

Revealing the uncultivated majority: combining DNA stable-isotope probing, multiple displacement amplification and metagenomic analyses of uncultivated *Methylocystis* in acidic peatlands

Yin Chen,¹ Marc G. Dumont,^{1†} Josh D. Neufeld,^{1‡} Levente Bodrossy,² Nancy Stralis-Pavese,² Niall P. McNamara,³ Nick Ostle,³ Maria J. I. Briones⁴ and J. Colin Murrell^{1*}

¹Department of Biological Sciences, the University of Warwick, Coventry, CV4 7AL, UK.

²Department of Bioresources, Austrian Research Centre, GmbH, A-2444 Seibersdorf, Austria.

³Centre for Ecology and Hydrology, Lancaster Environment Centre, Lancaster, LA1 4AP, UK.

⁴Departamento de Ecología y Biología Animal, Facultad de Biología, Universidad de Vigo, 36310 Vigo, Spain.

Summary

Peatlands represent an enormous carbon reservoir and have a potential impact on the global climate because of the active methanogenesis and methanotrophy in these soils. Uncultivated methanotrophs from seven European peatlands were studied using a combination of molecular methods. Screening for methanotroph diversity using a particulate methane monooxygenase-based diagnostic gene array revealed that *Methylocystis*-related species were dominant in six of the seven peatlands studied. The abundance and methane oxidation activity of *Methylocystis* spp. were further confirmed by DNA stable-isotope probing analysis of a sample taken from the Moor House peatland (England). After ultracentrifugation, ¹³C-labelled DNA, containing genomic DNA of these *Methylocystis* spp., was separated from ¹²C DNA and subjected to multiple displacement amplification (MDA) to generate sufficient DNA for the preparation of a fosmid metagenomic library. Potential bias of MDA was detected by fingerprint analysis of 16S rRNA using denaturing gradient gel electrophoresis for low-template amplification (0.01 ng template). Sufficient

template (1–5 ng) was used in MDA to circumvent this bias and chimeric artefacts were minimized by using an enzymatic treatment of MDA-generated DNA with S1 nuclease and DNA polymerase I. Screening of the metagenomic library revealed one fosmid containing methanol dehydrogenase and two fosmids containing 16S rRNA genes from these *Methylocystis*-related species as well as one fosmid containing a 16S rRNA gene related to that of *Methylocella/Methylocapsa*. Sequencing of the 14 kb methanol dehydrogenase-containing fosmid allowed the assembly of a gene cluster encoding polypeptides involved in bacterial methanol utilization (*mxoFJGIRSAC*). This combination of DNA stable-isotope probing, MDA and metagenomics provided access to genomic information of a relatively large DNA fragment of these thus far uncultivated, predominant and active methanotrophs in peatland soil.

Introduction

Despite continuous developments in bacterial cultivation and isolation techniques in the last few decades (Janssen *et al.*, 2002; Kaeberlein *et al.*, 2002; Leadbetter, 2003; Keller and Zengler, 2004; Giovannoni and Stingl, 2007), it is still a challenge to cultivate the majority of microorganisms present in the environment. Thus, the metabolic functions of many uncultivated microorganisms remain largely unknown. In particular, a more in-depth understanding of the role of uncultivated microorganisms in trace gas production and consumption is important, especially within the context of global change (Schimel and Gullede, 1998). Metagenomics is a method to study microorganisms without the prerequisite for cultivation (Handelsman *et al.*, 1998) and can be used to retrieve genetic information directly from the environment by either direct large-scale DNA sequencing or by functional screening of relevant bioactive compounds (reviewed in Handelsman, 2005; Lasken, 2007; Schmeisser *et al.*, 2007; and references therein). For example, large-scale sequencing projects of environmental DNA have been performed (Tyson *et al.*, 2004; Venter *et al.*, 2004; Tringe *et al.*, 2005; Rusch *et al.*, 2007) and have allowed the

Received 25 February, 2008; accepted 7 May, 2008. *For correspondence. E-mail J.C.Murrell@warwick.ac.uk; Tel. (+44) 02476 523553; Fax (+44) 02476 523568. Present addresses: †Max Planck Institute for Terrestrial Microbiology, D-35043 Marburg, Germany., ‡Department of Biology, University of Waterloo, Waterloo, Ontario, N2L 3G1, Canada.

reconstruction of the metabolic pathways of microorganisms that are recalcitrant to cultivation (Tyson *et al.*, 2004; Martin *et al.*, 2006). A number of industrially useful enzymes and pharmacologically valuable compounds have also been isolated from the environment by the so-called 'functional metagenomics' approach (reviewed in Handelsman, 2005; Schmeisser *et al.*, 2007).

Although powerful, current metagenomic approaches are also associated with problems. For example, the environmental shotgun sequencing approach is heavily biased towards the most abundant species in the environment (Rusch *et al.*, 2007), and may miss less abundant species which may be of interest. Furthermore, reconstructing individual genomes from the massive number of mixed gene sequences is akin to assembling an enormous jigsaw puzzle which is missing most of its pieces. In addition, it is still expensive, despite the continuous technical improvements in DNA sequencing (Margulies *et al.*, 2005; Bentley, 2006; and references therein). Finally, the application of metagenomics for the discovery of industrial and pharmacologically relevant compounds from the environment, another key application of metagenomics, has also been hindered because of low success rates (Schmeisser *et al.*, 2007).

DNA stable-isotope probing (DNA-SIP) is a method for studying active microorganisms involved in a certain bioprocess (Radajewski *et al.*, 2000; Dumont and Murrell, 2005). Labelling of microorganisms with stable isotopes (e.g. ^{13}C , ^{15}N) followed by separation of their DNA from those that are unlabelled serves as a filter to enrich for relevant microorganisms, yet still bypasses cultivation steps. There is considerable interest in combining DNA-SIP with metagenomics (Schloss and Handelsman, 2003; Wellington *et al.*, 2003; Friedrich, 2006; Neufeld *et al.*, 2007a) to function as a filter to isolate DNA from functionally relevant microorganisms for the construction of metagenomic libraries, or for direct parallel sequencing (Lasken, 2007). Combining DNA-SIP and metagenomics can yield genetic information of unknown and uncultivated microorganisms that may exist at low relative abundance but are actively involved in a particular metabolic process (Dumont *et al.*, 2006; Neufeld *et al.*, 2008). In addition, by combining DNA-SIP and metagenomics, gene detection frequencies are significantly enhanced (Dumont *et al.*, 2006; Schwarz *et al.*, 2006; Neufeld *et al.*, 2008) compared with direct screening without SIP incubation and separation, where 250 000 fosmid clones were screened yielding two positive clones (Ricke *et al.*, 2005). Thus, this approach may also have great potential in finding novel biocatalysts and useful microbial secondary metabolites, where both cloning of large gene clusters and improvement of gene detection frequencies are essential (Schwarz *et al.*, 2006; Schmeisser *et al.*, 2007).

Peatlands are important terrestrial ecosystems in terms of methane production and carbon storage and harbour diverse groups of methanotrophs that consume methane before it is released into the atmosphere. For some peatland ecosystems, it is estimated that up to 90% of methane that is produced by methanogens is consumed by methanotrophs (Shannon *et al.*, 1996). Methanotrophs can significantly reduce the release of this greenhouse gas. Using culture-independent molecular methods, we and others have shown that *Methylocystis* spp. are particularly abundant in peatlands (McDonald *et al.*, 1996; 1999; McDonald and Murrell, 1997a,b; Dedysch *et al.*, 2001; 2003; 2006; Morris *et al.*, 2002; Jaatinen *et al.*, 2005), and are directly involved in methane oxidation *in situ* as they express particulate methane monooxygenase (pMMO; Chen *et al.*, 2008). This enzyme catalyses the first step of the methane oxidation pathway in aerobic methanotrophic bacteria (Murrell *et al.*, 2000). Two moderately acid-tolerant *Methylocystis* species (optimum pH of 5.8–6.2) have been isolated (Dedysch *et al.*, 2007). However, suitable cultivation conditions for those acidophilic *Methylocystis* present in peatlands have not yet been established.

Here we present a combination of DNA-SIP, multiple displacement amplification (MDA) and metagenomics to study *Methylocystis*-related species in peatlands. A diagnostic *pmoA* (encoding a subunit of pMMO)-based microarray (Bodrossy *et al.*, 2003) was applied to analyse the diversity of methanotrophs present in seven peatlands across Europe. Microarray analysis revealed the predominance of *Methylocystis*-related species in these environments. The activity of *Methylocystis* spp. in methane oxidation was further confirmed by DNA-SIP analyses using $^{13}\text{CH}_4$. By combining DNA-SIP, MDA and metagenomics, it was possible for the first time to gain access to the genetic information of relatively large DNA fragment of these thus far uncultivated methanotrophs that play an important role in reducing methane emissions from peatlands.

Results

Presence of Methylocystis in acidic peatlands

Soil samples to 10 cm depth were taken from seven acidic peatlands across Europe (pH ranges from 4.2 to 4.9, Table 1). A *pmoA* diagnostic microarray (Bodrossy *et al.*, 2003) was used to screen for methanotroph diversity within these acidic peatland samples. This version of the *pmoA* array contained 135 oligonucleotide probes, targeting all known pMMO-containing methanotrophs except recently identified thermoacidophilic methanotrophs from the *Verrucomicrobia* phylum (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008).

Table 1. Properties of peat sampling sites.

Site	Latitude and longitude	Altitude (m)	C : N	Peat depth (m)	Water content (%)	pH	Net CH ₄ flux (µg CH ₄ -C m ⁻² h ⁻¹) ^a
Moor House (England)	54°42'N 2°18'W	290	26	2–3	92.0	4.5	25.8 (2)
Auchencorth (Scotland)	55°47'N 3°14'W	270	21	0.6	82.2	4.2	33.5 (1.9)
Jyvaskyla (Finland)	62°11'N 25°40'E	165	32	< 0.5	89.8	4.4	42.5 (3.7)
Plynlimon (Wales)	52°26'N 3°46'W	648	19	1–2	87.6	4.5	25.5 (2.4)
Xistral (Spain)	42°48'N 8°6'W	1039	21	2	78.6	4.9	51.1 (5.9)
Caithness (Scotland)	58°08'N 0°36'W	1.5	30	0.1–4	81.6	4.5	nd
Clara Bog (Ireland)	53°19'N 7°39'W	60	31	8	95.8	4.7	nd

a. Numbers in brackets represent standard error of the mean for six replicates. nd, not determined.

The *pmoA* microarray results are shown in Fig. 1. As representative *Methylocella* spp. do not contain pMMO (Theisen *et al.*, 2005), they will not be detected by *pmoA* microarray analyses. Type II methanotrophs closely related to *Methylocystis* (probes Mcy413, Mcy522, Mcy459) were dominant in six of the seven peatlands. Strong signals associated with the peat-subgroup *Methylocystis* (targeted by probe peat264) (Bodrossy *et al.*, 2003) were also obtained. These *pmoA* clone sequences were previously retrieved from peatlands (I.R. McDonald and J.C. Murrell, unpubl. data) and signals associated with probe peat264 were only seen in some peatland samples. Strong signals from probes (probe MsT214, Msi232) targeting methanotrophs related to *Methylosinus trichosporium* were also seen in some of the peatland samples. In general, the abundance of type I (γ -*Proteobacteria*) methanotrophs was low, as indicated by weak (or undetectable) hybridization signals for *pmoA* probes targeting *pmoA* from type I methanotrophs. The result from Caithness peat (Scotland) was different from other peat samples in that only the peat-subgroup *Methylocystis* (targeted by probe peat264). Probes for *Methylocystis* and general probes for type II methanotrophs hybridized poorly to target nucleic acid from the Caithness peat sample.

DNA-SIP analysis of methanotrophs in peatlands

To confirm that these *Methylocystis* spp. associated with peatlands were actively involved in oxidizing methane, DNA-SIP was applied to label these microorganisms in a UK peatland (Moor House, England). We chose Moor House as it has been a primary site for our previous investigations (McDonald *et al.*, 1996; Ward *et al.*, 2007; Chen *et al.*, 2008; McNamara *et al.*, 2008). No additional nutrients were added into the SIP microcosm, as they may have caused a shift in the methanotroph community (Cébron *et al.*, 2007). However, this resulted in a relatively long ¹³CH₄ incubation (16 days) for sufficient labelling (140 µmol of CH₄ consumed per g of wet soil).

The 16S rRNA gene denaturing gradient gel electrophoresis (DGGE) fingerprints for 'heavy' ¹³C-DNA (fraction 7, density 1.725 g ml⁻¹) and 'light' ¹²C-DNA (fraction 11, density 1.708 g ml⁻¹) revealed that two predominant bands (bands 1 and 2) appeared after ¹³CH₄ labelling (Fig. 2A). Other less predominant bands which appeared in other fractions of the DGGE gel were not sequenced. Band 3 was present in fractions 7–12 and the sequence was related to the 16S rRNA gene of an *Acidobacterium* clone (93%, EF076217). This is probably due to the effect of the relatively high GC content of these organisms. The 16S rRNA sequence of band 1 was closely related to 16S rRNA gene sequences from *Methylocella* (97%, AJ555244) and *Methylocapsa* (96%, AJ278726). The sequence of band 2, however, was closely related (97%–98% identity) to 16S rRNA gene sequences from several uncultivated bacteria (AM162432–AM162435, AY080911, AY080912, AY080914, AY080917) retrieved from *Sphagnum* peat (Dedysh *et al.*, 2006) and acidic forest soil (Radajewski *et al.*, 2002) (Fig. 2B). They also showed ~97% identity to the newly isolated moderate acid-tolerant methanotroph, *Methylocystis heyeri* (Dedysh *et al.*, 2007). These uncultivated *Methylocystis* spp. are probably globally important as they are reported to be present in a number of samples taken from different peatlands (McDonald *et al.*, 1996; 1999; McDonald and Murrell, 1997a,b; Dedysh *et al.*, 2001; 2003; 2006; 2007; Morris *et al.*, 2002; Radajewski *et al.*, 2002; Jaatinen *et al.*, 2005; Raghoebarsing *et al.*, 2005; Chen *et al.*, 2008).

MDA of 'heavy' ¹³C-DNA, determination and minimization of the amplification bias

To construct a metagenomic library using the ultracentrifugation-separated ¹³C-DNA in order to gain access to the genome information of relatively large DNA fragments of these uncultivated *Methylocystis* spp., microgram quantities of ¹³C-DNA were required. However, the minimum time required to achieve successful labelling of DNA with ¹³C was used in order to minimize potential

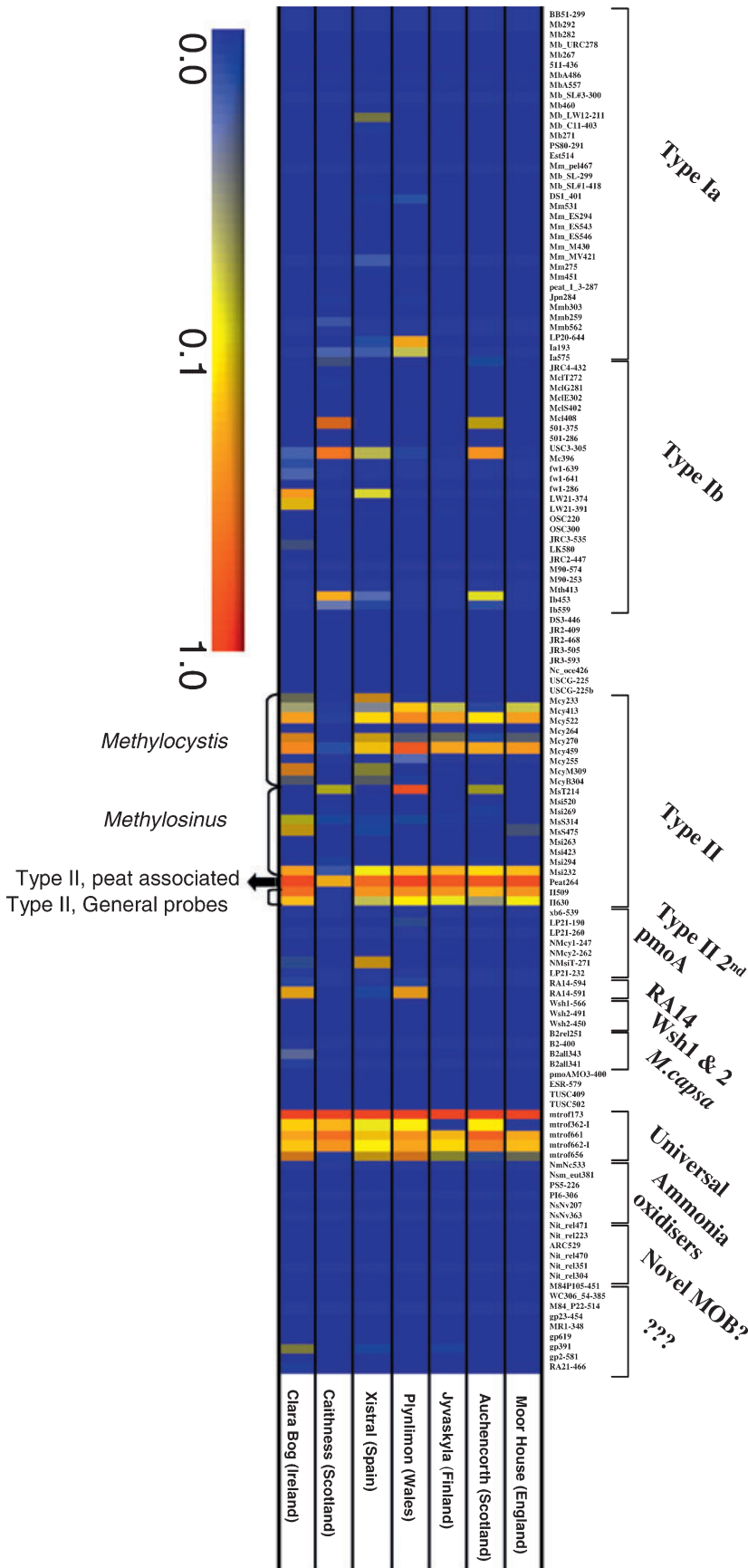


Fig. 1. Methanotroph community analyses using a *pmoA* microarray. Results of hybridizations were normalized to control probes and to the reference values determined individually for each probe. Colour coding is indicated below the figure; a value of 1.0 (red) indicates maximum achievable signal for an individual probe, and a value of 0.1 (yellow) indicates that 10% of the total PCR product hybridized to that probe. *M. capsa*, *Methylocapsa*; type II 2nd *pmoA*, second copy of *pmoA* sequences present in some *Methylocystis* strains (Tchawa Yimga *et al.*, 2003); RA14, *pmoA* sequences which were suggested to be present in methanotrophs that utilize atmospheric concentrations of methane (Holmes *et al.*, 1999); Wsh1 and 2, *pmoA* sequences retrieved from watershed and flooded upland soil (Ogram *et al.*, 2006). ??? refers to environmental clones with uncertain taxonomic affiliation (Stralis-Pavese *et al.*, 2004).

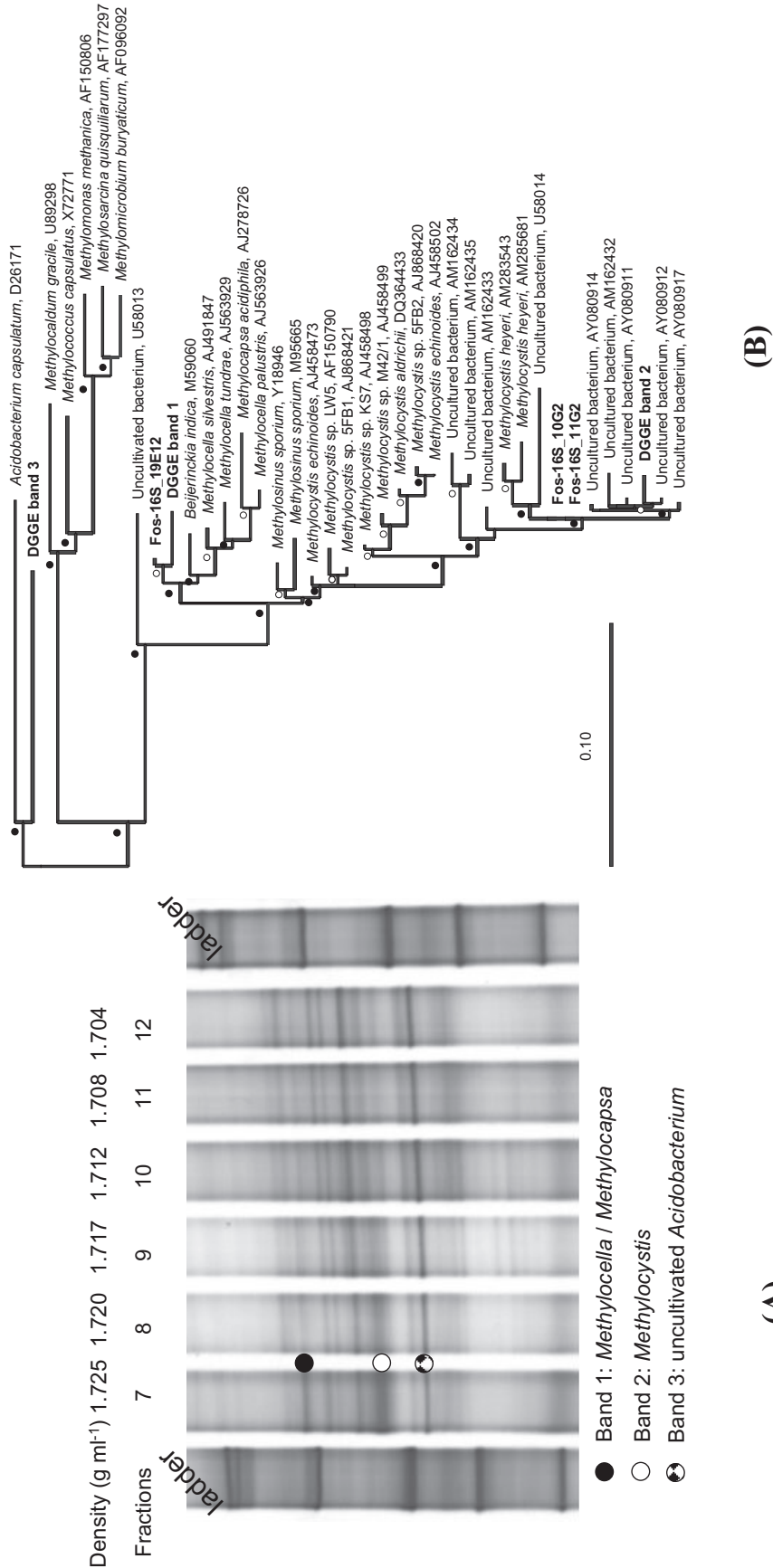


Fig. 2. A. DGGE analysis of 16S rRNA genes in fractions 7–12 from Moor House peat SIP incubations using primers 341F_GC/907R. The sequences from bands 1–3 were closely related to *Methylocella* (97%, AJ55244)/*Methylocapsa* (96%, AJ278726), uncultivated *Methylocystis* (98%, AM162432) and an *Acidobacterium* clone (93%, EF076217) respectively. B. Neighbour-joining phylogenetic analysis of 16S rRNA gene sequences (511 nucleotides) amplified from the DGGE gel (Fig. 2A) and fosmids 10G2, 11G2 and 19E12. The scale bar represents one substitution per 10 nucleotides. Bootstrap values were indicated by either filled circles (> 50%) or open circles (< 50%).

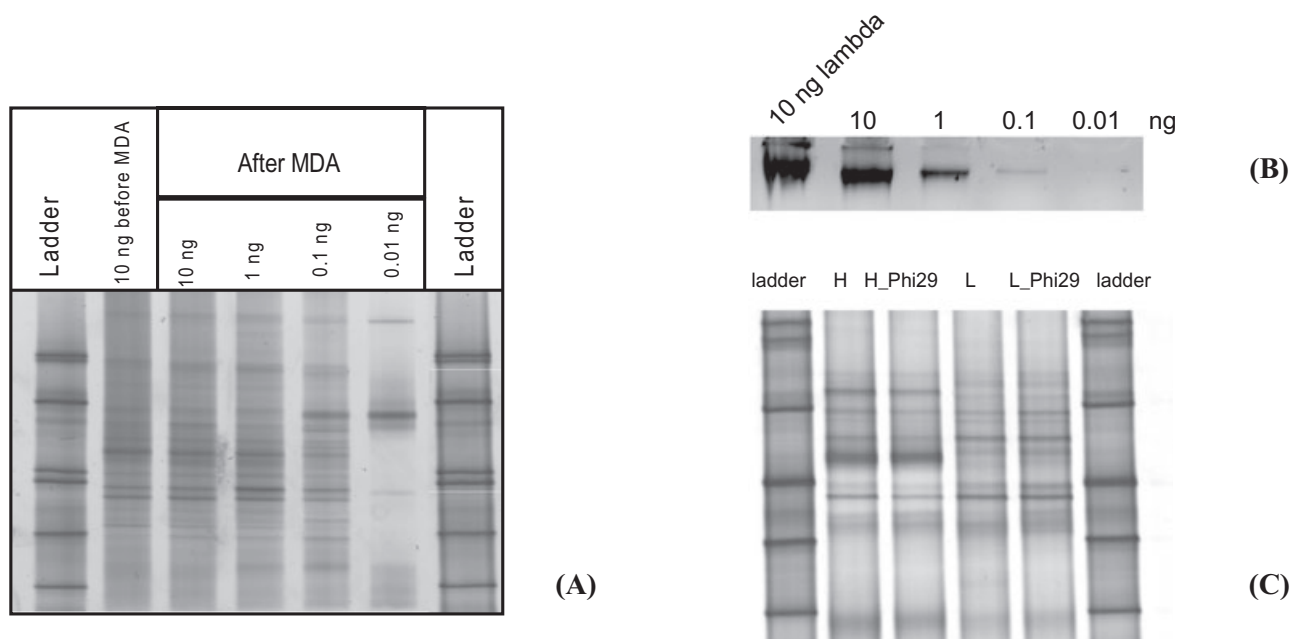


Fig. 3. A. DGGE fingerprint analysis of 16S rRNA genes in total crude soil DNA after SIP incubation, before and after MDA. DNA was extracted from soil taken from Moor House (England) after labelling with $^{13}\text{C}_4$ and diluted to 0.01, 0.1, 1 and 10 ng, which were then used as templates for MDA. One microlitre of 10^{-1} diluted MDA-generated DNA was used for PCR amplification using primers 341F_GC/907R. B. Agarose gel electrophoresis quantification of 1 μl of DNA after MDA with 0.01, 0.1, 1 and 10 ng of DNA as templates. A control was set up using 10 ng of lambda DNA as template and 1 μl of the amplified DNA was loaded on the gel. C. DGGE fingerprint analysis of 16S rRNA genes from ^{13}C -DNA (fraction 7) and ^{12}C -DNA (fraction 11) before (lanes H and L) and after MDA (lanes H_Phi29 and L_Phi29), showing minimum bias during MDA.

cross-feeding of ^{13}C (Dumont and Murrell, 2005; Friedrich, 2006; Neufeld *et al.*, 2007a). Thus, only nanogram quantities of ^{13}C -labelled 'heavy' (fraction 7) were available. To generate sufficient DNA for construction of a metagenomic library, MDA was performed (Blanco *et al.*, 1989; Dean *et al.*, 2002). Before MDA of 'heavy' ^{13}C -DNA (fraction 7), potential bias associated with Phi29 DNA polymerase amplification was assessed using a serial dilution of template DNA (extracted from ^{13}C -labelled Moor House SIP incubations). The DGGE fingerprints of total DNA before and after MDA with 0.1–10 ng of template were highly similar (Fig. 3A). However, bias towards DNA from certain sequences was observed when using 0.01 ng of template. Further, the yield of DNA from MDA was substantially reduced with a decrease in template from 10 to 0.01 ng (Fig. 3B). At least 1 ng of total DNA was needed for MDA in order to minimize any potential amplification bias and to yield sufficient DNA for construction of fosmid libraries using the MDA conditions chosen for this study.

One microlitre (~1–5 ng) of DNA from fraction 7 (^{13}C -labelled 'heavy' DNA, density 1.725 g ml^{-1}) was used as template for MDA. The DGGE analysis of 16S rRNA genes from bacteria in 'heavy' DNA after MDA showed that there was no detectable bias during the amplification procedure (Fig. 3C). The size of MDA-generated DNA ranged from 5 to 50 kb (Fig. S1). In order to minimize

potential chimera formation during cloning of the MDA- ^{13}C -DNA into *Escherichia coli*, the amplified DNA was subjected to several enzyme treatments to digest single-strand DNA and for debranching, as recommended by Zhang and colleagues (2006). Enzyme treatments reduced the yield of MDA-generated DNA (data not shown), but did not significantly reduce the fragment size of the amplified ^{13}C -DNA (Fig. S1). After enzyme treatments, the MDA-generated DNA from fraction 7 ('heavy' DNA) was size-selected (30–50 kb) and used for the preparation of a fosmid library.

Fosmid library construction, screening and shotgun sequencing

Using 50 ng of size-selected DNA, a fosmid library of 2300 clones was constructed (average size ~30 kb). Screening of the 2300 clones for *mxoA*, *mnoX*, *pmoA* [encoding a subunit of methanol dehydrogenase, soluble methane monooxygenase, (sMMO) and pMMO respectively] and 16S rRNA gene for type II methanotrophs (using primer set type IIF/type IIR, Chen *et al.*, 2007) by PCR yielded four positive clones. Three clones contained 16S rRNA genes (fosmid 10G2, 11G2 and 19E12) related to type II methanotrophs and one contained *mxoA* (fosmid 16A2). No fosmid containing a *pmoA* or *mnoX* gene was

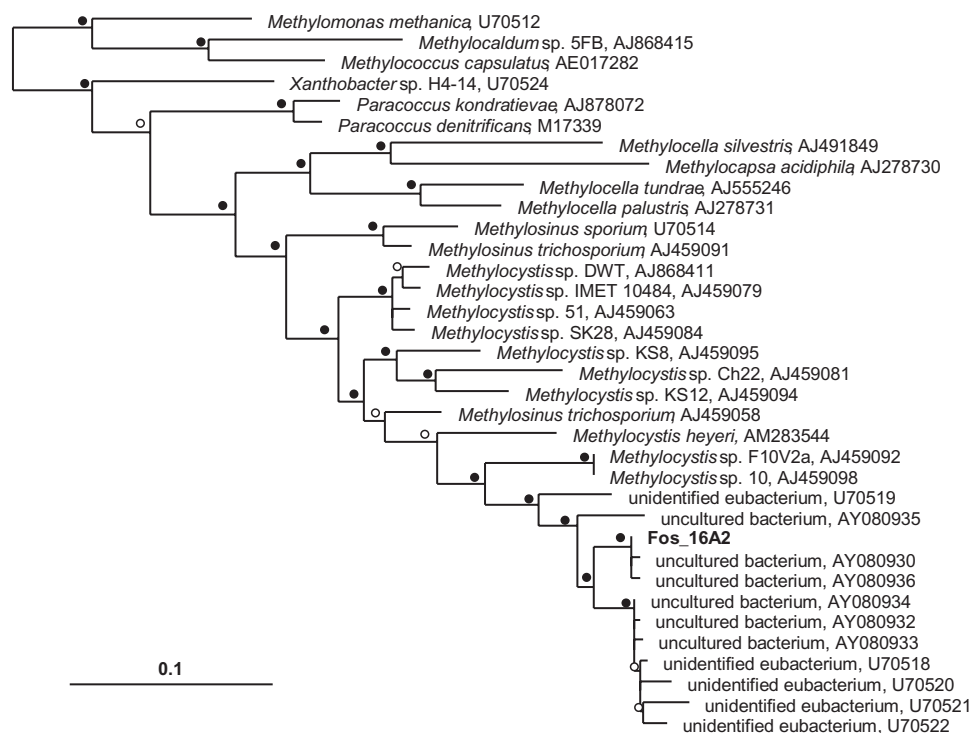


Fig. 4. Neighbour-joining phylogenetic analysis of deduced MxaF sequence from fosmid 16A2 (148 amino acids; amplified with 1003F/1555R) (Neufeld *et al.*, 2007c). The scale bar represents one substitution per 10 nucleotides. Bootstrap values were indicated by either filled circles (> 50%) or open circles (< 50%).

found by either PCR-based screening (using primers targeting *pmoA* and *mmoX* genes respectively) or colony hybridization using ^{32}P -labelled PCR fragments. The 16S rRNA fragments of fosmids 10G2 and 11G2 were identical and closely related to the 16S rRNA gene sequence from band 2 retrieved from the DGGE gel (Fig. 2B), which is associated with *Methylocystis* spp. The 16S rRNA gene sequence from fosmid 19E12 was closely related to the 16S rRNA gene sequence retrieved from band 1 (Fig. 2B), which was identified as a sequence from *Methylocella/Methylocapsa*.

The deduced protein sequence of MxaF from fosmid 16A2 was related to *Methylocystis heyeri* (95%, CAK95254) and most closely related (96%–98%) to MxaF sequences retrieved from acidic forest soils and blanket bog peat cores (Fig. 4). The *mxoF*-containing fosmid (16A2) was shotgun-sequenced and this allowed the assembly and identification of a 14 kb contig containing a cluster of genes (*mxoFJGIRSAC*) known to be involved in bacterial methanol utilization (Table 2). A similar arrangement of these methanol oxidation genes has also been obtained in *Methylobacterium extorquens* (Amaratunga *et al.*, 1997), *Methylococcus capsulatus* (Ward *et al.*, 2004) and *Methylobacillus flagellatus* (Chistoserdova *et al.*, 2007) genomes. Four open reading frames (ORFs) located immediately upstream of *mxoF* were identified.

The ORF1 was 76% identical to a transposase from *Methylobacterium dichloromethanicum* which has been suggested to play a role in the acquisition of dichloromethane dehalogenases by horizontal gene transfer (Schmid-Appert *et al.*, 1997). The ORF2, ORF3 and ORF4 encode hypothetical polypeptides closely related to three polypeptides from the *Bradyrhizobium japonicum* (59% identity), *Rhodobacter sphaeroides* (37% identity) and *M. extorquens* (56% identity) genomes respectively.

Discussion

Molecular ecology studies suggested that *Methylocystis* spp. are abundant and involved in methane oxidation in peatlands. These include the analyses of PCR-amplified functional genes (*mxoF*, *pmoA*) (McDonald and Murrell, 1997a,b), sequencing of 16S rRNA (McDonald *et al.*, 1999; Dedysh *et al.*, 2001; 2003; 2006), DNA-SIP (Morris *et al.*, 2002), characterization of *pmoA* transcripts (Chen *et al.*, 2008) and the analyses of phospholipid fatty acids (Sundh *et al.*, 1995a,b; 1997). *Methylocella* spp. are also present in peatlands and they are not captured by *pmoA* microarray analyses as they do not possess pMMO (Theisen *et al.*, 2005). Quantification of *Methylocella* populations in peatlands using fluorescence *in situ* hybridization showed that they are one of the dominant

Table 2. Putative genes corresponding to each ORF on fosmid 16A2.^a

Name	Location in fosmid 16A2	Protein name or role of closest BLASTX match	Source	% Identity ^b	e-value
Orf1	754-2046	Putative transposase	<i>Methylobacterium dichloromethanicum</i>	76	2E-171
Orf2	2645-2235	Hypothetical protein	<i>Bradyrhizobium japonicum</i>	59	1E-22
Orf3	3059-3967	Putative diheme cytochrome c	<i>Rhodobacter sphaeroides</i>	37	2E-27
Orf4	4963-3397	Hypothetical protein	<i>Methylobacterium dichloromethanicum</i>	56	9E-66
<i>mxoF</i>	5192-7147	Methanol dehydrogenase, large subunit	<i>Methylobacterium nodulans</i>	82	0
			Uncultivated bacterium (AY080930) ^c	99	1E-110
<i>mxoJ</i>	7219-8205	Methanol oxidation protein, MxoJ	<i>Methylobacterium extorquens</i>	57	2E-84
<i>mxoG</i>	8215-8826	Cytochrome c, class I	<i>Methylobacterium extorquens</i>	54	3E-37
<i>mxoI</i>	8886-9182	Methanol dehydrogenase, small subunit	<i>Methylococcus capsulatus</i>	66	1E-28
<i>mxoR</i>	9321-10340	ATPase	<i>Methylobacterium extorquens</i>	65	2E-120
<i>mxoS</i>	10337-11182	Conserved hypothetical protein, MxoS	<i>Methylobacterium extorquens</i>	54	3E-70
<i>mxoA</i>	11179-12099	Methanol oxidation protein, MxoA	<i>Methylobacterium extorquens</i>	40	5E-46
<i>mxoC</i>	12096-12971	Methanol utilization protein, MxoC	<i>Methylobacterium extorquens</i>	60	1E-86

a. Gene abbreviations and closest BLASTX relatives were obtained from GenBank.

b. Per cent identity is reported for amino acid sequences.

c. BLASTX searched using nucleotide region between the primers 1003F/1555R (Neufeld *et al.*, 2007c).

methanotrophs with 10^6 – 10^7 cells per g of wet peat (Dedysh *et al.*, 2001). However, attempts to isolate these organisms from peatlands have been largely unsuccessful (Dedysh *et al.*, 2007). It has been observed that some enriched acidophilic methanotrophs have relatively slow growth rates, do not grow on agar plates and grow only in low-ionic-strength medium, which are some of the reasons why they were more difficult to capture using standard laboratory conditions (Dedysh *et al.*, 2002; 2007). By using *pmoA*-based microarray and DNA-SIP analyses, our study demonstrated that *Methylocystis* spp. were one of the numerically and functionally predominant methanotrophs in European peatlands. For unknown reasons, Caithness in Scotland was the sole exception. Only the peat-subgroup *Methylocystis* (targeted by probe peat264) were detected. This site had some differences compared with the others that may have contributed to this finding. First, this site is close to the Atlantic and exposed to moderate inputs of marine aerosols; second, while Caithness is characterized by deep blanket peat (1–2 m), the sample was taken from an area where the peat was much shallower (10–15 cm) than the other European sites (Table 1).

Multiple displacement amplification, which was previously used primarily for diagnostic application in medical fields, has recently been applied in microbial ecology studies to generate sufficient DNA from low amounts of template (Erwin *et al.*, 2005; Kalyuzhnaya *et al.*, 2006; Mußmann *et al.*, 2007; Podar *et al.*, 2007; Stepanauskas and Sieracki, 2007; reviewed in Binga *et al.*, 2008). Two technical aspects need to be taken into consideration when using this method, namely amplification bias and chimera formation (Lasken, 2007). Our results and those of others have shown that when the initial amount of DNA is less than 1 ng, bias can be severe (Kalyuzhnaya *et al.*, 2006; Zhang *et al.*, 2006). As bias also tends to occur in a

random manner (Zhang *et al.*, 2006), greater depths need to be reached in order to complete the genome when MDA was applied to amplify DNA from single or few cell(s) (Mußmann *et al.*, 2007; Podar *et al.*, 2007; Stepanauskas and Sieracki, 2007), which makes MDA still a technical challenge for single-cell analyses. Besides, formation of chimeric structures during MDA results in fragmented genes and difficulties in assembling large genomic contigs. It has been suggested that treatment of MDA-generated DNA with a series of enzymes to remove hyperbranched structure and single-strand DNA helped to reduce chimeric sequences (Zhang *et al.*, 2006). In our study, we followed this suggestion and carefully estimated the size of the enzymatically treated DNA. Although enzyme treatment reduced the yield, no detectable reduction of DNA length was observed, which makes it still suitable for cloning of large fragments. However, chimeric sequences were still observed at both ends of the contig in our fosmid (data not shown), which was a similar finding to the fosmid insert sequenced in a recent marine SIP–MDA–metagenomics study (Neufeld *et al.*, 2008). A recent study showed that chimeras may form by the amplification process itself and an enzyme treatment during the early stage of amplification may be the best way to prevent forming chimeras (Lasken and Stockwell, 2007). The amplification and enzyme treatment process requires further optimization to minimize chimera formation during MDA.

By screening 2300 clones, we did not identify either *pmoA*- or *mmoX*-containing fosmids (encoding key subunit of pMMO and sMMO respectively). As only a few *Methylocystis* strains possess the sMMO enzyme (Heyer *et al.*, 2002), it is probably not surprising that no *mmoX*-containing fosmids were identified. The failure to detect *pmoA* genes in this library is more surprising, given that the library size should be sufficient (~70 Mb) and that two

fosmids containing 16S rRNA genes and one fosmid containing an *mxoF* from those organisms were identified. It has been reported that pMMO-encoding gene clusters are difficult to clone possibly as a result of disruption of *E. coli* cell membranes by expression of membrane-bound pMMO (Semrau *et al.*, 1995; Gilbert *et al.*, 2000). However, Ricke and colleagues (2005) and Dumont and colleagues (2006) retrieved *pmoCAB* clusters from soil metagenomes with single-copy vectors, as used in this study, after screening 250 000 fosmids and 2300 BAC clones respectively. Nonetheless, it is possible that pMMO genes may have lower cloning efficiency and were not captured by our metagenome library.

By combining DNA-SIP and metagenomics, we identified a gene cluster encoding methanol oxidation genes, which is probably from uncultivated *Methylocystis* present in peatlands. We also managed to identify 16S rRNA genes of these microorganisms in the fosmid library. Compared with PCR-based methodologies, the combination of DNA-SIP and metagenomics is capable of accessing genome information of genes flanking the target sequence. Further screening of functional genes involved in one-carbon transfer and intermediary metabolism will be carried out in future to study the *Methylocystis* spp. in peatlands. However, with progress in single-cell genomics and sequencing (Zhang *et al.*, 2006; Marcy *et al.*, 2007a,b; Mußmann *et al.*, 2007; Podar *et al.*, 2007; Stepanauskas and Sieracki, 2007), it is now possible to study these uncultivated organisms at single-cell level. Nevertheless, combining DNA-SIP, MDA and metagenomics is powerful in cloning large DNA fragment from unknown and uncultivated microorganisms, and may have potential use in finding novel bioactive compounds from a metagenome as it significantly enhances gene detection frequency. Advances in high-throughput sequencing also make it possible to combine single-cell amplification using Phi29 DNA polymerase with direct sequencing, without the prerequisite of cloning (Mußmann *et al.*, 2007). Such an approach may help to minimize the accumulation of infrequent chimeras in genome assemblies (Binga *et al.*, 2008). Future single-cell genomic studies of these peatland microorganisms could provide a wealth of information that may help to identify suitable growth conditions to facilitate the isolation of these bacteria and enable the study of their physiology within the peatland environment.

Experimental procedures

Peatland sampling

Soil cores (10 cm depth, 6.3 cm inner diameter) were sampled from peatlands across Europe during 2005 and 2006 (Table 1). Within the UK and Ireland, we sampled five peatlands with similar characteristic vegetation being *Sphagnum* spp., *Eriophorum* spp. and *Calluna vulgaris*. We also sampled

a 'relic' peatland from the Xistral Mountains in North-west of Spain with characteristic vegetation being *Eriophorum angustifolium*, *Carex durieui*, *Carex echinata*, *Molinia caerulea*, *Erica mackaiana* and *Calluna vulgaris*. Finally, we sampled a forested (*Betula pubescens* and *Picea abies*) shallow peat in Finland with characteristic shrubs being *Sphagnum angustifolium*, *Sphagnum girgensohnii*, *Polytrichum commune*, *Carex* sp., *Calamagrostis purpurea*, *Equisetum sylvaticum*, *Vaccinium myrtillus* and *Vaccinium vitis-idaea*. After collection, cores were frozen at -23°C until DNA was extracted. For DNA extraction, each core was separated into two well-mixed 5 cm sections (0–5 and 5–10 cm) and DNA was extracted from the bottom 5–10 cm layer, which is usually the most active in methane oxidation (data not shown). Soil water content, pH, % C and % N were determined for each soil sample. The pH was determined by mixing 1 g of wet soil with 10 g of distilled water. pH was measured in the resulting slurry. To determine the soil moisture content, 10 g of soil was dried at 85°C and weighed. Total carbon and nitrogen in soil was analysed using an Elemental Vario EL elemental analyser (Elementar). General characteristics of the sites are given in Table 1.

Net CH_4 core fluxes

Net CH_4 fluxes in cores were measured for all sites except Clara Bog (Ireland) and Caithness (Scotland). Soil cores were adjusted to 60% water holding capacity prior to gas measurements. For each site, six replicate cores were incubated in gas-tight 1.8 l containers at 4.5°C and headspace gas samples were taken five times over 4 h. Methane concentrations were analysed using a Perkin Elmer Autosystem XL Gas Chromatograph (PerkinElmer) fitted with a flame ionization detector operated at 350°C . Methane was separated isothermally on a 4 m Poropak Q packed column at 40°C , with N_2 as the carrier gas flowing at $30\text{ cm}^3\text{ min}^{-1}$. Results were calibrated against certified gas standards comprising 10 p.p.m. methane (Air Products, UK). Methane flux rates were calculated using the approach of Holland and colleagues (1999) after applying linear regression to the methane concentration versus time data.

pmoA-based microarray analysis

The diversity of methanotrophs in these peatlands was investigated using a *pmoA*-based microarray analysis (Bodrossy *et al.*, 2003). DNA extracted from peat soils was used as template to amplify *pmoA* fragments using the primer set a189f/T7-mb661r (Bodrossy *et al.*, 2003). A touch-down programme was performed in order to minimize non-specific amplification during PCR. The programme was: 94°C for 5 min, followed by 15 cycles of 45 s at 94°C ; 1 min at 60°C with decrease of 0.5°C every cycle; 1 min at 72°C ; followed by 25 cycles of 45 s at 94°C ; 1 min at 53°C ; 1 min at 72°C . Final extension was 5 min at 72°C . The labelling and hybridization conditions were identical to those previously described (Bodrossy *et al.*, 2003).

$^{13}\text{CH}_4$ labelling, DNA extraction and purification

A total of 5 g of soil from Moor House (England) was incubated in a 125 ml crimp-top serum vial with 2% (v/v) of

$^{13}\text{CH}_4$. No additional nutrients were added. The serum vial was left in the dark at room temperature ($\sim 20^\circ\text{C}$). The soil was collected when 10, 50 and 140 $\mu\text{mol g}$ (wet weight soil) $^{-1}$ $^{13}\text{CH}_4$ were consumed, and distinct DGGE fingerprints in 'heavy' fractions (compared with DGGE fingerprints from 'light' fractions) only appeared when 140 $\mu\text{mol g}$ (wet weight soil) $^{-1}$ $^{13}\text{CH}_4$ was consumed after 16 days (data not shown).

DNA extraction from peat soils was carried out using the Bio 101 FastDNA spin kit for soil (QBiogene) according to the instruction manual. The protocol for high-molecular-mass DNA extraction from Moor House SIP incubations was based on the method of Zhou and colleagues (1996), with slight modifications (Dumont *et al.*, 2006).

Ultracentrifugation and gradient fractionation

Two micrograms of purified DNA was added to CsCl solutions and subjected to density-gradient ultracentrifugation in order to separate ^{13}C -DNA from ^{12}C -DNA (average density 1.725 g ml^{-1}). Gradient fractionation was carried out as previously described (Neufeld *et al.*, 2007b). Densities of all 12 400 μl fractions were determined using a digital refractometer (Reichert AR200, Reichert Analytical Instruments). DNA was purified from each fraction and suspended in 50 μl of TE buffer.

Multiple displacement amplification

Multiple displacement amplification was carried out using the GenomiPhi V2 DNA Amplification Kit (GE Healthcare) according to the manufacturer's protocol. Reactions were carried out in 20 μl volumes. To test the potential bias in amplification of DNA by Phi29 enzyme, a serial dilution of total DNA (DNA extracted from Moor House SIP incubations), with 10, 1, 0.1 and 0.01 ng of templates, was used in MDA reactions. One-microlitre aliquots of the amplified product were run on agarose gels (1%, w/v) in order to estimate the efficiency of the amplification. The MDA product was diluted 10 times and 1 μl was used as template for PCR/DGGE analysis as described below.

PCR and DGGE

PCR amplifications were performed in a final volume of 50 μl containing 1–10 ng of template DNA. PCR primer set 341f_GC/907r (Muyzer *et al.*, 1993) was used to determine fingerprint patterns before and after MDA of total DNA after SIP incubations, 'heavy' DNA (fraction 7, density 1.725 g ml^{-1}) and 'light' DNA (fraction 11, density 1.708 g ml^{-1}). The DGGE was also used for the amplification of bacterial 16S rRNA genes from DNA-SIP fractions using the same primer set.

The DGGE was performed as described previously (Chen *et al.*, 2007) with gels of 8% (v/v) of polyacrylamide and 30–70% of denaturant gradient. Bands from DGGE gels (indicated in Fig. 2) were excised, re-amplified, purified and sequenced by cycle sequencing with BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems).

Enzyme treatments of MDA-generated 'heavy' DNA

Five reactions of MDA-generated 'heavy' DNA (fraction 7) were combined and treated with enzymes as described by Zhang and colleagues (2006) to minimize possible chimeras produced during cloning of MDA DNA into *E. coli*. Briefly, five reactions (100 μl) of MDA-generated 'heavy' DNA were first purified using a Microcon YM-30 column (Millipore), then incubated with 10 U of Phi29 DNA polymerase (Fermentas) at 30°C for 2 h without adding random hexamer primers. The reaction was stopped by heating at 65°C for 10 min and the DNA was purified and incubated with 200 U of S1 nuclease (Fermentas) at 37°C for 30 min. The reaction was stopped by adding 50 μl of 0.5 M EDTA and heating at 70°C for 10 min. DNA was then extracted with phenol : chloroform : isoamyl alcohol (25:24:1 v/v), again with chloroform : isoamyl alcohol (24:1 v/v) and then precipitated with ethanol. The pellet was air-dried and dissolved in 100 μl of nuclease-free water, to which 20 U of DNA polymerase I was added. The reaction was incubated at 25°C for 1 h and then stopped by heating at 75°C for 10 min. The enzyme-treated MDA-generated DNA was then end-repaired using the End-Repair enzyme mix from CopyControl Fosmid Library Production Kit (Epicentre).

Fosmid library construction and screening

The end-repaired, MDA-generated DNA was then subjected to pulsed field gel electrophoresis, using a 1% (w/v) low-melting agarose gel for size selection of DNA fragments. The gel was subsequently stained with ethidium bromide, scanned with a FLA-5000 scanner (Fujifilm) and 30–50 kb DNA fragments was excised from the gel. DNA was purified from the gel using GELase and precipitated with ethanol according to the manufacturer's protocol (Epicentre). The DNA pellet was dissolved in 30 μl of TE buffer and quantified using a ND-1000 spectrophotometer (NanoDrop). Fosmid library construction was carried out according to the instruction manual (Epicentre) with 6 μl (~ 50 ng) of purified size-fractionated DNA.

The resulting 2300 colonies were transferred into 96-well culture plates containing 50 μl of Luria-Bertani (LB)/chloramphenicol broth. Cultures from each well of every five culture plates were pooled and used as template for colony PCR screening for the following genes: *mxoA* (1003F/1555R, Neufeld *et al.*, 2007c), 16S rRNA from type II methanotrophs (type IIF/type IIR, Chen *et al.*, 2007), *pmoA* (189f/mb661r, Holmes *et al.*, 1995; Costello and Lidstrom, 1999) and *mmoX* (206f/886r, Hutchens *et al.*, 2004). PCR amplification were carried out in a Tetrad thermocycler (Bio-Rad) with annealing temperatures at 55°C for *mxoA* and *pmoA* and 60°C for 16S rRNA and *mmoX* respectively. PCR products were run in a 1% (w/v) agarose gel and amplicons with the correct sizes were further screened for individual clones. The presence of target genes from those positive fosmids was confirmed by PCR amplification of corresponding genes with extracted fosmids as templates. PCR amplicons were purified and sequenced.

The resulting 2300 colonies were also screened by colony blotting and hybridization as described by Dumont and colleagues (2006). Briefly, the 2300 colonies were transferred onto a Nylon Hybond N⁺ membrane (Amersham) placed on

the surface of selective LB agar plates. The plates were incubated at 37°C overnight and then lysed on the membrane. DNA probes were labelled by random priming using random hexanucleotide primers (Roche) and dNTP. The gene probes were amplified from the crude DNA extracted from ¹³CH₄-labelled Moor House peat sample by PCR using the A189f/682r *pmoA* primers (Holmes *et al.*, 1995; Costello and Lidstrom, 1999), mmoX206f/mmoX886r *mmoX* primers (Hutchens *et al.*, 2004) and the 1003f/1555r *mxoF* primers (Neufeld *et al.*, 2007c). Twenty-five to fifty nanograms of agarose gel-purified DNA fragment was labelled with 50 µCi of [α -³²P]-dGTP for 1–2 h at 37°C with DNA polymerase I Klenow fragment (Fermentas). The probes were denatured by the addition of NaOH to a final concentration of 0.4 M and incubated for 2 min at room temperature before adding to the hybridization solution. Hybridizations were carried out overnight at 55°C. The hybridization solution was discarded and the membrane washed twice with 100 ml of 2× saline-sodium citrate buffer at 55°C. The membrane was removed and scanned with a Geiger Müller detector to estimate the amount of bound probe. If necessary, the membrane was washed at higher stringency. Fuji 100NIF Medical X-ray Film (Fuji film) was exposed to hybridized membranes between intensifying screens in autoradiography cassettes at –80°C. The length of exposure varied between 1 and 72 h, based on the signal intensity. Autoradiograph film was developed and fixed in accordance with the manufacturer's instructions.

Shotgun sequencing of fosmid inserts and phylogenetic analyses

Only the *maxF*-containing fosmid insert was subcloned and sequenced, using the TOPO Shotgun Cloning Kit (Invitrogen). Individual clones were PCR-amplified using vector-specific flanking primers. PCR products were purified and sequenced using BigDye 3.1 chemistry on an ABI3730 (Applied Biosystems). Sequence reads were assembled using SeqMan II (version 6, DNASTAR). The ORFs were identified using the ORF Finder programme (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) and compared with GenBank database with BLASTX. DNA sequence alignment and phylogenetic analyses were performed as described previously (Chen *et al.*, 2007). Sequences from this study were submitted to GenBank with the accession numbers EU362857–EU362863.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Pulsed field gel electrophoresis analysis of enzyme-treated MDA-generated DNA from fraction 7. Lanes 1 and 9: high-molecular-weight ladder (Invitrogen); lanes 2 and 8: 1 kb ladder (Invitrogen); lanes 3 and 7: lambda DNA (40 kb); lane 4: MDA-generated DNA of fraction 7; lanes 5 and 6: enzyme-treated, MDA-generated DNA.

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