Methanogenesis In Mclean Bog, An Acidic Peat Bog In Upstate New York: Stimulation By H2/CO2 In The Presence Of Rifampicin, Or By Low Concentrations Of Acetate

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Abstract

Acidic peat bog soils produce CH4 and although molecular bio-logical studies have demonstrated the presence of diverse methano-genic populations in them, few studies have sustained methano-genesis by adding the CH4 precursors H2/CO2 or acetate, and few indigenous methanogens have been cultured. McLean Bog is a small (ca. 70 m across), acidic (pH 3.4–4.3) Sphagnum-dominated bog in upstate New York. Although addition of H2/CO2 or 10 mM acetate stimulated methanogenesis in soils from a nearby circumneutral-pH fen, neither of these substrates led to sustained methanogenesis in McLean Bog soil slurries. After a brief period of stimulation by H2/CO2, methanogenesis in McLean Bog soil declined, which could be attributed to buildup of large amounts of acetic acid pro-duced from the H2/CO2 by acetogens. Addition of the antibiotic rifampicin inhibited acetogenesis (carried out by Bacteria) and al-lowed methanogenesis (carried out by Archaea)tocontinue. Using rifampicin, we were able to study effects of temperature, pH, and salts on methanogenesis from H2/CO2 in McLean Bog soil samples. The enriched H2/CO2-utilizing methanogens showed an optimum for activity near pH 5, and a temperature optimum near 35 ◦ C. Methanogenesis was not stimulated by addition of 10 mM acetate, butit was stimulated by 1 mM acetate, and multiple additions were consumed at increasing rates and nearly stoichiometrically converted to CH4.Inconclusion, we have found that both hydro-gentrophic and aceticlastic methanogens are present in McLean Bog soils, and that methanogenic activity can be stimulated using H2/CO2 in the presence of rifampicin, or using low concentrations of acetate.

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INTRODUCTION

Peatlands cover approximately 346–500 Mha of land in northern (>40°N) latitudes (Wieder 2001). Some of these wetlands, like those in Western Canada that have been studied more extensively, have an estimated annual net primary production of roughly 69–1249 g of carbon (C) m^{-2} y^{-1}. (Wieder 2001). Thus, up to 6 billion metric tons (6 Pg) of C are produced each year through primary production in northern peatlands. Only a small fraction of this C accumulates instead of being decomposed, yet an estimated 1/3 of terrestrial C (450 Pg) is sequestered in these northern peatland sites (Post et al. 1982; Gorham 1991). While a major sink for CO_2, peatlands are also a significant source of CH_4, and contribute approximately 20% of the total estimated annual CH_4 emissions (Cicerone and Oremland 1988). An important question is whether such northern peatlands will continue to be C sinks or become sources of atmospheric C in the face of predicted climatic changes. Total CH_4 concentration in the atmosphere has increased more than twofold, from 0.7 ppm to 1.7 ppm in the past 300 years (Bartlett and Harriss 1993). As a greenhouse gas, CH_4 has nearly 21 times the power of CO_2 to absorb IR radiation on a molecule per molecule basis. Thus, it is important that we understand factors, which influence the rates of methane production in these northern peatlands.

Biological CH_4 forms via the splitting of acetate (the aceticlastic reaction), reduction of CO_2 using H_2 or other reductants such as formate or alcohols, or from methanol, methylamines, methyl sulfides, and carbon monoxide. Acetate and CO_2 are usually the most quantitatively important CH_4 precursors, typically accounting for two-thirds and one-third, respectively, of methanogenesis in non-gastrointestinal habitats (Zinder 1993; Madigan et al. 2000). Although it is clear that acidic peat bogs produce CH_4, and molecular ecological techniques have identified a rich diversity of methanogens (Hales et al. 1996; Basiliko et al. 2003; Horn et al. 2003; Sizova et al. 2003), the indigenous methanogens have resisted culture and isolation.

Numerous attempts at culturing H_2/CO_2-utilizing methanogens from peatlands at low pH using standard culture media have failed, although methanogens growing at higher pH have been found (Williams and Crawford 1985; Goodwin and Zeikus 1987; Horn et al. 2003). Recently Sizova et al. (2003) obtained stable slow-growing H_2/CO_2 enrichments from Siberian peat, but were unable to isolate methanogens in pure culture. Media and growth conditions more closely resembling *in situ* conditions need to be designed to support growth of indigenous methanogens, so that more of these methanogens can be studied. Our initial attempts to study H_2/CO_2-utilizing methanogens were hampered by high activity of H_2/CO_2-utilizing acetogens that produced toxic amounts of acetic acid. We describe here the use of the antibiotic rifampicin to reduce acetogenesis (below detectable levels) allowing us to study methanogenic populations.

Acetate is the dominant CH_4 precursor in most freshwater anaerobic soils, and often accounts for 60% or more of total methanogenesis (Zinder 1993; Schulz and Conrad 1996; Conrad et al. 2002), similar to results from anaerobic digestors. However some studies measuring $^{12}C/^{13}C$ isotopic ratios in CH_4 in oligotrophic bogs and fens indicated that a higher percentage of total CH_4 formation came from H_2/CO_2 (Lansdown et al. 1992; Popp and Chanton 1999; Chasar et al. 2000). In addition, acetate has been found to accumulate ephemerally in some sites (Shannon and White 1996; Hines et al. 2001; Duddleston et al. 2002), and it is unclear to what extent aceticlastic methanogenesis is occurring in low pH peatland ecosystems. Studies have attempted unsuccessfully to enrich for aceticlastic methanogens in acidic peat (Horn et al. 2003; Sizova et al. 2003), and many have found additions of acetate inhibitory, or non-stimulatory in peat microcosms (Williams and Crawford 1984; Goodwin and Zeikus 1987; Bridgham and Richardson 1992; Watson and Nedwell 1998; Blodau et al. 2002; Horn et al. 2003). Using ^{14}C-labeled acetate, Goodwin and Zeikus (1987) demonstrated that aceticlastic methanogenesis accounted for a significant fraction of methanogenesis in Crystal Bog, Wisconsin, USA, but were unable to enrich for aceticlastic methanogens. Thus, the role of acetate in C flow to CH_4 in acidic bogs remains unclear.

The main objectives in this study were to examine the factors that favor methanogenesis including temperature, pH, and ionic conditions and to use this information to enrich for aceticlastic and hydrogenotrophic methanogens in acidic peat soils.

METHODS

Study Sites

We used peat from three sites located within 40 km of Ithaca, NY (42° 30' N, 76° 30'W), where mean annual precipitation is 932 mm and mean annual temperature is 7.8°C. The principle study site, McLean Bog, is an ombrotrophic (rain fed) kettle hole bog that has been described previously (Osvald 1970). It has a peat depth of 8 meters and the vegetation is dominated by *Sphagnum* moss (*S. angustifolium*, and *S. magellanicum*) and ericaceous shrubs (*Chamaedaphne calyculata*, and *Vaccinium corymbosum*). Another similar site, which was used for comparison, is Chicago Bog, also described by Osvald (1970). Chicago bog is an oligotrophic floating bog associated with open water. The vegetation is dominated primarily by *Chamaedaphne calyculata* and *Sphagnum* species. During sampling, the pH at ca.15 cm depth was 3.4-4.3 for McLean Bog and 4.1 for Chicago Bog. Acetate, which is important at low pH, was not detected in the pore water (detection limit < 150 μM). Both bogs are ombrotrophic, have low mineral content and are acidic, owing (at least in part) to the uronic acids in *Sphagnum*, which have high cation exchange capacity (Rydin and Clymo 1989; Mitch and Gosselink 2000). In contrast to the acidic bogs, the third study site, Michigan Hollow is a minerotrophic sedge fen with circumneutral soil pH and a relatively shallow layer of peat, ca. 1 m. Michigan Hollow has been described by Bernard and Macdonald (1974) and the vegetation is dominated by the tall lakeside sedge, *Carex lacustris*. During sampling in January 2002, the pH of the soil was 6.7.

Sample Collection

Soils were collected from depths approximately 15–25 cm below the soil surface and placed directly into airtight jars. Within 2 hours of sampling, jars were taken into the anaerobic chamber and any air pockets were removed. Jars were stored at room temperature as storing jars at lower temperatures led to a permanent and irreversible reduction in rates of methanogenesis (data not shown). Samples from McLean Bog were collected approximately every two months from August 2001–August 2003. For comparative purposes, additional soil samples were collected from Michigan Hollow in January 2002, and from Chicago Bog in August 2003.

Soil Slurry Incubations

Inside an anaerobic glove box, an aliquot of peat, 1 g (wet weight) for McLean Bog and Chicago Bog peat, and 5 g (wet weight) of Michigan Hollow peat, was weighed into each 150 × 18 mm crimp-top tube (Bellco Glass Co, Vineland, NJ, USA) on a Harvard Trip balance. Anaerobic deionized water was added for an approximate final volume of 10 mL and the tubes were sealed with gray butyl rubber stoppers and crimped. We made high dilutions of the acidic peats for two reasons: first, to facilitate the extraction of liquid samples for analysis by HPLC, since lower dilutions often formed a gel-like mass, and second, to allow microbial growth in the soil slurries so that enrichments could be made in subsequent studies. Deionized water was used as a diluent because the use of anaerobic peat pore water led to a decrease in rates of methanogenesis in preliminary studies. Tubes were then flushed on the bench for 5 minutes with sterile O_2-scrubbed N_2/CO_2 (70%/30%, certified standard mixture from Messer, Mixed Gas Industries, Morrisville, PA, USA). Anaerobic stock solutions were added as indicated to the following final concentrations: rifampicin (10 mg L^{-1}), sodium acetate (1, 2, 3, 5, or 10 mM), methanol (12.5 mM), pipes buffer

Table 1
Initial and final (after 21 days incubation) pH values for the soil slurries presented in Figure 4

Treatment	Initial pH	Final pH	Final pH for H_2/CO_2
No buffer	4.54 ± 0.05	4.24 ± 0.08	3.53 ± 0.01
5 mM pipes (pH 3.5)	3.37 ± 0.01	3.39 ± 0.05	3.30 ± 0.01
5 mM homopipes (pH 4)	4.19 ± 0.01	4.18 ± 0.02	3.86 ± 0.00
5 mM homopipes (pH 5)	4.89 ± 0.03	4.79 ± 0.04	4.13 ± 0.01
1.5 mM bicarbonate	5.19 ± 0.06	4.97 ± 0.08	3.72 ± 0.01
6 mM bicarbonate	6.18 ± 0.04	5.95 ± 0.07	4.59 ± 0.13

Initial pH values represent the average of all treatments ± standard deviation. Final pH values represent the average of all treatments excluding the H_2/CO_2 treatments in the absence of rifampicin.

or homopipes buffer (both 5 mM with NaOH added to achieve a final pH near 3.5, 4, or 5 in the soil slurries, see Table 1), and/or $NaHCO_3$ (1.5 or 6 mM). Sodium acetate instead of acetic acid was added to allow our results to be comparable with most other studies, which used sodium acetate. Sterile O_2-scrubbed H_2/CO_2 (70%/30%, or 80%/20%, 0.1–0.7 atm, certified standard mixture from Messer, Mixed Gas Industries, Morrisville, PA, USA) was added to appropriate tubes as needed throughout the course of the incubation.

Unless otherwise indicated, tubes for soil slurry incubations were prepared in triplicate and were incubated at 28°C in the dark. Where indicated, tubes were shaken to allow for rapid diffusion of H_2/CO_2 from the headspace as we found much higher rates of methanogenesis from H_2/CO_2 in tubes that were shaken (data not presented). Initial pH was determined by preparing two additional duplicate tubes for each incubation condition. Final pH was determined at the end of the experiment. Soil slurries did not need to be diluted before measuring pH; however for fresh soil samples, pH was determined by diluting the soils 1:3 in distilled water and shaking vigorously to equilibrate the solution before recording the pH value using a 211 microprocessor pH meter (Hanna Instruments, Woonsocket, RI, USA). Moisture content was determined by weighing soils before and after incubation at 60°C for 48 hours. Endogenous rates of methanogenesis at room temperature (ca. 22°C) were determined by measuring the amount of CH_4 produced in soil slurries (10 mL as described above) with no added CH_4 precursors incubated under an N_2/CO_2 headspace (70%/30%). CO_2:CH_4 ratios were determined under a headspace of N_2. Soil pH, moisture content, and endogenous rates of methanogenesis were determined for each sample jar within 48 hours of sampling (each measurement was taken in duplicate).

Analytical Methods

Alcohols and organic acids, including acetate were determined by high performance liquid chromatography using a Rabbit HP solvent delivery system (Rainin Instrument Co., Inc., Emeryville USA) to regulate flow through a BioRad Fast Acid Analysis Column (100×7.8 mm, BioRad Life Science Research, Hercules, CA, USA). The system was equipped with a 20 μL injection loop and a Knauer differential refractometer. The method detection limit for acetate was 150 μM. Samples taken from soil slurries for HPLC were stored at −20°C until analyzed for fatty acids. Then the samples were thawed, vortexed thoroughly and centrifuged for approximately 7 minutes before HPLC analysis. The gas headspace was analyzed for CH_4 using a Perkin Elmer 3920B gas chromatography column with a flame ionization detector (Phoenix Equipment Inc., Rochester, NY, USA). CO_2:CH_4 ratios as well as endogenous rates of methanogenesis were determined using a GOW MAC Series 550 gas chromatograph equipped with a thermal conductivity detector (Bridgewater, NJ, USA) as described previously (Zinder et al. 1984). Soil slurries were vortexed for 30 s prior to analyzing the gaseous headspace to allow the gas in the liquid phase to equilibrate.

Statistical Analyses

One-way ANOVA and Tukey's multiple comparisons tests ($\alpha = 0.05$), unless otherwise stated were used to determine statistical differences between treatments (pH, temperature, H_2/CO_2, rifampicin, etc.). Data were analyzed using Minitab Statistical Software version 13.1.

RESULTS

Methanogenesis in McLean Bog Soils verses Michigan Hollow Soils in Response to Additions of Methanogenic Precursors

Additions of H_2 initially stimulated methanogenesis in McLean Bog soil slurries; however, after 1–3 weeks, methanogenesis decreased significantly. Total CH_4 production with H_2 additions (1.95 ± 0.95 mmol L^{-1}) was not significantly greater than without H_2 additions (1.27 ± 0.83 mmol L^{-1}), and was never greater than 3.77 mmol L^{-1} CH_4 in 10 different experiments with incubation times of 21–48 days. In these experiments, acetic acid accumulated in samples to which H_2/CO_2 was added (5.00 ± 2.23 mM), but was undetectable in control samples (see Figure 1 as an example). Further additions of H_2 did not stimulate methanogenesis. Addition of methanol (12.5 mM, data not shown), or acetate (10 mM, see results next and Figure 7) did not stimulate methanogenesis in McLean Bog soil slurries.

Soil slurries from Michigan Hollow, a neutral pH site, were used for comparative purposes. In contrast to results found using McLean Bog soil slurries, addition of H_2 (0.1 atm, initial addition; 0.3 atm, multiple subsequent additions), methanol (12.5 mM, multiple additions), or acetate (10 mM, multiple additions) to Michigan Hollow soil slurries led to considerable and sustained stimulation of methanogenesis compared to control samples receiving no addition (Figure 2) and the

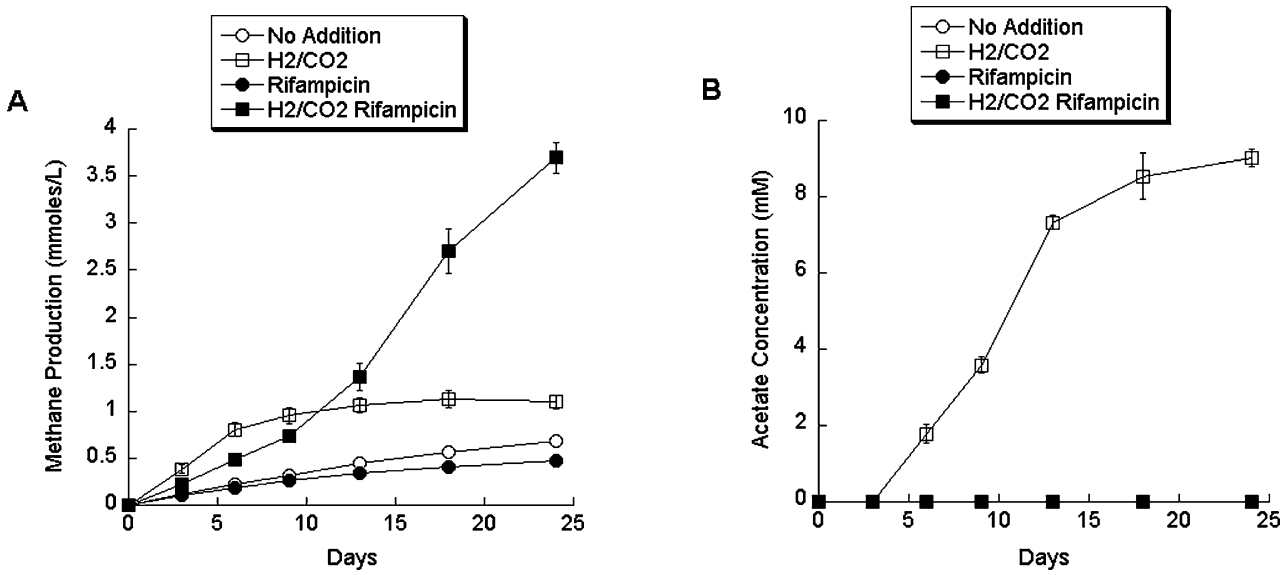

Figure 1. Time course for CH_4 production (A) and acetate concentration (B) for slurries of McLean Bog soil collected in April 2003 in response to H_2/CO_2 addition with and without rifampicin. Slurries were incubated in a shaker at 28°C. Each data point represents an average of triplicate tubes and the bars represent standard error. Symbols that are not visible are obscured by the squares; this indicates that acetate was not detected.

slurries converted multiple additions of these substrates to CH_4.

Methanogenesis and Acetogenesis from H_2/CO_2 in McLean Bog Soil and Inhibition of Acetogenesis by Rifampicin

We hypothesized that the inhibition of methanogenesis from H_2/CO_2 in McLean Bog soil was caused by acid buildup due to the activity of acetogens converting H_2/CO_2 to acetic acid. Because acetogens are *Bacteria* whereas methanogens are *Archaea*, we examined several different antibiotics for their ability to inhibit acetogenesis but not methanogenesis. Of those tried, rifampicin, a bacterial RNA polymerase inhibitor known to be acid stable, gave consistent results in all six experiments that were conducted. Other antibiotics such as vancomycin and kanamycin failed to inhibit acetogenesis, possibly due to acid liability. In each of the six experiments with rifampicin, methanogenesis in samples incubated with H_2/CO_2 and rifampicin was always more than 1.5 times that in samples incubated with H_2/CO_2 without rifampicin, and on average was six times greater than samples with H_2/CO_2 without rifampicin. In these experiments, McLean Bog slurries incubated with H_2/CO_2 produced greater than 2 mM acetic acid (5.67 ± 2.33 mM), whereas acetic acid was undetectable in the presence of rifampicin (see Figure 1 as an example).

The final pH was measured for tubes in the experiment shown in Figure 1. The final pH in samples incubated with H_2/CO_2 without rifampicin was 3.50 ± 0.03, and that for samples incubated with rifampicin was 4.13 ± 0.04, close to the initial pH value. Moreover, in all these experiments, methanogenesis ceased in 1–3 weeks in samples incubated with H_2/CO_2 without rifampicin; however, methanogenesis continued at increasing rates in the presence of rifampicin. Indeed, in two experiments, CH_4 in slurry samples incubated with H_2/CO_2 and

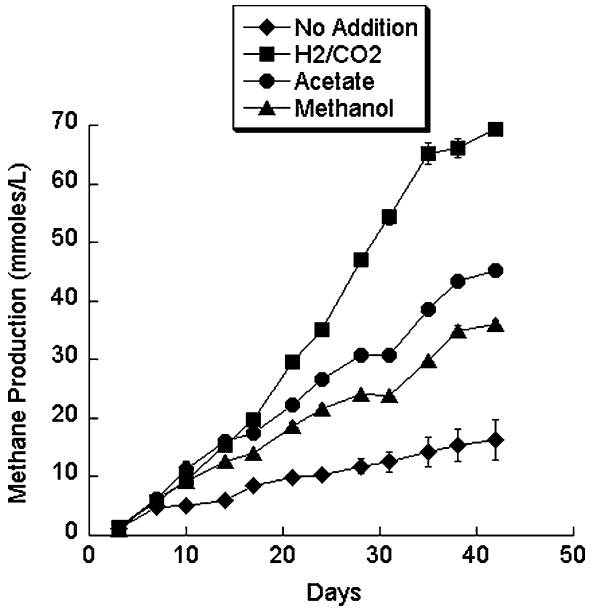

Figure 2. Time course for CH_4 production for slurries of Michigan Hollow soil collected in January 2002 in response to additions of H_2/CO_2, acetate or methanol. Slurries were incubated at room temperature. Each data point represents an average of duplicate tubes and the bars represent the range around that average.

rifampicin reached levels exceeding 15 mmol L^{-1}. For slurries of McLean Bog soil collected in August 2002, tubes incubated in the presence of H_2/CO_2 produced 2.57 ± 2.47 mmol L^{-1} CH_4 and 5.21 ± 0.40 mM acetate after 42 days, whereas tubes incubated in the presence of H_2/CO_2 and rifampicin produced 17.42 ± 0.87 mmol L^{-1} CH_4 and did not produce detectable levels of acetate after 42 days. Using soil collected from Chicago Bog in August 2003, tubes incubated in the presence of H_2/CO_2 produced 7.47 ± 2.04 mmol L^{-1} CH_4 and 4.41 ± 0.60 mM acetate after 28 days. As in McLean Bog soil slurries, rifampicin addition also inhibited acetic acid buildup (acetate was not detected) in Chicago Bog soil slurries and allowed sustained methanogenesis from H_2/CO_2 (12.74 ± 0.25 mmol L^{-1} CH_4 were produced). In all of our experiments, rifampicin had only a modest inhibitory effect on methanogenesis from endogenous substrates in samples incubated under N_2/CO_2 (Figure 1A), indicating that organisms converting substrates to CH_4 precursors were less susceptible to this antibiotic than the acetogens. Thus, addition of rifampicin allowed us to study the response to various environmental parameters by organisms from McLean Bog peat carrying out methanogenesis from H_2/CO_2.

Temperature Effects on Methanogenesis from H_2/CO_2 in McLean Bog Soil

Temperature optima for endogenous production of CH_4 in slurries incubated 13 days in the absence of added H_2/CO_2 with or without rifampicin were similar, ca. 37°C (Figure 3A). Compared to samples without rifampicin, samples incubated with rifampicin and added H_2/CO_2 showed considerably greater methanogenesis near the optimum temperature of 33–37°C and acetate did not accumulate. In the presence of H_2/CO_2 without rifampicin, the temperature optimum appeared closer to 33°C. In those samples, acetic acid accumulated with an optimum near 37°C (Figure 3B), indicating interference with methanogenesis by acetic acid in the absence of rifampicin. All samples showed limitation of methanogenesis at 4 and 15°C, and essentially no CH_4 or acetate was detected in any of the samples incubated at 45°C.

pH Effects on Methanogenesis and Acetogenesis from H_2/CO_2 in McLean Bog Soil

CH_4 was produced by McLean Bog soil slurries over a pH range of 3.4–6.1, and the lowest rates of methanogenesis were at pH 3.4 in all samples (Figure 4). In samples incubated for 13 days in the presence or absence of rifampicin, rates of methanogenesis from endogenous substrates were similar at all pH values, and no acetic acid buildup was detected at any pH value. Similar CH_4 production rates were measured for samples with and without buffer indicating that the buffers were not inhibitory or stimulatory. H_2/CO_2 caused some stimulation of methanogenesis and considerable stimulation of acetogenesis at all pH values in the absence of rifampicin. Methanogenesis from H_2/CO_2 in the absence of rifampicin did not show a discernable pH optimum, whereas acetogenesis was greatest when buffered at pH 6.2. This may be due to the greater buffer capacity at higher pH values allowing greater accumulation of acetic acid. In the presence of rifampicin, no acetic acid was detected in samples incubated with H_2/CO_2, and methanogenesis was greatly stimulated and showed a clear optimum near pH 5.

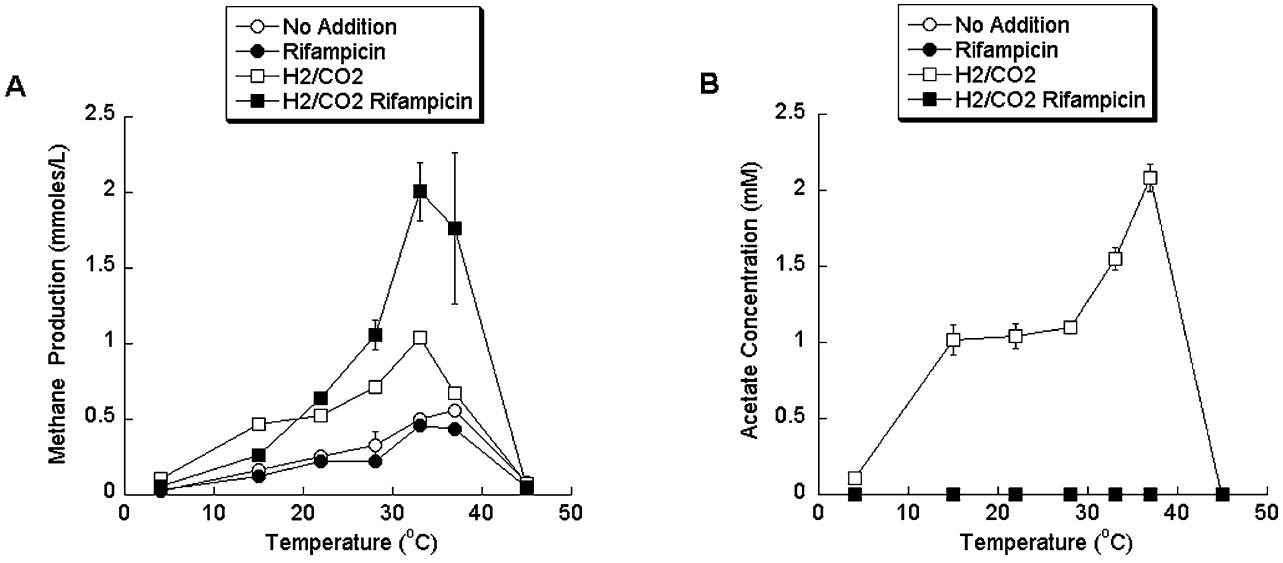

Figure 3. CH_4 production (A) and acetate concentration (B) after 13 days incubation for slurries of McLean Bog soil collected in April 2003 incubated across a range of temperatures from 4 to 45°C with additions of H_2/CO_2, with and without rifampicin. Each data point represents an average of triplicate tubes and the bars represent standard error. Symbols that are not visible are obscured by the squares; this indicates that acetate was not detected.

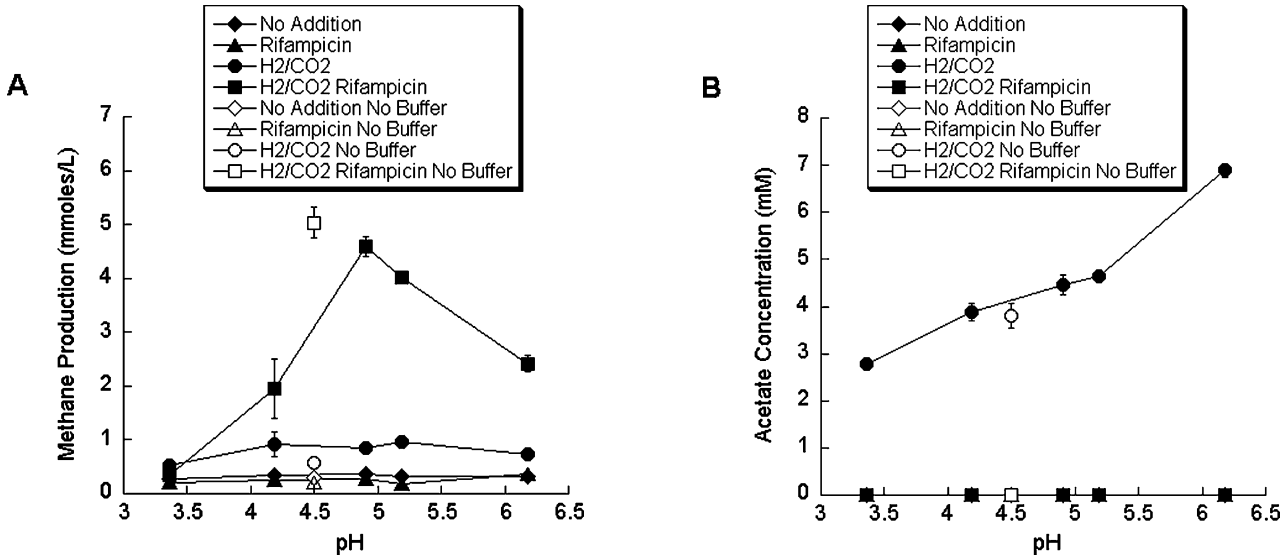

Figure 4. CH_4 production (A) and acetate concentration (B) after 13 days incubation in slurries of McLean Bog soil collected in June 2003 in response to various pH changes, 3.4–6.2, and in response to H_2/CO_2 additions with or without rifampicin. Slurries were incubated in a shaker at 28°C. Each data point represents an average of triplicate tubes and the bars represent standard error. Symbols that are not visible are obscured by the squares; this indicates that acetate was not detected.

The final pH values for samples incubated with H_2/CO_2 in the absence of rifampicin were significantly lower, ($\alpha = 0.01$), when compared to the other treatments for all buffers except those buffered at pH 3.5 (Table 1). In all other treatments, the pH value did not change more than 0.2 pH units when a buffer was used, and changed only 0.3 pH units in the absence of a buffer.

Response of McLean Bog Soil to Additions of Sodium Chloride and Potassium Chloride

Concentrations of NaCl or KCl as low as 2 mM inhibited methanogenesis from H_2/CO_2 in samples incubated 19 days with H_2/CO_2 in the presence of rifampicin, indicating that the organisms were initially adapted to low ionic strength (Figure 5). However, after 46 days, samples incubated in the presence of as

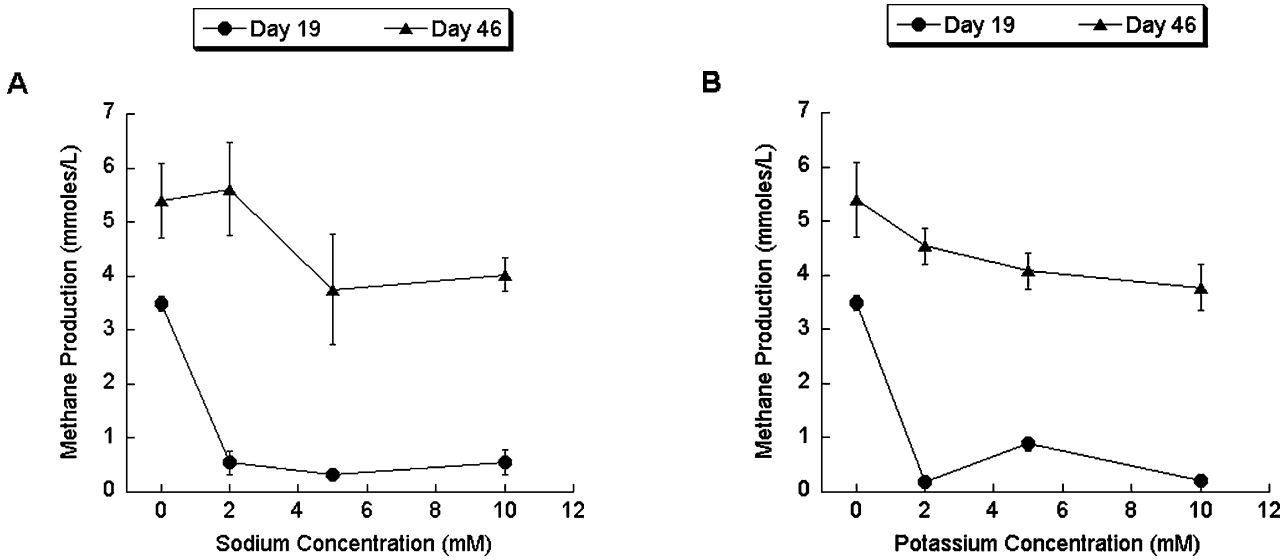

Figure 5. CH_4 production at day 19 and day 46 for slurries of McLean Bog soil collected in April 2003 in response to additions of sodium (A) or potassium (B) in an $N_2/H_2/CO_2$ atmosphere and in the presence of rifampicin while incubated in a shaker at 28°C. Each data point represents an average of triplicate tubes and the bars represent standard error.

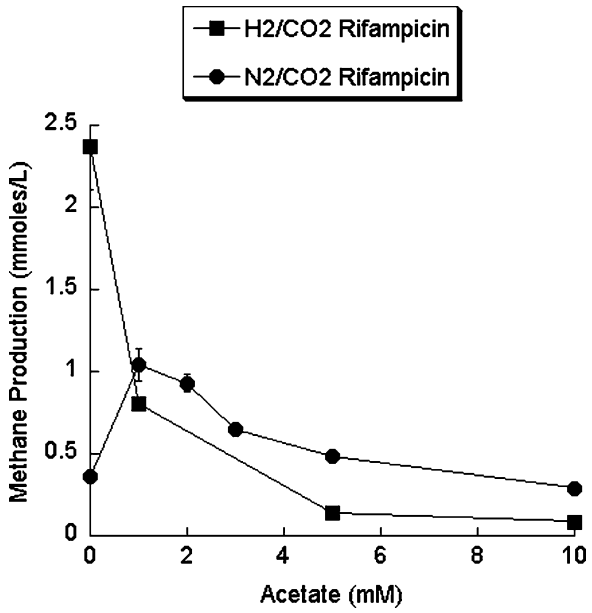

Figure 6. CH_4 production after 19 days for slurries of soil collected from McLean Bog in June 2003 in response to various concentrations of sodium acetate, with and without H_2/CO_2 addition and in the presence of rifampicin. Slurries were incubated in a shaker at 28°C. Each data point represents an average of triplicate tubes and the bars represent standard error.

much as 10 mM NaCl or KCl showed significant methanogenesis. In control samples incubated with N_2/CO_2, total CH_4 production was less than 0.25 mmol L^{-1} at day 19 and less than 0.65 mmol L^{-1} at day 46, so that nearly all of the CH_4 in Figure 5 was derived from added H_2/CO_2.

Methanogenesis from Acetate in McLean Bog Soil

Samples receiving 1 mM acetate and incubated for 19 days under N_2/CO_2 produced 0.68 ± 0.09 mmol L^{-1} more CH_4 than samples with no acetate added (Figure 6), whereas higher concentrations stimulated methanogenesis less. Methanogenesis in samples with 10 mM acetate was nearly equal to that of the control. At the low pH values of the acidic peat, a fraction of the acetate will become acetic acid, based on its pK value of 4.7. Buffer was not added to soil slurries, thus the pH was affected by acetate addition. Samples to which no acetate was added had final pH values near 4.16 ± 0.02, samples with 1 mM acetate were near pH 4.31 ± 0.07, and those with 10 mM acetate were near pH 5.12 ± 0.04.

We also examined the effect of acetate on samples incubated with H_2/CO_2 in the presence of rifampicin, where concentrations as low as 1 mM sodium acetate caused significant inhibition of methanogenesis. In contrast to experiments with H_2/CO_2, rifampicin and 5 or 10 mM NaCl (Figure 5), samples incubated 46 days with H_2/CO_2, rifampicin and 5 or 10 mM sodium acetate did not show any recovery of methanogenesis; however, samples incubated with H_2/CO_2, rifampicin and 1 mM sodium acetate did show stimulation of methanogenesis after prolonged incubation periods greater than 30 days (data not shown).

Stimulation of methanogenesis by additions of low concentrations of acetate under N_2/CO_2 indicated that acetate was being converted to CH_4. In addition, the ratio of endogenous rates of CO_2:CH_4 production was 1.10 ± 0.04 for McLean Bog soil slurries in and N_2 atmosphere, indicating that the soil is sufficiently reduced to allow for the anaerobic conversion of acetate to CH_4 and CO_2. We examined this further by making multiple additions of acetate (1mM final concentration). As shown in Figure 7, several doses of acetate were actively consumed

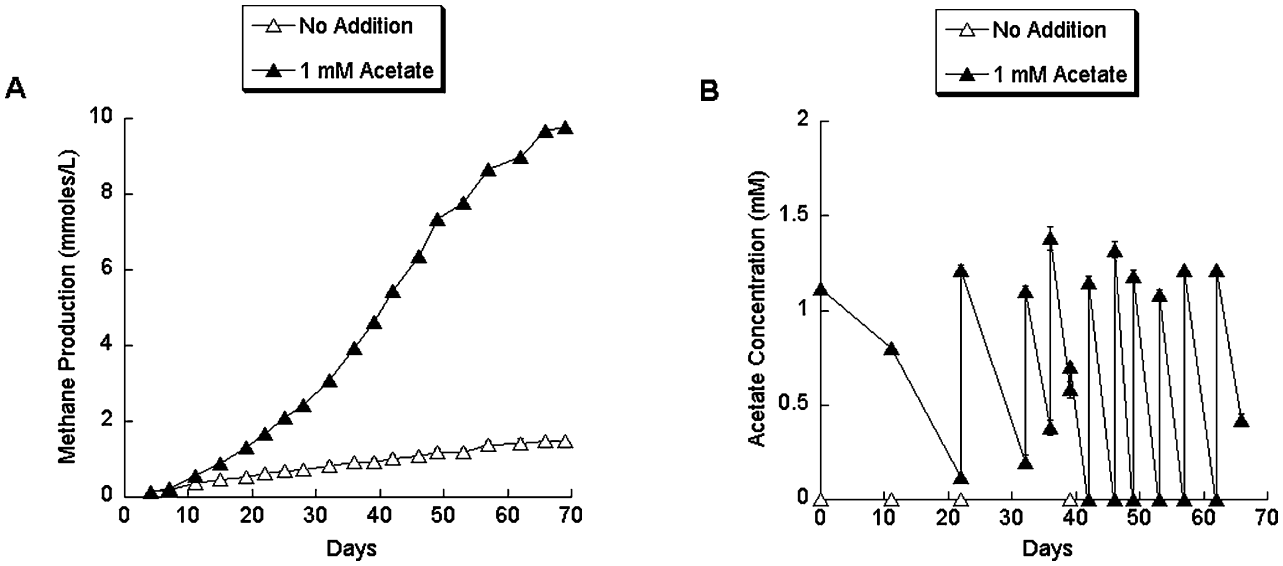

Figure 7. Time course for CH_4 production (A) and utilization of acetate (B) for slurries of soil collected from McLean Bog in June 2003 in response to 10 additions of acetate (1 mM) in the absence of rifampicin, and in the absence of other methanogenic precursors. Slurries were incubated in a shaker at 28°C. Each data point represents an average of triplicate tubes and the bars represent standard error.

in these samples and methanogenesis was stimulated to an extent nearly stoichiometric with the amount of acetate consumed (0.99 ± 0.16). The rates of acetate consumption and methanogenesis both increased, indicative of growth. In comparison to soil slurries without rifampicin, the presence of rifampicin lowered the endogenous rates of CH_4 production in samples not receiving acetate, but did not affect the stoichiometry or rate of reaction in samples receiving acetate additions (data not shown). In subsequent experiments we found that after 3 doses of sodium acetate, acetic acid instead of sodium acetate could be added to samples and converted to CH_4, thus preventing any further increase in pH (data not presented).

DISCUSSION

Inhibition of Methanogenesis from Added H_2/CO_2 in McLean Bog Samples by Acetic Acid and Use of Rifampicin to Inhibit Acetogenesis

Addition of H_2/CO_2 to acidic McLean Bog soil slurries caused only a modest and short-lived stimulation of methanogenesis versus controls incubated with N_2/CO_2, whereas samples from neutral-pH Michigan Hollow showed sustained stimulation of methanogenesis in response to addition of CH_4 precursors. Analyses of fatty acids demonstrated that acetic acid accumulated to concentrations of several mM in McLean Bog samples incubated with H_2/CO_2. This buildup undoubtedly was caused by acetogenic bacteria carrying out the reaction $4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$. Accumulation of acetic acid caused the pH in the samples to drop to ca. 3.5 in McLean Bog samples, and as discussed below, acetic acid is toxic at low pH. Thus, addition of H_2 caused toxic acidification of the samples. This acidification interfered with our ability to examine the effect of various environmental parameters on the metabolism and growth of H_2/CO_2-utilizing methanogens in the peat.

We found that the transcription inhibitor rifampicin was an efficient inhibitor of acetogenesis in our samples from McLean Bog. It appeared to be stable under the acid conditions in the peat, since acetogenesis remained inhibited after prolonged incubations. Rifampicin targets the β subunit of bacterial (B) core RNA polymerase (RNAP) and therefore selectively inhibits the growth of bacteria (Campbell et al. 2001). There are cases of archaeal sensitivity to rifampicin reported in the literature (Gonzalez et al. 1995; Jeanthon et al. 1999; Watrin et al. 1996) but in general archaea are considered resistant. Methanogens appear to be rifampicin-resistant in these samples, since methanogenesis from H_2/CO_2 or acetate was not inhibited by rifampicin. Methanogenesis from endogenous substrates was only mildly inhibited by rifampicin, indicating that the organisms converting organic material in the peat to methanogenic precursors, most likely members of the *Bacteria*, were not as sensitive to rifampicin as were the acetogens. Interestingly, it was recently shown that certain spirochetes, a bacterial group known to be resistant to rifampicin (Stanton and Canale-Parola 1979), are capable of acetogenesis (Leadbetter et al. 1999). While we observed spirochete-like cells in some samples from McLean Bog incubated with yeast extract and rifampicin, we did not observe them in samples incubated with H_2/CO_2 (data not presented).

Other studies have used selective inhibition by antibiotics as a tool in attempts to isolate or enrich for methanogens (Drake et al. 1996; Williams and Crawford 1985), but antibiotics have been used less often as a tool for studying methanogenic processes. Sanz et al. (1996) in their study of antibiotic effects in anaerobic digestion processes, found that rifampicin inhibited the breakdown of C3 and C4 fatty acids by bacteria, without significantly affecting the utilization of acetate, presumably carried out by aceticlastic methanogens. It is not clear to what extent that the phenomenon of acetogenesis interfering with methanogenesis is a problem in other acidic peat samples to which H_2/CO_2 was added. Acetogenesis from H_2/CO_2 at rates comparable to methanogenesis was also observed by Horn et al. (2003) in acidic peat samples from Germany. Svensson (1984) did not detect significant stimulation of methanogenesis by H_2/CO_2 additions in Swedish peat samples, although acetate concentrations were not measured in those studies. Williams and Crawford (1985) detected a four-fold stimulation of CH_4 production in Minnesota peat samples, indicating a lack of acetogenesis-related inhibition, but detailed time courses for methanogenesis and measurements of acetate were not presented. We obtained similar results to those from McLean Bog using soil slurries prepared from the acidic Chicago Bog located a few km from McLean Bog: buildup of acetic acid in samples incubated with H_2/CO_2, and rifampicin addition inhibiting acetic acid buildup and allowing sustained methanogenesis from H_2/CO_2. Thus, inhibition of methanogenesis from added H_2/CO_2 by acetic acid accumulation may occur in other acidic peat bog systems and rifampicin may be a useful tool to inhibit acetogenesis in those systems.

Physiology of Hydrogenotrophic Methanogens in McLean Bog

Despite the fact that many freshwater soils remain cool for most of the year, many studies have found the temperature optimum for methanogenesis to be close to 35°C, as reviewed by Zinder (1993). McLean Bog soils also revealed a temperature optimum for endogenous methanogenesis near 37°C, indicating that methanogenesis is severely temperature limited under field conditions. In the presence of H_2/CO_2, the temperature optimum appeared closer to 33°C in the absence of rifampicin. However the large production of acetic acid at higher temperatures ($\geq$33°C) clearly interfered with methanogenesis, since there was much greater methanogenesis from H_2/CO_2 in the presence of rifampicin with an optimum near 37°C under those conditions, similar to the optimum for methanogenesis in most other temperate systems.

Attempts to isolate H_2/CO_2 utilizing methanogens that grow at pH values near the *in situ* pH values of acidic peat bogs have

failed. The most acid tolerant methanogenic isolate described thus far is *Methanobrevibacter acidurans* (Savant et al. 2002), which was isolated from a low-pH anaerobic digestor and can grow at pH 5.0. Several pure culture strains of *Methanosarcina* are able to tolerate a minimum pH value of 4.3, but only in the presence of methanol and H_2 (Maestrojuan and Boone 1991). Organisms isolated from peat bogs have typically shown growth at pH values considerably higher than the *in situ* pH (Williams and Crawford 1985; Horn et al. 2003). Methanogenesis from H_2/CO_2 in McLean Bog soil slurries incubated in the presence of rifampicin showed a clear optimum near pH 5, indicating that methanogenic populations are adapted to acidic conditions and are not just acid tolerant. H_2 consumption in Crystal Bog in Wisconsin (pH 4.9) was shown to have an optimum for H_2 uptake near pH 5.6 (Goodwin and Zeikus 1987), although both methanogens and acetogens could be contributing to this process. Recently, enrichment cultures capable of growth near pH 4.9 were cultured from acidic bogs (Sizova et al. 2003) further supporting the existence of acidophilic methanogens. It is notable that endogenous methanogenesis showed a nearly flat response to environmental pH except for some inhibition at pH 3.4. Methanogens in McLean Bog soil appear to have a pH optimum near 5, however endogenous methanogenesis did not show a clear pH optimum. Thus, we speculate that endogenous rates of methanogenesis are limited by the production of methanogenic substrates, and that processes leading to those substrates, especially hydrolysis of plant material, are not sensitive to pH in the range tested.

The ionic strength of acidic peat bog water is often extremely low. Indeed, McLean Bog porewater samples taken while we performed these studies showed Na^+ and K^+ concentrations near 2 μM and 10 μM respectively (M. Dettling and J. Yavitt, personal communication). However most methanogens require 5 mM Na^+ or greater (Jarrell and Kalmokoff 1988) because of a sodium-dependent step in their metabolism (Deppenmeier et al. 1999). We found that both NaCl and KCl at concentrations as low as 2 mM inhibited methanogenesis from H_2/CO_2 in McLean Bog soil slurries during the first 19 days of incubation although eventually CH_4 production at concentrations up to 10 mM of either salt nearly equaled CH_4 production at low salt. It is not clear whether it was primarily the cations or the chloride ion that was responsible for the inhibition in these cases. However, we can conclude from these studies that the methanogens in McLean Bog are adapted to low ionic strength conditions for growth.

Using rifampicin to inhibit acetogenesis has allowed us to examine the effects of temperature, pH, and ionic composition on microbial populations actively converting H_2/CO_2 to CH_4, and on methanogenic populations that were actively growing, as demonstrated by the increasing rates of methanogenesis. This approach can be used to further examine other conditions for their ability to support growth of hydrogenotrophic methanogens.

Methanogenesis from Acetate

Our results show that it was possible to stimulate methanogenesis from acetate in McLean Bog soil slurries using concentrations of acetate near 1 mM and that higher concentrations were less stimulatory. It is unclear why acetate-utilizing methanogens were not stimulated in other studies of acidic bogs, but in most of those studies, acetate concentrations of 5–10 mM were used. Acetic acid and other fatty acids are known to be toxic at low pH (Russell 1991). At pH values below 6.0, a greater fraction of total acetate will be present as acetic acid ($pK = 4.7$) which can permeate cell membranes causing acidification of the cell interior or anion buildup (Russell 1991). CH_4 production by rumen methanogens has been shown to decrease dramatically with pH in the presence of high concentrations of acetate (100 mM), or at a single pH value (6.0), to decrease with increasing acetate concentrations (Van Kessel and Russell 1996). Thus, while acetate may be utilized at it's natural concentrations in the micromolar range, doses of 5–10 mM are likely to be inhibitory at low pH.

In the presence of both H_2 and 1 mM acetate, not only was CH_4 production lower, but acetate utilization was also low, indicating that the presence of 1 mM acetate may have inhibited hydrogenotrophic methanogenesis, and/or the presence of H_2 may have inhibited aceticlastic methanogenesis. Both the former (Conrad et al. 1987) and the latter (Baresi et al. 1978; Boone 1982; Ferguson and Mah 1983) phenomena have been observed and reported in the literature.

McLean Bog soil apparently has only minor amounts of electron acceptors other than CO_2, since the CO_2:CH_4 production ratio was near 1:1, the ratio expected for complete methanogenic conversion of glucose to CH_4 and CO_2 under ideal conditions. In comparison, the Alaskan peats studied by Duddleston et al. (2002), had CO_2:CH_4 ratios up to 530:1 indicating the presence of large amounts of electron acceptors other than CO_2. In that study as well as others, acetate was found to accumulate seasonally (Duddleston and others 2002; Hines et al. 2001; Shannon and White 1996) followed by oxidation, and it has been proposed that aceticlastic methanogenesis may be absent or inhibited at these sites. The low temperature and presence of oxidants at these sites may be factors limiting methanogenesis from acetate.

In the current study, the stimulation of CH_4 production by low concentrations of acetate (1 and 2 mM) was observed in less than two weeks. This provides strong evidence that a microbial population capable of utilizing acetate was present in the soil community. While it is likely that this population consists of aceticlastic methanogens, especially since the activity was resistant to rifampicin, it is also possible that a two-membered consortium consisting of an acetate-oxidizing organism coupled to a H_2/CO_2-utilizing methanogen (Zinder 1994) is responsible for the activity we observed.

The only genera known to utilize acetate, *Methanosarcina* spp. and *Methanosaeta* spp., are members of the order *Methanosarcinales*. Molecular studies have indicated the presence of

16S rRNA gene sequences in the order *Methanosarcinales* in northern peatland ecosystems (Hales et al. 1996; Edwards et al. 1998; Nercessian et al. 1999; Galand et al. 2002), but none that clustered within *Methanosarcina* and *Methanosaeta*, and since this order includes methylotrophic and H_2/CO_2 utilizing methanogens (Garcia et al. 2000) the roles of organisms harboring those sequences is not clear. Recently, Basiliko et al. (2003) found sequences directly related to both genera in McLean Bog soil, although they represented a relatively small fraction of the total methanogenic clones (13/86). These results are consistent with methanogenesis from acetate occurring in McLean Bog soil.

SUMMARY

This study has demonstrated the utility of rifampicin as a selective inhibitor of acetogenesis to allow study of the process of methanogenesis from H_2/CO_2 in McLean Bog soil. The results of this study indicate that a mildly acidophilic (pH optimum near 5) community of hydrogenotrophic methanogens is present in acidic peat soil. We have also demonstrated that methanogenesis from acetate can be stimulated by low concentrations of acetate.

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