

Depth related diversity of methanogen Archaea in Finnish oligotrophic fen

›Pierre E. Galand^a, Sanna Saarnio^b, Hannu Fritze^c, Kim Yrjälä^{a,*}

^a Department of Biosciences, Division of General Microbiology, P.O. Box 56, University of Helsinki, FIN-00014 Helsinki, Finland

^b Department of Biology, University of Joensuu, P.O. Box 111, FIN-80101 Joensuu, Finland

^c Finnish Forest Research Institute, Vantaa Research Center, P.O. Box 18, FIN-01301 Vantaa, Finland

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Abstract

The annual rate of CH₄ release and potential CH₄ production has recently been studied in the Salmisuo fen in eastern Finland but the microbiota responsible for the CH₄ production has not been examined. The diversity of the methane producing Archaea was analysed, at different depths, in the most representative microsite (*Eriophorum* lawn) of the fen. Methanogen populations were studied using primers amplifying a region of the methyl-coenzyme M reductase gene. PCR products were analysed by denaturing gradient gel electrophoresis and restriction fragment length polymorphism (RFLP) analysis of clone libraries. A representative of each RFLP group was sequenced. The study revealed a change of the methanogen populations with depth. Sequences from the upper layers of the fen grouped in a novel ‘Fen cluster’ and were related to Methanomicrobiales. Sequences retrieved from the deeper layers of the fen were related to Methanosarcinales via the Rice Cluster-I.

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1. Introduction

Carbon dioxide (CO₂) and methane (CH₄) are the two main greenhouse gases responsible for global warming. Although the methane concentration in the atmosphere is low compared to that of carbon dioxide (1.745 ppm versus 0.367 ppm), the ability of methane to absorb and radiate energy back to Earth makes it 21 times more efficient than CO₂ as greenhouse gas. The methane concentration has increased with 151% since 1750 and is actually still increasing [1]. Wetlands, including peatlands, are the main source of natural methane emission with an estimated 100–200 Tg per year [2]. Microbial activity is almost exclusively responsible for methane production both from natural and human-engineered systems [3].

In Finland, the proportion of land covered by mires is bigger than in any other country; mires or peatlands constitute a third of the land area of Finland [4]. The exis-

tence of peatlands is directly dependent on their water inputs and outputs [5]. A global climate change will inevitably induce hydrological changes and therefore threaten these vulnerable ecosystems. As a consequence of the predicted rise of the CO₂ concentration in the atmosphere and the average temperature on the globe, the methane production in these ecosystems is expected to increase, if other conditions (hydrology, vegetation etc.) remain rather unchanged [6,7]. In order to understand the process leading to methane production, it is crucial to monitor the methanogen organisms in these complex habitats.

The methanogens, which produce methane during the anaerobic digestion of organic matter, belong exclusively to the domain Archaea. Actually, 26 genera and more than 80 species of methanogens have been recorded [8]. The methanogens utilise a limited number of substrates with methyl coenzyme-M reductase (MCR) as their key enzyme [9]. The MCR catalyses the reduction of methyl-coenzyme M accompanied by release of methane [10]. The biological activity correlated to the MCR gene is easy to measure by determining the potential methane production in soil samples. Unlike other enzymes in the methanogenic metabolism, the MCR appears to be unique to the meth-

* Corresponding author. Tel.: +358 (9) 19 15 92 20; Fax: +358 (9) 19 15 92 62.

E-mail address: kim.yrjala@helsinki.fi (K. Yrjälä).

anogens [11,12]. Parts of the MCR operon are also highly conserved and all MCR operons appear to have evolved from a common source [13,14]. This functional gene is therefore a helpful tool for an ecological and phylogenetic survey of a microbial population with known activity.

Molecular biology tools enable the study of microbial diversity without a cultivation step. The diversity of methanogens has previously been described in different habitats, such as peat bogs [15–19] and rice field soils [20–22], but never in boreal fens. In Finland, the dynamics of methane release and production has been widely investigated in the Salmisuo fen [6,7,23–25] but the diversity of methane producing organisms has never been assessed. In this study, we examined the methanogen biodiversity, at different depths, in this well-characterised, undisturbed oligotrophic fen.

2. Materials and methods

2.1. Environmental site and sample collection

Peat samples were collected in June 2001 from the Salmisuo mire complex in eastern Finland (62°47'N, 30°56'E). The site is a minerogenic, oligotrophic low-sedge *Sphagnum papillosum* pine fen. Four different types of microsites have been described at the Salmisuo fen: *Eriophorum* lawn, Flark, Hummock and *Carex* lawn. They are defined by their nutritional statuses and their average water table level. Samples were taken from *Eriophorum* lawns, the most common microsite covering 86% of the mire surface. On the *Eriophorum* lawn microsite the water level is mainly 5–20 cm below the peat surface and vegetation is dominated by *Sphagnum* mosses, *Eriophorum vaginatum*, *Vaccinium oxycoccos* and *Andromeda polifolia* (for more details, see [7,24]). Three parallel peat profiles (E1–E3), 20 cm distant from each other, were collected from the microsite with a box sampler (8×8×100 cm). Soil samples for DNA analyses and potential methane production measurement were taken each 10 cm (± 2 cm) of the core (Table 1). All together the sampling resulted in 12 peat samples from which the measurements were taken.

2.2. Measurement of potential methane production

The potential CH₄ production is the maximum produc-

tion measured in the laboratory under defined temperature conditions. Fifteen millilitres of peat were added to 100-ml, oxygen free, infusion bottles containing 30 ml of distilled water. The bottles were flushed with 99.96% nitrogen in order to obtain anoxic conditions [23] and sealed with butyl rubber septa. The glass vials were allowed to stand at +4°C for 5 days to allow the gas present in the peat core section to equilibrate with the headspace. The vessels were then flushed with nitrogen and kept unshaken in the dark for 170 h. The incubation temperature was +10°C (in situ temperature). Four times during the incubation, subsamples were taken from the headspace for analysis of the methane concentration. Volumes of 100 μ l of gas, taken in a Hamilton syringe, were injected into a Perkin-Elmer Sigma II gas chromatograph equipped with a flame ionisation detector and a 1.5-m column packed with Porapak Q 80:100 mesh. N₂ was used as a carrier gas at a flow rate of 30 ml min⁻¹. The injector, oven and detector temperatures were set at 35°C, 40°C and 350°C, respectively.

The rate of methane production was calculated from the slope of the linear regression given by the CH₄ concentration increase over time. No non-linear ($r^2 < 90\%$) cases were found. After incubation, the peat samples were dried to constant weight at 60°C and the methane production was normalised to the dry weight of the peat samples.

2.3. DNA extraction

DNA was extracted directly from 0.25 g of peat samples by chemical (detergent) and mechanical (bead beating) cell lysis with the Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA), following the manufacturer's suggested protocol. No further purifications were needed. This extraction method has been shown to hold high reproducibility and efficiency when compared with other methods [26].

2.4. PCR amplification

The α -subunit of the MCR gene was used as molecular marker to study the methanogen diversity. The α -, β - and γ -subunit genes constitute the MCR gene coding for the key enzyme in all pathways for biogenic methane production. The primer pair ME1–ME2 [27] was used to amplify specifically a 760-bp-long region of the MCR α -subunit. A 40-nucleotide-long GC clamp was added to the forward

Table 1

Sampling site, water table depth (WT), peat temperature and average potential CH₄ production, for three separate peat profiles, at the Salmisuo fen in June 2001

Microsite and WT	Sample depth (cm below WT)	Peat temperature (°C)	Average potential CH ₄ production (nmol g ⁻¹ h ⁻¹) and S.E.
<i>Eriophorum</i> lawns; WT: -6 cm	-10	11.5	15.2 (3.6)
	-20	10.8	17.4 (7)
	-30	10	8.3 (5.1)
	-40	9	0.7 (0.4)

primer to enable denaturing gradient gel electrophoresis (DGGE) analysis [28]. The 50- μ l PCR mixture contained 25 pmol of the appropriate primer pairs, 200 μ M dNTPs, 1 U Red Hot polymerase (Advanced Biotechnologies, Epsom, UK), PCR reaction buffer and 3 μ l of template (concentration determined empirically). Bovine serum albumin (0.1 μ g μ l⁻¹) was used to prevent PCR inhibition. The reaction conditions were 30 cycles of 94°C for 45 s, 50°C for 45 s, 72°C for 2 min. The PCR was performed using a Gene Amp thermal cycler (Perkin Elmer, Inc., Wellesley, MA, USA). Products were analysed on 1% agarose gels with ethidium bromide staining.

2.5. DGGE fingerprinting

DGGE was performed on all 12 samples with a D-code system (Bio-Rad, Inc., Hercules, CA, USA) following the manufacturer's instructions. Samples were loaded on 6% (w/v) polyacrylamide gels in 1 \times TAE buffer. The denaturing gradients ranged from 40 to 56%. 100% denaturant corresponds to 7 M urea and 40% (v/v) deionised formamide. The gel was run at 60°C, at a constant voltage of 160 V for 7 h. After electrophoresis, the gel was stained for 30 min with SYBR Green I (1/10 000 dilution; Molecular Probes, Eugene, OR, USA) and digitised in UV light using a Fluor-S MultiImager system (Bio-Rad, Inc., Hercules, CA, USA).

2.6. Cloning, restriction fragment length polymorphism (RFLP) and sequencing of PCR product

Two gene libraries were constructed using DNA extracted and amplified from two depths (–10 and –40 cm) of the E2 sample. PCR products obtained with ME primers (amplification described above) without GC clamp were analysed by gel electrophoresis, purified with β -agarase (New England BioLab, Beverly, MA, USA) and cloned into a pGEM-T vector plasmid using JM109 competent cells (Promega, Mannheim, Germany) according to the manufacturer's instructions. MCR insert sequences were amplified directly with ME primers. Colonies dispersed in H₂O were used as templates. The products were digested with *Msp*I for RFLP analysis.

The method of Good was used to calculate the percentage of coverage of the clone libraries [29,30]. Forty-nine and 52 clones were analysed from the depth of 10 and 40 cm, respectively.

The Shannon diversity index [31] was calculated as:

$$H' = -\sum_{i=1}^S p_i \ln(p_i)$$

where p_i is the proportion of clones belonging to the i th restriction group and S is the total number of restriction groups.

Colonies showing distinct fingerprint patterns were se-

lected for sequencing. Plasmid DNA was isolated with the GFX Micro Plasmid Prep Kit (Pharmacia Biotech Inc., N. Peapack, NJ, USA) and sequenced using the vectors' universal primers. To assess if the RFLP groups contain closely related sequences, two representatives from each of the biggest RFLP groups were sequenced and compared by pairwise alignment.

In order to relate the results obtained by DGGE fingerprinting with those from RFLP analysis of clone libraries, cloned MCR sequences representing each RFLP group were re-amplified with GC clamp and run in DGGE. Additionally, two bands from the total community DGGE fingerprint (D1 and D4, Fig. 2) were cut and kept overnight at 4°C in 20 μ l H₂O. Five microlitres of the suspension were used as template for PCR and the amplified DNA product was cloned as described above. Twelve clones containing the correct insert were analysed by RFLP.

The MCR sequences obtained in this study were deposited in the EMBL database under accession Nos. AJ489761–AJ489782.

2.7. Phylogenetic analysis

Sequences were compared to those in the EMBL database using Fasta3 [32] at the European Bioinformatics Institute (EBI) URL (www.ebi.ac.uk; Hinxton Hall, Cambridge, UK). Amino acid sequences were inferred from the ME nucleotide sequences. Sequences of approximately 250 amino acids were aligned using CLUSTAL W package [33] and checked manually. A phylogenetic tree was estimated using the PHYLIP package (v.3.57c; J. Felsenstein, University of Washington, <http://evolution.genetics.washington.edu/phylip.html>). PROTDIST was used to compute a distance matrix with Dayhoff PAM 001 as amino acid replacement model. The distance matrix was estimated by FITCH with global rearrangement of branches and randomised species input order. Even though full sequences should be used for phylogeny determination [34], partial sequences can be used to determine the closest relatives of unknown sequences [35,36]. The dendrogram was verified by neighbour-joining and maximum parsimony methods.

3. Results and discussion

3.1. Potential CH₄ production

The water level and the peat temperature (Table 1), measured in June 2001 during the sample collection, were similar to previous data available for the Salmisuo pine fen [24].

CH₄ production was detected in all peat samples but one (E3, –40 cm, Fig. 1). Profiles E2 and E3 had the highest potential production 10 cm below the water table whereas profile E1 had its maximum at –20 cm. The sin-

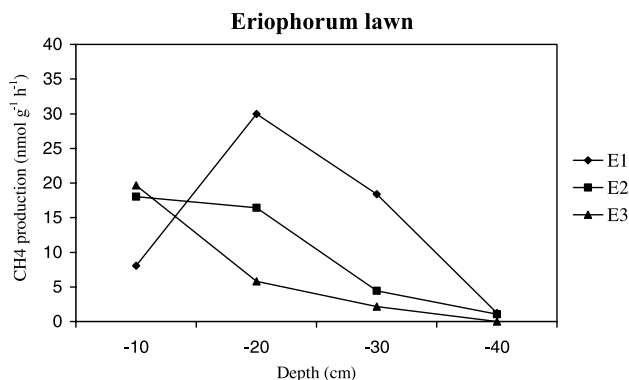


Fig. 1. Potential CH₄ production at four different depths in three separate peat profiles (E1, E2, E3) taken from the *Eriophorum* lawn at Salmisuo fen.

gularity of E1 is probably due to sampling bias. The characteristic softness of the peat profile could have induced an outstretching of the E1 peat colon. In all three profiles the minimum rate of CH₄ production was detected in samples taken from the depth of 40 cm (Fig. 1). All profiles showed a maximum production in the upper water-saturated layers and a decrease of the potential production with depth. This trend is typical for mires and has been shown in previous studies [23,37]. The highest potential CH₄ production at the *Eriophorum* lawn varied from 18 to 30 nmol g h⁻¹. Those rates are in a similar range as the one measured earlier at the Salmisuo fen [23] and at two different bogs [15,17].

In anoxic conditions with constant temperature, the potential CH₄ production depends on substrate availability in the peat column, activity of methane producing Archaea and the presence of possible substrate competitors [8]. At the depth where potential methane production is observed, the oxygen concentration in the peat was low enough to enable methanogens activity. The presence of O₂ through the peat profile is directly correlated to the water level. When the water table fluctuates, the potential CH₄ production follows that variation with lag [23]. The average water table depth in the *Eriophorum* lawns has been shown to have seasonal variations during the snow free months of the year (May–September). The water table is generally at its minimum during the hottest summer months (July, August) and at its maximum with snow melting during the beginning of the spring (May, June) and autumn (September, October) [6,24,38]. At the sampling time, in June, the water table had been at its maximum for several weeks, enabling methanogen activity in the water saturated layers.

The observed potential CH₄ production also shows that the peat contains a sufficient amount of substrates (e.g. acetate, hydrogen, carbon dioxide) to enable methane production by Archaea at the incubation temperature (+10°C). In the anoxic layers of the mire, the organic matter (litter) is completely degraded in four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis [8].

Methanogens occupy the terminal position in the anaerobic decomposition chain and are dependent on the degradation of complex organic compounds by chemoheterotrophic bacteria. The main carbon sources are an accumulation of suitable carbon during autumn and early winter (when the temperature is too low for high methanogenic activity), a new anoxic degradation of litter when the thawing of the peat begins and exudates of carbon compounds from living plants [24].

Most of the degradation of organic matter occurs in the surface of the mire. The low potential methane production in the deeper layers of the peat can be explained by the lack of suitable substrate or the presence of less favourable substrate for the methanogens at those depths [39,40]. Additionally, lower potential CH₄ production can also be explained by the presence of other microorganisms out-competing the methanogens. It has been, for example, shown that homoacetogenic bacteria compete against hydrogenotrophic methanogens for the use of H₂ excreted by fermentative bacteria [41,42].

3.2. Diversity analyses by DGGE

The depth related variation of the MCR gene diversity was monitored by DGGE analysis. In Fig. 2, results from three different profiles (E1, E2 and E3) are shown. In each of the profiles, a difference in the banding pattern can be seen between the upper layers (–10, –20 cm) and the deeper layers (–30, –40 cm, Fig. 2). This difference is characterised by a variation in the number of bands and their relative intensity. More bands are present in the deeper layers than in the upper ones (6–7 bands at –10 cm and 8–12 at –40 cm). Some bands are typically found in the deep layer (D bands, Fig. 2), others are characteristic for the upper layer (U bands, Fig. 2) and some are found at all depth (UD bands, Fig. 2). The singularity of the fingerprint observed in E1 is probably due to a sampling bias (see explanations above).

The MCR gene diversity has not earlier been studied by DGGE and the depth dependent variation of methanogen communities has not been shown before. Some works have been studying potential methanogen stratification with depth, but none of them found depth related variations in the diversity [17–19].

It has been shown that DGGE cannot detect populations whose abundance is less than 1% of the total community [43,44]. In order to enhance the precision of our data for the comparison of community through the peat, the PCR products from the two different depths were cloned. The construction of a clone library also allows sequencing of the MCR genes and determination of the phylogenetic relationships between sequences.

3.3. Diversity analyses by RFLP of clone libraries

The two depths (–10 and –40 cm) chosen for the con-

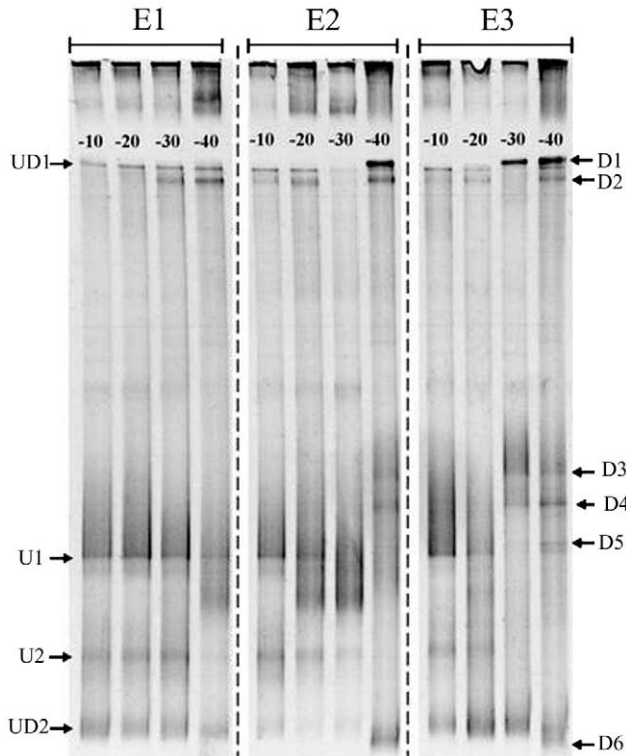


Fig. 2. DGGE of methanogen MCR sequences (760 bp). Sequences from four different depths (-10, -20, -30, -40 cm) in three separate peat profiles (E1, E2, E3) are shown. The urea-formamide gradient ranges from 40 to 56%. Arrows marked U show bands representative for the upper layers of the fen, arrows marked D show bands representative for the deeper part of the fen and arrows marked UD show bands found at both depths.

struction of clone libraries represent high and low potential methane production (Fig. 2) and are characteristic for a superior and deep layer methanogen community. Because the sequence diversity variation with depth was observed in all three parallel samples by DGGE, those libraries were thought to be representative for the *Eriophorum* lawn.

Clones from the two libraries were screened and grouped on the basis of their RFLP patterns. Twenty-one unique RFLP patterns were found altogether and plasmid DNA from each unique RFLP profile was extracted and sequenced. Additionally, a second representative from each of the six biggest RFLP groups (F, G, T, E, J and N) was sequenced. The level of similarity (amino acid) between the two representatives of each RFLP group was 100% for five of the groups and 99.2% for one (N). We therefore assume that sequences within each RFLP group are closely related to each other. The clone libraries had a coverage of 94% and 87% for E2-10 and E2-40, respectively. It means that in a similar clone library of infinite size, the clones obtained in this study will represent 94% and 87%, respectively, of the restriction groups of the library.

The RFLP patterns given by the functional gene show a

clear difference between -10 and -40 cm (Fig. 3). Sequences amplified from the deeper layer show a much higher number of RFLP patterns compared with the sequences from the upper layer. Moreover, the most common RFLP groups differ between the two depths. The Shannon diversity index (H') had a lower value for the upper layer (1.25) than for the deeper layer (2.43). This confirms the results obtained by DGGE fingerprint where the largest diversity was observed at 40 cm below the water table (Fig. 2). At this depth, 18 different patterns are present with E, J and N being the most common group of sequences (50% of all clones). At -10 cm, only seven unique profiles are seen and F is the main group (60% of

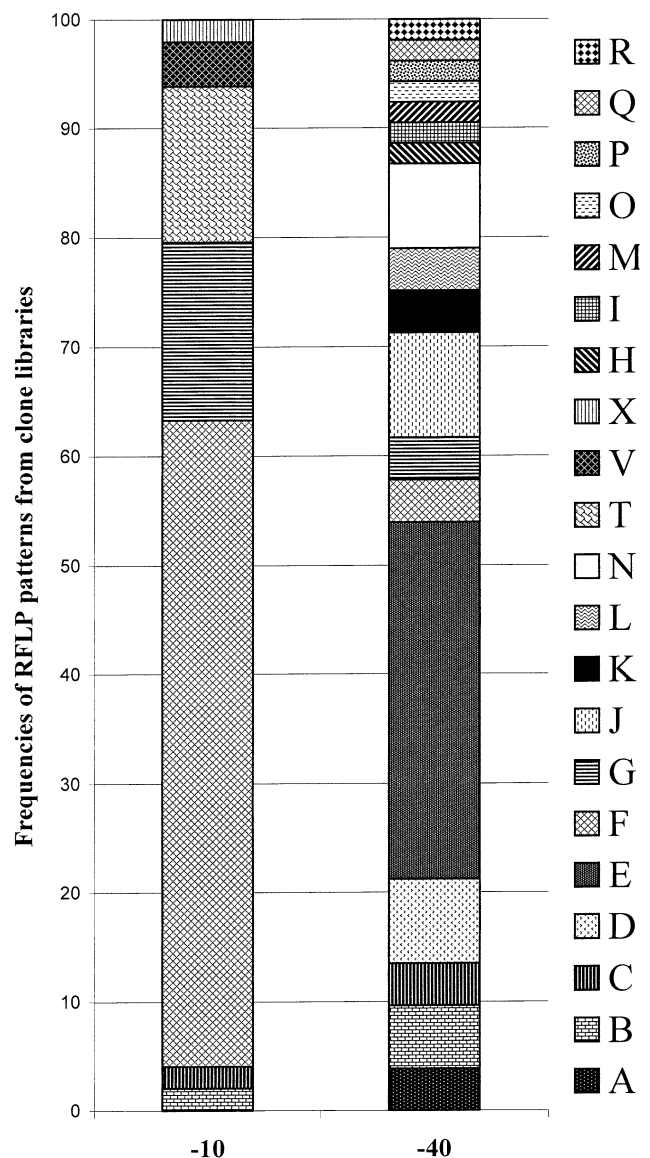


Fig. 3. RFLP analysis of MCR clone libraries. The sequences were obtained from peat samples taken 10 and 40 cm below the water table. Forty-nine and 52 clones were analysed from the depth of 10 and 40 cm, respectively. Letters in the legend represent the different RFLP groups.

all clones). The variation of the number of patterns with depth may reflect a phenotypic adaptation to the biotic and abiotic conditions found throughout different depths of the fen.

The number of functional sequence groups found at the Salmisuo fen could have been higher if the MCR primers [14] would additionally been used. These primers amplify a shorter segment of the MCR gene but have been shown to have higher diversity coverage. They are known to detect sequences within the families of Methanosaetaceae and Methanobacteriaceae which the ME primers do not detect [22]. Due to difficulties in consistently getting proper amplicons, the MCR primers were not used in our study.

Some orders of methanogens (Methanobacteriales and Methanococcales) have been shown to contain, additionally to the MCR, an isoenzyme called methyl coenzyme-M reductase two (MCR-II) [45–47]. Since ME primers are known not to amplify the sequence coding for MCR-II [22], possible MCR-II sequences present in the samples would not have been detected in this study. Consequently, the high number of functional sequence groups found at –40 cm cannot be explained by the possible presence of MCR-II sequences.

Sequences representing each of the RFLP groups were run in DGGE. Out of 21 sequences analysed, 18 appeared within the gel. Five bands were situated in the upper part of the gel, 13 in the lower part and the missing three sequences probably migrated out of the gel. Many bands migrated to positions very close to each other. RFLP analyses of clones obtained from the fingerprint bands (D1 and D4) revealed that several sequences can be present in each band (up to six different RFLP patterns in one band). This confirms that some sequences are immobilising at positions very close to each other and are not separated properly. The imperfect separation of the sequences is a possible explanation for the difference observed between the diversity estimated by DGGE fingerprinting (up to 12 different bands observed in the gel) and the one obtained by RFLP analyses (21 different restriction groups). This underlines the need to use an approach combining DGGE fingerprinting and RFLP analyses.

3.4. Phylogenetic analysis

After sequencing a representative of each RFLP group, a phylogenetic tree was constructed. The 21 sequences obtained from the Salmisuo fen, from 10 and 40 cm depth, group in six distinct clusters (Fig. 4). Four clusters (I–IV) appear as a novel group of sequences with closest phylogenetic connection to members of the order Methanomicrobiales (Fig. 4). These MCR sequences are clearly distinct from other published ones with maximum sequence similarity of amino acids ranging from $\approx 85\%$ (sequence D) to $\approx 87\%$ (sequence K) to *Methanospirillum hungatei*. This novel MCR cluster was named ‘Fen cluster’ (FC) and was divided into four sub-groups (FC-I to FC-IV). Two

other clusters (V and VI) are related to sequences from Rice cluster I (RC-I). This MCR group of sequences retrieved from Italian rice field soils is related to the Methanosarcinales and Methanomicrobiales [22]. With sequences related to two orders of Euryarchaeota, the phylogenetic diversity of the MCR gene observed at the Salmisuo fen is higher than the one earlier shown in an English bog [18] but lower than diversity found in Italian rice soil [22].

Sequences representing the deeper layers of the fen (restriction groups E, J, N) all group in clusters related to RC-I, whereas the main sequences from the upper layer are all gathered within the new FC group. Sequences from three of the novel sub-clusters group according to the depth they were retrieved from. FC-I contains only sequences from the deeper layer, FC-II contains only sequences retrieved from the upper layers of the fen and FC-IV (which contains only one sequence) is from the upper part of the fen (Fig. 4). This depth related, or dependent, variation of functional gene diversity has not been shown before in mires.

The major methanogen groups found, in our study, in the upper part of the peat (restriction groups F, G, T) form a branch with species belonging to Methanomicrobiales. Since the order Methanomicrobiales consists of hydrogenotrophic methanogens, the methanogens most commonly found in the upper layers of the fen may belong to a family forming methane by oxidising H_2 and reducing CO_2 . Hydrogenotrophic methanogens receive their substrate (H_2) directly from fermentative bacteria and interspecies transfers [48,49]. This nutritional reaction is thermodynamically the more favourable, in terms of free energies ($\Delta G'_0$), of those inducing methane synthesis [8]. It has been shown that H_2 dependent methanogenesis dominates in bogs and other studied environments [50]. Since the maximum potential CH_4 production is observed in the upper layer of the peat profiles and the organisms found at that depth are related to H_2/CO_2 utilising methanogens, hydrogenotrophy could also be the main pathway for CH_4 production in the Salmisuo fen.

The dominant groups (E, J, N) found in the deeper layers are within the phylogenetic radiation of the order Methanosarcinales via the RC-I group (Fig. 4). The level of sequence similarity (amino acids) for this lineage is $\approx 83\%$ to members of Methanosarcinales and $\approx 81\%$ to members of Methanomicrobiales. The relatively new Methanosarcinales order [51] regroups acetotrophic and/or methylotrophic methanogens. The organisms most commonly found in the deeper part of the fen, in this study, may then belong to the group using acetate as methane precursor. The stratification of methanogens with depth could be explained by the difference in temperature found between the superior and deeper layers of the peat profile (Table 1) [6]. Several studies have shown that acetotrophic organisms have a lower optimal temperature than hydrogenotrophic methanogens [52,53]. This could be

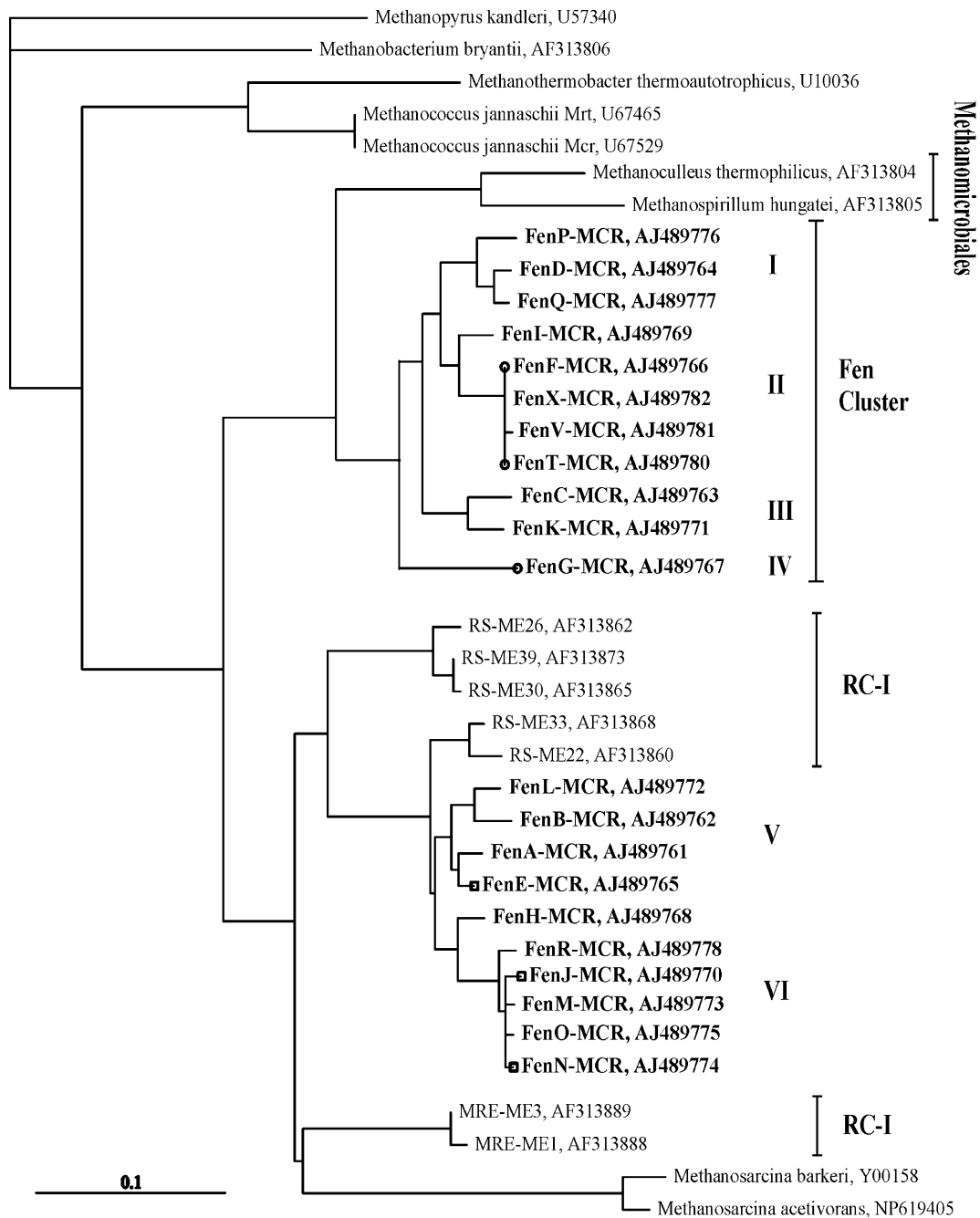


Fig. 4. Phylogenetic dendrogram representing the relationship between MCR sequences, retrieved from boreal fen, and other related methanogenic Archaea. Sequences (bold face) were retrieved from libraries constructed with ME primers. The capital letter in the sequence name corresponds to the RFLP pattern. The tree was constructed from inferred amino acid sequences using FITCH distance matrix analysis. *Methanopyrus kandleri* was used as outgroup. ○ marks the main sequences found in the upper part of the peat and ◻ marks the main sequences found in the deeper part. The scale bar represents 10% sequence divergence. GenBank accession numbers are indicated for all sequences.

one reason explaining a dominance of methanogens related to Methanomicrobiales in the upper part of the peat and acetate utilising methanogens, as Methanosarcinales, in the deeper part of the fen.

On the other hand, members of the RC-I have been selectively enriched with H_2/CO_2 as energy sources [22], indicating that this group contains hydrogenotrophic

methanogens. The phylogenetic clusters IV and V, grouping sequences from the deeper part of the fen, are closely related to RC-I. Methanogens from the deeper part of the fen could therefore include hydrogenotrophs. Studies focusing on the physiology of these newly discovered fen methanogens are needed in order to determine the nutritional status of those organisms.

4. Conclusion

(1) The study of the diversity of the functional MCR gene in a lawn microsite of an undisturbed boreal fen revealed a novel lineage of methanogens. The new 'Fen cluster' was phylogenetically related to the order Methanomicrobiales.

(2) The study showed a change in the methanogen populations with depth. The genotypic diversity was much higher at a depth of 40 cm than at a depth of 10 cm below the water table.

(3) The main groups present in the upper layer were most related to the order Methanomicrobiales. This order is composed of hydrogen utilising methanogens.

(4) In the deeper layers of the fen the dominant groups were most related to Methanosarcinales via the RC-I group. Methanosarcinales use acetate as precursor but members of RC-I have previously been enriched with H₂/CO₂. The nutritional affiliation of the methanogens found in the Salmisuo fen remains therefore unclear.

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References

- [1] IPCC (2001) Climate Change 2001 (The Scientific Basis). Cambridge University Press, Cambridge.
- [2] IPCC (1995) Climate Change 1994 (Radiative Forcing of Climate Change). Cambridge University Press, Cambridge.
- [3] PSAB (2000) Environmental change: microbial contributions, microbial solutions. American Society for Microbiology. <http://www.asmsa.org/pasrc/reports.htm>.
- [4] Lappalainen, E. (1996) Mires of Finland and their use. In: Global Peat Resources (Lappalainen, E., Ed.), pp. 69–74. International Peat Society, Jyväskylä.
- [5] Ross, S., Gilvear, D.J., Grieve, I.C. and Wilby, N. (1998) Hydrochemical-vegetation interactions within Scottish fens. In: Hydrology in a Changing Environment (Wheeler, H. and Kirby, C., Eds.), pp. 431–444. John Wiley and Sons, Chichester.
- [6] Kettunen, A., Kaitala, V., Alm, J., Silvola, J., Nykänen, H. and Martikainen, P.J. (1996) Cross-correlation analysis of the dynamics of methane emissions from a boreal peatland. Glob. Biogeochem. Cycles 10, 457–471.
- [7] Saarnio, S., Saarinen, T., Vasander, H. and Silvola, J. (2000) A moderate increase in the annual CH₄ efflux by raised CO₂ or NH₄NO₃ supply in a boreal oligotrophic mire. Glob. Change Biol. 6, 137–144.
- [8] Garcia, J.L., Patel, B.K.C. and Ollivier, B. (2000) Taxonomic phylogenetic and ecological diversity of methanogenic Archaea. Anaerobe 6, 205–226.
- [9] Rmrler, U., Grabarse, W., Shima, S., Goubeaud, M. and Thauer, R.K. (1997) Crystal structure of methyl-coenzyme M reductase: the key enzyme of biological methane formation. Science 278, 1457–1462.
- [10] Ellermann, J., Hedderich, R., Bocher, R. and Thauer, R.K. (1988) The final step in methane formation. Investigations with highly purified methyl-CoM reductase (component C) from *Methanobacterium thermoautotrophicum* (strain Marburg). Eur. J. Biochem. 172, 669–677.
- [11] Thauer, R.K. (1998) Biochemistry of methanogenesis: a tribute to Marjory Stephenson. Microbiology 144, 2377–2406.
- [12] Chistoserdova, L., Vorholt, J.A., Thauer, R.K. and Lidstrom, M.E. (1998) C1 transfer enzymes and coenzymes linking methylotrophic bacteria and methanogenic Archaea. Science 281, 99–102.
- [13] Nolling, J., Elfiner, A., Palmer, J.R., Steigerwald, V.J., Pihl, T.D., Lake, J.A. and Reeve, J.N. (1996) Phylogeny of *Methanopyrus kandleri* based on methyl coenzyme M reductase operons. Int. J. Syst. Bacteriol. 46, 1170–1173.
- [14] Springer, E., Sachs, M.S., Woese, C.R. and Boone, D.R. (1995) Partial gene sequences for the A subunit of methyl-coenzyme M reductase (mcrI) as a phylogenetic tool for the family Methanosarcinaceae. Int. J. Syst. Bacteriol. 45, 554–559.
- [15] Krumholz, L.R., Hollenback, J.L., Roskes, S.J. and Ringelberg, D.B. (1995) Methanogenesis and methanotrophy within a Sphagnum peatland. FEMS Microbiol. Ecol. 18, 215–224.
- [16] Lloyd, D., Thomas, K.L., Hayes, A., Hill, B., Hales, B.A., Edwards, C., Saunders, J.R., Ritchie, D.A. and Upton, M. (1998) Micro-ecology of peat: minimally invasive analysis using confocal laser scanning microscopy, membrane inlet mass spectrometry and PCR amplification of methanogen-specific gene sequences. FEMS Microbiol. Ecol. 25, 179–188.
- [17] McDonald, I.R., Upton, M., Hall, G., Pickup, R.W., Edwards, C., Saunders, J.R., Ritchie, D.A. and Murrell, J.C. (1999) Molecular ecological analysis of methanogens and methanotrophs in blanket bog peat. Microb. Ecol. 38, 225–233.
- [18] Nercessian, D., Upton, M., Lloyd, D. and Edwards, C. (1999) Phylogenetic analysis of peat bog methanogen populations. FEMS Microbiol. Ecol. 173, 425–429.
- [19] Upton, M., Hill, B., Edwards, C., Saunders, J.R., Ritchie, D.A. and Lloyd, D. (2000) Combined molecular ecological and confocal laser scanning microscopic analysis of peat bog methanogen populations. FEMS Microbiol. Lett. 193, 275–281.
- [20] Chin, K.-J., Lukow, T. and Conrad, R. (1999) Effect of temperature on structure and function of the methanogenic archaeal community in an anoxic rice field soil. Appl. Environ. Microbiol. 65, 2341–2349.
- [21] Grosskopf, R., Janssen, P.H. and Liesack, W. (1998) Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Appl. Environ. Microbiol. 64, 960–969.
- [22] Lueders, T., Chin, K.-J., Conrad, R. and Friedrich, M. (2001) Molecular analyses of methyl-coenzyme M reductase alpha-subunit (mcrA) genes in rice field soil and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage. Environ. Microbiol. 3, 194–204.
- [23] Kettunen, A., Kaitala, V., Lehtinen, A., Lohila, A., Alm, J., Silvola, J. and Martikainen, P.J. (1999) Methane production and oxidation potentials in relation to water table fluctuations in two boreal mires. Soil Biol. Biochem. 31, 1741–1749.
- [24] Saarnio, S., Alm, J., Silvola, J., Lohila, A., Nykänen, H. and Martikainen, P.J. (1997) Seasonal variation in CH₄ emissions and production and oxidation potentials at microsites on an oligotrophic pine fen. Oecologia 110, 414–422.
- [25] Saarnio, S., Alm, J., Martikainen, P.J. and Silvola, J. (1998) Effects of raised CO₂ on potential CH₄ production and oxidation in, and CH₄ emission from, a boreal mire. J. Ecol. 86, 261–268.
- [26] Niemi, R.M., Heiskanen, I., Wallenius, K. and Lidstrom, K. (2001) Extraction and purification of DNA in rhizosphere soil samples for PCR-DGGE analysis of bacterial consortia. J. Microbiol. Methods 45, 155–165.
- [27] Hales, B.A., Edwards, C., Ritchie, D.A., Hall, G., Pickup, R.W. and Saunders, J.R. (1996) Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. Appl. Environ. Microbiol. 62, 668–675.
- [28] Muyzer, G., Brinkhoff, T., Nubel, U., Santegoeds, C., Schafer, H.

- and Wawer, C. (1998) Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. In: *Molecular Microbial Ecology Manual* (Akkermans, A.D.L. et al., Eds.), pp. 1–27. Kluwer Academic Publishers, Dordrecht.
- [29] Mullins, T.D., Britschgi, T.B., Krest, R.L. and Giovannoni, S.J. (1995) Genetic comparisons reveal the same unknown bacterial lineages in Atlantic and Pacific bacterioplankton communities. *Limnol. Oceanogr.* 40, 148–158.
- [30] Good, I.J. (1953) The population frequencies of species and the estimation of the population parameters. *Biometrika* 40, 237–264.
- [31] Shannon, C.E. and Weaver, W. (1963) *The Mathematical Theory of Communication*. University of Illinois Press, Urbana, IL.
- [32] Pearson, W.R. and Lipman, D.J. (1988) Improved tools for biological sequence analysis. *Proc. Natl. Acad. Sci. USA* 85, 2444–2448.
- [33] Higgins, D., Thompson, J., Gibson, T., Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
- [34] Ludwig, W. and Schleifer, K.H. (1994) Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. *FEMS Microbiol. Rev.* 15, 155–173.
- [35] Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M. and Schleifer, K.H. (1998) Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* 19, 554–568.
- [36] Stackebrandt, E. and Rainey, F.A. (1995) Partial and Complete 16S rDNA Sequences, their Use in Generation of 16S rDNA Phylogenetic Trees and their Implications in Molecular Ecological Studies, pp. 1–17. Kluwer Academic Publishers, Dordrecht.
- [37] Williams, R.T. and Crawford, R.L. (1984) Methane production in Minnesota peatlands. *Appl. Environ. Microbiol.* 47, 1266–1271.
- [38] Alm, J., Talanov, A., Saarnio, S., Silvola, J., Ikkonen, E., Aaltonen, H., Nykänen, H. and Martikainen, P.J. (1997) Reconstruction of the carbon balance for microsites in a boreal oligotrophic pine fen, Finland. *Oecologia* 110, 423–431.
- [39] Valentine, D.W., Holland, E.A. and Schimel, D.S. (1994) Ecosystem and physiological controls over methane production in northern wetlands. *J. Geophys. Res.* 99, 1563–1571.
- [40] Svensson, B.H. and Sundh, I. (1992) Factors affecting methane production in peat soils. *Suo* 43, 183–190.
- [41] Abram, J.W. and Nedwell, D.B. (1978) Inhibition of methanogenesis by sulfate reducing bacteria competing for transferred hydrogen. *Arch. Microbiol.* 117, 89–92.
- [42] Kotsyurbenko, O.R., Glagolev, M.V., Nozhevnikova, A.N. and Conrad, R. (2001) Competition between homoacetogenic bacteria and methanogenic archaea for hydrogen at low temperature. *FEMS Microbiol. Ecol.* 38, 153–159.
- [43] Muyzer, G., De Waal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59, 695–700.
- [44] Casamayor, E.O., Schafer, H., Baneras, L., Pedros-Alio, C. and Muyzer, G. (2000) Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: comparison by microscopy and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 66, 499–508.
- [45] Bult, C.J., White, O., Olsen, G.J., Zhou, L., Fleischmann, R.D., Sutton, G.G., Blake, J.A., FitzGerald, L.M., Clayton, R.A., Gocayne, J.D., Kerlavage, A.R., Dougherty, B.A., Tomb, J.F., Adams, M.D. and Venter, J.C. (1996) Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. *Science* 273, 1058–1073.
- [46] Lehmacher, A. and Klenk, H.P. (1994) Characterization and phylogeny of mcrII, a gene cluster encoding an isoenzyme of methyl coenzyme M reductase from hyperthermophilic *Methanothermobacter ferredoxinus*. *Mol. Gen. Genet.* 243, 198–206.
- [47] Pihl, T.D., Sharma, S. and Reeve, J.N. (1994) Growth phase-dependent transcription of the genes that encode the two methyl coenzyme M reductase isoenzymes and N super(5)-methyltetrahydromethanopterin: coenzyme M methyltransferase in *Methanobacterium thermoautotrophicum* Delta H. *J. Bacteriol.* 176, 6384–6391.
- [48] Thiele, J.H. and Zeikus, J.G. (1988) Control of interspecies electron flow during anaerobic digestion: significance of formate transfer versus hydrogen transfer during syntrophic methanogenesis in flocs. *Appl. Environ. Microbiol.* 54, 20–29.
- [49] Lovley, D.R. (1985) Minimum threshold for hydrogen metabolism in methanogenic bacteria. *Appl. Environ. Microbiol.* 49, 1530–1531.
- [50] Lansdown, J.M., Quay, P.D. and King, S.L. (1992) CH₄ production via CO₂ reduction in a temperate bog: a source of 13-C-depleted CH₄. *Geochim. Cosmochim. Acta* 56, 3493–3503.
- [51] Boone, D.R., Whitman, W.B. and Rouvière, P. (1993) Diversity and taxonomy of methanogens. In: *Methanogenesis* (Ferry, J.G., Ed.), pp. 35–80. Chapman and Hall, New York.
- [52] Wagner, D. and Pfeiffer, E.M. (1997) Two temperature optima of methane production in a typical soil of the Elbe river marshland. *FEMS Microbiol. Ecol.* 22, 145–153.
- [53] Fey, A. and Conrad, R. (2000) Effect of temperature on carbon and electron flow and on the archaeal community in methanogenic rice field soil. *Appl. Environ. Microbiol.* 66, 4790–4797.