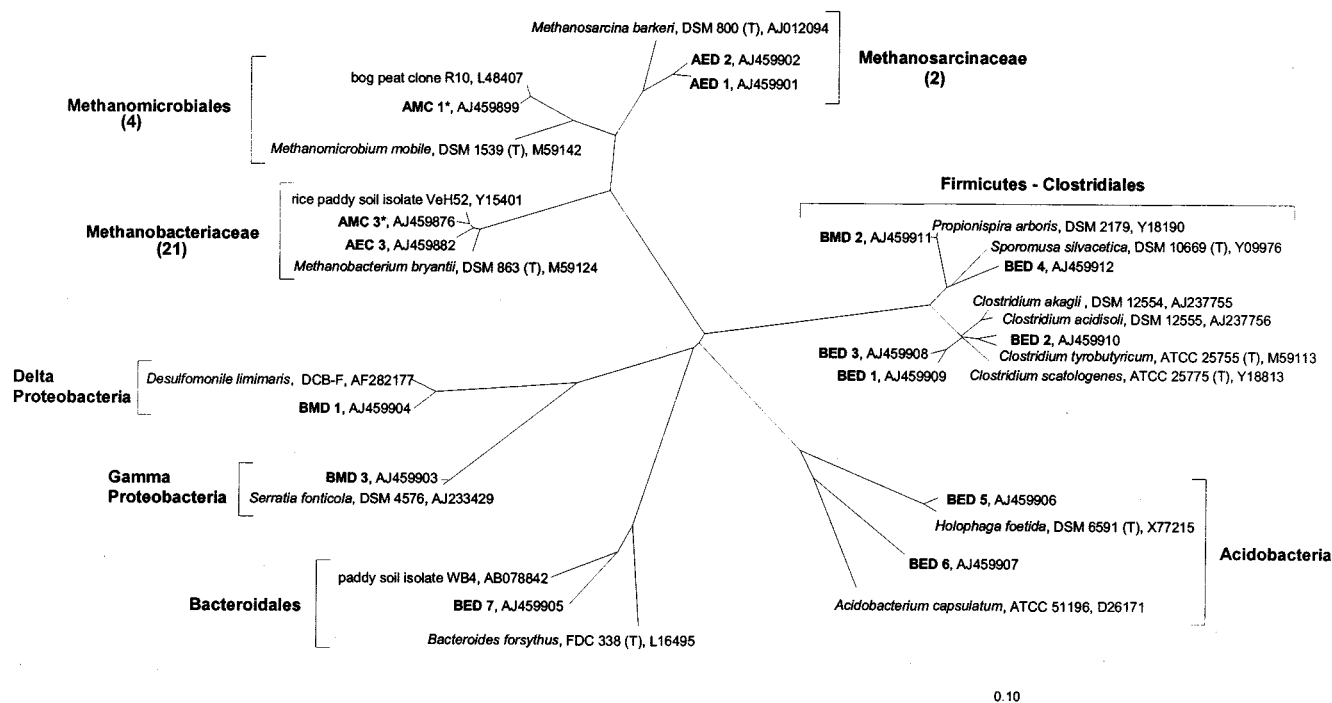


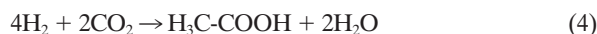
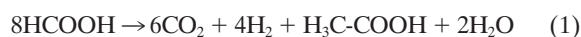
FIG. 5. Phylogenetic positions of bacterial and representative archaeal sequences obtained in this study (bold) as inferred from comparative analysis of 16S rRNA gene sequence data. Values in parentheses are the number of sequences obtained for a given group. The codes used for the sequences obtained in this study are as follows: A, archaeal; B, bacterial; M, from highest CH_4 -positive MPN dilution of bog peat; E, from methanogenic enrichment culture; C, sequences (1,400 bp) from clone library; D, sequences (approximately 400 bp) from excised bands of DGGE analyses. Sequences that are almost complete (i.e., approximately 1,400 bp) are marked with an asterisk. The bar represents a 0.1 estimated change per nucleotide.

essential to the normal flow of both carbon and reductant in this acidic bog peat.

Effects of volatile fatty acids and pH. Methanogens can tolerate up to 100 mM volatile fatty acids at neutral pH; however, they are very sensitive to volatile fatty acids at mildly acidic pH (56, 57). It was therefore not surprising that acetate and other volatile fatty acids inhibited methanogenesis in bog peat at pH 4.5 but not at pH 6.5 (Table 2; Fig. 4). The methanogenic capacities of tundra wetland soils and mildly acidic peat are likewise inhibited by volatile fatty acids under mildly acidic conditions (26, 29, 62). At an extracellular pH_{out} of <4.7 ($\text{pK}_{\text{a}}^{\text{acetic acid}} = 4.7$), undissociated acetic acid is abundant and can permeate the cellular membrane. Inside the cell, at an assumed near-neutral intracellular $\text{pH}_{\text{in}} \gg \text{pK}_{\text{a}}^{\text{acetic acid}}$, the acid dissociates and leads to a decoupling of the membranous proton motive force. The resulting decrease in the intracellular pH in poorly buffered cells and the decoupling of the proton motive force can be lethal to the cell (31, 46). Thus, the decrease in methanogenesis in TSB-supplemented microcosms was probably due to the inhibitory effects of the fatty acids that were produced from TSB (which contained glucose). Microorganisms capable of producing volatile fatty acids and H_2 include clostridial (23) and *Propionispira* (49) species; both types of bacteria were detected in bog peat (Fig. 5). Novel, acid-tolerant clostridial strains have been isolated from similar bog peats (27, 35).

The inhibition of methanogenesis by formate was relieved after formate had been consumed. The ratio of formate con-

sumed to CO_2 , H_2 , and acetate produced approximated 8:6:4:1 (reaction 1), a stoichiometry that is consistent with the combined activities of formate dehydrogenase and hydrogenase (reaction 2) and formate- or H_2 -dependent acetogenesis (reactions 3 and 4 respectively) (12):



Although these results suggest that acetogens occur in this acidic habitat, it should be noted that most known acetogens have relatively neutral pH optima (12).

Most known methanogens grow optimally at near-neutral pH (4, 65). However, an unclassified *Methanobacterium* isolate (63) and a strain of *Methanosarcina barkeri* (5) can tolerate pH 4. The in situ emission of CH_4 from the bog at pH 4.3 to 4.5 and the relative abundance of culturable methanogens in the acidic peat (approximately 1% of the general fermentative anaerobes at a cultivation pH of 4.5 [Table 3]) demonstrated that the methanogens of this bog are functional under these acidic conditions. Indeed, an increase in pH from 4.5 to 6.5 did not result in higher rates of CH_4 production by methanogenic enrichments (Fig. 4). In contrast, methanogens obtained from other peats or acidic bog sediments are significantly stimulated

by neutral pH and account for only 0.1% of the cultured anaerobes (17, 64).

Effect of antibiotics. The production of CH₄ by cultured methanogens was inhibited by streptomycin, kanamycin, and the β-lactam antibiotic penicillin (Table 3 and data not shown), indicating that the cultured methanogens were sensitive to at least one of these antibiotics. Some methanogens are sensitive to the antibiotics used. *Methanotrix soehngenii* and the moderately acid-tolerant *Methanobacterium espanolae* are sensitive to β-lactam antibiotics and kanamycin, respectively (25, 43). Additional antibiotics that inhibit protein synthesis also inhibit certain methanogens (21). Thus, antibiotics do not always selectively inhibit only bacteria and cannot be used to definitively differentiate between bacterial and methanogenic activities.

Phylogenetic identification of methanogens. Much of the experimental evidence outlined above indicated that H₂-CO₂ was the main precursor for methanogenesis in the peat, and the main goal of the cultivation experiments was to identify in situ relevant hydrogenotrophic methanogens. Most of the archaeal sequences obtained from MPN dilutions and enrichments were phylogenetically related to *Methanobacteriaceae* and *Methanomicrobiales*; most known species of these two groups are capable of hydrogenotrophic methanogenesis (4, 16). *Methanobacteriaceae*- and *Methanomicrobiales*-related methanogens were also detected in an acidic Welsh peat and a Minnesota peatland (41, 63), and three of the archaeal bog peat sequences (AMC 1, AMC 2, and AEC 1) obtained in the present study were closely affiliated with a *Methanomicrobiales*-related sequence that was derived from one of these peats (20). Based on the MPN analyses and 16S rRNA gene sequences that were retrieved from the highest CH₄-positive MPN dilutions of bog peat, cultivated cell numbers of *Methanobacterium*- and *Methanomicrobiales*-related species approximated 3 × 10⁷ and 1 × 10⁶ organism g (dry wt) of soil⁻¹, respectively, indicating that these organisms contribute to the in situ production of CH₄ in this bog peat.

In contrast, *Methanosarcinales*-related species appear to occur only in small numbers in this acidic peat since the sequences that were retrieved from the CH₄-positive MPN dilutions were not affiliated with the *Methanosarcinales* cluster and since (semi) specific PCR amplifications were necessary to obtain *Methanosarcinales*-related sequences (AED 1 and AED 2) from the methanogenic bog peat enrichments. *Methanosarcinales*-related species might play a role in other peatlands (20, 36). Regional differences in the structure and function of the methanogenic communities in acidic peats have not been investigated.

In conclusion, the results of the present study indicate that in situ relevant acid-tolerant methanogens of this acidic peat belong to the *Methanomicrobiales* and *Methanobacteriaceae*, are hydrogenotrophic, and are inhibited by small amounts of acetate or other volatile fatty acids at in situ pH. Hydrogenotrophic methanogens may be symbiotically associated with hydrogen-producing anaerobes and keep H₂ levels low during the normal flow of carbon and reductant in this bog peat.

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