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Identification of Novel Methane-, Ethane-, and Propane-Oxidizing Bacteria at Marine Hydrocarbon Seeps by Stable Isotope Probing▽†

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Marine hydrocarbon seeps supply oil and gas to microorganisms in sediments and overlying water. We used stable isotope probing (SIP) to identify aerobic bacteria oxidizing gaseous hydrocarbons in surface sediment from the Coal Oil Point seep field located offshore of Santa Barbara, California. After incubating sediment with ^{13}C-labeled methane, ethane, or propane, we confirmed the incorporation of ^{13}C into fatty acids and DNA. Terminal restriction fragment length polymorphism (T-RFLP) analysis and sequencing of the 16S rRNA and particulate methane monooxygenase (*pmoA*) genes in ^{13}C-DNA revealed groups of microbes not previously thought to contribute to methane, ethane, or propane oxidation. First, ^{13}C methane was primarily assimilated by *Gammaproteobacteria* species from the family *Methylococcaceae*, *Gammaproteobacteria* related to *Methylophaga*, and *Betaproteobacteria* from the family *Methylophilaceae*. Species of the latter two genera have not been previously shown to oxidize methane and may have been cross-feeding on methanol, but species of both genera were heavily labeled after just 3 days. *pmoA* sequences were affiliated with species of *Methylococcaceae*, but most were not closely related to cultured methanotrophs. Second, ^{13}C ethane was consumed by members of a novel group of *Methylococcaceae*. Growth with ethane as the major carbon source has not previously been observed in members of the *Methylococcaceae*; a highly divergent *pmoA*-like gene detected in the ^{13}C-labeled DNA may encode an ethane monooxygenase. Third, ^{13}C propane was consumed by members of a group of unclassified *Gammaproteobacteria* species not previously linked to propane oxidation. This study identifies several bacterial lineages as participants in the oxidation of gaseous hydrocarbons in marine seeps and supports the idea of an alternate function for some *pmoA*-like genes.

Hydrocarbon seeps are widespread along continental margins and emit large amounts of oil and gas into the surrounding environment. This gas is primarily composed of methane, a powerful greenhouse gas, and marine hydrocarbon seeps are estimated to contribute 20 Tg year^{-1} methane to the atmosphere, representing about 5% of the total atmospheric flux (21, 39). Seeps of thermogenic gas also release an estimated 0.45 Tg year^{-1} ethane and 0.09 Tg year^{-1} propane to the atmosphere (20). Each of these three fluxes would be substantially larger if not for microbial oxidation in the sediments and water column (68). Methane, ethane, and propane are subject to anaerobic oxidation in anoxic sediments and water columns (44, 53, 68) or to aerobic oxidation in oxic and suboxic water columns and oxygenated surface sediment (10, 47, 53, 80). We focus here on aerobic oxidation.

The majority of known aerobic methane-oxidizing bacteria are members of either *Gammaproteobacteria* (type I) or *Alphaproteobacteria* (type II) (29), though several strains of highly acidophilic methanotrophic *Verrucomicrobia* have also been recently isolated (63). Most methanotrophs are capable of growth only on methane or other one-carbon compounds (17, 29), using a methane monooxygenase (MMO) enzyme to oxidize methane to methanol. There are two known forms of this enzyme: soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO). sMMO is a soluble, di-iron-containing monooxygenase found only in certain methanotrophs and typically expressed only under low-copper conditions (57). In contrast, pMMO is a membrane-bound enzyme believed to contain copper and iron (26). It is found in all known methanotrophs, with the exception of species of the genus *Methylocella* (16). *pmoA*, the gene encoding the α subunit of pMMO, is often used to identify methanotrophic bacteria (54). Very few methanotrophs from marine environments have been cultured (22, 49, 72, 74), but several previous studies of marine methanotrophs (35, 62, 77, 82, 85) have been performed with culture-independent methods and have almost exclusively detected type I methanotrophs. Many of the *pmoA* sequences from methane seep sites are quite different from those of cultured organisms, suggesting that these environments may contain many novel methanotrophs (77, 82, 85).

Even less is known about the organisms that oxidize ethane or propane in marine environments. The number of such isolates, which primarily represent high G+C Gram-positive bacteria (*Nocardia*, *Pseudonocardia*, *Gordonia*, *Mycobacterium*, and *Rhodococcus*) or *Pseudomonas* species, is limited (70). Nearly all of these strains were isolated from soil and selected

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for their ability to grow on propane or *n*-butane as the sole carbon source. Most propane-oxidizing strains can oxidize butane, as well as a range of longer chain *n*-alkanes, but differ in the ability to oxidize ethane. These strains show little, if any, ability to oxidize methane, and none have been shown to grow with methane as the sole carbon source (13, 27, 38, 45, 65). As with methane metabolism, the first step in aerobic ethane and propane metabolism is the oxidation of the alkane to an alcohol (70). Several different enzymes are known to catalyze this step. *Thauera butanivorans* uses a soluble di-iron butane monooxygenase related to sMMO to oxidize C_2 through C_9 *n*-alkanes (18, 73). *Gordonia* sp. strain TY-5, *Mycobacterium* sp. strain TY-6, and *Pseudonocardia* sp. strain TY-7 contain soluble di-iron propane monooxygenases that are capable of both terminal and subterminal propane oxidation and differ in their substrate ranges (45, 46). *Nocardioides* sp. strain CF8 is believed to possess a copper-containing monooxygenase similar to pMMO and ammonia monooxygenase (27, 28). An alkane hydroxylase typically used to oxidize longer-chain *n*-alkanes has also shown some ability to oxidize propane and butane but not ethane (38). The variety of enzymes and their substrate ranges make it difficult to identify ethane or propane oxidizers with a single functional gene.

In order to identify the organisms responsible for methane, ethane, and propane oxidation at hydrocarbon seeps, we used stable isotope probing (SIP). SIP allows the identification of organisms actively consuming a ^{13}C-labeled substrate of interest, based on the incorporation of ^{13}C into biomass, including DNA and lipids (67). We collected sediment from the Coal Oil Point seep field and incubated sediment-seawater slurries with ^{13}C methane, ethane, or propane. Samples were removed at three time points, chosen to ensure sufficient ^{13}C incorporation into DNA while minimizing the spread of ^{13}C through the community as a result of cross-feeding on metabolic byproducts. ^{13}C-DNA was separated from ^{12}C-DNA by CsCl density gradient ultracentrifugation, and we used the fractionated DNA for terminal restriction fragment length polymorphism (T-RFLP) and clone library analysis. We also measured ^{13}C incorporation into fatty acids in order to confirm significant ^{13}C enrichment in membrane lipids, to determine the carbon labeling pattern for each substrate and lipid, and to further characterize the composition of the microbial community.

MATERIALS AND METHODS

Study site and sample collection. Sediment for SIP incubations was collected from Shane Seep in the Coal Oil Point seep field, offshore of Santa Barbara, CA (34°24.370′N, 119°53.428′W; 21 m depth). Coal Oil Point is one of the world's most prolific areas of hydrocarbon seepage, emitting 2×10^{10} g year^{-1} methane, 1.9×10^9 g year^{-1} ethane, 1.4×10^9 g year^{-1} propane, and 5.8×10^6 liters year^{-1} oil (9, 31). The shallow, nearshore seeps are highly dynamic environments, with variable gas flux levels and seasonal changes in sediment deposition (43). A neighboring seep, Brian Seep, has been described in detail by Kinnaman et al. (43) and Treude and Ziebis (78); Shane Seep is very similar, though the sediment contains more oil than at Brian Seep. At Brian Seep, convective pore water transport allows oxygen penetration through the top centimeter of sediment; the high methane oxidation rates observed in this layer have been attributed to aerobic methane oxidation (78). The distribution of chemicals in sediment pore fluids at Shane Seep suggests such that convective processes are also active (42). For this study, divers collected sediment from the top 1 to 2 cm in areas of active bubbling at Shane Seep; this sediment had no detectable sulfide odor. Sediment and the overlying seawater were collected in sterile polypropylene tubes, and samples were kept on ice until return to the laboratory. SIP incubations were prepared immediately.

SIP incubations. SIP incubations were carried out with ^{13}C-labeled methane, ethane, or propane (Isotec) (99% ^{13}C). Parallel incubations used natural abundance levels of ^{13}C for each hydrocarbon substrate (referred to as ^{12}C controls). No hydrocarbon was added to an additional control sample. Sediment and overlying seawater from six 50-ml collection tubes were pooled and mixed to form a slurry, of which ~25 g was added to each 125-ml serum bottle. Bottles were sealed with butyl rubber stoppers and aluminum crimp caps. A 5-ml volume of hydrocarbon gas was injected into each bottle, and air was added for overpressure to allow removal of samples for gas analysis. Samples were incubated at room temperature in the dark, with shaking at 75 rpm. Every 24 to 72 h, headspace concentrations of hydrocarbons, oxygen, and carbon dioxide were measured with a 3000A MicroGC system (Agilent) equipped with a thermal conductivity detector. When the hydrocarbon gas had nearly been consumed, the headspace was purged with air and an additional 5 ml of hydrocarbon gas was added. Oxygen concentrations in the headspace remained above 10% at all times. Sediment samples (3 ml) were removed at 3 days and 6 days and after the second 5-ml aliquot of hydrocarbon had been consumed (9 days for methane and ethane, 14 days for propane). Sediment was stored frozen until analysis.

Phospholipid fatty acid extraction, quantification, and isotopic analysis. Samples were lyophilized and then extracted in dichloromethane (DCM)/methanol (9:1) by the use of a microwave-assisted extraction system (CEM Mars5) at 100°C for 20 min with stirring. The total lipid extract was collected and filtered using a GFF-A filter, and the solvent was removed under dry N_2 conditions. The lipid extract was resuspended in 0.5 ml of hexane, and fatty acid fractions were purified by solid-phase extraction (Phenomenex Sepra NH2) (0.5 g), with elution by the use of 8.0 ml of 2% formic acid in DCM. Fatty acids were converted to their methyl esters by reaction with BF_3-methanol at 70°C for 20 min and were extracted into 10 ml of 3× hexane.

Fatty acid structure and abundance were quantified by gas chromatography/mass spectrometry/flame ionization detection (GC/MS/FID) using a Thermo Finnigan Trace/DSQ system at Caltech. GC separation employed a ZB-5ms column (Phenomenex) (30 m by 0.25 mm; 0.25 μm pore size) with a programmable-temperature vaporization (PTV) injector. The effluent from the GC column was split 80/20 between the MS and FID, allowing for simultaneous structure identification by MS and quantitation using the FID signal by comparison to an internal standard (palmitic acid isopropyl ester). No correction for relative response factors in the FID was made.

Stable carbon isotope ratios (δ^{13}C values) were measured using a Thermo Finnigan GC-isotope ratio mass spectrometer (IRMS) at the Marine Science Institute Analytical Lab, University of California (UC), Santa Barbara, CA. This system used a Trace GC sytem with an Omegawax 250 capillary column (Supelco) (30 m by 0.25 mm; 0.25 μm pore size) with a splitless injector, a GC combustion III interface, and a Delta Plus XP mass spectrometer. The GC combustion III unit catalyzed the oxidation of organic analytes to CO_2 over Cu/Ni/Pt wire heated to 950°C, and water was removed through a selectively permeable membrane. The carrier gas was helium, with a flow rate of 2.4 ml min^{-1}. Peaks eluted from the GC column were converted into CO_2 and analyzed by IRMS. Carbon isotope ratios for each compound were measured relative to a CO_2 working standard with a nominal δ^{13}C of −32.6‰. All δ^{13}C values were normalized to a decanoic acid methyl ester (C10:0) isotope standard obtained from Arndt Shimmelmann at Indiana University and then corrected for the addition of the methyl group from methanol, assuming $\delta^{13}C_{methanol} = -25‰$. Each sample was analyzed twice; in all cases, the atomic %^{13}C was within ±0.5% ^{13}C between duplicates for ^{13}C-labeled samples and within ±0.01% ^{13}C for ^{12}C controls.

CsCl gradient fractionation. Sediment DNA was extracted with a FastDNA spin kit for soil (MP Biomedicals). ^{12}C-DNA and ^{13}C-DNA were separated by ultracentrifugation and density gradient fractionation as described by Neufeld et al. (60). Briefly, ~5 to 7 μg of DNA was added to a CsCl solution and centrifuged in a VTi 65.2 rotor (Beckman) at 177,000 × *g* for 48 to 60 h at 20°C. Gradients were separated into 12 425-μl fractions, where fraction 1 was the heaviest and fraction 12 was the lightest. The density of selected fractions was checked with a refractometer (Reichert). DNA was precipitated with polyethylene glycol and glycogen, washed with 70% ethanol, and eluted in 30 μl of Tris-EDTA buffer. The amount of DNA in each fraction was quantified with a PicoGreen double-stranded DNA (dsDNA) assay kit (Invitrogen).

T-RFLP. T-RFLP analysis was used for initial characterization of the gradient DNA. For fractions 2, 4, 6, 8, 10, and 12 of each sample, the 16S rRNA gene was PCR amplified using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′; labeled with 6-carboxyfluorescein) and 1392R (5′-ACGGGCGGTGTGTAC-3′). Each 50-μl reaction mixture contained 1× buffer, 1.5 mM $MgCl_2$, 200 μM each dNTP (deoxynucleoside triphosphate), 0.5 μM each primer, 0.2 mg/ml bovine serum albumin (BSA), 1.25 U of *Taq* (Qiagen), and 2 or 4 μl of DNA (2 μl for

the three "light" fractions and 4 μl for the three "heavy" fractions). Thermal cycling conditions were as follows: initial denaturation at 95°C for 2 min; 30 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C for 90 s; and a final extension at 72°C for 5 min. Duplicate PCRs were pooled and cleaned with a Wizard SV DNA purification kit (Promega). DNA (100 ng) was digested with MspI or HhaI for 3 h at 37°C, followed by heat inactivation for 20 min at 65°C. Digests were purified using a Montage PCR centrifugal filter device kit (Millipore) and analyzed at the UC Berkeley DNA Sequencing Facility. T-REX (10) was employed for data processing, using a clustering threshold of 1 bp. Relative abundances were calculated using peak areas. Preliminary samples were run in duplicate. The relative abundances of all peaks that were >1% of the total peak area were within ±2% of the total peak area of the duplicates.

16S rRNA and *pmoA* gene clone libraries. 16S rRNA gene clone libraries were constructed from the initial sediment and at least one fraction of "heavy" DNA (fraction 4 or 6; 1.73 or 1.75 g ml^{-1}) from each substrate at each time point. Clone libraries were also created from selected controls: "light" DNA (fraction 10; 1.68 g ml^{-1}) from ^{13}C incubations and "heavy" DNA from ^{12}C control incubations. PCR conditions were the same as those for T-RFLP, except that an unlabeled forward primer was used.

pmoA clone libraries were constructed from the initial sediment and heavy DNA from day 3 of the methane incubation using the primers pmoA189f and mb661r (14). For the ethane incubations, we used additional degenerate primers designed to amplify the highly divergent *pmoA* sequences listed in GenBank for *Methylococcaceae* ET-HIRO and ET-SHO (AB453960 to AB453963) as well as other *Methylococcaceae* species. Heavy DNA from day 6 of the ethane incubation was amplified with the following pairs of primers: pmoA189f and mb661r, ethmo189f (5′-GGYGAYTGGGAYTTYTGG-3′) and ethmo661r (5′-CTGGA GCMACGTCTTTAC-3′), and ethmo189f and ethmo649r (5′-GTWCCMCGC TCNAYCATBC-3′). Reaction concentrations were the same as those described above, and thermal cycling conditions were as follows: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 60 s, 54°C for 90 s, and 72°C for 60 s; and a final extension at 72°C for 5 min. For the ethane sample, the template volume was limited and yields were low in all three reactions, so the PCR products were pooled.

For both 16S rRNA and *pmoA*, PCR products were cleaned with a Wizard SV DNA purification kit (Promega) and cloned with a PCR cloning kit (Qiagen). Plasmid DNA was isolated from randomly selected clones with an UltraClean 6-minute Mini Plasmid Prep kit (Mo Bio) and sequenced at the UC Berkeley DNA Sequencing Facility.

Phylogenetic analysis and assignment of T-RFs. Sequences were edited and assembled with Sequencher (Gene Codes Corp.). 16S rRNA gene sequences were screened for potential chimeras with Bellerophon (32), CHIMERA CHECK (12), and Pintail (1) software; suspected chimeras were not considered in further analysis. Phylogenetic affiliations of 16S rRNA genes and their relative abundances were determined with the RDP Classifier tool (81). Representative 16S rRNA and *pmoA* sequences and related reference sequences were aligned using CLUSTALW, and neighbor-joining phylogenetic trees were constructed with MEGA software (76).

Sequences were further classified by *in silico* digestions performed with MspI and HhaI to predict terminal restriction fragment (T-RF) lengths. These were used to assign identities to T-RFs from the T-RFLP analysis. 16S rRNA gene sequences from both ^{13}C-DNA and controls were used to create a list of sequences with each predicted T-RF length. Predicted and observed T-RFs correlated closely, provided that each observed T-RF length was corrected by 4 bp. Differences between observed and predicted T-RF lengths have often been observed in T-RFLP studies (64, 69), and can differ based on purine content or fragment length (41), but in this case appeared to take the form of a constant 4-bp offset. The T-RF lengths shown represent the observed T-RF length plus 4 bp. T-RFs were then combined to form six major groups, based on the clone library sequences with these predicted T-RF lengths.

Nucleotide sequence accession numbers. These sequence data have been submitted to the GenBank database under accession numbers GU584298 to GU584873 (16S rRNA gene) and GU584229 to GU584297 (*pmoA*).

RESULTS

^{13}C enrichment in fatty acids. Fatty acids were extracted from the initial sediment used for inoculation and from the sediment remaining after the final time point of each SIP incubation. In all cases, the most abundant fatty acids were the 16- and 18-carbon-saturated fatty acids (16:0 and 18:0) and monounsaturated fatty acids (16:1 and 18:1; multiple positional isomers were present for each but were not differentiated) (data not shown). In all cases, the ^{13}C incubations showed very high levels of ^{13}C incorporation into individual fatty acids (up to 63%), while the ^{12}C controls showed no significant ^{13}C enrichment (<1.25% ^{13}C) (Fig. 1A). The 16:1, 16:0, and 18:1 fatty acids were heavily enriched with ^{13}C in all samples, while the 18:0 and 14:0 fatty acids showed much less ^{13}C incorporation. In all samples, >50% of the ^{13}C incorporated into fatty acids was found in the 16:1 fatty acid, ~20% in the 16:0 fatty acid, and <3% in the 18:0 fatty acid. The 18:1 fatty acid accounted for ~20% of the ^{13}C in the ethane and propane incubations but for only 4% in the methane incubation (Fig. 1B). Mass spectra for the highly labeled fatty acids, particularly for the 16:1 fatty acid, indicated a bimodal pattern of isotopic distribution, where most molecules either contained a natural abundance of ^{13}C (no ^{13}C or one ^{13}C per molecule) or were fully labeled with ^{13}C (Fig. 2). Mass spectra from unlabeled (^{12}C) controls showed no appreciable incorporation of ^{13}C, which was in agreement with the $\delta^{13}C$ values measured by GC/IRMS.

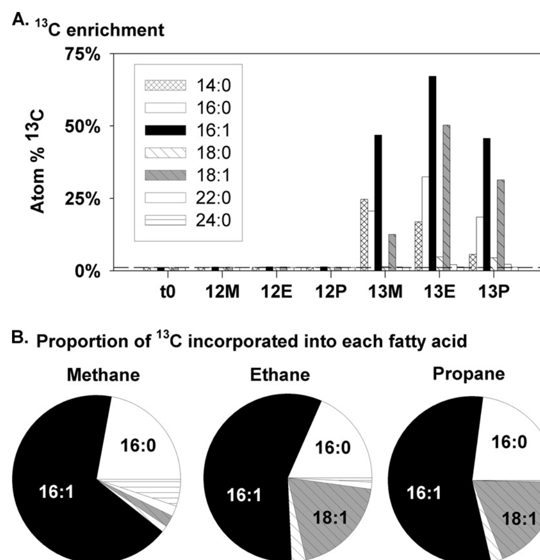

FIG. 1. (A) ^{13}C enrichment of phospholipid fatty acids of ^{13}C methane (M), ethane (E), and propane (P) incubations and ^{12}C controls in initial sediment (t0) and at final time points. The 16:1 and 18:1 isomers were not differentiated. The dashed line indicates natural abundance levels of ^{13}C. (B) Proportion of ^{13}C incorporated into each fatty acid.

16S rRNA gene clone libraries. DNAs from each ^{13}C sample and its ^{12}C control were separated by density gradient ultracentrifugation into 12 fractions, with the buoyant densities ranging from 1.66 g ml^{-1} to 1.78 g ml^{-1}). 16S rRNA gene clone libraries were constructed from at least one heavy DNA fraction (1.73 or 1.75 g ml^{-1}) from each gas at each time point, as well as from a heavy DNA fraction from selected ^{12}C controls and a light DNA fraction (1.69 g ml^{-1}) from selected ^{13}C samples. Because we were primarily interested in the most abundant phylotypes, we sequenced 24 to 48 clones from most fractions, for a total of 595 sequences. The phylogenetic affiliations of these sequences are shown in Fig. 3 and 4.

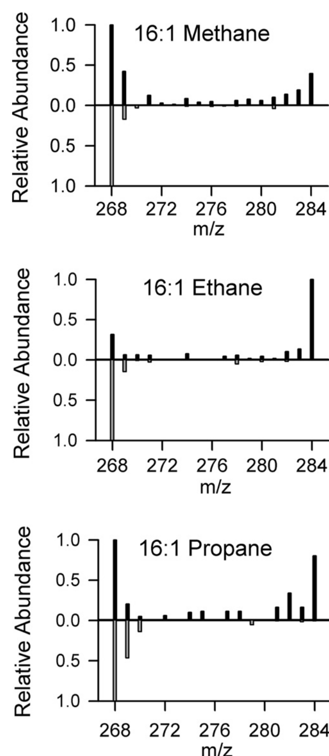

FIG. 2. Mass spectra for the 16:1 fatty acid molecular ion peak, showing the extent of ^{13}C labeling at the final time point of the methane, ethane, and propane incubations. The *m/z* values for the spectra span the full range from no ^{13}C incorporation (*m/z* = 268) to full ^{13}C labeling (*m/z* = 268 + 16 = 284). The spectra oriented upward correspond to incubations with ^{13}C labeling, whereas the inverted spectra correspond to control incubations with substrate lacking ^{13}C labeling. Each spectrum is normalized to the height of its tallest peak.

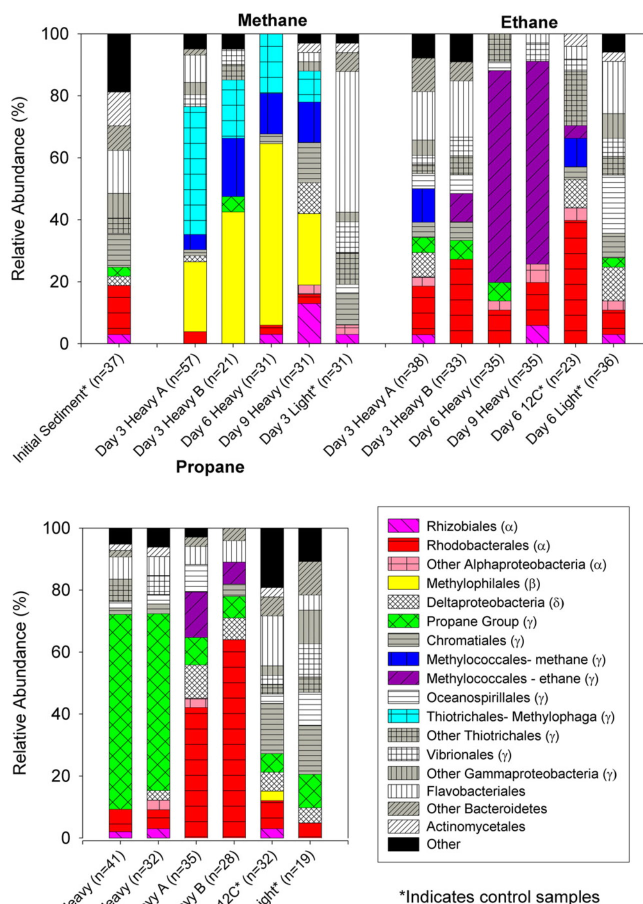

FIG. 3. Relative abundances of 16S rRNA sequences in clone libraries from heavy DNA (fraction 4 or 6 [noted as Heavy A or Heavy B] or both) from the three time points of the ^{13}C methane, ethane, and propane incubations, selected controls (light DNA from the ^{13}C incubations and heavy DNA from the ^{12}C controls), and the initial sediment. Sequences were grouped using the RDP Classifier tool; "other" combines sequences that, classified at the order level, represented less than 5% of the clones in any individual clone library. Representative sequences from each of the groups indicated in methane, ethane, or propane oxidation are included in the phylogenetic tree in Fig. 4.

Methane. The most abundant sequences in the heavy DNA from all three time points of the methane incubation were from species of a group of *Gammaproteobacteria* related to *Methylophaga* and a group of *Betaproteobacteria* in the family *Methylophilaceae*, followed by a group of *Gammaproteobacteria* in the family *Methylococcaceae*. These three groups accounted for 81% of sequences in the heavy fraction 6 from day 3, 91% in the heavy fraction from day 6, and 45% in the heavy fraction from day 9, with sequences of *Methylophaga* and the *Methylophilaceae* bacteria making up the majority at all time points. By day 9, two additional groups comprised at least 10% of the heavy DNA sequences: *Alphaproteobacteria* in the order *Rhizobiales* and *Gammaproteobacteria* in the order *Chromatiales*. In comparison, the most common sequences in the light DNA clone library were *Flavobacteriales* (45%), followed by *Thiotrichales*, *Vibrionales*, and *Chromatiales*; among the 31 clones, there were no sequences affiliated with the *Methylococcaceae*, *Methylophilaceae*, or *Methylophaga*.

Ethane. At day 3, the heavy DNA sequences from the ^{13}C ethane incubations were similar to those of the ^{12}C control heavy DNA and the light DNA from the ^{13}C sample, predominately representing *Rhodobacterales*, *Oceanospirillale*s, *Thiotrichales*, and *Flavobacteriale*s species. By days 6 and 9, though, the majority of heavy DNA sequences were from a group of *Gammaproteobacteria* bacteria in the family *Methylococcaceae.* Like the methane *Methylococcaceae* sequences, their closest cultured relatives were *Methylobacter* sp. BB5.1, *Methylobacter luteus* NCIMB 11914, and *Methylobacter marinus* strain A45. However, the ethane *Methylococcaceae* sequences all clustered separately from the methane *Methylococcaceae* sequences and could be distinguished by their predicted MspI T-RF (496 bp for ethane and 491 bp for methane). The most similar sequences in GenBank were from "*Methylococcaceae* bacterium ET-HIRO" (AB453957.1) and "*Methylococcaceae* bacterium ET-SHO" (AB453956.1).

Propane. The majority of sequences in the heavy DNA clone libraries from days 3 and 6 of the propane incubation belonged to species from a group of unclassified *Gammaproteobacteria* most closely related to the genera *Marinimicrobium*, *Microbulbifer*, and *Saccharophagus.* This group accounted for 61% and 56% of heavy DNA sequences at days 3 and 6, respectively, but only 6% of

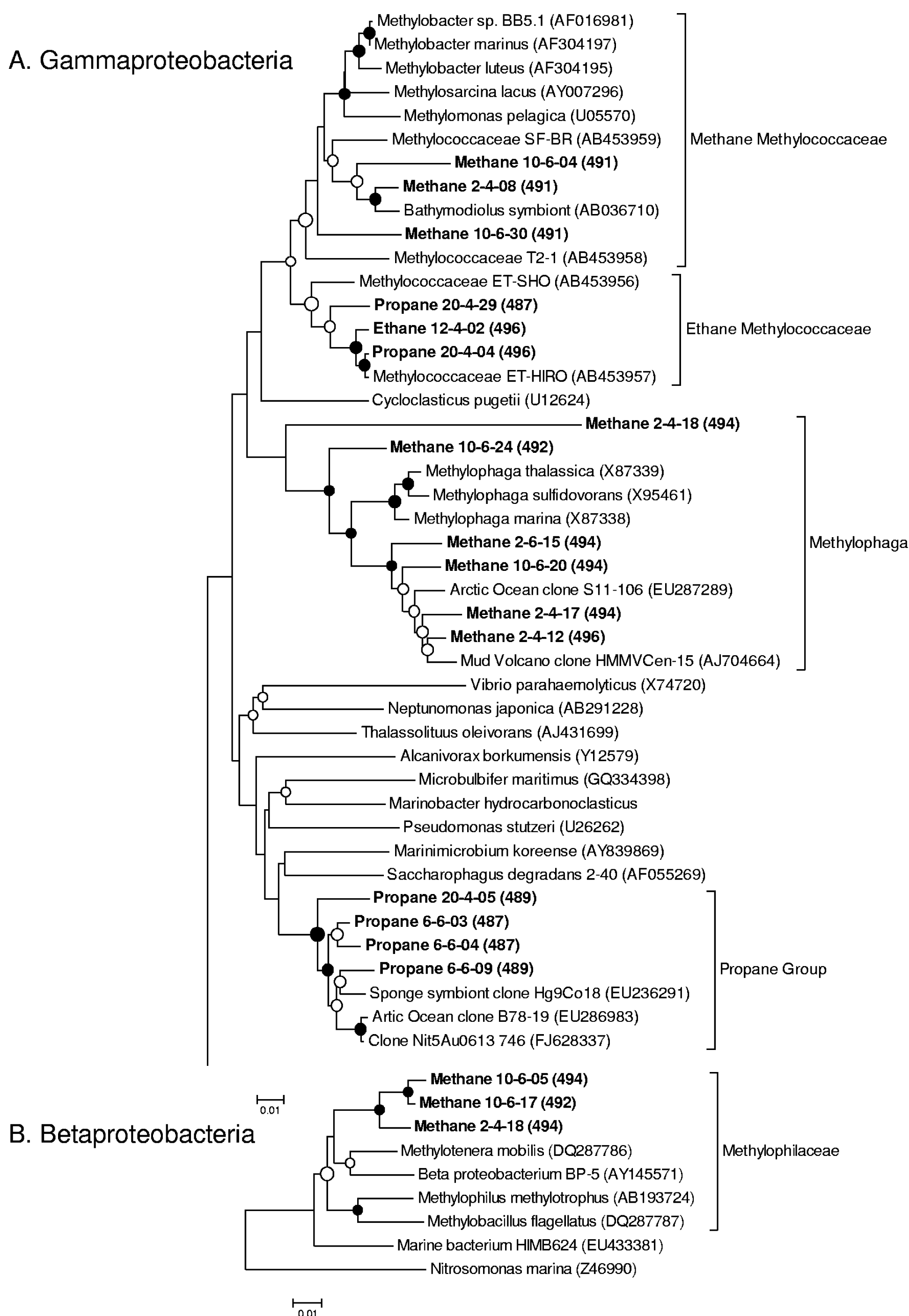

FIG. 4. Neighbor-joining phylogenetic trees of the 16S rRNA gene sequences from groups involved in methane, ethane, or propane oxidation, based on their abundance in both the heavy DNA clone libraries and the heavy T-RFLP fractions, relative to light DNA and ^{12}C controls. (A) *Gammaproteobacteria*; (B) *Betaproteobacteria*. Sequences from this study are shown in bold, with predicted MspI T-RF lengths in parentheses. Reference sequences from GenBank are shown with accession numbers in parentheses. Filled circles indicate bootstrap values above 90% and open circles bootstrap values above 50% (2,000 replicates).

heavy DNA from the ^{12}C control. By day 14, however, they made up less than 10% of the sequences in fractions 4 and 6, and *Rhodobacterales* sequences were most abundant instead (43% and 65%, respectively). *Rhodobacterales* sequences made up only 5% of the light DNA sequences from the last time point.

***pmoA* and *pmoA*-like gene clone libraries.** In order to further characterize the *Methylococcaceae* species in the ethane incubations, we created small *pmoA* clone libraries by the use of degenerate primers designed to amplify highly divergent *pmoA* sequences. The clone library with heavy DNA from the ethane

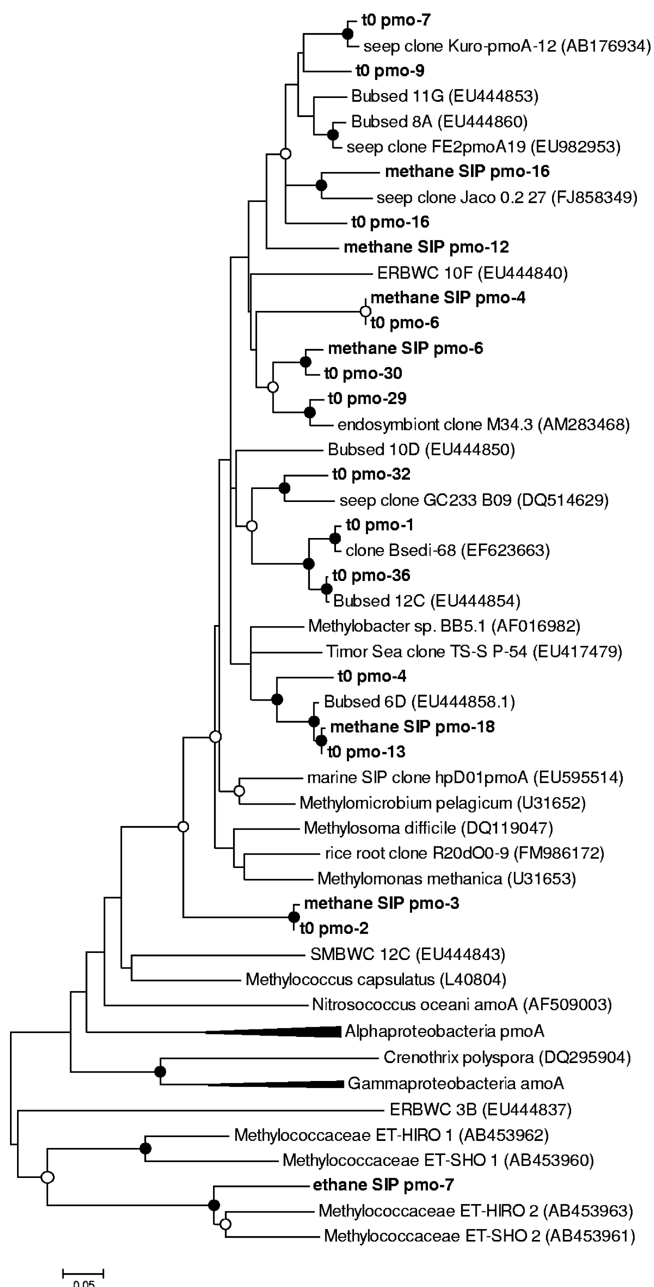

FIG. 5. Neighbor-joining phylogenetic tree of *pmoA* gene sequences from seep sediment (t0) and methane and ethane heavy DNA sequences, plus reference sequences from GenBank (accession numbers in parentheses). Only the divergent sequences from the ethane SIP sample are shown; others were identical to those in the t0 and methane SIP samples. Filled circles indicate bootstrap values above 90% and open circles bootstrap values above 50% (2,000 replicates). The alphaproteobacterial *pmoA* and gammaproteobacterial *amoA* sequences are condensed for clarity.

incubation contained several highly divergent *pmoA* sequences, which grouped with the ET-HIRO and ET-SHO sequences (Fig. 5). We also created small *pmoA* clone libraries from the initial sediment (46 clones) and heavy DNA from the methane incubations (10 clones) by employing the commonly used primers pmoA189f and mb661r. Representative sequences are also shown in Fig. 5. These sequences all grouped with the gammaproteobacterial *pmoA* sequences and were most closely related to those from other uncultured marine methanotrophs.

T-RFLP fingerprinting of density gradient fractions and correlation with clone libraries. Though clone libraries provide a more detailed representation of the 16S rRNA genes present in each fraction, T-RFLP is useful for characterizing a broader range of gradient fractions from both ^{13}C incubations and ^{12}C controls. From six fractions of each sample, 16S rRNA genes were amplified and digested with the restriction enzymes MspI and HhaI. The two restriction enzymes produced similar results; only those from MspI are shown here (Fig. 6; see also Fig. S1 to S3 in the supplemental material). It was possible to amplify the 16S rRNA gene from nearly every fraction, but the amount of PCR product was insufficient for further analysis in some of the heavy fractions.

In order to correlate T-RFLP and clone library data, we performed *in silico* digestions of all suitable 16S rRNA sequences from the clone libraries and grouped them by T-RF length. The MspI T-RF lengths clearly distinguished the species of *Rhodobacterales* and other *Alphaproteobacteria* (439 or 441 bp) from those of the *Flavobacteriales* and other *Bacteroidetes* (91, 93, or 95 bp). All members of the "propane group" of unclassified *Gammaproteobacteria* species had a predicted T-RF of 485, 487, or 489 bp, though other high-abundance *Gammproteobacteria* (*Oceanospirillales*) sequences shared these T-RF lengths. All of the *Methylococcaceae* sequences from the methane incubation had a predicted T-RF of 491 bp, while all of the *Methylococcaceae* sequences from the ethane incubation had a T-RF of 496 bp. The 496 bp T-RF was also predicted for some *Methylophaga* sequences, though the rest of the *Methylophaga* sequences had T-RFs of 492 or 494 bp. It was not possible to differentiate *Methylophaga* from *Methylophilaceae*; these sequences also all had a predicted T-RF of 492 or 494 bp. Sequences of several other groups of *Gammaproteobacteria* (predominately *Thiotrichales* and *Chromatiales*) also had a predicted T-RF of 492, 494, or 496, but these sequences never represented more than 10% of the sequences in the heavy DNA clone libraries (Fig. 3). They were, however, more abundant in the clone libraries from the control samples, so the presence of these fragment lengths in T-RFLP samples from the light fractions was also expected, and these groups of *Gammaproteobacteria* and the *Methylophilaceae* could not be clearly differentiated. Digestion with HhaI led to better differentiation of a few groups of *Gammaproteobacteria* but more poorly resolved others and still did not differentiate between sequences of the methylotrophic *Piscirickettsiaceae* (*Gammaproteobacteria*) and *Methylophilaceae* (*Betaproteobacteria*) species.

Based on these observations, we combined our T-RFs into six main groups. All remaining T-RFs were combined and are represented as "other"; the relative abundances of the individual T-RFs in this group were never more than 5% in the ^{13}C-labeled heavy fractions. Figure 6 shows T-RFLP profiles from one time point each of the methane, ethane, and propane incubations. The other two time points for each substrate are shown in the supplemental material. In all cases, the T-RFs corresponding to the most abundant sequences in the heavy DNA clone libraries were also most abundant in the heavy fractions of the T-RFLP profiles.

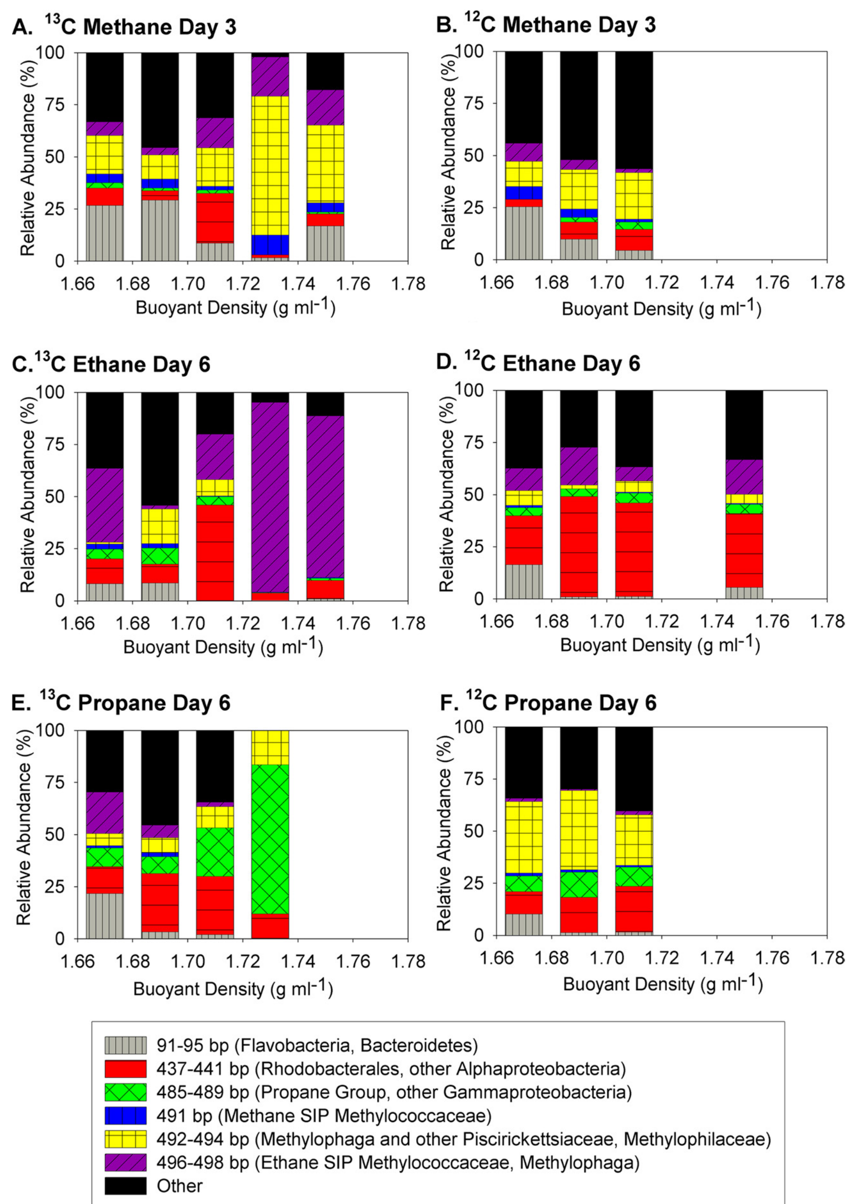

FIG. 6. T-RFLP fingerprinting of the 16S rRNA gene in density gradient fractions from day 3 of the ^{13}C (A) and ^{12}C (B) methane, day 6 of the ^{13}C (C) and ^{12}C (D) ethane, and day 6 of the ^{13}C (E) and ^{12}C (F) propane incubations. Major T-RFs were identified by *in silico* digestions of clone library sequences with MspI. Less-abundant T-RFs (<5% of the total peak area in any of the heavy fractions) were combined and are shown as "other." Fractions with insufficient DNA for analysis are left blank.

DISCUSSION

Factors to be considered with the SIP technique. Though SIP can be useful in linking metabolic function to identity, there are challenges in designing these experiments and correctly interpreting the results. A successful SIP study requires sufficient incorporation of ^{13}C into biomass, such that ^{13}C-labeled DNA can be differentiated from the background of unlabeled DNA distributed across all fractions (51, 58). Longer incubations, which increase the extent of labeling, also lead to the spread of ^{13}C throughout the community by cross-feeding involving metabolic byproducts or dead cells (52). Longer incubations and higher substrate concentrations also increase the possibility of selecting organisms adapted for higher substrate concentrations rather than those active *in situ* (58).

We attempted to minimize these problems in two ways. First, we sampled each incubation at three points in time, increasing the odds of catching the incubations at a point where there was sufficient ^{13}C incorporation but minimal cross-feeding. Second, we ran parallel incubations with ^{12}C methane, ethane, and propane. Though there was DNA present in most of the heavy fractions of the ^{12}C controls, the amount recovered from fractions 6 and 7 was generally smaller than that from the ^{13}C samples (see Fig. S4 in the supplemental material). T-RFLP analysis of ^{12}C samples typically showed similar

communities in the heavy and light fractions, while ^{13}C samples showed distinct communities. A distinctive "heavy" community was also observed in the clone libraries, though accompanied by a smaller number of sequences from the most abundant light groups, such as those of *Rhodobacterales*, *Flavobacteriales*, *Thiotrichales*, *Vibrionales*, and *Chromatiales* species. While it is possible that these organisms actually incorporated ^{13}C, it is more likely that most of these sequences were from contaminating ^{12}C-DNA. Only groups that were much more abundant in the heavy fractions of the ^{13}C incubations than in the heavy fractions of the ^{12}C incubations and light fractions of the ^{13}C incubations, based on both T-RFLP and clone libraries, were considered ^{13}C labeled. The bimodal distribution of ^{13}C content in fatty acids (Fig. 2) provided additional confirmation of ^{13}C uptake by a subset of the microbial community and supported the results of the clone library and T-RFLP data.

Methane. From both the T-RFLP data and the clone libraries, it appears that the major consumers of ^{13}C in the methane incubations were affiliated with the *Methylophilaceae* or *Methylophaga*, with a smaller contribution from the *Methylococcaceae*. The *Methylophilaceae* and *Methylophaga* are both groups of non-methane-oxidizing methylotrophs, consuming other C1 compounds such as methanol and methylamine (2); previous SIP studies have shown that members of the *Methylophaga* group were the primary consumers of methanol and methylamine in marine environments (56, 59, 61). Most methane SIP studies have observed some ^{13}C uptake by organisms more closely related to nonmethanotrophic methylotrophs than to known methanotrophs, a results which has been attributed to cross-feeding (8, 33, 50). However, methylotroph sequences in two marine studies were much less abundant than sequences related to known methanotrophs (37, 56), in contrast to the pattern of relative abundance we observed. In a study of rice roots, Qiu et al. (66) found that most of the sequences in a ^{13}C-DNA clone library were affiliated with *Methylophilales* species, but their long (20-day) incubations make cross-feeding the likely explanation. In our study, the sequences affiliated with *Methylophaga* and *Methylophilales* were far more abundant than the *Methylococcaceae* sequences after just 3 days and remained more abundant over the 9-day incubation period. The early and sustained representation of methylotrophs in the heavy fractions suggests that these *Methylophaga* and *Methylophilales* species are either directly incorporating methane or are involved in a closely coupled relationship with methanotrophic *Methylococcaceae.* In all three of these groups, the most abundant fatty acids are 16:1 and 16:0 (3, 36, 40, 79), which is consistent with the "heavy" labeling of these fatty acids observed at the end of the incubation.

The *pmoA* data, though limited, show a number of sequences not closely related to cultured methanotrophs. The sequence of the most common phylotype in both the initial sediment and the heavy DNA from the methane incubation was only 81% similar to any other sequence in GenBank. The most closely related sequences came from several uncultured bacteria and two cultured but unclassified *Methylococcaceae* species. The other *pmoA* sequences in our methane clone library also clustered with the *Methylococcaceae* sequences, particularly with those of representatives from methane seep environments like the Eel River Basin (77). We found no sequences affiliated with type II methanotrophs, and very few sequences closely related to cultured type I methanotrophs. Based on the affiliation of these sequences with the gammaproteobacterial *pmoA* sequences, they likely belong to members of the *Methylococcaceae* observed in the 16S rRNA clone libraries, though it is also possible that they belong to the *Methylophaga*-like bacteria, representing a novel group of methanotrophs.

Ethane. ^{13}C ethane was primarily consumed by a group of organisms from the family *Methylococcaceae*. These organisms have previously been thought to be obligate methanotrophs (3), capable of growing only on methane or a few other C1 compounds. Without pure cultures, we cannot determine whether these ethane-oxidizing *Methylococcaceae* species are also capable of growth on methane. However, the ethane *Methylococcaceae* sequences were not detected in any of the heavy DNA clone libraries from the methane incubations, indicating that they were not a major contributor to methane oxidation under the conditions of the SIP incubation. Interestingly, five similar sequences from the last time point of the propane incubation were found in the heavy DNA clone library, raising the possibility that these bacteria also have the ability to consume propane or one of its metabolic byproducts.

Based on the co-occurrence of 16S rRNA genes from members of the family *Methylococcaceae* and a highly divergent *pmoA*-like gene in the heavy DNA, we hypothesize that this gene encodes part of a novel ethane monooxygenase enzyme active in some *Methylococcaceae* species. While molecular genetic and biochemical studies are needed to test this hypothesis, there is some corroboration in existing GenBank sequence entries (AB453956 to -65). These GenBank entries include 16S rRNA and *pmoA* gene sequences reported to be from the isolated cultures of the "*Methylococcaceae* bacterium" species ET-HIRO, ET-SHO, T2-1, and SF-BR. They are grouped under the title "Discovery of ethane monooxygenase in marine bacteria belongs to Methylococcaceae that could grow on ethane, but not methane," but additional information has yet to be published in the peer-reviewed literature. These *pmoA*-like sequences group outside those of all known *pmoA* genes from *Alphaproteobacteria* and *Gammaproteobacteria* species and *amoA* genes from *Betaproteobacteria* and *Gammaproteobacteria* species. They are also distantly related to the highly divergent sequence observed by Tavormina et al. (77) in the water column above methane seeps in the Eel River Basin. The prevalence and function of both these enzymes are currently unknown, but they may also play a role in the oxidation of other common biogenic molecules such as methane, ammonia, or ethene.

It is difficult to determine the environmental importance of these putative oxidizers of ethane. Both methane- and ammonia-oxidizing bacteria have long been known to oxidize small amounts of ethane (11, 34), but this has only been observed during methane- or ammonia-supported growth. High concentrations of ethane are typically accompanied by propane and even higher concentrations of methane (20, 83), making it unlikely that the bacteria are ever exposed to an environment with ethane as the most abundant carbon source. However, ethane oxidation was detectable in our incubations within 24 h, suggesting that these bacteria required little adaptation to use

ethane as the major carbon source. By the final time point, all four of the major fatty acids in the incubations were more enriched in ^{13}C than those in the methane and propane incubations. The ethane incubations also showed the highest levels of 100% ^{13}C-labeled fatty acids—50% of the 16:1 fatty acids were 100% ^{13}C labeled. As mentioned above, *Methylococcaceae* species typically contain high levels of 16:1 and 16:0 fatty acids (3), which could account for much of the ^{13}C ethane incorporation into fatty acids. In contrast to the methane incubations, though, ~20% of the ^{13}C was incorporated into 18:1 fatty acids, which are not commonly found at high levels in *Methylococcaceae* species (4). This result may have been due to different levels of fatty acid content in these *Methylococcaceae* species or to the presence of cross-feeding bacteria. By the final time point, the second-most-abundant group of T-RFs in the heavy fractions was that assigned to the species of *Rhodobacterales*, many of which contain predominately 18:1 fatty acids (25, 71, 75). Thus, the most parsimonious explanation for the lipid distributions is that 16:0 and 16:1 fatty acids were primarily produced by ethane-fed *Methylococcaceae* species throughout the incubation, whereas the 18:1 fatty acid was primarily produced by cross-feeding *Rhodobacterales* species near the end of the incubation. Elevated levels of intermediate ^{13}C labeling in 18:1 compared to 16:0 and 16:1 fatty acids further support this explanation.

Propane. Propane was primarily consumed by members of a group of unclassified *Gammaproteobacteria* whose closest cultured relatives are found in the genera *Marinimicrobium*, *Microbulbifer*, and *Saccharophagus* (16S rRNA genes ~92% similar). These bacteria are typically known for degradation of complex polysaccharides such as cellulose, chitin, and agar (19, 24), and no members of these genera have previously been linked to propane oxidation or hydrocarbon oxidation in general. However, members of this group also showed ~90% 16S rRNA gene similarity to some *Pseudomonas* species, which have been shown to oxidize propane (70), and to obligate hydrocarbon degraders such *Alcanivorax* and *Marinobacter* spp. (84).

At the last time point, the clone libraries and the T-RFLP data both showed the *Gammaproteobacteria* giving way to *Rhodobacterales* species in the heavy fractions. The *Rhodobacterales* were not very abundant in the light fractions, so this result appears to have been due to ^{13}C uptake rather than to the presence of contaminating ^{12}C-DNA, perhaps due to a change in community composition over time or, more likely, to cross-feeding. As with ethane, the idea of ^{13}C uptake by this group is supported by the fact that 18:1 fatty acids contained ~20% of all ^{13}C incorporated into fatty acids; 18:1 fatty acids are abundant in *Rhodobacterales* species (25, 71, 75). An increase in the abundance of certain members of the *Rhodobacterales* has often been observed in oil-contaminated environments or oil-amended mesocosm experiments (6, 15, 55). Whether these organisms are actually able to degrade hydrocarbons is unclear (7, 48), though at least one member of this group has been shown to degrade C_{10} through C_{13} *n*-alkanes (30). *Rhodobacterales* species have not been linked to ethane or propane oxidation, but some species are able to consume ethanol or propanol (23), likely sources of ^{13}C through cross-feeding. It may be that many of the oil-associated *Rhodobacterales* species are not capable of direct consumption of alkanes but instead feed on the byproducts of alkane metabolism, as appears to have been the case in our incubations.

As in the ethane incubations, the majority of ^{13}C propane was incorporated into 16:1 and 16:0 fatty acids, presumably belonging to the primary propane oxidizers, but the lack of cultured bacteria closely related to these unclassified *Gammaproteobacteria* species makes comparison impossible. The heavy DNA from the last time point also included several *Methylococcaceae* sequences similar to those from the ethane incubations, which may account for some of the heavily labeled 16:1 or 16:0 fatty acids. These sequences appeared only in the final samples, suggesting that the ethane oxidizers required an adaptation period before beginning propane oxidation or that they were incorporating a byproduct of propane oxidation. Most previously described ethane oxidizers can oxidize propane, and some propane oxidizers can oxidize ethane (70), so some overlap between the two is not surprising, but ethane and propane oxidation appear to be primarily performed by different communities.

Lipids. One common and striking feature of the newly produced fatty acids in each experiment is the dominance of monounsaturates, specifically, 16:1 and 18:1. High percentages of these fatty acids in membrane lipids are typical of methanotrophs and are thought to be associated with the extensive network of stacked membranes common in these organisms (5). The gammaproteobacterial (type I) methanotrophs typically contain mostly 16:1 fatty acids, while the alphaproteobacterial (type II) methanotrophs typically contain mostly 18:1 fatty acids (2, 5). The predominant incorporation of ^{13}C-methane into 16:1 fatty acids provides additional evidence that methane was consumed by gammaproteobacterial methanotrophs. The high level of incorporation into 16:1 fatty acids observed in the ethane incubation also supports our interpretation from investigations of 16S rRNA and *pmoA* clone libraries that the responsible organisms share numerous similarities with methanotrophs, potentially including stacked membranes. A second striking feature determined from these results is the large extent of fully ^{13}C-labeled lipids and low levels of any specific intermediately labeled lipid. These results suggest a high degree of metabolic specificity for all three substrates, though this may include cross-feeding relationships.

Conclusions. This report demonstrates the ability of SIP to identify previously unknown groups of methane-, ethane-, and propane-oxidizing bacteria at a marine hydrocarbon seep, with a distinct group of bacteria found to be responsible for each process. These results may have implications for our understanding of methane oxidation in marine environments. Novel groups of marine bacteria may contain monooxygenases related to those of terrestrial bacteria but with different substrate affinities, affecting our ability to detect them with common primer sets and to predict biogeochemical function based on the presence or absence of these genes. The multitude of hydrocarbon compounds present in seep environments could support a range of hydrocarbon-oxidizing bacteria, but relatively little is known about which organisms consume which compounds and under what conditions. Further work, with both culturing and culture-independent techniques like SIP, is necessary to improve understanding of these processes and their roles in biogeochemical cycling.

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