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Shift from Acetoclastic to H_2-Dependent Methanogenesis in a West Siberian Peat Bog at Low pH Values and Isolation of an Acidophilic *Methanobacterium* Strain[▽]

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Methane production and archaeal community composition were studied in samples from an acidic peat bog incubated at different temperatures and pH values. H_2-dependent methanogenesis increased strongly at the lowest pH, 3.8, and *Methanobacteriaceae* became important except for *Methanomicrobiaceae* and *Methanosarcinaceae*. An acidophilic and psychrotolerant *Methanobacterium* sp. was isolated using H_2-plus-CO_2-supplemented medium at pH 4.5.

Wetlands are considered to be the largest natural sources of atmospheric CH_4. Acidic peatlands are the most typical type of northern wetlands and are responsible for about 60% of total wetland emission (26). Peat bogs are characterized by low concentrations of mineral salts, low pH, and low temperature. Various factors have been identified as important controls of methanogenesis, with temperature, water table level, and content of organic matter being the most notable ones (4, 9, 12, 27, 32, 35, 38). However, there is little information on how pH influences the composition and functioning of the methanogenic community.

In peatlands, hydrogentrophic methanogenesis is the predominant pathway of CH_4 formation, especially in deeper layers, accounting for 50 to 100% of total CH_4 production (12, 18, 28, 40). However, acetoclastic methanogenesis has also been found to play an important role in acidic bogs (1, 2, 21). Relatively little is known about the archaeal communities inhabiting peatlands. Recent studies of different wetlands revealed the presence of methanogens belonging to the *Methanomicrobiaceae*, *Methanobacteriaceae*, *Methanococcaceae*, *Methanosarcinaceae*, and *Methanosaetaceae* as well as new archaeal lineages within the *Euryarchaeota* (3, 7, 8, 13, 17, 34, 36, 37). However, the role of the methanogenic populations in CH_4 production under different in situ conditions is unknown.

Attempts to isolate acidophilic or acidotolerant methanogens in pure culture have failed until very recently, although acid-tolerant enrichment cultures have been reported (6, 11, 15, 34, 41). It was only after we finished our study that Bräuer and coworkers reported the successful isolation of a moderately acidophilic methanogen belonging to the *Methanomicrobiales* order (5).

The aim of the present study was to investigate how high acidity and low temperature can affect the functioning of the methanogenic community, its structure, and, hence, methane production in a peat bog, as well as to obtain a pure culture of an acidophilic methanogen. We used the same bog samples as in our previous study (21).

We obtained peat samples from Bakchar Bog, which is located in West Siberia (57°N, 83°E). The main unforested part of the bog is covered with continuous *Sphagnum* moss and patches of vascular plants (*Carex*, *Menyanthes*, and *Equisetum* spp.). The detailed location of the bog and structure of the plant community have been described earlier (21, 29). The samples were taken in July 1999 at a depth of 30 to 50 cm below the water table from the site covered with *Equisetum*. The peat pH values were in a range of 3.5 to 5.5, with pH 4.8 at the sampling site. The bog surface temperature varied during the summer season from 5 to 35°C but never exceeded 15°C at the depth of sampling.

The samples were transferred into sterile plastic bottles, transported to the laboratory, and then stored at 4°C for up to 3 months before analysis. The peat samples were handled anoxically under a N_2 atmosphere as described before (21). An aliquot (15 ml) of the samples was placed into a sterile serum bottle (60 ml), gassed with N_2, closed with a sterile black butyl rubber stopper, and then incubated in darkness without shaking. The samples were adjusted to different pH values by the addition of either 1 M HCl or 1 M NaOH. The total incubation time depended on temperature and pH and varied from 20 to 360 days at 25°C (pH 4.8) and 4°C (pH 3.8), respectively. The production rates of CH_4 were determined as described previously (21). Experiments were done in duplicate or triplicate. The $\delta^{13}C$ of CH_4 and CO_2 was determined at the end of the incubations as described before (21).

Radioactive tracer experiments were done similarly to those described above but with Hungate tubes (20 ml). The tubes

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TABLE 1. Acetate turnover and methanogenesis in peat samples incubated at different temperatures and pHs[a]

Temp (°C)	pH	Maximum fraction of acetate converted to CH_4 and CO_2	Acetate turnover time (h)	Acetate turnover rate (nmol liter^{-1} h^{-1})	Respiratory index	CH_4 production due to acetate turnover (nmol liter^{-1} h^{-1})	Total CH_4 production (nmol liter^{-1} h^{-1})	Fraction of acetate-dependent CH_4 production	Fraction of CO_2-dependent CH_4 production
25	6.0	0.83	27.3 ± 8.3	44 ± 12	0.12 ± 0.04	39 ± 12	62 ± 17	0.63 ± 0.28	0.38 ± 0.09
	4.8[b]	0.93	43.8 ± 12.2	26 ± 8	0.10 ± 0.02	24 ± 9	38 ± 10	0.63 ± 0.29	0.38 ± 0.07
	3.8	0.78	177 ± 48	13 ± 7	0.06 ± 0.02	12 ± 5	18 ± 6	0.68 ± 0.32	0.36 ± 0.12
15	6.0	0.87	43.9 ± 6.9	24 ± 7	0.07 ± 0.03	22 ± 7	33 ± 9	0.68 ± 0.26	0.37 ± 0.11
	4.8[b]	0.77	46.3 ± 8.5	18 ± 5	0.07 ± 0.02	17 ± 6	26 ± 7	0.65 ± 0.29	0.43 ± 0.09
	3.8	0.93	862 ± 212	2.0 ± 0.6	0.21 ± 0.08	1.7 ± 0.9	14 ± 5	0.12 ± 0.07	0.92 ± 0.08
4	6.0	0.85	131.7 ± 43.5	1.8 ± 0.7	0.31 ± 0.11	1.2 ± 0.5	1.8 ± 0.6	0.68 ± 0.27	0.35 ± 0.09
	4.8[b]	0.93	309.5 ± 57.5	3 ± 1	0.47 ± 0.12	1.5 ± 0.7	2.5 ± 0.8	0.60 ± 0.34	0.35 ± 0.06
	3.8	0.72	1,460 ± 380	0.09 ± 0.03	0.97 ± 0.05	$2.7 \times 10^{-3} \pm 0.8 \times 10^{-3}$	0.06 ± 0.02	0.04 ± 0.02	0.98 ± 0.05

[a] The data shown represent means ± standard deviations.
[b] The data in this row were taken from the study by Kotsyurbenko et al. (21) for comparison.

were filled with 10-ml peat samples, evacuated, gassed with N_2, and closed with rubber stoppers. The tubes were incubated at different temperatures, and the concentrations of intermediates were monitored until they reached steady state (21). The experiments were initiated by injecting 0.5 ml of carrier-free ^{14}C-labeled Na-[2-^{14}C]acetate (1×10^6 dpm, 53 mCi mmol^{-1}; Amersham) or Na$H^{14}CO_3$ (1×10^6 dpm, 54 mCi mmol^{-1}) into each tube. Radioactive and nonradioactive CH_4 and CO_2 were analyzed in samples taken from the headspace. Acetate turnover rates, the respiratory index [$^{14}CO_2/(^{14}CO_2 + {}^{14}CH_4)$] for degradation of [2-^{14}C]acetate, the fraction of acetate-dependent CH_4 production, and the fraction of CH_4 produced from H_2 and CO_2 were determined as described before (21). All experiments were done in triplicate.

The sampling for DNA extraction and terminal restriction fragment length polymorphism (T-RFLP) analysis of archaeal 16S rRNA genes was carried out using duplicate peat samples taken at the end of incubations (using all except those incubated at 4°C), and the samples were stored at −20°C. The procedure was described in detail before (21).

Methanogenic archaea were enriched in a low-ionic-strength liquid medium containing (in milligrams per liter of distilled water) NH_4Cl (16.5), $MgCl_2 \cdot 6H_2O$ (25), $CaCl_2$ (8.4), KCl (16.4), KH_2PO_4 (16.8), $NaHCO_3$ (100), Na-acetate (30), yeast extract (20), and resazurin (10), as an indicator of anoxic conditions. The medium was supplemented with 0.5% (vol/vol) of a vitamin solution (42), trace element stock solutions (21), and H_2 plus CO_2 (80:20, vol:vol) as substrate. The medium was buffered with morpholinoethanesulfonic acid (10 mM) and reduced with titanium citrate (0.5 mM). The initial pH of the medium was adjusted to 4.5 using 0.1 N HCl. Peat samples from different research sites were used as inocula. As soon as visual turbidity developed, CH_4 production was measured and an aliquot was transferred to fresh medium of the same composition. The same medium was used to assay the pH and temperature dependence of the cultures.

The procedure used for DNA extraction from methanogenic cultures has been described previously (16). Primers Ar12f (25) and Ar1542r (19) were used to amplify the nearly complete 16S rRNA gene of the isolated methanogens. Primer combinations ME1/ME2 and MCRf/MCRr were used to amplify *mcrA* fragments. The PCR mixtures and the other thermal cycling parameters were as previously described (24, 33) but with 32 cycles and an annealing temperature of 50°C for the amplification of the 16S rRNA gene. PCR products were purified with a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Sequencing of PCR products was performed using a BigDye Terminator cycle sequencing kit on an ABI 377A DNA sequencer (Applied Biosystems, Foster City, CA). Sequences were assembled using the Lasergene software package (DNAStar, Madison, WI). Phylogenetic analysis of the sequence data was made using the ARB software package and its databases (23) as previously described (24). The 16S rRNA gene and *mcrA* sequences obtained in this study have been designated in the GenBank database under the accession numbers DQ677518 and DQ677519, respectively.

Rates of CH_4 production, acetate turnover, and fractions of the relative contribution of acetoclastic and hydrogenotrophic methanogenesis were measured in peat samples incubated at different pH values and temperatures (Table 1). Rates of CH_4 production decreased with decreasing pH values and temperatures. Under most incubation conditions, acetoclastic and hydrogenotrophic methanogenesis contributed to total CH_4 production at ratios of about 65 and 35%, respectively, as expected from our previous study of the same peat bog (21), the results of which are included in Table 1. However, hydrogenotrophic methanogenesis became the dominant path of CH_4 formation (>92%) when the peat samples were incubated at pH 3.8 and at low temperatures (4 or 15°C) (Table 1). Note that these are realistic in situ conditions. The relative increase of the role of H_2-dependent methanogenesis was confirmed by the $\delta^{13}C$ of produced CH_4, which became increasingly depleted in ^{13}C with decreasing pH values (Fig. 1). The ratio of $\delta^{13}CO_2$ to $\delta^{13}CH_4$ gives an approximation of the apparent fractionation between CO_2 and CH_4, which can be calculated as follows: $(\delta^{13}CO_2 + 1{,}000)/(\delta^{13}CH_4 + 1{,}000)$. The ratio of $\delta^{13}CO_2$ to $\delta^{13}CH_4$ increased from 1.053 at pH 6 to up to 1.072 at pH 3.8, confirming a relative increase of hydrogenotrophic methanogenesis at low pH values (18, 39).

The archaeal community structure in the samples incubated at different temperatures and pH values was determined by T-RFLP analysis. Most of the incubations exhibited the same T-RFLP patterns that had been reported in our previous study

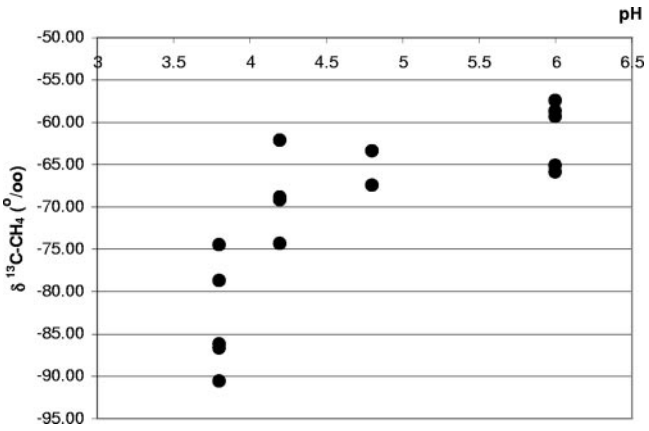

FIG. 1. $\delta^{13}CH_4$ values measured in peat samples (*Equisetum* site) adjusted to different pH values and incubated at 4°C.

(21), i.e., the predominance of members of the methanogenic families *Methanosarcinaceae* (T-RF, 185 bp) and *Methanomicrobiaceae* (T-RF, 392 bp). Incubation at 15°C versus 25°C affected the T-RFLP patterns only a little, but pH values showed a marked effect (Fig. 2). Notably, at the lowest pH, 3.8, at 15°C, we observed the appearance of a substantial T-RF of 91 bp which represents members of *Methanobacteriaceae*. Also, at pH 6.0 and 15°C, a novel T-RF (257 bp) appeared which, however, has not yet been identified.

The stimulation of hydrogenotrophic methanogenesis at low pH values prompted us to attempt the isolation of acidophilic methanogens. Three methanogenic strains, MB2, MB3, and MB4, were enriched in a low-ionic-strength medium supplemented with H_2 plus CO_2 at pH 4.5 and subsequently isolated as pure cultures after repeated transfers. All three strains were from different samples collected at different peat bog research sites covered with *Carex*, *Equisetum*, and *Menyanthes* species (21). The organisms have similar morphologies and are long rods, growing both as single cells and in associations (Fig. 3). The cells are often irregularly twisted. All strains had identical 16S rRNA gene sequences and thus belong to the same species. Phylogenetic analysis revealed that the new strains represent a new species within the genus *Methanobacterium* (Fig. 4); the closest relative was *Methanobacterium congolense* with 97% sequence identity. The McrA amino acid sequence of strain MB4 was most similar to that of *Methanothermobacter thermautotrophicus* X07794 as the closest cultivated relative (90% sequence similarity). The isolated strains grew only on

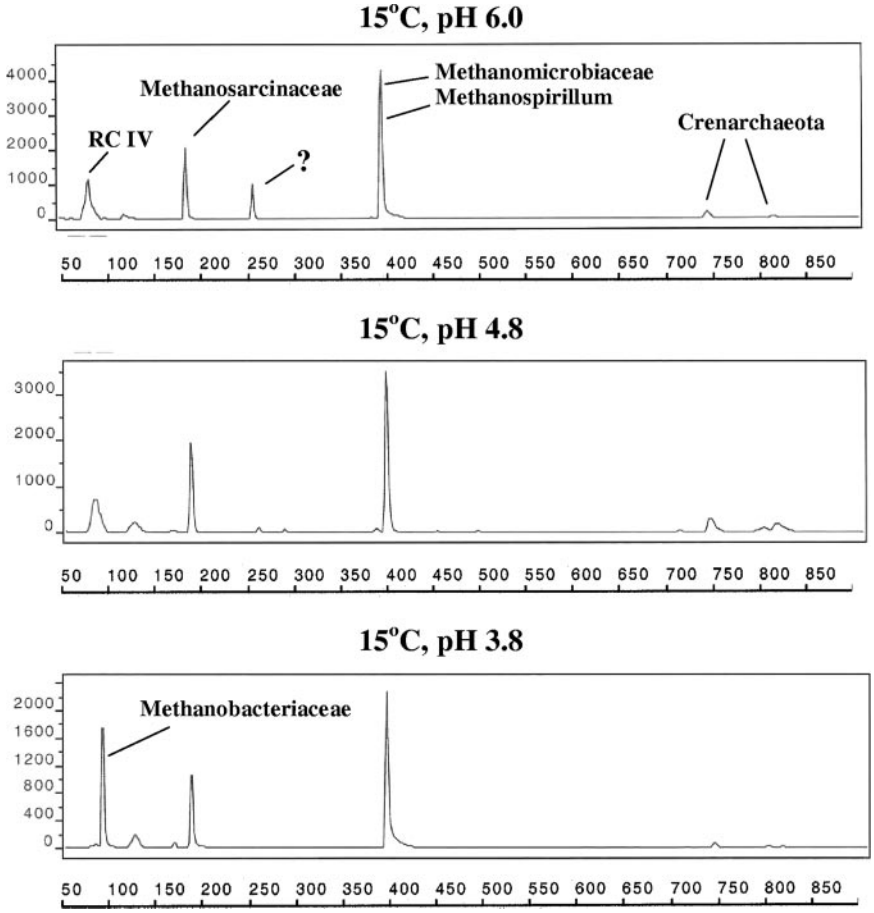

FIG. 2. T-RFLP patterns of archaeal small-subunit rRNA gene fragments amplified from DNA extracts obtained from peat samples (*Equisetum* site) incubated at 15°C and different pH values. The *x* axis shows the lengths (bp) of the T-RFs. The *y* axis shows the intensities of the bands in arbitrary units. RC IV, a crenarchaeotal lineage detected on rice roots.

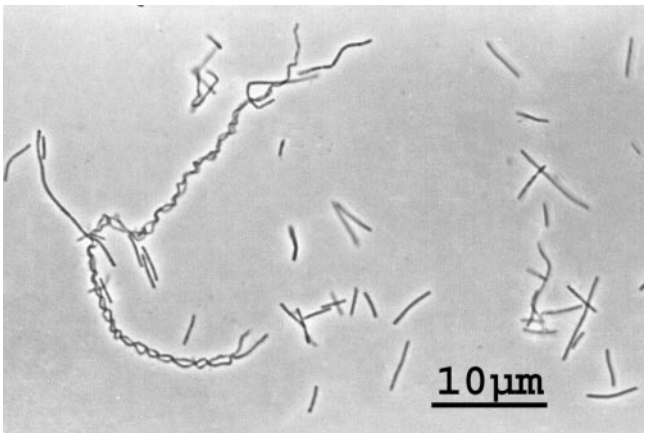

FIG. 3. Phase-contrast microscopy of a methanogenic archaeon, strain MB4, isolated from Bakchar Bog.

H_2-CO_2 or formate as the energy source when a mineral medium that contained tiny amounts of yeast extract (0.002%) and acetate (0.003%) as additional carbon sources was used. The growth rate was very low (on the order of days), as the cultures were fully grown after about 2 weeks. Most notably, the cultures grew at low pH values and low temperatures and produced CH_4 over a range of pH values (3.8 to 6.0) and temperatures (5 to 30°C), with maximal rates at pH 5.0 to 5.5 and 25 to 30°C.

Our study shows that pH is an important factor influencing not only the rate of methanogenesis but also the CH_4 production pathway and the methanogenic archaeal community. A shift from acetoclastic to H_2-dependent methanogenesis occurred between pH 4.7 and 3.8 (Table 1). The predominance of hydrogenotrophic methanogenesis has also been observed in other acidic peatlands (10, 22, 27) and is possibly explained by the existence of acetic acid in its free form at a pH of <4.7. Acetic acid can pass through the cell membrane and act as a decoupler of the proton motive force. However, this is probably not the only explanation, since acetoclastic methanogenesis has occasionally been observed in acidic peatlands (1, 2, 21). Furthermore, hydrogenotrophic methanogens and other microorganisms are also exposed to the acetic acid present in their environment and thus must have mechanisms to compensate for decoupling by acetic acid.

A strong signal of a T-RF of 91 bp, characteristic for *Methanobacteriaceae*, was detected in samples at pH 3.8 and 15°C, indicating that the relative proportion of this methanogenic group increased under these conditions. Importantly, all species of the *Methanobacteriaceae* family grow with H_2 plus CO_2 as the substrate to produce CH_4. Hence, we conclude that *Methanobacteriaceae* became prevalent at low pH values, thus explaining the predominance of H_2-dependent CH_4 production observed in the isotopic experiments.

This conclusion is in agreement with the successful enrichment and isolation of a *Methanobacterium* sp. capable of growth with H_2 and CO_2 as a sole source of carbon and energy. The unique feature of this organism is its ability to grow at a pH as low as 3.8. No known methanogenic archaeon has such a low minimal pH for growth (5, 14, 31). The isolation of virtually the same *Methanobacterium* sp. from different field sites indicates its widespread distribution and possible ecological importance in Bakchar Bog. The phylogenetic affiliation of the isolated methanogen to a new species within the genus *Methanobacterium* indicates that members of this methanogenic genus may have a large capacity for adaptation to extreme pH conditions. It should be noted that the genus *Methanobacterium* already contains two alkaliphilic and one moderate acidophilic species (20, 30, 43), thus collectively having the widest growth range concerning pH (from 3.8 to 9.9). However, there are also reports on enrichments (6, 34) of acidiphilic archaea belonging to novel archaeal clusters. One of these was recently isolated (5). Like our isolate, this archaeon is also a hydrogenotrophic methanogen but belongs to a novel cluster within the order *Methanomicrobiales*.

Our study expands our knowledge about the mechanisms of CH_4 formation and the diversity of methanogenic archaea in acidic peat bogs.

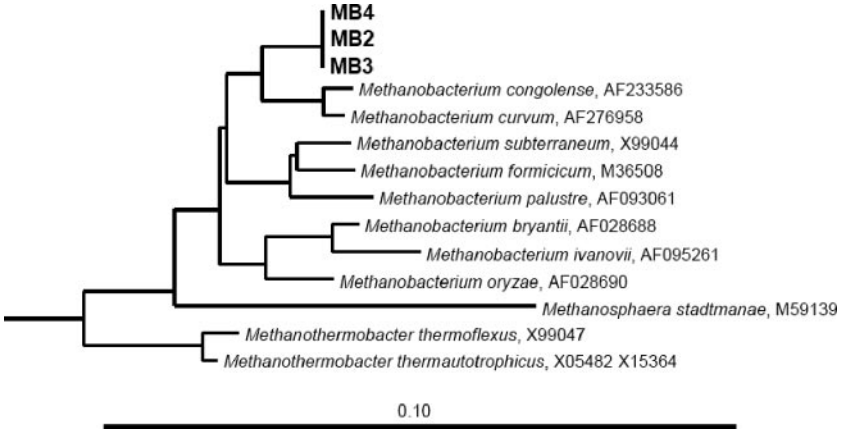

FIG. 4. Phylogenetic positions of strain MB4 and two further new strains of acidophilic methanogens. The 16S rRNA gene tree was constructed from a distance matrix using neighbor-joining analysis. The scale bar indicates 0.1 base pair changes per nucleotide sequence position.

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