DNA-, rRNA- and mRNA-based stable isotope probing of aerobic methanotrophs in lake sediment

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Summary

**A stable isotope probing (SIP) approach was used to
study aerobic methane-oxidizing bacteria (methan-
otrophs) in lake sediment. Oligotrophic Lake Stechlin
was chosen because it has a permanently oxic sedi-
ment surface. 16S rRNA and the *pmoA* gene, which
encodes a subunit of the methane monooxygenase
enzyme, were analysed following the incubation of
sediment with $^{13}CH_4$ and the separation of ^{13}C-labelled
DNA and RNA from unlabelled nucleic acids. The
incubation with $^{13}CH_4$ was performed over a 4-day
time-course and the *pmoA* genes and transcripts
became progressively labelled such that approxi-
mately 70% of the *pmoA* genes and 80% of the tran-
scripts were labelled at 96 h. The labelling of *pmoA*
mRNA was quicker than *pmoA* genes, demonstrating
that mRNA-SIP is more sensitive than DNA-SIP;
however, the general rate of *pmoA* transcript labelling
was comparable to that of the *pmoA* genes, indicating
that the incorporation of ^{13}C into ribonucleic acids
of methanotrophs was a gradual process. Labelling of
Betaproteobacteria was clearly seen in analyses of
16S rRNA by DNA-SIP and not by RNA-SIP, suggest-
ing that cross-feeding of the ^{13}C was primarily
detected by DNA-SIP. In general, we show that the
combination of SIP approaches provided valuable
information about the activity and growth of the
methanotrophic populations and the cross-feeding of
methanotroph metabolites by other microorganisms.**

Introduction

Stable isotope probing (SIP) methods can identify micro-
organisms within a community that use a particular growth
substrate. It is performed by adding isotopically labelled
compounds to an environmental sample and then analys-
ing the isotope-labelled biomarkers that are produced in
the target organisms. The approach has been applied to
lipids (Evershed *et al.*, 2006), nucleic acids (Neufeld
et al., 2007) and proteins (Jehmlich *et al.*, 2008). With
respect to nucleic acids, DNA-SIP and rRNA-SIP are well-
established methods (Radajewski *et al.*, 2000; Manefield
et al., 2002; Neufeld *et al.*, 2007; Whiteley *et al.*, 2007),
but mRNA-SIP has only been reported once (Huang *et al.*,
2009). Although ^{13}C-labelled substrates have been most
commonly used for SIP, the method has also been estab-
lished for ^{15}N-labelled substrates (Cadisch *et al.*, 2005;
Buckley *et al.*, 2007; Cupples *et al.*, 2007; Addison *et al.*,
2010) and in principle can be performed with other iso-
topes of atoms present in nucleic acids (Radajewski *et al.*,
2003; Schwartz, 2007).

DNA-SIP and rRNA-SIP each have their advantages.
The primary advantage of rRNA-SIP is the increased sen-
sitivity resulting from earlier and more rapid accumulation
of isotope into RNA than DNA (Manefield *et al.*, 2002).
Also, at least in theory, RNA can be labelled in non-
growing cells, whereas isotope will mostly be incorporated
into DNA during replication of the genome associated with
cell division. The key advantage of DNA-SIP over rRNA-
SIP is that complete genomes are labelled allowing func-
tional genes to be targeted by PCR. As an alternative to
PCR, the labelled metagenomic fragments recovered with
DNA-SIP can be cloned and sequenced (Dumont *et al.*,
2006; Chen *et al.*, 2008; Kalyuzhnaya *et al.*, 2008;
Neufeld *et al.*, 2008; Sul *et al.*, 2009), enabling what has
been termed 'high-resolution metagenomics' of a target
group (Kalyuzhnaya *et al.*, 2008).

In principle, mRNA-SIP would combine the strengths of
the methods: high sensitivity and functional information.
For enzymes that are regulated at the level of transcrip-
tion, the detection of mRNA transcripts will also indicate
that the corresponding biochemical pathway is functioning
in the cell. As such, mRNA-SIP will be a powerful method
in microbial ecology as it can both identify the organisms
that are assimilating a substrate and also indicate which
genes are being expressed in order to do so. Huang and
colleagues (2009) labelled organisms in groundwater
using ^{13}C-naphthalene and were able to detect naphtha-
lene dioxygenase (NDO) mRNA from both light and heavy
fractions of a caesium trifluoracetate (CsTFA) density

gradient. This suggested that NDO transcripts were stable during the gradient density centrifugation and that the buoyant densities of labelled and unlabelled NDO mRNA corresponded to that of the labelled and unlabelled rRNA respectively. This was an encouraging first demonstration of the possibility of mRNA-SIP.

Methanotrophic bacteria can use methane as a sole source of carbon and energy (Hanson and Hanson, 1996). The known aerobic methanotrophic bacteria are found in the *Proteobacteria* and *Verrucomicrobia* and those in the *Proteobacteria* are frequently referred to as either type I or II methanotrophs. The type I methanotrophs are members of the family *Methylococcaceae* and belong to the γ-*Proteobacteria* (Bowman *et al.*, 1993). The type II methanotrophs are α-*Proteobacteria* and include several genera in the family *Methylocystaceae* (Bowman *et al.*, 1993) and the genera *Methylocapsa* (Dedysh *et al.*, 2002) and *Methylocella* (Dedysh *et al.*, 2000) in the family *Beijerinckiaceae*. The type I methanotrophs can be subdivided into two phylogenetic clades: the type Ia, which primarily includes the mesophilic genera, and type Ib (also referred to as type X), which includes some moderately thermophilic genera. Currently, the known methanotrophs from the phylum *Verrucomicrobia* are extremophiles and are restricted to some unique high-temperature environments (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008). The oxidation of methane is catalysed by a methane monooxygenase enzyme (MMO), of which there are two distinct types: a particulate (pMMO) and soluble (sMMO) enzyme (Hakemian and Rosenzweig, 2007). Almost all methanotrophs possess the pMMO and the *pmoA* gene, encoding the β-subunit, is often used as a functional and phylogenetic marker for methanotrophs (McDonald *et al.*, 2008).

The objective of the study was to demonstrate the possibility of performing mRNA-SIP and to compare results obtained by the simultaneous analysis of labelled DNA, rRNA and mRNA of a target group. We used $^{13}CH_4$ to target aerobic methanotrophs, which are organisms that play an important role limiting the release of methane from many environments including lakes (Bastviken *et al.*, 2008).

Results

Diversity of methanotrophs in Lake Stechlin sediment

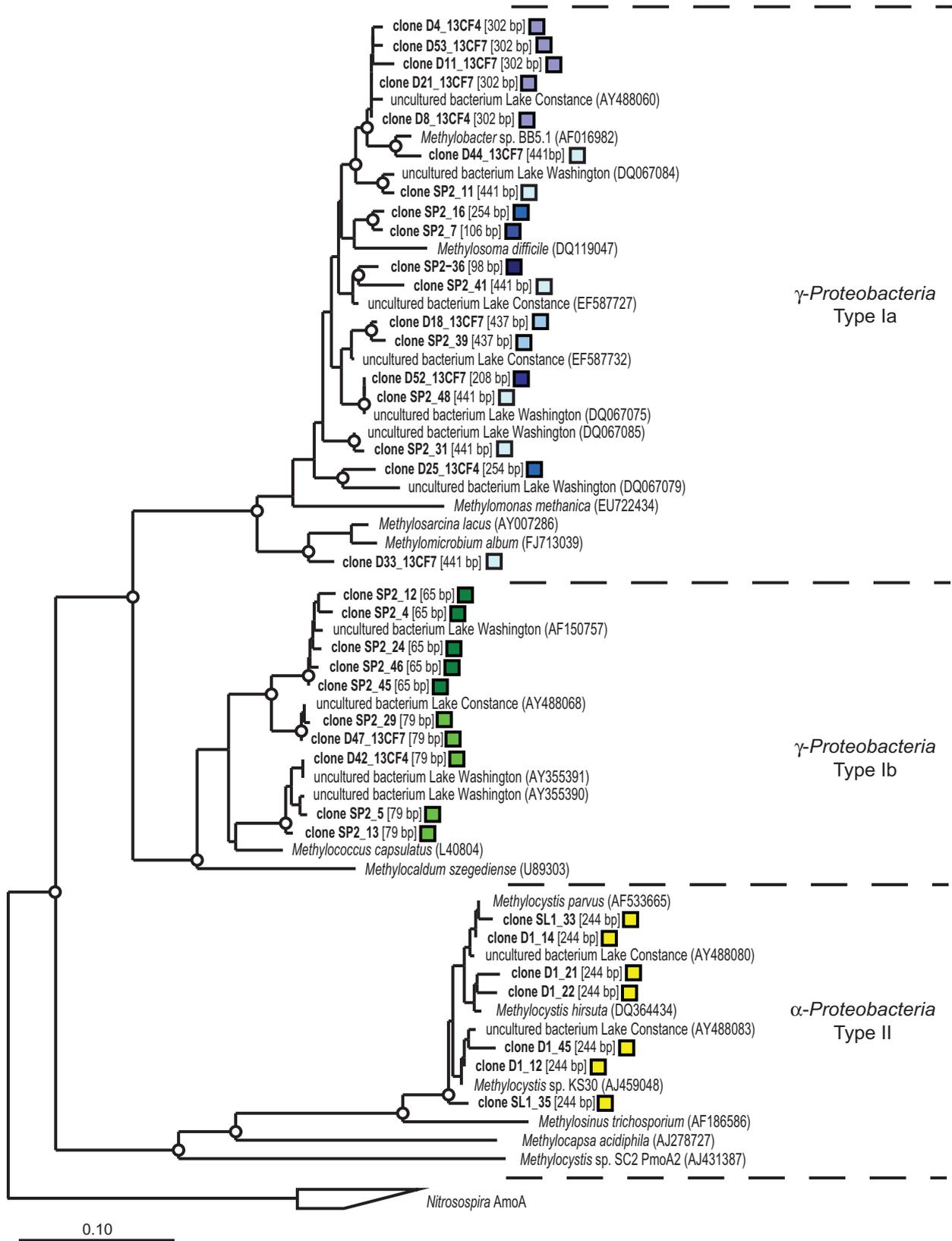PCR, cloning and sequencing of *pmoA* genes from Lake Stechlin sediment DNA indicated that representatives of type Ia, type Ib and type II methanotrophs were present (Fig. 1). With the exception of type II *pmoA* gene sequences that grouped closely with those from some cultivated *Methylocystis* species, closely affiliated *pmoA* sequences from cultivated representatives were not present for most of the sequences detected. Instead, many of the sequences clustered with those retrieved from Lake Washington and Lake Constance sediments, suggesting they belonged to yet uncultivated lake sediment-adapted methanotrophs. In particular, the type Ib *pmoA* sequences lack closely related cultivated representatives. A library of 45 *pmoA* clones from Lake Stechlin profundal sediment contained 18 type Ia clones, 27 type Ib and no type II sequences. Some additional clone sequences from Lake Dagow, which is located adjacent to Lake Stechlin, are also included in the phylogenetic tree (Fig. 1) because the clones matched some of the minor terminal restriction fragments (T-RFs) observed in the terminal restriction fragment length polymorphism (T-RFLP) analyses, as described below.

Terminal restriction fragment length polymorphism of *pmoA* was used to assess the relative abundance of methanotrophs in individual samples. T-RFLP of *pmoA* has been used in several studies, including rice field soil (Horz *et al.*, 2001; Shrestha *et al.*, 2008) and lake sediment (Pester *et al.*, 2004; Rahalkar and Schink, 2007). The size of the *pmoA* T-RFs generated from a double-digest with the enzymes MspI and RsaI restriction enzymes are indicated and colour-coded in the phylogenetic tree (Fig. 1); the same colour-coding is used for T-RFLP results depicted elsewhere in this study. In most instances the assignment of T-RFs is straightforward, particularly for the type Ib (65 and 79 bp) and type II (244 bp) *pmoA* sequences. The type Ia sequences possess numerous T-RF sizes (98, 106, 208, 254, 302, 437, 441 bp).

Time-course SIP of methanotrophs in Lake Stechlin sediment

A time-course labelling experiment with $^{13}CH_4$ was performed to monitor the incorporation of ^{13}C into the DNA, RNA and mRNA of the methanotroph community in Lake Stechlin sediment. Initially the incubation was performed at 4°C, which is close to the *in situ* sediment temperature; however, the CH_4 uptake rate was relatively low (Fig. S1) and no labelling of *pmoA* could be detected by subsequent SIP analysis (results not shown). Therefore, the

Fig. 1. Neighbor-joining phylogenetic tree of deduced PmoA sequences from Lakes Stechlin and Dagow. Reference sequences from the database and their corresponding GenBank accession numbers are shown. The names of sequences retrieved during this study are shown in bold and the size of the T-RF generated from a double-digest with RsaI and MspI is given in square brackets and colour-coded. The clone names beginning with 'D' were obtained from Lake Dagow sediment, those with 'SP' from Lake Stechlin profundal and those with 'SL' from Lake Stechlin littoral sediment. AmoA sequences from *Nitrosospira* were used as outgroup. Circles at branch nodes indicate those that were also present in the consensus tree of a bootstrapped maximum-likelihood analysis.
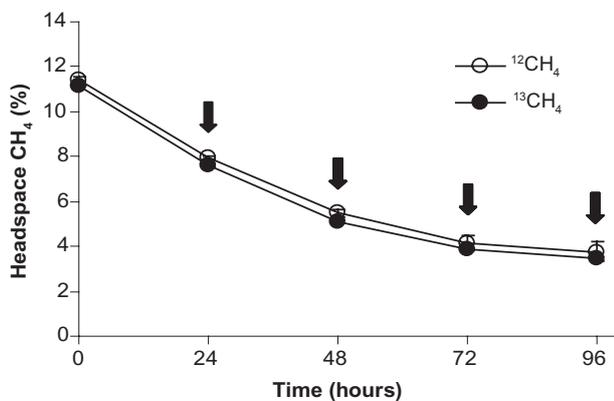
clone D4_13CF4 [302 bp] ■
clone D53_13CF7 [302 bp] ■
clone D11_13CF7 [302 bp] ■
clone D21_13CF7 [302 bp] ■
uncultured bacterium Lake Constance (AY488060)
clone D8_13CF4 [302 bp] ■
Methylobacter sp. BB5.1 (AF016982)
clone D44_13CF7 [441bp] ■
uncultured bacterium Lake Washington (DQ067084)
clone SP2_11 [441 bp] ■
clone SP2_16 [254 bp] ■
clone SP2_7 [106 bp] ■
Methylosoma difficile (DQ119047)
clone SP2−36 [98 bp] ■
clone SP2_41 [441 bp] ■
uncultured bacterium Lake Constance (EF587727)
clone D18_13CF7 [437 bp] ■
clone SP2_39 [437 bp] ■
uncultured bacterium Lake Constance (EF587732)
clone D52_13CF7 [208 bp] ■
clone SP2_48 [441 bp] ■
uncultured bacterium Lake Washington (DQ067075)
uncultured bacterium Lake Washington (DQ067085)
clone SP2_31 [441 bp] ■
clone D25_13CF4 [254 bp] ■
uncultured bacterium Lake Washington (DQ067079)
Methylomonas methanica (EU722434)
Methylosarcina lacus (AY007286)
Methylomicrobium album (FJ713039)
clone D33_13CF7 [441 bp] ■

γ-Proteobacteria
Type Ia

clone SP2_12 [65 bp] ■
clone SP2_4 [65 bp] ■
uncultured bacterium Lake Washington (AF150757)
clone SP2_24 [65 bp] ■
clone SP2_46 [65 bp] ■
clone SP2_45 [65 bp] ■
uncultured bacterium Lake Constance (AY488068)
clone SP2_29 [79 bp] ■
clone D47_13CF7 [79 bp] ■
clone D42_13CF4 [79 bp] ■
uncultured bacterium Lake Washington (AY355391)
uncultured bacterium Lake Washington (AY355390)
clone SP2_5 [79 bp] ■
clone SP2_13 [79 bp] ■
Methylococcus capsulatus (L40804)
Methylocaldum szegediense (U89303)

γ-Proteobacteria
Type Ib

Methylocystis parvus (AF533665)
clone SL1_33 [244 bp] ■
clone D1_14 [244 bp] ■
uncultured bacterium Lake Constance (AY488080)
clone D1_21 [244 bp] ■
clone D1_22 [244 bp] ■
Methylocystis hirsuta (DQ364434)
uncultured bacterium Lake Constance (AY488083)
clone D1_45 [244 bp] ■
clone D1_12 [244 bp] ■
Methylocystis sp. KS30 (AJ459048)
clone SL1_35 [244 bp] ■
Methylosinus trichosporium (AF186586)
Methylocapsa acidiphila (AJ278727)
Methylocystis sp. SC2 PmoA2 (AJ431387)

α-Proteobacteria
Type II

Nitrosospira AmoA

0.10

Fig. 2. CH_4 concentrations in bottles for the Lake Stechlin time-course SIP labelling. The times when sediment was removed for nucleic acid extraction are indicated with arrows. Open circles correspond to bottles incubated with unlabelled CH_4 and filled circles with 99 atom% $^{13}CH_4$.

incubation was performed at 25°C. Methane was added to bottles to a final concentration of ~11% and was consumed by the sediment without an observed lag phase (Fig. 2). Samples were taken every 24 h over 4 days and nucleic acids were purified. Copies of *pmoA* genes were measured by real-time PCR and found to be 3.88×10^4 ($\pm 6.81 \times 10^3$) ml^{-1} of fresh sample before the start of the incubation to 7.51×10^4 ($\pm 3.96 \times 10^4$) ml^{-1} by 96 h. The relatively small increase in size of the methanotroph population in relation to the amount of methane consumed suggests they may have been consumed by protozoa (Murase and Frenzel, 2008). The number of *pmoA* transcripts was quantified and found to be 1.48×10^4 ($\pm 9.49 \times 10^3$) at time zero and 5.51×10^4 ($\pm 9.85 \times 10^3$) copies ml^{-1} (wet weight) at 96 h; the absolute quantities of *pmoA* transcripts may have been underestimated as a result of inhibition from reverse transcriptase enzyme (Chandler *et al.*, 1998). The methanotroph community was monitored over the incubation by T-RFLP of the *pmoA* gene (Fig. S2).

Analysis of ^{13}C-enrichment of pmoA *by DNA-SIP and RNA-SIP*

The DNA and RNA from each time point were resolved by isopycnic gradient centrifugation in CsCl and CsTFA gradients respectively. After centrifugation and fractionation of the gradients, the *pmoA* genes and mRNA transcripts present in each gradient fraction were quantified by real-time PCR. The maximum quantities of *pmoA* genes detected from gradient fractions were on the order of 10^5 copies per fraction and the maximum quantities of *pmoA* transcripts detected were 10^4 copies. The total *pmoA* transcript abundance is limited by the total amount of RNA (most of which is rRNA) that can be loaded into

the gradient, which is limited because it precipitates at elevated concentrations (Lueders *et al.*, 2004a). Also, the quantities varied between replicate gradients (Fig. S3) probably as a result of slight inaccuracy in the determination of the initial nucleic acid concentration loaded into the gradient or differences in the amount of nucleic acid that was lost by adhesion to the wall of the polyallomer centrifugation tube. This made it impossible to perform meaningful comparisons between absolute abundances of a target obtained from different gradients and therefore results are always presented as relative abundances, as has been done in other studies (Lueders *et al.*, 2004a,b).

The relative abundance of *pmoA* genes and transcripts in each gradient fraction is shown in Fig. 3. Gradients containing either DNA or RNA from sediment samples incubated with $^{12}CH_4$ only contained single peaks of *pmoA* genes (Fig. 3A) or transcripts (Fig. 3B). Incubation with $^{13}CH_4$ resulted in the appearance of 'heavy' *pmoA* genes (density > 1.74 g ml^{-1}) and mRNA (density > 1.82 g ml^{-1}). At 24 h there was only a slight enrichment of *pmoA* genes at density 1.75 g ml^{-1}, whereas the presence of heavy *pmoA* transcripts was already pronounced. The proportion of *pmoA* genes and transcripts appearing at denser fractions increased with incubation time, and at 96 h a large proportion of the genes (~70%) and an even larger proportion of the transcripts (~80%) were ^{13}C-labelled.

The labelled and unlabelled *pmoA* genes and transcripts were characterized by T-RFLP analysis. The T-RFLP profiles for the *pmoA* genes in CsCl gradients are shown in Fig. 4A. The phylogenetic affiliation of the T-RFLP profiles of the unlabelled *pmoA* genes did not show major changes over the time-course and resembled the methanotroph community composition in the unincubated (0 h) sediment; at all time points these profiles were composed of 70–85% type Ia T-RFs, 15–30% type Ib T-RFs and no detectable type II methanotroph *pmoA* T-RFs. In contrast, the T-RFLP profiles of labelled *pmoA* genes were clearly different from those of the unlabelled methanotroph community. Comparing the T-RFLP profiles with those from unlabelled *pmoA*, the most striking difference is the relatively high proportion of type II methanotrophs (5–18%). There was also a relatively large proportion of the 79 bp T-RF in the heavy fraction.

The T-RFLP profiles for the *pmoA* transcripts are shown in Fig. 4B; each time point corresponds to the same nucleic acid extract that was used for the *pmoA* gene analysis shown in Fig. 4A, but were treated with DNase and *pmoA* sequences were amplified by RT-PCR. The *pmoA* T-RFLP profiles in the 'light fractions', corresponding to unlabelled transcripts, changed over the time-course (Fig. 4B). In particular, there was an increased relative abundance of the type Ib T-RFs (65 and 79 bp) from 2% at 24 h to 20% at 96 h. In addition, the 244 bp
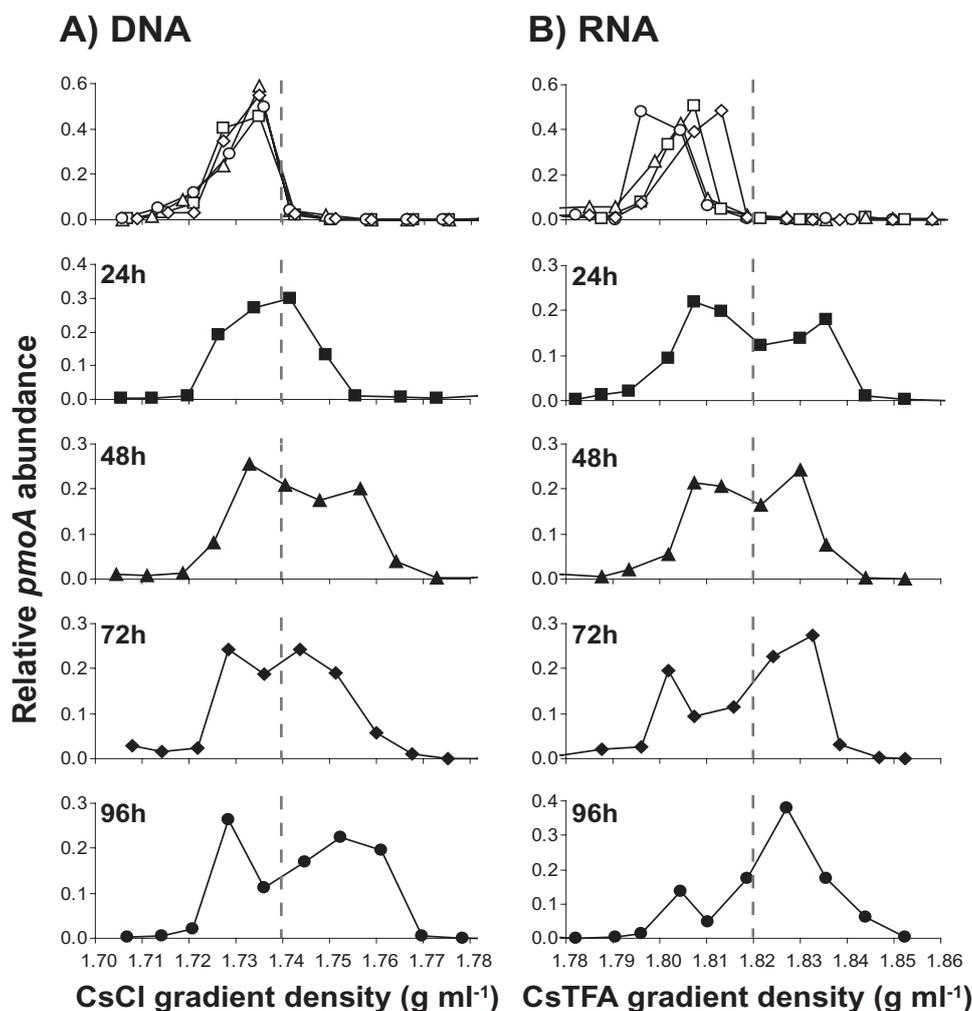
A) DNA

B) RNA

Fig. 3. Relative *pmoA* gene abundances recovered from CsCl gradient fractions (A) and *pmoA* mRNA abundances from CsTFA gradient fractions (B). Quantities were determined by quantitative PCR for genes and quantitative RT-PCR for mRNA. The *y*-axis indicates the relative abundance at each gradient fraction, with the total quantity detected from a gradient equal to 1.0. The uppermost panel in both (A) and (B) correspond to the gradients containing nucleic acids from the $^{12}CH_4$ control incubations; symbols indicate 24, 48, 72 and 96 h incubation. Lower panels show the data for nucleic acids recovered from $^{13}CH_4$ incubations over the time-course, as indicated. Dashed lines indicate the boundary between what were considered 'light' and 'heavy' fractions.

T-RF (corresponding to type II methanotrophs) became detectable from 48 h onwards. The T-RFLP profiles from the 'heavy fractions', corresponding to labelled transcripts, were composed primarily of type Ia T-RFs (Fig. 4B, right-side panel). They contained a small amount of the 79 bp type Ib T-RFs and, at 72 and 96 h, about 2% and 4% of the type II T-RF.

There was a striking lack of correlation between the T-RFLP profiles of labelled *pmoA* genes (Fig. 4A, 'Heavy Fraction') and labelled *pmoA* transcripts (Fig. 4B, 'Heavy Fraction'). For example, abundance of type Ib (65 and 79 bp) and type II (244 bp) T-RFs were relatively low in the mRNA. In contrast, the 254 bp T-RF was high in the mRNA profiles and low in the DNA profiles. To test for a bias in reverse transcription, we quantified the amount of

cDNA obtained from the reverse transcription of type Ia and type II *pmoA in vitro* transcripts and obtained equivalent amounts from the same starting quantities (Fig. S4), suggesting that there is no bias in the reverse transcription of type Ia and II *pmoA* mRNA.

Analysis of ^{13}C-enrichment of 16S rRNA by DNA-SIP and RNA-SIP

The 16S rRNA clone libraries from heavy fractions of CsCl and CsTFA gradients from incubations with $^{13}CH_4$ showed high numbers of methanotrophs: 29% of the 16S rRNA clones from the heavy fractions of a DNA gradient at 96 h, 90% from a heavy fraction of an RNA-SIP gradient at 24 h and 70% at 96 h. A phylogenetic tree of representative
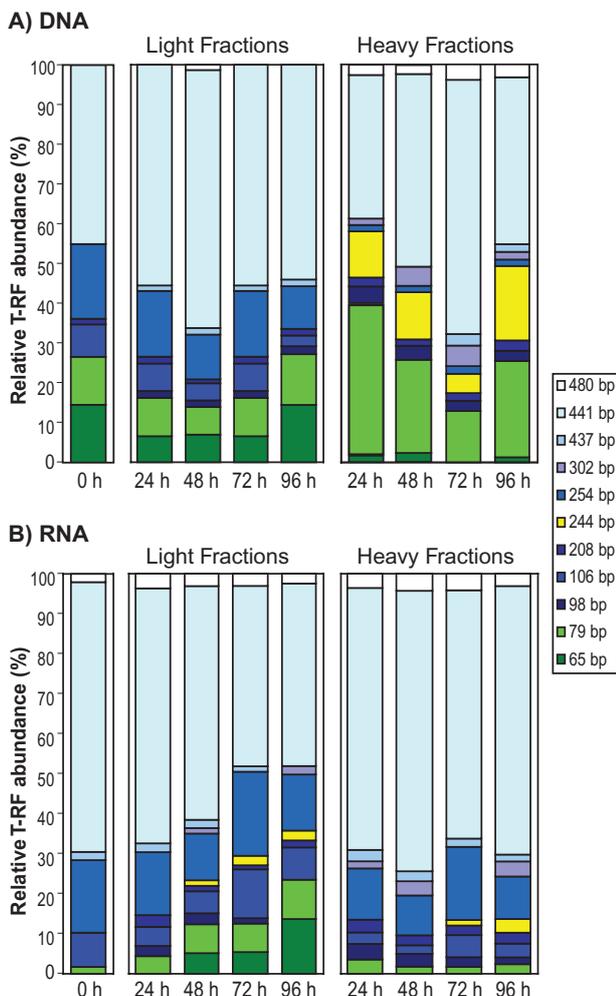
A) DNA

B) RNA

Fig. 4. T-RFLP analyses of *pmoA* genes (A) and mRNA (B). The profiles obtained from the sediment immediately prior to the incubation are shown (0 h). The plots labelled 'Light Fraction' and 'Heavy Fraction' refer to *pmoA* profiles from gradients containing nucleic acids from sediment incubated with $^{13}CH_4$ for various times, as indicated. The legend indicates the size of the T-RFs, from which representative sequences can be found in Fig. 1. The 480 bp T-RF could not be assigned to any clones.

sequences from these clone libraries is shown in Fig. S5. The majority of methanotroph 16S rRNA clones obtained (114 sequences of 116) belonged to the type Ia methanotrophs. The remaining methanotroph clones consisted of one clone distantly related type Ib sequences (clone R13; 92% identity to *Methylococcus capsulatus* 16S rRNA) and one type II sequence (clone R25; maximum 96% identity to *Methylocystis heyeri* 16S rRNA). In addition, the clone library from heavy DNA fractions had numerous sequences belonging to the *Betaproteobacteria*.

Terminal restriction fragment length polymorphism analyses of 16S rRNA genes (Fig. 5) and molecules (Fig. 6) both showed the enrichment of T-RFs in the heavy fractions (Figs 5A and 6A) and a relatively complex com-

munity patterns in the light fractions (Figs 5B and 6B). A similar analysis with nucleic acids from the $^{12}CH_4$ incubations (an important control) did not show enrichment of any particular T-RF in the heavy fractions (Fig. S6). Most of the T-RFs in the heavy fractions (Figs 5A and 6A) could be matched to sequences in the clone libraries, with the exception of the 145 and 225 bp T-RFs in the heavy fractions of DNA-SIP analyses. The 445, 456 and 489 bp T-RFs, which were the primary peaks associated with heavy RNA, correspond to the T-RF size of most type Ia methanotroph sequences (Fig. S5). A small 151 bp T-RF appeared in the heavy RNA fractions from 72 h and corresponds to the T-RF size of the type II clone; this was not seen in the heavy DNA profiles. A 438 bp T-RF, which corresponds to that of *Hyphomicrobium* sequences in the clone libraries, was present throughout the time-course in the DNA-SIP analysis, but only appeared in the RNA profile at 96 h. The DNA-SIP analyses also showed prominent T-RFs corresponding to betaproteobacterial clones (115, 140 and 493 bp), but these were not detected in the RNA-SIP profiles.

Discussion

In this study we labelled methanotrophs in Lake Stechlin sediment with $^{13}CH_4$ and examined *pmoA* and 16S rRNA sequences and samples were taken over a time-course. Although both DNA-SIP and rRNA-SIP have been performed simultaneously from the same labelled environmental sample (Lueders *et al.*, 2004b; Héry *et al.*, 2008), a direct comparison the two approaches over a time-course has not been previously reported. In addition to performing the concomitant analysis of DNA and rRNA, we also analysed the ^{13}C-labelling of mRNA – more specifically the *pmoA* gene transcripts. This study provided unique information about the sensitivity of SIP approaches and the turnover of these molecules in the methanotroph community.

Sensitivity of SIP of DNA, rRNA and mRNA

Manefield and colleagues (2002) showed that ^{13}C from labelled phenol accumulates more rapidly in RNA than DNA. This is not surprising because DNA synthesis is mostly associated with cell division and there is more RNA than DNA in a cell. But, it should be considered that first and foremost, SIP requires the catabolism of a labelled substrate (e.g. ^{13}C) coupled to the anabolism of ^{13}C-nucleotides. Although we would anticipate that mRNA-SIP would be the most sensitive approach given the high turnover rate of mRNA, it is important to consider that ^{13}C-nucleotide synthesis in the cell is the essential first step; only after the production of ^{13}C-nucleotides does the

A) Heavy fractions

T-RF length (bp)

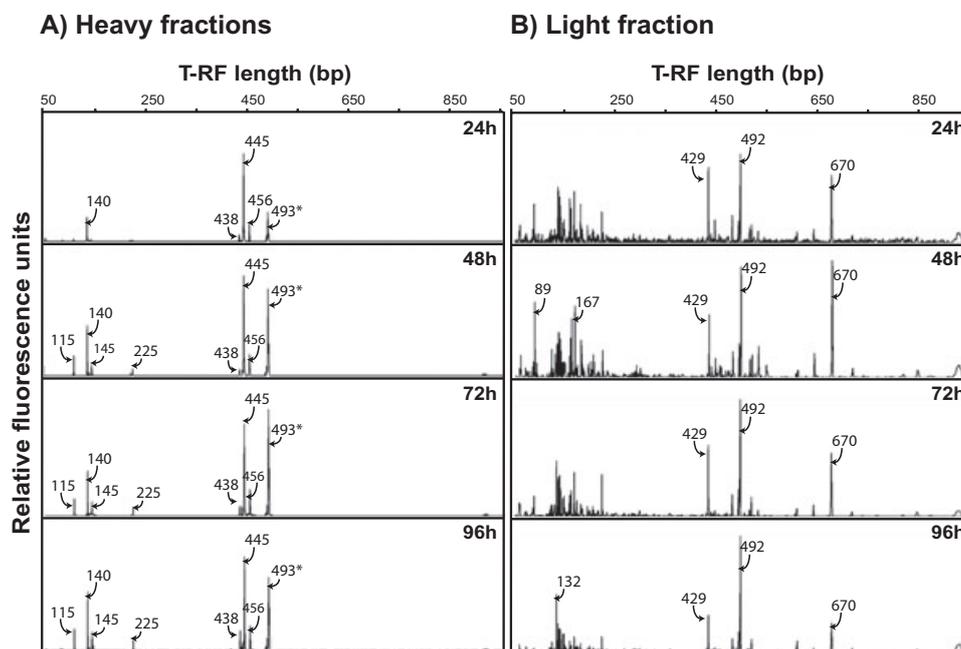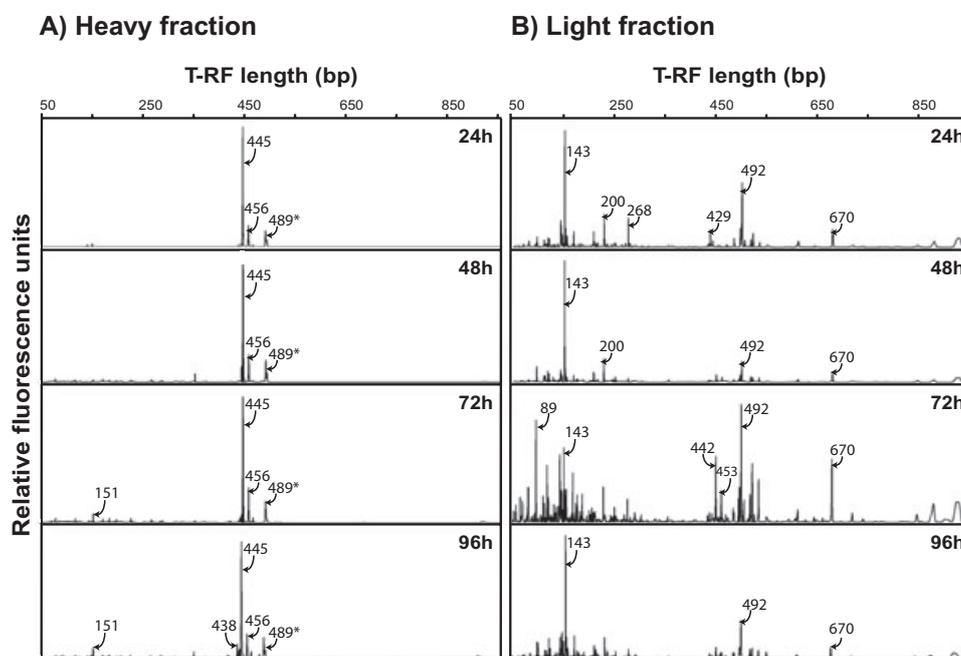## B) Light fraction

T-RF length (bp)

Fig. 5. Bacterial 16S rRNA T-RFLP analyses of DNA recovered from CsCl gradients. Heavy fractions correspond to DNA with density between 1.74 and 1.76 g ml^{-1} (A) and light fractions to DNA with density between 1.72 and 1.74 g ml^{-1} (B). An asterisk is present at 493 bp to indicate that a smaller peak of 489 bp is also present.

turnover rate of the nucleic acid become relevant to the sensitivity of the SIP approach.

Nucleotide synthesis in all organisms proceeds by either salvage or *de novo* pathways, with the former pre-ferred as *de novo* synthesis is energetically more costly (Nyhan, 2005). The *de novo* synthesis of nucleotides can be considered a requisite for nucleic acid SIP since the salvage pathways in an organism catabolizing a ^{13}C-

A) Heavy fraction

T-RF length (bp)

B) Light fraction

T-RF length (bp)

Fig. 6. Bacterial 16S rRNA T-RFLP analyses of RNA from CsTFA gradients. Heavy fractions correspond to RNA with density between 1.82 and 1.84 g ml^{-1} (A) and light fractions to DNA with density between 1.80 and 1.82 g ml^{-1} (B). An asterisk is present at 489 bp to indicate that a smaller peak of 493 bp is also present.

substrate would only result in unlabelled, or at most partly labelled, nucleotides. Therefore, to predict and understand the labelling of DNA and RNA by SIP, it is necessary to understand when *de novo* synthesis of nucleotides occurs. Essentially, *de novo* biosynthesis pathways are directly regulated by the levels of the nucleotides in the cell (Kilstrup *et al.*, 2005; Turnbough and Switzer, 2008). As a result, labelled nucleotides will be produced in cells that are metabolizing the ^{13}C-substrate only if the cellular pool size of nucleotides is low. Of course, this will occur during cell division since nucleotides are consumed during genome replication and increased RNA synthesis. To what extent *de novo* nucleotide synthesis occurs in non-dividing cells is unknown.

The incorporation of ^{13}C into transcripts is likely to increase sooner than genes given the high turnover rate of mRNA. Moreover, transcripts should be more abundant than genes; however, in reality the complete recovery of mRNA from environmental samples might not be possible. A comparison of *pmoA* transcript and gene abundances in this study and in Steenbergh and colleagues (2010) show ratios less than one, suggesting incomplete *pmoA* mRNA recovery or reverse transcription. It is also possible that *pmoA* levels were too low to quantitatively measure as a result of inhibition by reverse transcriptase enzyme (Chandler *et al.*, 1998). Regardless, the mRNA should be labelled sooner and more rapidly than genes. Indeed, the proportion of labelled *pmoA* mRNA was greater than *pmoA* genes at each time point (Fig. 3). Furthermore, a direct comparison of the density of *pmoA* genes and transcripts after 24 h of incubation showed that the maximum increase in buoyant density of the *pmoA* transcripts was greater than that of the *pmoA* genes. The partial shift in DNA density can be explained the semi-conservative nature of DNA replication resulting in a maximum of 50% genome labelling after the first cell division (Meselson and Stahl, 1958). Therefore, after 24 h of incubation with ^{13}CH$_4$ the methanotrophs had completed a maximum of one cell division; however, even at this point some *pmoA* transcripts were present in the gradient at the maximum density observed for mRNA (> 1.82 g ml^{-1}), indicating that mRNA was more rapidly labelled than DNA.

16S rRNA genes and 16S rRNA molecules from the gradients were also analysed. Universal bacterial 16S rRNA primers were used since methanotrophs are polyphyletic. Therefore, the sensitivity of detecting ^{13}C-labelled *pmoA* by quantitative PCR from gradient fractions was greater than 16S rRNA because there is no background of the non-methanotrophic community when targeting *pmoA*. As such, it was not possible to clearly detect a peak of 16S rRNA genes in the heavy gradient fractions in this study (results not shown). Longer incubations with ^{13}C-substrate – or a more abundant target community –

would increase the accumulation of labelled 16S rRNA in heavy fractions of gradients; for example, it was possible to visualize a peak of 16S rRNA after 6-day incubation of rice field soil with ^{13}C-methanol (Lueders *et al.*, 2004b). Also, many DNA-SIP studies used sufficient ^{13}C-substrates to enable the visual detection of labelled DNA in gradients using ethidium bromide staining (McDonald *et al.*, 2005). In this study we wanted to minimize the labelling to prevent enrichment artefacts and therefore incubated a maximum of 4 days. Although a peak of 16S rRNA was not detectable in either the RNA or DNA gradients after 4 days, it was possible to detect enrichment of 16S rRNA sequences in the heavy fractions using T-RFLP fingerprinting and by clone library analysis even after 24 h, corresponding to less than 20 μmol of ^{13}C consumed g^{-1} wet weight sediment. This indicates that both DNA-SIP and rRNA-SIP allow relatively sensitive detection of methanotrophic bacteria in lake sediments.

Comparison of pmoA *gene and transcript labelling*

One of the most conspicuous findings in this study was the large discrepancy between the *pmoA* T-RFLP profiles from labelled DNA and RNA (Fig. 4). Given that both *pmoA* genes and transcripts were being labelled during the time-course, the T-RFLP profiles of transcripts should have matched that of the genes if all active methanotrophs maintained *pmoA* transcripts at the same level in the cell. Instead, the labelled transcripts of the type Ib and type II methanotrophs were very low compared with their abundance among labelled genes, suggesting that the *pmoA* transcripts per cell were considerably higher for the type Ia methanotrophs. We performed controls using *in vitro* transcripts of cloned *pmoA* genes from type Ia and II methanotrophs and observed no bias in the efficiency of reverse transcription and T-RFLP (Fig. S4); however, this does not exclude the possibility of differences in the reverse transcription efficiency of native *pmoA* mRNA targets, as there could be conformational effects with the full-length transcripts or they may contain modified bases which differentially influence the RT-PCR.

The relative abundance of gene transcripts between species is a subject which needs to be examined more carefully as transcriptomic analyses become more widely performed. Methanotrophs typically possess two copies of the *pmoA* gene (Murrell *et al.*, 2000) and therefore the relative abundance of *pmoA* genes should reflect the abundance of the methanotroph species. In *Methylosinus trichosporium* OB3b it has been shown that *pmoA* transcript abundance is proportional to the whole-cell pMMO activity (Han and Semrau, 2004); however, transcription is dynamic and expression of *pmoA* is upregulated by copper (Nielsen *et al.*, 1997; Knapp *et al.*, 2007). The final concentration in a cell will be determined by the

rate of *pmoA* transcription and degradation, the result of which could be unique for a species. Therefore, it is plausible that the transcript abundance is higher in the type Ia than type Ib and II methanotrophs in Lake Stechlin sediment.

Several studies agree with the hypothesis that relatively few *pmoA* transcripts are recovered from type II compared with type Ia methanotrophs. Krause and colleagues (2010) monitored the succession of methanotrophs in a microcosm system by analysing the relative abundance of *pmoA* genes and transcripts and found that type II *pmoA* genes were often predominant, but the associated transcripts were always relatively low or below the detection limit. Relatively low *pmoA* transcript copies per methanotroph cell were also reported for type II methanotrophs in a river floodplain soil (Steenbergh *et al.*, 2010) and type II *pmoA* transcripts were relatively low in a landfill cover soil (Chen *et al.*, 2007) and Lake Washington sediment (Nercessian *et al.*, 2005). Incidentally, type II methanotroph-associated *mxaF* gene transcripts were abundant in the landfill cover soil and *fae* genes in Lake Washington sediment, indicating that the low relative abundance of *pmoA* transcripts in type II methanotrophs might be unique to *pmoA* and not a general pattern of lower gene transcript abundance in these organisms. In conclusion, this would mean that mRNA abundance may not always be a good proxy for the relative activity of the encoded enzyme, particularly when comparing between genera.

In this study we predicted, and observed, a gradual increase in the proportion of labelled *pmoA* genes over the time-course, presumably as the methanotrophs divided and their genomes became labelled. It was not clear beforehand how the labelling of *pmoA* transcripts would occur. Like *pmoA* genes, this turned out to be a gradual process with unlabelled *pmoA* transcripts still present at the end of the time-course (Fig. 3). This indicates that there were methanotrophs expressing *pmoA* for up to 96 h in the presence of $^{13}CH_4$, but still not incorporating ^{13}C into their nucleotides. This is noteworthy considering that the presence of *pmoA* transcripts is considered diagnostic for methane oxidation activity. Although the results do not necessarily contradict this, they indicate that the presence of *pmoA* transcripts does not necessarily mean the anabolic incorporation of methane into all the components of the cell. One explanation for this, as discussed above, is that the synthesis of ^{13}C-nucleotides in the methanotrophs was linked to cell division. As such, the mRNA transcripts would have remained unlabelled in methanotrophs that had not entered a cell division process, even if they were metabolizing the $^{13}CH_4$ and the mRNA was being continuously transcribed and degraded in the cell. The other possibility is that these unlabelled *pmoA* transcripts belonged to methanotrophs using another (unlabelled) growth substrate. This is possible

given the recent description of facultative methanotrophs (Dedysh *et al.*, 2005; Belova *et al.*, 2010; Dunfield *et al.*, 2010), but methanotrophs for the most part prefer methane. Incidentally, Huang and colleagues (2009), in the only previous mRNA-SIP report, also showed the presence of both labelled and unlabelled NDO transcripts after incubation of their sample with ^{13}C-naphthalene.

The unlabelled *pmoA* transcripts should constitute a dynamic pool that would be depleted if these methanotrophs switch to synthesizing ^{13}C-nucleotides or replenished if dormant methanotrophs become active and start expressing *pmoA* from a cellular pool of unlabelled nucleotides. Indeed, T-RFLP analysis of the light fractions did show a change in the relative abundance of T-RFs over the time-course; most notably, there was a relative accumulation of some type Ib *pmoA* transcripts (65 bp T-RF). This suggests that this population of methanotrophs was not incorporating the $^{13}CH_4$, or was doing so much more slowly than the other methanotrophs. As a result, their *pmoA* transcripts were accumulating in this light fraction as the *pmoA* transcripts of other organisms became ^{13}C-labelled and moved to the heavier gradient fractions. In agreement with this, the 65 bp T-RF was generally not seen in the heavy fractions of either DNA or RNA gradients. There are several possible reasons why labelling of the 65 bp T-RF *pmoA* group was not seen: these organisms were inactive but still maintaining some *pmoA* transcripts in their cells; they were actively metabolizing the $^{13}CH_4$ but not incorporating ^{13}C into their nucleotides; or, they are facultative methanotrophs that constitutively express *pmoA* while consuming another carbon substrate.

Comparison of 16S rRNA and rDNA labelling

The analysis of 16S rRNA labelling was important to monitor the incorporation of ^{13}C into all species, which will include the primary utilizers (methanotrophs) and organisms cross-feeding on the ^{13}C products of their metabolism (Lueders *et al.*, 2004b; Manefield *et al.*, 2007; Murase and Frenzel, 2007; Cébron *et al.*, 2007b; Qiu *et al.*, 2008). This would not be revealed by the analysis of *pmoA* sequences, which is restricted to methanotrophs. Targeting other functional genes might also overlook species and would not provide the phylogenetic resolution of 16S rRNA. Therefore, the concomitant analysis of 16S rRNA was important in this study.

The most striking finding of the analyses of 16S rRNA was the relatively high abundance of betaproteobacterial sequences observed by DNA-SIP and not RNA-SIP. The 16S rRNA associated with the heavy fractions of RNA gradients were primarily those of type Ia methanotrophs. There are a number of reasons why the profiles would be different between the methods. The primary factor is that

the ribosome abundance can vary dramatically in a cell depending on the growth rate of the organism (Bremer and Dennis, 1987). Also, the maximum ribosome content of a cell can be species specific (Fegatella *et al.*, 1998; Geissinger *et al.*, 2009). These differences in ribosome contents between organisms will heavily influence the 16S rRNA relative abundances in the RNA-SIP analyses. On the other hand, the abundance of 16S rRNA genes will be fixed by the *rrna* operon copy number. Therefore, the explanation for the absence of betaproteobacterial 16S rRNA sequences in the RNA-SIP profiles could be that they had fewer ribosomes than the type Ia methanotrophs, either because of comparatively low growth rates or because of lower maximal ribosome contents.

Betaproteobacterial sequences are often obtained by DNA-SIP with $^{13}CH_4$ (Morris *et al.*, 2002; Radajewski *et al.*, 2002; Cébron *et al.*, 2007a). At present there are no peer-reviewed publications reporting the existence of methanotrophs belonging to the *Betaproteobacteria*, but the possibility exists and a description of one such isolate is reported in a thesis (Islam, 2009). It is likely that at least some of the betaproteobacterial labelling observed in this study was obtained by cross-feeding of methanotroph metabolites. Sequences related to *Hyphomicrobium* species, which are methylotrophic alphaproteobacteria known to contaminate methanotroph enrichment cultures presumably by cross-feeding on methanotroph metabolites (Calhoun and King, 1998), were also detected in both DNA-SIP and RNA-SIP analyses. There are methylotrophic genera within the *Betaproteobacteria*, such as *Methylibium* and *Methyloversatilis* (Kelly and Wood, 2010), and therefore the leakage of labelled one-carbon compounds from the active methanotroph cells could theoretically be incorporated by these organisms. Also, cross-feeding may have arisen via predation of methanotrophs by protozoa (Murase and Frenzel, 2007; 2008). The T-RFs associated with betaproteobacterial 16S rRNA gene sequences were absent or relatively low at 24 h incubation in the time-course but became progressively dominant, which would be consistent with the hypothesis that they were dependent on the methanotrophs for the supply of ^{13}C-metabolites. This is also consistent with the theory that relatively low growth rates of the *Betaproteobacteria* resulted in lower ribosome contents and their 16S rRNA sequences not being detected by RNA-SIP.

Conclusions

We have demonstrated that SIP can also be applied to mRNA molecules. This will complement DNA-SIP studies, particularly if using an RNA-Seq approach, by indicating which genes are being expressed by the target organisms. We have shown that the DNA-SIP, rRNA-SIP and

mRNA-SIP methods provide complementary information, but the relative abundance of labelled molecules can vary between phylotypes when using the different approaches. Therefore, caution should be taken when drawing conclusions about the relative activity of a phylotype based on data from a single SIP method.

Experimental procedures

Sample collection and stable isotope labelling

Oligotrophic Lake Stechlin is located in Brandenburg Germany and has been extensively studied and described (Casper *et al.*, 2003). The uppermost 1–2 cm of the sediment is permanently oxic (Sass *et al.*, 1997) and methanogenesis occurs in deeper sediment. Samples were collected using a gravity corer (Uwitec®) from the profundal sediment (35 m depth southern sampling station) of Lake Stechlin on 18 June 2009. Additional samples from eutrophic Lake Dagow (Casper, 1996) were collected on the same day. Sediment from the top 10 cm was stored in closed bottles at 4°C until the start of the experimental incubations (approximately 6 weeks).

The Lake Stechlin sample contained about 50% overlying lake water. Aliquots (20 ml) of the mixed sediment/water sample were added to 120 ml serum bottles and the headspaces were evacuated and flushed with air to remove methane and oxygenate the sediment. The bottles were capped with black butyl stoppers. CH_4 (Messer) or 99 atom% $^{13}CH_4$ (Sigma Isotec) were added to a final concentration of 11% in air. The incubations were performed in duplicate and a control without added CH_4 was also performed. Bottles were incubated at 25°C on a shaker (100 r.p.m.) in the dark. Methane concentrations were measured daily by gas chromatography. Oxygen was also monitored by gas chromatography to ensure that the bottles did not become anoxic during the incubations. The bottles were sampled every 24 h for 4 days by removing 2 ml aliquots with an 18-gauge needle attached to a 3 ml syringe. The samples were immediately transferred to 2 ml screw-cap tubes, centrifuged in a benchtop microcentrifuge at maximum speed for 5 min at 25°C to pellet the sediment and bacterial cells; the aqueous supernatants were removed and the pellets (~0.4 g) were immediately frozen in liquid nitrogen and stored at −80°C until nucleic acid extraction.

Escherichia coli JM109 was cultivated in either LB medium (as a standard medium containing unenriched ^{12}C-substrate) or M9 medium (Sambrook and Russell, 2001) containing 10 mM ^{13}C-glucose (99 atom% ^{13}C, Sigma Isotec). Ten millilitres of overnight cultures were centrifuged for 5 min at 25°C and at maximum speed in a benchtop microcentrifuge (Eppendorf 5417R™) and the RNA was extracted from cell pellets with the same protocol used for RNA purification from sediment (see below).

Nucleic acid purification

Total nucleic acids were purified from 0.4 g of frozen sediment pellets using a method described previously (Bürgmann

et al., 2003), except that 2.5% sodium dodecyl sulfate (SDS) replaced CTAB in the extraction buffer. The precipitation of nucleic acids was performed in 2 ml non-stick RNase-free tubes (Applied Biosystems) and dissolved in a final volume of 100 µl of nuclease-free water (Applied Biosystems). For RNA purification, 50 µl of the crude extract was treated with RNase-free DNase I (Qiagen) according to the manufacturer's instructions and purified using the RNeasy MinElute Cleanup Kit (Qiagen). Nucleic acids were routinely quantified using a NanoDrop 1000 spectrophotometer (Thermo). DNA removal was verified by PCR and an inability to obtain products without the addition of reverse transcriptase enzyme (see below).

The mRNA-Only Prokaryotic mRNA Isolation Kit (Epicentre) was used to remove rRNA from *E. coli* mRNA.

Isopycnic centrifugation

The mRNA-SIP approach was first tested with pure cultures, the results of which are presented in *Supporting information* (Fig. S7).

The isopycnic centrifugation of DNA in CsCl and RNA in CsTFA gradients was performed as described previously (Lueders *et al.*, 2004a), with some slight modifications to the latter where an average density of 1.80 g ml^{-1} and slightly higher formamide concentrations were used. CsTFA gradients were prepared as follows: first, 2.0 g ml^{-1} CsTFA (Amersham), buffer (100 mM Tris-HCl, pH 8.0; 100 mM KCl; 1 mM EDTA) and RNA (maximum 500 ng) were combined to give a solution with density 1.825 g ml^{-1} and a refractive index (RI), measured with a Reichert AR200 instrument, of 1.3702 (the exact volumes depended on the initial CsTFA concentration, which varied between batches); second, a volume of Hi-Di™ formamide (Applied Biosystems) corresponding to 3.59% of the CsTFA/buffer/RNA solution was added, resulting in an RI of 1.3725 and density of 1.80 g ml^{-1} measured gravimetrically. If necessary, adjustments were made by the addition of small volumes of CsTFA/buffer solution (RI 1.3702) or formamide. Centrifugation and gradient fractionation was performed as described previously (Lueders *et al.*, 2004a). To precipitate RNA, 1 µl (20 µg) of glycogen (Sigma), 40 µl of 3 M sodium acetate (pH 5.2) and 1 ml of ethanol were added to tubes and incubated at least 1 h at −20°C. Tubes were centrifuged at maximum speed (20 817 *g*) for 30 min at 4°C in a benchtop microcentrifuge (Eppendorf 5417R™). RNA pellets were rinsed with cold 70% ethanol, dried, dissolved in 5 µl of nuclease-free water (Applied Biosystems) containing 1 U µl^{-1} RNase inhibitor (SUPERase-In, Applied Biosystems) and stored at −80°C. *Escherichia coli* RNA was visualized using the Agilent RNA 6000 Pico Kit and the Agilent Bioanalyzer™ 2100 instrument according to the manufacturer's instructions.

The G+C content of DNA is known to affect the buoyant density in CsCl gradients (Buckley *et al.*, 2007). To minimize bias from G+C content when amplifying genes from 'light' or 'heavy' regions of the gradient, fractions in each region were always pooled prior to PCR amplification: CsCl gradient fractions with densities between 1.72 and 1.74 g ml^{-1} were considered light fractions and those between 1.74 and 1.76 g ml^{-1} were considered heavy fractions. No G+C effect was observed for the *pmoA* transcripts in CsTFA gradients (Fig. S8).

Reverse transcription of RNA

RNA was converted to single-stranded cDNA by reverse transcription. RNA from gradients was used directly as template, but RNA extracted directly from sediment without CsTFA centrifugation was diluted (1:10, 1:20, 1:50 and 1:100) in nuclease-free water. The reactions were performed in 0.2 ml PCR tubes as follows: 1 µl of RNA, 5 µl of 50 ng µl^{-1} random hexamer (Invitrogen), 5 µl of 2 mM dNTP (Fermentas) and 2.5 µl of nuclease-free water were incubated at 65°C for 5 min in a thermocycler and then placed on ice; a mixture containing 4 µl of 5× Superscript III reaction buffer (supplied), 1 µl of DTT (supplied), 1 µl of Superase-In (Applied Biosystems), 0.5 µl of 20 mg ml^{-1} BSA (Roche) and 20 U (0.1 µl) of SuperScript III (Invitrogen) was added to the denatured RNA to a final volume of 20 µl; finally, the reactions were incubated at 25°C for 5 min, 50°C for 60 min and 70°C for 15 min. The cDNA was stored at −20°C. It was found that 20 U of SuperScript III enzyme, instead of 200 U recommended by the manufacturer, generated equivalent quantities of *pmoA* and 16S rRNA cDNA copies from our samples. Control reactions were performed as above, but with water instead of SuperScript III RT enzyme.

PCR, T-RFLP, cloning and sequence analysis

PCR amplification of *pmoA* and 16S rRNA was achieved using the A189F and mb661R primers (Costello and Lidstrom, 1999) and 27F and 907R primers (Lueders *et al.*, 2004b) respectively. PCRs were performed in 50 µl volumes containing 1× PCR Buffer (Invitrogen), 0.2 mM dNTPs (Fermentas), 1.5 mM MgCl$_2$ (Invitrogen), 10 pmol each primer, 10 µg of BSA (Roche), 2 U of Platinum *Taq* DNA polymerase (Invitrogen) and 1 µl of template. Cycling was performed on a GeneAmp PCR System 9700 Instrument (Applied Biosystems) with an initial denaturation at 94°C for 4 min followed by 32 cycles: 94°C 1 min, 55°C 1 min, 72°C 1 min, and a final extension at 72°C for 10 min; additional cycles for *pmoA* (up to 40) were sometimes required to obtain sufficient product, particularly when using the FAM-labelled primer for T-RFLP. For sequencing, PCR products were ligated into the pGEM-T Easy vector and transformed into *E. coli* JM109 competent cells (Promega).

For profiling of *pmoA* using T-RFLP analysis, the A189F primer was labelled with FAM (6-carboxyfluorescein). PCR products were purified using the GenElute PCR Clean-up kit (Sigma) and approximately 100 ng of purified product was digested in a 10 µl volume containing 1× Tango Buffer with BSA (Fermentas) and 5 units each of both RsaI and MspI enzymes (Fermentas); reactions were incubated for 3 h at 37°C. Reactions were subsequently desalted using SigmaSpin™ Post-Reaction Clean-up Columns (Sigma) and 3 µl of the desalted fragments were mixed with 11 µl of Hi-Di™ formamide (Applied Biosystems), 0.3 µl of ROX-labelled MapMarker 1000 + 30, 40 (BioVentures), incubated for 3 min at 94°C and cooled on ice. Size separation was performed using a 3130 Genetic Analyzer (Applied Biosystems). The DNA quantities between samples were normalized (Dunbar *et al.*, 2000) and a peak height of 20 was used as the noise-threshold. Peak areas were used in the resulting data set to calculate T-RF relative abundance and a cut-off of 1% was

used. 16S T-RFLP was performed as describe previously (Lueders *et al.*, 2004b)

Phylogenetic analyses were performed using the ARB software package (Ludwig *et al.*, 2004). Analysis of *pmoA* sequence data was performed using the 155-amino-acid positions, excluding the primer regions, that were present in all sequences. 16S rRNA clone sequences were aligned against the SILVA ref database (Pruesse *et al.*, 2007) and checked manually. Neighbor-joining analyses were performed within ARB. Maximum-likelihood analyses were performed using the web implementation of RAxML (Stamatakis *et al.*, 2008). A core maximum-likelihood 16S rRNA tree was constructed using near full-length sequences and was used as a constraint tree when including sequences less than 1400 nt. Highly variable positions were excluded from the phylogenetic analyses of 16S rRNA sequences. Sequence data were deposited with GenBank under Accession No. HM216814 to HM216893.

Real-time PCR

The relative abundance of 16S rRNA and *pmoA* transcripts within gradients was determined by real-time PCR using the SYBR Green JumpStart Taq ReadyMix System (Sigma). The assays were performed using an iCycler instrument (Bio-Rad) and the associated software. Bacterial 16S rRNA copies were quantified with the primers 519F and 907R as described previously (Stubner, 2002). *pmoA* transcripts were quantified using the A189F and mb661R primers as described (Kolb *et al.*, 2003). For determination of absolute quantities of *pmoA* transcripts directly extracted from sediment, the real-time PCR was performed using 1 µl of cDNA template prepared from the RNA dilution series described above. The standard was prepared from an *in vitro* transcript of two *pmoA* clones from Lake Stechlin sediment using the Riboprobe *in vitro* Transcription System (Promega) according to the manufacturer's instructions; the T7 or SP6 enzyme was used depending on the orientation of clone in the pGEM-T Easy vector. The *in vitro* transcript was purified by phenol-chloroform extraction and quantified using the RiboGreen RNA quantification kit (Invitrogen). A total of 1×10^9 transcripts were reverse transcribed as described above. For the quantification, a dilution series (factor 10) corresponding to 5×10^6 to 5×10^0 *in vitro* transcripts from two *in vitro* transcription reactions was used as a standard. The cDNA template from sediment was also assayed in duplicate.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. CH_4 consumption by Lake Stechlin sediment incubated at 4°C.

Fig. S2. T-RFLP profiles of *pmoA* genes recovered from the sediment during the SIP time-course incubations. At each time point, the profiles from the $^{12}CH_4$ incubation (left bar) and $^{13}CH_4$ incubation (right bar) are shown.

Fig. S3. Absolute values of *pmoA* gene copies per CsCl gradient fraction.

Fig. S4. Quantitative PCR (A) test for bias against reverse transcription of type II *pmoA* mRNA. RNA was produced by *in vitro* transcription of a type II *pmoA* clone (SL1-3) and type Ia clone (SL1-4). A total of 5×10^8 RNA copies of each clone were reverse transcribed. The cDNA was serially diluted and used as templates in quantitative PCR. The threshold cycles were the same for each sequence, indicating that equivalent amounts of cDNA was generated from both RNA sequences. In (B) we performed a similar test using T-RFLP by mixing equivalent amounts of the *in vitro* transcripts (top panel) and performing reverse transcription followed by PCR and T-RFLP; as a comparison, the two sequences types were mixed after the reverse transcription (lower panel). Both showed nearly equivalent amounts of the sequence types (SL1-3 T-RF is 244 bp and SL1-4 T-RF is 441 bp) and identical results if the reverse transcription was performed as a mixture or separately.

Fig. S5. Maximum-likelihood phylogenetic tree of representative 16S rRNA sequences obtained from the heavy fraction of SIP gradients. The clone names are indicated in bold type and the observed T-RF size for the clones is indicated in square brackets. The clone names beginning with 'R' originate from RNA-SIP gradients and those with 'D' from DNA-SIP gradients. Representative sequences from various bacterial phyla were used as outgroup (not shown). The GenBank accession numbers of reference sequences are shown in brackets.

Fig. S6. 16S rRNA T-RFLP profiles in the heavy fractions of gradients from $^{12}CH_4$-incubated control samples. DNA-SIP (A) and RNA-SIP (B) gradients are shown. The characteristic T-RFs associated with the heavy fractions from $^{13}CH_4$ incubations (Figs 5 and 6) are not present, indicating that those T-RFs are indeed from labelled nucleic acids.

Fig. S7. *Escherichia coli* RNA analysis with a Bioanalyzer™. The illustration is a simulated gel image produced by the accompanying software. Lanes: 1, RNA size standard; 2, total RNA uncentrifuged; 3, total RNA centrifuged and recovered from the CsTFA gradient; 4, enriched mRNA uncentrifuged; 5, enriched mRNA centrifuged and recovered from CsTFA gradient.

Fig. S8. Investigation of *pmoA* distribution in caesium gradients containing Lake Stechlin DNA (A) or mRNA (B). The uppermost plots show the relative abundance of *pmoA* in each of the gradient fractions as determined by quantitative PCR and quantitative RT-PCR respectively. The lower plots show the *pmoA* T-RFLP data for the gradient fractions containing *pmoA* genes (A) and transcripts (B). The type Ia *pmoA* sequences (blue T-RFs) have lower G+C contents than the type Ib (green) and type II (yellow), which was reflected in the distribution of DNA (A), but not RNA (B). The results show a strong effect of G+C content on the distribution of *pmoA* genes in CsCl gradients, but no such effect of the *pmoA* transcripts in CsTFA gradients.

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