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1 **Combining metagenomics and metaproteomics reveals metabolic pathways used by an**  
2 **uncultivated marine methanol utiliser**

3

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16 Running title: metagenomics, metaproteomics and methylotrophy

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19

## 20 **Summary**

21 In order to better understand how the activity of microbial organisms influences and regulates  
22 all major biogeochemical cycles, a great deal of effort has been put into characterizing the  
23 physiology and metabolism of key representatives available in culture. Such effort is often  
24 limited by the difficulties associated with isolating novel microorganisms from the  
25 environment and cultivating them in the laboratory. To overcome this problem, a variety of  
26 culture-independent techniques have been developed that can be used in conjunction with the  
27 above to investigate natural microbial populations. In this study, we combined DNA-stable  
28 isotope probing with metagenomics and metaproteomics to characterize an as yet  
29 uncultivated marine methylotroph that actively incorporated carbon from  $^{13}\text{C}$ -labeled  
30 methanol into biomass. By metagenomic sequencing of the heavy DNA, we retrieved  
31 virtually the whole genome of this bacterium and identified through protein-stable isotope  
32 probing the metabolic pathways used to assimilate methanol. This proof-of-concept study is  
33 the first in which both DNA- and protein-stable isotope probing has been used to characterize  
34 the metabolism of an uncultivated bacterium from the marine environment and thus provides  
35 a powerful approach to access the genome and proteome of uncultivated microbes involved  
36 in key processes in the environment.

## 37 **Introduction**

38 One of the main challenges in microbial ecology is to directly access the genomes and  
39 understand the metabolism of key microbes involved in biogeochemical cycling. An ideal  
40 scenario is to isolate model organisms and then characterize them using conventional  
41 physiological and biochemical techniques. However, since many microbes are difficult to  
42 cultivate in the laboratory, focussed cultivation-independent techniques are also required. To  
43 address these challenges, DNA-Stable Isotope Probing (DNA-SIP), involving the use of  $^{13}\text{C}$ -  
44 labeled substrates that are incorporated into the biomass of active microbes, has been  
45 developed (Radajewski *et al.*, 2000, Dumont and Murrell, 2005, Neufeld *et al.*, 2007b). This  
46 technique enables the separation of heavy ( $^{13}\text{C}$ -labeled) from light (unlabeled) DNA, thus  
47 allowing the isolation of  $^{13}\text{C}$ -DNA from microbes that have assimilated the target  $^{13}\text{C}$ -labeled  
48 substrate from those that have not. The identity of active cells can then be determined by  
49 interrogating the heavy DNA via PCR using 16S rRNA or genes encoding key steps in  
50 microbial metabolism.

51 Further information regarding the metabolic potential and the metabolic pathways actually  
52 being used by target microorganisms during SIP incubations can be obtained by  
53 complementary metagenome analysis of the heavy DNA combined with metaproteome  
54 analysis, i.e., protein-SIP. This approach yields quantitative data on the incorporation of  
55 heavy isotopes of carbon, nitrogen or sulfur into peptides (Seifert *et al.*, 2012) and their  
56 sequence analysis results on information on both the phylogeny and physiology of microbial  
57 organisms (von Bergen *et al.*, 2013).

58 In this study, we provide proof-of-concept experiments to show that DNA- and protein-SIP  
59 can be combined with metagenomics to characterize the metabolism of an as yet uncultivated  
60 marine bacterium. We chose to use methanol and methylotrophic bacteria in the marine  
61 environment to develop these techniques because in our previous studies we showed that

62 marine methylotrophs of the genus *Methylophaga* were present and active in coastal seawater  
63 environments (station L4 of the Western Channel Observatory, Plymouth, UK) and that we  
64 could use DNA-SIP to recover genes involved in methanol oxidation from this uncultivated  
65 *Methylophaga* species (Neufeld *et al.*, 2007a, Neufeld *et al.*, 2008b, Neufeld *et al.*, 2008a).  
66 We previously showed that methanol is metabolized *in situ* at station L4 (Dixon *et al.*, 2011,  
67 Sargeant, 2013) and therefore in this study we used methylotrophy as a model system with  
68 which to combine for the first time DNA- and protein-SIP approaches to access the  
69 metabolism of a marine *Methylophaga* which we have failed to isolate and cultivate in the  
70 laboratory.

71 A key enzyme involved in methanol metabolism by methylotrophs, methanol dehydrogenase  
72 (MDH), catalyses the conversion of methanol to formaldehyde (Anthony, 1982,  
73 Chistoserdova, 2011). The gene coding for the large subunit of the classical MDH, *mxoF*, has  
74 been well characterized (Anthony *et al.*, 1994). A homologue of *mxoF* gene, *xoxF*, which can  
75 also be involved in methanol metabolism, is present in all known methylotrophs and several  
76 non-methylotrophic organisms (Chistoserdova and Lidstrom, 1997, Chistoserdova *et al.*,  
77 2009, Chistoserdova, 2011). Multiple *xoxF* genes, sometimes belonging to more than one of  
78 the five distinct *xoxF* clades that have been described (Chistoserdova, 2011, Keltjens *et al.*,  
79 2014), can often be found in a single methylotroph genome, making it difficult to  
80 unequivocally assign a functional role to this gene (Chistoserdova, 2011). Based on  
81 sequencing data, specific PCR primer sets have been designed to target *mxoF* (McDonald and  
82 Murrell, 1997, Neufeld *et al.*, 2007a) and *xoxF* genes (Taubert *et al.*, *in revision*) and thus  
83 determine the distribution and diversity of methylotrophic bacteria in the environment. The  
84 presence of these functional biomarkers alone does not however imply that they are  
85 metabolically active.

86 In this study, a combination of DNA-SIP and protein-SIP, metagenomics and  
87 metaproteomics, 16S rRNA gene, *mxoF* and *xoxF* functional gene amplicon sequencing was  
88 used to identify the phylogenetic affiliation and methanol utilization pathways of a marine  
89 methylotroph. We estimate that more than 90% of the genome of a marine *Methylophaga*  
90 species from the English Channel was obtained and concomitant metaproteomics analysis  
91 revealed the pathways of carbon assimilation used by this uncultivated methylotroph.

## 92 **Results and discussion**

93 SIP incubations were carried out in duplicate over three days using surface seawater from  
94 station L4 in the English Channel, with <sup>13</sup>C-labeled and unlabeled (<sup>12</sup>C) methanol (control) as  
95 substrate. The purpose of this study was to use SIP to access the genome and proteome of an  
96 uncultivated marine methylotroph, so we chose a substrate concentration that we could  
97 confidently measure to monitor methanol consumption throughout the experiment, i.e., 100  
98 μM. Given that surface methanol concentrations at station L4 are in the 16-78 nM range  
99 (Beale *et al.*, 2015), it could be argued that this concentration is not environmentally relevant.  
100 However, we have previously shown that even 1 μM stimulates the activity of *Methylophaga*  
101 from station L4 (Neufeld *et al.*, 2008a). We therefore believe that the methanol concentration  
102 used here is suitable for the purpose of the proof-of-concept study presented here.

103 Total DNA was extracted from seawater at the beginning and end of the experiment and used  
104 to determine bacterial diversity. To assess the metabolic potential of the bacterial community,  
105 protein was also extracted from SIP incubations after three days. After separating <sup>13</sup>C-DNA  
106 from <sup>12</sup>C-DNA, the former was used to determine the diversity of active methylotrophs in  
107 DNA-SIP incubations by isolation and analysis of 16S rRNA, *mxoF* and *xoxF* gene sequences  
108 (Supplementary Table S1, for the total number of sequences retrieved from each sample). <sup>13</sup>C-  
109 DNA was also amplified using multiple displacement amplification to generate sufficient

110 material for metagenome sequencing and analysis of DNA of the dominant methylotroph in  
111 duplicate SIP incubations.

112 **Combining DNA- and Protein-SIP.** Bacterial community composition at the beginning of  
113 the incubations (T0) was determined by analysis of 16S rRNA gene sequences from duplicate  
114 DNA samples. Contributions of different bacterial groups to the total 16S rRNA gene  
115 sequences retrieved from each sample (Supplementary Table S1) were virtually identical (not  
116 shown), so average values are given below. 16S rRNA gene sequence analysis showed that at  
117 T0, the bacterial community was mainly dominated by Alphaproteobacteria (e.g., *Candidatus*  
118 *Pelagibacter* constituted 21% of all 16S rRNA gene sequences retrieved), Betaproteobacteria  
119 (e.g., *Achromobacter*, 15%), and Flavobacteria (e.g., *Formosa*, 15%) (Figure 1A). These  
120 results agree with previous studies carried out at station L4 where Alphaproteobacteria 16S  
121 rRNA gene sequences, particularly those belonging to the SAR11 clade (Pelagibacteraceae)  
122 are predominant throughout most of the year followed by Flavobacteria, with Beta- and  
123 Gammaproteobacteria also being present (Gilbert *et al.*, 2009, Gilbert *et al.*, 2012, Sargeant,  
124 2013). Eighty different bacterial genera that constituted less than 5% of the total number of  
125 sequences could also be identified at the beginning of the SIP incubations (combined under  
126 “Others” in Figure 1A), with potential methanol utilizers, such as *Methylophaga*, *Ruegeria*  
127 and *Roseovarius* representing <0.5% of the total 16S rRNA gene sequences analyzed.

128 After incubating for three days with 100  $\mu$ M methanol, changes in community composition  
129 were assessed based on 16S rRNA gene sequences retrieved from unfractionated DNA  
130 obtained from duplicate  $^{13}\text{C}$  and  $^{12}\text{C}$  methanol incubations (Supplementary Table S1).  
131 Compared with T0, the bacterial community in all four experiments was significantly  
132 enriched in Gammaproteobacteria (75-87% of the sequences; Supplementary Figure S1). At  
133 the methanol concentration used in these SIP incubations, 84% of the 16S rRNA gene  
134 sequences retrieved from the unfractionated  $^{13}\text{C}$ -labeled samples belonged to the genus

135 *Methylophaga* (Figure 1A), which only represented 0.01% of the sequences at T0 (included in  
136 “Others” in Figure 1A). Other bacteria present at the start of the SIP incubations, such as  
137 *Candidatus Pelagibacter* (5%) and *Owenweeksia* (3%), were also present in the unfractionated  
138 DNA (Figure 1A).

139 CsCl density gradient centrifugation was used to separate heavy ( $^{13}\text{C}$ -labeled) from light ( $^{12}\text{C}$ ,  
140 unlabeled) DNA extracted from  $^{13}\text{C}$  methanol incubations, following the protocol described in  
141 Neufeld et al. (Neufeld et al., 2007b).  $^{13}\text{C}$ -DNA was subsequently used to determine the  
142 phylogenetic affiliation of active methylotrophs by targeting 16S rRNA gene, as well as *mxoF*  
143 and *xoxF* functional genes. Most of the 16S rRNA gene sequences present in  $^{13}\text{C}$ -DNA  
144 samples belonged to *Methylophaga*, thus indicating rapid incorporation of  $^{13}\text{C}$  from methanol  
145 into *Methylophaga* biomass (Figure 1B). *mxoF* and *xoxF* gene sequences obtained from  $^{13}\text{C}$ -  
146 DNA 454 data (not shown) confirmed that the enriched group was most closely related to  
147 *Methylophaga thiooxydans* DMS010 (Schäfer, 2007, Boden *et al.*, 2010). The amplicon  
148 sequencing results presented above agree with previous DNA-SIP experiments carried out at  
149 station L4 using methanol and other  $\text{C}_1$  substrates, such as mono- and dimethylamine,  
150 dimethylsulfide and methyl bromide, that showed that *Methylophaga* spp. present in the  
151 marine environment are capable of metabolizing these compounds (Neufeld et al., 2007a,  
152 Neufeld et al., 2008a, Neufeld et al., 2008b). In  $^{12}\text{C}$ -DNA samples, only 16% of the total 16S  
153 rRNA gene sequences retrieved from duplicate incubations were affiliated to *Methylophaga*  
154 whereas *Candidatus Pelagibacter* dominated (60%), despite being present at only 0.1% in the  
155  $^{13}\text{C}$ -DNA fraction (Figure 1B). Although this might seem high, the proportion of *Candidatus*  
156 *Pelagibacter* sequences at the end of the incubations (unfractionated DNA in Figure 1A) was  
157 lower than at the beginning (T0 in Figure 1A).

158 To complement the DNA-SIP data, proteins were extracted from  $^{13}\text{C}$ -labeled and unlabeled  
159 (controls) methanol SIP incubations after three days. Tryptic peptides were measured using a



160 high resolution Orbitrap mass spectrometer and further identified using the OpenMS pipeline  
161 (Kohlbacher *et al.*, 2007, Sturm *et al.*, 2008) via the OMSSA search engine (Geer *et al.*,  
162 2004). Protein identity and taxonomic affiliation were determined using a customised NCBI nr  
163 database (Supplementary Information). 79% of the peptides identified from these protein  
164 samples were assigned to *Methylophaga* species and most of the peptides affiliated with this  
165 group showed <sup>13</sup>C incorporation from methanol (Figure 1C; Supplementary Dataset S1), with  
166 an average relative isotope abundance of 88.8% +/- 2.8%. No unlabeled *Methylophaga*  
167 peptides were detected in <sup>13</sup>C-labeled incubations, showing that the majority of *Methylophaga*  
168 biomass (>99% based on the detection limit of the instrument, not shown) was produced after  
169 the addition of labeled methanol. This confirms that *Methylophaga* constituted only a minor  
170 fraction of the bacterial community at the beginning of the experiment, as observed with 16S  
171 rRNA gene sequences data. Finally, ~13% of all peptides found to have <sup>13</sup>C incorporation  
172 patterns related to crossfeeding were identified as SAR11 peptides (not shown). This suggests  
173 that SAR11 cells were still active during the three day incubation with 100 µM methanol,  
174 having incorporated <sup>13</sup>C-labeled carbon into their peptides through crossfeeding but not by the  
175 direct use of this substrate as a carbon source. This is consistent with previous reports  
176 showing that members of the SAR11 clade to which *Candidatus Pelagibacter* found in <sup>12</sup>C-  
177 DNA belongs can oxidize methanol to CO<sub>2</sub>, but do not seem to use it as a carbon source (Sun  
178 *et al.*, 2011).

179 **Metagenomics of <sup>13</sup>C-labeled DNA.** In order to investigate the metabolic potential of the  
180 organisms that were actively incorporating methanol into their biomass, heavy DNA from the  
181 <sup>13</sup>C-labeled experiment from two biological replicates was amplified using multiple  
182 displacement amplification and the amplified <sup>13</sup>C-DNA was used for metagenome sequencing  
183 on the Illumina MiSeq DNA sequencing platform (Supplementary Information).

184 16S rRNA gene sequences retrieved from <sup>13</sup>C-DNA metagenome datasets again showed the  
185 dominance of *Methylophaga* species, with >50% of them (in both replicates) having been  
186 assigned to *M. thiooxydans* using the Ribosomal Database Project (RDP) database  
187 (Supplementary Figure S2). Metagenome data were assembled in BaseSpace using SPAdes  
188 Genome Assembler v3.0 and the assembled contigs were annotated in RAST, followed by  
189 manual correction. The assembly of the whole metagenome dataset yielded 8 large contigs  
190 clearly belonging to *Methylophaga* with a total length of 2.60 Mb, an average coverage of 98x  
191 and a GC content of 45.7% (Supplementary Dataset S2). Of the remaining reads, assembled  
192 into 5,557 much smaller contigs (4.28 Mb and 13x coverage in total), less than 1% belonged  
193 to *Methylophaga*. The *Methylophaga* L4 genome derived from the SIP metagenome dataset  
194 was most closely related to the genome of *Methylophaga thiooxydans* DMS010 (Schäfer,  
195 2007, Boden et al., 2010), a strain originally isolated from an enrichment culture of the  
196 coccolithophore *Emiliana huxleyi* (Schäfer, 2007). First described as *Methylophaga* sp. strain  
197 DMS010, this species grows on dimethylsulfide (DMS) and a variety of other C<sub>1</sub> compounds  
198 (Schäfer, 2007). *Methylophaga* sp. strain DMS010 was renamed *M. thiooxydans* DMS010  
199 after a new pathway of DMS metabolism (DMS oxidation to tetrathionate) was discovered in  
200 this organism (Boden et al., 2010).

201 Although *M. thiooxydans* DMS010 has a genome size of 3.05 Mb (Boden et al., 2011), closer  
202 investigation of this genome uncovered a series of identical phage-like regions that when  
203 removed left a core genome of 2.59 Mb. Hence, the 8 contigs of the *M. thiooxydans* strain L4  
204 genome assembled from the metagenome very likely cover > 90% of the genome of this SIP-  
205 enriched *Methylophaga* species (Supplementary Figure S3). This SIP-metagenome-derived  
206 genome of *M. thiooxydans* strain L4 is also comparable in size with the genomes of  
207 *Methylophaga* strains *M. nitratireducenticrescens* JAM1 (3.1 Mb) and *M. frappieri* JAM7  
208 (2.7 Mb) (Villeneuve et al., 2012, Villeneuve et al., 2013). Finally, given the high level of

209 coverage of the genome, the use of DNA-SIP targeted metagenomic would seem to be a  
210 useful complementary molecular ecology technique to single-cell whole genome sequencing.  
211 Analysis of the *M. thiooxydans* strain L4 genome assembled here revealed the presence of key  
212 genes involved in one-carbon metabolism (Supplementary Dataset S3). The entire gene  
213 cluster coding for the small (*mxal*) and large (*mxaf*) subunits of the pyrroloquinoline quinone  
214 (PQQ)-dependent methanol dehydrogenase and accessory genes was found (Figure 2). The  
215 full length *mxaf* gene retrieved from the assembled genome was 98% and 99% percent  
216 identical to the *mxaf* gene sequence and the derived amino acid sequence of *M. thiooxydans*  
217 DMS010, respectively (Supplementary Figure S4A). Four copies of the *mxaf* homolog *xoxF*  
218 were also identified (Supplementary Figure S5). Their sequences were 93-97% identical to *M.*  
219 *thiooxydans* DMS010 (Supplementary Figure S4B).

220 Two gene clusters containing genes involved in the conversion of methylamine to  
221 formaldehyde via two different pathways were also identified. One gene cluster,  
222 *mauFBEDAGLMN*, contains genes encoding the large (*mauB*) and small (*mauA*) subunit of  
223 methylamine dehydrogenase, a TTQ-dependent dehydrogenase, as well as further accessory  
224 genes required for the activity of this enzyme (Supplementary Figure S6; Anthony, 1982).  
225 Methylamine dehydrogenase catalyses the direct oxidation of methylamine to formaldehyde  
226 and ammonium and is typically found in Proteobacteria that use methylamine as a carbon  
227 source (Wischer *et al.*, 2014). The second gene cluster encodes genes for gamma-  
228 glutamylmethylamide (GMA) synthetase (*gmaS*), N-methylglutamate (NMG) synthase  
229 (*mgsABC*) and NMG dehydrogenase (*mgdABCD*). In this pathway, methylamine is oxidised  
230 in a stepwise conversion via the methylated amino acids GMA and NMG to 5,10-  
231 methylenetetrahydrofolate (Latypova *et al.*, 2010, Chen *et al.*, 2010; Supplementary Figure  
232 S6). This pathway is found in methylotrophs as well as non-methylotrophs that use  
233 methylamine as nitrogen source (Wischer *et al.*, 2014).

234 Further analysis of the *M. thiooxydans* strain L4 genome enabled reconstruction of key steps  
235 in carbon and nitrogen metabolism (Table 1). Carbon fixation from formaldehyde could occur  
236 via the ribulose monophosphate (RuMP; Johnson and Quayle, 1965) cycle (Entner-Doudoroff  
237 variant), as all the necessary genes are present in the genome. Genes encoding key enzymes of  
238 other potential carbon fixation pathways, such as the serine and Calvin-Benson-Bassham  
239 (CBB) cycles were missing, indicating the likely absence of these pathways in *M. thiooxydans*  
240 strain L4 (Table 1). Both Entner-Doudoroff and pentose phosphate pathways were complete,  
241 but no gene encoding 6-phosphofructokinase, a key enzyme of the glycolysis pathway, was  
242 found. Genes encoding the 2-oxoglutarate dehydrogenase complex of the tricarboxylic acid  
243 (TCA) cycle, *sucA* and *sucB*, were also missing (Table 1), while the *lpd* gene encoding the  
244 lipoamide dehydrogenase was only found in conjunction with the pyruvate dehydrogenase  
245 complex. All other genes of the TCA cycle were detected. These results are consistent with  
246 the notion that bacteria using the RuMP cycle as their major carbon assimilation pathway tend  
247 to have an incomplete TCA cycle (Anthony, 1982). The resulting incomplete TCA cycle  
248 could still channel carbon from pyruvate to all intermediates that may be required for central  
249 biosynthetic pathways, including oxoglutarate and succinyl-CoA, but cannot be used to  
250 generate energy by oxidising carbon compounds to CO<sub>2</sub>. To produce reducing power through  
251 the oxidation of formaldehyde to CO<sub>2</sub>, two potential pathways were present: the dissimilatory  
252 hexulose phosphate (HuP) cycle (Chistoserdova *et al.*, 2000) and the tetrahydromethanopterin  
253 (H<sub>4</sub>MPT)-dependent oxidation pathway (Vorholt *et al.*, 2000). Regarding nitrogen  
254 metabolism, genes for assimilatory nitrate reduction to ammonium, including a assimilatory  
255 nitrate reductase and a NAD(P)H-dependent nitrite reductase, were present in the genome of  
256 *M. thiooxydans* strain L4, but no genes linked to dissimilatory nitrate reduction were found.  
257 The glutamine synthase/glutamine 2-oxoglutarate amidotransferase (GS/GOGAT) pathway  
258 for ammonia assimilation (Trotsenko and Murrell, 2008), as well as alanine dehydrogenase

259 and glutamate dehydrogenase genes, were also present. For sulfur metabolism, all genes  
260 required for assimilatory reduction of sulfate to sulfide were present in the genome, and  
261 furthermore all genes required for cysteine biosynthesis were found. No genes specific for  
262 dissimilatory sulfate reduction were present. Finally, the biosynthetic pathway for ectoine, a  
263 common bacterial osmolyte, was found, coinciding with the marine habitat of *M. thiooxydans*  
264 strain L4.

265 Using a similar targeted metagenomics approach, Kalyuzhnaya and collaborators  
266 (Kalyuzhnaya *et al.*, 2008) were able to retrieve the near complete genome of a novel  
267 methylotroph, *Methylothera mobilis*, from Lake Washington sediment. By complementing  
268 this culture-independent approach with metaproteomic analyses, here we further determined  
269 which of the metabolic pathways identified in the reconstructed genome were being expressed  
270 by *Methylophaga thiooxydans* strain L4 during growth on methanol, thus gaining a deeper  
271 understanding of the metabolism of this organism.

272 **Metaproteomics and metabolic reconstruction.** An LC-MS/MS analysis of proteolytic  
273 peptides lysates from proteomes isolated after three day SIP incubations was performed and  
274 analyzed using the predicted proteins of the *M. thiooxydans* strain L4 genome as reference  
275 database. Of the 2,522 protein-encoding genes predicted in the eight contigs of the *M.*  
276 *thiooxydans* strain L4, 737 were identified, accounting for 29% of the proteome of this  
277 *Methylophaga* species (Supplementary Dataset S4). Based on these data, metaproteomic  
278 reconstruction of the central carbon metabolic pathways of *M. thiooxydans* strain L4 growing  
279 on methanol was achieved (Figure 3). The large subunit MxaF of methanol dehydrogenase  
280 was detected along with some of the accessory proteins, MxaD, MxaE, MxaJ, MxaG, MxaR  
281 and MxaL, indicating expression of the inducible *mxafJGIRSACKL* operon (Amaratunga *et*  
282 *al.*, 1997, Toyama *et al.*, 1998), as predicted from the metagenome. An additional  
283 *mxarsacklc* operon (Beck *et al.*, 2014) and at least three of the four alternative methanol

284 dehydrogenase genes *xoxF* were expressed, together with the associated *xoxJ*. Since four out  
285 of the five methanol dehydrogenase genes identified in the metagenome-derived genome of  
286 *Methylophaga thiooxydans* strain L4 were expressed, the role of individual *xoxF* genes in  
287 methanol oxidation by this methylotroph remains unclear.

288 In *M. thiooxydans* strain L4, the formaldehyde resulting from oxidation of methanol is  
289 assimilated into cell material via the RuMP cycle (Johnson and Quayle, 1965). This was not  
290 surprising since all members of the Piscirickettsiaceae family (Chistoserdova and Lidstrom,  
291 2013), including *Methylophaga* spp. (Janvier *et al.*, 1985), have been reported to use this  
292 carbon assimilation pathway. All proteins required for the Entner-Doudoroff variant of this  
293 cycle and for the transketolase/transaldolase system were detected, including the key  
294 enzymes 3-hexulosephosphate synthase and 2-keto-3-dexoy-6-phosphogluconate (KDPG)  
295 aldolase (Trotsenko and Murrell, 2008, Chistoserdova, 2011). Formaldehyde is also oxidised  
296 to CO<sub>2</sub> via the H<sub>4</sub>MPT-dependent pathway to provide this methylotroph with energy for  
297 biosynthesis. In contrast, no 6-phosphogluconate dehydrogenase of the oxidative branch of  
298 the HuP pathway was detected, possibly indicating preferential use of the direct  
299 formaldehyde oxidation pathway via H<sub>4</sub>MPT.

300 The variant of the RuMP cycle detected in the metaproteome fixes three moles of  
301 formaldehyde into one mole of pyruvate as the central intermediate (Anthony, 1982). For the  
302 conversion of pyruvate, and also phosphoenolpyruvate (PEP), to further central intermediates  
303 such as acetyl-CoA and oxaloacetate, several enzymes were detected that connect to the TCA  
304 cycle, including PEP carboxylase, the pyruvate dehydrogenase complex, oxaloacetate  
305 decarboxylase and pyruvate carboxyl transferase. Also, all enzymes of the lower part of the  
306 Entner-Doudoroff pathway, following KDPG aldolase, were present, enabling the conversion  
307 of glyceraldehyde 3-phosphate to pyruvate and vice versa, via PEP synthase, establishing a  
308 further connection between carbon fixation and central carbon metabolism in *M. thiooxydans*

309 strain L4. Finally, the proteins required for oxidation of methylamine, from both the *mau* and  
310 the *gma/mgs/mgd* gene cluster, have not been identified in the proteome data. This is not  
311 surprising, as the incubations were carried out with methanol as growth substrate and these  
312 pathways are only induced in the presence of methylamine.

313 In this study, we combine for the first time DNA- and protein- stable isotope probing with  
314 metagenomics and functional gene amplicon sequencing to investigate the methanol  
315 metabolism of an uncultivated marine methylotroph. This focussed metagenomic approach  
316 yielded a near complete genome of an uncultivated *Methylophaga* species, *M. thiooxydans*  
317 strain L4, and metaproteomics analysis established the pathways of methanol metabolism in  
318 this bacterium. This focussed 'omics approach using <sup>13</sup>C-labeled substrates will have  
319 significant utility in cultivation-independent studies in microbial ecology.

## 320 **Experimental Procedures**

321 **Stable Isotope Probing (SIP) experimental set up.** Surface seawater for SIP experiments  
322 was collected from station L4 in the English Channel (50°15.0'N; 4°13.0'W) on September  
323 the 2<sup>nd</sup>, 2013. Four litres of seawater were filtered in duplicate through 0.22 µm Sterivex TM  
324 filters (Merck Millipore) using a peristaltic pump (Watson-Marlow 502S, 1 ml min<sup>-1</sup>), to  
325 extract DNA for analysis of the bacterial community composition at the start of the SIP  
326 experiment. Four 2 L gas-tight glass bottles were filled with 0.75 L of the same seawater,  
327 inoculated with 75 µmol of <sup>13</sup>C-labeled (2 bottles) or (<sup>12</sup>C) unlabeled (2 control bottles)  
328 methanol and incubated at 25°C in a shaking incubator (50 rpm) for three days. Methanol  
329 concentration in the incubation bottles was measured every day using an Agilent 7890A gas  
330 chromatograph (GC) equipped with a 7693A autosampler and a HP-5 column (see  
331 Supplementary Information). After incubation for three days, no methanol could be detected,  
332 indicating that sufficient <sup>13</sup>C had been incorporated during the SIP experiment. Seawater  
333 from all four SIP incubations was filtered through Sterivex filters using a 50 ml syringe. All

334 filters were stored at -20°C before extracting DNA and proteins within two weeks of the start  
335 of the experiment.

336 **DNA extraction.** DNA was extracted from Sterivex filters by adding 1.6 ml of SET buffer  
337 (0.75 M sucrose, 40 mM EDTA, 50 mM Tris-HCl pH 9) and 0.2 ml of 10% (w/v) SDS and  
338 incubating with rotation in a hybridization oven (Hybaid, Waltham, MA, USA) at 55°C for  
339 2.5 h. After incubation, two phenol:chloroform:isoamyl alcohol (25:24:1) extractions and a  
340 single chloroform:isoamyl extraction were performed before precipitating the DNA overnight  
341 at -20°C with a glycogen solution (Roche, Basel, Switzerland), 7.5 M ammonium acetate and  
342 ethanol, as previously described by Neufeld and collaborators (Neufeld et al., 2007a; see  
343 Supplementary Information). DNA was pelleted by centrifugation at 4,500 x g for 30 min  
344 before washing twice with 80% (v/v) ethanol, drying for 15 min at room temperature and  
345 resuspending in 50 µl of nuclease-free water.

346 **Protein extraction.** The phenol phases from the DNA extraction were combined and mixed  
347 with a five-fold volume of ice-cold 100 mM ammonium acetate in methanol and also left at  
348 -20°C overnight for precipitation. Samples were centrifuged for 30 min at 4,500 x g and  
349 protein pellets were washed twice with 100 mM ammonium acetate in methanol, twice with  
350 ice cold 80% (v/v) acetone and once with ice cold 70% (v/v) ethanol and finally dried at  
351 room temperature.

352 **DNA-SIP centrifugation and fractionation.** For each sample, 5 µg of DNA extracted from  
353 SIP incubations were added to a mixture of 7.163 M CsCl and Gradient Buffer (0.1 M Tris,  
354 0.1 M KCl and 1 mM EDTA) set to a final density of 1.725 g ml<sup>-1</sup> before centrifugation for  
355 40 hrs at 20°C and 44,100 rpm (~177,000 x g) with vacuum, maximum acceleration and no  
356 brake, using a Vti 65.2 rotor and a Optima™ LE-80K Ultracentrifuge (Beckman Coulter).  
357 Fractionation of CsCl gradients was done using a low-flow peristaltic pump as described in  
358 Neufeld and colleagues (Neufeld et al., 2007b). A total of twelve CsCl fractions, each of



359 425 µl, were obtained, ranging from heavy to light DNA. DNA from all fractions was  
360 precipitated by adding 20 µg of linear polyacrylamide (LPA) and 900 µl of PEG-NaCl 6000  
361 solution (30%/1.6M) and left at room temperature overnight before centrifugation at 13,000 x  
362 g for 30 min. DNA pellets were washed with 500 µl of 70% (v/v) ethanol, centrifuged for  
363 another 10 min, air-dried for 15 min and resuspended in 50 µl of TE buffer (10 mM Tris-HCl,  
364 1 mM EDTA).

365 **DNA amplicon sequencing.** Primer sets used in this study were: 27Fmod (5'  
366 AGRGTTTGATCMTGGCTCAG 3') and 519Rmodbio (5'-GTNTTACNGCGGCKGCTG-3')  
367 to amplify 16S rRNA gene, 1003F/1555R (Neufeld et al., 2007a) to amplify *mxoF* gene and a  
368 primer set targeting clade 5 to amplify *xoxF* gene (Taubert *et al.*, *in revision*). In each case,  
369 three independent PCR products were combined. Purified PCR products were sequenced by  
370 454 pyrosequencing (GS FLX Titanium system, MR DNA, Shallowater, TX, USA). 16S  
371 rRNA gene data analysis was done according to Dowd *et al.*, 2011) and DeSantis *et al.*,  
372 2006). *mxoF* and *xoxF* functional gene amplicon sequencing data was analyzed using  
373 software packages mothur (Schloss *et al.*, 2009) and USEARCH (Edgar, 2013). See  
374 Supplementary Information for details.

375 **Metagenome sequencing.** DNA from the heavy fraction of duplicate <sup>13</sup>C experiments was  
376 amplified using REPLI-g Mini Kit (Qiagen), using 5-10 ng of DNA (or nuclease-free water  
377 for negative controls) as starting material, following instructions provided by the  
378 manufacturer. The amplified DNA was purified using LPA and PEG-NaCl 6000 solution (see  
379 Supplementary Information) and 4 µg from each sample were sent for MiSeq, 2 x 300 bp,  
380 Illumina sequencing (2 million reads; MR DNA, Shallowater, TX, USA). The metagenome  
381 data received was analyzed using MG-RAST (Meyer *et al.*, 2008) to determine the  
382 phylogenetic classification of the reads based on the Ribosomal Database Project (RDP)  
383 database (Wang *et al.*, 2007), assembled in BaseSpace (basespace.illumina.com) using

384 SPAdes Genome Assembler v3.0 (Bankevich *et al.*, 2012) and annotated in RAST (Aziz *et*  
385 *al.*, 2008).

386 **Sequence data deposition.** Nucleotide sequences from 454 amplicon pyrosequencing  
387 obtained in this study were deposited in the GenBank nucleotide sequence database under  
388 accession numbers KM657588 (*mxoF*) and KM657641 - KM657644 (*xoxF*). Raw data from  
389 454 amplicon pyrosequencing of 16S rRNA and functional gene amplicons have been  
390 deposited in the Sequence Read Archive (SRA) of NCBI under accession numbers  
391 SRR1576828, SRR1576831, SRR1584480 - SRR1584483, SRR1584485, SRR1584486,  
392 SRR1584503, SRR1584504, SRR1584506 and SRR1584507. Annotated genome sequences  
393 of *M. thiooxydans* strain L4 are available in the GenBank Whole Genome Shotgun (WGS)  
394 database under accession number JRQD01000000. Raw Illumina MiSeq data were deposited  
395 at BaseSpace (<https://basespace.illumina.com/s/eiGGwUvz6xBP>).

396 **Protein-SIP analyses.** Protein extracts were denatured by incubation in SDS sample buffer  
397 (62.5 mM Tris/HCl pH 6.8, 10% glycerol (v:v), 2% SDS (w:v), 5% mercaptoethanol (v:v),  
398 0.005% bromophenol blue) at 90°C for 10 minutes, followed by centrifugation at 13,000 x *g*  
399 for 10 minutes. Supernatants were subjected to one-dimensional SDS polyacrylamide gel  
400 electrophoresis for prefractionation as described previously (Taubert *et al.*, 2012). Gel lanes  
401 were cut into four bands each. Bands were destained and dehydrated, followed by reduction  
402 with 10 mM dithiothreitol for 30 minutes at room temperature and subsequently alkylation  
403 with 100 mM iodoacetamide for 30 minutes at room temperature. Proteolysis with trypsin was  
404 performed overnight at 37°C. Extracted peptides were desalted and concentrated using  
405 ZipTip- $\mu$ C18 (Merck Millipore, Darmstadt, Germany). Solvents were evaporated under  
406 vacuum and samples were resuspended in 0.1% formic acid for LC-MS/MS analysis. Mass  
407 spectrometry analysis was performed by an Orbitrap Fusion instrument (Thermo Fisher  
408 Scientific, Waltham, MA, USA) coupled to a TriVersa NanoMate (Advion, Ltd., Harlow,

409 UK). In total, 5  $\mu$ L of the peptide lysates were separated via a Dionex Ultimate 3000 nano-  
410 LC system (Dionex/Thermo Fisher Scientific, Idstein, Germany). Raw data files were  
411 converted to peak lists and analyzed using TOPPAS v1.11.0 and OpenMS pipeline  
412 (Kohlbacher et al., 2007, Sturm et al., 2008), with the OMSSA search algorithm v2.1.8 (Geer  
413 et al., 2004). Two databases were used, one consisting of protein sequences obtained from the  
414 NCBI database and one consisting of the predicted protein sequences from the  
415 metagenome. Only peptides with a false discovery rate (FDR) <2%, estimated by a decoy  
416 database, and peptide rank equal 1 were considered as identified (see Supplementary  
417 Information).

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566

### 567 **Table and figure legends**

568 Table 1. Central pathways for carbon and nitrogen metabolism detected in the metagenome  
569 dataset obtained from <sup>13</sup>C-DNA of the <sup>13</sup>C-labeled incubations. (1) No 6-  
570 phosphofructokinase, (2) No 2-oxoglutarate dh complex, (3) Via 2-keto-3-  
571 desoxyphosphogluconate aldolase (KDPGA) and transketolase/transaldolase (TK/TA), (4)  
572 Via glutamine synthase/ glutamine 2-oxoglutarate aminotransferase (GS/GOGAT).

573 Figure 1. Phylogenetic diversity of the total bacterial community (A) at the beginning (T0)  
574 and end (unfractionated DNA) of the Stable Isotope Probing (SIP) experiment, (B) of <sup>13</sup>C-  
575 labeled (heavy) and unlabeled (light) DNA and (C) in total peptides and peptides labeled by  
576 methylotrophy from SIP samples incubated for three days with 100 μM of <sup>13</sup>C-labeled  
577 methanol. Results presented in A and B are based on 16S rRNA gene pyrosequencing data,  
578 whereas in (C) they are based on protein SIP analysis of the <sup>13</sup>C samples.

579 Figure 2. The methanol dehydrogenase gene cluster of *Methylophaga thiooxydans* strain L4  
580 (Genbank accession LP43\_0439 to LP43\_0425) (*M. t.* L4) retrieved from assembled  
581 metagenomic sequences, compared with sequences from the available genomes of three other  
582 *Methylophaga* species: *M. thiooxydans* DMS010 (*M. t.* DMS010), *M.*  
583 *nitratireducenticrescens* strain JAM1 (*M. n.* JAM1) and *M. frappieri* strain JAM7 (*M. f.*  
584 JAM7). Different colours correspond to different genes. *mxoF* and *mxoI* correspond to the  
585 large and small subunit of methanol dehydrogenase, *mxoG* encodes the associated  
586 cytochrome and *mxoJ* is a gene of unknown function required for activity. *mxoDE* and  
587 *mxoYX* have regulatory functions in gene expression, *mxoRSACKL* are required for  
588 maturation and activation of the enzyme.

589 Figure 3. Key steps in carbon metabolism of *M. thiooxydans* strain L4 identified by  
590 combining metagenomics and metaproteomics: coloured arrows indicate pathways that were  
591 present in the metagenome and were detected completely (green), partially (yellow) or not  
592 detected (red) in the metaproteomics data. Key proteins are: (1) methanol dehydrogenase, (2)  
593 formaldehyde activating enzyme, (3) D-arabino-3-hexulose 6-phosphate formaldehyde lyase,  
594 (4) 6-phospho-3-hexuloisomerase, (5) 2-keto-3-deoxyphosphogluconate aldolase. PP and ED  
595 are pentose phosphate and Entner-Doudoroff pathway, respectively. TCA, tricarboxylic acid  
596 cycle; H<sub>4</sub>MPT, tetrahydromethanopterin; HuP, dissimilatory hexulose phosphate cycle;  
597 RuMP, ribulose monophosphate cycle; GS/GOGAT, glutamine synthase/glutamine 2-  
598 oxoglutarate amidotransferase.