Vertical Profiles Of Methanogenesis And Methanogens In Two Contrasting Acidic Peatlands In Central New York State, USA

By: Hinsby Cadillo-Quiroz, Suzanna Bräuer, Erika Yashiro, Christine Sun, Joseph Yavitt and Stephen Zinder

Abstract

Northern acidic peatlands are important sources of atmospheric methane, yet the methanogens in them are poorly characterized. We examined methanogenic activities and methanogen populations at different depths in two peatlands, McLean bog (MB) and Chicago bog (CB). Both have acidic (pH 3.5–4.5) peat soils, but the pH of the deeper layers of CB is near-neutral, reflecting its previous existence as a neutral-pH fen. Acetotrophic and hydrogenotrophic methanogenesis could be stimulated in upper samples from both bogs, and phylotypes of methanogens using H2/CO2 (Methanomicrobiales) or acetate (Methanosarcinales) were identified in 16S rRNA gene clone libraries and by terminal restriction fragment length polymorphism (T-RFLP) analyses using a novel primer/restriction enzyme set that we developed. Particularly dominant in the upper layers was a clade in the Methanomicrobiales, called E2 here and the R10 or fen group elsewhere, estimated by quantitative polymerase chain reaction to be present at ~108 cells per gram of dry peat. Methanogenic activity was considerably lower in deeper samples from both bogs. The methanogen populations detected by T-RFLP in deeper portions of MB were mainly E2 and the uncultured euryarchaeal rice cluster (RC)-II group, whereas populations in the less acidic CB deep layers were considerably different, and included a Methanomicrobiales clade we call E1-E1¢, as well as RC-I, RC-II, marine benthic group D, and a new cluster that we call the subaqueous cluster. E2 was barely detectable in the deeper samples from CB, further evidence for the associations of most organisms in this group with acidic habitats.
Introduction

Although peatlands in northern latitudes account for 5–10% of the methane emitted into the Earth’s atmosphere (Whalen, 2005), little is known about the composition and abundance of the microorganisms responsible for methane production in these systems. A better understanding of these methanogens is needed to predict how emissions of atmospheric methane from northern peatlands will respond to global environmental changes (Wieder and Yavitt, 1994; Brown, 1998).

Northern peatlands include a wide range of ecosystems, each with a characteristic peat soil derived from partially decaying plant material and with little or no rock-derived minerals. At one end are the most acidic systems, called bogs, characterized by low mineral nutrient concentrations and dominated by Sphagnum mosses and a few ericaceous shrub species (Crum, 1992). Increasing pH is associated with less Sphagnum and dominance by Carex sedges and graminoid plants, resulting in near-neutral-pH systems, called fens (Crum, 1992). A characteristic of many northern peatlands is that the plant species composition of surface vegetation can be quite different from the remains of plants in the peat that dominated in the past (Barber, 1981).

Numerous methanogens have been cultivated and characterized from diverse habitats (Garcia, 1990), but initial attempts at cultivation of indigenous methanogens from bogs were unsuccessful (Williams and Crawford, 1985; Goodwin and Zeikus, 1987). However, the application of molecular techniques based on 16S rRNA and mcrA (encoding a subunit of the methylreductase involved in methanogenesis) gene sequences has revealed a diversity of methanogens as well as related Euryarchaeota in bogs (Hales et al., 1996; Basiliko et al., 2003; Kotsyurbenko et al., 2004; Galand et al., 2005). At this point in time, none of the sequences in acidic peat bogs match those of isolated methanogens, although related sequences have recently been detected in enrichment cultures (Horn et al., 2003; Sizova et al., 2003).

In this study, we examined the depth (vertical) distribution of methanogenic activity and methanogenic Archaea in two northern peatlands in New York State. One, known locally as ‘Chicago bog’ (CB) has acidic bog plants growing over what was once a neutral-pH fen so that lower layers are less acidic. The other site, known locally as
‘McLean bog’ (MB), was acidic throughout the profile. We demonstrate here that terminal restriction fragment length polymorphism (T-RFLP) analyses of methanogen populations of all samples from MB showed dominance of a clade in the *Methanomicrobiales* associated with acidic habitats, whereas methanogen populations in lower, more neutral, samples from CB differed considerably from the acidic upper layers.

**Results**

**Physicochemical characteristics of vertical profiles of peat cores**

Stratigraphy of the cores from both sites indicated a 10 cm cap of light brown coloured sphagnum peat underlain by a more degraded softer in consistency, dark brown peat at 15–20 cm. At depths below 40 cm, the peat was highly decomposed and darker in colour indicating highly reduced conditions. The peat in deeper layers (40 and 65 cm depth) of CB had decreasing amounts of *Sphagnum* remains and was herbaceously derived, i.e. mostly *Carex* rhizomes, whereas deeper layers in MB consisted mostly of sphagnum remains. The pH in deeper layers of MB remained low, but in CB it increased to 4.9 and 5.7, respectively, at 40 and 65 cm depths (Table 1). The dry matter content was greater at lower depths in both cores, with a greater increase in the CB core.

**CH₄ production by core samples**

Rates of CH₄ production from endogenous substrates in samples from the 40 cm depth were less than 15% of those in the 15–20 cm depth (Fig. 1), and even slower rates occurred in samples from 65 cm depth (data not presented). Added acetate had no effect on rates of CH₄ production until approximately 20 days of incubation when CH₄ production in the acetate-amended samples from upper layers of MB and the 15 cm layer of CB (Fig. 1A and B) increased relative to unamended samples. Added acetate did not stimulate CH₄ production in the 10 cm layer of CB or in the 40 cm (Fig. 1C) and 65 cm samples from either bog during the entire 63-day incubation time.

A previous report at the same study sites showed that the build-up of acetic acid inhibited CH₄ production in peat amended with H₂/CO₂, and that the addition of rifampicin avoided this interference by acetogenic organisms (Bräuer et al., 2004). CH₄ production in upper layers by samples amended with H₂/CO₂ and rifampicin was significantly greater than the rifampicin-only controls. Rates of methanogenesis increased during the first days of incubation for both sites (Fig. 1A and B), indicative of methanogen growth. In deeper layers, the CH₄ production in H₂/CO₂-amended samples started to increase above the unamended control only after more than 20–30 days of incubation (Fig. 1C). Samples from 65 cm presented similar, although slower, response compared with those from 40 cm (not shown).

**16S rRNA phylogenetic diversity**

In order to study methanogen community composition we used cloning and sequence analyses of the 16S rRNA gene. We utilized the 1AF-1100R set of primers (Hales et al., 1996) because our initial studies on clone libraries developed with different archaeal primer sets indicated a good coverage of methanogenic *Archaea* in our study sites (H. Cadillo-Quiroz, unpublished). Comparison of reported sequences for shallow layers of MB (Basiliko et al., 2003) and sequences from our clone libraries for CB shallow layers showed a similar phylogenetic composition of methanogenic archaeal groups (Fig. 2). Sequences were associated with *Methanosarcinaceae* and *Methanosaetaceae* families, and the *Methanomicrobiales*. Uncultured groups such us rice cluster (RC)-I and -II were present in both bogs. Clones with phylotypes associated with marine benthic group D (MBD) (Vetriani et al., 1999) were recovered only from the deeper layers of CB. Additionally, we observed a cluster of sequences that were not positioned within the other previously described groups. Those sequences formed a distinct cluster related mainly to environmental phylotypes retrieved from immersed or subaqueous environments like lake sediments, Siberian deep wells, deep-sea chimneys and a nearby neutral fen (J. Yavitt, unpublished). This cluster of sequences, which we have named the subaqueous-

<table>
<thead>
<tr>
<th>Depth (cm)</th>
<th>Chicago bog</th>
<th>McLean bog</th>
<th>Chicago bog</th>
<th>McLean bog</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3.92 ± 0.07</td>
<td>3.58 ± 0.42</td>
<td>5.42 ± 0.09</td>
<td>4.22 ± 1.08</td>
</tr>
<tr>
<td>15/20</td>
<td>4.06 ± 0.06</td>
<td>4.03 ± 0.06</td>
<td>5.25 ± 0.06</td>
<td>7.88 ± 0.21</td>
</tr>
<tr>
<td>40</td>
<td>4.87 ± 0.13</td>
<td>4.19 ± 0.02</td>
<td>12.12 ± 0.11</td>
<td>9.12 ± 0.4</td>
</tr>
<tr>
<td>65</td>
<td>5.71 ± 0.12</td>
<td>4.21 ± 0.04</td>
<td>11.91 ± 0.21</td>
<td>7.5 ± 0.23</td>
</tr>
</tbody>
</table>

Peat samples were taken at the indicated depths; mean ± SD; n = 3.
ous cluster (SC), represents a new group of uncharacterized euryarchaeota.

**T-RFLP analysis of community structure**
We used T-RFLP analysis to obtain a description of the dominant groups in the archaeal community accessible through our primer set. The 1Af-1100R set of primers have not been used previously in T-RFLP analysis, and in this study we defined T-RFLP parameters that minimized ambiguous identification. A T-RFLP protocol using two restriction enzymes (HhaI and Sau96I) was designed by

![Fig. 1. Time-course for methane production by peat slurries from Chicago bog and McLean bog vertical profiles. (A) 10 cm deep, (B) 15/20 cm deep and (C) 40 cm deep incubated at approximately 23°C; mean ± SD; n = 3.](image)

![Fig. 2. Phylogenetic tree for archaeal 16S rRNA gene clones from McLean bog (MB) and Chicago bog (CB). Methanosarcinales: Methanosarcinaceae (MS); Methanosetaeaceae (MT); subaqueous cluster (SC); rice cluster-I (RC-I); rice cluster-II (RC-II); Methanomicrobiales: group E1, group E1', group E2 and group E2' (classification based on Basiliko *et al.*, 2003); marine benthic group D (MBD) (based on Vetriani *et al.*, 1999). Euryarchaeal and crenarchaeal nearly complete 16S rRNA gene sequences were used to construct tree with the neighbour joining and quartet PUZZLE methods. Tree topology was confirmed with maximum likelihood algorithm, and bootstrap values (100 trees) greater than 50 are indicated. GenBank accession numbers of added sequences are indicated. Clones from CB shallow and deeper layers are indicated by the initials CBs and CBd respectively. 6A correspond to methanogenic culture from MB (Bräuer *et al.*, 2006).](image)
performing multiple *in silico* sequence analyses of published sequences and our clone libraries, and confirming the predicted fragment size by amplifying and T-RFLP analysing clones. Table 2 presents the identity match and predicted terminal restriction fragment (T-RF) size from the *in silico* digestion of our sequences, and Fig. 3 shows two representative profiles and their corresponding peak identifications. We were able to resolve the groups A-F described by Basiliko and colleagues (2003) and in many cases obtain better resolution. For example, group B, equivalent to the *Methanosarcinaeacea*, could be resolved into three groups, and group E, equivalent to the *Methanomicrobiales*, could be resolved into two groups E1 and E2, which were not entirely monophyletic and were subdivided into E1, E1’, E2 and E2’ (Fig. 2). E1’ was only found in clone libraries of deeper layers of CB, and E2’ sequences were not recovered in the libraries and correspond to cultured *Methanomicrobiales* (Fig. 2). Of other groups, the SC cluster shared the same T-RF size with a subcluster of RC-I. According to our clone libraries, both would only occur in samples from deeper layers of CB.

Assessment of the vertical community structure in duplicate core samples from both study sites is summarized in Fig. 4. The community profiles from both sites were similar.

### Table 2. Predicted terminal restriction fragment (T-RF) length of archaeal 16S rRNA gene sequences from clone libraries and their phylogenetic affiliation.

<table>
<thead>
<tr>
<th>Phylogenetic association</th>
<th>T-RF length (bp)</th>
<th>Clone name</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Methanosarcinaeacea</em></td>
<td>271</td>
<td>MB13, MB09, CBs-a3H, CBs-b2A</td>
</tr>
<tr>
<td><em>Methanosoaetacea</em></td>
<td>88</td>
<td>MB04, MB18, CBs-a2H, CBs-b3C</td>
</tr>
<tr>
<td></td>
<td>118</td>
<td>CBd-4610G, CBd-366G</td>
</tr>
<tr>
<td></td>
<td>397</td>
<td>MB12</td>
</tr>
<tr>
<td>Subaqueous cluster</td>
<td>363</td>
<td>CBd-466G, CBd-461H, CBd-462E, CBd-465B</td>
</tr>
<tr>
<td>Rice cluster-I</td>
<td>230</td>
<td>MB14, CBs-b1D, CBs-b3B, CBs-c1F</td>
</tr>
<tr>
<td>Rice cluster-II</td>
<td>100</td>
<td>MB05, MB08, CBs-b2C, CBd-366D, CBd-464H, CBd-308E, CBd-361E</td>
</tr>
<tr>
<td><em>Methanomicrobiales</em> group E2</td>
<td>233</td>
<td>MB16, MB19, MB02b, MB 17, MB03, CBs-e2B, CBs-c2H, CBs-c1H, CBd-364F, CBd-471E, CBd-362C, 6A8</td>
</tr>
<tr>
<td><em>Methanomicrobiales</em> group E1</td>
<td>292</td>
<td>MB06, MB10, CBs-e2E, CBs-a1D, CBd-304B, CBd-472G</td>
</tr>
<tr>
<td><em>Methanomicrobiales</em> group E1'</td>
<td>292</td>
<td>LH 01, LH03, CBd-463F, CBd-472C, CBd-303D</td>
</tr>
<tr>
<td>Marine benthic group D</td>
<td>337</td>
<td>CBd-472B, CBd-305F, CBd-302C</td>
</tr>
</tbody>
</table>

Fragment size is predicted from 3’ end for fluorescently labelled reverse primer 1100R (Hales *et al.*, 1996).
The dominant T-RF in these layers was that of group Methanomicrobiales E2, in agreement with its high abundance in clone libraries (data not shown). Group Methanomicrobiales E1 was present as a smaller fraction and was greater in MB than in CB. Methanosarcinaceae, Methanosaetaceae and RC-II T-RFs were present in upper layers at both sites as a very small fraction of the community.

The community structure at 40 and 65 cm depth clearly changed compared with the upper layers (Fig. 4), but the differences not only corresponded with depth but also between sites. Chicago bog harboured a greater number of T-RF types than MB, where MBD, RC-I and SC were consistently observed at deep layers of CB only. Group E2 decreased in dominance at both sites. However, E2 changed from being the most dominant T-RF to a very small fraction of the community (close to the technique’s limit of detection) in CB; while in MB, E2 was still one of the major components in the profiles. Group E1/E1’ (Fig. 2) increased in CB, particularly at 65 cm where E1’ sequences were mainly recovered, and slightly decreased in MB. In addition, a unique Methanosaetaceae T-RF was present only at lower depths of CB and it had a greater abundance than the others found in higher layers particularly at 40 cm. The increase of the RC-II fraction at 40 cm depth was the only common finding between the two sites at their deeper layers.

Abundance estimation by quantitative polymerase chain reaction

We attempted to complement the T-RFLP’s qualitative information with a quantitative polymerase chain reaction (qPCR) approach focused on the abundance of total Archaea, and groups E2 and E1. Because of the limitations imposed by the close phylogenetic relationships of group E2 and E1, we could not design primers to quantify each group separately but instead developed a single primer set that quantifies both groups together but excludes E1’- and E2’-associated sequences. For total archaeal quantification, we used universal primers that have been successfully used in qPCR in other studies (Riley-Buckley, 2001). Both sets of primers were tested for specificity and coverage and the results are summarized in Table 3.

Results of qPCR (Fig. 5A) indicated that total archaeal numbers did not strongly decline with depth in both sites (10^8–10^9 normalized gene copy number per gram of dry peat). These results, although higher in order of magnitude as they are expressed per gram of dry instead of fresh peat, were similar to those previously obtained with fluorescent in situ hybridization (FISH) in a Siberian acidic peatland (Kotsyurbenko et al., 2004). Attempts to apply FISH to these peat samples were unsuccessful partly because of substantial autofluorescence of the peat material.

The quantification results for group E2-E1 (Fig. 5B) showed that E2 and E1 roughly constituted approximately 55% of total archaeal targets in the upper layers of both sites, with E2 as the main contributor as indicated by T-RFLP E2-E1 target decreased to a different degree at 40 and 65 cm at both sites: in CB it decreased by roughly 2.5 orders of magnitude (~0.15% of total Archaea), while in MB it decreased by 1.3 orders of magnitude (~2% of total Archaea).

Fig. 4. Normalized T-RFLP profiles of 16S rRNA gene fragments amplified from peat samples from vertical profile of Chicago bog (CB) and McLean bog (MB). Sampling depths are indicated in each set of profiles. Peaks matching its predicted group were arranged in the same order as in their phylogenetic tree, and the Methanosaetaceae peaks were merged into a single group for simplicity. The archaeal groups represented by distinctive T-RFs are: Methanosarcinaceae (MS), Methanosaetaceae (MT), rice cluster-I (RC-I), rice cluster-II (RC-II), marine benthic group D (MBD) and Methanomicrobiales: group E1 (E1), group -E1’ (E1’), group E2 (E2). Profiles were normalized to a total of 100 units. Mean ± SD; n = 3. The asterisk (*) indicates E1’ sequences were only observed in CB at 40 cm and 65 cm.
Discussion

McLean bog and CB are typical of acidic ombrotrophic (ombro = rain fed; Crum, 1992) bogs with low mineral content (Blodau, 2002). Upper layer at both sites shared similar physicochemical characteristics, but their deeper layers exhibited significant differences, particularly pH (Table 1) and peat composition. In MB and CB, methanogenic activity in lower layers using endogenous substrates was 1–1.5 orders of magnitude lower and there was little if any initial stimulation by addition of methanogenic substrates, indicating that the methanogenic populations were at low levels and had low activity.

Hydrogenotrophic and aceticlastic, i.e. H₂/CO₂ and acetate based, methanogenesis are frequently observed in wetlands (Whalen, 2005). In acidic ombrotrophic bogs, the former has been commonly reported as the predominant process (Williams and Crawford, 1984; Lansdown et al., 1992; Avery et al., 1999; 2003; Chasar et al., 2000; Hornibrook et al., 2000; Duddleston et al., 2002; Nakagawa et al., 2002; Horn et al., 2003; Galand et al., 2005), although the latter has recently been shown as the main

![Graph](image_url)

**Fig. 5.** Real-time qPCR quantification of 16S rRNA gene copy number of total *Archaea* (A) and the E2-E1 group (B) in DNA extracts from peat samples from the vertical profile of Chicago bog (CB: black bars) and McLean bog (MB: white bars). The x-axis represents the number of gene copies normalized by two rRNA operon copies and expressed in logarithmic scale; y-axis indicates the depth at which the sample was taken.

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**Table 3.** Specificity and coverage of 16S rRNA gene primers used to quantify total *Archaea* and the *Methanomicrobiaceae* E2-E1 group.

<table>
<thead>
<tr>
<th>Group/genus</th>
<th>Organisms tested</th>
<th>Reaction with Archaeal primers</th>
<th>E2-E1 primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Bulkholderia</em> sp., <em>Chromobacterium</em> sp., <em>Clostridium</em> sp., <em>Yersinia</em> sp., <em>Azospirillum</em> sp., <em>Magnetospirillum</em> sp.</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Archaea</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Thermoplasma</em></td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td><em>Methanococcus</em></td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td><em>Methanosarcina</em></td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td><em>Methanogenium</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td><em>Methanocorpusculum</em></td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td><em>Methanofollis</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td><em>Methanospirillum</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>E2 culture</strong></td>
<td>Methanogenic culture 6A</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><strong>Environmental clones</strong></td>
<td>CBs-c1H (group E2 clone)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CBs-e2E (group E1 clone)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CBs-b3C (Methanosaeta clone)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**a.** Bacterial isolates from MB classified by their closest phylogenetic association to bacterial groups based on their 16S rRNA gene sequence.  
**b.** Chromosomal DNA from microorganisms cultures in PCR reaction was 0.5 ng µl⁻¹.  
**c.** Plasmid DNA for environmental clones was used in 10⁶ copies µl⁻¹.
contributor in a pH 4.8 Siberian bog (Kotsyurbenko et al., 2004). Our incubation results showed that H$_2$/CO$_2$-amended samples produced significantly more CH$_4$ than those amended with acetate, indicating a greater potential for this pathway. The most methanogenically active layers, the upper 20 cm, just below the water table level, had a minimal lag time and rates increased within a few days after the incubations begun (Fig. 1A and B). This supports the existence of active and rapidly responding hydrogenotrophic methanogen populations at upper layers. The long lags demonstrated by samples from lower layers incubated with H$_2$/CO$_2$ indicated that populations of hydrogenotrophic methanogens were low and that perhaps only a specific subpopulation could be stimulated by addition of substrate.

On the other hand, our results for potential aceticlastic methanogenesis showed stimulation after a 20-day lag period in both upper layers of MB’s and CB’s 15 cm (Fig. 1). This indicates that the acetate-utilizing populations were near saturation for acetate, which we have measured as < 50 µM (S. Bräuer, unpublished) and are slow to respond to substrate addition. Although aceticlastic methanogens in MB can be stimulated with low doses of acetate (Bräuer et al., 2004; our results), and Kotsyurbenko and colleagues (2004) demonstrated a case in which this pathway is responsible for twice as much methanogenesis as the hydrogenotrophic one, typical of neutral-pH habitats, the contribution of aceticlastic methanogens was low and that perhaps only a specific subpopulation could be stimulated by addition of substrate.

The numerical dominance of hydrogenotrophic methanogens in the upper layers suggested by T-RFLP was confirmed with our real-time qPCR 16S rRNA gene target quantification, which estimated that the abundance of groups E2 and E1 together (although E1 contribution was small, particularly in CB samples) roughly accounted for ~55% of total archaeal gene targets in the upper layers, with their populations reaching a size on the order of 10$^8$ cells per gram of dry peat. These results clearly indicate the importance of group E2 for CH$_4$ production by CO$_2$ reduction in these Sphagnum-dominated sites. The E2 group is not unique to our study sites in the northeast USA, but instead has a widespread distribution in peatlands in the northern hemisphere. Phylotypes clustering within group E2 have been found in bogs from Germany (Horn et al., 2003), Finland (Galand et al., 2003), the UK (Edwards et al., 1998) and Canada (J. Yavitt, unpub-
lished), making up a significant fraction of clones in libraries or peak heights in T-RFLP analysis. An exception appears to be a pH 4.8 Siberian peat sample (Kotsyubenko et al., 2004) in which E2 was only a small proportion of the methanogenic community. We speculate that this group is a significant contributor to methanogenesis in highly acidic peatlands. This hypothesis is in agreement with the significant presence of the ‘fen cluster’ in Finnish peatlands (Galand et al., 2002; 2005; Juottonen et al., 2005) whose mcrA sequences are phylogenetically associated with the E2 culture (96% similarity; Bräuer et al., 2006). The population size and activity of group E2 at additional sites is required in order to confirm this findings.

In the vertical profiles, group E2 decreased in proportion of methanogens in T-RFLP (Fig. 4) and in numbers (Fig. 5) at both sites in samples from 40 and 65 cm. These deep samples also showed low rates of endogenous and substrate-stimulated methanogenesis. In MB, E2 still represented about half of the total peak height, whereas it was barely detectable in the more neutral CB deep samples, consistent with its being associated with low-pH habitats. Group E2’s preference for acidic conditions is supported by the observations of Bräuer and colleagues (2006). The population size and activity of group E2 at additional sites is required in order to confirm this findings.

Other significant changes in the community structure at depths below 40 cm were related to an increase in RC-II at both sites, and the presence of RC-I, MBD, SC and increase of E1 in CB. Rice cluster-II became an important fraction of the euryarchaeal community at both sites regardless of the pH differences, suggesting it has members that thrive in a broad range of pH values; this is supported by the fact that its sequences have been retrieved from acidic as well as neutral sites (Chin et al., 1999; Basiliko et al., 2003; Kemnitz et al., 2004). No isolated representative of RC, MBD or SC groups have been obtained up until now; although RC-I has been highly enriched with H2/CO2 from peatland and rice paddy soil samples with some initial physiological and genomic characterization (Lueders et al., 2001; Sizova et al., 2003; Erkel et al., 2005). It is interesting that the samples from CB lower levels, despite having low rates of methanogenesis, show considerably higher diversity than the upper levels, perhaps remnants of methanogenic populations present from a neutral-pH fen.

Experimental procedures

Study sites and sampling

McLean bog and CB are located within 40 km of Ithaca, New York (42°30’N, 76°30’W) in the northeast USA, and both sites were initially described by Osvald (1970). McLean bog is an ombrotrophic kettle hole bog approximately 70 m across (0.04 km²), the peat is 8 m deep, and the vegetation is dominated by Sphagnum moss (S. angustifolium and S. magellanicum). Vascular plants including ericaceous shrubs (Chamaedaphne calyculata, and Vaccinium corymbosum), three-way sedge (Dolichium spp.) and pitcher plants (Sarracenia purpurea) are also present in significant numbers. Chicago bog is an oligotrophic floating bog of approximately 0.05 km², and is associated with a pond on its east side. Its current surface is sphagnum dominated, and its earlier stages of peat development were sedge derived (Dettling, 2005). The Sphagnum species covering this bog (e.g. S. fuscum, S. magellanicum) are responsible for its hummocky surface; and the drier hummocks support ericaceous shrubs (C. calyculata) which are dominant in the site.

Sampling was performed on 28 June in CB and 4 October for MB in 2004. Duplicate cores were taken at both sites using a polyvinyl chloride (PVC) coring device with airtight seals. Sample points along vertical profile were determined by considering the physical characteristics of the peat in a parallel core obtained with a Russian style peat corer device. Airtight sealed cores were immediately transported to the laboratory and sampled inside an anaerobic glove box (Coy Laboratory Products, USA) with an atmosphere of N2 and 2–4% H2. Peat samples from each of the two cores taken from each site were individually assayed. As the replicate cores had similar results, only those from one core from each site are presented.

CH4 production

Peat slurry incubations with or without substrate additions were performed as described by Bräuer and colleagues (2004). Briefly, inside an anaerobic glove box, 1 g of peat was added to anaerobic deonized water to a volume of 10 ml in 18 × 150 mm crimp-top tubes. The tubes were sealed with butyl rubber stoppers and were flushed with sterile O2-scrubbed N2/CO2 (70%/30%, Mixed Gas Industries, USA). Anaerobic stock solutions were added as indicated to the following final concentrations: sodium acetate (1 mM), rifampicin (10 mg l−1). Sterile O2-scrubbed H2/CO2 (80%/20%, 70.7 kPa, Mixed Gas Industries, USA) was added to appropriate tubes. Incubations were performed at room temperature (−23°C) in the dark, under static conditions for samples with no addition or 1 mM acetate, and shaking (225 r.p.m.) for samples with rifampicin, or rifampicin + H2/CO2. Presented data represent the averages of triplicate samples.

Chemical analyses

Headspace gas was analysed for CH4 using a Perkin Elmer 3920B gas chromatography column with a flame ionization detector (Phoenix Equipment, USA). Peat slurries were vortexed for 30 s before headspace analysis. Dry weight and pH of triplicate peat samples were determined as described elsewhere (Bräuer et al., 2004) within 24 h after sampling.

DNA extraction and PCR amplification

Triplicate samples from each depth in the peat core were extracted with the Power Soil™ DNA kit (MoBio, USA) using
the manufacturer’s protocol with some slight modifications. Briefly, 0.5 g of peat was mixed with beads and disrupting solution, and after adding the C1 solution we also added 50 µl of a sterile 200 mM AlNH₄(SO₄)₂ solution to avoid humic acid co-purification (Braid et al., 2003). The mixture was subjected to 1 min 20 s of bead beating at maximum speed (MiniBeadbeater™, Biospect Products, USA). The quality of recovered DNA solution was examined by 2% agarose gel electrophoresis and spectrophotometer readings at 230 and 260 nm; minimal DNA shearing and A₂₆₀/A₂₃₀ ratios close to 2 were observed in almost all samples.

A fragment from positions 1–1100 (Escherichia coli numbering) of the 16S rRNA gene was amplified using the archaeal-specific 1AF (5′-TCY GKT TGC TGG YGS CRG AG-3′)-1100R (5′-TGG GTC TCG CTC GTT G-3′) set of primers (Hales et al., 1996). The PCR mixture contained the following components at its reactants concentrations per µl: 1× Taq buffer with 1.5 mM MgCl₂ (Eppendorf, USA), 0.2 mM deoxy- nucleotide triphosphates (dNTP), 0.25 µM forward and reverse primers, 1.2 U of Taq Polymerase (Eppendorf, USA), 0.2 µg of bovine serum albumin (BSA) and 0.1–0.3 ng of extracted DNA. The PCR conditions were as described by Hales and colleagues (1996) with 25 amplification cycles. Amplification products were examined by electrophoresis on 1% agarose gels for size verification.

Cloning, sequencing and phylogenetic analysis

16S rRNA gene clone libraries were constructed as described by Basiliko and colleagues (2003). Six clone libraries were constructed for samples from 15 cm, from 40 and 65 cm depth in CB. Using the TA Cloning kit® (Invitrogen, USA) and m13 primer screening, 60 clones per library were selected for restriction analysis with HaeIII and HhaI enzymes (New England Biolabs, USA). Clones displaying unique restriction patterns were sequenced with an ABI 3730 automated sequencer (Bio Resources Center, Cornell University). Sequences were compared against the GenBank database (Benson et al., 2004) in order to ensure that newly reported relatives were included in our database.

Phylogenetic analyses of the sequences were performed using the ARB software (Ludwig et al., 2004) with the latest 16S rRNA sequences database release (released August 2003; http://www.arb-home.de) and an ‘archaeal database’ with 2500 complete and partial archaeal sequences (Jurgens, 2002). Phylogenetic placement was performed using the quartet PUZZLE method implemented in ARB in comparison with reference archaeal sequences. A nucleotide base frequency filter that included positions with more than 50% invariance (1020 valid columns) was used to avoid possible treeing artefacts. Sequences were added to the tree reference sequence tree using the ARB parsimony tool without altering global tree topology. Tree topology was confirmed using maximum likelihood, neighbour joining and FITCH methods (as implemented in the ARB package).

The sequences of the 16S rRNA gene clones obtained in this study have been deposited in the GenBank nucleotide sequence database under the Accession No. DQ301878 to DQ301915.

T-RFLP analysis

We used the PCR primers and conditions described above. The 1100R reverse primer was fluorescently labelled on its 5′ end with Carboxyfluorescein (5′-/6-FAM). Terminal restriction fragment length polymorphism analysis was performed as described elsewhere (Marsh, 1999) with some modifications. Briefly, 30 µl of triplicate PCR reaction per sample was pooled and purified with the Quick Step™ PCR Purification Kit (Edge Biosystems, USA), and 70 ng was digested with a mix of HhaI (15 U) and Sau96I (10 U) enzymes (New England Biolabs, USA) for 3 h at 37°C. Digested DNA was purified with the Performa® DTRV3 96-Well Short Plate Kit (Edge Biosystems, USA). Purified products were concentrated in a vacuum centrifuge, and then resuspended with a mix of Hi Di-Formamide (Applied Biosystems, USA) and Gene Scan 500-Liz marker (12 µl µl⁻¹; Applied Biosystems, USA). Fragments were resolved with an Applied BioSystems 3730xl DNA Analyser (Bio Resources Center, Cornell University).

Terminal restriction fragment sequence length, peak height and area were determined using the GeneScan Analysis Software (Applied Biosystems, 2000). GeneScan's results containing peak size, height and area were exported for profile standardization of each sample. Using a Java-based routine, the relative fluorescent units (RFU) of peaks with size 50–500 bases were added by height or area and normalized to 100 total RFU. The standardization step minimized peak height and area variation from sample to sample and did not affect the profiles’ morphologies. Standardized profiles from samples taken in triplicate at each depth were averaged by their correspondent peak heights, and standard deviations were calculated.

Real-time quantitative PCR

Primer selection for total Archaea, and primer design for group E2-E1, was performed considering previously described optimal conditions (Suzuki et al., 2000; Nadkarni et al., 2002) and primer efficiency and coverage. For total Archaea, the Arch 967 F primer 5′-AAATGTCGCGGGAGCCAGC-3′ (Amann et al., 1990; Riley-Buckley, 2001) combined with Arch-1060R – the reverse complement of A1040F (Renyenbach and Pace, 1995) – had a broad euryarchaeal and crenarchaeal coverage with no matches for bacterial or eukaryotic sequences as reviewed for A1040F (Baker et al., 2003). For group E2-E1 joint quantification, we used the A-45E632 (5′-ACTCGAGT GCG GTT WAA ATC-3′) and A-45Es50aR (5′-AGT AAT AGT GGC CAC CAC TCG AGC-3′) set of primers, designed in this study with the primer-design and probe-match tool of ARB software. Predicted coverage and specificity analyses used the latest ARB database, the ‘Archaeal database’ (Jurgens, 2002) and Ribosomal Database Project II (Cole et al., 2003), confirming that all the archaeal phylotypes retrieved from our study sites could be amplified by the archaeal primers and that the E1-E2 primers only amplified members from this group (data not shown). Both primers were challenged with unspecific and specific targets as shown in Table 3.

Primer concentration was optimized using iTaq SYBR Green Supermix with ROX (Bio-Rad, USA). Polymerase
chain reactions (30 µl) contained 1x Supermix, 0.25 and 0.2 µM forward and reverse primers (for total Archaea and E2-E1 respectively), 0.1 g l\(^{-1}\) BSA and 3 µl of extracted DNA. Amplifications were performed on duplicate samples at two dilutions (1:5 and 1:10) using an ABI Prism® 7000 Sequence Detector (Applied Biosystems, USA) with the following settings: 2 min at 50°C, 10 min at 95°C, and 38 cycles of 15 s at 95°C with 1 min at 60°C. This was followed by a dissociation protocol to check for proper dissociation profiles, otherwise reactions were rejected. Plasmid DNA external standards with 10^8–10^10 target copies per microlitre were constructed for quantification as described by Fey and colleagues (2004).

Real-time PCR results were normalized to per cent peat dry weight at each depth and by the arbitrary value of two rRNA gene operons per genome, the closest integer to the currently reported archaeal average in the Ribosomal RNA Operon Copy Number Database (Kaplenbach et al., 2001).

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