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Identification of a complete methane monooxygenase operon from soil by combining stable isotope probing and metagenomic analysis

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Summary

Stable isotope probing (SIP) allows the isolation of nucleic acids from targeted metabolically active organisms in environmental samples. In previous studies, DNA-SIP has been performed with the one-carbon growth substrates methane and methanol to study methylotrophic organisms. The methylotrophs that incorporated the labelled substrate were identified with polymerase chain reaction and sequencing of 16S rRNA and 'functional genes' for methanotrophs (*mxaF*, *pmoA*, *mmoX*). In this study, a SIP experiment was performed using a forest soil sample incubated with $^{13}CH_4$, and the ^{13}C-DNA was purified and cloned into a bacterial artificial chromosome (BAC) plasmid. A library of 2300 clones was generated and most of the clones contained inserts between 10 and 30 kb. The library was probed for key methylotrophy genes and a 15.2 kb clone containing a *pmoCAB* operon, encoding particulate methane monooxygenase, was identified and sequenced. Analysis of the *pmoA* sequence suggested that the clone was most similar to that of a *Methylocystis* sp. previously detected in this forest soil. Twelve other open reading frames were identified on the clone, including the gene encoding beta-ribofuranosylaminobenzene 5′-phosphate synthase, which is involved in the biosynthesis of the 'archaeal' C_1-carrier, tetrahydromethanopterin, which is also found in methylotrophs. This study demonstrates that relatively large DNA fragments from uncultivated organisms can be readily isolated using DNA-SIP, and cloned into a vector for metagenomic analysis.

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Introduction

Microbial ecology is a particularly challenging discipline given that most microorganisms have so far eluded cultivation. The current estimate is that there are 52 bacterial phyla, half of which lack a single cultivated representative (Rappé and Giovannoni, 2003). As a result, it is impossible to infer the function of many microorganisms. In addition, because the conditions in laboratory media are likely to be different from those *in situ,* inferring the role of microorganisms based on laboratory studies could be misleading. Therefore, a useful strategy to study microbial ecology is to use cultivation-independent methods such as stable isotope probing (SIP) (Radajewski *et al*., 2000; Manefield *et al*., 2002), which can link microorganism identity (e.g. 16S rRNA sequences) in an environmental sample to the incorporation of a growth substrate. Metagenomics, the genomic analysis of uncultivated microorganisms, may also be useful in studying microbial ecology through the interpretation of nucleotide sequence data (Handelsman, 2004).

Stable isotope probing is a technique for studying the active organisms in environmental samples. The DNA-SIP technique was originally developed using CH_4 and CH_3OH substrates (Radajewski *et al*., 2000; Morris *et al*., 2002), but has since been used with CO_2 (Whitby *et al*., 2001), methyl halides (Miller *et al*., 2004; Borodina *et al*., 2005) and several multicarbon compounds such as naphthalene, phenol, caffeine, glucose, salicylate and phenanthrene (Jeon *et al*., 2003; Padmanabhan *et al*., 2003; Singleton *et al*., 2005). In previous DNA-SIP studies, the microbial populations that incorporated the isotopically heavy substrate were characterized by polymerase chain reaction (PCR) amplification of 16S and 18S rRNA genes and functional genes (for example, genes encoding methane monooxygenase and methanol dehydrogenase) using the ^{13}C-DNA as template (Radajewski *et al*., 2002; Ginige *et al*., 2004; Hutchens *et al*., 2004; Lin *et al*., 2004; Lueders *et al*., 2004; Gallagher *et al*., 2005; Lu *et al*., 2005).

A difficulty in analysing a metagenomic library is the high degree of complexity that results from the huge microbial diversity in many environmental samples. Estimates of microbial diversity in a forest soil using single-stranded DNA reassociation kinetics indicated 12 000–18 000 different species (Torsvik *et al*., 1990). There is

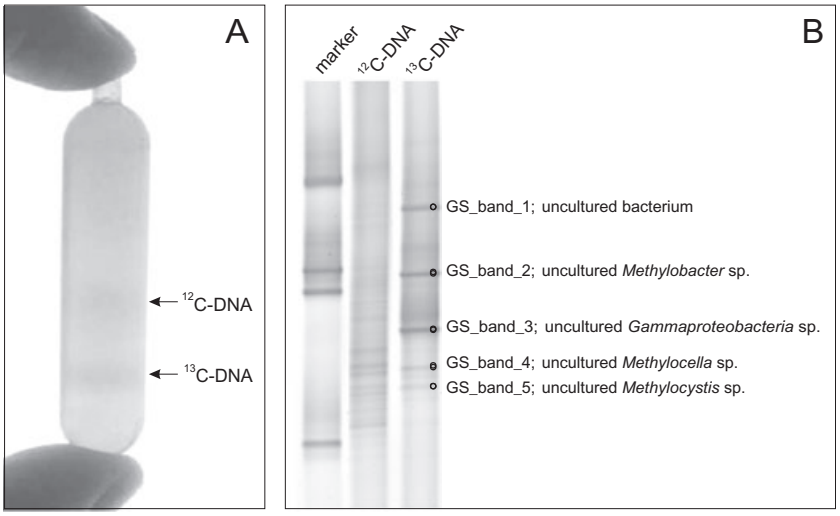

Fig. 1. A. The CsCl-ethidium bromide gradient containing ^{12}C-DNA and ^{13}C-DNA from the DNA-SIP experiment with forest soil that consumed $^{13}CH_4$. The DNA was visible and photographed in ambient light and the position of the ^{12}C-DNA and ^{13}C-DNA bands is indicated by the arrows.
B. Denaturing gradient gel electrophoresis analysis of the ^{12}C-DNA and ^{13}C-DNA recovered from the gradient. The marker contained a mixture of 16S rRNA gene products from pure bacterial cultures. The predominant DGGE bands from the ^{13}C-DNA were sequenced and a genus name is indicated where the per cent identity of the sequence was greater than 98% to that of a cultivated representative. The GS_band_3 sequence belongs to an uncultivated *Gammaproteobacteria* that was also PCR amplified in a previous DNA-SIP analysis of this soil (Radajewski *et al.*, 2002). GS_band_1 possesses similarity to *Bacteroidetes* 16S rRNA sequences.

considerable interest in the possibility of combining DNA-SIP with metagenomic studies to solve this dilemma (Schloss and Handelsman, 2003; Wellington *et al.*, 2003; Dumont and Murrell, 2005a). In a metagenomic library constructed from ^{13}C-DNA from a DNA-SIP experiment, the cloned DNA would originate from genomes of organisms that have obtained the majority of their carbon, either directly or indirectly via cross-feeding, from the ^{13}C-substrate. This limits the library to genome fragments of organisms involved in a specific metabolic process of interest and increases the likelihood of obtaining and sequencing genes that are integral to the process.

In this study, a metagenomic library was constructed using ^{13}C-DNA from a DNA-SIP experiment with $^{13}CH_4$. The objective was to perform an experiment to determine the feasibility of cloning larger fragments of the ^{13}C-DNA and identifying a clone containing methylotrophy genes. A clone library was made from ^{13}C-DNA using a bacterial artificial chromosome (BAC) plasmid and a clone containing a complete *pmoCAB* operon, encoding the particulate methane monooxygenase (pMMO) enzyme, was identified and sequenced. The *pmoA* gene was similar to that of an uncultivated *Methylocystis* sp. previously identified in this soil by DNA-SIP and PCR analysis. A very brief preliminary report of these data was published in a review of DNA-SIP (Dumont and Murrell, 2005a).

Results

Stable isotope probing with $^{13}CH_4$ and construction of a metagenomic library

The SIP experiment was performed in a manner similar to that described previously (Radajewski *et al.*, 2002) and with sample taken from the same forest soil site. Soil was collected from the acidic oak forest soil and incubated with $^{13}CH_4$ until a total of 50 ml had been consumed. The DNA was extracted from the $^{13}CH_4$-labelled soil and the DNA was visible in ambient light after isopycnic centrifugation in CsCl (Fig. 1A). Therefore, the ^{13}C-DNA band could be collected without exposure to DNA-damaging UV irradiation. The ^{13}C-DNA was analysed by pulsed-field gel electrophoresis (PFGE) and the majority of fragments were between ~15 kb and ~100 kb in length (results not shown).

Restriction enzyme activity is sensitive to inhibition by soil organics which are co-extracted with DNA (Zhou *et al.*, 1996). Although several rounds of CsCl gradient centrifugation had been used to purify the DNA, the gradient-purified DNA was not sufficiently pure to be digested with restriction enzyme BamHI (results not shown). Therefore, further purification was achieved by electrophoresis of the ^{13}C-DNA through a 1% (w/v) agarose gel. The agarose gel electrophoresis had two convenient purposes: first, it eliminated contaminants from the DNA so that it could be digested with restriction enzymes, and second, the ^{13}C-DNA formed a tight band that could be excised in an agarose plug in which restriction enzyme digestion of the ^{13}C-DNA could be performed. This minimized additional DNA shearing that could potentially occur when pipetting the DNA solution.

Partially digested ^{13}C-DNA was resolved on a standard agarose gel (20 cm × 20 cm) rather than a PFGE system, because standard electrophoresis resulted in a more tight distribution of the desired size fragments within the gel and easier elution from the gel. As described in the *Experimental procedures* section, extreme care was taken to avoid exposure of the DNA to UV irradiation. The partially BamHI-digested and size-selected DNA was recovered from the agarose and ligated into plasmid pCC1BAC™ and a library of 2300 clones was generated using the

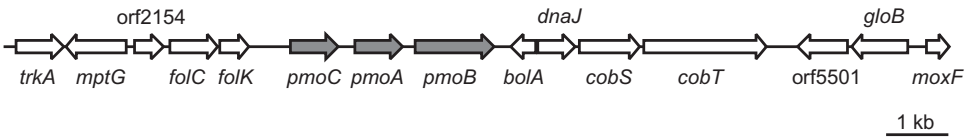

Fig. 2. Genetic map of ORFs identified on clone GSC357. The fragment is 15.2 kb in length. The assigned names represent the closest known genes (refer to Table 1).

E. coli TransforMax EC300™ cells provided with the vector (Epicentre).

Analysis of the SIP labelling and the metagenomic library

Bacterial 16S rRNA genes were amplified from the ^{12}C-DNA and ^{13}C-DNA by PCR and analysed by denaturing gradient gel electrophoresis (DGGE) (Fig. 1B). The DGGE pattern for the ^{12}C-DNA was complex, as expected for a soil bacterial community. The DGGE pattern for the ^{13}C-DNA showed less than 10 predominant bands. Sequencing of the dominant ^{13}C-DNA DGGE bands indicated the presence of the methanotrophic genera *Methylobacter* (GS_band_2), *Methylocella* (GS_band_4) and *Methylocystis* (GS_band_5) (Fig. 1B). The *Methylocystis* 16S rRNA sequence was very similar to the UP4, UP5, UP6 and UP7 16S rRNA sequences (AY080911, AY080917, AY080912, AY080914 respectively) that together constituted 96% of the 16S rRNA gene sequences from the ^{13}C-DNA in the original $^{13}CH_4$ SIP analysis of this forest soil (Radajewski *et al*., 2002). In addition to the methanotroph 16S rRNA gene sequences, a predominant sequence type (GS_band_1) was present with similarity to the *Bacteroidetes* and a second type (GS_band_3) with similarity to a *Gammaproteobacteria*; the latter was also retrieved in the previous SIP study of this forest soil (Radajewski *et al*., 2002).

After construction of the metagenomic library from ^{13}C-DNA, the plasmids were isolated from 48 clones chosen at random and analysed by digestion with BamHI followed by agarose gel electrophoresis. Most clones contained inserts between 10 and 30 kb and two of the 48 plasmids contained no visible insert DNA. Also, nearly half of the clones analysed had additional BamHI sites within the insert fragment (results not shown), which indicated that the cloned ^{13}C-DNA had been incompletely digested as intended, or alternatively had been partially protected from cutting by methylation. The sequencing primers T7 and RP2 annealed adjacent to the cloning site of pCC1BAC™ and were used to sequence the ends of several of the cloned inserts. The sequence data from the clones were compared with the GenBank database by BLAST analysis (Altschul *et al*., 1990) and were different from each other, with the exception of two clones that appeared to be identical. None of the genes identified by end sequencing were of obvious relevance to methylotrophy and these clones were not sequenced further.

The metagenomic library was screened by colony hybridization for *pmoA, mmoX* and *mxaF*, which encode subunits of the pMMO, soluble methane monooxygenase and methanol dehydrogenase respectively. Polymerase chain reaction primers were used to amplify the probe using the ^{13}C-DNA as template. As the probes were amplified from ^{13}C-DNA, there was a high probability that the homologous probe would be present for a cloned gene. The three gene hybridizations were performed separately and a total of four membranes each with approximately 575 clones were hybridized with ^{32}P-GTP random primed PCR probes.

The *mmoX* and *mxaF* probes did not hybridize with any of the clones in the library. Two clones, GSC357 (Gisburn Soil Clone) and GSC1346, hybridized strongly to the *pmoA* probe and the *pmoA* gene could be amplified from these clones by PCR (results not shown). The restriction fragment length polymorphism (RFLP) patterns of GSC357 and GSC1346 digested with NotI were identical (results not shown). GSC357 was completely sequenced using a shotgun approach. The complete sequence was 15 230 bp and a summary of the genes contained on this plasmid and their arrangement is given in Fig. 2 and Table 1. GSC1346 was partially sequenced and found to be very similar to the sequence of GSC357. It is likely that GSC357 and GSC1346 were from different strains of the same species.

Analysis of the pmoCAB *operon on clone GSC357*

GSC357 contained a complete *pmoCAB* operon, with *pmoC, pmoA* and *pmoB* of 765 bp, 759 bp and 1257 bp respectively. The intergenic region between *pmoC* and *pmoA* was 269 bp and between *pmoA* and *pmoB* was 227 bp. The sequences of *pmoCAB* operons and derived polypeptide sequences demonstrated considerable similarity to those of *Methylocystis* sp. strain M (Gilbert *et al*., 2000), *Methylosinus trichosporium* OB3b (Gilbert *et al*., 2000), *Methylocystis* sp. strain SC2 (Ricke *et al*., 2004) and *Methylococcus capsulatus* (Bath) (Semrau *et al*., 1995; Stolyar *et al*., 1999) (Table 2). Putative ribosome binding sites were present upstream (5′) of all three *pmo* genes on the clone. The *pmoCAB* operon in *Methylocystis*

Table 1. Putative genes corresponding to each ORF on GSC357.

Name	Protein name or role of closest BLASTX match	Accession No.	Source	% ID	'*e*-value'
trkA	Component of K^+ transport system	NP_422290	*Caulobacter crescentus*	50	$5e^{-35}$
mptG	β-ribofuranosylaminobenzene 5′-phosphate synthetase	AAS86339	*Hyphomicrobium zavarzinii*	42	$7e^{-54}$
orf2154	pterin-4α-carbinolamine dehydratase	ZP_00034540	*Burkholderia fungorum*	63	$9e^{-42}$
folP	dihydropteroate synthase	ZP_00050905	*Magnetospirillum magnetotacticum*	53	$6e^{-62}$
folK	2-amino-4-hydroxy-hydroxymethyldihydopteridine pyrophosphokinase (Hppk)	NP_102516	*Mesorhizobium loti*	46	$2e^{-29}$
pmoC	**pMMO subunit C**	AAF37896	*Methylocystis* sp. M	95	$1e^{-110}$
pmoA	**pMMO subunit A**	AAL87439	Uncultivated *Methylocystis* PmoA P12.9	98	$7e^{-102}$
pmoB	**pMMO subunit B**	CAE47801	*Methylocystis* sp. SC2	92	0.0
bolA	BolA-like protein	NP_945854	*Rhodopseudomonas palustris*	40	$2e^{-07}$
dnaJ	DnaJ-family molecular chaperone	ZP_00052274	*Magnetospirillum magnetotacticum*	48	$8e^{-44}$
cobS	Cobalt insertion protein	NP_766820	*Bradyrhizobium japonicum*	84	$1e^{-162}$
cobT	Cobyrinic acid synthase	NP_945849	*Rhodopseudomonas palustris*	61	0.0
orf5501	Predicted secreted protein	NP_772837	*Bradyrhizobium japonicum*	55	$2e^{-71}$
gloB	Zn-dependent hydrolase (GloB)	NP_772836	*Bradyrhizobium japonicum*	54	$2e^{-25}$
moxF	Methanol dehydrogenase large subunit-like protein	NP_772853	*Bradyrhizobium japonicum*	63	$1e^{-40}$

The enzyme, accession number and the organism corresponding to the closest relative in the GenBank database is given, as well as the per cent identity and the '*e*-value' of the BLAST algorithm (Altschul *et al*., 1990).

strains has been shown to be expressed from a σ^{70} promoter upstream of the *pmoC* and the transcriptional start has been mapped by primer extension for copies of *pmoCAB* (Gilbert *et al*., 2000; Ricke *et al*., 2004). A putative −35, −10 σ^{70} consensus sequence was present upstream of the *pmoC* gene on GSC357 and had the consensus sequence TTGTTT-N17-AATTGT and was located 311 bp from the start of *pmoC*.

The closest relatives of *pmoA* on GSC357 were genes from uncultivated methanotrophs previously detected in this soil by SIP and PCR analysis (Radajewski *et al*., 2002). The P12.9 (AY080955) *pmoA* gene sequence (531 bp) retrieved in the previous study was identical to the corresponding sequence from GSC357, with the exception of an ambiguous residue in P12.9 and four mismatches in the primer region used to amplify P12.9; these coding differences correspond to two mismatches in the derived PmoA polypeptide sequences and are likely to be PCR artefacts. The P13.7 (AY080951) *pmoA* sequence was the next closest match with seven nucleotide mismatches. P12.9 and P13.7 represented 9% and 26% of the ^{12}C-DNA and ^{13}C-DNA *pmoA* libraries in the previous study of this soil (Radajewski *et al*., 2002). A tree representing the phylogeny of the inferred PmoA sequence from GSC357 is shown (Fig. 3).

Additional open reading frames on GSC357

In addition to *pmoCAB*, a further 12 open reading frames (ORFs) were identified on GSC357 (Table 1). Previously published studies have identified genes flanking *pmoCAB* operons in *Methylocystis* sp. strain SC2 (Ricke *et al*., 2004), *Methylococcus capsulatus* Bath (Semrau *et al*., 1995; Ward *et al*., 2004) and *Methylosinus trichosporium* OB3b (Gilbert *et al*., 2000). None of the ORFs previously identified in the vicinity of *pmoCAB* operons correspond to those found on GSC357; however, unpublished data (I.R. McDonald and J.C. Murrell) for the second copy of *pmoCAB* in *Methylosinus trichosporium* OB3b indicate that, like GSC357, the *folP* and *folK* genes are immediately upstream of *pmoC* in this organism. Therefore it is possible that these *pmoCAB* operons are situated within similar regions of the chromosome in these organisms.

Table 2. Comparison of the derived PmoC, PmoA and PmoB polypeptide sequences from clone GSC357 with the corresponding sequences from cultivated methanotrophs.

Organism	PmoC (%)	PmoA (%)	PmoB (%)
Methylocystis strain M	93	92	88
Methylocystis strain SC2 (copy 1)	83	94	87
Methylocystis strain SC2 (copy 2)	65	64	58
Methylosinus trichosporium OB3b	85	88	77
Methylococcus capsulatus (Bath)	52	57	47

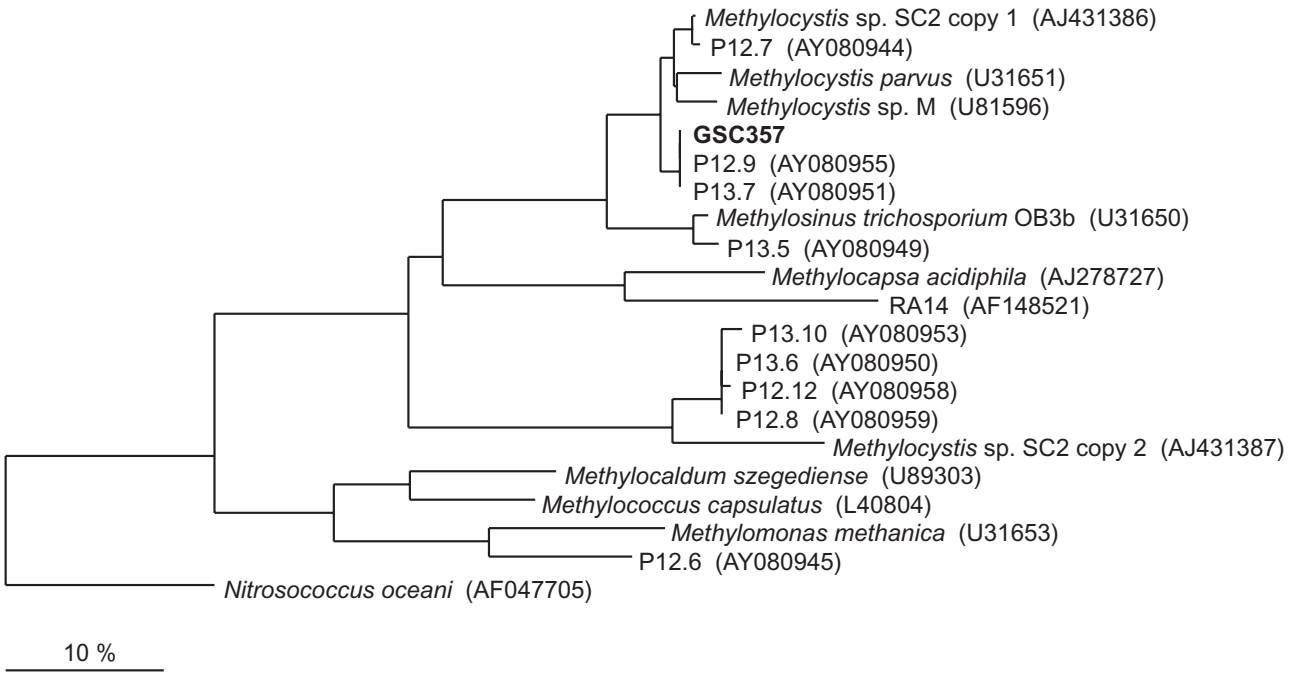

Fig. 3. Phylogenetic tree of the derived PmoA sequence from the GSC357 clone (shown in bold text). Representative PmoA sequences retrieved in the previous DNA-SIP analysis of this soil (Radajewski *et al*., 2002) and PmoA sequences from selected methanotrophs were included as references. The tree was constructed as previously described (Radajewski *et al*., 2002). The scale bar represents 10 amino acid substitutions per 100 residues.

Three of the 12 putative genes, *mptG*, *folP* and *folK,* have proposed roles in one-carbon metabolism (Chistoserdova *et al*., 2003). Dihydropteroate synthase (FolP) and 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydopteridine pyrophosphokinase (FolK) are involved in folate biosynthesis. In methylotrophs that use the serine pathway for formaldehyde fixation, a tetrahydrofolate cofactor is involved in transferring the methyl group, and on the genome of the facultative methylotroph, *Methylobacterium extorquens* AM1, these genes are located within a cluster of genes essential for growth on methanol (Chistoserdova *et al*., 2003). The *mptG* gene encodes β-ribofuranosylaminobenzene 5′-phosphate synthetase (β-RFAP synthase), which is involved in methanopterin biosynthesis (Scott and Rasche, 2002). Tetrahydromethanopterin is an 'archaeal' cofactor that is also present in methylotrophic bacteria and involved in the transfer of the methyl group during oxidation or assimilation of formaldehyde (Chistoserdova *et al*., 1998; 2000). A '*moxF′*-like gene is present downstream (3′) of the *pmoCAB* operon. These *moxF* ORFs are apparent homologues of *mxaF,* which encode the large subunit of the methanol dehydrogenase. Similar *mxaF* homologues have been identified in the methylotrophs *Methylobacterium extorquens* (AAB58890) and *Methylococcus capsulatus* (AAU90462) and organisms not known to incorporate one-carbon compounds, such as *Bradyrhizobium japonicum* (NP_772853) and *Sinorhizobium meliloti* (NP_436713). The ORF between *mptG* and *folP,* designated orf2154, is similar to genes encoding pterin-4α-carbinolamine dehydratase, which is involved in the biosynthesis of the tetrahydrobiopterin (BH4) cofactor (Thöny *et al*., 2000).

Discussion

Traditionally, DNA-SIP has been used in conjunction with PCR of rRNA and 'functional' genes to identify the organisms that have incorporated the heavy substrate. Although only a small number of genes are usually targeted by PCR and analysed, DNA-SIP results in the retrieval of the complete genomic DNA complement of the labelled population. This is the first study in which the ^{13}C-DNA from a DNA-SIP experiment has been cloned into a vector in order to directly capture genomic fragments of the active population, without needing to first isolate the genes by PCR. There is great potential for this technique to provide a large amount of useful genetic and metabolic data from an active population occupying a specific ecological niche within a habitat, particularly if the funds for a large sequencing effort are available.

In this study, a library of 2300 clones was constructed from ^{13}C-DNA obtained from a DNA-SIP experiment with $^{13}CH_4$. A clone, designated GSC357, was identified that contained a *pmoCAB* operon, which encodes the pMMO

enzyme that catalyses the first step in the metabolism of methane by methanotrophic bacteria (Semrau *et al*., 1995; Hanson and Hanson, 1996). Phylogenetic analysis of the *pmoA* gene sequence indicated that it is most closely related to the sequences from *Methylocystis* species and the gene was virtually identical to a sequence previously detected in this forest soil (Radajewski *et al*., 2002). This demonstrates that it is feasible to retrieve key genomic fragments from a target population by combining SIP and metagenomic analysis. The majority of the ^{13}C-DNA purified was between 15 kb and 100 kb in length, and the maximum insert size observed in the clone library was approximately 30 kb. Previous studies have shown that with extensive optimization it is possible to obtain clones from soil with inserts as large as 80 kb (Rondon *et al*., 2000). Pulsed-field gel electrophoresis analysis of the ^{13}C-DNA obtained in our study indicated that DNA fragments larger than 100 kb were present, and therefore it should be possible to obtain ^{13}C-DNA clones significantly larger than 30 kb, or at least that which could be packaged using a cosmid or fosmid system.

In addition to *pmoA*, the metagenomic clone library was also screened for genes encoding the soluble methane monooxygenase (*mmoX*) and the methanol dehydrogenase (*mxaF*). Neither the *mmoX* nor *mxaF* gene was detected, which indicates that the library screen was insufficient to provide coverage of these genes. The clone library in this study was generated by partial digestion with BamHI, which recognizes a 5′-GGATTC-3′ sequence, and the library will have been biased towards BamHI fragments that fall within the 10–30 kb size range; if two BamHI sites were not within 30 kb of each other in a genome, it is unlikely that this region of DNA would be represented in the library. Two of the 48 clones that were end-sequenced appeared to be identical, which may also suggest that the cloning was non-random. Also, GSC357 and GSC1346 appeared to be the same fragment from similar strains of a *Methylocystis* species. This possible bias associated with using an enzyme, such as BamHI, that recognizes a six-base sequence could be reduced by increasing the size of the ligated ^{13}C-DNA fragments, or by generating several libraries each with a different enzyme. Preferably, an enzyme that cuts DNA more frequently, such as Sau3AI, should be used. Some vectors, including fosmids, will ligate blunt ends produced by random shearing and end-repair, and should produce the most random library.

A major criticism of the DNA-SIP technique is the long incubation periods and artificially elevated substrate concentrations often used to label the sample, which may result in an enrichment effect and cross-feeding of the label to other members of the community. The sensitivity of RNA-SIP has permitted the use of less ^{13}C-substrate and more recent studies have performed successful DNA-SIP incubations by pulse-labelling samples with more realistic concentrations of ^{13}C-substrate (Lueders *et al*., 2004). DNA-SIP incubations for the purpose of constructing a metagenomic library may require greater ^{13}C-DNA than is required as template for PCR analysis and the amount of $^{13}CH_4$ used in this study far exceeded the concentration this forest soil would normally encounter. The PCR amplification of 16S rRNA genes from the ^{13}C-DNA related to the *Bacteroidetes* (GS_band_1) and an uncultivated *Gammaproteobacteria* (GS_band_2), which are organisms not known to oxidize methane, may be evidence of cross-feeding. Ideally, SIP conditions should be optimized empirically to limit enrichment and cross-feeding, but still provide adequate ^{13}C-DNA for construction of the metagenomic library.

The clone GSC357 that contained a *pmoCAB* operon also carried 12 additional ORFs, some of which are related to genes encoding enzymes known to be involved in growth on one-carbon compounds. Analysis of the genome sequence of the facultative methylotroph, *Methylobacterium extorquens* AM1, indicated that many methylotrophy genes are linked in clusters on the chromosome (Chistoserdova *et al*., 2003). With the possible exception of *moxF*, none of the genes situated downstream (3′) of the *pmoCAB* operon on GSC357 appear to be specifically related to methylotrophic growth (Table 1). The functions of many of the genes surrounding *pmoCAB* on GSC357 have been addressed in previous culture-based studies. Upstream of the *pmoC* were *folC*, *folK* and *mptG,* which are all necessary for growth on one-carbon compounds (Chistoserdova and Lidstrom, 1997; Chistoserdova *et al*., 2003; Kalyuzhnaya *et al*., 2005). The presence of a gene encoding pterin-4α-carbinolamine dehydratase (*orf2152*) located upstream of methylotrophy genes was intriguing as it may imply that it had a function related to growth on one-carbon compounds; however, a null mutant of the homologous gene was obtained in *Methylobacterium extorquens* AM1 and found to have no effect on growth with methanol (Kalyuzhnaya *et al*., 2005). Chistoserdova and Lidstrom (1997) were unable to generate a null mutant of *folC* in *Methylobacterium extorquens* AM1, presumably because folate is unconditionally essential for growth. The *mptG* gene that encodes β-RFAP synthetase, which is involved in the synthesis of the H_4MPT cofactor, is present in many Gram-negative methylotrophs (Chistoserdova *et al*., 2000; 2003; Kalyuzhnaya *et al*., 2005). An *mptG* null mutant of *Methylobacterium extorquens* AM1 was incapable of growth on methanol (Kalyuzhnaya *et al*., 2005). The *mptG* gene has been shown to be situated within gene islands containing the genes involved in the biosynthesis of H_4MPT and the genes involved directly in H_4MPT-linked C_1 transfer reactions (Kalyuzhnaya *et al*., 2005). The genes identified by Kalyuzhnaya and colleagues that normally flank *mptG* were not identified on

the soil clone and therefore the position of the *mptG* gene on this clone from an uncultivated *Methylocystis* sp. is unusual. Within the genome of *Methylococcus capsulatus* (Bath) (AE017282), the *mptG* gene is also positioned in close proximity to a *pmoCAB* operon, but is separated by four genes, *fhcC, fhcD, fhcA* and *fhcB*, that encode subunits of the formyltransferase/hydrolase complex for C_1 transfer.

In summary, the retrieval of SIP-generated ^{13}C-DNA from environmental samples provides access to the genomes of bacteria in the environment that were involved in specific metabolic processes. As shown in this study, the genes encoding metabolic pathways are often linked on chromosomes and therefore it is possible by combining DNA-SIP and metagenomics to retrieve targeted genetic information with minimal sequencing effort. With continued advances in genomics and DNA sequence analysis, it may soon become more feasible to reconstruct the complete genomes of microbial populations and consortia directly from the environment (Venter *et al*., 2004). Until now, metagenomic studies have been largely restricted to the most abundant organisms in the community, but DNA-SIP offers a breakthrough means by which ecologically relevant, and potentially subdominant, community members may be characterized and their metabolic functions revealed.

Experimental procedures

Soil sampling and SIP

The characteristics of the Gisburn forest soil were described previously (Radajewski *et al*., 2002). Soil was collected in August 2002 and SIP experiments were performed essentially as described previously (Radajewski *et al*., 2002). Briefly, a soil slurry was made by adding 10 ml of ANMS medium (NMS medium (Whittenbury *et al*., 1970) containing 0.5 g NH_4Cl, 0.5 g KNO_3 and buffered with 4 mM phosphate, pH 3.5) to 5 g of soil. The slurry was incubated in a 125 ml serum vial sealed with a butyl stopper on a rotary shaker (~100 rpm) at room temperature (20–25°C) and in the dark. The soil DNA was harvested after consumption of a total of 50 ml of $^{13}CH_4$ (Linde) added in 10 ml aliquots to the soil slurry microcosm (Radajewski *et al*., 2002). The headspace was flushed with air between injections to limit the accumulation of $^{13}CO_2$ from complete oxidation of $^{13}CH_4$ by methanotrophs.

DNA extraction from soil

The protocol for DNA extraction from soil was based on the method previously described (Zhou *et al*., 1996), with several modifications. The soil slurry was placed in a 35 ml Oakridge tube and centrifuged at 6000 *g* in a JA20 rotor. The soil pellet was suspended in 13.5 ml of extraction buffer [100 mM Tris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 1% (w/v) CTAB] to which 100 μl of fresh Proteinase K (10 mg ml^{-1}) was added. The tube was placed horizontally on a 200 rpm shaker at 37°C for 30 min. 1.5 ml of 20% (w/v) SDS was added and the tube was placed at 65°C for 2 h and mixed by inversion every 15 min. The supernatant was decanted into a clean tube after centrifugation for 10 min at 6000 *g* at 25°C. The soil pellet was again suspended in 4.5 ml of extraction buffer and 0.5 ml of 20% (w/v) SDS, incubated at 65°C, centrifuged as before and the supernatant added to the first aliquot. The crude extract (~20 ml) was gently extracted with chloroform [containing 4% (v/v) isoamyl alcohol to minimize foaming] and centrifuged at 16 000 *g* for 10 min at 25°C. The aqueous phase was transferred to a clean tube with a wide bore 5 ml pipette tip, made by cutting ~5 mm from the tip end. Care was taken to leave the interface undisturbed. The DNA was precipitated from the aqueous phase by adding 0.6 vol. (~10.5 ml) of 2-propanol, mixing gently and incubating for 1 h at room temperature. The DNA was pelleted by centrifugation at 16 000 *g* for 20 min at 20°C. The DNA pellet was rinsed with 5 ml of 70% (v/v) ethanol and air-dried for 20 min.

Ultracentrifugation

Total DNA from the extraction was dissolved in 20 ml of TE buffer (Sambrook and Russell, 2001) at 4°C for 16 h. The volume of DNA solution was measured and exactly 1 g ml^{-1} CsCl was dissolved by gentle mixing. Five hundred microlitres of ethidium bromide (10 mg ml^{-1}) was added and the solution transferred to a 25 mm × 89 mm polyallomer Quick-Seal™ ultracentrifuge tube (Beckman). Unfilled tube volume was filled by adding 1 g ml^{-1} CsCl in TE buffer. The tube was centrifuged at 180 000 *g* (45 000 rpm) for 20 h. If the position of the DNA in the gradient could not be seen in visible light, the tube was exposed to long wavelength UV (365 nm) for a minimum time (< 2 s); it was found that indiscriminate UV exposure subsequently made it impossible to clone the DNA. The portion of gradient containing the DNA was collected using a 16-gauge needle and 2.5 ml syringe (Sambrook and Russell, 2001). Another 250 μl of ethidium bromide (10 mg ml^{-1}) was added to the DNA solution, which was transferred to a new 25 mm × 89 mm Quick-Seal™ tube and centrifuged as before. DNA bands were collected with a needle and syringe as before. The DNA was then transferred to a 13 mm × 51 mm polyallomer Quick-Seal™ tube and centrifuged at 265 000 *g* (55 000 rpm) in a VTi65 rotor at 20°C. The bands were collected independently (^{13}C-DNA followed by ^{12}C-DNA) and each placed in a new 13 mm × 51 mm Quick-Seal™ tube and centrifuged as before. The repeated centrifugations were performed to ensure that the ^{13}C-DNA was purified from contaminating ^{12}C-DNA and coextracted soil organics. Ethidium bromide was removed from DNA preparations by repeated extractions with water-saturated 1-butanol (Sambrook and Russell, 2001). The CsCl was removed by dialysis against TE buffer and the DNA stored at 4°C.

The desalted DNA was purified by electrophoresis on a 1% (w/v) low melting point agarose gel in 1× TAE buffer without added ethidium bromide. Low melting point agarose (Gibco) was used because it has a large pore size which allows the restriction enzyme to penetrate the agarose plug (Sambrook and Russell, 2001). To minimize DNA shearing, 3 mm was

cut off from the end of the pipette tips to increase the bore of the opening for all pipetting of ^{13}C-DNA. A Nile Blue staining protocol, which forms a complex with DNA visible in white light, was used in place of ethidium bromide to eliminate any further exposure of the DNA to UV radiation (Adkins and Burmeister, 1996; Yang *et al.*, 2000). The gel was stained by soaking overnight in 15 μg ml^{-1} Nile Blue in water. The gel fragment (~3 mm thick) containing the DNA was excised with a clean scalpel and stored in TE buffer at 4°C.

Partial restriction enzyme digestion of DNA

Agarose gel fragments containing ~2 μg DNA were washed three times at room temperature in 20 ml of TE buffer on a tube roller. The agarose plugs were suspended in 1 ml of 1× restriction enzyme buffer and incubated for 1 h at 37°C. The equilibration in restriction enzyme buffer was repeated with fresh buffer. The plugs were transferred to 2 ml microcentrifuge tubes containing 500 μl of cold 1× restriction enzyme buffer with *n* units of restriction enzyme. For each experiment, a range of enzyme concentrations was used; for example, with BamHI $n = 0$, 5, 10, 25, 50, 100, 200 and 500 units. The enzyme was allowed to penetrate the agarose matrix for 1 h on ice. The tubes were then incubated for 1 h in a 37°C water bath so that the restriction enzyme could cut the DNA molecules. The same method was used with Sau3AI restriction enzyme, but ultimately the libraries generated were not used as they contained many clones with tiny inserts.

Immediately after restriction enzyme digestion, the agarose plugs were inserted into the wells of a 20 cm 1% (w/v) agarose TAE gel. DNA markers of an appropriate size were included on the gel and loaded on both sides of the lanes containing the agarose plugs. A relatively complex staining protocol was followed to circumvent exposing the partially digested DNA to UV radiation. The lanes containing the DNA markers were cut from the gel and stained by soaking in 0.5 μg ml^{-1} ethidium bromide. The stained gel was placed on a UV transilluminator and the positions of the DNA bands of the markers were indicated by placing strips of paper on the gel surface. The DNA marker lanes were then replaced alongside the unstained gel. Using the paper strips as a guide, the gel region containing 30–50 kb partially digested DNA was derived and the regions removed with a scalpel from each lane. The remainder of the gel was stained in ethidium bromide and photographed on a UV transilluminator. The gel slices containing size-selected DNA were identified as those having DNA both ahead and behind the removed region; for BamHI, this was found to be those treated with 75 units of enzyme.

Ligation of DNA into pCC1BAC™

High molecular mass DNA was recovered from agarose gels by electroelution. Dialysis tubing was prepared according to Sambrook and Russell (2001). The gel segment containing the DNA to be recovered was excised with a scalpel and clamped inside the dialysis tubing with approximately 250 μl of 1× TAE buffer. The bag was submerged in 1× TAE inside an electrophoresis unit and 5 V cm^{-1} applied for 45 min. The current was reversed for 1 min to free DNA retained on the tubing wall. To minimize mechanical shearing of the DNA, the solution was removed from the tubing using wide bore 200 μl Gilson pipette tips constructed by cutting ~3 mm from the tip end. The DNA was purified by drop dialysis against 0.5× TE buffer using VSWP membranes (Millipore) according to the method of Sambrook and Russell (2001). The DNA was ligated into pCC1BAC™ in a 100 μl volume according to the manufacturer's instructions (Epicentre).

Electrotransformation of E. coli *TransforMax EC300™*

Transformations of DNA into *E. coli* TransforMax EC300™ were performed according to the supplier's instructions (Epicentre). The ligations were desalted by drop dialysis against 0.5× TE buffer using VSWP membranes (Millipore) according to the method of Sambrook and Russell (2001). The electroporation was performed in 0.1 cm cuvettes at the following settings: 2.5 kV cm^{-1} at 25 μF and 100 Ω on a Bio-Rad GenePulser™.

Construction of a soil DNA clone library

White colonies were picked from the Luria–Bertani (LB) [40 μg ml^{-1} X-gal, 0.4 mM IPTG, 12.5 μg ml^{-1} chloramphenicol (Cm)] agar plates using sterile toothpicks and transferred to individual wells of 96 well plates containing 150 μl per well of freezing medium [LB broth, 7.5% (v/v) glycerol, 12.5 μg ml^{-1} Cm]. The plates were incubated for 24 h at 37°C and subsequently stored at −80°C.

Nylon Hybond N+ membranes (Amersham) were inoculated from the 96 well plates using a 96-pin replicating device (Boekel). The gene probes were amplified from the ^{13}C-DNA by PCR using the A189f/682r *pmoA* primers (Holmes *et al.*, 1995), mmoX206f/mmoX886r *mmoX* primers (Hutchens *et al.*, 2004) and the 1003f/1561r *mxaF* primers (McDonald and Murrell, 1997) according to described protocols (Dumont and Murrell, 2005b). The PCR gene probes were labelled by random priming (Feinberg and Vogelstein, 1983; 1984) using hexanucleotide primers and dNTPs from Roche according to the manufacturer's instructions. Between 25 and 50 ng of agarose gel-purified DNA fragment was labelled with 50 μCi of [α-^{32}P]dGTP for 1 h at 37°C with Klenow polymerase (Invitrogen). Unincorporated label was removed using a MicroSpin™ Column (Amersham Pharmacia Biotech) according to the manufacturer's instructions. 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.

Membranes were rolled in mesh and placed in a Hybaid tube containing 20 ml of prewarmed prehybridization solution [0.5 M sodium phosphate (pH 7.2), 7% (w/v) SDS, 5 mM EDTA] and incubated in a Hybaid oven for 30 min at 55°C. The prehybridization solution was discarded and replaced with 20 ml of fresh 55°C solution, to which the denatured radiolabelled DNA probe was added. Hybridizations were carried out overnight at 55°C.

The hybridization solution was discarded and the membrane washed twice with 100 ml of 2× SSC 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 greater stringency by incre-

mentally decreasing the salt concentration and/or increasing the wash temperature. In most instances, a wash with 0.5× SSC at 65°C was adequate to remove background hybridization signals.

Pulsed-field gel and denaturing gradient gel electrophoresis

Pulsed-field gel electrophoresis was performed using a Bio-Rad Chef Mapper™. Denaturing gradient gel electrophoresis was performed using a DCode universal mutation detection system (Bio-Rad Laboratories). Bacterial 16S rRNA genes were amplified using the 341F-GC and 907RM PCR primers (Schäfer and Muyzer, 2001). A polyacrylamide gel with a gradient of 30–70% denaturant was used. Gels were run for 18 h at 100 V at 60°C and were stained with Sybr green nucleic acid stain.

DNA sequencing and analysis

The selected BAC clone was sequenced using a shotgun sequencing method. The cloned insert DNA was excised from the vector and gel-purified using the GenecleanII Kit (Bio101). The purified insert DNA was partially digested for 1 h at 37°C with *Bsp*143I by incubating with a limiting quantity of enzyme (~0.05 unit, optimized empirically). The digested fragments were resolved by agarose gel electrophoresis and fragments of 0.8–1.5 kb were gel-purified and ligated into pUC19, which had been linearized with BamHI and dephosphorylated using calf intestinal alkaline phosphatase (Roche). *Escherichia coli* transformants were isolated, the clones purified by plasmid miniprep and analysed by RFLP to ensure the inserts were 0.8–1.5 kb. Clones were chosen at random and the plasmid DNA sequenced using the M13f primer (Invitrogen). Approximately sixfold sequence data were accumulated and analysed using the SEQMANII software program (DNASTAR Inc). To join contiguous sequences and re-sequence regions of poor data, the original (full length) clone was sequenced using custom-designed primers (Invitrogen).

The assembled GSC357 sequence was submitted to GenBank with the Accession Number DQ379514 and the 16S rRNA sequences with Accession Numbers DQ379509–DQ379513.

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