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ORIGINAL ARTICLE

Bacterial metabolism of methylated amines and identification of novel methylotrophs in Movile Cave

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Movile Cave, Romania, is an unusual underground ecosystem that has been sealed off from the outside world for several million years and is sustained by non-phototrophic carbon fixation. Methane and sulfur-oxidising bacteria are the main primary producers, supporting a complex food web that includes bacteria, fungi and cave-adapted invertebrates. A range of methylotrophic bacteria in Movile Cave grow on one-carbon compounds including methylated amines, which are produced via decomposition of organic-rich microbial mats. The role of methylated amines as a carbon and nitrogen source for bacteria in Movile Cave was investigated using a combination of cultivation studies and DNA stable isotope probing (DNA-SIP) using ^{13}C-monomethylamine (MMA). Two newly developed primer sets targeting the gene for gamma-glutamylmethylamide synthetase (*gmaS*), the first enzyme of the recently-discovered indirect MMA-oxidation pathway, were applied in functional gene probing. SIP experiments revealed that the obligate methylotroph *Methylotenera mobilis* is one of the dominant MMA utilisers in the cave. DNA-SIP experiments also showed that a new facultative methylotroph isolated in this study, *Catellibacterium* sp. LW-1 is probably one of the most active MMA utilisers in Movile Cave. Methylated amines were also used as a nitrogen source by a wide range of non-methylotrophic bacteria in Movile Cave. PCR-based screening of bacterial isolates suggested that the indirect MMA-oxidation pathway involving GMA and *N*-methylglutamate is widespread among both methylotrophic and non-methylotrophic MMA utilisers from the cave.
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Introduction

Most ecosystems rely on phototrophic carbon fixation, or, in the absence of light, an external supply of phototrophically-fixed carbon into the ecosystem. Exceptions are deep sea hydrothermal vents, where carbon is derived from chemosynthesis using energy sources other than light (reviewed by Lutz and Kennish, 1993; Van Dover *et al.*, 2002; Campbell, 2006). Movile Cave, located near the coast of the Black Sea in Mangalia, Romania, is an underground cave system that has been completely sealed off from the outside world for several million years (Sarbu *et al.*, 1996). Unlike other cave systems, where dissolved and particulate organic carbon enters the cave with meteoric waters from above, the food web in Movile Cave is sustained exclusively by non-phototrophic carbon fixation. Since its discovery in 1986, Movile Cave has provided an excellent natural ecosystem to study a highly unusual, light-independent, microbially-driven food web (Sarbu *et al.*, 1994; Sarbu and Kane, 1995; Sarbu *et al.*, 1996; Vlasceanu *et al.*, 1997; Rohwerder *et al.*, 2003; Hutchens *et al.*, 2004; Porter *et al.*, 2009; Chen *et al.*, 2009). Movile Cave harbours rich and diverse populations of cave-adapted invertebrates, all of which are sustained by chemolithoautotrophic microorganisms that thrive along the redox interface created between the oxygenated atmosphere and the high concentrations of reduced compounds such as hydrogen sulfide (H_2S) and methane (CH_4) present in the water (Sarbu and Kane, 1995). Microbial mats composed of bacteria, fungi and protists float on the water surface (kept afloat by CH_4 bubbles) and also grow on the limestone walls of the cave (Sarbu *et al.*, 1994).

Methylotrophs are organisms capable of using one-carbon (C1) compounds, that is, compounds lacking carbon–carbon bonds, as their sole source of carbon and energy (Anthony, 1982; Lidstrom, 2006; Chistoserdova *et al.*, 2009). In addition to CH_4, C1 compounds such as methanol and methylated amines are important carbon and energy sources for a range of methylotrophic bacteria in Movile Cave (Hutchens *et al.*, 2004; Chen *et al.*, 2009). Methylated amines are typically associated with

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saline environments (Gibb *et al.*, 1999; Fitzsimons *et al.*, 2006) where they are formed by the degradation of glycine betaine and trimethylamine *N*-oxide, osmolytes commonly found in marine organisms (Barrett and Kwan, 1985; Lin and Timasheff, 1994). There are fewer studies on the distribution of methylated amines in terrestrial and freshwater environments, although the dissolved organic nitrogen fraction as a whole is increasingly being recognised as an important source of microbial nitrogen nutrition (Berman and Bronk, 2003, Worsfold *et al.*, 2008). Generally, environments with high concentrations of organic matter have a high potential for dissolved organic nitrogen generation (Neff *et al.*, 2003). We hypothesise that in Movile Cave, degradation of the extensive, organic-rich microbial mats produces large amounts of methylated amines, which are used as growth substrates by certain microorganisms that are the subject of this study.

Methylotrophs that use methylated amines as a carbon source are phylogenetically diverse, ubiquitous in the environment and often metabolically versatile (for example, Bellion and Hersh, 1972; Colby and Zatman, 1973; Levering *et al.*, 1981; Anthony, 1982; Bellion and Bolbot, 1983; Brooke and Attwood, 1984; Kalyuzhnaya *et al.*, 2006b; Boden *et al.*, 2008). New methylotrophs are still being identified from a wide range of environments, including genera not previously associated with methylotrophy, and novel metabolic pathways (see recent reviews by Chistoserdova *et al.*, 2009; Chistoserdova, 2011).

Methylated amines are also a nitrogen source for a wide range of non-methylotrophic bacteria. While utilisation of monomethylamine (MMA) as a bacterial nitrogen source was reported over 40 years ago (Budd and Spencer, 1968; Bicknell and Owens, 1980; Anthony, 1982; Murrell and Lidstrom, 1983; Glenn and Dilworth, 1984), details of the metabolic pathways involved have only recently been identified (Chen *et al.,* 2010b).

The key intermediates in methylotrophic metabolism are formaldehyde or formate, respectively, as they represent the branching point at which carbon is either oxidised further to CO_2, or assimilated into cell carbon. Carbon is assimilated from formaldehyde via the ribulose monophosphate cycle, or from formate via the serine cycle (Anthony, 1982; Chistoserdova *et al.*, 2009; Chistoserdova, 2011). In the metabolism of methylated amines, there are two possible pathways for the oxidation of MMA (Supplementary Figures S1a and b): In the well-characterised, direct MMA-oxidation pathway, a single enzyme oxidises MMA to formaldehyde, releasing ammonium. In methylotrophic Gram-positive bacteria the enzyme responsible is MMA oxidase, while in Gram-negative methylotrophs it is MMA dehydrogenase (Anthony, 1982). PCR primers are available for *mauA* (Neufeld *et al.*, 2007a), the gene coding for the small subunit of MMA dehydrogenase. However, these primers do not detect all MMA-utilising bacteria. An alternative, indirect pathway oxidises MMA not to formaldehyde but to 5,10-methylenetetrahydrofolate ($CH_2=THF$) in a stepwise conversion via the methylated amino acids gamma-glutamylmethylamide (GMA) and/or *N*-methylglutamate (NMG) (Latypova *et al.*, 2010; Chistoserdova, 2011). Although this pathway has been known since the 1960s (Kung and Wagner, 1969), the enzymes and genes involved have only recently been characterised (Latypova *et al.*, 2010; Chen *et al.*, 2010a): MMA is converted to GMA by GMA synthetase (*gmaS*), GMA is then converted to NMG by NMG synthase (*mgsABC*), and finally to $CH_2=THF$ by NMG dehydrogenase (*mgdABCD*). A variation of this pathway is found in *Methyloversatilis universalis* FAM5, where *gmaS* is not essential for oxidation of MMA to $CH_2=THF$ via NMG (Latypova *et al.*, 2010). Importantly, the GMA-/NMG-mediated pathway is also found in bacteria that use MMA only as a nitrogen (but not carbon) source (Chen *et al.*, 2010b; Chen 2012). In a recent study (Chen, 2012), PCR primers targeting *gmaS* from marine *Roseobacter* clade (MRC) bacteria were developed for the detection of MMA utilisers in marine environments, highlighting the potential of the *gmaS* gene as a biomarker for MMA utilisation.

The objectives of this study were to determine the role of methylated amines as carbon and nitrogen sources for microorganisms in Movile Cave, and to identify active MMA utilisers in this unique ecosystem using DNA stable isotope probing (DNA-SIP) (Radajewski *et al.*, 2000; Murrell and Whiteley, 2011). DNA-SIP has been successfully applied in the study of methanotrophic and autotrophic communities in Movile Cave (Hutchens *et al.*, 2004; Chen *et al.*, 2009). Time-course SIP experiments with ^{13}C-labelled MMA were set up in order to monitor changes in the methylotrophic community. Cultivation-based studies were also used to isolate and characterise methylated amine-utilising bacteria from the cave. The distribution of genes for the GMA-dependent MMA-oxidation pathway in Movile Cave microbes was examined using new PCR primer sets developed to target *gmaS* from non-marine bacteria.

Material and methods

Study site and sampling

Movile Cave near Mangalia on the coast of the Black Sea is located in an area rich in hydrothermal activity with numerous sulfurous springs and lakes, as well as creeks bubbling with methane. The cave consists of a network of passages, including a dry, upper level and a lower level, which is partly flooded by thermal sulfidic waters (for a detailed cross-section of the cave see Supplementary Figure S2). A small lake room (ca 3 m in diameter) is located between the dry and the flooded sections of the cave, and two air bells are located in the submerged region. The temperature in the cave is a constant 21 °C (Sarbu and Kane, 1995). The atmosphere in the air bells shows O_2 depletion (7–10% v/v) and is rich in CO_2 (2.5% v/v) and CH_4 (1–2% v/v) (Sarbu and Kane, 1995). The water contains H_2S (0.2–0.3 mM), NH_4^+ (0.2–0.3 mM) and CH_4 (0.02 mM) and is buffered by high amounts of bicarbonate from the limestone walls at ~pH 7.4 (Sarbu, 2000). Dissolved O_2 decreases to less than 1 μM after the first few centimetres from the water surface, with the deeper water being essentially anoxic (Sarbu, 2000). Methylamine concentrations in the cave water were measured by our recently developed ion chromatography method with a detection limit of ~1 μM for MMA (Lidbury *et al.*, 2014). Preliminary measurements carried out using this assay suggested that the *in situ* concentration of MMA in Movile Cave water is below the detection limit of 1 μM, which could indicate rapid turnover of MMA by bacteria in the cave.

Water and floating mat samples for enrichment and isolation experiments were collected from the lake room and the two air bells in October 2009, stored at 4 °C in the nearby field station and processed within 48 h. Biofilm covering the limestone walls of both air bells was scraped off into sterile tubes. Similar samples for further isolation experiments, SIP enrichments and nucleic acid extractions were obtained from Movile Cave in April 2010. Material for DNA work was concentrated by centrifugation within 1 h of sampling and frozen at −20 °C for storage until processing.

DNA-SIP with ^{13}C-MMA

SIP incubations were set up at the field station in Mangalia, within 1 h of sampling, using water from Airbell 2. For each incubation, a 20 ml aliquot of cave water was added to a pre-sterilised 120 ml serum vial containing 50 μmol of labelled (^{13}C) or unlabelled MMA–HCl (dissolved in 0.2 ml sterilised distilled water). Control incubations with no added MMA (referred to as ‘no-substrate controls’ from here on) were also set up. All serum vials were immediately sealed with a butyl rubber cap and an aluminium crimping lid and incubated at 21 °C in the dark. Samples for t_0 ($t = 0$ days) were prepared by centrifugation of 20 ml of cave water, discarding the supernatant and freezing the pellet at −20 °C. SIP incubations and no-substrate controls were harvested in the same way at time intervals of 48 h (t_1), 96 h (t_2) and 4 weeks (t_3). In future SIP experiments, the recently developed ion chromatography method for measuring MMA (see above, Lidbury *et al.*, 2014) could be used to monitor consumption of substrate over time. From each sample, up to 1 μg of total extracted DNA was added to caesium chloride (CsCl) solutions for isopycnic ultracentrifugation and gradient fractionation following published protocols (Neufeld *et al.*, 2007b).

Enrichment and isolation of methylated amine-utilising bacteria from Movile Cave

Methylotrophic bacteria capable of using methylated amines as a carbon and nitrogen source were selectively enriched using MMA, dimethylamine (DMA) and trimethylamine (TMA). Separate enrichments were set up for each of the three substrates by adding a final concentration of 1 mM substrate to 20 ml cave water in sterile 120 ml serum vials. For mats and biofilms, 2 g sample material was placed into 27 ml serum vials and made up to a final volume of 4 ml with nitrogen-free dilute basal salts (DBS) medium. DBS medium (modified after Kelly and Wood, 1998) contained (per litre): 0.1 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.11 g K_2HPO_4, 0.085 g KH_2PO_4, adjusted to pH 7. The medium was supplemented with a vitamins solution as described by Kanagawa *et al.* (1982) and 1 ml of a trace element solution (modified after Kelly and Wood, 1998) containing (per 1 L): 50 g EDTA, 11 g NaOH, 5 g $ZnSO_4 \cdot 7H_2O$, 7.34 g $CaCl_2 \cdot 2H_2O$, 2.5 g $MnCl_2 \cdot 6H_2O$, 0.5 g $CoCl_2 \cdot 6H_2O$, 0.5 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 5 g $FeSO_4 \cdot 7H_2O$, 0.2 g $CuSO_4 \cdot 5H_2O$, adjusted to pH 6.0. After flushing the headspace of each vial with N_2, the headspace was made up to a final concentration of 7% (v/v) O_2 and 3.5% (v/v) CO_2 to resemble the cave atmosphere. Enrichments were incubated at 21 °C in the dark. After 4 weeks, 10 ml (for water samples) or 4 ml (for mat samples) of fresh DBS medium were added and cultures were spiked with 20 mM MMA, 10 mM DMA or 10 mM TMA. After amending the headspace as previously, enrichment cultures were incubated at 21 °C in the dark. When enrichments became turbid (after a further 2 weeks), dilutions were spread onto agar plates (DBS medium, 1.5% agar) containing 5 mM MMA, DMA, or TMA as the only added carbon and nitrogen source. Plates were incubated at 21 °C in the dark until colonies became visible (2–10 days). In order to achieve isolation of a variety of methylotrophs, individual colonies were examined by microscopy and a selection of morphotypes was transferred onto fresh plates containing the same substrates as before. Cells were observed at ×1000 magnification in phase-contrast under a Zeiss Axioskop 50 microscope (Carl Zeiss Ltd, Cambridge, UK). Isolates were submitted to a series of transfers on plates and microscopy was used routinely to check purity before transferring individual isolates into liquid media (containing 5 mM MMA, DMA or TMA). Once grown in liquid (2–7 days), isolates were transferred back onto methylated amine plates.

In a separate enrichment approach, non-methylotrophic bacteria capable of using methylated amines as a nitrogen (but not carbon) source were isolated. These enrichments were set up in the same manner and using the same sample material as described above for the methylotrophs. In addition to 1 mM of MMA, DMA or TMA, a mixture of alternative carbon compounds (comprising glucose, fructose, succinate, glycerol, pyruvate and acetate) was added to a final concentration of 5 mM. Isolates obtained in this way were additionally tested for growth in liquid medium containing no alternative carbon source to detect any co-enriched methylotrophs, as well as in liquid medium containing carbon sources but no methylated amines to eliminate the possibility that they might be fixing N_2 rather than using methylated amines as nitrogen source.

DNA extraction and PCR amplification of bacterial 16S rRNA genes

DNA from cave samples, SIP enrichments and bacterial isolates was extracted as previously described (Neufeld *et al.*, 2007a). DNA from soil and lake sediment samples retrieved from the University of East Anglia campus (used for *gmaS* primer validation, see later) was extracted using the FastDNA SPIN Kit for soil by MP Biomedicals LLC (Santa Ana, CA, USA). Bacterial 16S ribosomal RNA (rRNA) genes from SIP enrichments were amplified using primer set 341f-GC/907r (Muyzer *et al.,* 1993; Lane, 1991) for analysis by denaturing gradient gel electrophoresis (DGGE). For cloning and sequencing, bacterial 16S rRNA genes from isolates were amplified with primer set 27f/1492r (DeLong, 1992; Lane *et al.*, 1985).

Denaturing gradient gel electrophoresis (DGGE)

DGGE analysis of bacterial 16S rRNA gene fragments was carried out as described by Neufeld *et al.* (2007a) using the DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). After electrophoresis for 16 h at 60 °C and 80 V, gels were stained using SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Paisley, UK) and viewed under the Bio-Rad Gel Doc XR gel documentation system using Amber Filter 5206 (Bio-Rad). For gene sequence analysis, well-defined DNA bands were physically excised from the gel for re-amplification using the same PCR conditions and primers described above, followed by sequencing analysis using primer 341f (Muyzer *et al.*, 1993).

DGGE, when compared with amplicon pyrosequencing, is a relatively low resolution technique. However, the DGGE technique enabled us to accurately compare SIP enrichments across different CsCl gradient fractions (heavy to light) and also to compare ^{13}C-incubations to ^{12}C-incubated controls. This first study on MMA degraders in Movile Cave thereby allowed us to identify key players in the microbial food web. Building on data obtained in this study, more detailed studies involving pyrosequencing of amplicons can be carried out in the future.

Functional gene PCR and development of gmaS primers

mauA genes were amplified using PCR primer set *mauA*f1/*mauA*r1 (Neufeld *et al.,* 2007a). Currently there is one *gmaS* PCR primer set available (Chen, 2012) which targets specifically the MRC. This PCR primer set therefore may not detect *gmaS* from non-marine bacteria. Three new *gmaS* PCR primers were designed in this study, based on multiple alignment of 34 *gmaS* sequences derived from (i) methylotrophic isolates confirmed to use the NMG-/GMA-mediated pathway and (ii) bacterial genomes published on the Integrated Microbial Genomes (IMG) platform (Markowitz *et al.*, 2010) of the Joint Genome Institute (JGI) Genome Portal (http://genome.jgi.doe.gov). Genomes were screened for *gmaS*-related sequences using *gmaS* from *Methylocella silvestris* as a query sequence (Chen *et al.*, 2010a). Corresponding full length sequences included both *gmaS* and glutamine synthetase type III (*glnA*) sequences, due to the high level of sequence similarity between the two genes. In order to identify genuine *gmaS* sequences, the gene neighbourhood of all obtained sequences was manually inspected for predicted neighbouring open reading frames typically found adjacent to *gmaS* (genes encoding NMG dehydrogenase and NMG synthase). Confirmed *gmaS* sequences included many sequences apparently mis-annotated as *glnA*. For primer design, multiple sequence alignments of chosen sequences were established with the Clustal X program (Thompson *et al.*, 1997) and viewed using the GeneDoc software (Nicholas *et al.*, 1997). Because of their sequence similarity to *gmaS*, a number of *glnA* sequences were included in the alignment in order to identify suitable primer-binding regions specific only to *gmaS* (for a complete list of all *gmaS* and *glnA* sequences used for primer design, see Supplementary Table S1). The resulting forward primer *gmaS*_557 f (5′-GARGAYGCSAACGGYCAGTT-3′) was used in all cases, with the reverse primers α_*gmaS*_970r (3′-TGGGTSCGRTTRTTGCCSG-5′) and β_γ_*gmaS*_1332r (3′-GTAMTCSAYCCAYTCCATG-5′) being used to target the *gmaS* gene of non-marine *Alphaproteobacteria* and that of *Beta*- and *Gammaproteobacteria*, respectively. Touchdown PCR protocols for *gmaS* amplification were used as follows: for *gmaS*_557f/α_*gmaS*_970r, an initial step at 94 °C for 5 min was followed by 10 cycles of denaturation at 94 °C for 45 s, annealing at variable temperatures for 45 s and extension at 72 °C for 1 min. In the first cycle, the annealing temperature was set to 60 °C, and for each of the nine subsequent cycles the annealing temperature was decreased by 1 °C. This was followed by 30 cycles of 45 s at 94 °C, 45 s at 56 °C and 1 min at 72 °C, and a final extension time of 8 min at 72 °C. For *gmaS*_557 f/β_γ_*gmaS*_1332r, a modification of the first touchdown protocol was used; the annealing temperature was set to 55 °C in the first cycle and decreased by 1 °C for each of the nine subsequent cycles. The first 10 cycles were followed by 35 cycles with an annealing temperature of 52 °C.

The primer sets were tested for their specificity by (i) amplification and sequencing of *gmaS* sequences from genomic DNA of the following bacterial strains known to use the indirect MMA-oxidation pathway: *Sinorhizobium meliloti* 1021, *Mesorhizobium loti* MAFF303099, *Rhizobium leguminosarum* bv. viciae 3841, *Agrobacterium tumefaciens* C58 and *Pseudomonas fluorescens* SBW25 (Chen *et al.*, 2010b). For further validation of the primers, *gmaS* was amplified from DNA extracted from (ii) MMA enrichments from Movile Cave, (iii) fresh Movile Cave mat and (iv) soil and freshwater sediment from a small lake (the 'Broad') on the University of East Anglia campus. *gmaS*-based clone libraries were constructed for (ii)–(iv) and a total of 30 clones were randomly selected for sequencing.

DNA sequencing and phylogenetic analysis

DNA sequencing employed the Sanger method on a 3730A automated sequencing system (PE Applied Biosystems, Warrington, UK). To determine approximate phylogenetic affiliations, partial 16S rRNA gene sequences were analysed with the Basic Local Alignment Search Tool (BLAST) on the NCBI GenBank database (Altschul *et al.*, 1990). Amino acid and nucleotide-based phylogenetic trees were established using the MEGA5 program (Tamura *et al.*, 2011). The evolutionary history was inferred by neighbour-joining (Saitou and Nei, 1987). For nucleotide-based trees (Supplementary Figures 1a and b), the evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2004). For amino-acid-based trees, the evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965). All positions containing gaps and missing data were eliminated. Bootstrap analysis (1000 replicates) was performed to provide confidence estimates for phylogenetic tree topologies (Felsenstein, 1985). Phylogenetic analysis of *gmaS* genes was carried out at the amino-acid level (135–250 amino-acyl residues).

Nucleotide sequence accession numbers

Nucleotide gene sequences obtained from this study were deposited in the GenBank nucleotide sequence database under the accession numbers KM083620–KM083705.

Results

Active methylotrophic bacteria identified by DNA-SIP with ^{13}C-MMA

DNA-SIP enrichments with ^{13}C-labelled MMA were set up from Movile Cave water in order to identify active, methylotrophic bacteria capable of using MMA as a carbon source. DNA was extracted from microcosms enriched with ^{13}C-labelled and unlabelled MMA after 48 h (t_1), 96 h (t_2) and 4 weeks (t_3). The bacterial communities in the microcosms were investigated by DGGE analysis of bacterial 16S rRNA gene fragments. Comparison of DGGE profiles from unfractionated DNA from the different time points revealed a significant shift in the bacterial community over time, which was similar between ^{12}C-MMA and ^{13}C-MMA incubations (Figure 1).

For identification of active methylotrophs, DNA extracted from all time points was subjected to density gradient centrifugation and fractionation, allowing separation of ^{13}C-labelled DNA (contained in heavy fractions) from unlabelled ^{12}C-DNA (contained in light fractions). Bacterial 16S rRNA gene fragments were amplified from all DNA fractions and analysed by DGGE and sequencing. Time point t_1 (48 h) did not show any significant enrichment in ^{13}C-DNA and was therefore not further analysed. DGGE analysis of heavy and light DNA fractions from time points t_2 and t_3 (^{13}C-MMA incubation) revealed major differences in the community profiles of the heavy fractions (Figures 2a and c): A single band dominated the heavy fractions at t_2 (96 h, Figure 2a) but was absent at t_3 (4 weeks, Figure 2c). Sequence analysis of the excised band revealed that the sequence affiliated with *Methylotenera mobilis* (99% identity), an obligate methylotroph (Kalyuzhnaya *et al.*, 2006a) known to be abundant in Movile Cave from previous studies (Chen *et al.*, 2009). At t_3, several different phylotypes appeared in the heavy fractions of the ^{13}C-MMA incubation (Figure 2c), that is, a more diverse bacterial community had incorporated the label following extended incubation with MMA.

Bacterial 16S rRNA gene sequences from these DGGE bands affiliated with well-characterised methylotrophs such as *Methylobacterium extorquens* (100% identity) and *Methylovorus* (97% identity to

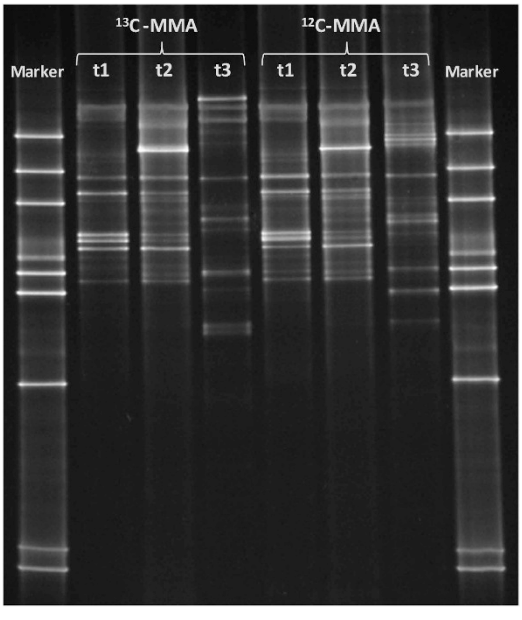

Figure 1 Denaturing gradient gel electrophoresis analysis of bacterial 16S rRNA gene fragments in native (unfractionated) DNA from incubations of Movile Cave water with ^{13}C-MMA (left) and unlabelled MMA (right), after 48 h (*t*1), 96 h (*t*2) and 4 weeks (*t*3).

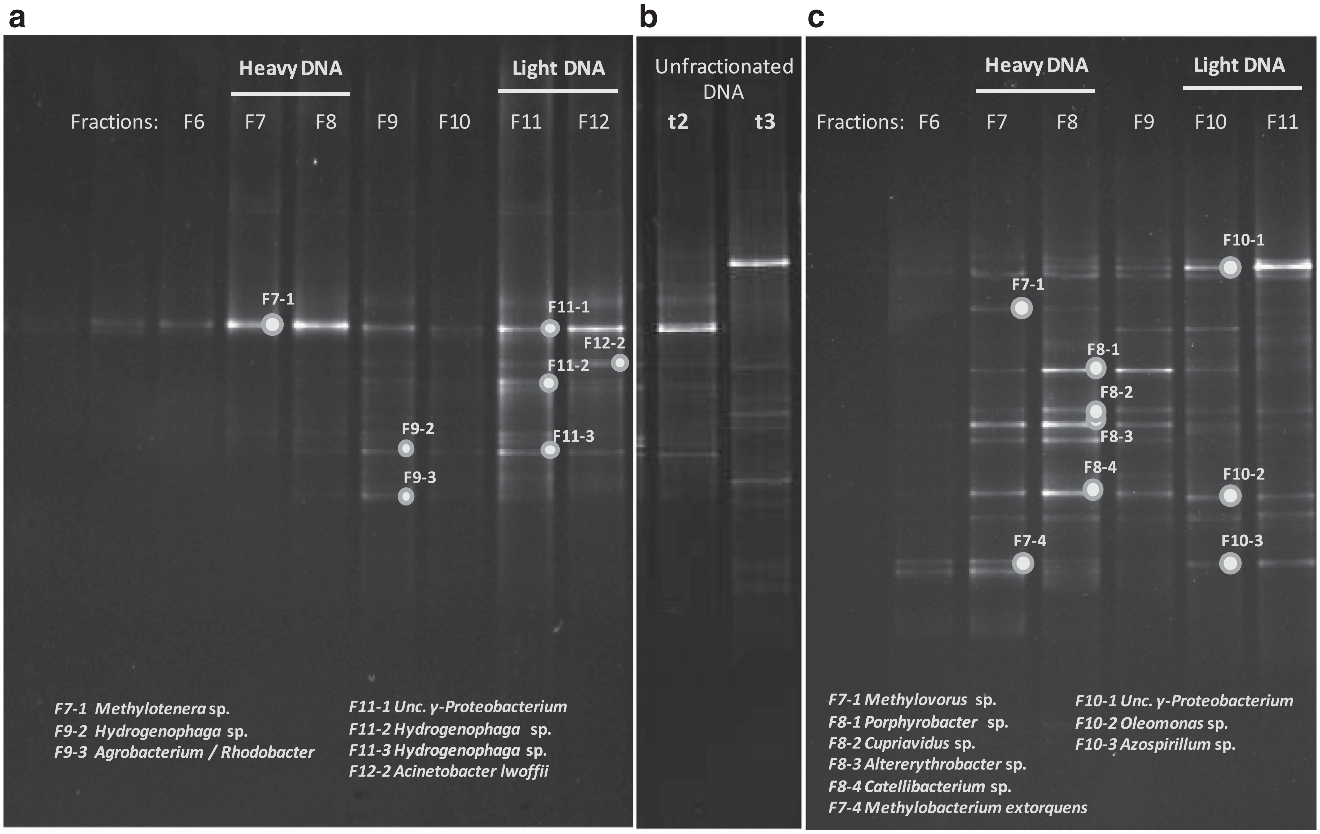

Figure 2 Denaturing gradient gel electrophoresis analysis of bacterial 16S rRNA gene fragments in light and heavy DNA fractions from ^{13}C-MMA incubations of Movile Cave water after 96 h (**a**) and 4 weeks (**c**) DGGE profiles of unfractionated DNA of both time points (**b**) are shown for reference.

Methylovorus menthalis), but also included *Catellibacterium* (98% identity to *Catellibacterium caeni*), *Cupriavidus* (99% identity to *Cupriavidus necator*), *Porphyrobacter* (99% identity to *Porphyrobacter neustonensis*) and *Altererythrobacter* (99% identity to *Altererythrobacter epoxidivorans*), none of which have previously been reported to grow methylotrophically. The *Catellibacterium* sequence identified from DGGE shared 98–100% sequence identity with a novel organism subsequently isolated from Movile Cave during this study (see below) and cloned 16S rRNA gene sequences from ^{13}C-labelled DNA from t_3 (data not shown, refer to Supplementary Figure S3a).

The non-methylotrophic bacterial community co-enriched in ^{13}C-MMA incubations was investigated by PCR-DGGE of 16S rRNA bacterial genes present in the light fractions (^{12}C-DNA). Light fractions harboured a diversity of mostly heterotrophic bacterial sequences (Figure 2a and b), namely *Rhodobacter, Acinetobacter, Azospirillum, Oleomonas* and *Hydrogenophaga* and a number of sequences not closely related to cultivated representatives (as little as 84–87% identity).

All bacterial sequences obtained from DGGE bands in lanes loaded with heavy DNA (that is, ^{13}C-labelled organisms) were exclusive to MMA enrichments, and not seen in no-substrate controls (data not shown). Two sequences detected in the light fractions from MMA incubations (*Acinetobacter* and *Azospirillum*) also appeared to be absent in the no-substrate controls, suggesting that these bacteria may have been selectively enriched due to their capability of using MMA as a nitrogen source (but not as a carbon source, and so their DNA was not labelled). One of these sequences (*Acinetobacter lwoffi*) did indeed correspond to a bacterium isolated from Movile Cave in this study with MMA as the only nitrogen source (see below).

Methylotrophic and non-methylotrophic isolates from Movile Cave

To complement data from ^{13}C-MMA-SIP experiments, methylated amine-utilising bacteria were isolated from different locations (lake room, Airbell 1 and Airbell 2) in Movile Cave. Methylotrophs were isolated with DBS medium containing MMA, DMA or TMA as sole added source of carbon, energy and nitrogen. A selection of isolates differing in colony and cell morphology was transferred into liquid DBS medium containing the respective methylated amine (to distinguish true methylotrophs from organisms growing on agar). Seven methylotrophic strains were isolated, identified based on 16S rRNA gene sequencing analysis (Table 1, Supplementary Figure S3a). The highest diversity of methylotrophs was obtained on MMA enrichments (based on morphology and 16S rRNA gene sequencing data),

Table. 1 Growth of bacterial isolates from Movile Cave on methylated amines with and without added carbon

Isolates	*Phylogeny*	*Identity*	*Growth on methylated amines*					
		(%)	*MMA +C*	*DMA +C*	*TMA +C*	*MMA*	*DMA*	*TMA*
Alphaproteobacteria								
2W-7	*Methylobacterium extorquens*	100	+	+	+	+	+	+
LW-13	*Xanthobacter tagetidis*	100	+	+	+	+	+	+
A2-1D	*Paracoccus yeei*	100	+	+	+	+	+	+
2W-61	*Paracoccus yeei*	98	+	+	+	+	+	+
2W-12	*Aminobacter niigataensis*	100	+	+	+	+	+	+
LW-1	*Catellibacterium caeni*	99	+	−	+	+	+	+
1M-11	*Mesorhizobium loti*	99	+	+	+	+	+	+
A2-41x	*Shinella yambaruensis*	98	+	−	−	−	NA	NA
1W-5	*Rhodobacter blasticus*	96	+	+	+	−	−	−
O1	*Oleomonas sagaranensis*	98	+	+	−	−	−	−
O3	*Oleomonas sagaranensis*	99	+	−	−	−	−	−
Gammaproteobacteria								
1W-58	*Acinetobacter johnsonii*	100	+	+	+	−	−	−
2W-62	*Acinetobacter lwoffii*	100	+	+	−	−	−	−
1W-57Y	*Pseudomonas oleovorans*	99	+	−	−	−	−	−
Betaproteobacteria								
A2-14M	*Zoogloea caeni*	100	+	+	+	−	−	−

Abbreviations: C, carbon mixture; DMA, dimethylamine; MMA, monomethylamine; NA, not analysed; TMA, trimethylamine.
Carbon mixture consists of sucrose, glucose, fructose, glycerol, pyruvate and acetate. Carbon sources were supplied at 5 mM, nitrogen sources at 1 mM.

while DMA and TMA enrichments were dominated by *Xanthobacter tagetidis* (Padden *et al.*, 1997). Notably, no *Methylotenera* isolates were obtained (even after using a variety of different cultivation media which are commonly used for methylotrophic bacteria, changing incubation conditions such as temperature, pH, ionic strength of media and dilution-to-extinction experiments), despite the active role of this methylotroph in MMA metabolism as determined by DNA-SIP results (see above), and its apparent abundance in Movile Cave (Chen *et al.*, 2009). In addition to well-characterised methylotrophs such as *M. extorquens*, two novel methylotrophs were also isolated. A member of the relatively new genus *Catellibacterium* (Tanaka *et al.*, 2004; Liu *et al.*, 2010, Zheng *et al.*, 2011; Zhang *et al.*, 2012), provisionally named *Catellibacterium* sp. LW-1 was isolated from lake water enrichments with MMA. 16S rRNA gene sequences relating to this organism were also detected in heavy DNA fractions from ^{13}C-MMA enrichments (see above, Figure 2c, Supplementary Figure S1b), indicating that *Catellibacterium* may have a significant role in the cycling of methylated amines in Movile Cave. In addition, a new member of the genus *Mesorhizobium* (a genus not currently known to contain any methylotrophic species), was isolated from an MMA enrichment set up with floating mat from Airbell 1. All methylotrophic isolates were facultative, that is, also able to use sugars or carboxylic acids for growth. Notably, all methylotrophs could use all three methylated amines as sole growth substrates, with the exception of *Catellibacterium* sp. LW-1 which did not grow on DMA (Table 1).

In a separate experiment, heterotrophic bacteria capable of using methylated amines as a nitrogen but not carbon source were enriched and isolated using the same sample material as above. MMA, DMA or TMA were the only added nitrogen sources in these enrichments and a mixture of sugars and carboxylic acids were added as carbon and energy source. A diversity of non-methylotrophic methylated amine-utilising bacteria was obtained—in total eight bacterial species, as determined by 16S rRNA gene sequencing analysis (Table 1, Supplementary Figures S3a and b). All of these isolates used MMA as a nitrogen source, while only some could use DMA and TMA (Table 1), suggesting that many lack the enzymes for de-methylation of secondary and tertiary methylated amines to MMA. None of the isolates grew methylotrophically with MMA, DMA or TMA. While all methylotrophic isolates obtained in this study belonged to the *Alphaproteobacteria*, non-methylotrophic MMA utilisers also included *Beta*- and *Gammaproteobacteria* (Table 1). *A. lwoffi,* isolated from Airbell 2 water with MMA as a nitrogen source, was also detected in ^{12}C-DNA fractions from MMA-SIP incubations (see above), while not seen in control incubations without added MMA. These results suggest that *Acinetobacter* (and other non-methylotrophs) may have an active role in the cycling of methylated amines in Movile Cave.

Development and validation of functional gene primer sets targeting gmaS

The gene *gmaS* codes for GMA synthetase, the enzyme catalysing the first step in the conversion of MMA to

$CH_2{=}THF$ in the recently characterised indirect MMA-oxidation pathway (Latypova *et al.*, 2010; Chen *et al.*, 2010a, 2010b). We selected *gmaS* as a functional biomarker to assess the distribution of this pathway among MMA-utilising bacteria. Since currently available *gmaS* primers are specific to the MRC (Chen, 2012), we designed two new primer sets covering *gmaS* of non-marine bacteria. Suitable primer regions were identified by alignment of *gmaS* sequences obtained from (i) isolates confirmed to use the GMAS-/NMG-mediated pathway and (ii) published bacterial genomes. Due to sequence similarity between the two genes, a number of *glnA* gene sequences were included in the alignment to enable identification of suitable *gmaS* primer-binding regions not found in *glnA*.

Sequence alignment and establishment of nucleotide-based and amino-acid-based phylogenetic trees clearly separated *glnA* from *gmaS* genes and revealed two distinct *gmaS* clusters dividing (i) *Alphaproteobacteria* and (ii) *Beta*- and *Gammaproteobacteria* (Figure 3). The alphaproteobacterial *gmaS* cluster was further split into two major subgroups: ‘Group 1’ contained MRC-associated sequences (in a separate sub-cluster), as well as sequences belonging to soil and freshwater bacteria from the orders *Rhodobacterales* and *Rhizobiales,* while ‘Group 2’ contained only *gmaS* sequences from non-marine bacteria of the orders *Rhodospirillales*, *Rhizobiales* and *Sphingomonadales* (Figure 3). For primer design, sequences associated with the MRC were removed from the alignment as they were too divergent from the other sequences to be targeted by the same primers. A common region shared by all remaining *gmaS* sequences was used to design the forward primer (*gmaS*_557f). Two different reverse primers were designed for *Alphaproteobacteria* (α_*gmaS*_970r) and *Beta*- and *Gammaproteobacteria* (β_γ_*gmaS*_1332r) because no further region of sufficient similarity shared by both groups could be identified (alignments in Supplementary Figures S4a–c).

Specificity of these PCR primer sets was confirmed by amplification and sequencing of *gmaS* from (i) five bacteria known to use the *gmaS*-dependent pathway (as specified in Material and methods) (ii) MMA enrichments from Movile Cave (iii) Movile Cave biofilm and (iv) soil and lake sediment from a different environment (UEA campus; as described in Material and methods). All PCR products obtained were of the expected size, that is, ~410 bp (alphaproteobacterial *gmaS*) and ~770 bp (beta- and gammaproteobacterial *gmaS*). With DNA from MMA enrichments, a slightly larger, second band was obtained in addition to the *gmaS* product when using 557f/1332r. This gene fragment shared high sequence identity with a viral coat protein and could not be eliminated by using more stringent PCR conditions due to extremely high similarity with the target gene in the primer-binding regions. This alternative amplification product was restricted to Movile Cave enrichment DNA and was avoided by gel excision of the *gmaS* band. All sequences obtained from genomic DNA (i) and clone libraries (a total of 30 randomly selected clones from (ii), (iii) and (iv)) were identified as *gmaS* (Figure 3, Supplementary Figure S5), confirming specificity of the primers.

The *gmaS* sequences obtained from Movile Cave DNA affiliated with *gmaS* from both methylotrophic and non-methylotrophic bacteria—namely *Methylobacterium*, *Catellibacterium, Pseudomonas* and *Acinetobacter* (99–100% similarity, Figure 3)—identified by DNA-SIP and isolation work in this study. A further sequence loosely affiliated with *Methylotenera*, *Methylovorus* and *Methylophaga* (89–90% similarity with all three genera). A final *gmaS* sequence was related to *gmaS* from the methylotroph *Hyphomicrobium* (99% similarity) which had not been detected by DNA-SIP or isolation.

Distribution of gmaS and mauA genes in Movile Cave isolates

To assess the distribution of the direct and indirect MMA-oxidation pathways in Movile Cave, bacterial isolates were screened for the presence of *mauA* and *gmaS* genes. While the *mauA*-dependent, direct MMA-oxidation pathway is so far only known to exist in bacteria using MMA as a carbon source (that is, methylotrophs), the *gmaS*-dependent, indirect pathway has recently been shown to also exist in bacteria using MMA for nitrogen nutrition only (that is, non-methylotrophs) (Chen *et al.*, 2010b). Using the *gmaS* primer sets developed in this study, PCR and sequence analysis of DNA from isolates revealed the presence of *gmaS* in all eight non-methylotrophic MMA-utilising bacteria and in all seven methylotrophic MMA utilisers (Table 2). Phylogenetic analysis placed the retrieved *gmaS* sequences within the alphaproteobacterial and the beta-/gammaproteobacterial clusters as expected. Interestingly however, *gmaS* from *Aminobacter, Paracoccus, Catellibacterium, Mesorhizobium* and *Rhodobacter* formed a distinct subgroup within the *Alphaproteobacteria*, separate from the other freshwater and soil group, and separate from the marine group (Figure 3). *mauA* was detected in addition to *gmaS* in four of the seven methylotrophic isolates. These data indicate that the *gmaS* gene is widespread among MMA-utilising bacteria in Movile Cave.

Discussion

Methylated amine-utilising methylotrophs in Movile Cave

The combination of SIP and cultivation proved very effective for the identification of methylotrophs. DNA-SIP results revealed *M. mobilis* as one of the major MMA-utilising methylotrophs in Movile Cave, which is in agreement with previous studies which showed high abundance of this organism (Chen *et al.*, 2009). While resisting all isolation

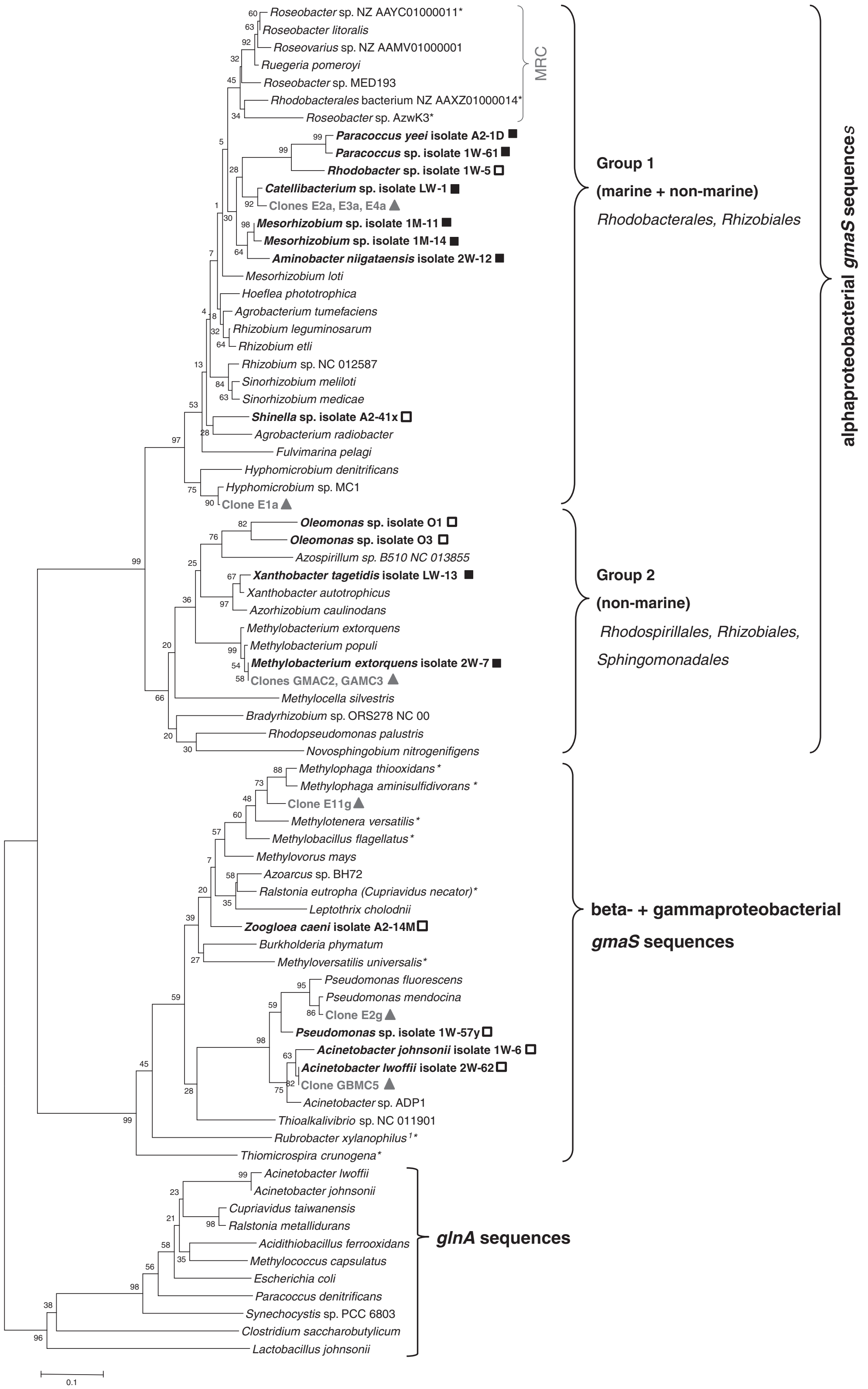

Figure 3 Phylogenetic relationship of *gmaS* sequences (135–250 amino acids) derived from published bacterial genomes, methylotrophic (solid rectangles/orange font) and non-methylotrophic (hollow rectangles/blue font) bacterial isolates and clone library sequences (triangles/bold print) from Movile Cave. *glnA* sequences present the outgroup. The tree was established using the neighbour-joining method (1000 bootstrap replicates) and the Poisson correction method for computing evolutionary distances. [1]*Rubrobacter xylanophilus* is a member of the *Actinobacteria* although its *gmaS* sequence affiliates with the beta- and gammaproteobacterial cluster. **gmaS* sequences containing a total of more than two mismatches across the forward/reverse primer set designed for the respective *gmaS* clusters are marked with an asterisk. MRC, marine Roseobacter clade.

Table 2 Methylated amine metabolism and the presence of functional gene markers in Movile Cave isolates

Isolate		*Methylated amines used as*		*Functional genes*	
		N-source	*C-source*	gmaS	mauA
Alphaproteobacteri*a*					
1	*Methylobacterium extorquens* 2W-7	+	+	+	+
2	*Xanthobacter tagetidis* LW-13	+	+	+	+
3	*Paracoccus yeei* A2-1D	+	+	+	+
4	*Paracoccus* sp. 1W-61	+	+	−	+
5	*Aminobacter niigataensis* 2W-12	+	+	+	−
6	*Catellibacterium* sp. LW-1	+	+	+	−
7	*Mesorhizobium* sp. 1M-11	+	+	+	−
8	*Shinella* sp. A2-41x	+	−	+	−
9	*Rhodobacter* sp. 1W-5	+	−	+	−
10	*Oleomonas* sp. O1	+	−	+	−
11	*Oleomonas* sp. O3	+	−	+	−
Gammaproteobacteria					
12	*Acinetobacter johnsonii* 1W-6	+	−	+	−
13	*Acinetobacter lwoffii* 2W-62	+	−	+	−
14	*Pseudomonas* sp. 1W-57Y	+	−	+	−
Betaproteobacteria					
15	*Zoogloea caeni* A2-14M	+	−	+	−

Overview of bacterial isolates from Movile Cave, their capability of using methylated amines as a carbon (C) and/or nitrogen (N) source, and presence of functional genes indicating the direct (*mauA*) or indirect (*gmaS*) methylamine oxidation pathway.

attempts, at 96 h in SIP incubations *M. mobilis* was the first organism that responded to addition of MMA.

The combination of cultivation-based studies and SIP furthermore revealed that a new methylotroph, *Catellibacterium* sp. LW-1, is an active MMA utiliser in Movile Cave. Growth studies were essential in consolidating DNA-SIP results and confirming *Catellibacterium* as a novel methylotroph and active MMA-utilising bacterium in Movile Cave. These results also highlight the benefit of analysing SIP enrichments at different time points.

Data from SIP enrichments also suggested that *Cupriavidus*, *Porphyrobacter* and *Altererythrobacter* might have a major role in methylotrophic MMA utilisation alongside known methylotrophs such as *Methylobacterium* and *Methylovorus*. While these organisms were not isolated from the cave and have hence not been tested for growth with methylated amines, published genomes of some *Cupriavidus*/*Ralstonia* species contain *gmaS* (refer to trees in Figure 3, Supplementary Figure S5).

Use of methylated amines by non-methylotrophic bacteria in Movile Cave

The large variety of bacterial isolates in Movile Cave using methylated amines as nitrogen sources but not as carbon sources is intriguing, considering the relatively high standing concentrations of ammonium present in Movile Cave water. It is possible that ammonium-depleted areas exist within the microbial mats where utilisation of MMA is advantageous. The fact that nitrogen in the mat is isotopically light while ammonium in the cave water is heavy (Sarbu *et al.*, 1996) could be explained by isotopic fractionation during ammonium assimilation and nitrification. However, it may also indicate that a nitrogen source other than ammonium is used. When growing methylotrophically, some bacterial species have been shown to use the nitrogen of MMA, even when high ammonium concentrations are present (Bellion *et al.*, 1983). The high concentrations of ammonium may even be partly due to the release of excess nitrogen by bacteria using MMA as both a carbon and nitrogen source.

Distribution of the gmaS gene and its use as a biomarker

The newly developed PCR primers targeting *gmaS* were successful in the detection of MMA-utilising bacteria not covered by currently available primers which target *mauA*-containing methylotrophs. Results from functional gene screening of non-methylotrophic Movile Cave isolates support previous findings (Chen *et al.*, 2010a) which showed that the *gmaS*-dependent pathway is used by the non-methylotroph *Agrobacterium tumefaciens*. Taken together, these results suggest that the *gmaS* pathway may be the major mode of MMA utilisation in bacteria using MMA as a nitrogen, but not as a carbon, source. Based on our results, the *gmaS*-dependent pathway also appears to be present in the majority of methylotrophic MMA-utilising bacteria. The direct MMA dehydrogenase (*mauA*)-dependent pathway, which was detected in a number of methylotrophic isolates in addition to *gmaS*, seems to be restricted to certain groups of methylotrophic bacteria. It will be interesting to understand how the two pathways are regulated under different growth conditions in organisms containing both.

Conclusions

Combining DNA-SIP and isolation studies, key methylotrophs in Movile Cave were identified and it was shown that methylated amines are important intermediates in Movile Cave, serving as a source of carbon, energy and/or nitrogen for a wide range of bacteria. The GMAS-/NMG-mediated pathway appears to be widespread among both methylotrophic and non-methylotrophic MMA utilisers and newly developed primer sets targeting *gmaS* have great potential as biomarkers for identification of MMA-utilising bacteria.

Conflict of Interest

The authors declare no conflict of interest.

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