

## Substrate-Specific Clades of Active Marine Methylotrophs Associated with a Phytoplankton Bloom in a Temperate Coastal Environment<sup>∇†</sup>

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Marine microorganisms that consume one-carbon (C<sub>1</sub>) compounds are poorly described, despite their impact on global climate via an influence on aquatic and atmospheric chemistry. This study investigated marine bacterial communities involved in the metabolism of C<sub>1</sub> compounds. These communities were of relevance to surface seawater and atmospheric chemistry in the context of a bloom that was dominated by phytoplankton known to produce dimethylsulfoniopropionate. In addition to using 16S rRNA gene fingerprinting and clone libraries to characterize samples taken from a bloom transect in July 2006, seawater samples from the phytoplankton bloom were incubated with <sup>13</sup>C-labeled methanol, monomethylamine, dimethylamine, methyl bromide, and dimethyl sulfide to identify microbial populations involved in the turnover of C<sub>1</sub> compounds, using DNA stable isotope probing. The [<sup>13</sup>C]DNA samples from a single time point were characterized and compared using denaturing gradient gel electrophoresis (DGGE), fingerprint cluster analysis, and 16S rRNA gene clone library analysis. Bacterial community DGGE fingerprints from <sup>13</sup>C-labeled DNA were distinct from those obtained with the DNA of the nonlabeled community DNA and suggested some overlap in substrate utilization between active methylotroph populations growing on different C<sub>1</sub> substrates. Active methylotrophs were affiliated with *Methylophaga* spp. and several clades of undescribed *Gammaproteobacteria* that utilized methanol, methylamines (both monomethylamine and dimethylamine), and dimethyl sulfide. rRNA gene sequences corresponding to populations assimilating <sup>13</sup>C-labeled methyl bromide and other substrates were associated with members of the *Alphaproteobacteria* (e.g., the family *Rhodobacteraceae*), the *Cytophaga-Flexibacter-Bacteroides* group, and unknown taxa. This study expands the known diversity of marine methylotrophs in surface seawater and provides a comprehensive data set for focused cultivation and metagenomic analyses in the future.

Methylotrophic bacteria represent an important functional guild, contributing to the metabolism and assimilation of one-carbon (C<sub>1</sub>) compounds. Because the carbon sources that these bacteria depend on in the marine environment are present at low concentrations, characterizing marine methylotrophs has involved the use of enrichment and cultivation approaches with a variety of C<sub>1</sub> substrates. The C<sub>1</sub> substrates of relevance to the marine environment include methane, methanol, methylated amines, methyl halides, and methylated sulfur compounds. Methane is supersaturated in surface seawater, and several studies have isolated methanotrophs from the marine environment (14, 16, 28, 47). Methyl halides are produced by a number of phytoplankton species (42), and these ozone-depleting compounds have been used to isolate methylotrophic *Alphaproteobacteria* that belonged to the *Roseobacter* clade (43, 45). Methanol represents a marine C<sub>1</sub> substrate derived from phytoplankton (13) and the atmosphere (7) which may be actively metabolized by marine methylotrophs (21). Methanol concentrations have been estimated at between 100 nM (48) and 300 nM (10) and were directly measured in one study,

ranging between 50 and 250 nM in several tropical Atlantic samples (54). Enrichment and isolation studies using methanol as the sole carbon source have generated molecular fingerprint phylotypes and have characterized isolates of *Methylophaga* spp. (*Gammaproteobacteria*). *Methylophaga* spp. have also been isolated using dimethyl sulfide (DMS) (8, 44) and can grow on monomethylamine (23), both of which occur at nanomolar concentrations in surface seawater (11, 24). Together, these cultivation-based approaches have revealed the presence of organisms capable of C<sub>1</sub> cycling in the marine environment. Their involvement in methylotrophic metabolism in situ can be experimentally addressed using stable isotope probing (SIP) (40).

DNA SIP recently identified *Methylophaga*-like organisms as active methylotrophs that assimilated methanol and methylamine in surface waters of the English Channel (36). That study also demonstrated that 16S rRNA gene sequences representing clades of uncultivated *Gammaproteobacteria* were also retrieved from the heavy DNA for each of these compounds that clustered close to *Methylophaga*. A DNA SIP experiment with methanol substrate dilution to concentrations anticipated to reflect those in situ (34) confirmed the involvement of *Methylophaga* spp. in methanol consumption and retrieved functional genes involved in methanol metabolism from these active methylotrophs using metagenomic libraries.

The goal of the current study was to extend our previous observations that were made under nonbloom conditions by studying methylotrophic populations in the context of a phytoplankton bloom dominated by *Emiliania huxleyi* and *Karenia mikimotoi* (formerly *Gyrodinium aureolum*). Both the cocco-

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lithophores (e.g., *Emiliania*) and the small dinoflagellates (e.g., *Karenia*) are associated with dimethylsulfoniopropionate production (22, 29), and phytoplankton blooms are known to produce relevant  $C_1$  compounds or their precursors, including methanol (13), methylated sulfur compounds (24), and methyl halides (42). As with our previous marine SIP studies (34, 36), seawater samples were incubated with methanol and methylamine, and in this investigation, SIP incubations were also carried out with  $^{13}C$ -labeled methane, dimethylamine, methyl bromide, and DMS to identify microbial populations that are actively involved in the cycling of these  $C_1$  compounds during phytoplankton blooms in situ.

## MATERIALS AND METHODS

**Bloom sampling.** A transect across a phytoplankton bloom dominated by *E. huxleyi* and *K. mikimotoi* (D. Schroeder, personal communication) was sampled in the English Channel bordering the southern coast of the United Kingdom. Surface seawater was taken from inside the bloom (49.3222 N, 5.1446 W to 49.5105 N, 5.1217 W), at the edge of the bloom (49.5472 N, 4.3966 W to 49.5523 N, 4.4045 W), and outside the bloom area (50.1158 N, 4.1998 W to 50.1053 N, 4.2062 W). The distances between the beginnings and ends of sampled areas differed for the three sampling stations and were 1.3 km, 0.8 km, and 20 km for the outside, the edge, and the inside of the bloom, respectively. All samples were taken between 1000 h and 2200 h on 26 July 2007. Water samples were returned to the laboratory, and aliquots were taken for filtration (for DNA extraction) and to establish SIP incubations on 27 July 2006. Multiple aliquots of approximately 1 liter were filtered through 0.2- $\mu$ m Sterivex filters (Durapore, Millipore) and frozen at  $-80^\circ C$  until processed for nucleic acid extraction.

**Incubation with  $^{13}C$ -labeled substrates.** Samples taken from the edge of the bloom were chosen to set up SIP incubations with several  $^{13}C$ -labeled  $C_1$  substrates. Seawater sample aliquots of 750 ml were added to 1-liter serum bottles with the addition of 0.1% (750  $\mu$ l) marine ammonium mineral salts medium (modified from that described in reference 12) and substrate. A total of 75  $\mu$ mol of  $^{13}C$ -labeled substrate was added to bottles for methanol, monomethylamine, dimethylamine, methyl bromide, and methane (final concentration of 100  $\mu$ M, assuming complete dissolution). For DMS, 187.5  $\mu$ mol of substrate was added to make up a final concentration of 250  $\mu$ M. All serum bottles were crimp sealed with butyl rubber bungs to prevent the loss of volatile substrates. All  $^{13}C$ -labeled compounds had a purity of 99% or higher and were obtained from Cambridge Isotope Laboratories (Hook Hampshire, United Kingdom), except methylated amines (Sigma, Gillingham, United Kingdom). [ $^{13}C_2$ ]DMS was prepared by a method adapted from that described for labeled dimethyl sulfoxide synthesis (5). Sodium sulfide nonahydrate (6.5 g) was dissolved in 6.5 ml of sterile deionized water in a glass test tube and cooled to  $0^\circ C$  in an ice-water bath, with vigorous stirring. Subsequently, 5 g of [ $^{13}C$ ]methyl iodide (Cambridge Isotope Laboratories LTD, Andover, MA) was added dropwise over a period of 30 min prior to incubating the reaction mixture at  $0^\circ C$  for 5 h with stirring. Five milliliters each of 2 M sodium hydroxide solution and 1 M sodium thiosulfate solution was added, the reaction mixture vessel was then connected to a receiving tube held at  $-170^\circ C$  in liquid nitrogen, and the reaction mixture was allowed to warm to  $40^\circ C$  in a water bath. The [ $^{13}C_2$ ]DMS was distilled from the reaction mixture for 90 min and then redistilled into a sterile receiving vessel for 1 h. Sterile deionized water was added to the receiving vessel to dissolve the [ $^{13}C_2$ ]DMS, and the resulting solution was transferred, with washings, to a sterile 1-liter serum vial which was then sealed with a butyl rubber bung. The concentration and purity of the [ $^{13}C_2$ ]DMS solution were assessed by gas chromatography with a flame ionization detector. A total of 250 ml of a 7 mM solution of pure [ $^{13}C_2$ ]DMS was obtained.

For all substrates, parallel incubations were set up as  $^{12}C$ -unlabeled controls and  $^{13}C$ - and  $^{12}C$ -labeled substrate incubations were harvested at a single time point. With the exception of monomethylamine and dimethylamine, substrate utilization was monitored by gas chromatography using a flame ionization detector. Measurement of DMS and methyl bromide concentrations in sterile seawater controls confirmed that the degradation observed for SIP incubations was due to biological processes and not to chemical decomposition. The concentrations of the methylamines were assumed to mirror those of methanol; recovery of [ $^{13}C$ ]DNA from methylamine and dimethylamine incubations confirmed that methylated amines had been assimilated. Following substrate deple-

tion, SIP incubations were filtered through 0.22- $\mu$ m Sterivex filters and frozen at  $-80^\circ C$  until processed for nucleic acid extraction.

**DNA extraction, SIP gradient centrifugation, and fractionation.** Total nucleic acids were extracted directly from Sterivex filters according to a previously described protocol (36). Briefly, lysozyme, proteinase K, and sodium dodecyl sulfate were used to lyse cells, and lysates were transferred to 15-ml phase lock tubes (Qiagen, West Sussex, United Kingdom) for phenol-chloroform and chloroform extractions. Purified DNA was quantified on a 1% (wt/vol) agarose gel. Aliquots (1 to 5  $\mu$ g) of DNA extracts from each of the SIP incubations were added to cesium chloride (CsCl) solution (average density of  $\sim 1.725$  g ml $^{-1}$ ) and transferred to an ultracentrifuge gradient tube for centrifugation and fractionation as previously described (37). Briefly, tubes were added to a Vti 65.2 rotor (Beckman Coulter, Fullerton, CA) and centrifuged at 44,100 rpm ( $177,000 \times g_{av}$  [average]) for 40 h at  $20^\circ C$ . Gradients were fractionated from bottom (fraction 1, highest density) to top (fraction 12, lowest density) into 425- $\mu$ l fractions. DNA was purified from CsCl and quantitatively recovered by precipitation with glycogen (20  $\mu$ g) and polyethylene glycol (30% PEG 6000 and 1.6 M NaCl). Purified DNA was suspended in 30  $\mu$ l of sterile LoTE buffer (3 mM Tris [pH 8], 0.2 mM EDTA), and 5- $\mu$ l aliquots were run on a 1% (wt/vol) agarose gel for quantification and to identify the distribution of [ $^{13}C$ ]DNA relative to background, unlabeled [ $^{12}C$ ]DNA (see Fig. S1 in the supplemental material). These data indicated that the  $^{13}C$  labeling of DNA was very high for methanol, monomethylamine, and DMS incubations; most of the DNA for these  $^{13}C$ -incubated samples eluted in heavy fractions (fractions 7 and 8,  $\sim 1.725$  to 1.730 g ml $^{-1}$ ). The detection of  $^{13}C$ -labeled DNA confirmed that the substrate was incorporated into microbial biomass. For dimethylamine and methyl bromide incubations, the extent of DNA labeling was less pronounced. For methyl bromide incubations, there was almost no difference between the smears of DNA across gradients associated with  $^{12}C$ - and  $^{13}C$ -methyl bromide SIP incubations (see Fig. S1 in the supplemental material).

**DGGE.** For each fractionated gradient, two fractions were selected for analysis of the "heavy" [ $^{13}C$ ]DNA (fractions 7 and 8,  $\sim 1.725$  to 1.730 g ml $^{-1}$ ), and one fraction was selected for the characterization of "light" [ $^{12}C$ ]DNA (either fraction 11 or 12,  $\sim 1.710$  to 1.705 g ml $^{-1}$ ). One-microliter aliquots of gradient fractions were used as the template for PCR to obtain 16S rRNA gene fragments suitable for denaturing gradient gel electrophoresis (DGGE) analysis. Each 50- $\mu$ l reaction mixture consisted of 25 pmol of primers 341f-GC and 534r (32),  $1 \times (NH_4)_2SO_4$  buffer (Fermentas, York, United Kingdom), 1.5 mM MgCl $_2$ , 33.6  $\mu$ g nonacetylated bovine serum albumin (Sigma, Gillingham, United Kingdom), 40 nmol of each deoxynucleoside triphosphate, 1.25 U *Taq* polymerase (Fermentas). The reaction mixture tubes were loaded directly into the block at  $95^\circ C$  (simplified hot start), followed by an initial denaturation at  $95^\circ C$  for 5 min and 30 cycles of  $94^\circ C$  for 1 min (denaturing),  $55^\circ C$  for 1 min (annealing), and  $72^\circ C$  for 1 min (extension). A final extension at  $72^\circ C$  for 7 min was followed by a holding step at  $10^\circ C$ . Five-microliter aliquots were quantified on a 1% (wt/vol) agarose gel.

For DGGE, 5- $\mu$ l aliquots (100 to 300 ng) were run on a 10% polyacrylamide gel with a 30 to 70% denaturing gradient (100% denaturant is 7.0 M urea and 40% deionized formamide) according to the D-Code system instructions (Bio-Rad, Hercules, CA). Gels were run overnight (14 h) at 85 V and then stained for 1 h in SYBR Green I (Invitrogen, Paisley, United Kingdom). Gel images were captured with a FLA-5000 imaging system (Fujifilm, Tokyo, Japan). Bands selected for sequence analyses were sampled from the gel by means of sterile pipette tips and amplified from the gel, using the PCR conditions described above for DGGE. Sequencing was done with the 341f primer and a BigDye terminator version 3.1 kit (Applied Biosystems, Foster City), and the sequencing products were run on an ABI Prism 3130 genetic analyzer (Applied Biosystems) by the Molecular Biology Service, University of Warwick, Warwick, United Kingdom. DGGE band sequences were approximately 150 bases in length.

For determining the relatedness of the DGGE fingerprints, gels were imported into Gelcompar II (Applied Maths, Sint-Martens-Latem, Belgium) and normalized to ladder bands and additional internal standard bands. A dendrogram produced with the unweighted-pair group method using average linkages was generated by performing a Pearson correlation with background-subtracted densitometric curves, which takes band intensities into account. The output of the clustering analysis was independent of the input order of DGGE fingerprints.

**16S rRNA gene libraries.** Clone libraries of bacterial 16S rRNA genes were generated from the original seawater samples (outside of bloom, edge of bloom, inside of bloom; 36 clones sequenced from each) and for the heavy DNA associated with the five substrates (methanol, monomethylamine, dimethylamine, methyl bromide, and DMS) that yielded  $^{13}C$ -labeled DNA (24 clones sequenced from each). The PCR to amplify the 16S rRNA gene used primers 27f and 1492r (25) and the same amplification reaction as that used for DGGE,

except with an extension time of 1.5 min. Products were cloned into the TOPO-TA vector according to the manufacturer's protocol (Invitrogen). Screening was done as described previously (38), and cloned 16S rRNA gene inserts were sequenced at the Edinburgh node of the NERC Molecular Genetics Facility, using the 27f primer. Pintail (3) software was used to identify suspected chimeras and identified one heavy-band sequence which was likely chimeric in origin and several water library sequences that were likely chimeric; these sequences were excluded from further analyses. For seawater samples, classification of 16S rRNA gene sequences was done using an RDP-II classifier (53) after manually verifying base calls. For the 16S rRNA gene libraries constructed using [<sup>13</sup>C]DNA from SIP experiments, manually verified 16S rRNA gene sequences were compared to those in GenBank (6) to retrieve the three closest matches for each library sequence. Sequences were aligned within ARB (30) software, and an alignment was exported to MEGA4 (49). Evolutionary distances were computed using the maximum-composite likelihood method (50) and are presented in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). There was a total of 466 nucleotide positions in the final data set. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (9). The tree was drawn to scale, with branch lengths given in the same units as those of the evolutionary distances used to infer the phylogenetic tree. In the absence of cultivated methylotrophic organisms that fell within the groups of 16S rRNA genes derived from SIP experiments, clades were defined based on the consistent association with particular substrates, but a specific cutoff value was not used.

**Nucleotide sequence accession numbers.** All sequences were deposited in GenBank for the marine samples taken from the edge (accession no. EU399242 to EU399272), the inside (accession no. EU399273 to EU399306), and the outside (accession no. EU399307 to EU399340) of the phytoplankton bloom. The 16S rRNA gene clone library sequences from heavy DNA were deposited with the following accession numbers for SIP incubations with dimethylamine (accession no. EU399341 to EU399364), DMS (accession no. EU399365 to EU399386), methyl bromide (accession no. EU399387 to EU399407), monomethylamine (accession no. EU399408 to EU399428), and methanol (accession no. EU399429 to EU399451). DGGE band sequences from heavy DNA were deposited with the following accession numbers for SIP incubations with methanol (accession no. EU399452 to EU399457), monomethylamine (accession no. EU399458 to EU399464), dimethylamine (accession no. EU399465 to EU399469), DMS (accession no. EU399470 to EU399474), and methyl bromide (accession no. EU399475 to EU399477).

## RESULTS

**Phytoplankton bloom microbial community analysis.** This study was conducted with samples from an extensive phytoplankton bloom with a predominance of both *Emiliania huxleyi* and *Karenia mikimotoi* (D. Schroeder, personal communication). Based on remotely sensed observations obtained from the day prior to sampling, three sampling stations within the western English Channel were selected to represent areas with varying chlorophyll concentrations (Fig. 1A), indicating regions internal to the bloom ("inside"), on the edge of the bloom ("edge") and external to the bloom ("outside"). Prior to assessing the methylotrophs in the bloom (edge sample), we assessed the background bacterial community composition of the three water samples, using 16S rRNA gene fingerprinting (Fig. 1B) and clone libraries (Fig. 1C). The DGGE profiles indicate that the bacterial communities of these three water samples were represented by unique predominant band phylogenotypes, although several bands were shared among the three samples (Fig. 1B). Almost all sequences collected from the 16S rRNA gene clone libraries were most similar to GenBank sequences derived from other marine surface water samples, reflecting a composition similar to that described in previous studies (data not shown). All libraries were dominated by *Alphaproteobacteria* and *Cyanobacteria*, although *Bacteroidetes* organisms were also prevalent in the "inside" and "edge" li-

braries (Fig. 1C). Overall, the communities shared similar division-level compositions, but analysis also indicated that local sample heterogeneity existed across this relatively short bloom transect.

**DNA SIP incubations.** Enrichment incubations with six C<sub>1</sub> substrates were established on the day following sampling (day 0), with substrate concentrations of 100 μM (250 μM for DMS). Substrate had been depleted by day 3 in methanol incubations, and these were filtered for DNA extraction as were those containing methylamines. Approximately 83 μmol of [<sup>12</sup>C] and [<sup>13</sup>C]DMS were consumed by the fourth day (data not shown), and these incubations were subsequently sacrificed for DNA extraction. Methyl bromide incubations (with <sup>12</sup>C and <sup>13</sup>C) had consumed >90% of the 75 μmol of substrate originally present by day 18 and were filtered for DNA extraction. Changes in headspace concentrations of methane (100 μM total in the bottle; ~0.63% in the headspace) for seawater incubations with methane were unchanged for several months (data not shown), and these incubations were not analyzed further.

**16S rRNA gene fingerprinting of DNA from SIP experiments.** DGGE was used to profile the bacterial communities associated ["heavy"] and ["light"] fractions for both the <sup>12</sup>C-labeled control samples and the <sup>13</sup>C-labeled incubated samples. The <sup>12</sup>C-labeled incubated samples showed no profile differences between the "heavy" and the "light" fractions (data not shown), whereas unique fingerprints were evident for all C<sub>1</sub> substrate incubations (Fig. 2). As expected, the fingerprints for the light fractions of all tubes clustered together. The "background" bacterial communities in each SIP incubation were more similar to one another than to the "heavy" <sup>13</sup>C-labeled fraction fingerprints of the same incubation. However, for the methyl bromide SIP incubation, the fingerprint of the "heavy" DNA was less clearly unique for the light DNA than for the other substrate incubations, reflecting the possibility that only a small amount of DNA was labeled and was just detectable above background [<sup>12</sup>C]DNA. All other DGGE fingerprints from heavy fractions (fractions 7 and 8) of <sup>13</sup>C-labeled substrate incubations clustered in distinct clades apart from the "light" DNA, with monomethylamine and methanol fingerprints clustering closely, with some similarity to the dimethylamine fingerprints. Dimethylsulfide [<sup>13</sup>C]DNA fingerprints were distinct from all other patterns in this study, reflecting the unique composition of active methylotrophs enriched in these SIP incubations. Individual bands from fingerprints representing "heavy" DNA from <sup>13</sup>C<sub>1</sub> incubations were selected for PCR reamplification and sequencing. These sequences were used to assign band sequences to specific SIP-related 16S rRNA gene clades derived from this study and from a previous SIP study that was carried out under nonbloom conditions in the English Channel (36). The results indicate that *Methylophaga* spp. were associated with methyl bromide, methanol, and methylamine SIP incubations, whereas additional clades were affiliated with dimethylamine and DMS, likely contributing to their more distinct fingerprint profiles (Fig. 2).

**16S rRNA gene clone libraries of [<sup>13</sup>C]DNA.** As the diversity of active methylotrophs was anticipated to be relatively low, 24 clones were sequenced from each library associated with SIP incubations with each of the five substrates analyzed in this study. The results of the sequencing confirmed the relatively

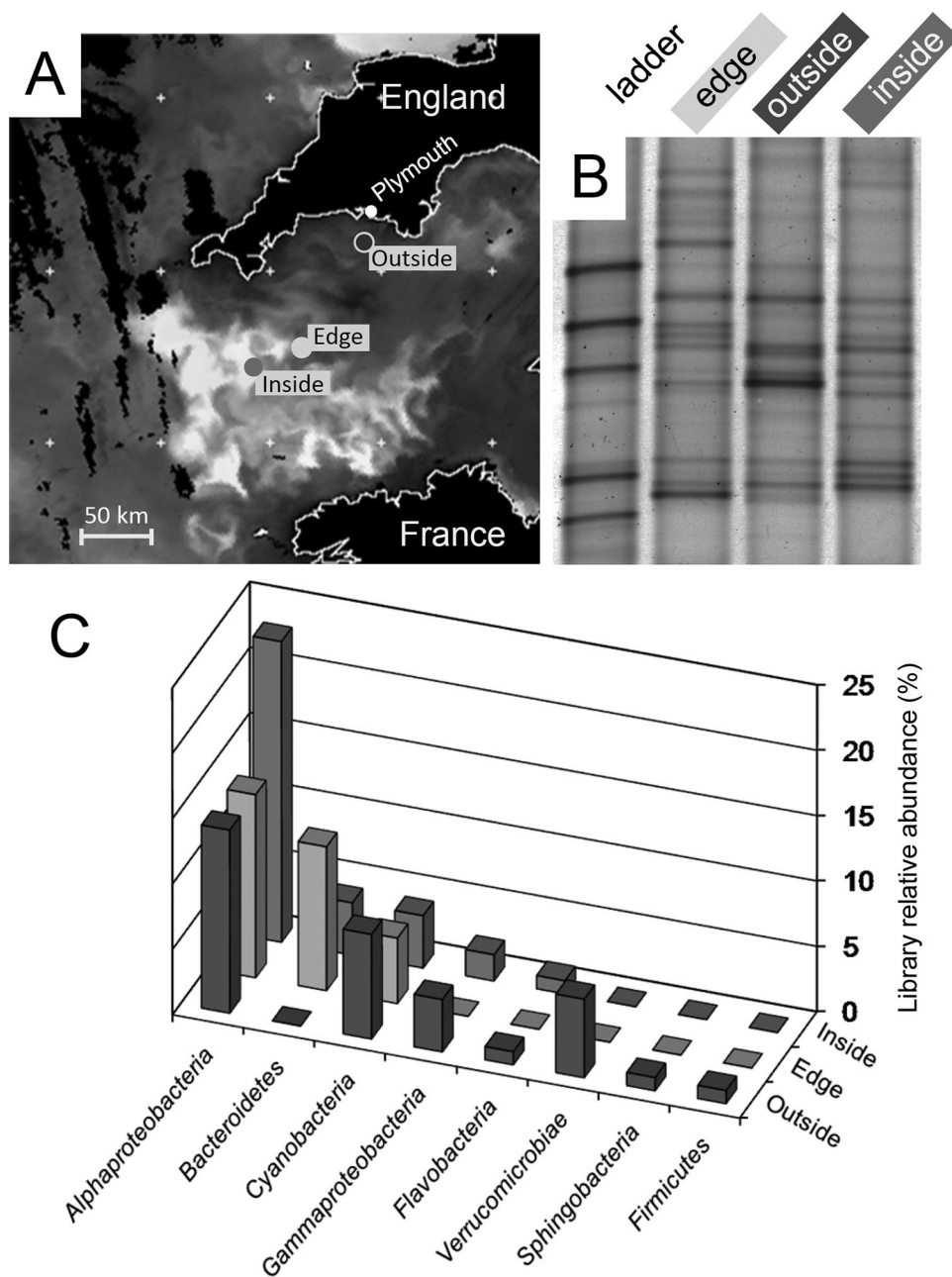


FIG. 1. Physical location and bacterial composition of samples. (A) A grayscale Aqua-MODIS satellite radiance image generated from a mixed-wavelength composite image (551-nm, 488-nm, and 443-nm wavebands) on 26 July 2006. *Emiliania huxleyi* coccolithophore reflectance is readily visible in this image. A chlorophyll *a* satellite image from the same day shows chlorophyll abundance corresponding to the *Karenia* bloom adjacent to the bloom on the north side of the English Channel (image not shown). Satellite data were received and processed by the NERC Earth Observation Data Acquisition and Analysis Service (NEODAAS) at Dundee University and Plymouth Marine Laboratory ([www.neodaas.ac.uk](http://www.neodaas.ac.uk)). (B) Bacterial DGGE fingerprints of samples taken from the locations indicated in panel A. (C) Frequency of 16S rRNA gene clones belonging to major phylogenetic groups across the different gene libraries analyzed.

low diversity of methylotrophs within each SIP incubation, but across the different substrates applied indicated a broad diversity of active marine methylotrophs in this study.

**Methanol-assimilating phylotypes.** Phylogenetic analysis demonstrated that sequences associated with SIP incubations with methanol clustered in the *Methylophaga* species clade of the *Gammaproteobacteria* together with sequences from a previous SIP incubation (36) and with several characterized

*Methylophaga* isolates (Fig. 3 and see Fig. S2 in the supplemental material). In addition, one cloned 16S rRNA gene sequence obtained from the methanol SIP was a member of a clade of unknown phylogenetic affiliation, which also contained one DMS and DMA-SIP-derived cloned 16S rRNA gene. The methanol clade identified in a previous methanol SIP experiment (Fig. 3; see Fig. S2 in the supplemental material) has the closest affiliation to *Gammaproteobacteria* sequences in GenBank that

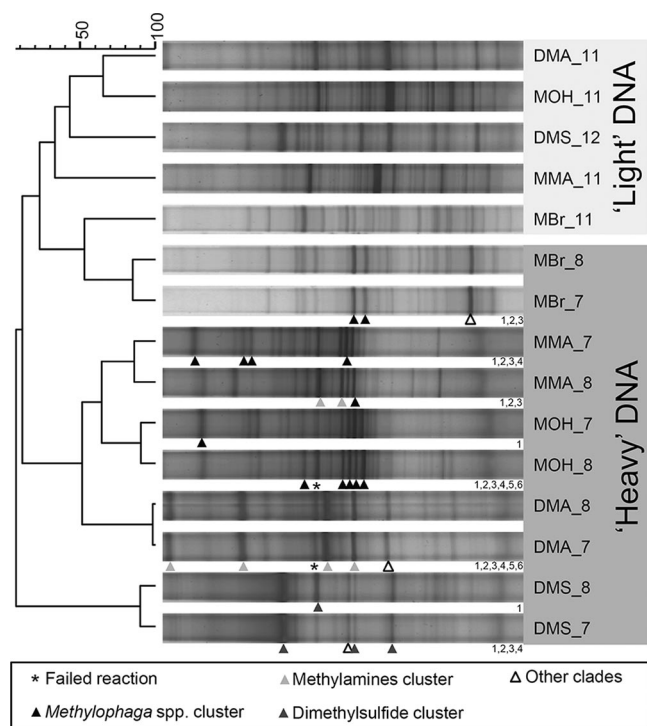


FIG. 2. DGGE fingerprint comparison of “light” and “heavy” DNA associated with DNA SIP incubations with different carbon sources (MMA, monomethylamine; DMA, dimethylamine; MOH, methanol; MBr, methyl bromide; and DMS). The dendrogram scale bar refers to the percent similarity of Pearson correlations between fingerprint densitometric curves. Shading of the triangle pointers indicates the phylogenetic affiliations of sequenced bands, most of which were associated with clades in Fig. 3. Numbers at the bottom right of the fingerprints correspond to sequenced bands submitted to GenBank. For example, the open triangle for fraction 7 of the [ $^{13}\text{C}$ ]methylbromide SIP (MBr<sub>7</sub>) is labeled MBr<sub>7\_3</sub> for the GenBank submission. Several bands were not associated with clades but were affiliated with sequences in Fig. 3, as follows: MBr<sub>7\_3</sub> is identical to MBr<sub>587\_7</sub>; DMA<sub>7\_6</sub> is closest to MBr<sub>587\_24</sub>; DMS<sub>7\_2</sub> is identical to DMS<sub>584\_3</sub>.

were retrieved from multiple marine Arctic surface sediments or detected on the surface of submerged artificial substrates incubated in marine water near China. This clade, however, was not detected in the current study.

**Phylotypes assimilating methylated amines.** As with the methanol SIP incubation, a previously characterized clade of sequences associated with a monomethylamine SIP incubation (36) was also represented by sequences from the monomethylamine SIP from the current study and also from the dimethylamine SIP incubation. In particular, 22 of the 24 sequences generated from the dimethylamine SIP incubation and most of the corresponding DGGE band sequences (Fig. 2) fell within this clade (Fig. 3 and see Fig. S3 in the supplemental material). This clade also contained several sequences isolated from arctic sediment (H. Li, Y. Yu, W. Luo, Y. Zeng, and B. Chen, unpublished), a mangrove ecosystem (27), and a deep-sea coral ecosystem (K. Penn, D. Wu, J. Eisen, and N. Ward, unpublished) and a strain isolated from the Yellow Sea (H. Kim and J.-C. Cho, unpublished; GenBank accession no. EF468718). Additional 16S rRNA gene sequences obtained

from the monomethylamine SIP incubation belonged to the *Methylophaga* clade, many species of which can grow on methylated amines.

**Phylotypes assimilating DMS.** Almost all 16S rRNA gene sequences derived from the DMS SIP “heavy” DNA were nearly identical and formed an additional clade with low relative diversity (Fig. 3; see Fig. S4 in the supplemental material). The DMS clade was most closely related to the methanol SIP clade associated with the class *Gammaproteobacteria* and identified in a previous study (35); it shared close similarity (96%) with sequences retrieved from clone libraries associated with DMS-enriched seawater samples from the Sargasso Sea (52) and was approximately 91% similar to the *Methylophaga* sp. clade, based on the percent similarity between sequences of DMS<sub>584\_22</sub> and *Methylophaga marina* (GenBank accession no. X95459) over 722 bases. Another sequence from the “heavy” DNA of the DMS SIP was affiliated with a clade of unknown phylogeny.

## DISCUSSION

The study site was chosen based on the mixed *Emiliania* and *Karenia* bloom that occurred in the English Channel in July 2006. The growth of phytoplankton in oceanic surface water has been associated with the direct or indirect production of methanol (13), methylamines (33), methyl halides (2, 4, 31, 42), methylated sulfur compounds (19, 20, 26), and methane, through decomposition (15, 39). In sampling from the edge of the bloom for SIP analysis (Fig. 1), the objective was to retrieve sequences of methylotrophs relevant to bloom  $\text{C}_1$  substrate production. Although the sample chosen was relevant to  $\text{C}_1$  metabolism, it is important to note that the substrate concentrations (100  $\mu\text{M}$ ) were far higher than those normally present in marine surface water samples. This was done because for a previous bloom in Bergen, Norway, the application of  $\text{C}_1$  substrates at low  $\mu\text{M}$  concentrations did not result in the detection of  $^{13}\text{C}$ -labeled DNA, possibly due to relatively high bacterial biomass associated with the bloom (J. D. Neufeld, R. Boden, H. Moussard, H. Schäfer, and J. C. Murrell, unpublished). In the present study, the objective was to identify phylotypes associated with the use of labeled  $\text{C}_1$  substrates, and the use of elevated substrate concentrations may have biased the results obtained. Typically, SIP experiments require substrate concentrations that exceed those found naturally, and the data may have to be interpreted with caution (35). Nonetheless, a comparison of near in situ substrate concentrations (1  $\mu\text{M}$ ) with a marine methanol SIP incubation detected the same *Methylophaga* species phylotypes as detected in the present study (34). As a result, for  $\text{C}_1$  substrates in the marine environment, the results may be consistent despite the range of substrate concentrations used. In all SIP incubations thus far, the incubation times were extended to days, and an addition of nutrients may have also selected for fast-growing species of methylotrophs. However, the uncultivated methylotrophs detected here are consistently present, which suggests that they do play an active role in  $\text{C}_1$  metabolism in coastal marine environments.

This study represents a comprehensive survey of active methylotrophs in a marine surface water sample during a bloom of phytoplankton associated with the production of dimethylsulfoniopropionate. The methylotrophs detected in this

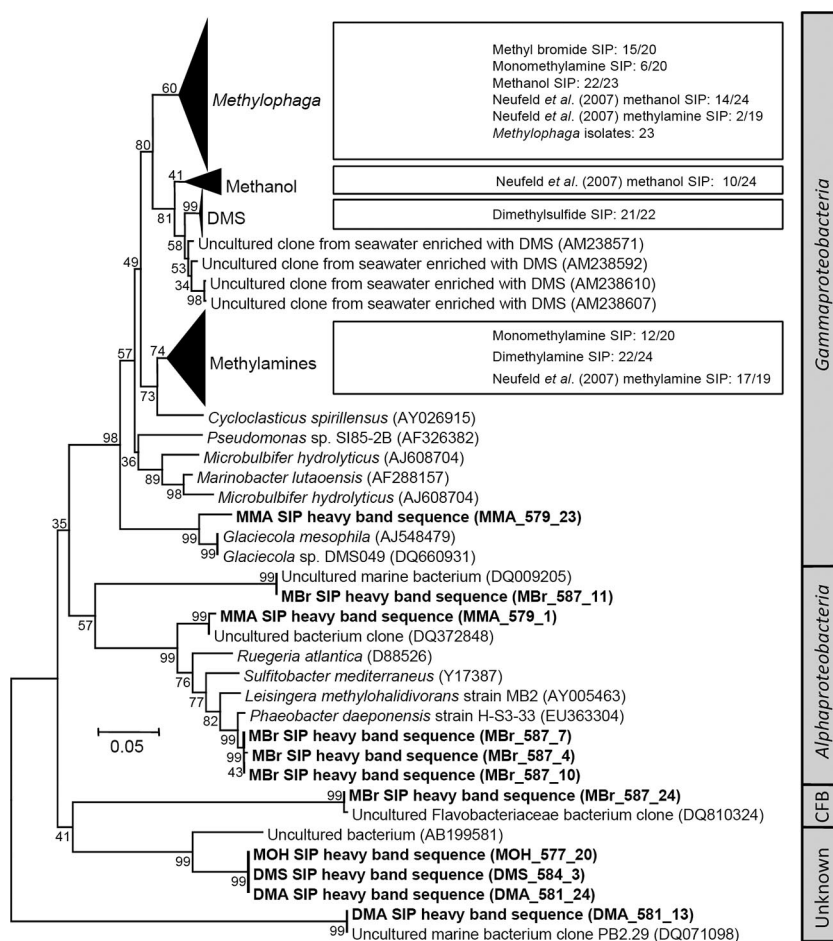


FIG. 3. Phylogenetic affiliations of 16S rRNA gene sequences obtained by  $^{13}\text{C}$ -labeled SIP incubations with methanol (MOH), monomethylamine (MMA), dimethylamine (DMA), methyl bromide (MBr), and DMS SIP incubations. Selected GenBank sequences from uncultivated clones and reference strains are included for comparison. Bootstrap values are included for all branch points on this neighbor-joining tree. GenBank accession numbers are shown in parentheses. The scale bar (in the tree) represents 5% sequence divergence. The collapsed clades are expanded in Fig. S2, S3, and S4 in the supplemental material. The division-level affiliation of sequences is indicated in the boxes at the right. CFB, *Cytophaga-Flavobacterium-Bacteroides*.

survey are consistent with the results of our pilot study with only methanol and monomethylamine under nonbloom conditions obtained a year prior to the current sampling event (36); however, the use of a wider range of  $\text{C}_1$  substrates allowed the identification of a larger diversity of methylotrophs than that found previously, including populations that assimilate dimethylamine, DMS, and methyl bromide. The DMS SIP clones obtained were most closely related to clones obtained from DMS enrichments from Pensacola, FL, and the Sargasso Sea by Vila-Costa and colleagues (52), suggesting that the latter had similar metabolic activities and indeed represented DMS-degrading populations. Those sequences were classified as “uncultivated *Methylophaga*”; however, given the relatively low similarity of the 16S rRNA gene sequences of these cloned 16S rRNA gene sequences to those of *Methylophaga* isolates (around 92%) and their distinct clustering, supported by bootstrap analysis (see Fig. S2 and S4 in the supplemental material), it is also possible that these represent DMS-degrading populations belonging to a different genus. Conversely, none of the DMS SIP clones was closely related to previously isolated

DMS-degrading *Methylophaga* isolates (44), which belonged to the *Methylophaga* clade detected with methanol, monomethylamine, and methyl bromide. This strongly suggests that populations closely related to the isolated strains may have a preference for other  $\text{C}_1$  substrates and/or are outcompeted by those represented by the DMS clade under the specific incubation conditions. The methyl bromide SIP sequences indicate that methyl bromide may be used by members of the genus *Methylophaga* and an organism with a 16S rRNA gene sequence most similar to that of *Phaeobacter gallaeciensis* (formerly *Roseobacter*) within the family *Rhodobacteraceae*. The notion that the *Phaeobacter*-related population degraded methyl halides would be supported by previous cultivation-based identification of marine methyl halide-degrading organisms which were closely related (43, 45, 46); however, screening of several *Methylophaga* isolates has failed to show their ability to degrade methyl halides (H. Schäfer, unpublished). The observation of *Methylophaga*-like sequences in the [ $^{13}\text{C}$ ]methyl bromide incubation could therefore be due to the slow hydrolytic conversion of methyl bromide to methanol (1) and subsequent

utilization of the resulting methanol by these organisms. If *Methylophaga* populations in the methyl bromide incubations became labeled with  $^{13}\text{C}$  due to the uptake of methanol produced by conversion of methyl halides to methanol, this would further underline their ability to take up methanol at ambient concentrations and support using SIP incubations with elevated substrate concentrations to investigate substrate-responsive populations in seawater. Together, these data suggest that marine waters harbor a diverse suite of active methylotrophs that, apart from *Methylophaga* spp., have been unnoticed by previous cultivation studies (8, 17, 23, 43–45) and are almost completely without representation in marine clone libraries. The sequences represented here are important targets for directed cultivation and focused activity-based studies of marine methylotrophy.

Given the focus of past marine metagenomic studies on abundant community members, it is perhaps not surprising that few genes (phylogenetic or “functional”) have reflected the predominance of methylotrophic bacteria. Although formaldehyde oxidation genes were identified in the Sargasso Sea metagenomic libraries (51), genes for methane, methylamine, and methanol oxidation were not detected (18). Furthermore, the only presumed methylotroph 16S rRNA gene sequences identified in a marine metagenomic library was from *Methylophilus* spp., and these sequences occurred at ~0.4% of the total 16S rRNA gene data set from the global ocean survey (41). The contribution of *Methylophilus* to marine  $\text{C}_1$  cycling remains unclear, and *Methylophilus* spp. have not been detected in [ $^{13}\text{C}$ ]DNA from the incubations carried out in this study. One possibility is that *Methylophilus* spp. represent *K*-selected organisms, which adapted to concentrations of carbon and nutrients that are lower than those used in this study. Cultivation-based approaches (17), enrichment cultures (44, 52), and SIP incubations (current study; 34, 36) have all demonstrated that *Methylophaga* spp. and related *Gammaproteobacteria* from multiple disparate marine samples (including estuary sediment; unpublished data) are present in the seawater samples and rapidly respond to the presence of  $\text{C}_1$  substrates. It is possible that these organisms may represent low-abundance and *r*-selected bacteria capable of opportunistic growth in the presence of relatively high concentrations of growth substrates during phytoplankton blooms, for example.

This study represents a comprehensive cultivation-independent survey of active marine methylotrophs and demonstrates that previously unrecognized bacterial groups are present in seawater, which are capable of responding to the presence of added  $\text{C}_1$  substrates. The presence of numerous clades of presumed substrate-specific methylotrophs presents a challenge to microbiologists to focus cultivation and quantitative molecular approaches to better understand the metabolism and distribution dynamics of these organisms with potentially enormous biogeochemical significance.

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