

ORIGINAL ARTICLE

Stable-isotope probing implicates *Methylophaga* spp and novel *Gammaproteobacteria* in marine methanol and methylamine metabolism

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The metabolism of one-carbon (C₁) compounds in the marine environment affects global warming, seawater ecology and atmospheric chemistry. Despite their global significance, marine microorganisms that consume C₁ compounds *in situ* remain poorly characterized. Stable-isotope probing (SIP) is an ideal tool for linking the function and phylogeny of methylotrophic organisms by the metabolism and incorporation of stable-isotope-labelled substrates into nucleic acids. By combining DNA-SIP and time-series sampling, we characterized the organisms involved in the assimilation of methanol and methylamine in coastal sea water (Plymouth, UK). Labelled nucleic acids were analysed by denaturing gradient gel electrophoresis (DGGE) and clone libraries of 16S rRNA genes. In addition, we characterized the functional gene complement of labelled nucleic acids with an improved primer set targeting methanol dehydrogenase (*mxhA*) and newly designed primers for methylamine dehydrogenase (*mauA*). Predominant DGGE phylotypes, 16S rRNA, methanol and methylamine dehydrogenase gene sequences, and cultured isolates all implicated *Methylophaga* spp, moderately halophilic marine methylotrophs, in the consumption of both methanol and methylamine. Additionally, an *mxhA* sequence obtained from DNA extracted from sea water clustered with those detected in ¹³C-DNA, suggesting a predominance of *Methylophaga* spp among marine methylotrophs. Unexpectedly, most predominant 16S rRNA and functional gene sequences from ¹³C-DNA were clustered in distinct substrate-specific clades, with 16S rRNA genes clustering with sequences from the *Gammaproteobacteria*. These clades have no cultured representatives and reveal an ecological adaptation of particular uncultured methylotrophs to specific C₁ compounds in the coastal marine environment.

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Introduction

Microbially mediated carbon metabolism in the marine ecosystem represents an enormous source and sink for many compounds of environmental significance. One-carbon (C₁) compounds influence marine and atmospheric chemistry, affect global

warming and impact cloud formation. C₁ compounds of relevance to the marine environment include methane, methanol, methylated amines (mono-, di- and trimethylamine), methyl halides (methyl chloride, methyl bromide and methyl iodide) and methylated sulphur compounds (dimethylsulphide (DMS), methanesulphonate and methanethiol). Despite their global significance, the organisms and enzymes involved in the metabolism of C₁ compounds in the marine environment are poorly characterized. Nonetheless, the presence of these compounds suggests a role for methylotrophs in their consumption.

Knowledge of marine methylotrophs is largely based on enrichment and cultivation studies using defined media and high concentrations of substrate. For example, several studies have enriched and isolated marine methylotrophs using

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CH₄ as a carbon source. Sieburth *et al.* (1987) used CH₄ to isolate *Methylomonas pelagica* (Type I methanotroph; *Gammaproteobacteria*) from multiple samples taken from within the Sargasso Sea. Lidstrom (1988) isolated four Type I methanotrophs from sewage outfall on the coast of Los Angeles. Holmes *et al.* (1996) prepared CH₄ enrichment and isolations using a water sample from a Welsh shoreline, resulting in the isolation of a Type I methanotroph of the genus *Methylomonas*. Methyl halide enrichments have produced isolates within the *Alphaproteobacteria*, including *Leisingera methylohalidivorans* (Schaefer *et al.*, 2002) and other distinct isolates from within the *Roseobacter* clade (Schäfer *et al.*, 2005). Together, enrichment and isolation studies using a variety of C₁ sources (methanol, methylamine and DMS) have generated molecular fingerprint phylotypes and characterized isolates of *Methylophaga* spp, moderately halophilic bacteria from the *Piscirickettsiaceae* family in the *Gammaproteobacteria* (Strand and Lidstrom, 1984; Janvier *et al.*, 1985; De Zwart *et al.*, 1996; Doronina *et al.*, 1997, 2003a, b, 2005; Vila-Costa *et al.*, 2006; Schäfer, 2007). However, the enrichment and isolation of particular organisms from the marine environment and demonstration of their ability to grow on defined media in the laboratory does not necessarily indicate methylophagous activity *in situ*.

Stable-isotope probing (SIP) provides a means by which environmental samples may be incubated with labelled substrates of interest, producing labelled nucleic acids from organisms responsible for the assimilation of those compounds. Isolation on defined media is obviated and in the case of DNA-SIP, phylogenetic (for example 16S rRNA) and enzyme-encoding (for example methanol dehydrogenase) gene sequences help characterize the organisms associated with labelled DNA. The use of DNA-SIP with C₁ compounds for the study of methylophagy has been applied to a variety of environments with a wide range of substrates (reviewed in Neufeld *et al.*, 2007c). Nercessian *et al.* (2005) at the University of Washington used SIP to analyse a wide range of C₁ compounds in freshwater sediment and identified specific populations of methylophagous that responded to individual substrates. This current study represents the first application of SIP to the marine environment to characterize the methylophagous associated with the metabolism of methanol and methylamine, two C₁ compounds of importance for marine and atmospheric chemistry (Van Neste *et al.*, 1987; Heikes *et al.*, 2002).

The ocean is considered as a large reservoir for methanol (Jacob *et al.*, 2005) because this C₁ compound is deposited, produced and consumed in the marine environment (Heikes *et al.*, 2002; Carpenter *et al.*, 2004). Methylamine represents a source of both carbon and nitrogen and its flux from the marine environment is thought to be limited by microbial metabolism (Fitz-Gibbon and House, 1999; Naqvi *et al.*, 2005). Despite their importance

to marine C₁ cycling, the marine methylophagous involved in the metabolism of methanol and methylamine have not been identified with cultivation-independent techniques before this study.

The goal of this study was to investigate active marine methylophagous using DNA-SIP. The use of two substrates (methanol and methylamine) helped assess the extent of niche differentiation that was apparent in methylophagous populations in the English Channel. We demonstrate that *Methylophaga*-related 16S rRNA, methanol dehydrogenase (*mxhA*), and methylamine dehydrogenase (*mauA*) gene sequences were associated with heavy DNA fractions. Surprisingly, a large proportion of sequences representing active marine methylophagous were associated with substrate-specific clades with no close affiliations with sequences from cultured bacteria, providing ideal targets for future cultivation and metagenomic approaches.

Materials and methods

SIP conditions

Approximately 30 l of surface sea water from 1-m depth was collected on 28 November 2005 from the L4 sampling station (50°15'N, 4°13'W). L4 is located approximately 10 km off the coast of Plymouth (UK) in the English Channel. Within 2 h of sampling, 1.5 l of sea water was filtered through a 0.2- μ m Sterivex filter (Durapore; Millipore, Billerica, MA, USA) for DNA extraction. SIP incubations were prepared within 2–3 h of sampling. One-litre serum bottles were filled with 750 ml sea water, and supplemented with 0.2% (1.5 ml) marine ammonium mineral salts medium (MAMS; modified from Goodwin *et al.*, 2001), and supplemented to 17.5 μ M with either ¹²C or ¹³C-methanol or -methylamine (> 99% label, CK Gas Products Ltd., Hook, Hampshire, UK). Bottles were sealed with butyl rubber stoppers and incubated for 4 days at 19°C. Each day, bottles were supplemented with an additional 1.5 ml MAMS and 13.13 μ mol of the appropriate labelled substrate. Bottles were sacrificed on days 1 (24 h), 3 (72 h) and 4 (96 h) by filtration through a 0.2 μ m Sterivex filter (Durapore, Millipore). Filters were immediately stored at –80°C. On day 4, diluted aliquots from completed methanol and methylamine SIP incubations were plated onto MAMS plates (1.5% agar) with 5 mM methylamine. Representative colonies were added directly to tubes of sterile water for 16S rRNA gene colony PCR.

DNA extraction

DNA was extracted from Sterivex filters by a modification of two previously published protocols (Somerville *et al.*, 1989; Murray *et al.*, 1998). Briefly, 1.6 ml of SET buffer (40 mM ethylenediamine tetraacetic acid, 50 mM Tris-HCl pH 9 and 0.75 M sucrose) and 180 μ l of fresh lysozyme solution (990 μ l sterile

water, 9 mg lysozyme and 9 μ l 1 M Tris-HCl pH 8) were added and the filter was incubated with rotation in a hybridization oven (Hybaid, Waltham, MA, USA) at 37°C for 30 min. After the addition of 200 μ l of 10% (w/v) SDS and 55 μ l of fresh proteinase K solution (950 μ l sterile water, 50 μ l 1 M Tris-HCl pH 8 and 20 mg proteinase K) the filter was then incubated at 55°C for 2 h with rotation. Lysates were withdrawn into 5-ml syringes, filters rinsed with 1 ml of SET buffer and the rinsed buffer combined with the lysate. Pooled lysates were added to 15-ml phase lock tubes (Eppendorf, Hamburg, Germany). Two phenol:chloroform:isoamyl alcohol (25:24:1) extractions and a single chloroform extraction were performed with a 2-ml addition of organic solvent to the same tube according to the manufacturer's instructions. Subsequently, the 2–3 ml of aqueous phase was decanted into a 25-ml centrifuge tube. Five-microlitres of a 20- μ g μ l⁻¹ glycogen solution (Roche, Basel, Switzerland), 0.5 volumes of 7.5 M ammonium acetate and two volumes of 95% (v/v) ethanol were added to the tube. Precipitations were carried out overnight at -20°C. Samples were centrifuged at 48 000 g to pellet the nucleic acid, washed twice with 80% (v/v) ethanol, dried for 30 min at room temperature, and suspended in 200 μ l of sterile water. DNA was quantified on a 1% (w/v) agarose gel.

DNA-SIP gradient fractionation

From each sample, 1–5 μ g of total extracted DNA was added to caesium chloride (CsCl) solutions for isopycnic ultracentrifugation and gradient fractionation similarly to a previously described protocol (Neufeld *et al.*, 2007b). Briefly, DNA, gradient buffer and CsCl were combined to a final density of 1.725 g ml⁻¹ and centrifuged at 177 000 g_{av} in a Vti 65.2 vertical rotor (Beckman Coulter, Fullerton, CA, USA) at 20°C for 40 h. Gradients were fractionated into ~425- μ l fractions and DNA was precipitated with 20 μ g glycogen and two volumes of polyethylene glycol (30% PEG 6000 and 1.6 M NaCl). DNA was suspended in 20 μ l of nuclease-free water and aliquots were run on a 1% (w/v) agarose gel.

Denaturing gradient gel electrophoresis of 'heavy' and 'light' DNA

From each tube, 1 μ l of fraction 7 (heavy ¹³C-DNA) and 1 μ l of fraction 11 (light ¹²C-DNA) were used as template for PCR and denaturing gradient gel electrophoresis (DGGE) as described previously (Leckie *et al.*, 2004). Briefly, PCR (30 cycles) amplification of ~490-bp fragment was carried out with primers 63f-GC and 517r. PCR products were quantified by comparison to a 1-kb ladder (Invitrogen, Paisley, UK) on a 1.5% (w/v) agarose gel and 100–300 ng PCR product was loaded in each lane of the DGGE. DGGE electrophoresis was performed

using the D-Code System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's directions. Gels had a denaturing gradient of 40–70% (100% denaturant contains 7.0 M urea and 40% deionized formamide). After electrophoresis for 14 h at 60°C and 85 V, gels were stained with SYBR Green I (Molecular Probes (Invitrogen), Paisley, Scotland, UK) at a 1:10 000 dilution for 1 h. Gels were scanned with a FLA-5000 imaging system (Fujifilm, Tokyo, Japan). Specific bands were amplified from the gel with the same PCR conditions as described above, but without a GC clamp. Sequencing of PCR products was done with the 63f primer, BigDye terminator version 3.1 (Applied Biosystems, Foster City, CA, USA) and reaction products were run on an ABI PRISM 3130 \times 1 Genetic Analyser (Applied Biosystems) by the Molecular Biology Service (University of Warwick).

16S rRNA gene library

Clone libraries were generated from DNA taken from the L4 marine sampling site, and from the heavy DNA from day 3 (methanol SIP) or day 4 (methylamine SIP) incubations. PCR with primers 27f and 1492r (Lane, 1991) was carried out as for DGGE but with an extension time of 1.5 min. Products were cloned into the TOPO-TA cloning vector (Invitrogen) according to the manufacturer's protocol. From the original L4 DNA, 36 colonies were screened by colony PCR as previously described (Neufeld *et al.*, 2004), but in 50- μ l reactions using primers -21M13 (TGTAACGACGGCCAGT) and M13 reverse (CAGGAAACAGCTATGACC). In the same way, 24 colonies were screened from each of the SIP heavy DNA samples. Sequencing of PCR products was done with the 27f primer at the Edinburgh node of the NERC Molecular Genetics Facility. The affiliations of sequences from the L4 library were assigned using the Classifier function of RDP-II (Cole *et al.*, 2003). Sequences from the SIP heavy DNA libraries were aligned in Arb (Ludwig *et al.*, 2004) with related GenBank (Benson *et al.*, 2000) reference sequences. Cropped alignments (660 bp) were exported from Arb as a Phylip (Felsenstein, 1989) file and used for tree construction. The Jukes–Cantor model (Jukes and Cantor, 1969) was used to construct a neighbour-joining tree, which was bootstrapped 100 times using TREECON (Van de Peer and De Wachter, 1994). The relative locations of DGGE sequences were determined by adding the ~490 bp sequences to the final tree using the parsimony method in Arb (Ludwig *et al.*, 2004).

Methanol dehydrogenase gene (*mxoF*) library

The methanol dehydrogenase gene (*mxoF*) primer set, originally designed by McDonald and Murrell (1997), was revised based on additional full-length sequences available in GenBank as of November 2005. The Sargasso Sea data set (Venter *et al.*, 2004)

was also mined for *mxoF* by Kalyuzhnaya *et al.* (2005), but none were found to help with primer design. The forward primer was unchanged (1003f) but the reverse primer (1561r) was modified to target additional *mxoF* sequences (1555r; 5'-CATGAABGGCTCCCATCCAT-3'). Using 1003f and 1555r, clone libraries were generated with DNA from the L4 marine sampling site, and from day 3 (methanol SIP) or day 4 (methylamine SIP) 'heavy' DNA retrieved from the SIP incubations. PCR was performed as for DGGE but with an annealing temperature of 50°C. PCR products of the appropriate size (~550 bp) from L4 DNA and SIP heavy band DNA samples were purified from agarose gels using the QIAquick gel extraction kit (Qiagen, Crawley, West Sussex, UK), and cloned into the TOPO-TA cloning vector (Invitrogen) according to the manufacturer's protocol. Twenty-four colonies were selected for colony PCR and sequencing clones from each library was conducted as described above (except using M13r as a sequencing primer) on clones with inserts of a suitable size. Derived amino acid sequences from the L4 marine sampling site DNA and the SIP heavy DNA libraries were aligned in Arb with related GenBank reference sequences. Cropped alignments (148 amino acids) were exported from Arb as a Phylip (Felsenstein, 1989) file and used for tree construction. The Kimura model (Kimura, 1980) was used to construct a neighbour-joining tree, which was bootstrapped 100 times using TREECON (Van de Peer and De Wachter, 1994).

Methylamine dehydrogenase gene (*mauA*) library

The seven known gene sequences encoding the small subunit of methylamine dehydrogenase were retrieved from GenBank and aligned in Arb in May 2006. New primers, forward (*mauAf1*; ARKCYTGYGABTAYTGCG) and reverse (*mauAr1*; GARAYVGTGCARTGRTARGTC), were designed to amplify these *mauA* gene sequences. Using the same PCR conditions as for the amplification of *mxoF*, 310-bp portions of the *mauA* gene were amplified from DNA from the L4 marine sampling site, from day 3 (methanol SIP) or day 4 (methylamine SIP) heavy DNA retrieved from SIP incubations and from pure culture DNA from the University of Warwick culture collection. PCR products from the L4 marine sampling site DNA and from SIP heavy DNA were cloned into the TOPO-TA cloning vector (Invitrogen) according to the manufacturer's protocol. Twenty-four colonies were selected for colony PCR from each sample, and sequencing of each library was conducted as described above (using M13r as a sequencing primer) on clones with inserts of a correct size. Derived amino acid sequences from the L4 marine sampling site DNA and the SIP heavy DNA libraries were aligned in Arb with related GenBank reference sequences. Cropped alignments (87 amino acids) were exported from Arb as a Phylip (Felsenstein, 1989) file and used for tree construc-

tion. The Kimura model (Kimura, 1980) was used to construct a neighbour-joining tree, which was bootstrapped 100 times using TREECON (Van de Peer and De Wachter, 1994).

Sequence accession numbers

The following are GenBank accession numbers for DGGE band sequences (EU001661–EU001664), 16S rRNA gene sequences for L4 (EU001665–EU001695), methanol SIP heavy DNA (EU001696–EU001719) and methylamine SIP heavy DNA (EU001720–EU001738). Also deposited were *mxoF* sequences for L4 (EU001807–EU001812), methanol SIP heavy DNA (EU001813–EU001836), methylamine SIP heavy DNA (EU001837–EU001859), and *mauA* sequences for L4 (EU001751–EU001761), methylamine SIP heavy DNA (EU001762–EU001784), methanol SIP heavy DNA (EU001785–EU001803). Additional accession numbers for isolates and lab strains are indicated in the figures and include the accession numbers EU001739–EU001740, EU001744–EU001750 and EU001860–EU001862.

Results

The incubation of sea water with methanol and methylamine proceeded for 4 days, with individual bottles sacrificed on days 1, 3 and 4. These time points were chosen because enrichment cultures and initial SIP experiments with excessive substrate concentrations demonstrated either activity (measured by gas chromatography) or growth (measured by optical density) of the microbial community on these two C-sources after 3 days (Neufeld *et al.*, 2007a). From the filtered samples, extracted DNA was centrifuged, and light and heavy DNA samples from gradients were characterized by DGGE fingerprinting (Figure 1). Unlabelled ¹²C control incubations confirmed that the fingerprints associated

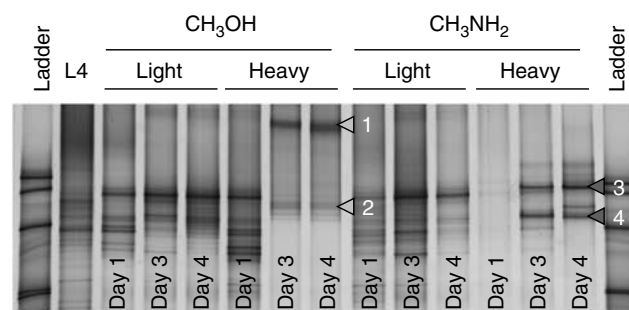


Figure 1 Denaturing gradient gel electrophoresis (DGGE) fingerprint profiles for 'light' and 'heavy' DNA from SIP gradients. Fingerprints are shown for stable-isotope probing (SIP) incubations with ¹³C-methylamine or -methanol, for samples retrieved on days 1, 3 and 4. A fingerprint from a marine DNA extract from the beginning of the experiment is included for comparison. Bands chosen for sequencing are indicated. Ladders were generated by mixing PCR products from 16S rRNA genes, cloned from cultured isolates or previously run DGGE fingerprints.

with the heavy DNA were due to ^{13}C -labelled incorporation into the community (data not shown). CsCl gradient fractions 7 ($\sim 1.730\text{ g ml}^{-1}$) and 11 ($\sim 1.715\text{ g ml}^{-1}$) were characterized over time. By days 3 and 4, a unique pattern emerged in the heavy fractions for both methanol and methylamine SIP incubations (Figure 1), representing phylotypes that had incorporated ^{13}C -labelled methanol or methylamine. Further, the patterns were stable over days 3 and 4, and were distinct for each substrate, suggesting that specific methylotrophic populations were associated with the metabolism of each of these carbon sources. Figure 1 indicates DGGE bands that were retrieved from the gel, PCR amplified and sequenced directly. All four bands yielded clear 16S rRNA gene sequence data and were in agreement with clone library sequences, grouping within the *Gammaproteobacteria* (Figure 2).

16S rRNA gene libraries

Clone libraries were generated with PCR products from the original L4 seawater DNA, and from the heavy DNA associated with both ^{13}C -methanol (day 3) and -methylamine SIP (day 4) incubations (Figure 2). High-quality sequences (31 total) from the L4 sampling site (Figure 2a) were associated with *Proteobacteria* (21 sequences), *Cyanobacteria* (6 sequences), *Bacteroidetes* (3 sequences) and *Firmicutes* (1 sequence). Unsurprisingly, most of the sequences were closely affiliated with uncultured clones or characterized organisms previously isolated from the marine environment (data not shown). With the largest sequence representation in the *Alphaproteobacteria* (17 sequences; $\sim 55\%$) and only low representation of the *Gammaproteobacteria* (3 sequences; $\sim 10\%$), the small L4 16S rRNA gene clone library was sufficiently sampled to demonstrate a clear contrast with libraries prepared from SIP heavy DNA (Figure 2b). All 16S rRNA gene sequences derived from heavy DNA were affiliated with the *Gammaproteobacteria* and were different from the L4 *Gammaproteobacteria* sequences. Most of the ^{13}C -DNA sequences from the ^{13}C -methanol SIP clustered within the *Methylophaga* clade, along with two of the sequences from the ^{13}C -methylamine SIP experiment. In addition to sequences from the ^{13}C -labelled DNA, marine isolates represented by sequences AM2 and AM3 (this study) and from DMS enrichments (*Methylophaga* spp isolates DMS010 and DMS021; Schäfer, 2007) were contained within the *Methylophaga* cluster (Figure 2b). Finally, sequences from DGGE bands 2 (methanol SIP; Figure 1) and 4 (methylamine SIP; Figure 1) were also affiliated with the *Methylophaga* clade.

In addition to the *Methylophaga* clade, almost half of the ^{13}C -methanol SIP heavy sequences (10 of 24) and the sequence for DGGE band 1 (methanol SIP; Figure 1) clustered with environmental sequences from a variety of marine and other aquatic

environments, lacking close affiliation with cultured representatives. These sequences likely represent one or more organisms able to utilize methanol. With only $\sim 90\%$ identity to *Methylophaga marina*, these may represent organisms of a novel and uncultivated genus of marine methylotrophs.

Another distinct and uncultured clade was composed of almost identical sequences retrieved from the ^{13}C -methylamine SIP heavy DNA. The large proportion of these sequences (15 of 19), and representation by DGGE band 3 (methylamine SIP; Figure 1) suggests the ability of these organisms to utilize methylamine as a carbon source. Given that the identity of these sequences to *M. marina* 16S rRNA gene is 84% and to the closest cultured representative (*Microbulbifer salipaludis*; a moderate halophile; Yoon *et al.*, 2003) is 91% over the $\sim 660\text{ bp}$ fragment, these sequences are likely to represent a novel uncultured genus of marine methylotrophs. Additional sequences associated with the methylamine SIP heavy DNA (33-7_H09 and H10) clustered distantly, revealing additional uncultured methylamine-utilizing *Gammaproteobacteria* phylotypes.

Methanol dehydrogenase gene (*mxoF*) libraries

To minimize the bias of the *mxoF* libraries, we modified the original 1003f and 1561r *mxoF* primer set (McDonald and Murrell, 1997). The original primers were designed using three available *mxoF* sequences (*Methylobacterium extorquens* strain AM1, *Methylobacterium organophilum* strain XX and *Paracoccus denitrificans*). In November 2005, seven additional *mxoF* sequences were available in GenBank that spanned a greater portion of the gene than that amplified by 1003f and 1561r, respectively (Table 1). Primer 1003f was unaltered as it was a perfect match to 7 of the 10 spanning sequences and other mismatches were mostly $>10\text{ bp}$ from the 3' end (Table 1). Primer 1561r required modification as only 5 of 13 available sequences were a perfect match to the primer. To replace 1561r, primer 1555r was designed as a perfect match to 12 of the 13 available sequences. The only mismatch was toward the 5' end of the primer for *Hyphomicrobium methylovorum*. The new *mxoF* primer set (1003f and 1555r) was tested with PCR against DNA extracted from 14 *mxoF*-containing methylotrophs and methanotrophs in the Warwick culture collection and six negative control strains. The primers amplified products of the correct size for all *mxoF*-containing organisms and for none of the negative controls (Cox, 2005).

Using the improved *mxoF* primer pair, clone libraries were prepared for the original L4 DNA sample and from the methanol and methylamine SIP heavy DNA. A derived amino-acid neighbour-joining tree confirmed the results from the 16S rRNA gene clone libraries, demonstrating that several of the methanol and methylamine SIP sequences

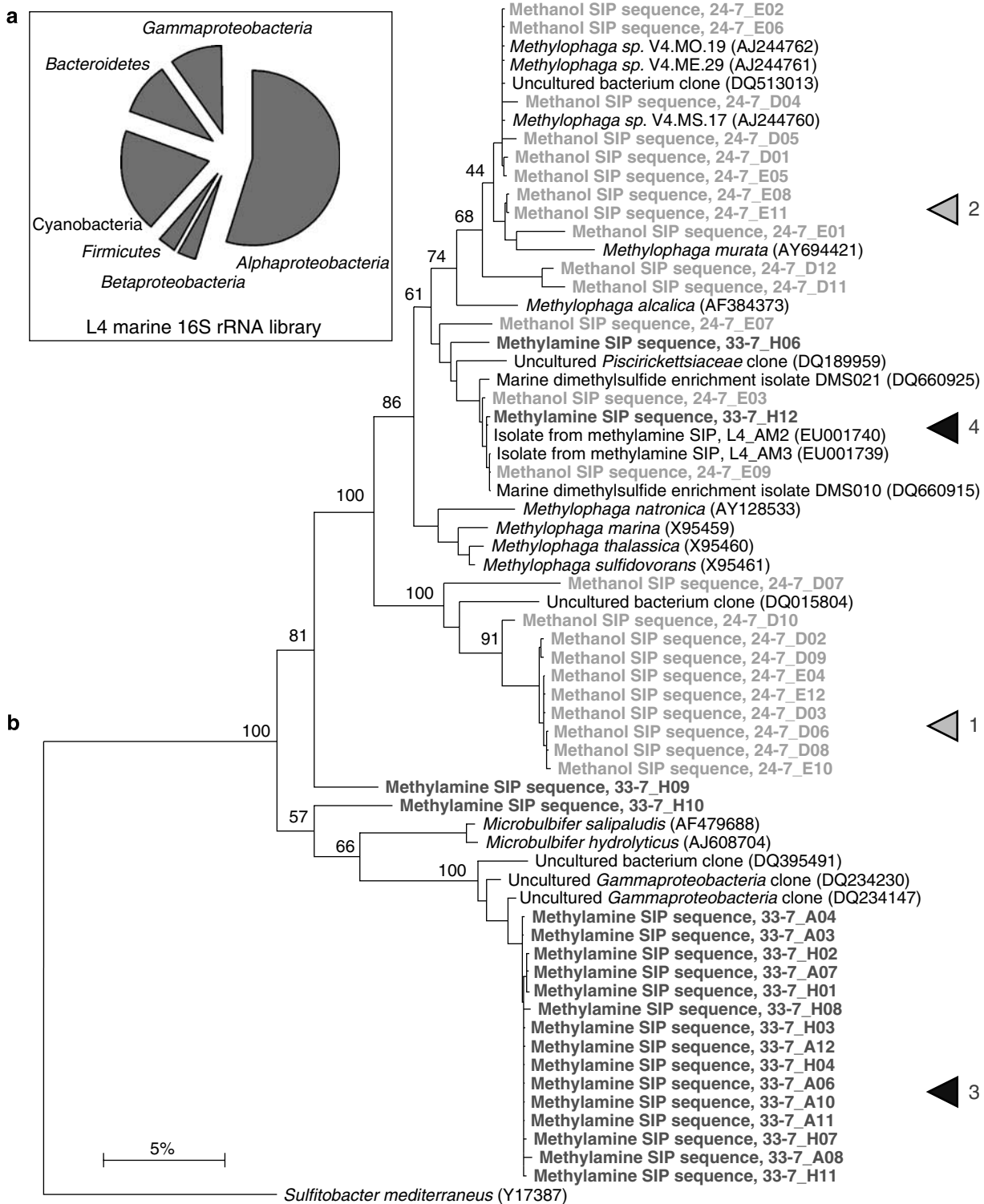


Figure 2 Phylogenetic affiliations of 16S rRNA gene sequences. (a) Division-level diversity representing all sequences from the L4 marine 16S rRNA gene library. (b) All sequences from stable-isotope probing (SIP) gradient ‘heavy’ DNA and closest GenBank reference strains. All sequences were affiliated with the *Gammaproteobacteria* and bootstrap values are indicated for major branch points on this neighbour-joining tree. GenBank accession numbers for reference sequences are included within parentheses. The scale bar represents 10% sequence divergence and the numbered symbols along the right side indicate the approximate phylogenetic affiliations of the corresponding denaturing gradient gel electrophoresis (DGGE) band sequences from Figure 1.

clustered in a *Methylophaga*-like clade but that additional substrate-specific clades existed (Figure 3). The majority of the methanol SIP

sequences clustered with *Methylophaga*-like sequences and the majority of the methylamine-SIP sequences were associated with a distinct clade. L4

Table 1 Evaluation of *mxoF* primers 1003f and 1561r and an improved reverse primer, 1555r

Organism	Accession no.	Primer 1003f		Primer 1561r		Primer 1555r		
		Match	Mismatch > 10 bases from 3'	Match	Mismatch > 10 bases from 3'	Match	Mismatch > 10 bases from 3'	Mismatch < 10 bases from 3'
<i>Methylobacterium extorquens</i> ^a	MEU72662	●		●		●		
<i>Methylobacterium organophilum</i> ^a	M22629	●		●		●		
<i>Paracoccus denitrificans</i> ^a	M17339	●		●		●		
<i>Methylobacterium nodulans</i>	AF220764	●		●		●		
<i>Methylophilus methylotrophus</i>	U41040		●		●		●	
<i>Hyphomicrobium methylovorum</i>	AB004097							
<i>Methylororus</i> sp. SS1	AF184915	●		●		●		
<i>Methylococcus capsulatus</i> (Bath)	AE017282							
<i>Methylobacillus</i> sp. SK-5	AF494424	●		●		●		
<i>Alibacter methylovorans</i>	AJ878069	●		●		●		NA
<i>Methylopila capsulata</i>	AJ878071	NA	NA	NA	NA	NA	NA	NA
<i>Methylorhabdus multivorans</i>	AJ878070	NA	NA	NA	NA	NA	NA	NA
<i>Beijerinckia mobilis</i>	AJ563936	NA	NA	NA	NA	NA	NA	NA
<i>Methylobacterium dichloromethanicum</i>	AJ878068	NA	NA	NA	NA	NA	NA	NA

Abbreviations: *mxoF*, methanol dehydrogenase; NA, not applicable, the sequence length was insufficient for primer design.

Black circles indicate either perfect matches or mismatches for primers and the listed sequences.

^aOriginal sequences used for the design of 1003f and 1561r.

seawater sequences were broadly distributed throughout the *MxaF* tree although one L4 *MxaF* sequence clustered within the *Methylophaga* clade. An additional methanol SIP sequence from the *mxoF* clone library clustered most closely with *XoxF* sequences. *XoxF* is an *MxaF* homologue with no known function (Kalyuzhnaya *et al.*, 2005), but is commonly associated with the genomes of methylotrophic bacteria.

Methylamine dehydrogenase gene (mauA) libraries

One pathway by which methylamine is metabolized by methylotrophic bacteria is via methylamine dehydrogenase (Anthony, 1982). However, primers had not yet been designed for the gene encoding this enzyme. Almost complete *mauA* sequences were available for six sequences as of February 2006: *P. denitrificans*, *Paracoccus versutus*, *Methylobacillus flagellatus*, *Methylophilus methylotrophus*, *M. extorquens* strain AM1 and *Methylophaga thalassica*. With several degenerate bases, primers *mauAf1* and *mauAr1* matched all six *mauA* DNA sequences. The primer set correctly amplified *mauA* amplicons from seven pure cultures of methylamine utilizers (Figure 4). DNA from other organisms known to utilize methylamine did not amplify (*Methylobacterium thiocyanatum*, *P. denitrificans* strain 381, *M. thalassica*), suggesting that alternative methylamine utilization pathways may be present in these organisms.

The newly designed *mauA* primer set was used to generate clone libraries from the original L4 DNA sample and from the methanol and methylamine SIP heavy DNA. A derived amino-acid neighbour-joining tree demonstrated that all of the SIP heavy DNA sequences were associated with *Methylophaga*-like *MauA* sequences, with most of the methanol SIP heavy band sequences being in a different *Methylophaga* cluster to the majority of the methylamine SIP heavy band sequences (Figure 4). While substrate-specific *MauA* sequences were evident, tight clustering of these enzyme sequences to *Methylophaga* indicates that either the organisms associated with the uncultured 16S rRNA gene and *MxaF* clades metabolize methylamine via a different pathway or the phylogenetic relatedness of *MauA* sequences do not mirror those of 16S rRNA gene or methanol dehydrogenase sequences. The derived amino acid sequences from the original L4 seawater DNA cluster distantly from the other known *MauA* sequences (Figure 4). These marine sequences may represent highly divergent *MauA* sequences or may simply be amplified products from a distantly related paralog. Nonetheless, the majority of the key amino acids representing probable enzyme active site residues or active site cavity lining (Chen *et al.*, 1998) were conserved in the L4 clusters (data not shown), suggesting an evolutionary constraint due to a functional homology between these derived amino-acid sequences.

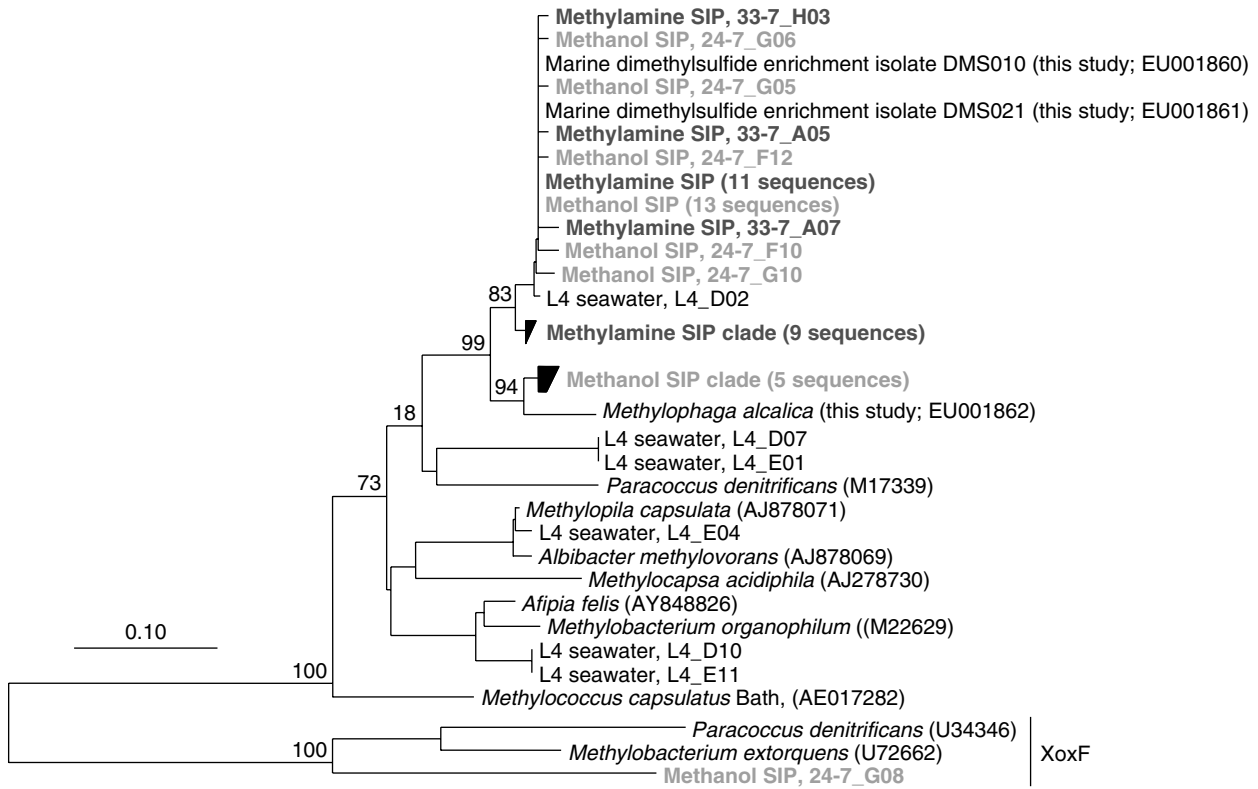


Figure 3 Phylogenetic analysis of derived methanol dehydrogenase (MxaF) sequences. This unrooted neighbour-joining tree was generated with partial *mxnF* sequences and closest GenBank reference sequences (accession numbers shown in parentheses). The scale bar represents 10% sequence divergence. Bootstrap values are indicated for major branch points.

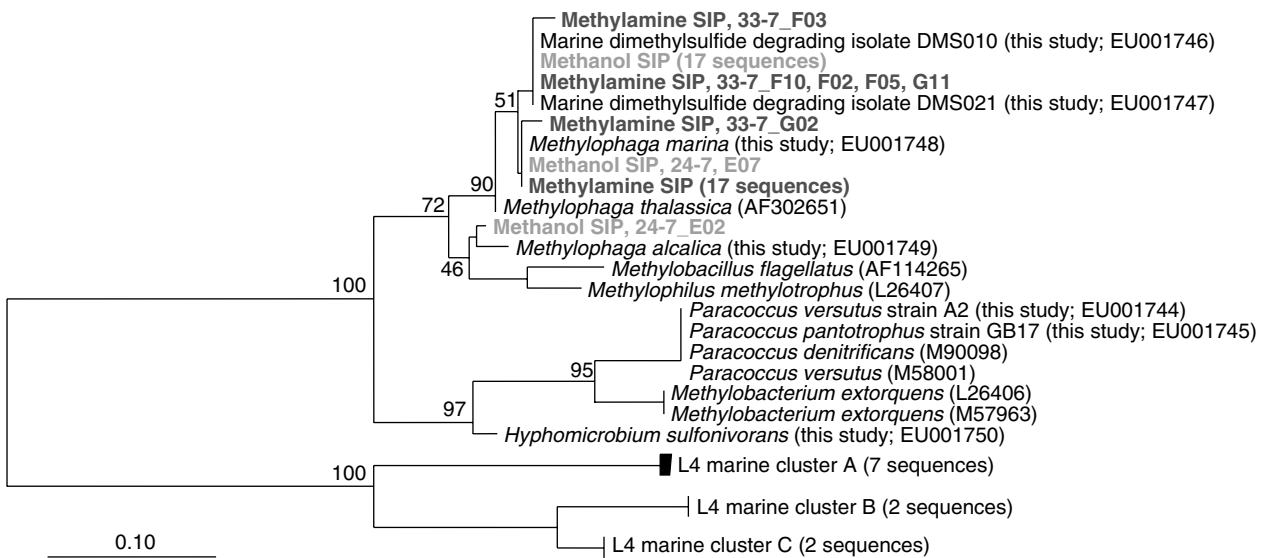


Figure 4 Phylogenetic analysis of derived methylamine dehydrogenase (MauA) sequences. This unrooted neighbour-joining tree was generated with partial *mauA* amplicons and closest GenBank reference sequences of known methylamine utilizers (accession numbers shown in parentheses). The scale bar represents 10% sequence divergence. Bootstrap values are indicated for major branch points.

Discussion

Methylamine and methanol are two important C₁ compounds available to microorganisms in the marine environment. Methylamine is produced

from the breakdown of trimethylamine-*N*-oxide, an osmoregulatory compound that accumulates in algae, invertebrates and fish in response to salinity and stress (reviewed by Gibb and Hatton, 2004). Methylamine is a biogenic carbon and nitrogen

source for microbial metabolism and has atmospheric influences in common with DMS. Aerosol particles of both methylamine and DMS promote cloud formation, which leads to greater solar reflection and a reduced greenhouse effect. In sea water, the average dissolved concentrations of methylamine in the coastal marine environment can reach 66 nM, with averages reported between 12 and 22 nM (reviewed in Gibb and Hatton, 2004). Methylamine represents the most abundant methylamine species (of mono-, di- and trimethylamine) measured in those studies.

Methanol is a volatile organic compound, second in abundance to methane in the atmosphere. Of importance to atmospheric chemistry, methanol reacts with hydroxyl radicals to produce formaldehyde, hydrogen radicals and ozone (Heikes *et al.*, 2002). Additionally, photochemistry in cloud water produces formic acid, which contributes to the background acidity of cloud and rain water (Monod *et al.*, 2000). Given their high aqueous solubility, oceans represent an enormous reservoir for methanol. Estimates suggest that oceans contain approximately 230 Tg methanol, which is 66 times the atmospheric content (Galbally and Kirstine, 2002). While methanol concentrations have not been directly measured in surface sea water, estimates based on atmospheric concentrations and solubility range from 100 (Singh *et al.*, 2003) to 300 nM (Galbally and Kirstine, 2002). The lifetime of methanol in the marine mixed layer is unknown. An underlying difficulty in characterizing the fate of methanol is the unknown abundance and activity of methylotrophs responsible for its conversion into biomass and CO₂.

As with all SIP experiments to date, the substrate concentrations used here exceeded those present in the natural environment. Samples were incubated at an initial concentration of 17.5 μ M for the SIP experiments described here. On the basis of the above discussion, the *in situ* concentrations of methylamine and methanol were likely to be 100–1000-fold lower than those used in our SIP experiments. Further, incubating samples in bottles and supplementing with nutrients represents additional conditions that differ from the *in situ* environment. While these incubation conditions will certainly bias the results to some extent, this work represents an essential first step towards characterizing the active methylotrophs in marine surface waters.

In contrast to the L4 sample clone library, the 16S rRNA genes that were retrieved from heavy DNA clustered entirely within the *Gammaproteobacteria* (Figure 2). Most of the ¹³C-methanol SIP-associated sequences were closely related to characterized strains of *Methylophaga*. This group of methylotrophs uses the ribulose monophosphate pathway for assimilation of C₁ compounds. They are moderately halophilic, requiring Na⁺, Mg²⁺ and vitamin B₁₂ for growth and have a low G+C content (reviewed in Janvier and Grimont, 1995). Characterized

Methylophaga spp have been isolated from a wide variety of environments, including coastal marine water (*M. thalassica*; Janvier *et al.*, 1985), marine mud (*M. marina*; Janvier *et al.*, 1985), saline soda lakes (*M. alcalica*; Doronina *et al.*, 2003b, *M. natronica*; Doronina *et al.*, 2003a), Black Sea liman (*M. limanica*; Doronina *et al.*, 1997), a microbial mat (*M. sulfidovorans*; De Zwart *et al.*, 1996) and from deteriorating marble in the Kremlin (*M. murata*; Doronina *et al.*, 2005). With the exception of *M. sulfidovorans*, all of these moderately halophilic methylotrophs were isolated with methanol as a C₁ substrate. Thus, by using ¹³C-labelled methanol as an SIP substrate, the identified 16S rRNA genes (Figure 2b) reflect marine methanol-consuming isolates. The presence of novel uncultured clades identified by ¹³C-methanol and -methylamine SIP reveals additional and unexplored marine methylotroph diversity, underlining the importance of vigorous cultivation attempts with additional substrates and incubation conditions.

Many of the 16S rRNA gene phylotypes (Figure 2) and functional gene sequences (Figures 3 and 4) retrieved from labelled DNA clustered into substrate-specific clades. This indicates that unique methylotrophic communities were responsible for methanol and methylamine metabolism, providing first evidence that competition for C₁ compounds in the oligotrophic marine environment has resulted in the coexistence of related *Gammaproteobacteria* that differ in their substrate preference. Even the 16S rRNA gene DGGE fingerprints representing ¹³C-methanol and -methylamine SIP heavy DNA were distinct (Figure 1). Cultivation-based surveys of marine methylotrophs may not be suitable to reveal these trends; the two unique methylamine isolates we obtained were affiliated with ¹³C-methanol SIP sequences in the *Methylophaga* cluster.

This study generated the first *mxoF* sequences from the *Methylophaga* genus (Figure 3). Surprisingly, this characterized marine methylotroph had no published *mxoF* sequences deposited in GenBank. Using an improved *mxoF* primer set (Table 1), we amplified the gene encoding the large subunit of methanol dehydrogenase from several *Methylophaga* spp and demonstrated their affiliation with sequences from the L4 marine sample and SIP heavy DNA (Figure 3). The L4 marine *mxoF* library contains sequences that cluster with a variety of methylotrophic bacteria, indicating potentially high methylotroph diversity in the marine environment. Importantly, an L4 sequence clustered with *Methylophaga*-like sequences retrieved with many of the methanol and methylamine SIP sequences. This provides evidence that *Methylophaga*-like bacteria are numerically predominant marine methylotrophs in addition to being metabolically active in SIP incubations.

The *soxB* (*mxoF'*) sequence that was retrieved from the labelled DNA represents a puzzling observation (Figure 3). This enzyme is commonly associated with methylotrophic organisms and

isolates not necessarily known as methylotrophic. Its function remains completely unknown (Kalyuzhnaya *et al.*, 2005). The improved *mxoF* primer set presented here (1003f and 1555r) shares some identity with several homologous *xoxF* sequences (data not shown). However, DNA-SIP data must be interpreted cautiously. While *XoxF* could well be involved in an aspect of methylotrophic metabolism, its presence in heavy DNA only implies that organisms identified with ^{13}C -methanol SIP incubations contain a copy of *xoxF* within labelled genome fragments. Of course, the same caution applies to the *mxoF* and *mauA* genes identified here (Figures 3 and 4). The transcription and translation of functional genes are not confirmed by their detection in labelled DNA. A desired goal for SIP-based methodological development is the characterization of labelled mRNA (Dumont *et al.*, 2006; Neufeld *et al.*, 2007a). Such an approach would be helpful in confirming the transcription of genes during growth on particular substrates but has not yet been demonstrated as a feasible technique.

Interpreting the significance of the *mauA* gene is challenging. The methylamine dehydrogenase gene is not a universal marker for methylamine metabolism. Methylamine can be metabolized through at least three different pathways, each with corresponding key enzymes (methylamine dehydrogenase: E.C.1.4.99.3, methylamine oxidase: E.C.1.4.3.6, methylamine-glutamate *N*-methyltransferase: E.C.2.1.1.21). Nonetheless, we chose to develop methylamine dehydrogenase genes for primer design as this is the most well-characterized microbial pathway for methylamine metabolism and several gene sequences are known, permitting the development of a consensus primer set. The *mauA* gene (also known as *madB*) encodes the structural gene for the small subunit of the methylamine dehydrogenase. This subunit also contains the active site of this enzyme and has active site-associated regions of high sequence conservation, useful for primer design (Chistoserdov *et al.*, 1994; Chen *et al.*, 1998). The *mauA* gene sequences shown in Figure 4 represent all methylamine dehydrogenase small subunit sequences available to date. The *mauA* sequences associated with methanol and methylamine SIP heavy DNA were detected from marine organisms that metabolized methanol and methylamine. However, it is not possible to determine whether the *mauA* gene was expressed in the organisms or which *mauA* (or *mxoF*) sequence were linked to which 16S rRNA gene sequences. Linking of 16S rRNA gene sequences and C_1 metabolism genes may be possible in future studies involving metagenomics and cultivation attempts.

This study demonstrates the importance of conducting marine SIP experiments using C_1 substrates. Not only do these compounds affect marine and atmospheric chemistry, influence global carbon cycling and contribute either positively or negatively to global warming, but the 16S rRNA gene sequences associated with labelled DNA represent

novel and deeply branching uncultured bacteria. These data enable future research to confirm the *in situ* activity and ecology of these phylotypes. We are currently testing additional C_1 compounds of relevance to the marine environment to help broaden our understanding of oceanic methylotroph diversity. Marine methylotrophy is an important component of global carbon cycling and climate regulation. This study represents an initial exploration into the marine methylotrophs that contribute to these global phenomena.

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References

- Anthony C. (1982). *The Biochemistry of Methylotrophs*. Academic Press: New York.
- Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Rapp BA, Wheeler DL. (2000). Genbank. *Nucleic Acids Res* **28**: 15–18.
- Carpenter LJ, Lewis AC, Hopkins JR, Read KA, Longley ID, Gallagher MW. (2004). Uptake of methanol to the North Atlantic Ocean surface. *Global Biogeochem Cycles* **18**: GB4027.
- Chen L, Doi M, Durley RCE, Chistoserdov AY, Lidstrom ME, Davidson VL *et al.* (1998). Refined crystal structure of methylamine dehydrogenase from *Paracoccus denitrificans* at 1.75 Å resolution. *J Mol Biol* **276**: 131.
- Chistoserdov AY, Chistoserdova LV, McIntire WS, Lidstrom ME. (1994). Genetic organization of the *mau* gene cluster in *Methylobacterium extorquens* AM1: complete nucleotide sequence and generation and characteristics of *mau* mutants. *J Bacteriol* **176**: 4052–4065.
- Cole JR, Chai B, Marsh TL, Farris RJ, Wang Q, Kulam SA *et al.* (2003). The ribosomal database project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* **31**: 442–443.
- Cox MJ. (2005). *Marine Methyl Bromide-Utilising Bacteria*. Department of Biological Sciences: University of Warwick.
- De Zwart J, Nelisse P, Kuenen J. (1996). Isolation and characterization of *Methylophaga sulfidovorans* sp. nov.: an obligately methylotrophic, aerobic, dimethylsulfide oxidizing bacterium from a microbial mat. *FEMS Microbiol Ecol* **20**: 261–270.

- Doronina N, Darmaeva T, Trotsenko Y. (2003a). *Methylophaga natronica* sp. nov., a new alkaliphilic and moderately halophilic, restricted-facultatively methylotrophic bacterium from Soda Lake of the southern Transbaikal region. *Syst Appl Microbiol* **26**: 382–389.
- Doronina NV, Darmaeva TD, Trotsenko YA. (2003b). *Methylophaga alcalica* sp. nov., a novel alkaliphilic and moderately halophilic, obligately methylotrophic bacterium from an East Mongolian saline soda lake. *Int J Syst Evol Microbiol* **53**: 223–229.
- Doronina NV, Krauzova VI, Trotsenko YA. (1997). *Methylophaga limanica* sp. nov., a new species of moderately halophilic, aerobic, methylotrophic bacteria. *Mikrobiologiya* **66**: 434–439.
- Doronina NV, Lee TD, Ivanova EG, Trotsenko YA. (2005). *Methylophaga murata* sp. nov.: a haloalkaliphilic aerobic methylotroph from deteriorating marble. *Microbiology* **74**: 440–447.
- Dumont MG, Neufeld JD, Murrell JC. (2006). Isotopes as tools for microbial ecologists. *Curr Opin Biotechnol* **17**: 57–58.
- Felsenstein J. (1989). PHYLIP—phylogeny inference package (version 3.2). *Cladistics* **5**: 164–166.
- Fitz-Gibbon ST, House CH. (1999). Whole genome-based phylogenetic analysis of free-living microorganisms. *Nucleic Acids Res* **27**: 4218–4222.
- Galbally IE, Kirstine W. (2002). The production of methanol by flowering plants and the global cycle of methanol. *J Atmos Chem* **43**: 195–229.
- Gibb S, Hatton A. (2004). The occurrence and distribution of trimethylamine-*N*-oxide in Antarctic coastal waters. *Mar Chem* **91**: 65–75.
- Goodwin KD, Varner RK, Crill PM, Oremland RS. (2001). Consumption of tropospheric levels of methyl bromide by C_1 compound-utilizing bacteria and comparison to saturation kinetics. *Appl Environ Microbiol* **67**: 5437–5443.
- Heikes BG, Chang W, Pilson MEQ, Swift E, Singh HB, Guenther A *et al.* (2002). Atmospheric methanol budget and ocean implication. *Global Biogeochem Cycles* **16**: 80–81.
- Holmes AJ, Owens NJP, Murrell JC. (1996). Molecular analysis of enrichment cultures of marine methane oxidising bacteria. *J Exp Mar Biol Ecol* **203**: 27–38.
- Jacob DJ, Field BD, Li Q, Blake DR, Gouw Jd, Warneke C *et al.* (2005). Global budget of methanol: constraints from atmospheric observations. *J Geophys Res* **110**: D08303.
- Janvier M, Frehel C, Grimont F, Gasser F. (1985). *Methylophaga marina* gen. nov., sp. nov. and *Methylophaga thalassica* sp. nov., marine methylotrophs. *Int J Syst Bacteriol* **35**: 131–139.
- Janvier M, Grimont PAD. (1995). The genus *Methylophaga*, a new line of descent within phylogenetic branch γ of *Proteobacteria*. *Res Microbiol* **146**: 543–550.
- Jukes TH, Cantor CR. (1969). Evolution of protein molecules. In: Munro HN (ed). *Mammalian Protein Metabolism*. Academic Press: New York, pp 21–132.
- Kalyuzhnaya MG, Nercessian O, Lapidus A, Chistoserdova L. (2005). Fishing for biodiversity: novel methanopterin-linked C_1 transfer genes deduced from the Sargasso Sea metagenome. *Environ Microbiol* **7**: 1909–1916.
- Kimura M. (1980). A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. *J Mol Evol* **16**: 111–120.
- Lane DJ. (1991). 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds). *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley & Sons Inc.: Chichester, UK, pp 115–175.
- Leckie SE, Prescott CE, Grayston SJ, Neufeld JD, Mohn WW. (2004). Characterization of humus microbial communities in adjacent forest types that differ in nitrogen availability. *Microb Ecol* **48**: 29–40.
- Lidstrom ME. (1988). Isolation and characterization of marine methanotrophs. *Antonie Van Leeuwenhoek* **54**: 189–199.
- Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar *et al.* (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363–1371.
- McDonald IR, Murrell JC. (1997). The methanol dehydrogenase structural gene *mxoA* and its use as a functional gene probe for methanotrophs and methylotrophs. *Appl Environ Microbiol* **63**: 3218–3224.
- Monod A, Chebbi A, Durand-Jolibois R, Carlier P. (2000). Oxidation of methanol by hydroxyl radicals in aqueous solution under simulated cloud droplet conditions. *Atmos Environ* **34**: 5283–5294.
- Murray AE, Preston CM, Massana R, Taylor LT, Blakis A, Wu K *et al.* (1998). Seasonal and spatial variability of bacterial and archaeal assemblages in the coastal waters near Anvers Island, Antarctica. *Appl Environ Microbiol* **64**: 2585–2595.
- Naqvi SWA, Bange HW, Gibb SW, Goyet C, Hatton AD, Upstill-Goddard RC. (2005). Biogeochemical ocean-atmosphere transfers in the Arabian Sea. *Prog Oceanogr* **65**: 116.
- Nercessian O, Noyes E, Kalyuzhnaya MG, Lidstrom ME, Chistoserdova L. (2005). Bacterial populations active in metabolism of C_1 compounds in the sediment of Lake Washington, a freshwater lake. *Appl Environ Microbiol* **71**: 6885–6899.
- Neufeld JD, Dumont MG, Vohra J, Murrell JC. (2007a). Methodological considerations for the use of stable isotope probing in microbial ecology. *Microb Ecol* **53**: 435–442.
- Neufeld JD, Vohra J, Dumont MG, Lueders T, Manefield M, Friedrich MW *et al.* (2007b). DNA stable-isotope probing. *Nat Protoc* **2**: 860–866.
- Neufeld JD, Wagner M, Murrell JC. (2007c). Who eats what, where and when? Isotope-labelling experiments are coming of age. *ISME J* **1**: 103–110.
- Neufeld JD, Yu Z, Lam W, Mohn WW. (2004). Serial analysis of ribosomal sequence tags (SARST): a high-throughput method for profiling complex microbial communities. *Environ Microbiol* **6**: 131–144.
- Schaefer JK, Goodwin KD, McDonald IR, Murrell JC, Oremland RS. (2002). *Leisingera methylohalidivorans* gen. nov., sp. nov., a marine methylotroph that grows on methyl bromide. *Int J Syst Evol Microbiol* **52**: 851–859.
- Schäfer H. (2007). Isolation of *Methylophaga* spp from marine dimethylsulfide-degrading enrichment cultures and identification of polypeptides induced during growth on dimethylsulfide. *Appl Env Microbiol* **73**: 2580–2591.
- Schäfer H, McDonald IR, Nightingale PD, Murrell JC. (2005). Evidence for the presence of a CmuA methyltransferase pathway in novel marine methyl halide-oxidizing bacteria. *Environ Microbiol* **7**: 839–852.
- Sieburth JN, Johnson PW, Eberhardt MA, Sieracki ME, Lidstrom M, Laux D. (1987). The first methane-oxidizing bacterium from the upper mixing layer of

- the deep ocean: *Methylomonas pelagica* sp. nov. *Curr Microbiol* **14**: 285–293.
- Singh HB, Tabazadeh A, Evans MJ, Field BD, Jacob DJ, Sachse G *et al.* (2003). Oxygenated volatile organic chemicals in the oceans: inferences and implications based on atmospheric observations and air–sea exchange models. *Geophys Res Lett* **30**: 1862.
- Somerville CC, Knight IT, Straube WL, Colwell RR. (1989). Simple, rapid method for direct isolation of nucleic acids from aquatic environments. *Appl Environ Microbiol* **55**: 548–554.
- Strand SE, Lidstrom ME. (1984). Characterization of a marine methylotroph. *FEMS Microbiol Lett* **21**: 247–251.
- Van de Peer Y, De Wachter R. (1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Appl Biosci* **10**: 569–570.
- Van Neste A, Duce RA, Lee C. (1987). Methylamines in the marine atmosphere. *Geophys Res Lett* **14**: 711–714.
- Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA *et al.* (2004). Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304**: 66–74.
- Vila-Costa M, del Valle DA, Gonzalez JM, Slezak D, Kiene RP, Sanchez O *et al.* (2006). Phylogenetic identification and metabolism of marine dimethylsulfide-consuming bacteria. *Environ Microbiol* **8**: 2189–2200.
- Yoon J-H, Kim I-G, Shin D-Y, Kang KH, Park Y-H. (2003). *Microbulbifer salipaludis* sp. nov., a moderate halophile isolated from a Korean salt marsh. *Int J Syst Evol Microbiol* **53**: 53–57.