Methylamine as a Nitrogen Source for Microorganisms from a Coastal Marine Environment

Martin Taubert[a,b], Carolina Grob[b], Alexandra M. Howat[b], Oliver J. Burns[c], Jennifer Pratscher[b], Nico Jehmlich[d], Martin von Bergen[d,e,f], Hans H. Richnow[g], Yin Chen[h,1], J. Colin Murrell[b,1]

[a]Aquatic Geomicrobiology, Institute of Ecology, Friedrich Schiller University Jena, Dornburger Str. 159, 07743 Jena, Germany

[b]School of Environmental Sciences, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK

[c]School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK

[d]Department of Molecular Systems Biology, Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany

[e]Institute of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, University of Leipzig, Brüderstraße 32, 04103 Leipzig, Germany

[f]Department of Chemistry and Bioscience, University of Aalborg, Fredrik Bajers Vej 7H, 9220 Aalborg East, Denmark.

[g]Department of Isotope Biogeochemistry, Helmholtz-Centre for Environmental Research – UFZ, Permoserstrasse 15, 04318 Leipzig, Germany

[h]School of Life Sciences, University of Warwick, Coventry, CV4 7AL, UK

[1]To whom correspondence should be addressed. J. Colin Murrell, Phone: +44 (0)1603 59 2959, Email: J.C.Murrell@uea.ac.uk, and Yin Chen, Phone: +44 (0)24 76528976, Email: Y.Chen.25@warwick.ac.uk

Keywords: marine methylotrophs, ^{15}N stable isotope probing, methylamine, metagenomics, metaproteomics

Classification: BIOLOGICAL SCIENCES/Microbiology
Short title: Methylamine as a Nitrogen Source for Marine Microbes

Originality-Significance Statement

Methylated amine compounds such as methylamine are very important sources of nitrogen for microorganisms in seawater. In the marine environment, methylamine is generated by degradation of organic matter, and this volatile one-carbon compound can escape to the atmosphere where it affects global climate processes. Microbes which can use methylamine as a nitrogen source prevent the escape of this climate-active molecule to the atmosphere and recycle organic nitrogen compounds released from complex organic matter. We know little about the identity and activity of methylamine-degrading microorganisms occurring in marine environments. In these experiments, we combine ^{15}N stable isotope probing, metagenomics and metaproteomics to detect and identify bacteria from a coastal environment that utilize methylamine and employ it as a nitrogen source.

Abstract

Nitrogen is a key limiting resource for biomass production in the marine environment. Methylated amines, released from the degradation of osmolytes, could provide a nitrogen source for marine microbes. Thus far, studies in aquatic habitats on the utilization of methylamine, the simplest methylated amine, have mainly focussed on the fate of the carbon from this compound. Various groups of methylotrophs, microorganisms that can grow on one-carbon compounds, use methylamine as a carbon source. Non-methylotrophic microorganisms may also utilize methylamine as a nitrogen source, but little is known about their diversity, especially in the marine environment. In this proof-of-concept study, stable isotope probing (SIP) was used to identify microorganisms from a coastal environment that assimilate nitrogen from methylamine. SIP experiments using ^{15}N methylamine combined with metagenomics and metaproteomics facilitated identification of active methylamine-utilizing Alpha- and Gammaproteobacteria. The draft genomes of two methylamine utilizers were obtained and their metabolism with respect to methylamine was examined. Both bacteria identified in these SIP experiments used the γ-glutamyl-methylamide pathway, found in both methylotrophs and non-methylotrophs, to metabolize methylamine. The utilization of ^{15}N methylamine also led to the release of ^{15}N ammonium that was used as nitrogen source by other microorganisms not directly using methylamine.

Introduction

Nitrogen is one of the major limiting elements for biological productivity in the marine environment (Gruber, 2008). Dissolved organic nitrogen compounds, including methylated amines, are likely to be an important source of nitrogen for marine microorganisms (Capone et al., 2008). In the oceans, methylated amines are produced in large amounts, resulting in the release of 0.6 Tg N per annum into the atmosphere (Lee, 1988). Little is known, however, about the turnover of methylated amines in the marine environment. The low standing concentrations of methylated amines found in the open ocean, typically in the nanomolar range (Naqvi et al., 2005), may be what remains after microbial utilization.

Methylamine, the simplest alkylated amine, is released through the biodegradation of proteins and N-containing osmolytes (Barrett and Kwan, 1985; Neff et al., 2002). Containing carbon and nitrogen, this compound constitutes a direct link between the biogeochemical cycles of the two elements. Certain microbes can grow on methylamine as sole source of carbon and energy (Anthony, 1982). As methylamine is a one-carbon (C1) compound, these microbes are classified as methylotrophs. Phylogenetically diverse, ubiquitous and often metabolically versatile, methylotrophs play major roles in C1-cycling in marine habitats (Anthony, 1982; Strand and Lidstrom, 1984; Neufeld et al., 2007a; Giovannoni et al., 2008; Chen, 2012). A wide range of non-methylotrophic organisms, some of which can be found in marine environments, can also degrade methylamine to CO_2 and ammonium, the latter being used as a nitrogen source by these and other microorganisms (Budd and Spencer, 1968; Bicknell and Owens, 1980; Anthony, 1982; Murrell and Lidstrom, 1983; Chen et al., 2010a; Wischer et al., 2015).

The ability to utilize methylamine is found mainly in the Gram-negative phylum Proteobacteria, but also in Gram-positive methylotrophs such as some Actinobacteria and *Bacillus* species (McIntire et al., 1990; McTaggart et al., 2015), and in Eukaryotes such as the fungus *Aspergillus niger* (Frebort et

al., 1999). Gram-positive bacteria and Eukaryotes typically employ a copper-containing methylamine oxidase to cleave methylamine to formaldehyde and ammonium (Anthony, 1982). Within the Proteobacteria, two different pathways for methylamine utilization are known. The methylamine dehydrogenase (MaDH) pathway employs a tryptophan tryptophyl-quinone (TTP)-dependent, periplasmic dehydrogenase catalysing the oxidative cleavage of methylamine to formaldehyde and ammonium. The alternative pathway proceeds via formation of the methylated amino acids γ-glutamyl-methylamide (GMA) and *N*-methylglutamate (NMG) (Anthony, 1982; Chen et al., 2010a; Chen et al., 2010b; Latypova et al., 2010; Good et al., 2015). This cytoplasmic pathway typically requires three enzymes, a GMA synthetase (GmaS), an NMG synthase (MgsABC) and an NMG dehydrogenase (MgdABCD). The GMA pathway transfers the C1 group of methylamine to tetrahydrofolate, and also releases ammonium. The eight polypeptides required for this pathway are typically encoded in one gene cluster in bacterial genomes (Chen, 2012).

Both the MaDH and the GMA pathway are present in methylotrophs that grow on methylamine as sole carbon and energy source. Some methylotrophs, such as *Methylophaga* species or *Methylobacterium extorquens* strains, possess both pathways (Vuilleumier et al., 2009; Grob et al., 2015). The GMA pathway is also present in non-methylotrophs that use methylamine as a nitrogen source (Chen et al., 2010a; Chen, 2012; Nayak et al., 2016) and its presence in some Gram-positive bacteria has been suggested (McTaggart et al., 2015). Little is known, however, about the distribution of microbes using the GMA pathway in the marine environment. Initial investigations in aquatic ecosystems revealed a high diversity of *gmaS* genes, suggesting an important role for the GMA pathway in methylamine utilization (Chen, 2012; Wischer et al., 2015).

To identify active microorganisms in environmental samples, where classical enrichment and isolation experiments have proven to be difficult, the technique of stable isotope probing (SIP) has been established. In this cultivation-independent method, substrates labelled with heavy isotopes such as ^{13}C are used, leading to the incorporation of these isotopes in the biomass of active microbes. The application of SIP in combination with analysis of DNA and RNA, i.e., DNA- and RNA-SIP

(Radajewski et al., 2003; Neufeld et al., 2007b), as well as proteins, i.e., Protein-SIP, (Jehmlich et al., 2010), has enabled the detection of the heavy isotopes in a variety of biomolecules. Active marine methylotrophs utilizing methylamine, such as *Methylophaga* spp., have previously been identified by ^{13}C stable isotope probing (SIP) experiments (Neufeld et al., 2007a). However, non-methylotrophic organisms that utilize methylamine as an nitrogen source would be missed in ^{13}C SIP studies, as only those who have assimilated methylamine derived carbon into biomass would be detected (Neufeld et al., 2007b).

In this study, a SIP experiment using ^{15}N labelled methylamine was combined with metaproteomics and metagenomics to identify microbes from a coastal marine habitat that are capable of utilizing methylamine and assimilating methylamine-derived nitrogen. The quantification of ^{15}N incorporated into proteins after incubation with ^{15}N methylamine showed a clear link between nitrogen uptake and the pathways used for methylamine utilization, and revealed the identity of different methylamine utilizing bacteria. A “blueprint” for the central metabolism of two of these key species was reconstructed from metagenomic sequence data generated from ^{15}N labelled DNA from the same samples, and then validated using metaproteomics.

Results and Discussion

The seawater samples used in this study were obtained from Station L4 of the Western Channel Observatory (WCO, Plymouth, UK). Genomic DNA and proteins were extracted after incubating seawater with 100 µM ^{14}N or ^{15}N methylamine for 3, 6 or 8 days in duplicate, and at the beginning of the incubation experiments (T0) to establish the initial microbial community composition in the seawater used. The aim of this SIP experiment was to explore the metabolism of microbes from coastal seawater capable of responding to an increase in the concentration of methylamine. Although the relatively large amount of substrate added here is not strictly environmentally relevant, and the resulting microbial activities are not necessarily representative of *in situ* conditions, it

enabled enriching for groups of microbes of interest. This in turn allowed evaluation of the potential of naturally occurring marine microbes to utilize methylamine without the need to cultivate them.

Composition of the microbial community in methylamine incubations

Microbial diversity in methylamine incubations was determined using 454 amplicon pyrosequencing targeting bacterial 16S rRNA genes in the total extracted DNA. Three of the samples (one incubated with ^{15}N methylamine for 3 days and two incubated with ^{14}N methylamine for 6 and 8 days) showed a distinct difference in community composition compared to the others (Fig. S1). In these samples, a single OTU related to the genus *Methylophaga* (Piscirickettsiaceae) was enriched up to 92%, whereas in the remaining samples, *Methylophaga* were present at <1% of relative abundance or not detected at all (T0).

Analysis of 16S rRNA gene diversity from the remaining DNA samples consistently yielded the same major phylogenetic groups (Fig. 1). In the seawater used to set up the incubation experiments (T0), the dominant operational taxonomic units (OTUs) were related to *Candidatus* Pelagibacter (Pelagibacteraceae, approximately 60%), but this group decreased in relative abundance to between 1 and 27% after incubation. *C.* Pelagibacter belong to the SAR11 cluster, first described in the Sargasso Sea, and are commonly found at high abundance in marine habitats (Morris et al., 2002; Rappe and Giovannoni, 2003). OTUs related to Rhodobacteraceae increased in relative abundance from approximately 5% at T0 to between 20 and 70% after incubation. Up to 50% of the Rhodobacteraceae OTUs were related to *Leisingera*, the remaining OTUs being mostly related to *Roseobacter*, *Ruegeria* and *Phaeobacter*. OTUs related to the Gammaproteobacterium strain IMCC2047 also increased in abundance after 3 days of incubation, reaching up to 24%. This OTU was found in low abundance (0.2%) at T0, and decreased again at later time points to around 1%.

The changes observed in the microbial community composition were most likely caused by the relatively high methylamine concentration used for the SIP experiment, leading to an enrichment of the most capable and rapidly growing organisms during incubation. Other organisms, such as the

slow growing Pelagibacter (Carini et al., 2013), seemed to disappear as they were outcompeted by fast growing methylamine utilizers. After 6 and 8 days of incubation, 16S rRNA genes of other bacterial families, such as Flavobacteriaceae and Cryomorphaceae, increased in abundance. This diversification is likely to be a result of cross-feeding and metabolic processes other than methylamine utilization. We thus focused our investigation on those microorganisms that first responded to the addition of methylamine: *Leisingera* sp. and the IMCC2047-related Gammaproteobacterium.

Retrieval of genomes of enriched methylamine utilizers by ^{15}N DNA-SIP

The incorporation of ^{15}N into DNA leads to an increase of its density that can be detected by density gradient centrifugation. While for ^{13}C DNA of microbial communities, this increase is large enough to allow a complete separation from ^{12}C DNA, separation of ^{14}N and ^{15}N DNA is not possible, as DNA contains less nitrogen than carbon, and DNA density is also influenced by GC content, resulting in an overlap of GC-rich ^{14}N DNA and AT-rich ^{15}N DNA (Fig. S2) (Cupples et al., 2007). Here, density gradient centrifugation of a DNA sample from seawater incubated for 3 days with ^{15}N methylamine was used to enrich for DNA of OTUs related to Rhodobacteraceae and strain IMCC2047 that were presumed to be involved in methylamine utilization. The enrichment was quantified by a comparison of 16S rRNA gene profiles obtained by amplicon pyrosequencing from unfractionated DNA and from DNA fractions obtained after density gradient centrifugation. For the IMCC2047-related Gammaproteobacterium, a relative 16S rRNA gene abundance of 34.9% was observed in the fraction with a density of 1.695 g ml^{-1}, corresponding to a ~2-fold enrichment compared to unfractionated DNA (see Fig. S2). For Rhodobacteraceae, an abundance of 98.7% was observed in the fraction with a density of 1.704 g ml^{-1}, corresponding to a ~1.6-fold enrichment. These two fractions were selected for metagenomic sequencing, allowing a targeted reconstruction of the genomes of the corresponding organisms that would not have been possible without the fractionation process. The completeness of the genomes obtained was assessed with CheckM (Parks et al., 2015) after removal of phylogenetically unrelated sequences.

The genome of the IMCC2047-related Gammaproteobacterium constructed was estimated to be approximately 89% complete (Table 1), based on the presence of 401 out of 452 single-copy core genes defined by CheckM for this phylogenetic group. Based on the abundance of multiple versions of these single copy genes, the genome was estimated to contain approximately 3% of genes likely related to other taxa. The genome obtained from the fraction enriched in OTUs related to Rhodobacteraceae was phylogenetically classified as belonging to the genus *Leisingera*, and was most closely related to *Leisingera aquimarina* (Vandecandelaere et al., 2008). All of the 626 core genes of this phylogenetic group (based on CheckM analysis) were present (Table 1). However, a high number of these single-copy genes were found multiple times, showing more than 90% protein sequence similarity to each other. This indicates the presence of genomic sequences from more than one *Leisingera* strain, but for simplicity, these were treated as one phylogenetic entity in the following analysis. The *Leisingera* genome contained about 8% of genes which were likely to be related to other taxa based on the CheckM analysis. In summary, metagenomic sequencing of DNA fractions from the ^{15}N DNA-SIP experiment, containing a high enrichment of DNA from the organisms related to *Leisingera* and strain IMCC2047, led to the successful recovery of two almost complete microbial genomes of organisms rapidly responding to an increase in the availability of methylamine in seawater.

^{15}N incorporation into peptides confirms methylamine utilization by enriched microbes

The incorporation of ^{15}N from methylamine in microbial biomass, as evidence for an assimilation of methylamine derived nitrogen, was investigated in DNA and proteins obtained from seawater after 3 days of incubation with methylamine. To detect potential changes in density of the DNA of particular organisms between ^{14}N and ^{15}N methylamine incubations, DNA distribution profiles were calculated based on 16S rRNA gene amplicon pyrosequencing data of DNA fractions. For *Leisingera* and the IMCC2047-related Gammaproteobacterium, an increase in DNA density between ^{14}N to ^{15}N samples

was observed (Fig. S2). A similar increase was observed between ^{14}N DNA and ^{15}N DNA of reference strains investigated in control experiments (Fig. S2). OTUs related to *C.* Pelagibacter were also investigated, and showed a partial and minor increase in DNA density between the ^{14}N and ^{15}N sample. This was the first indication for ^{15}N assimilation from methylamine by *Leisingera* and the IMCC2047-related Gammaproteobacterium, but lack thereof to any appreciable extent, by *C.* Pelagibacter. However, as a complete separation of ^{14}N and ^{15}N DNA was not possible, mass spectrometric investigation of the ^{15}N incorporation in peptides was performed for validation, since this can quantify changes in ^{15}N abundance down to 0.1 at.% (Taubert et al., 2013).

Protein extracts obtained from seawater incubated for 3 days with methylamine were investigated by high resolution mass spectrometry (MS) after tryptic digestion, using the NCBInr database for peptide identification. A total of 8,184 non-redundant peptides were identified in the samples incubated with ^{14}N methylamine. Of these, 131 peptides, 997 peptides and 2,010 peptides were unique for *C.* Pelagibacter, Rhodobacteraceae (including *Leisingera*), and strain IMCC2047, respectively (Table S1). The ^{15}N at.% in peptides of these three phylogenetic groups was assessed in the samples incubated with ^{15}N methylamine to investigate whether these bacteria assimilated methylamine-derived nitrogen, or unlabelled ammonium that had been added to the incubations as an alternative nitrogen source. Under the conditions present in our SIP incubations, bacteria using methylamine directly as a nitrogen source should be almost completely labelled (i.e. close to 100 at.% ^{15}N). However, the breakdown of ^{15}N methylamine will also lead to an isotopic enrichment of the ammonium pool in the incubations. Due to the unlabelled ammonium in the incubations, however, organisms that assimilate ammonium, and are thus cross-feeding on methylamine-derived ammonium, should show a significantly lower ^{15}N labelling compared to those assimilating methylamine-N. All Rhodobacteraceae- and IMCC2047-related peptides were found to be more than 90% enriched in ^{15}N, which indicated that under the SIP incubation conditions used, these bacteria mostly assimilated nitrogen from methylamine (Fig. 2, Table S2). *C.* Pelagibacter-related peptides contained a significantly lower amount ($p < 0.001$, *t*-test) of ^{15}N with an average of 44%. This low ^{15}N

incorporation suggests mostly assimilation of nitrogen from the ammonium pool in the incubations, which got only slightly enriched in ^{15}N by ammonium release from methylamine utilizing organisms. In the ^{15}N sample enriched in *Methylophaga*, a ^{15}N content of 70-75%, differing significantly from all other organisms investigated ($p < 0.001$, *t*-test), was found in peptides unique for *Methylophaga*, indicating a different route of nitrogen assimilation as compared to Rhodobacteraceae and the IMCC2047-related Gammaproteobacterium, most likely at least partially via ammonium. The ^{15}N incorporation patterns in peptides thus enabled a clear discrimination of bacteria using mostly methylamine as nitrogen source (Rhodobacteraceae and the IMCC2047-related Gammaproteobacterium), and organisms either additionally or exclusively using ammonium as nitrogen source (*C.* Pelagibacter and *Methylophaga*).

Metabolic pathways for methylamine utilization and ammonium assimilation

The genomes obtained from the two enriched methylamine utilizers related to *Leisingera* and IMCC2047 in the SIP incubations with ^{15}N methylamine were investigated for the presence of genes required for methylamine utilization and ammonium assimilation. In the assembled genomes, no genes of the MaDH pathway were present. Genes of the GMA pathway were found in both genomes. In the *Leisingera*-related genome, putative *gmaS* and *mgsABC* genes (encoding GMA synthetase and NMG synthase) were present in one gene cluster, and two separate *mghABCD* clusters (encoding NMG dehydrogenase) were found. In the genome of the IMCC2047-related Gammaproteobacterium, a single gene cluster containing all eight genes of the GMA pathway was present. The *gmaS* gene of the *Leisingera*-related genome was 93% identical to that of *Leisingera aquimarina*, while the *gmaS* gene of the genome of the IMCC2047-related Gammaproteobacterium was 96% identical to that of strain IMCC2047 (both at the nucleic acid level). An overview of the phylogenetic distribution of the derived GmaS-sequences is given in Fig. S3.

Protein sequences derived from the genomes obtained were used to reanalyze the metaproteomics data from the SIP incubations to verify the expression of proteins from the GMA pathway. Proteins

encoded by both GMA gene clusters related to *Leisingera* and the IMCC2047-related Gammaproteobacterium were found to be expressed (Fig. S4). This confirmed that both organisms used the GMA pathway for methylamine utilization. No peptides specific for GMA gene clusters related to *C.* Pelagibacter were detected. For comparison, we also checked for expression of proteins of GMA and MaDH gene clusters related to *Methylophaga*, using the samples where *Methylophaga* was enriched in 16S rRNA gene profiles (after 6 and 8 days of incubation). The published genome of *Methylophaga thiooxydans* strain L4 (Grob et al., 2015), the closest relative of the *Methylophaga* sp. detected in our incubations, was used as the reference. We detected 25 peptides encoded in the MaDH gene cluster, but only 5 peptides encoded in the GMA gene cluster. This strongly suggests that in our SIP incubations, *Methylophaga* used the MaDH pathway for methylamine utilization.

Genes involved in ammonium assimilation, encoding the glutamine synthetase/glutamine:oxoglutarate amidotransferase system (GS/GOGAT), the ammonium transporter *amtB* and the glutamate dehydrogenase, were present in the genomes of *Leisingera* and the IMCC2047-related Gammaproteobacterium. The corresponding proteins related to both organisms were also expressed. An alanine dehydrogenase gene was only present in the *Leisingera* genome, but no corresponding protein was detected, suggesting that the GS/GOGAT pathway was primarily used for ammonium assimilation by *Leisingera* during these SIP incubations.

In summary, the methylamine SIP experiment revealed the presence of two key methylamine utilizers related to *Leisingera* and Gammaproteobacterium strain IMCC2047, both employing the GMA pathway for methylamine utilization. Concomitantly, these organisms showed a high ^{15}N incorporation in their peptides (Table S2), indicating the use of methylamine as sole nitrogen source. The *Methylophaga* sp. enriched in three of our incubations, conversely, was found to use the MaDH pathway for methylamine utilization, employing the two-subunit methylamine dehydrogenase (MauAB). Peptide analysis revealed a lower incorporation of ^{15}N, indicating the additional uptake of unlabelled ammonium by this bacterium. A possible explanation for this difference is the cellular location of the pathways. Enzymes of the GMA pathway are found in the cytoplasm, thus the ^{15}N

ammonium is released directly inside of the cell, where it can be immediately assimilated via the GS/GOGAT pathway or by glutamate dehydrogenase (Fig. 3). The MaDH is located in the periplasm, and therefore ammonium is released to the outside of the cell. Any ^{15}N ammonium released in this way would thus need to be transported back into the cell (together with unlabelled ammonium from the seawater) before assimilation.

Genomic and proteomic analysis of the metabolism of methylamine utilizers

The draft genome sequences of the two key methylamine utilizers related to *Leisingera* and Gammaproteobacterium strain IMCC2047 that we obtained were analyzed in order to reconstruct their central metabolic pathways. Peptides identified were mapped to the protein sequences derived from these genomes to obtain data on gene expression in our SIP incubations.

The *Leisingera*-related organism expressed key proteins of the serine pathway for carbon assimilation, including serine hydroxymethyltransferase and malate thiokinase. Furthermore, the pathway for tetrahydrofolate-dependent reduction of formate as well as an S-(hydroxymethyl) glutathione dehydrogenase for the glutathione-dependent oxidation of formaldehyde to formate, and a formate dehydrogenase were expressed by this organism. It was thus presumably utilizing methylamine not only as nitrogen source, but also as an energy source, by oxidising formaldehyde derived from the GMA pathway to CO_2, and as carbon source by reduction of formate and assimilation via the serine pathway. This follows the classical mode of carbon utilization in alphaproteobacterial methylotrophs (Anthony, 1982). The Rhodobacteraceae, including the marine *Roseobacter* clade comprising up to 25% of marine microbial communities, contain a variety of organisms able to utilize C1 compounds, including methylated sulfur compounds (Buchan et al., 2005) and amines (Chen, 2012). The closest relatives of the *Leisingera*-related organism, *L. aquimarina* and *L. methylhalidivorans*, possess genes of the GMA pathway and are able to use methylamine as nitrogen source but not as carbon source (Chen, 2012). The observed enrichment of the *Leisingera*-related organism in our SIP experiment hints to the ability of this organism to employ

methylamine also as carbon source, indicating an interesting deviation from the metabolic capabilities found in other members of *Leisingera*. In the *Leisingera*-related genome, genes encoding enzymes involved in degradation of dimethylsulfoniopropionate (*dmdA*, *dddD*) (Todd et al., 2007; Reisch et al., 2008) and dimethyl sulfoxide (*dmsABC*) (Weiner et al., 1992) were present. Furthermore, this genome also contained a *sox* gene cluster encoding enzymes involved in thiosulfate oxidation (Friedrich et al., 2000). These genes are also present in other *Leisingera* species (Schaefer et al., 2002; Vandecandelaere et al., 2008), suggesting that these organisms can utilize various sulfur compounds, but none of these genes were found to be expressed under the incubation conditions used in our experiments.

The genome of the IMCC2047-related Gammaproteobacterium lacked genes encoding hydroxypyruvate reductase and malyl-CoA lyase, which are key enzymes of the serine pathway. Key genes of the ribulose monophosphate cycle, another methylotrophic pathway for the assimilation of carbon from methylamine (Anthony, 1982), were also missing. Alternatively, a ribulose-bisphosphate carboxylase and a phosphoribulokinase gene were found, suggesting that carbon might be assimilated into biomass at the level of CO_2 via the Calvin Benson Bassham (CBB) cycle. The protein products of these genes were not detected, so it remained uncertain whether the organism used methylamine or CO_2 as carbon source. The IMCC2047-related Gammaproteobacterium expressed various proteins involved in oxidation/reduction of C1 groups (methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase, formate:tetrahydrofolate ligase, S-(hydroxymethyl)glutathione dehydrogenase, S-formylglutathione hydrolase, and formate dehydrogenase). This indicated the likely use of methylamine as a source of reducing power and energy by the IMCC2047-related Gammaproteobacterium. No further genes encoding enzymes involved in C1 metabolism were found in the genome of this organism. The presence of a proteorhodopsin for light driven formation of a proton gradient is described in strain IMCC2047 (Kang et al., 2011). The combination of proteorhodopsin and the CBB cycle has been suggested to allow a photoheterotrophic growth of strain IMCC2047, with reducing power obtained from

exogenous chemical compounds (Pinhassi et al., 2016) such as methylamine. However, no photorhodopsin encoding gene was found in the genome of the IMCC2047-related Gammaproteobacterium obtained here, potentially due to its incompleteness.

Only a low number of peptides related to *C.* Pelagibacter were identified, and no evidence for the expression of *C.* Pelagibacter-specific proteins involved in methylamine utilization was found. However, *Candidatus* Pelagibacter ubique strain HTCC1062, the first cultivated representative of this genus (Giovannoni et al., 1990; Giovannoni et al., 2005), possesses genes of the GMA pathway for methylamine utilization and can oxidize methylamine for ATP production (Sun et al., 2011). Environments containing *C.* Pelagibacter strains are highly oligotrophic, and presumably these bacteria grow on the low concentrations of such compounds when they are released from dissolved organic matter (Tripp, 2013). When grown in culture, C. *Pelagibacter* strains have strict nutritional requirements for compounds such as pyruvate, glycine and reduced sulfur compounds, and typically have doubling times of more than 2 days even under optimal conditions (Carini et al., 2013). In our incubations, *C.* Pelagibacter was thus outcompeted by organisms capable of higher growth rates responding rapidly to methylamine addition. Nevertheless, as even slow growth with ^{15}N methylamine as nitrogen source would result in a measurable abundance of peptides highly enriched in ^{15}N (> 90% at.%), we still believe that C. Pelagibacter was not involved in methylamine utilization in our SIP incubations. *C.* Pelagibacter strains are adapted to nitrogen-limited conditions, and only switch to uptake of organic nitrogen sources under high nitrogen stress (Smith et al., 2013). Under the conditions present in our incubations, cross-feeding on ^{15}N ammonium released by other methylamine utilizers might thus have been sufficient to satisfy the nitrogen requirement of *C.* Pelagibacter (Fig. 3), which would also explain the amount of ^{15}N labelling observed in *C.* Pelagibacter peptides.

For *Methylophaga* sp., key enzymes of the ribulose monophosphate cycle for carbon assimilation and enzymes involved in the tetrahydromethanopterin- and tetrahydrofolate-dependent oxidation/reduction of C1 groups were detected. This resembles the metabolism of the closely

related *Methylophaga thiooxydans* strain L4 during growth on methanol, as previously described (Grob et al., 2015). *Methylophaga* spp. are known *bona fide* methylotrophs (Janvier et al., 1985), thus it is not surprising that the *Methylophaga* sp. in our incubations used methylamine as source of carbon and energy, while releasing ammonium as a by-product. Interestingly, *Methylophaga* species are commonly observed in enrichment cultures and SIP experiments where marine samples are incubated with C1 compounds such as methanol, methylamine or dimethylsulfide (Neufeld et al., 2007a; Moussard et al., 2009; Boden et al., 2010), even though they appear to be present only at very low relative abundance in 16S rRNA gene surveys of seawater samples (Janvier et al., 2003; Rusch et al., 2007; Grob et al., 2015). The bloom-like appearance of *Methylophaga* sp. in three of our incubations might indicate a preference for the high concentrations of methylamine used in our SIP experiment, in contrast to the ability of e.g. *C.* Pelagibacter to grow under highly oligotrophic conditions. The ecological niche of *Methylophaga* spp. in the environment, however, remains unknown.

In summary, the draft genomes of two methylamine-utilizing organisms were recovered after ^{15}N-SIP experiments. Both bacteria used the GMA pathway for methylamine utilization and assimilated nitrogen from the methylamine present. Based on the expressed proteins we detected, they probably also used methylamine as an energy source, but potentially employed different carbon uptake pathways, using either methylamine or CO_2 as carbon source.

Conclusion

In this study, we used a ^{15}N-SIP experiment to investigate the metabolism of methylamine in bacteria from a coastal environment. A combination of SIP, metagenomics and metaproteomics revealed that phylogenetically diverse methylamine utilizers of the Alpha- and Gammaproteobacteria assimilate the nitrogen from methylamine into biomass using the GMA pathway. Furthermore, ammonium released during methylamine utilization, e.g. via the MaDH pathway, can be used as a nitrogen source by bacteria not utilizing methylamine. Our study demonstrated that ^{15}N-SIP is a powerful

technique to detect bacteria present in seawater samples that are able to respond to an increase in the availability of methylamine, and facilitates retrieval of their genomes by metagenomic sequencing of ^{15}N labelled DNA. When coupled with proteomics, ^{15}N-SIP can be used to reconstruct putative metabolic pathways and assess the expression of key proteins involved in cycling nitrogen from methylated amines by these bacteria. Since measurements of the *in situ* concentrations of methylated amines in seawater are difficult, further development of process-based methods, coupled with molecular ecology techniques such as SIP and metagenomics, will be required to analyze in depth the exact contribution of bacteria in the cycling of methylated amines in the marine environment.

Experimental Procedures

^{15}N stable isotope probing experimental setup

Surface seawater was collected at the WCO Station L4 (50°15.0'N; 4°13.0'W) on 29th of September, 2014. Three sets of four 2 L gas-tight glass bottles were filled with 0.75 L of seawater, to which 75 µmol of ^{15}N or ^{14}N methylamine (two bottles each per set) were added (100 µM final concentration) as well as 750 µl marine ammonium mineral salt medium (MAMS, NH_4^+ 15 µM final concentration) (Schäfer et al., 2005). Bottles were incubated at 25°C in a shaking incubator at 50 rpm. Methylamine concentration in the incubation bottles was measured daily by ion chromatography (see Supp. Info). When methylamine concentrations were below the limit of detection (5 µM), again 75 µmol of ^{15}N or ^{14}N methylamine were added. Seawater from a set of bottles was filtered through 0.22 µm Sterivex^{TM} filters (Merck Millipore) after 3, 6 and 8 days of incubation, by which time a cumulative amount of 75 µmol, 150 µmol and 300 µmol of methylamine had been added per bottle, respectively. For T0, 3.4 L of seawater were filtered in duplicate through Sterivex filters within 24 h of collection using a peristaltic pump (Watson-Marlow 502S, 50 ml min^{-1}).

All filters were stored at -20°C for a maximum of two weeks before extraction of DNA and proteins, which was performed as described in (Grob et al., 2015).

Protein-SIP analyses

Protein extracts were subjected to SDS polyacrylamide gel electrophoresis followed by in-gel tryptic digestion and LC-MS/MS analysis (see Supp. Info). Peptide identification was performed in Proteome Discoverer v1.4 (Thermo Fisher Scientific) via the Mascot search algorithm (Koenig et al., 2008). Only peptides with a false discovery rate (FDR) <1% and peptide rank of 1 were considered as identified. Searches against two different reference databases were performed: the NCBInr database with taxonomy set to Bacteria and Archaea and a database consisting of the predicted protein sequences of metagenome-derived DNA sequence data. To exclude peptides that were conserved in multiple phylogenetic groups, the taxonomic range of all peptides was checked with Unipept (www.unipept.ugent.be). Proteins were considered as identified if at least one unique peptide was identified.

Quantification of ^{15}N incorporation in peptides

A subset of peptides from proteins identified in samples incubated with ^{14}N methylamine was selected for investigation of ^{15}N incorporation in phylogenetic groups of interest (see Supp. Info). Mass spectra from samples of the corresponding ^{15}N methylamine incubations were analyzed, the signals of the selected peptides were identified based on expected *m/z*, chromatographic retention time and MS/MS fragmentation pattern, and ^{15}N incorporation was quantified as previously described (Taubert et al., 2013).

DNA-SIP ultracentrifugation and fractionation

DNA extracted from the incubations with ^{14}N and ^{15}N methylamine after 3 days was fractionated by ultracentrifugation in CsCl density gradients. For comparison, ^{14}N and ^{15}N DNA from reference strains (*Methylophaga marina*, *Escherichia coli* DH5α, *Rhodococcus* AD45) was also investigated. The

gradients were prepared by adding 3 µg of DNA per sample to a mixture of 7.163 M CsCl solution and gradient buffer (0.1 M Tris, 0.1 M KCl and 1 mM EDTA) set to a final density of 1.700 g ml^{-1}. Ultracentrifugation at 40,900 rpm (164,000 x g max) for 64 hours at 20°C and with vacuum, maximum acceleration and no brake set was performed, using a VTi 65.2 rotor and an OptimaTM LE-80K Ultracentrifuge (Beckman Coulter) (see Supp. Info).

16S rRNA gene amplicon sequencing

To generate amplicons of the 16S rRNA gene from DNA fractions and unfractionated DNA, the primer set 27Fmod (5'-AGRGTTTGATCMTGGCTCAG-3') and 519Rmodbio (5'-GTNTTACNGCGGCKGCTG-3') was used. After amplification by PCR, 454 pyrosequencing was performed on a GS FLX Titanium system (MR DNA, Shallowater, TX, USA) followed by sequence analysis and phylogenetic classification (see Supp. Info).

Selection and preparation of DNA fractions for shotgun sequencing

Normalized distribution profiles of DNA from phylogenetic groups of interest along the density gradient were estimated in order to enable the selection of samples with the highest enrichment of DNA from a particular phylogenetic group for metagenome sequencing (see Supp. Info). Multiple displacement amplification (MDA) using the REPLI-g Mini Kit (Qiagen) was done to increase the low amounts of available DNA (see Supp. Info). A total of 4 µg of amplified DNA from each sample were sent for MiSeq, 2 x 300 bp, Illumina sequencing (2 million reads; MR DNA, Shallowater, TX, USA).

Genome reconstruction and analysis

The MiSeq sequencing datasets were assembled using SPAdes Genome Assembler v3.0 (Bankevich et al., 2012). Contigs below 1 kb were removed, the remaining contigs were binned based on tetranucleotide frequencies and % GC-content using VizBin (Laczny et al., 2015) and comparison with reference genomes using blastn (Altschul et al., 1997). Completeness of genomic bins was checked using CheckM (Parks et al., 2015). Selected genomic bins were annotated in RAST (Aziz et al., 2008),

followed by manual revision. Annotation of genes related to the GMA pathway was done as previously described ((Chen, 2012; Wischer et al., 2015), see Supp. Info).

Sequence data deposition

Raw data from 454 amplicon pyrosequencing of 16S rRNA gene amplicons have been deposited in the Sequence Read Archive (SRA) of NCBI under accession numbers SRR3183712-SRR3183751. Genome sequences obtained are available in the GenBank Whole Genome Shotgun (WGS) database under accession number LUKH00000000 (*Leisingera*) and LUKI00000000 (IMCC2047-related Gammaproteobacterium). Raw Illumina MiSeq data were deposited at BaseSpace (https://basespace.illumina.com/s/gDb2v3gnAbxU).

Acknowledgments

We thank the Captain and crew of the R.V. Plymouth Quest, as well as Michael Cunliffe and Kimberley Bird, for collecting the seawater used in this work. The authors are grateful for using the analytical facilities of the Centre for Chemical Microscopy (ProVIS) at the Helmholtz Centre for Environmental Research which is supported by European Regional Development Funds (EFRE – Europe funds Saxony) and the Helmholtz Association. This work was supported by the Gordon and Betty Moore Foundation Marine Microbiology Initiative Grant GBMF3303 to J. Colin Murrell and Yin Chen and through the Earth and Life Systems Alliance, Norwich Research Park, Norwich, UK.

The authors declare no conflict of interest.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389-3402.

Anthony, C. (1982) *The biochemistry of methylotrophs*. New York: Academic Press.

Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A. et al. (2008) The RAST server: Rapid annotations using subsystems technology. *BMC Genomics* **9**: 75.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S. et al. (2012) SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* **19**: 455-477.
Barrett, E.L., and Kwan, H.S. (1985) Bacterial reduction of trimethylamine oxide. *Annu Rev Microbiol* **39**: 131-149.
Bicknell, B., and Owens, J.D. (1980) Utilization of methyl amines as nitrogen-sources by non-methylotrophs. *J Gen Microbiol* **117**: 89-96.
Boden, R., Kelly, D.P., Murrell, J.C., and Schafer, H. (2010) Oxidation of dimethylsulfide to tetrathionate by *Methylophaga thiooxidans* sp. nov.: a new link in the sulfur cycle. *Environ Microbiol* **12**: 2688-2699.
Buchan, A., Gonzalez, J.M., and Moran, M.A. (2005) Overview of the marine *Roseobacter* lineage. *Appl Environ Microb* **71**: 5665-5677.
Budd, J.A., and Spencer, C.P. (1968) Ultilisation of alkylated amines by marine bacteria. *Mar Biol* **2**: 92-101.
Capone, D.G., Bronk, D.A., Mulholland, M.R., and Carpenter, E.J. (2008) *Nitrogen in the marine environment*: Academic Press.
Carini, P., Steindler, L., Beszteri, S., and Giovannoni, S.J. (2013) Nutrient requirements for growth of the extreme oligotroph '*Candidatus* Pelagibacter ubique' HTCC1062 on a defined medium. *ISME J* **7**: 592-602.
Chen, Y. (2012) Comparative genomics of methylated amine utilization by marine *Roseobacter* clade bacteria and development of functional gene markers (*tmm*, *gmaS*). *Environ Microbiol* **14**: 2308-2322.
Chen, Y., McAleer, K.L., and Murrell, J.C. (2010a) Monomethylamine as a nitrogen source for a nonmethylotrophic bacterium, *Agrobacterium tumefaciens*. *Appl Environ Microb* **76**: 4102-4104.
Chen, Y., Scanlan, J., Song, L.J., Crombie, A., Rahman, M.T., Schafer, H., and Murrell, J.C. (2010b) gamma-Glutamylmethylamide is an essential intermediate in the metabolism of methylamine by *Methylocella silvestris*. *Appl Environ Microb* **76**: 4530-4537.
Cupples, A.M., Shaffer, E.A., Chee-Sanford, J.C., and Sims, G.K. (2007) DNA buoyant density shifts during N-15-DNA stable isotope probing. *Microbiol Res* **162**: 328-334.
Frebort, I., Matsushita, K., Toyama, H., Lemr, K., Yamada, M., and Adachi, O. (1999) Purification and characterization of methylamine oxidase induced in *Aspergillus niger* AKU 3302. *Biosci Biotech Bioch* **63**: 125-134.
Friedrich, C.G., Quentmeier, A., Bardischewsky, F., Rother, D., Kraft, R., Kostka, S., and Prinz, H. (2000) Novel genes coding for lithotrophic sulfur oxidation of *Paracoccus pantotrophus* GB17. *J Bacteriol* **182**: 4677-4687.
Giovannoni, S.J., Britschgi, T.B., Moyer, C.L., and Field, K.G. (1990) Genetic diversity in Sargasso Sea bacterioplankton. *Nature* **345**: 60-63.
Giovannoni, S.J., Hayakawa, D.H., Tripp, H.J., Stingl, U., Givan, S.A., Cho, J.C. et al. (2008) The small genome of an abundant coastal ocean methylotroph. *Environ Microbiol* **10**: 1771-1782.
Giovannoni, S.J., Tripp, H.J., Givan, S., Podar, M., Vergin, K.L., Baptista, D. et al. (2005) Genome streamlining in a cosmopolitan oceanic bacterium. *Science* **309**: 1242-1245.
Good, N.M., Lamb, A., Beck, D.A., Martinez-Gomez, N.C., and Kalyuzhnaya, M.G. (2015) C1-pathways in *Methyloversatilis universalis* FAM5: Genome wide gene expression and mutagenesis studies. *Microorganisms* **3**: 175-197.
Grob, C., Taubert, M., Howat, A.M., Burns, O.J., Dixon, J.L., Richnow, H.H. et al. (2015) Combining metagenomics with metaproteomics and stable isotope probing reveals metabolic pathways used by a naturally occurring marine methylotroph. *Environ Microbiol* **17**: 4007-4018.
Gruber, N. (2008) The marine nitrogen cycle: overview and challenges. In *Nitrogen in the marine environment*: Academic Press, pp. 1-50.
Janvier, M., Regnault, B., and Grimont, P. (2003) Development and use of fluorescent 16S rRNA-targeted probes for the specific detection of *Methylophaga* species by *in situ* hybridization in marine sediments. *Res Microbiol* **154**: 483-490.

Janvier, M., Frehel, C., Grimont, F., and Gasser, F. (1985) *Methylophaga marina* gen. nov., sp. nov. and *Methylophaga thalassica* sp. nov., marine methylotrophs. *Int J Syst Bacteriol* **35**: 131-139.
Jehmlich, N., Schmidt, F., Taubert, M., Seifert, J., Bastida, F., von Bergen, M. et al. (2010) Protein-based stable isotope probing. *Nat Protoc* **5**: 1957-1966.
Kang, I., Kang, D., Oh, H.M., Kim, H., Kim, H.J., Kang, T.W. et al. (2011) Genome sequence of strain IMCC2047, a novel marine member of the gammaproteobacteria. *J Bacteriol* **193**: 3688-3689.
Koenig, T., Menze, B.H., Kirchner, M., Monigatti, F., Parker, K.C., Patterson, T. et al. (2008) Robust prediction of the MASCOT score for an improved quality assessment in mass spectrometric proteomics. *J Proteome Res* **7**: 3708-3717.
Laczny, C.C., Sternal, T., Plugaru, V., Gawron, P., Atashpendar, A., Margossian, H.H. et al. (2015) VizBin - an application for reference-independent visualization and human-augmented binning of metagenomic data. *Microbiome* **3**: 1.
Latypova, E., Yang, S., Wang, Y.S., Wang, T.S., Chavkin, T.A., Hackett, M. et al. (2010) Genetics of the glutamate-mediated methylamine utilization pathway in the facultative methylotrophic beta-proteobacterium *Methyloversatilis universalis* FAM5. *Mol Microbiol* **75**: 426-439.
Lee, C. (1988) Amino acid and amine biogeochemistry in marine particulate material and sediments. In *Nitrogen cycling in coastal marine environments*. Blackburn, T.H., and Sorensen, J. (eds). Chichester: John Wiley and Sons, pp. 125-141.
McIntire, W.S., Dooley, D.M., McGuirl, M.A., Cote, C.E., and Bates, J.L. (1990) Methylamine oxidase from *Arthrobacter* P1 as a prototype of eukaryotic plasma amine oxidase and diamine oxidase. In *Amine Oxidases and Their Impact on Neurobiology*. Riederer, P., and Youdim, M.H. (eds): Springer Vienna, pp. 315-318.
McTaggart, T.L., Beck, D.A., Setboonsarng, U., Shapiro, N., Woyke, T., Lidstrom, M.E. et al. (2015) Genomics of methylotrophy in gram-positive methylamine-utilizing bacteria. *Microorganisms* **3**: 94-112.
Morris, R.M., Rappe, M.S., Connon, S.A., Vergin, K.L., Siebold, W.A., Carlson, C.A., and Giovannoni, S.J. (2002) SAR11 clade dominates ocean surface bacterioplankton communities. *Nature* **420**: 806-810.
Moussard, H., Stralis-Pavese, N., Bodrossy, L., Neufeld, J.D., and Murrell, J.C. (2009) Identification of active methylotrophic bacteria inhabiting surface sediment of a marine estuary. *Env Microbiol Rep* **1**: 424-433.
Murrell, J.C., and Lidstrom, M.E. (1983) Nitrogen-metabolism in *Xanthobacter* H4-14. *Arch Microbiol* **136**: 219-221.
Naqvi, S.W.A., Bange, H.W., Gibb, S.W., Goyet, C., Hatton, A.D., and Upstill-Goddard, R.C. (2005) Biogeochemical ocean-atmosphere transfers in the Arabian Sea. *Prog Oceanogr* **65**: 116-144.
Nayak, D.D., Agashe, D., Lee, M.C., and Marx, C.J. (2016) Selection maintains apparently degenerate metabolic pathways due to tradeoffs in using methylamine for carbon versus nitrogen. *Curr Biol* **26**: 1416-1426.
Neff, J.C., Holland, E.A., Dentener, F.J., McDowell, W.H., and Russell, K.M. (2002) The origin, composition and rates of organic nitrogen deposition: A missing piece of the nitrogen cycle? *Biogeochemistry* **57**: 99-136.
Neufeld, J.D., Schafer, H., Cox, M.J., Boden, R., McDonald, I.R., and Murrell, J.C. (2007a) Stable-isotope probing implicates *Methylophaga* spp and novel Gammaproteobacteria in marine methanol and methylamine metabolism. *ISME J* **1**: 480-491.
Neufeld, J.D., Vohra, J., Dumont, M.G., Lueders, T., Manefield, M., Friedrich, M.W., and Murrell, J.C. (2007b) DNA stable-isotope probing. *Nat Protoc* **2**: 860-866.
Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., and Tyson, G.W. (2015) CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* **25**: 1043-1055.
Pinhassi, J., DeLong, E.F., Beja, O., Gonzalez, J.M., and Pedros-Alio, C. (2016) Marine bacterial and archaeal ion-pumping rhodopsins: genetic diversity, physiology, and ecology. *Microbiol Mol Biol Rev* **80**: 929-954.

Radajewski, S., McDonald, I.R., and Murrell, J.C. (2003) Stable-isotope probing of nucleic acids: a window to the function of uncultured microorganisms. *Curr Opin Biotech* **14**: 296-302.
Rappe, M.S., and Giovannoni, S.J. (2003) The uncultured microbial majority. *Annu Rev Microbiol* **57**: 369-394.
Reisch, C.R., Moran, M.A., and Whitman, W.B. (2008) Dimethylsulfoniopropionate-dependent demethylase (DmdA) from *Pelagibacter ubique* and *Silicibacter pomeroyi*. *J Bacteriol* **190**: 8018-8024.
Rusch, D.B., Halpern, A.L., Sutton, G., Heidelberg, K.B., Williamson, S., Yooseph, S. et al. (2007) The Sorcerer II Global Ocean Sampling expedition: Northwest Atlantic through Eastern Tropical Pacific. *PLOS Biol* **5**: 398-431.
Schaefer, J.K., Goodwin, K.D., McDonald, I.R., Murrell, J.C., and Oremland, R.S. (2002) *Leisingera methylohalidivorans* gen. nov., sp nov., a marine methylotroph that grows on methyl bromide. *Int J Syst Evol Micr* **52**: 851-859.
Schäfer, H., McDonald, I.R., Nightingale, P.D., and Murrell, J.C. (2005) Evidence for the presence of a CmuA methyltransferase pathway in novel marine methyl halide-oxidizing bacteria. *Environ Microbiol* **7**: 839-852.
Smith, D.P., Thrash, J.C., Nicora, C.D., Lipton, M.S., Burnum-Johnson, K.E., Carini, P. et al. (2013) Proteomic and transcriptomic analyses of "*Candidatus* Pelagibacter ubique" describe the first PII-independent response to nitrogen limitation in a free-living Alphaproteobacterium. *Mbio* **4**: e00133-00112.
Strand, S.E., and Lidstrom, M.E. (1984) Characterization of a new marine methylotroph. *FEMS Microbiol Lett* **21**: 247-251.
Sun, J., Steindler, L., Thrash, J.C., Halsey, K.H., Smith, D.P., Carter, A.E. et al. (2011) One carbon metabolism in SAR11 pelagic marine bacteria. *PLOS One* **6**.
Taubert, M., von Bergen, M., and Seifert, J. (2013) Limitations in detection of N-15 incorporation by mass spectrometry in protein-based stable isotope probing (protein-SIP). *Anal Bioanal Chem* **405**: 3989-3996.
Todd, J.D., Rogers, R., Li, Y.G., Wexler, M., Bond, P.L., Sun, L. et al. (2007) Structural and regulatory genes required to make the gas dimethyl sulfide in bacteria. *Science* **315**: 666-669.
Tripp, H.J. (2013) The unique metabolism of SAR11 aquatic bacteria. *J Microbiol* **51**: 147-153.
Vandecandelaere, I., Segaert, E., Mollica, A., Faimali, M., and Vandamme, P. (2008) *Leisingera aquimarina* sp nov., isolated from a marine electroactive biofilm, and emended descriptions of *Leisingera methylohalidivorans* Schaefer et al. 2002, *Phaeobacter daeponensis* Yoon et al. 2007 and *Phaeobacter inhibens* Martens et al. 2006. *Int J Syst Evol Micr* **58**: 2788-2793.
Vuilleumier, S., Chistoserdova, L., Lee, M.C., Bringel, F., Lajus, A., Zhou, Y. et al. (2009) *Methylobacterium* genome sequences: a reference blueprint to investigate microbial metabolism of C1 compounds from natural and industrial sources. *PLOS One* **4**.
Weiner, J.H., Rothery, R.A., Sambasivarao, D., and Trieber, C.A. (1992) Molecular analysis of dimethylsulfoxide reductase - a complex iron-sulfur molybdoenzyme of *Escherichia coli*. *Biochim Biophys Acta* **1102**: 1-18.
Wischer, D., Kumaresan, D., Johnston, A., El Khawand, M., Stephenson, J., Hillebrand-Voiculescu, A.M. et al. (2015) Bacterial metabolism of methylated amines and identification of novel methylotrophs in Movile Cave. *ISME J* **9**: 195-206.

Table and Figure Legends

Figure 1: Phylogenetic profiles of samples from the methylamine SIP experiment obtained by 16S rRNA gene amplicon sequencing. Relative abundance of taxonomic groups within each sample is shown at the family level as percentages and coloured bars. Profiles are derived from total DNA extracted from seawater samples collected at the Western Channel Observatory Station L4 (T0) and after incubating the same seawater with ^{14}N or ^{15}N methylamine and 0.1% MAMS (NH_4^+ 15 µM final conc.) for 3, 6 or 8 days, when 75, 150 and 300 µmol of methylamine had been consumed, respectively. Mean values from 3 replicates (incubation for 3, 6 and 8 days) or 2 replicates (T0) and standard deviations are shown. Families containing less than 3% of sequences are combined in the "unknown/other" category.

Figure 2: Incorporation of ^{15}N into peptides after 3 days of incubation of seawater with ^{15}N methylamine. Boxplots show median, first and third quartile for ^{15}N relative isotope abundance in unique peptides of Gammaproteobacterium strain IMCC2047, *Leisingera*, *C.* Pelagibacter and *Methylophaga*. Whiskers indicate minimum and maximum values. ***All four groups differ significantly from each other ($p < 0.001$, *t*-test).

Figure 3: Hypothetical overview of nitrogen utilization by the major phylogenetic groups identified in the ^{15}N methylamine SIP experiment. Red discs show release of ammonium by methylamine utilization. Enzymes shown in black have been detected by metaproteomics. Enzymes shown in grey have not been detected, but the corresponding organisms possess the genes encoding these enzymes. GmaS: γ-glutamylmethylamide synthetase, Mgs: *N*-methylglutamate synthase, Mgd: *N*-methylglutamate dehydrogenase, MauAB: methylamine dehydrogenase; GS/GOGAT: Glutamine synthetase/ Glutamine 2-oxoglutarate amidotransferase pathway of ammonium assimilation.

Table 1: Summary of genome statistics based on CheckM analysis (see Parks et al., 2015). N50/L50: length and number of the contig for which the collection of all contigs of at least that length contains at least half of the total length of the genome. Strain heterogeneity comprises the fraction of core genes present multiple times with a protein sequence similarity > 90%.

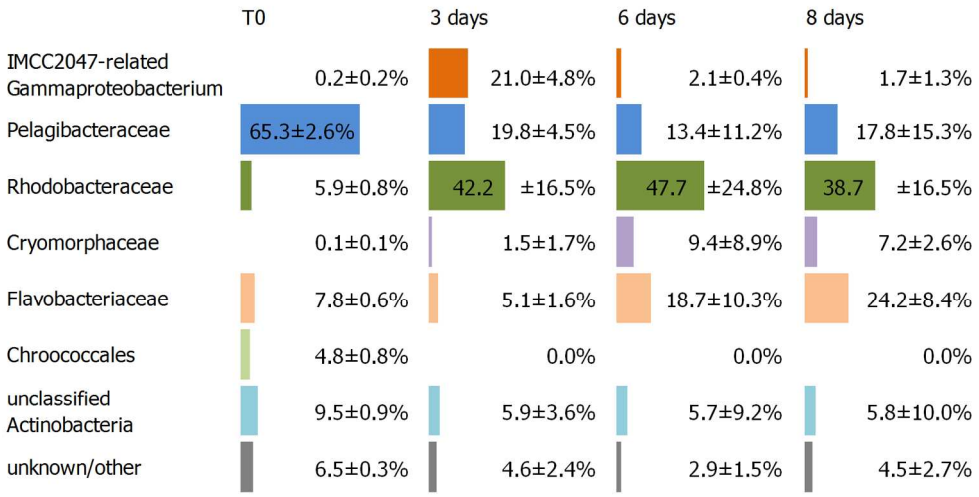

Figure 1: Phylogenetic profiles of samples from the methylamine SIP experiment obtained by 16S rRNA gene amplicon sequencing. Relative abundance of taxonomic groups within each sample is shown at the family level as percentages and coloured bars. Profiles are derived from total DNA extracted from seawater samples collected at the Western Channel Observatory Station L4 (T0) and after incubating the same seawater with ^{14}N or ^{15}N methylamine and 0.1% MAMS (NH_4^+ 15 µM final conc.) for 3, 6 or 8 days, when 75, 150 and 300 µmol of methylamine had been consumed, respectively. Mean values from 3 replicates (incubation for 3, 6 and 8 days) or 2 replicates (T0) and standard deviations are shown. Families containing less than 3% of sequences are combined in the "unknown/other" category.

Fig. 1
162x82mm (300 x 300 DPI)

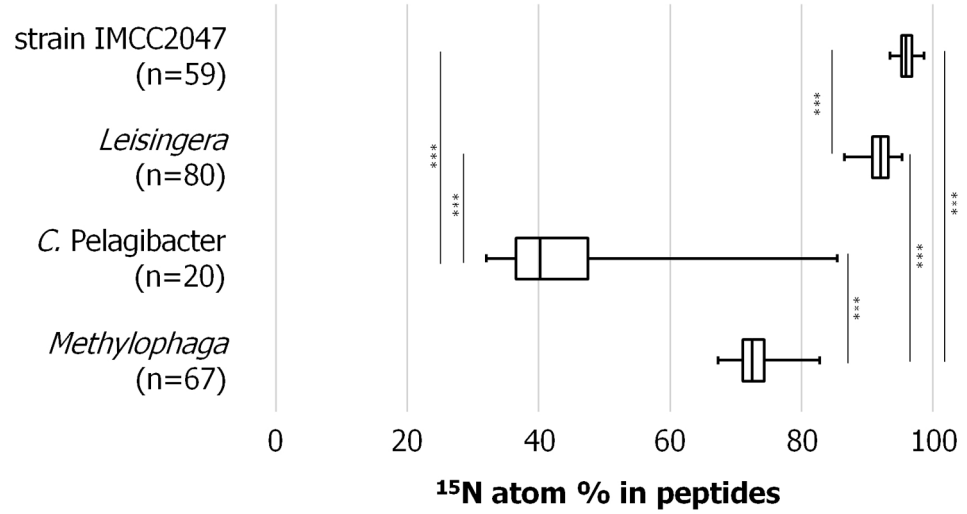

Figure 2: Incorporation of ^{15}N into peptides after 3 days of incubation of seawater with ^{15}N methylamine. Boxplots show median, first and third quartile for ^{15}N relative isotope abundance in unique peptides of Gammaproteobacterium strain IMCC2047, *Leisingera*, *C.* Pelagibacter and *Methylophaga*. Whiskers indicate minimum and maximum values. ***All four groups differ significantly from each other ($p < 0.001$, t-test).
Fig. 2
154x80mm (300 x 300 DPI)

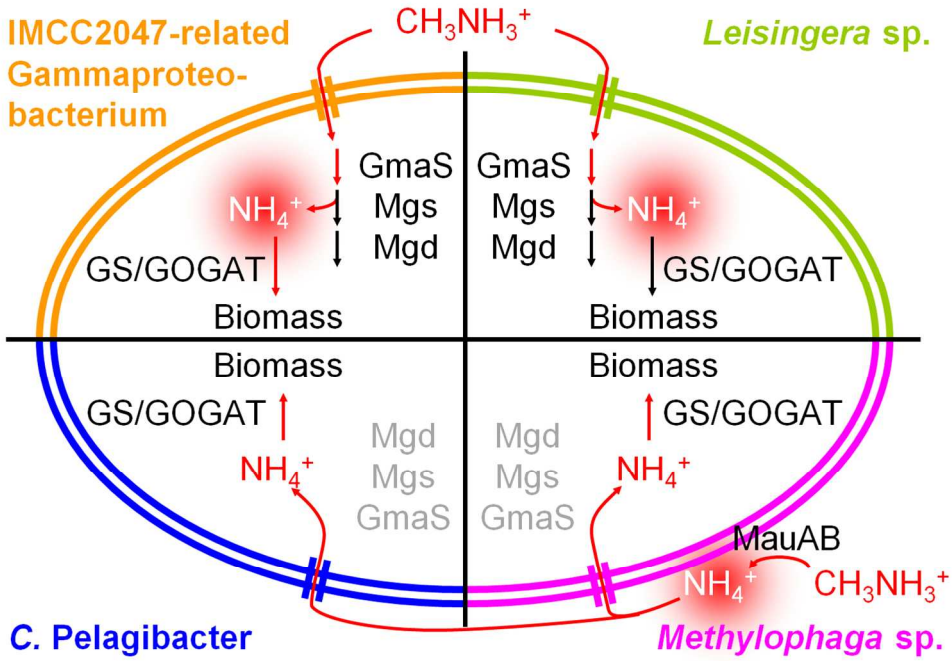

Figure 3: Hypothetical overview of nitrogen utilization by the major phylogenetic groups identified in the ^{15}N methylamine SIP experiment. Red discs show release of ammonium by methylamine utilization. Enzymes shown in black have been detected by metaproteomics. Enzymes shown in grey have not been detected, but the corresponding organisms possess the genes encoding these enzymes. GmaS: γ-glutamylmethylamide synthetase, Mgs: *N*-methylglutamate synthase, Mgd: *N*-methylglutamate dehydrogenase, MauAB: methylamine dehydrogenase; GS/GOGAT: Glutamine synthetase/ Glutamine 2-oxoglutarate amidotransferase pathway of ammonium assimilation.
Fig. 3
132x87mm (300 x 300 DPI)

Table 1: Summary of genome statistics based on CheckM analysis (see Parks et al., 2015). N50/L50: length and number of the contig for which the collection of all contigs of at least that length contains at least half of the total length of the genome. Strain heterogeneity comprises the fraction of core genes present multiple times with an identity of > 90% on amino acid level.

	fraction 5 *Leisingera*	fraction 7 IMCC2047[1]	*Methylophaga* str. L4[2]
no. of contigs	1488	214	8
total length / bp	7,813,916	2,494,546	2,589,653
N50 / bp	52,902	36,700	397,852
L50	32	22	3
GC / %	61.8%	48.5%	45.7%
no. of predicted genes	8,600	2,656	2,521
core genes present	100%	88.9%	100%
core genes present multiple times	66.1%	3.0%	0.3%
strain heterogeneity	87.9%	44.4%	66.7%
core genes related to other taxa	8.0%	1.7%	0.1%

[1]IMCC2047-related Gammaproteobacterium
[2]*Methylophaga thiooxydans* strain L4, Accession JRQD01, from Grob et al., 2015