The *mcrA* gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill

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Inferred amino acid sequences of the methyl coenzyme-M reductase (*mcrA*) gene from five different methanogen species were aligned and two regions with a high degree of homology flanking a more variable region were identified. Analysis of the DNA sequences from the conserved regions yielded two degenerate sequences from which a forward primer, a 32-mer, and a reverse primer, a 23-mer, could be derived for use in the specific PCR-based detection of methanogens. The primers were successfully evaluated against 23 species of methanogen representing all five recognized orders of this group of *Archaea*, generating a PCR product between 464 and 491 bp. Comparisons between the *mcrA* and 16S small subunit rRNA gene sequences using PHYLIP demonstrated that the tree topologies were strikingly similar. Methods were developed to enable the analysis of methanogen populations in landfill using the *mcrA* gene as the target. Two landfill sites were examined and 63 clones from a site in Mucking, Essex, and 102 from a site in Odcombe, Somerset, were analysed. Analysis revealed a far greater diversity in the methanogen population within landfill material than has been seen previously.

Keywords: methanogenic *Archaea*, methyl coenzyme-M reductase, detection, identification

INTRODUCTION

Methanogenic archaea are vital in the anaerobic microbial degradation of organic waste with the resultant production of methane, a gas with potential for environmental harm, but also possible value as a non-fossil fuel. However, despite their key role as the terminal oxidizers in a complex microbial community, controlling the flux of carbon through the anaerobic degradative pathway in landfill, very little is known about methanogen community structure. While some of the methanogenic species in landfill have been recognized (Fielding *et al.*, 1988; Ladapo & Barlaz, 1997; Maule *et al.*, 1994), no satisfactory method has been established for the rapid detection and identification of methanogens present in landfill samples. Current methods may involve an enrichment step, which can introduce bias, or they are technically complex and

time-consuming. A method extensively used to detect methanogens in sewage sludge, the monitoring of the fluorescent co-factor F_{420} , gives an overall idea of methanogenic potential (Gorris *et al.*, 1988), but does not provide any information on the composition of the methanogen communities present (Peck & Archer, 1989). Unfortunately, it has been found that recovery of F_{420} from landfill material is difficult, making detection from these samples imprecise (Archer & Peck, 1989).

Our chosen alternative was to develop PCR-based techniques to investigate naturally occurring methanogen populations. Whilst many groups have used the amplification and analysis of 16S rRNA genes to unravel the complexities of microbial populations in environmental samples, our approach was to simplify the analysis by using PCR to generate methanogen-specific DNA fragments. The DNA sequence of each fragment could then be established and these data used to construct phylogenetic trees to enable comparisons with 16S rRNA phylogenetic trees, for which there is far more sequence data available.

The target for this methanogen-specific PCR was the terminal enzyme complex in the methane generation

Abbreviations: MCR, methyl coenzyme-M reductase; OTU, operational taxonomic unit.

The GenBank accession numbers for the *mcrA* sequences reported in this paper are AF414034–AF414051 (see Fig. 2) and AF414007–AF414033 (environmental isolates in Fig. 3).

pathway, methyl coenzyme-M reductase (MCR), which catalyses the reduction of a methyl group bound to coenzyme-M, with the concomitant release of methane. This enzyme complex is thought to be unique to, and ubiquitous in, methanogens (Thauer, 1998), making it a suitable tool for their specific detection. Targeting an enzyme also offers the potential for the development of activity-based detection methods based on the analysis of messenger RNA. The MCR operon exists in two forms, MCRI and MCRII. The MCRI form is thought to be present in all methanogens, whilst the MCRII form has only been demonstrated to be present in members of the orders *Methanobacteriales* and *Methanococcales*. One peptide of the MCRI complex, encoded by the *mcrA* gene, was selected as a suitable candidate for the development of PCR-based detection of methanogens. Others have demonstrated the suitability of this marker for the analysis of one of the five recognized orders, the *Methanosarcinales* (Springer *et al.*, 1995).

Further studies have highlighted the use of the *mcrA* gene as the target for the detection of methanogens in a wide range of environments. These have included rice paddies (Lueders *et al.*, 2001), peat bogs (Hales *et al.*, 1996; Lloyd *et al.*, 1998; Nercessian *et al.*, 1999), the guts of termites (Ohkuma *et al.*, 1995) and marine environments (Bidle *et al.*, 1999). None of these studies have confirmed, however, that the primers will detect all known orders of methanogen. Lueders *et al.* (2001) successfully detected methanogens from a range of orders, but failed to detect members of the *Methanomicrobiales*, an order previously found in endosymbionts isolated from the same environment (Grosskopf *et al.*, 1998). They suggested that their *mcrA* primers used for PCR were not of sufficient utility to detect all the orders of the methanogens.

In this paper we describe the development and evaluation of the methanogen-specific PCR approach to the detection of all known orders of methanogen. We confirm that *mcrA* and small subunit rRNA gene phylogenies are remarkably similar, validating this approach. Subsequently, we describe the application of this approach to the phylogenetic analyses of two different landfill methanogen populations.

METHODS

Archaeal strains and culture conditions. All 23 culture collection strains used in this study were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. The majority were type strains as indicated: *Methanobacterium formicicum* DSM 1312, *Methanobacterium bryantii* DSM 863^T, *Methanotermobacter thermoautotrophicus* DSM 1053^T, *Methanotermobacter wolfeii* DSM 2970^T, *Methanobrevibacter ruminantium* DSM 1093^T, *Methanobrevibacter arboriphilicus* DSM 1125^T, *Methanococcus* (now *Methanotorris*) *igneus* DSM 5666^T, *Methanococcus* (now *Methanocaldococcus*) *jannaschii* DSM 2661^T, *Methanococcus* (now *Methanothermococcus*) *thermolithotrophicus* DSM 2095^T, *Methanococcus vannielii* DSM 1224^T, *Methanococcus voltae* DSM 1537^T, *Methanocorpusculum aggregans* DSM 3027^T, *Methanocorpusculum parvum* DSM 3823^T, *Methanoculleus bourgensis*

DSM 3045^T, *Methanofollis liminatans* DSM 4140^T, *Methanomicrobium mobile* DSM 1539^T, *Methanopyrus kandleri* DSM 6324^T, *Methanosaeta concilii* DSM 3671^T, *Methanosarcina barkeri* DSM 800^T, *Methanosarcina mazei* DSM 2053^T, *Methanosphaera stadtmanae* DSM 3091^T, *Methanospirillum hungatei* DSM 864^T and *Methanothermus fervidus* DSM 2088^T. Most cultures were grown in 100 ml medium in 160 ml Wheaton bottles for 7 days at the temperature and in the medium recommended by the DSMZ. The exceptions to this were the thermophiles, which were grown for a maximum of 48 h.

Landfill sample collection and processing. Landfill material was recovered from two landfills, the Cory Environmental Waste Management site at Mucking, Essex, and the Wyvern Waste Management site at Odcombe, Somerset. The Mucking material was new landfill, less than 3 years old, recovered from a depth of 1–10 m using an excavator. The Odcombe material was obtained during drilling operations to install gas abstraction wells. It was obtained from a depth of 5–20 m, it was 2–5 years old and appeared well digested. The landfill material was packed into double-lined gas impermeable plastic sacks and sealed to exclude as much air as possible. Both samples of landfill material were hand-sorted to remove recalcitrant material and placed into model systems incubated at 37 °C. The models were flushed with oxygen-free nitrogen for 10 min to accelerate the establishment of anaerobic conditions. Sterile distilled water was added to the top of these model systems and leachate was collected from a sample port located at the bottom. To maximize the numbers of methanogens, the samples were incubated in the models until methane production had peaked, when it was believed the methanogen population would be at its greatest. Leachate samples were then collected. Cells were recovered immediately from the Odcombe leachate sample. Leachate (50 ml) was centrifuged at 7000 g for 10 min at room temperature. The pellet was resuspended in 0.6 ml TE (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and the DNA was extracted using the RiboLyser technique described below. Leachate from the Mucking sample was inoculated into a methanogen growth medium (DSM 119) and grown for a further 2 weeks prior to processing as described above, for the Odcombe leachate sample.

DNA extraction and recovery. The RiboLyser (Hybaid), a high-speed reciprocal shaker which retains samples in screw-capped tubes containing ceramic and silica beads, was used for DNA isolation. The sample (0.5 ml) was added, together with 0.5 ml Tris/HCl-equilibrated phenol, pH 8.0, and 0.1 ml chloroform/isoamyl alcohol solution (25:1, v/v), to a 'blue' RiboLyser tube and placed into the RiboLyser. The tube was treated for 30 s at 6 m s⁻¹ to maximize the release of DNA, and cooled on ice for 10 s. The phases were separated by centrifugation (10000 g, 10 min, 4 °C) and the aqueous phase was recovered and washed with 0.3 ml chloroform/isoamyl alcohol solution. The aqueous phase was recovered as before and the DNA was precipitated with an equal volume of 2-propanol, followed by an ethanol wash, brief drying of the pellet in a vacuum desiccator and resuspension in 0.1 ml TE.

Oligonucleotide primers. Degenerate primers were designed based on the alignment of five published *mcrA* gene sequences (Allmansberger *et al.*, 1986; Bokranz *et al.*, 1988; Bokranz & Klein, 1987; Cram *et al.*, 1987; Weil *et al.*, 1988) and the identification of two regions with a high degree of homology flanking a more variable region (Fig. 1). The two oligonucleotide primers were a forward primer, 5'-GGTGGTGTMGGA-TTCACACARTAYGCWACAGC-3', and a reverse primer, 5'-TTCATTGCRTAGTTWGGRTAGTT-3'. These oligonucleotides were obtained from the Structural Sciences De-

	351				400
<i>Mc. voltae</i>	GGVGFTQYAT	ASYTDDILDD	FSYYGYEYVE	KKY.G..RCG	...TKA..T
<i>Mc. vannielii</i>	GGVGFTQYAT	AAYTDDILDD	FSYVALDYVE	KKY.G..RMG	...TKA..T
<i>Mt. fervidus</i>	GGVGFTQYAT	ASYTDNILD	FTYYGKEYVE	DKY.G....G	...LAEAPHD
<i>Mbt. thermoauto</i>	GGVGFTQYAT	AAYTDNILD	FTYFGKEYVE	DKY.....G	...LCEAPNT
<i>Msr. barkeri</i>	GGVGFTQYAT	AAYTDDILDN	NTYVDVDYIN	DKYNGAATVG	KDNKVKA..S
	401				450
<i>Mc. voltae</i>	MDVVEDIASE	VTLYALEQYD	EYPALLEDHF	GGSQRAAVAA	AAAGISVC.M
<i>Mc. vannielii</i>	MDVVEDIASE	VTLYSLEQYD	EYPALLEDHF	GGSQRAAVAA	AASGIGVC.M
<i>Mt. fervidus</i>	FDTILDVATE	VTLYSLDQYE	EYPTLMEDHF	GGSQRAAIAA	AASGCSTA.F
<i>Mbt. thermoauto</i>	MDTVLDVASE	VTFYGLEQYE	EYPALLEDQF	GGSQRAAVVA	AAAGCSTA.F
<i>Msr. barkeri</i>	LEVVKDIATE	STLYGIETYE	KFPTALEDHF	GGSQRAATVLA	AAAGVA.CSL
	451				500
<i>Mc. voltae</i>	ATGNSNAGVN	GWYLSQILHK	EYHSRLGFYG	YDLQDQCGAS	NSLAIRNDEA
<i>Mc. vannielii</i>	ATGNSNAGVN	GWYLSQILHK	EYHSRLGFYG	YDLQDQCGAS	NSLAIRNDES
<i>Mt. fervidus</i>	AAGNAQAGLS	GWYLSQILHK	ECHARLGFYG	YDLQDQCGAA	NTYSIRSDEG
<i>Mbt. thermoauto</i>	ATANAQTGLS	GWYLSMYLHK	EQHSRLGFYG	YDLQDQCGAS	NVFSIRGDEG
<i>Msr. barkeri</i>	ATGNANAGLS	GWYLSMYLHK	EAWGRLGFFG	FDLQDQCGAT	NVLSYQGDG
	501				
<i>Mc. voltae</i>	SPLELRGPNY	PNYAMN			
<i>Mc. vannielii</i>	APLELRGPNY	PNYAMN			
<i>Mt. fervidus</i>	LPLELRGPNY	PNYAMN			
<i>Mbt. thermoauto</i>	LPLELRGPNY	PNYAMN			
<i>Msr. barkeri</i>	LPDELRGPNY	PNYAMN			

Fig. 1. Alignment of partial amino acid sequences of the *mcrA* gene from five different methanogens, *Methanococcus* (*Mc.*) *voltae* (X07793), *Methanococcus* (*Mc.*) *vannielii* (M16893), *Methanothermobacter* (*Mt.*) *fervidus* (J03375), *Methanothermobacter* (*Mbt.*) *thermoautotrophicus* (U10036) and *Methanosarcina* (*Msr.*) *barkeri* (Y00158), using the PILEUP alignment tool of the GCG package, with Blosum62 as the scoring matrix and gap creation and gap extension penalties set to 1. Accession numbers in GenBank are shown in parentheses. Consensus sequences used for the design of PCR primers are indicated in bold.

partment, CAMR, and synthesized on an Applied Biosystems 380B DNA synthesizer.

PCR. PCR was performed using the protocol of Innis & Gelfand (1990). Denaturation, annealing and extension were carried out at 95, 55 and 72 °C, respectively, on a Uno II PCR machine (Biometra). Since the primers were degenerate, the ramp in temperature between the annealing temperature and the extension temperature was slowed to 0.1 °C s⁻¹ for the first five cycles of the 40-cycle programme to allow extension of mismatched primers (Compton, 1990). PCR products were analysed on 2% agarose gels using a standard protocol and molecular mass marker V (Boehringer Mannheim).

Cloning. PCR products were purified using GeneClean (Bio 101), ligated into the pCR2.1 plasmid vector of the TA cloning kit (Invitrogen) and transformed into the *E. coli* INVα-F' cells provided, following the manufacturer's instructions. Plasmid DNA was recovered from individual colonies using Gene-Releaser (Bioventures) and used directly in PCR reactions to generate material for restriction fragment analysis and DNA sequencing.

Restriction fragment analysis. PCR product amplified from single clones (10 µl) was digested in PCR reaction buffer by the addition of 10 U *TaqI* and incubation at 65 °C for 1 h. Digested products were run through a 4% agarose gel and visualized by ethidium bromide staining.

DNA sequencing. PCR products were sequenced using one of two methods.

Using the TAQuence cycle sequencing kit (USB). Fresh PCR products were purified using GeneClean and resuspended in a total volume of 40 µl TE. A sample (6 µl) of this DNA was used in the master reaction mix of the sequencing reaction along with 2 pmol ³²P end-labelled PCR primer and other reagents as per the manufacturer's instructions. Twenty cycles of the reaction were found to give the clearest bands when the reaction mixes were run on polyacrylamide gradient sequencing gels (Sambrook *et al.*, 1989).

Using an ABI377 automated fluorescence sequencer. PCR products were reamplified from single clones using chimeric primers, which consisted of the methanogen *mcrA* forward and reverse primers, 3' to the M13 forward (−21) and M13 reverse

primers respectively. The primer sequences were *mcrA*/M13F, 5'-TGTAACACGACGGCCAGTGGTGGTGTMGATT-CACACARTAYGCWACAGC-3', and *mcrA*/M13R, 5'-CAGGAAACAGCTATGACCTTCATTGCRTAGTTWGGRTAGTT-3'. The resultant PCR products were then used directly with M13 forward (−21) and M13 reverse primers in automated sequencing using the fluorescence sequencer.

Phylogenetic analysis. Gene sequences and inferred protein sequences of the *mcrA* gene obtained in this study, and 16S rDNA sequences from GenBank, were aligned using PILEUP in the GCG suite of programs (Wisconsin package version 10.1; Genetics Computer Group), using the Blosum 62 matrix, with gap creation and gap extension penalties both set to 1. Alignments were scrutinized and altered manually where appropriate. Phylogenetic trees were constructed by analysing the data with one of the following algorithms: DNADIST or PROTDIST followed by FITCH or NEIGHBOR; DNAPARS or PROTPARS followed by CONSENSE; all found in PHYLIP (Phylogeny Inference Package version 3.57c; Felsenstein, 1995). Trees were drawn using TREEVIEW, version 1.6.1 (Page, 1996). Bootstrap analysis was carried out using SEQBOOT to generate 100 random combinations of the alignments followed by the algorithms stated previously to generate the trees and CONSENSE to produce a consensus tree and bootstrap values. SEQBOOT is also found in PHYLIP. Bootstrap values were then transposed onto the phylogenetic trees.

RESULTS

Development of a detection method for methanogens

In examining 23 species of methanogens representing the five proposed phylogenetic lineages of methanogenic *Archaea*, we hoped to demonstrate the general applicability of this PCR technique to the detection of all methanogens. Also, as we were interested in developing techniques for all methanogens, we chose a physical shear method for the extraction of DNA. The method of choice involved the use of a RiboLyser, a high-speed bead-beater designed, along with a series of kits, to enable RNA extraction from a range of organisms.

Table 1. Operational taxonomic units (OTUs) and corresponding RFLPs of *TaqI*-digested *mcrA* PCR products from two landfill samples

Analysis of cloned *mcrA* gene PCR products cut with the restriction endonuclease *TaqI*. The fragment sizes were estimated against a 50 bp DNA ladder on 4% agarose gels and confirmed by analysis of the DNA sequences of representative clones from each OTU. The OTUs are shown in the order of initial detection. E and F were found only in the Mucking sample, whereas G–S were found only in the Odcombe sample. Numbers in parentheses indicate the distribution of clones in the same OTU between samples (Mucking, Odcombe). All DNA sequences were obtained in this study except that for *Methanosarcina thermophila* which was obtained from GenBank (accession no. U22250).

OTU	Size of DNA fragments after restriction (bp)	Total fragment size (bp)	Total no. of clones with same restriction pattern	Species with same restriction pattern
A	51, 53, 387	491	19 (18, 1)	<i>Methanofollis liminatans</i> , <i>Methanospirillum hungatei</i>
B	33, 53, 384	470	5 (1, 4)	–
C	9, 21, 170, 267	467	12 (10, 2)	<i>Methanobacterium formicicum</i>
D	464	464	60 (30, 30)	<i>Methanococcus</i> (now <i>Methanocaldococcus</i>) <i>jannaschii</i> , <i>Methanobacterium formicicum mrtA</i>
E	53, 417	470	2	–
F	45, 53, 93, 297	488	2	<i>Methanosarcina thermophila</i>
G	104, 387	491	17	<i>Methanocorpusculum bavaricum</i>
H	104, 185, 202	491	7	<i>Methanocorpusculum aggregans</i> , <i>Methanocorpusculum parvum</i>
I	77, 99, 297	473	2	–
J	104, 154, 233	491	6	–
K	104, 113, 120, 154	491	15	–
L	170, 294	464	3	–
M	491	491	2	–
N	77, 396	473	1	–
O	51, 53, 113, 120, 154	491	6	–
P	104, 184, 203	491	3	–
Q	6, 53, 67, 81, 263	470	1	–
R	86, 93, 288	467	1	–
S	21, 179, 267	467	1	–

Modifications of the standard protocols provided with the kits enabled DNA extraction from a wide range of sources, including liquid cultures and landfill leachate. This general technique, which is able to recover total DNA from a range of sources, would be critical if PCR were to be applied to the qualitative and quantitative analysis of environmental samples.

DNA was successfully extracted from all 23 species of methanogen using the RiboLyser. Once the DNA had been purified, the PCR methodology was evaluated. A single major PCR product of the expected size (464–491 bp) was generated for all 23 methanogen species using the primers described. These primers were those found to give the most consistent results, although other primer pairs were evaluated (data not shown).

To confirm the identity of PCR products, the DNA sequence of each was determined. These sequences were verified as the predicted part of the *mcrA* gene by comparison with the published sequences of the *mcrA* gene. Those evaluated included the five previously published DNA sequences for the *mcrA* gene used in the alignment for the design of the PCR primers.

Eight DNAs from non-methanogen species, seven eubacterial and one archaeal DNA (*Pyrococcus furiosus*) were also evaluated to see if they generated a PCR product with the same primers. Under the conditions outlined for the PCR, none of these DNAs generated a PCR product (data not shown).

Restriction enzyme analysis

Once purified DNA had been recovered from the model reactors, the mixed *mcrA* gene fragments were PCR-amplified and the population of fragments separated by cloning and transformation. A total of 63 Mucking clones and 102 Odcombe clones were screened for the presence of the *mcrA* gene fragment by restriction digestion with *TaqI*. In total six restriction patterns were obtained from the Mucking site and 17 from the Odcombe site, four of which were found in both (Table 1). Screening by restriction analysis allowed the clones to be grouped based on *TaqI* RFLP patterns, reducing the total number of clones that needed to be sequenced. However, several members of each pattern were sequenced to confirm the reliability of this pre-screening.

Fig. 2. Phylogenetic trees showing the relationship between 16S rDNA sequences (A) and partial *mcrA* DNA sequences (B) of methanogens. All 16S rRNA and some *mcrA* sequences were retrieved from GenBank; the remaining *mcrA* sequences, marked in bold, were determined in this study (GenBank accession numbers AF414034–AF414051). Alignments were made using 1532 bp of the 16S rRNA sequence and 440 bp of the *mcrA* sequence with the PILEUP program of the GCG package. Trees were constructed using the DNADIST program with the Kimura two-parameter model, followed by FITCH to construct the trees from the calculated distances. The outgroup used was *Methanopyrus kandleri*. Bootstrap values, indicated at nodes, were calculated from 100 resamplings (values < 50 not shown). All these programs were from PHYLIP (Felsenstein, 1995). The trees were visualized using TREEVIEW. The scalebar represents 0.1 substitutions per base position. Genus names have been abbreviated in agreement with those found in the Ribosomal Database Project as follows: *Mb.*, *Methanobacterium*; *Mbt.*, *Methanothermobacter*; *Mbb.*, *Methanobrevibacter*; *Mcc.*, *Methanococcoides*; *Mc.*, *Methanococcus*; *Mcr.*, *Methanocorpusculum*; *Mcu.*, *Methanoculleus*; *Mf.*, *Methanofollis*; *Mhl.*, *Methanohalobium*; *Mha.*, *Methanohalophilus*; *Mlo.*, *Methanolobus*; *Mm.*, *Methanomicrobium*; *Mp.*, *Methanopyrus*; *Mst.*, *Methanosaeta*; *Msr.*, *Methanosarcina*; *Mpr.*, *Methanosphaera*; *Msp.*, *Methanospirillum*; *Mt.*, *Methanothermus*.

In total, 20 of the 63 Mucking clones and 53 of the 102 Odcombe clones were sequenced.

Phylogenetic analysis

The fragment of the *mcrA* gene corresponding to the conserved Tyrosine amino acid residues between positions 363 and 510 in the alignment in Fig. 1 were used in DNA and protein sequence alignments. The size of the DNA fragment compared varied between 414 and 438 bases, depending on the species. In constructing the phylogenetic trees it was decided to root them. As only methanogens carry the *mcrA* gene, the outgroup chosen for these analyses had to be a methanogen. From

taxonomy and 16S rRNA phylogeny, the deepest branching methanogen so far identified is *Methanopyrus kandleri* (Burggraf *et al.*, 1991) and the *mcrA* gene sequence from this species was therefore chosen as the outgroup.

Phylogenetic trees based on DNA and amino acid sequences of the *mcrA* gene were compared to see if there were any major differences in the phylogenetic trees generated. No major differences were apparent between DNA and amino acid sequences, nor between the different algorithms used. The possibility of chimeric sequences were also checked by separate phylogenetic analyses on the N and C termini of the McrA proteins. No evidence for chimeras were seen (data not shown).

Fig. 3. Phylogenetic tree showing the relationship of predicted *mcrA* amino acid sequences from two landfill sites with characterized methanogens. This tree was constructed from an alignment of sequences made by the PILEUP program of the GCG package, using the PROTDIST (PAM-Dayhoff) and FITCH programs in PHYLIP (Felsenstein, 1995), with *Methanopyrus kandleri* as the outgroup, and visualized using TREEVIEW. Bootstrap values out of 100 resamplings are shown at each node (<50 not shown). Values in parentheses reflect the number of clones detected for each sequence represented and the corresponding OTU. The scalebar represents 0.1 substitutions per amino acid position. Genus names have been abbreviated in agreement with those found in the Ribosomal Database Project as described in the legend to Fig. 2. The GenBank accession numbers for the *mcrA* sequences of the environmental isolates are AF414007–AF414033.

We have chosen to present trees generated from DNA sequence data when comparing *mcrA* and 16S rRNA sequences from known species (Fig. 2), and inferred amino acid sequences when examining environmental samples (Fig. 3), all using DNADIST or PROTDIST followed by FITCH. The use of inferred amino acid sequences reduces the variation seen in the DNA sequence, principally in the third base of the codon, and simplifies the trees by grouping together those with identical amino acid sequence. This is suitable for our purpose, but does mean that some of the finer genetic variation enabling differentiation at the subspecies level is lost. It has been suggested, however, that the third codon position can introduce major bias into a phylogenetic analysis, particularly when the G + C content is similar in unrelated species (Gupta, 1998). The use of amino acid sequences avoids this potential source of error and is more appropriate as we are analysing a functional gene.

Closer consideration of the phylogenetic analysis applied to the landfill samples and comparisons at the

amino acid sequence level of the *mcrA* gene fragments reveals a number of associations. Three groups of *mcrA* sequences clustered within the *Methanobacteriales*. One group showed 92% similarity to *Methanobacterium formicicum*, a second group showed 92.8% similarity to *Methanothermobacter thermoautotrophicus* and a single sequence showed 92.2% to *Methanobrevibacter arboriphilicus*. The *mrtA* sequences of the *Methanobacteriales* were readily distinguishable from the *Methanobacteriales mcrA* sequences, grouping together in a tight cluster on the phylogenetic tree close to the *Methanococcales* and showing between 94.9 and 100% similarity to the *Methanobacterium formicicum mrtA* sequence.

Four major clusters of sequences grouped within the *Methanomicrobiales* and each showed strong similarity to a recognized species: 92.5% to *Methanoculleus bourgensis*, 93.8% to *Methanospirillum hungatei*, 92.4–96% to *Methanocorpusculum bavaricum* and 99.3% to *Methanocorpusculum parvum*. All the sequences grouping within the *Methanocorpusculaceae* were isolated

from the Odcombe site. A single clone, OS77, branching at a deeper point in the tree, showed 74.7% similarity to its closest recognized species, *Methanomicrobium mobile*. Two sequences grouping close to *Methanosarcina barkeri* showed 92.4–93.1% similarity. Another two sequences, OS18 and OS55, both from the Odcombe sample, formed a deep-branching cluster with only 60.7% similarity to their closest recognized species, *Methanosaeta concilii*.

DISCUSSION

The application of PCR-based technologies to the investigation of naturally occurring methanogen populations has many advantages over traditional culture-based methods. No culturing of these fastidious anaerobes is required and novel sequences may be detected, indicating new species, which may be at present unculturable, providing a more complete description of the methanogen community. However, molecular methods introduce their own bias, such as the favoured lysis of one cell type over another, leading to the recovery of unrepresentative DNA fractions or skewed PCR amplification, where certain sequences are favoured over others (Moré *et al.*, 1994; Suzuki & Giovannoni, 1996).

To minimize bias in DNA recovery, physical shear techniques were developed. The RiboLyser was successfully used in recovering DNA from the 23 pure cultures of methanogens covering the broad range of known methanogen phylogeny used in this study. On application to environmental samples, DNA could also be recovered from solid landfill and leachate. Additionally, slight modifications to the protocol enables the recovery of RNA using the RiboLyser, which can be used in studies of activity within methanogen populations.

Springer *et al.* (1995) previously reported that the phylogenetic trees of 16S rRNA, and part of the *mcrA* gene analysed, for the methanogen family *Methanosarcinaceae*, were broadly similar. The far more comprehensive analysis, in terms of the breadth of methanogen phylogeny, examined in this study (Fig. 2B), supports this view, with the five proposed orders of methanogens clearly distinguishable. Indeed, closer comparison at the genus and species level within orders reveals that there is a strong correlation between 16S rRNA and *mcrA* even at this level. This strongly supports the view that we can use the analysis of *mcrA* gene sequences to describe the community present in environmental samples without the need to recover these isolates in culture.

However, there are some anomalies. Whilst the reorganization of the taxonomy of the methanogens as suggested by Boone *et al.* (1993) is broadly supported by our data, some of the more specific taxonomic relationships are not confirmed. An example is the position of *Methanosphaera stadtmanae*, which is considered to be a member of the order *Methanobacteriales*, grouping within this order by 16S rRNA analysis, but not by *mcrA* analysis (Fig. 2). *Methanosphaera stadtmanae* is an unusual

member of the *Methanobacteriales*, having a coccoid shape, unlike all the rest which are rods, and growth is limited to the reduction of methanol by H₂. The *mcrA* sequence we obtained is more closely related to the *mrtA* sequences of other *Methanobacteriales* (Fig. 3). It is therefore possible that either our primers failed to amplify the *Methanosphaera stadtmanae mcrA* gene fragment, or it is no longer present, as we were only able to detect this *mrtA*-like sequence. The rest of the *Methanobacteriales* fall readily into the taxonomic organization proposed by Boone *et al.* (1993) and confirmed by Wasserfallen *et al.* (2000). Similarly, the reorganization of the *Methanococcales* into two families and four genera is supported.

The taxonomy of the *Methanomicrobiales* is more complex with not all of the nine established genera represented on our *mcrA* tree, making overall comparisons difficult. However, the *Methanocorpusculaceae* clearly group together in a coherent group and the recognition of *Methanocorpusculum aggregans* and *Methanocorpusculum parvum* as synonymous (Boone *et al.*, 1993) is supported. The application of the *mcrA* gene in the analysis of the *Methanosarcinales* has been considered before (Springer *et al.*, 1995). An additional observation, supported by our data, is the division of the *Methanosarcinales* into two families, the *Methanosaetaeae*, which is represented by only one species, *Methanosaeta concilii*, in our *mcrA* study, and the *Methanosarcinaceae*, which includes a large number of species.

A potential pitfall in using the *mcrA* gene for detection is the high degree of DNA sequence conservation between *mcrA* and *mrtA*, the equivalent gene in the MCRII complex, a complex found in a limited number of methanogen species. This means that detection based on primers for PCR designed to conserved regions in *mcrA* is also likely to detect *mrtA* sequences, complicating the analysis. Fortunately, the *mrtA* gene sequences form a coherent group close to the *Methanococcales* (Fig. 3) and therefore are readily distinguishable from *mcrA* gene sequences. This close proximity has been reported previously and led to the suggestion that the *mrtA* gene has arisen by lateral transfer from the *Methanococcales* rather than gene duplication (Reeve *et al.*, 1997). There were, however, noticeably more *mrtA* sequences than *mcrA* *Methanobacteriales* sequences, suggesting overrepresentation of this sequence type in the analysis. This is likely to be due to PCR bias, amplifying the *mrtA* sequences preferentially, rather than a wider distribution of *mrtA* genes in the methanogen community, as the *mrtA* gene sequences group closely with a single *Methanobacteriales mrtA* sequence from *Methanobacterium formicicum*.

The principal aim of this study was not to compare 16S rRNA and *mcrA* as rival phylogenetic approaches, but to confirm the validity of using the *mcrA* gene as an alternative phylogenetic tool in the specific detection and identification of methanogens. This was achieved successfully, a comparison of the phylogeny of the *mcrA* and 16S rRNA genes from the five recognized orders of methanogens clearly indicating a strong similarity (Fig.

2). Additionally, unlike other studies, where all the expected orders of methanogens have not been detected in environmental samples (Lueders *et al.*, 2001), our primers were able to amplify the *mcrA* gene from all the known orders using DNA extracted from type species (Fig. 2B), and all the expected orders using DNA extracted from environmental samples (Fig. 3).

The two landfill sites examined both had complex methanogen communities, reflected by a wide distribution of sequences, with a far greater diversity than had previously been described in the literature (Fielding *et al.*, 1988; Maule *et al.*, 1994). This was despite the extensive sample treatment undergone by the solid landfill material collected, which was necessary to convert it into artificial leachate. The reason for using leachate is that it is likely to yield a more representative sample of the microbial community compared to extracting DNA from a small, single solid sample (Barlaz, 1997). Additionally, the artificial leachate from the Mucking sample was then inoculated into a methanogen growth medium in an attempt to boost numbers further. It is therefore unlikely that the populations we examined in this study are accurate representations of the overall methanogen population in the original solid landfill samples, but they will be subsets of this population. No attempts have therefore been made to compare populations as we were seeking only to exemplify the methods developed. Additionally, taking single samples is not statistically valid for the purpose of making comparisons between populations.

Methanobacterium formicicum has been detected previously in landfill, as an endosymbiont of the ciliate protozoan *Metopus palaeformis* (Finlay & Fenchel, 1991) and by oligonucleotide probe analysis to the *mcrA* gene on leachate samples (Maule *et al.*, 1994). Microorganisms antigenically related to *Methanobrevibacter arborophilicus* have been detected in an anaerobic sludge blanket reactor (Grotenhuis *et al.*, 1991), but there are no reports of previous detection in landfill.

Of the four *Methanomicrobiales* group sequences, two have been detected before in landfill, *Methanoculleus bourgensis* and *Methanospirillum hungatei*, using species-specific probes against the *mcrA* gene (Luton, 1996). Fielding *et al.* (1988) isolated a coccoid methanogen from landfill that was thought to be a *Methanogenium* sp. or a *Methanocorpusculum* sp. Mori *et al.* (2000) also reported the isolation of *Methanocalculus pumilus*, a member of the *Methanocorpusculaceae* family, from landfill. The inclusion of the *mcrA* gene sequence from this recently isolated methanogen may have further resolved the large number of clones in these *Methanocorpusculum* clusters. The unclustered sequence, OS77, could represent a novel *Methanomicrobiales* lineage or could simply be a representative of an as yet unsequenced *mcrA* gene from a recognized species. No previous studies have described this level of diversity among the *Methanomicrobiales* in landfill. Additionally, a recent report by Lueders *et al.* (2001) using detection of the *mcrA* gene in rice field soil failed to detect any *Methanomicrobiales*.

Analysis revealed a low number of clones from both samples grouping with the acetoclastic methanogen family, the *Methanosarcinaceae*. In the Mucking site, 2 of the 66 clones examined aligned with the acetoclastic methanogen *Methanosarcina barkeri*; in the Odombe sample no acetoclastic methanogen clones were detected. However, these landfill samples were introduced into laboratory model systems which is likely to have had some effect on methanogen population composition. The Mucking sample, which was subsequently inoculated into a *Methanobacterium* medium, did yield some acetoclastic methanogen sequences as well as a range of *Methanomicrobiales* sequences. The medium therefore was not totally selective for *Methanobacteriales*. Reports of low numbers of acetoclastic methanogens as compared with hydrogenotrophic methanogens in landfill have been reported previously (Fielding & Archer, 1986). Alternatively, the low numbers of *Methanosarcinaceae* could be explained by bias in the PCR where some sequences are amplified in preference to others. Bias in PCR has been observed when amplifying 16S rRNA genes (Reysenbach *et al.*, 1992), when very low template concentration is used (Chandler *et al.*, 1997) or when using degenerate primers (Polz & Cavanaugh, 1998).

Further examination of the OS18 and OS55 clones, which formed a deep-branching cluster separate from the other five orders of methanogens, revealed amino acid sequence similarity of between 55 and 61% with these orders. An additional sequence, uncultured archaeon 85A (GenBank accession no. AF268654), isolated from an anaerobic digester and deposited in GenBank, showed 84% amino acid sequence similarity and also fell into this cluster when included in a phylogenetic analysis. The low values of sequence identity with other methanogenic orders support the conclusion that this cluster of sequences is distinct from any described species of methanogen and may represent an uncultured novel methanogenic lineage. The independent recovery of sequences within this cluster by two groups strengthens the validity of this observation.

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REFERENCES

- Allmansberger, R., Bollschweiler, C., Konheiser, U., Muller, B., Muth, E., Pasti, G. & Klein, A. (1986). Arrangement and expression of methyl CoM reductase genes in *Methanococcus voltae*. *Syst Appl Microbiol* 7, 13–17.
- Archer, D. B. & Peck, M. W. (1989). The microbiology of methane production in landfills. In *Microbiology of Extreme Environments and its Potential for Biotechnology*, pp. 187–204. Edited by M. S. Da Costa, J. C. Duarte & R. A. D. Williams. London: Elsevier.

- Barlaz, M. A. (1997).** Microbial studies of landfills and anaerobic refuse decomposition. In *Manual of Environmental Microbiology*, pp. 541–557. Edited by C. J. Hurst, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach & M. V. Walter. Washington, DC: American Society for Microbiology.
- Bidle, K. A., Kastner, M. & Bartlett, D. H. (1999).** A phylogenetic analysis of microbial communities associated with methane hydrate containing marine fluids and sediments in the Cascadia margin (ODP site 892B). *FEMS Microbiol Lett* **177**, 101–108.
- Bokranz, M. & Klein, A. (1987).** Nucleotide sequence of the methyl coenzyme M reductase gene cluster from *Methanosarcina barkeri*. *Nucleic Acids Res* **15**, 4350–4351.
- Bokranz, M., Baumner, G., Allmansberger, R., Ankel-Fuchs, D. & Klein, A. (1988).** Cloning and characterization of the methyl coenzyme M reductase genes from *Methanobacterium thermoautotrophicum*. *J Bacteriol* **170**, 568–577.
- Boone, D. R., Whitman, W. B. & Rouviere, P. (1993).** Diversity and taxonomy of methanogens. In *Methanogenesis: Ecology, Physiology, Biochemistry and Genetics*, pp. 35–80. Edited by J. G. Ferry. New York: Chapman & Hall.
- Burggraf, S., Stetter, K. O., Rouviere, P. & Woese, C. R. (1991).** *Methanopyrus kandleri*: an archaeal methanogen unrelated to all other known methanogens. *Syst Appl Microbiol* **14**, 346–351.
- Chandler, D. P., Fredrickson, J. K. & Brockman, F. J. (1997).** Effect of PCR template concentration on the composition and distribution of total community 16S rDNA clone libraries. *Mol Ecol* **6**, 475–482.
- Compton, T. (1990).** Degenerate primers for DNA amplification. In *PCR Protocols: a Guide to Methods and Applications*, pp. 38–45. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: Academic Press.
- Cram, D. S., Sherf, B. A., Libby, R. T., Mattaliano, R. J., Ramachandran, K. L. & Reeve, J. N. (1987).** Structure and expression of the genes, *mcrBDCGA*, which encode the subunits of component C of methyl coenzyme M reductase in *Methanococcus vannielii*. *Proc Natl Acad Sci USA* **84**, 3992–3996.
- Felsenstein, J. (1995).** PHYLIP (Phylogeny Inference Package) version 3.57c. Seattle: Department of Genetics, University of Washington.
- Fielding, E. R. & Archer, D. B. (1986).** Microbiology of landfill. Identification of methanogenic bacteria and their enumeration. *Inst Chem Eng Symp Ser* **96**, 331–341.
- Fielding, E. R., Archer, D. B., Conway de Macario, E. & Macario, A. J. L. (1988).** Isolation and characterization of methanogenic bacteria from landfills. *Appl Environ Microbiol* **54**, 835–836.
- Finlay, B. J. & Fenchel, T. (1991).** An anaerobic protozoon, with symbiotic methanogens, living in municipal landfill material. *FEMS Microbiol Ecol* **85**, 169–180.
- Gorris, L. G., De Kok, T. M., Kroon, B. M., Van der Drift, C. & Vogels, G. D. (1988).** Relationship between methanogenic co-factor content and maximum specific methanogenic activity of anaerobic granular sludges. *Appl Environ Microbiol* **54**, 1126–1130.
- Grosskopf, R., Janssen, P. H. & Liesack, W. (1998).** Diversity and structure of the methanogenic community in anoxic rice paddy soil microorganisms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl Environ Microbiol* **64**, 960–969.
- Grotenhuis, J. T., Smit, M., Plugge, C. M., Xu, Y. S., van Lammeren, A. A., Stams, A. J. & Zehnder, A. J. (1991).** Bacteriological composition and structure of granular sludge adapted to different substrates. *Appl Environ Microbiol* **57**, 1942–1949.
- Gupta, R. S. (1998).** Protein phylogenies and signature sequences: a reappraisal of evolutionary relationships among archaeobacteria, eubacteria and eukaryotes. *Microbiol Mol Biol Rev* **62**, 1435–1491.
- Hales, B. A., Edwards, C., Ritchie, D. A., Hall, G., Pickup, R. W. & 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.
- Innis, M. A. & Gelfand, D. H. (1990).** Optimization of PCRs. In *PCR Protocols: a Guide to Methods and Applications*, pp. 3–12. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: Academic Press.
- Ladapo, J. A. & Barlaz, M. A. (1997).** Isolation and characterization of refuse methanogens. *J Appl Microbiol* **82**, 751–758.
- Lloyd, D., Thomas, K. L., Hayes, A., Hill, B., Hales, B. A., Edwards, C., Saunders, J. R., Ritchie, D. A. & Upton, M. (1998).** Microecology 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.
- Lueders, T., Chin, K.-J., Conrad, R. & Friedrich, M. (2001).** Molecular analyses of methyl-coenzyme M reductase alpha-subunit (*mcrA*) gene in rice field soil and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage. *Environ Microbiol* **3**, 194–204.
- Luton, P. E. (1996).** *A study of landfill methanogens*. PhD Thesis, Liverpool John Moores University.
- Maule, A., Luton, P. & Sharp, R. (1994).** *A Microbiological and Chemical Study of the Broxborough Test Cells*. Oxfordshire, UK: Energy Technology Support Unit, Department of Trade and Industry.
- Moré, M. I., Herrick, J. B., Silva, M. C., Ghiorse, W. C. & Madsen, E. L. (1994).** Quantitative cell lysis of indigenous microorganisms and rapid extraction of DNA from sediment. *Appl Environ Microbiol* **60**, 1572–1580.
- Mori, K., Yamamoto, H., Kamagata, Y., Hatsu, M. & Takamizawa, K. (2000).** *Methanocalculus pumilus* sp. nov., a heavy-metal-tolerant methanogen isolated from a waste-disposal site. *Int J Syst Evol Microbiol* **50**, 1723–1729.
- Nercessian, D., Upton, M., Lloyd, D. & Edwards, C. (1999).** Phylogenetic analysis of peat bog methanogen populations. *FEMS Microbiol Lett* **173**, 425–429.
- Ohkuma, M., Noda, S., Horikoshi, K. & Kudo, T. (1995).** Phylogeny of symbiotic methanogens in the gut of the termite *Reticulitermes speratus*. *FEMS Microbiol Lett* **134**, 45–50.
- Page, R. D. M. (1996).** TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput Applic Biosci* **12**, 357–358.
- Peck, M. W. & Archer, D. B. (1989).** Methods for the quantification of methanogenic bacteria. *Int Ind Biotechnol* **9**, 5–12.
- Polz, M. F. & Cavanaugh, C. M. (1998).** Bias in template-to-product ratios in multitemplate PCR. *Appl Environ Microbiol* **64**, 3724–3730.
- Reeve, J. N., Nolling, J., Morgan, R. M. & Smith, D. R. (1997).** Methanogenesis: genes, genomes and who's on first? *J Bacteriol* **179**, 5975–5986.
- Reysenbach, A. L., Giver, L. J., Wickham, G. S. & Pace, N. R. (1992).** Differential amplification of rRNA genes by polymerase chain reaction. *Appl Environ Microbiol* **58**, 3417–3418.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Springer, E., Sachs, M. S., Woese, C. R. & 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.

Suzuki, M. T. & Giovannoni, S. J. (1996). Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl Environ Microbiol* **62**, 625–630.

Thauer, R. K. (1998). Biochemistry of methanogenesis: a tribute to Marjory Stephenson. 1998 Marjory Stephenson Prize Lecture. *Microbiology* **144**, 2377–2406.

Wasserfallen, A., Nolling, J., Pfister, P., Reeve, J. & deMacario, E. C. (2000). Phylogenetic analysis of 18 thermophilic *Methanobacterium* isolates supports the proposals to create a new genus,

Methanothermobacter gen. nov., and to reclassify several isolates in three species, *Methanothermobacter thermautotrophicus* comb. nov., *Methanothermobacter wolfeii* comb. nov. and *Methanothermobacter marburgensis* sp. nov. *Int J Syst Evol Microbiol* **50**, 43–53.

Weil, C. F., Cram, D. S., Sherf, B. A. & Reeve, J. N. (1988). Structure and comparative analysis of the genes encoding component C of methyl coenzyme M reductase in the extremely thermophilic archaeobacterium *Methanothermus fervidus*. *J Bacteriol* **170**, 4718–4726.

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