Global Change Biology (2003) **9,** 1451–1457

Molecular analysis of methanogenic archaeal communities in managed and natural upland pasture soils

GRAEME W. NICOL, L. ANNE GLOVER and JAMES I. PROSSER
Department of Molecular and Cell Biology, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, Scotland, UK

Abstract

Grassland management influences soil archaeal communities, which appear to be dominated by nonthermophilic crenarchaeotes. To determine whether methanogenic *Archaea* associated with the *Euryarchaeota* lineage are also present in grassland soils, anaerobic microcosms containing both managed (improved) and natural (unimproved) grassland rhizosphere soils were incubated for 28 days to encourage the growth of anaerobic *Archaea*. The contribution of potential methanogenic organisms to the archaeal community was assessed by the molecular analysis of RNA extracted from soil, using primers targeting all *Archaea* and *Euryarchaeota*. Archaeal RT-PCR products were obtained from all anaerobic microcosms. However, euryarchaeal RT-PCR products (of putative methanogen origin) were obtained only from anaerobic microcosms of improved soil, their presence coinciding with detectable methane production. Sequence analysis of excised denaturing gradient gel electrophoresis (DGGE) bands revealed the presence of euryarchaeal organisms that could not be detected before anaerobic enrichment. These data indicate that nonmethanogenic *Crenarchaeota* dominate archaeal communities in grassland soil and suggest that management practices encourage euryarchaeal methanogenic activity.

Keywords: Archaea, DGGE, diversity, methane, methanogenesis, RT-PCR, 16S rRNA, soil,

Received 6 December 2002; revised version received 18 April 2003 and accepted 21 July 2003

Introduction

Methane flux within the soil represents a balance between methane uptake, by methanotrophic *Bacteria*, and production, by methanogenic *Archaea*. Net methane emission occurs in soils experiencing anoxic conditions, e.g., paddy fields and peat bogs. Even in well-drained, oxic soils, however, anoxic microenvironments will exist and provide the conditions required for methanogenesis (West & Schmidt, 2002). A number of environmental factors, in particular water potential, are important in determining the balance between methane uptake and emission (Topp & Pattey, 1997; Wachinger *et al*., 2000). Methane production can be induced rapidly in grassland soils (Kammann *et al*., 2001), with net production reported in forest and grassland soils with a high water content (Castro *et al*., 2000; Hu *et al*., 2001). While environmental conditions will influence methane production rates, the potential for methanogenesis will also depend on the numbers of methanogens present when conditions become anaerobic. All cultivated methanogens are prokaryotes belonging to the kingdom *Euryarchaeota*, within the domain Archaea. They are strict anaerobes requiring low redox potentials for growth and activity (Le Mer & Roger, 2001) and have been isolated from submerged soils, animal digestive tracts, geothermal springs and sediments (Garcia *et al*., 2000), and well-drained soils (Peters & Conrad, 1995).

Recent molecular studies have demonstrated the presence of archaeal 16S rRNA gene sequences in agricultural, grassland and forest soils, but these are dominated by sequences typical of the other established archaeal kingdom, the *Crenarchaeota* (see, for example, Bintrim *et al*., 1997; Jurgens *et al*., 1997). In one such study, Nicol *et al*. (2003) analysed archaeal communities in grassland soils that had been subjected for 30 years to different management regimes, including sowing of

Correspondence: James I. Prosser, tel. + 44 1224 555848, fax + 44 1224 555844, e-mail: j.prosser@abdn.ac.uk

different grass species, inorganic N fertilization and sheep grazing. 16S rRNA gene fragments were amplified from environmental DNA and RNA by PCR and RT-PCR amplification, respectively, using primers designed to amplify archaeal 16S rRNA gene sequences. The analysis of cloned PCR products and excised bands indicated the presence of crenarchaeotal sequences, but euryarchaeotal sequences, if present, were not sufficiently abundant for detection under these conditions. Nicol *et al*. (2003) also used DGGE analysis to demonstrate the influence of the management regime on the relative abundance and relative activities of different members of the archaeal community. The sequences representative of methanogens may not have been detected from these soils for a number of reasons. Methanogens may not be present in these upland pasture rhizosphere soils, or they may be a minor component of the archaeal community relative to the crenarchaeotes. Bias associated with lysis or the choice of primer sets could also exist, resulting in the preferential amplification of crenarchaeotal sequences. In addition, cultured representatives of the nonthermophilic *Crenarchaeota* have not yet been obtained and their metabolic potential is unknown. Their role in methanogenesis cannot, therefore, be ruled out.

The aim of this study was to determine whether methanogenic *Archaea* were present in these grassland soils and, if so, to determine whether their relative abundance and methanogenic activity were influenced by the management regime. RT-PCR was used to determine changes in active members of the community, as rRNA levels are likely to respond more rapidly to changing conditions than the cell number, particularly if growth is slow. Experiments were carried out in controlled anaerobic soil microcosms, with methane production assessed by gas chromatography.

Methods and materials

Collection and storage of soil

Rhizosphere soil samples were collected in May 1999 from two grassland pasture areas at Fassett Hill, Sourhope Research Station, Borders region, Scotland (map reference NT 850 205) associated with different management practices designated improved and unimproved. The improved grassland vegetation was dominated by *Lolium perenne* (perennial ryegrass), *Trifolium repens* (white clover) and *Cynosurus cristatus* (crested dog's-tail) vegetation. The plots received three fertilizer additions annually – 50 kg N ha^{-1} in March and August and 40 kg N ha^{-1} N:P:K (40:20:20) in May – and were grazed intensively by sheep during the spring, summer and autumn. The unimproved grassland represents natural grassland, with plant communities dominated by *Festuca ovina* (fescue), *Agrostis capillaries* (common bent) and *Galium saxatile* (heath bedstraw). These plots received no fertilizer and were grazed by sheep in summer only. Ten soil cores (8 cm diameter × 8 cm depth) were sampled randomly from within a 5 m × 5 m quadrat, placed randomly in three different sub-plots. Cores were bulked and homogenized through a 3.35 mm sieve to remove large stones and plant material and homogenized soil was stored at 4 °C prior to establishing the microcosms within 2 weeks.

Preparation and monitoring of soil microcosms

Soil microcosms were established in triplicate and consisted of 50 mL Duran glass bottles containing 5 g (dry weight) homogenized rhizosphere soil from bulked, homogenized samples of improved and unimproved grasslands. Microcosms were incubated for 28 days at 25 °C with 20 mL of sterile phosphate buffer (50 mM KH_2PO_4, 17 mM NaCl, 0.2 mM $MgCl_2$, pH 7.0), which had previously been used to enrich methanogenic *Archaea* on rice roots (Lehmann-Richter *et al*., 1999). Bottles were capped with butyl rubber subaseals and the headspace gas was flushed with oxygen-free nitrogen (British Oxygen, Surrey, UK) for 5 min using two hypodermic needles for gas supply, through butyl rubber tubing, and evacuation to the atmosphere. The headspace gas was flushed in a similar manner at 5 day intervals to prevent the accumulation of gas and overpressure. A thin layer of silicon rubber was smeared on subaseals following gas sampling to seal holes and prevent leaks.

Analysis of methane production

Microcosms were destructively sampled immediately after they were established and after measurement of the headspace methane concentration at 28 days. The soil was then frozen at –20 °C prior to molecular analysis. The headspace methane concentrations were determined immediately after sampling by gas chromatography as described previously (James *et al*., 1998). Atmospheric temperature and pressure were recorded, allowing the measurement of methane concentrations at standard temperature and pressure.

Molecular analysis of archaeal populations

Two nested RT-PCR approaches were used for the molecular analysis of archaeal communities in the anaerobic microcosms, permitting characterization of both total archaeal and euryarchaeal communities. Ribosomal RNA was extracted from 1 g (dry weight) soil samples using a bead-beating protocol described

previously (Nicol *et al*., 2003). Two 8 μL aliquots of each individual 16S rRNA extract were treated with DNase (RQ1 Dnase; Promega, Southampton, UK) before performing reverse transcription (Superscript RNase H^- reverse transcriptase; Life Technologies, Strathclyde, UK) using primers Ar9r (Jurgens *et al*., 1997) and 1100Ar (Embley *et al*., 1992) to select for the total archaeal and euryarchaeal rRNA gene fragments, respectively. For subsequent PCR amplification of the total archaeal cDNA, the primer set Ar3f (Giovannoni *et al*., 1988)/Ar9r (Jurgens *et al*., 1997) was used with the following cycling conditions: 95 °C for 5 min; followed by 5 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; followed by 30 cycles of 92 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; followed by 72 °C for 10 min. This primer set has been used to amplify archaeal 16S rDNA sequences from both the *Crenarchaeota* and *Euryarchaeota* lineages (although it does not exhibit complete identity with all archaeal sequences). PCR amplification selective for the euryarchaeal community was performed with the primer set 1Af/1100Ar (Embley *et al*., 1992) using the following cycling conditions: 95 °C for 5 min; followed by 10 cycles of 94 °C for 1 min, 50 °C for 30 s, 72 °C for 2 min; followed by 30 cycles of 92 °C for 1 min, 55 °C for 30 s, 72 °C for 2 min 30 s; followed by 72 °C for 10 min. The PCR products from these primary amplifications of archaeal and euryarchaeal gene fragments were then nested using the primer set SAf (Nicol *et al*., 2003)/PARCH519r (Øvreås *et al*., 1997) producing amplicons 117 bp in length (excluding incorporated primers) with the following cycling conditions: 95 °C for 5 min; followed by 5 cycles of 94 °C for 30 s, 53.5 °C for 30 s, 72 °C for 1 min; followed by 30 cycles of 92 °C for 30 s, 53.5 °C for 30 s, 72 °C for 1 min; followed by 72 °C for 10 min. All the PCR amplifications were performed in 50 μL reactions on a Hybaid Omn-E thermal cycler with hot-lid (Hybaid, Middlesex, UK) using *Thermus flavus* Biopro DNA polymerase (Bioline, London, UK) at 1 unit per reaction, $MgCl_2$ at 2 mM, dNTPs at 1 mM and each primer at 0.4 μM. The DGGE analysis of nested PCR products was performed using a DCode Universal Mutation Detection System (Bio-Rad, Hertfordshire, UK), and DGGE bands were recovered, processed and sequenced as previously described (Nicol *et al*., 2003).

Results and discussion

Detection of methanogenic activity following anaerobic incubation

Soil microcosms were incubated for 28 days, during which the headspace gas was purged regularly with N_2 gas. The final purge was carried out 2 days before measuring the headspace methane concentrations, ensuring that the methane detected resulted from recent methanogenic activity. After 28 days, the production of gas bubbles was observed with improved soil microcosms, and concentrations of methane in the headspace gas of each microcosm after triplicate measurements were 27 (SE 4), 29 (SE 1) and 216 (SE 3) nmol mL^{-1}. These values were significantly greater than the limit of detection, which was $\sim$3 nmol mL^{-1}. Bubbles were not observed in microcosms containing unimproved soil, and methane could not be detected in the headspace. The limit of detection was approximately 40-fold greater than the atmospheric methane concentration (0.076 nmol mL^{-1}) and low levels of methane production in these microcosms would therefore have been undetectable. Methanogenesis could have occurred during the early stages of incubation in unimproved soil, and decreased to undetectable levels by 28 days. However, this is considered unlikely, as typical methane production kinetics in similar experimental systems result in continuous production for several weeks (Peters & Conrad, 1996). A lag period greater than 28 days would also have prevented the detection of methanogenesis. In addition, the use of phosphate buffer may have inhibited acetoclastic methanogenesis in both soil types (Conrad *et al*., 2000). Nevertheless, assuming any biases to be similar in different treatments, the data demonstrate a difference in the potential for methane production between the two soils, and their ability to produce significant quantities of methane should conditions become favourable through the generation of anoxic conditions.

Analysis of archaeal community structure by RT-PCR and DGGE analysis

Changes in the archaeal community were determined by analysis of 16S rRNA extracted at the beginning and end (28 days) of anaerobic incubation. Incubation in phosphate buffer altered the appearance and structure of the soil, which had a sediment-like consistency at the end of the incubation period. Agarose gel electrophoresis of crude nucleic acid extract showed a visually detectable decrease in the quantity of RNA present in 1 g (dry weight) soil samples after anaerobic incubation, indicating a decrease in metabolic activity and/or biomass concentration.

RNA was used as a template for RT-PCR and DGGE analysis to profile the archaeal community with greatest presumptive activity. To assess the contribution of methanogenic *Archaea* within the archaeal community, RT-PCR was performed twice on each extract. In the first, reverse transcription and first-round PCR were carried out using the primer set Ar3f/Ar9r, designed to

target all *Archaea*. In the second, the primer set 1Af/1100Ar (Embley *et al.*, 1992) was used to target the *Euryarchaeota* lineage, which contains all known methanogens. The RT-PCR products from each of these amplification reactions were then used as templates for the second round of a nested PCR amplification using the primer set SAf/PARCH519r, which is broadly specific to both *Euryarchaeota* and *Crenarchaeota* sequences. DGGE bands co-migrating after the analysis of a sample from both first-round primer sets would be of euryarchaeotal (and potentially methanogen) origin. In addition, within-lane differences in the relative intensity of bands provide information on changes in the relative abundance of different components of the archaeal community.

The PCR products were detected in all samples using primers Ar3f/Ar9r, which provide broad specificity to the archaeal domain (Jurgens *et al.*, 1997). However, using 1Af/1100Ar, which would be expected to target only euryarchaeotal sequences, nested PCR products were detected only in 28-day samples from anaerobic microcosms containing improved soil. This correlated with the detection of methane in headspace gas. While biases associated with PCR-based strategies, including nested amplification, are inevitable, and may have influenced representation of the archaeal community, PCR products that are representative of methanogenic organisms were detected only in those microcosms in which methane production was detected.

DGGE profiles of nested PCR products generated after first-round amplification using the primer set Ar3f/Ar9r, with broad specificity for the archaeal domain, indicated changes in the archaeal communities during anaerobic incubation (Fig. 1a). A marker lane was run alongside the samples containing PCR fragments generated from an archaeal clone library containing sequences that belonged to the *Crenarchaeota* lineage (Nicol *et al.*, 2003). Bands migrating to the position marked 'b' (co-migrating with the SUPA5 marker) showed the greatest relative (within-lane) intensity in control and anaerobic soil samples from both grassland types. In profiles from improved soil samples, however, a band migrating to position 'a' (co-migrating with marker SUPA2) generally decreased in relative intensity

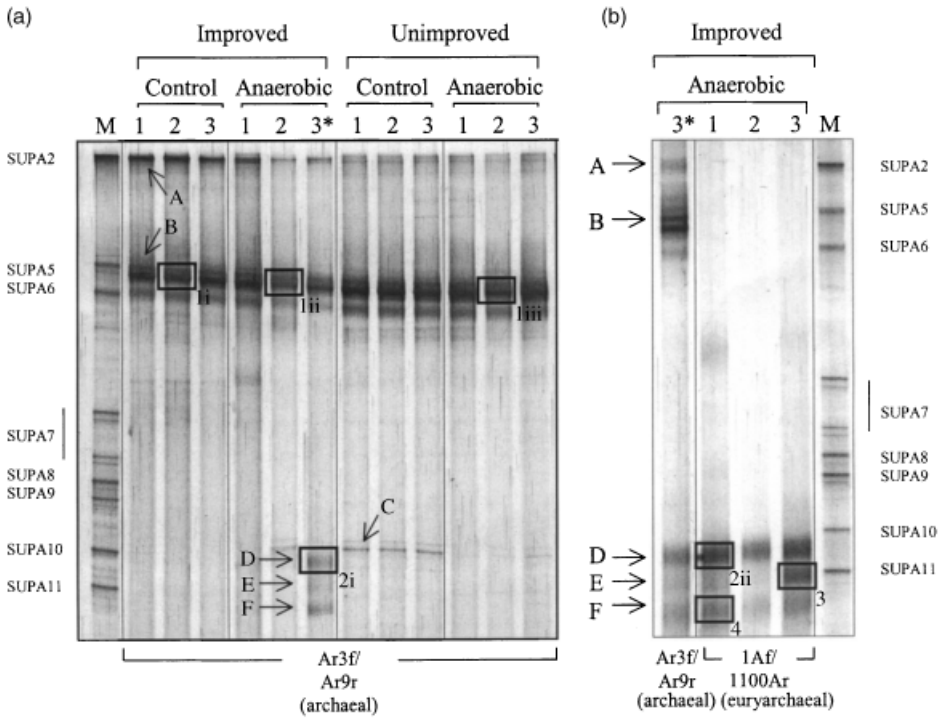

Fig. 1 DGGE analysis of 16S-rRNA-defined archaeal communities in triplicate improved and unimproved rhizosphere soil microcosms before and after anaerobic incubation for 28 days. Archaeal-specific nested amplification was performed using the primer set SAf/PARCH519r on first-round RT-PCR products, which used the primer set Ar3f/Ar9r to target *Archaea* (a) and 1Af/1100Ar to target *Euryarchaeota* (b). The Ar3f/Ar9r PCR products obtained from the third microcosm of anaerobically incubated improved soil (*) were run alongside 1Af/1100Ar PCR products. Band positions A–F are discussed in the text. Bands highlighted by a box were successfully excised, reamplified and sequenced. M denotes the marker lane composed of SAf/PARCH519r PCR products from cloned grassland archaeal 16S rDNA sequences SUPA2, 5, 6, 7, 8, 9, 10 and 11 (accession numbers AF512958 and AF512961 to AF512967).

following anaerobic incubation. Some bands were present in anaerobically incubated, improved soil profiles, but absent from control soil profiles. In improved anaerobic microcosm 3, bands appeared at positions 'd', 'e' and 'f'. The greater relative intensity of these three bands in improved anaerobic microcosm 3 correlated with greater methane production in this microcosm, compared with improved anaerobic microcosms 1 and 2, where bands at these positions could not be observed. Care must be exercised in attempting to quantify data from DGGE gels, as differences in PCR amplification kinetics, quantities of DNA loaded and other factors can lead to bands of different intensity. Qualitative information only may therefore be obtained by comparisons between lanes. However, comparison of band intensities within lanes enables evaluation of the relative abundance of different sequence types and may be used to assess changes in the relative abundance of different components of the population (Stephen *et al*., 1998).

No new band appeared in the profiles from unimproved samples after anaerobic incubation. This was consistent with the inability to detect methane production in these soils and the lack of detectable PCR products following amplification with the 1Af/1100Ar primer set. The only significant change in the DGGE profiles was a decrease in the relative intensity of bands migrating to position 'c' in anaerobic microcosms.

The DGGE profiles of nested PCR products from improved soil samples generated using the primer set 1Af/1100Ar are shown in Fig. 1b, where they are compared with the profile from microcosm 3 derived from the primer set Ar3f/Ar9r, which generated bands at positions 'd', 'e' and 'f'. The bands migrating to positions marked 'a' and 'b' (co-migrating with SUPA2 and SUPA5, respectively) have previously been demonstrated to be characteristic crenarchaeotal bands derived from improved grassland rhizosphere soil (Nicol *et al*., 2003) and, as expected, were not observed in *Euryarchaeota* biased profiles. The bands that appeared at positions 'd', 'e' and 'f' in the Ar3f/Ar9r (archaeal) profiles were dominant in the 1Af/1100Ar (euryarchaeotal) profiles. This would lead to the inference that these bands are from methanogenic *Archaea*, and illustrates that the primer set Ar3f/Ar9r can amplify sequences from both *Crenarchaeota* and *Euryarchaeota*, and that crenarchaeotes are the dominating *Archaea* in the upland pasture rhizosphere soil (Table 1).

To confirm the presence of both *Crenarchaeota* and *Euryarchaeota* in improved grassland rhizosphere soil, a limited number of bands, highlighted in Fig. 1, were excised from DGGE gels and sequenced. Sequences in the GenBank database with greatest similarity, indicated by BLASTn searches, are described in Table 2. Sequences of the three bands (1i, 1ii and 1iii), co-migrating with crenarchaeote sequence SUPA5, were identical over the length compared (the greatest being 109 bp). Sequences of the three bands excised and sequenced, which appeared only after anaerobic enrichment (2i/ii, 3 and 4), displayed high sequence similarity (98–100%) to methanogenic *Euryarchaeota* lineages found within flooded environments, e.g. rice field soil, freshwater lake and freshwater sediment (Chin *et al*., 1999; Jurgens *et al*., 2000; Stein *et al*., 2001). Sequences from bands 2 and 3 showed greatest similarity (100% and 98%, respectively) to sequences placed within the uncultivated 'Rice I cluster', members of which have been demonstrated to be methanogenic (Lueders *et al*., 2001), and band 4 showed greatest similarity (99%) to organisms placed within the *Methanosarcina*. Flooding of microcosms with phosphate buffer provided similar conditions, and the detection of similar sequences might therefore be expected. However, the results indicate that soil

Table 1 Detection of methane and nested RT-PCR products after first round amplification with archaeal (Ar3F/Ar9R) and euryarchaeal (1Af/1000Ar) 16S rRNA gene primers from improved and unimproved soil in triplicate microcosms before (control soil) and after (anaerobic soil) anaerobic incubation for 28 days

First round primers	Improved control soil			Improved anaerobic soil			Unimproved control soil			Unimproved anaerobic soil			RT and PCR controls		
	1	2	3	1	2	3	1	2	3	1	2	3	N^1	N^2	P
Ar3F/Ar9R	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+
1Af/1000Ar	–	–	–	+	+	+	–	–	–	–	–	–	–	–	+
Methane detected				+	+	+				–	–	–			

N^1 = negative control (no template), N^2 = negative control (no reverse transcriptase), P = positive control (*H. salinarum* DNA as template in PCR). + indicates PCR product obtained/methane detected; – indicates no PCR product obtained/methane not detected.

Table 2 Closest matches (name (*accession number*)) to database sequences of archaeal 16S rRNA gene fragment sequences excised from DGGE bands (Fig. 1) following analysis of improved soil samples incubated anaerobically for 28 days. Sequences (102 bp) were compared to those held in the GenBank database using the BLASTn search tool. The referenced phylogenetic placement of the most closely related sequences is described. The sequences of bands 1–4 have been deposited in the GenBank database with the prefix AGSA (anaerobic grassland soil *Archaea*) and the accession numbers AY176168 to AY176171

Band	Closest match	% Similarity	Kingdom	Lineage of closest relatives	Lineage reference
1 (i, ii, iii)	SUPA5 (*AF512961*) FRB32x2 (*AY016483*) FRC9 (*AY016496*) FRD25x2 (*AY016500*) FRD25B (*AY016499*)	100% (109/109)	*Crenarchaeota*	Group 1.1c	Nicol *et al.* (2003)
2 (i, ii)	VAL33-1 (*AJ131277*)	100% (102/102)	*Euryarchaeota*	VAL II 1 Rice cluster	Jurgens *et al.* (2000), Ramakrishnan *et al.* (2001)
3	S15-3 (*AJ236484*) S30-9 (*AJ236517*) AS17-14 (*AF225675*)	98% (100/102)	*Euryarchaeota*	Rice cluster 1 Rice cluster1 Rice cluster 1	Chin *et al.* (1999) Chin *et al.* (1999) Friedrich (2000)
4	ARG4 (*AF293015*) S15-15 (*AJ236496*) + 15 other sequences	99% (101/102)	*Euryarchaeota*	*Methanosarcina* *Methanosarcina*	Stein *et al.* (2001) Chin *et al.* (1999)

management practices resulted in differences in the relative abundance *of Archaea* in well-drained grassland soils.

In both sets of anaerobic microcosms, SUPA5-like crenarchaeotes were the dominant *Archaea* and both crenarchaeotal and euryarchaeotal sequences have been retrieved from methanogenic rice paddy soil microcosms (Großkopf *et al.*, 1998; Fey & Conrad, 2000). Soil crenarchaeotes could be methanogenic, but their presence in anaerobic microcosms with no detectable levels of methane and lack of increases in the relative abundance of crenarchaeotal sequences during methane production suggest that the crenarchaeotes present in these soil samples were not methanogenic.

In conclusion, this study provides preliminary data indicating a role for euryarchaeotal methanogens, but not crenarchaeotes, in methanogenesis in well-drained grassland soils. The data also indicate that management strategies applied to these grasslands, involving change in plant cover and application of inorganic nitrogen, may increase methanogenic populations and potential methanogenic activity. More extensive studies are required to determine the conditions leading to the growth and survival of methanogens in these soils and their activity when conditions become favourable for methanogenesis.

Acknowledgements

The authors would like to thank Dr Irene Watson-Craik (University of Strathclyde, UK) for assistance with gas chromatography. Graeme Nicol acknowledges receipt of a Natural Environment Research Council Postgraduate Research Studentship.

References

Bintrim SB, Donohue TJ, Handelsman J *et al.* (1997) Molecular phylogeny of archaea from soil. *Proceedings of the National Academy of Sciences, USA*, **94**, 277–282.

Castro MS, Gholz HL, Clark KL *et al.* (2000) Effects of forest harvesting on soil methane fluxes in Florida slash pine plantations. *Canadian Journal of Forest Research*, **30**, 1534–1542.

Chin K-J, Lukow T, Conrad R (1999) Effect of temperature on structure and function of the methanogenic archaeal community in an anoxic rice field soil. *Applied and Environmental Microbiology*, **65**, 2341–2349.

Conrad R, Klose M, Claus P (2000) Phosphate inhibits acetotrophic methanogenesis on rice roots. *Applied and Environmental Microbiology*, **66**, 828–831.

Embley TM, Finlay BJ, Thomas RH *et al.* (1992) The use of rRNA sequences and fluorescent probes to investigate the phylogenetic positions of the anaerobic ciliate *Metopus palaeformis* and its archaeobacterial symbiont. *Journal of General Microbiology*, **138**, 1479–1487.

Fey A, Conrad R (2000) Effect of temperature on carbon and electron flow and on the archaeal community in methanogenic rice field soil. *Applied and Environmental Microbiology*, **66**, 4760–4797.

Garcia JL, Patel BKC, Ollivier B (2000) Taxonomic, phylogenetic, and ecological diversity of methanogenic *Archaea*. *Anaerobe*, **6**, 205–226.

Giovannoni SJ, Delong EF, Olsen GJ (1988) Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial-cells. *J Bacteriol*, **170**, 720–726.

Großkopf R, Janssen PH, 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. *Applied and Environmental Microbiology*, **64**, 960–969.

Hu RG, Kusa K, Hatano R (2001) Soil respiration and methane flux in adjacent forest, grassland, and cornfield soils in Hokkaido, Japan. *Soil Science and Plant Nutrition*, **47**, 621–627.

James AG, Watson-Craik IA, Senior E (1998) The effects of organic acids on the methanogenic degradation of the landfill leachate molecules butyrate and valerate. *Water Research*, **32**, 792–800.

Jurgens G, Glockner FO, Amann R *et al*. (2000) Identification of novel *Archaea* in bacterioplankton of a boreal forest lake by phylogenetic analysis and fluorescent *in situ* hybridisation. *FEMS Microbiology Ecology*, **34**, 45–56.

Jurgens G, Linstrom K, Saano A (1997) Novel group within the kingdom *Crenarchaeota* from boreal forest soil. *Applied and Environmental Microbiology*, **63**, 803–805.

Kammann C, Grünhage L, Jäger H-J *et al*. (2001) Methane fluxes from differentially managed grassland study plots: the important role of CH_4 oxidation in grassland with a high potential for CH_4 production. *Environmental Pollution*, **115**, 261–273.

Le Mer J, Roger P (2001) Production, oxidation, emission and consumption of methane by soils: a review. *European Journal of Soil Biology*, **37**, 25–50.

Lehmann-Richter S, Großkopf R, Liesack W *et al*. (1999) Methanogenic *Archaea* and CO_2-dependent methanogenesis on washed rice roots. *Environmental Microbiology*, **1**, 159–166.

Lueders T, Friedrich M (2000) Archaeal population dynamics during sequential reduction processes in rice field soil. *Applied and Environmental Microbiology*, **66**, 2732–2742.

Lueders T, Chin K-J, Conrad R *et al*. (2001) Molecular analyses of methyl-coenzyme M reductase á-subunit (*mrcA*) genes in rice field soil and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage. *Environmental Microbiology*, **3**, 194–204.

Nicol GW, Glover LA, Prosser JI (2003) The impact of grassland management on archaeal community structure in upland pasture rhizosphere soil. *Environmental Microbiology*, **5**, 152–162.

Øvreås L, Forney L, Daae FL *et al*. (1997) Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Applied and Environmental Microbiology*, **63**, 3367–3373.

Peters V, Conrad R (1995) Methanogenic and other strictly anaerobic bacteria in desert soil and other oxic soils. *Applied and Environmental Microbiology*, **61**, 1673–1676.

Peters V, Conrad R (1996) Sequential reduction processes and initiation of CH_4 production upon flooding of oxic upland soils. *Soil Biology and Biochemistry*, **28**, 371–382.

Ramakrishnan B, Lueders T, Dunfeld PF *et al*. (2001) Archaeal community structures in rice soils from different geographical regions before and after initiation of methane production. *FEMS Microbiology Ecology*, **37**, 175–186.

Stein LY, LaDuc MT, Grundl TJ *et al*. (2001) Bacterial and archaeal populations associated with freshwater ferromanganous micronodules and sediments. *Environmental Microbiology*, **3**, 10–18.

Stephen JR, Kowalchuk GA, Bruns MAV *et al*. (1998) Analysis of beta-subgroup proteobacterial ammonia oxidizer populations in soil by denaturing gradient gel electrophoresis analysis and hierarchical phylogenetic probing. *Applied and Environmental Microbiology*, **64**, 2958–2965.

Topp E, Pattey E (1997) Soils as sources and sinks for atmospheric methane. *Canadian Journal of Soil Science*, **77**, 167–178.

Wachinger G, Fiedler S, Zepp K *et al*. (2000) Variability of soil methane production on the micro-scale: spatial association with hot-spots of organic material and archaeal populations. *Soil Biology and Biochemistry*, **32**, 1121–1130.

West AE, Schmidt SK (2002) Endogenous methanogenesis stimulates oxidation of atmospheric CH_4 in alpine tundra soil. *Microbial Ecology*, **43**, 408–415.