

Natural gas and temperature structured a microbial community response to the *Deepwater Horizon* oil spill

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Microbial communities present in the Gulf of Mexico rapidly responded to the *Deepwater Horizon* oil spill. In deep water plumes, these communities were initially dominated by members of *Oceanospirillales*, *Colwellia*, and *Cycloclasticus*. None of these groups were abundant in surface oil slick samples, and *Colwellia* was much more abundant in oil-degrading enrichment cultures incubated at 4 °C than at room temperature, suggesting that the colder temperatures at plume depth favored the development of these communities. These groups decreased in abundance after the well was capped in July, but the addition of hydrocarbons in laboratory incubations of deep waters from the Gulf of Mexico stimulated *Colwellia*'s growth. *Colwellia* was the primary organism that incorporated ¹³C from ethane and propane in stable isotope probing experiments, and given its abundance in environmental samples at the time that ethane and propane oxidation rates were high, it is likely that *Colwellia* was active in ethane and propane oxidation in situ. *Colwellia* also incorporated ¹³C benzene, and *Colwellia*'s abundance in crude oil enrichments without natural gas suggests that it has the ability to consume a wide range of hydrocarbon compounds or their degradation products. However, the fact that ethane and propane alone were capable of stimulating the growth of *Colwellia*, and to a lesser extent, *Oceanospirillales*, suggests that high natural gas content of this spill may have provided an advantage to these organisms.

methane | biodegradation | marine bacteria | archaea | alkane

The explosion and sinking of the *Deepwater Horizon* in April 2010 caused one of the world's largest marine oil spills, releasing at least 4 million barrels of oil before the well was capped in July 2010 (1). In addition to its size and duration, the *Deepwater Horizon* spill was different from previous oil spills for two major reasons, both of which are likely to influence the response of the microbial community. First, the spill released not just oil, but large amounts of natural gas (1, 2). The bacteria that typically consume methane, the primary component of natural gas, have limited abilities to consume multicarbon substrates (3), whereas many oil degraders are capable of growth only on larger hydrocarbons (4), and none are known to consume methane (5). The organisms that consume ethane and propane, the other major components of natural gas, remain largely unknown in marine environments, but may include organisms related to methanotrophs or oil-degraders (6). Second, because of the depth of the spill, nearly all of the gas (2, 7, 8) and some components of the oil (9) remained in the deep ocean, forming plumes of dissolved or dispersed hydrocarbons between 1,000 and 1,200 m (10–12). The temperature at these depths was just 4 °C to 6 °C, much colder than surface water. Temperature has a direct effect on microbial physiology and an effect on the physical properties of oil that influence its bioavailability; both factors will influence the response of hydrocarbon-degrading bacteria (13–15).

Crude oil is a complex mixture containing thousands of different hydrocarbon compounds. These compounds differ in solubility and volatility and are degraded at different rates; in general, the smaller, less substituted hydrocarbons are degraded faster than larger ones with more substituted groups (15, 16).

Although the ability to degrade hydrocarbons is found in many types of bacteria, the most abundant oil-degraders in marine environments are typically *Gammaproteobacteria*, particularly organisms such as *Alcanivorax*, which primarily degrades alkanes, or *Cycloclasticus*, which specializes in the degradation of aromatic compounds (16). However, most studies of microbial community response to hydrocarbons have been conducted in oil-amended mesocosm experiments with sediment, beach sand, or surface water (16), and little is known about the response to oil inputs in the deep ocean or the impact of natural gas on these communities. An initial report after the *Deepwater Horizon* spill showed that, in late May, an uncultivated group of *Oceanospirillales* were dominant in plume samples; 16 other groups of *Gammaproteobacteria* were also enriched in plume vs. out-of-plume samples (17). In June, plume samples were dominated by two different groups of *Gammaproteobacteria*, *Colwellia* and *Cycloclasticus* (2). The well was capped in mid-July, and by September, these groups were much less abundant and plumes were dominated by methylotrophs (*Methylococcaceae*, *Methyl-ophaga*, and *Methylphilaceae*), *Flavobacteria*, and *Rhodobacteriales* (18). Although the abundance of these taxa strongly suggests they played a role in hydrocarbon degradation, it is not possible to directly link any individual taxa to the degradation of a particular type of hydrocarbon with only the environmental sequence data. Here we present additional data on microbial communities in environmental samples taken over the course of the spill response, as well as the results of stable isotope probing (SIP) experiments linking *Colwellia* to the oxidation of ethane, propane, and benzene.

Results and Discussion

16S rRNA Clone Libraries from Environmental Samples. We collected samples on three cruises, referred to as “May” (May 26 to June 5, 2010), “June” (June 11–21, 2010), and “September” (September 7–17, 2010). We sequenced bacterial 16S rRNA genes from 29 deep samples and five surface samples; data from the five June samples and seven of the 12 September samples were previously published (2, 18), but we have since performed additional sequencing on some of these samples, and results from the full set of clone libraries are shown in Fig. 1. Deep water samples (below 800 m) were classified as “plume” or “non-plume” based on the presence or absence of a fluorescence anomaly indicative of aromatic hydrocarbons (11, 18–20). Non-plume samples were taken at plume depth within 5 km of a

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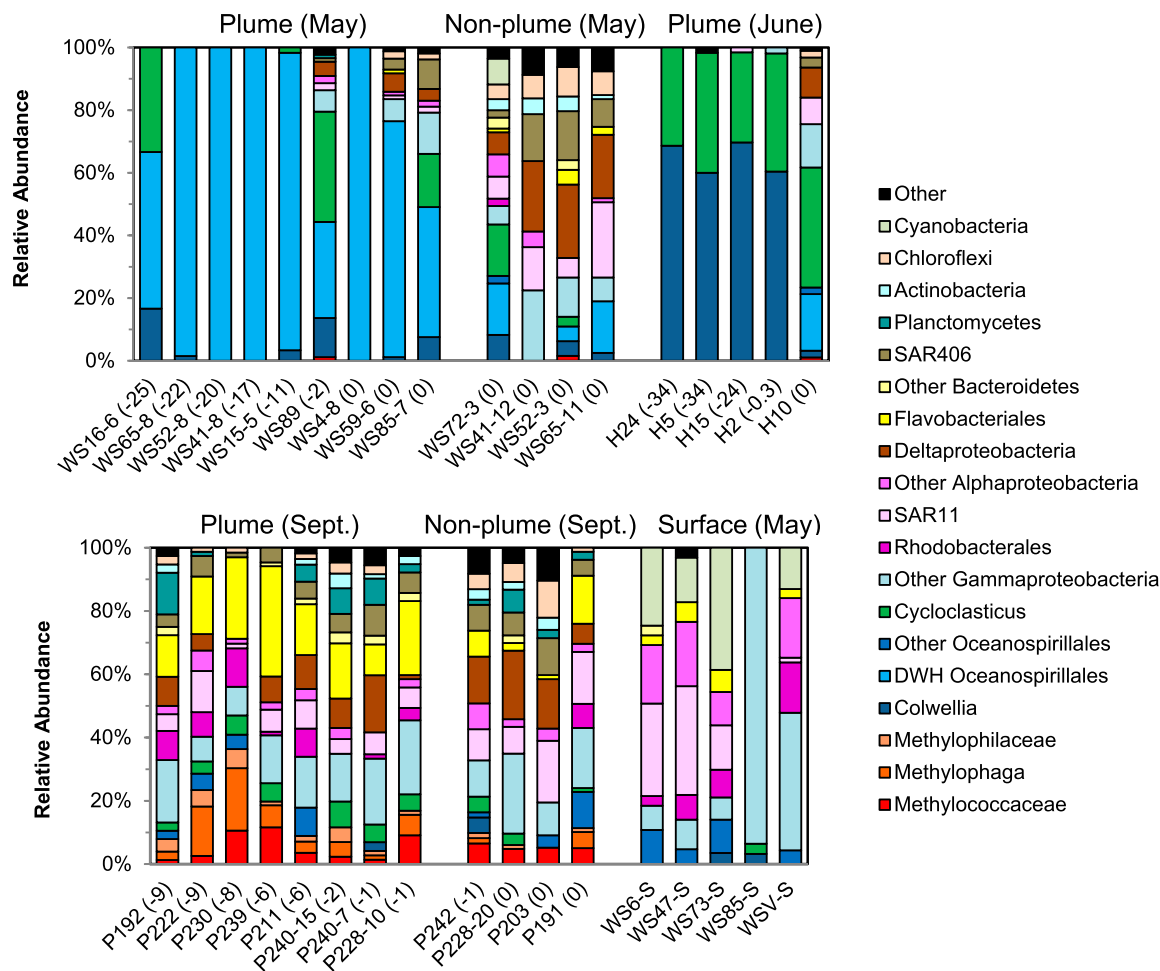


Fig. 1. Relative abundances in 16S rRNA gene clone libraries. Plume samples were taken from 1,000 to 1,300 m at sites with a fluorescence anomaly at that depth. Nonplume samples were from sites with no detectable fluorescence anomaly or from depths above or below the fluorescence anomaly (800–1,300 m). Oxygen anomalies ($\mu\text{mol/L}$) are shown in parentheses after the sample numbers. The number of sequences in each clone library is shown in Table S1 (average of 67 sequences per sample).

plume sample or at a plume site, 200 m above or below peak plume depth. Based on their proximity to plume samples, it is likely that these sites were influenced by low concentrations of hydrocarbons not detected fluorometrically and therefore should not be considered true control sites, but as comparison sites with lower exposure to hydrocarbons. Some plume samples also showed decreases in dissolved oxygen concentration indicative of recent respiration pulses. Oxygen anomalies, the difference between measured concentrations and background values (18), expressed in $\mu\text{mol/L}$, are shown in parentheses after the sample names in Fig. 1.

In all nine plume samples from the May cruise, the group of uncultivated *Oceanospirillales* observed by Hazen et al. (17) (subsequently referred to as DWH *Oceanospirillales*) accounted for more than 30% of sequences (Fig. 1). DWH *Oceanospirillales* accounted for more than 95% of sequences in five of those samples, including one sample with no detectable oxygen depletion (WS4-8). The remaining sequences from the May samples with oxygen anomalies more than 2 $\mu\text{mol/L}$ were affiliated with the genus *Cycloclasticus* or the genus *Colwellia*. In the plume samples with oxygen anomalies no greater than 2 $\mu\text{mol/L}$, the remaining sequences were *Cycloclasticus*, *Colwellia*, or a mix of other *Gammaproteobacteria*, *Deltaproteobacteria*, *Alphaproteobacteria*, *Chloroflexi*, and the SAR406 clade similar to those observed in the nonplume samples. Most sequences from the nonplume samples appear to be “typical” deep sea bacteria, nearly all of them closely

related to sequences from the deep Arctic Ocean (21). However, three of the four nonplume samples also contained DWH *Oceanospirillales*, *Cycloclasticus*, or *Colwellia*, accounting for 13% to 42% of sequences. Without widespread sampling before the spill, it is difficult to establish true background values for these groups, but given that no sequences closely related to the DWH *Oceanospirillales* were found in GenBank before the *Deepwater Horizon* spill, it is highly unlikely that these bacteria are regularly that abundant. Rather, these bacteria were likely responding to low concentrations of hydrocarbons in these samples, or result from extensive mixing of affected waters.

In contrast to the May samples, DWH *Oceanospirillales* were not detected in four of the five plume samples from June. Instead, *Cycloclasticus* and *Colwellia* accounted for more than 95% of sequences. The one sample (H10) in which DWH *Oceanospirillales* was detected was the closest sample to the wellhead and the one with no detectable oxygen anomaly, suggesting it had experienced the least biodegradation. Based on the observed progression in these samples and the greater abundance of the DWH *Oceanospirillales* in May, we suggest that the DWH *Oceanospirillales* bloomed first, followed by *Colwellia* and *Cycloclasticus*, although we lack definitive evidence because of the difficulty determining time of exposure. Fluctuations in ocean currents caused water near the wellhead to move away and then return, so the distance from the wellhead does not necessarily

correlate with the amount of time that water had been exposed to hydrocarbons (20, 22). The oxygen anomaly is a better indication of the amount of hydrocarbon degradation that has occurred in a given water mass, but it should be noted that oxygen concentrations never reached hypoxic levels, suggesting that, to some extent, mixing was able to replenish oxygen in plume samples (20). Microbial community composition appears to be a much more sensitive indicator of hydrocarbon exposure than oxygen depletion, given the abundance of these putative hydrocarbon degraders in both May and June samples with no detectable oxygen anomaly.

By September, oxygen anomalies could be traced several hundred kilometers to the southwest, and methane, ethane, and propane were lower than background concentrations for the Gulf of Mexico (18). DWH *Oceanospirillales* and *Colwellia* were no longer detectable in 11 of the 12 samples, but methanotrophs, other methylotrophs, *Flavobacteria* (mostly affiliated with the genera *Polaribacter* or *Owenweeksia*), and the *Alphaproteobacteria* order *Rhodobacterales* were much more abundant than they had been in plume or nonplume samples earlier in the summer. *Flavobacteria* in particular were more abundant in plume samples than nonplume samples (10–35% of sequences vs. 1–15%). *Flavobacteria* are abundant in the ocean and are often associated with the degradation of high molecular weight dissolved organic carbon compounds (23), but several methanol-oxidizing strains of *Flavobacterium* have been isolated (24) and they have often been observed in ^{13}C -labeled DNA from methane SIP studies (25). The *Flavobacteria* may therefore have been secondary consumers of methane, oil, or cellular decay products. The abundance of *Flavobacteria* may have been greater than indicated by the clone libraries, as has been observed when measured by techniques (e.g., FISH) that do not require PCR and cloning (26–28). Terminal restriction fragment length polymorphism (T-RFLP) analysis of these samples, which used the same PCR primers, but did not require cloning, showed the *Flavobacteria* accounted for as much as 56% of total peak area (Fig. S14). Although the relative abundances of all other groups of bacteria corresponded well between clone libraries and T-RFLP, the *Flavobacteria* were somewhat underrepresented in clone libraries (Fig. S2), possibly because of biases associated with cloning (29).

We also sequenced archaeal 16S rRNA genes from six deep water samples: plume/nonplume from May (WS41-8/WS41-12), low oxygen anomaly plume/high oxygen anomaly plume from June (H10/H24), and plume/nonplume from September (P203/P230). These samples contained different bacterial communities (WS41-12 and P203 showed relatively little hydrocarbon influence), but the archaeal communities in all six samples were very similar: more than 70% of sequences in each clone library were from one OTU affiliated with the Marine Group II *Euryarchaeota* (30), with the remainder related to other marine *Euryarchaeota* or *Thaumarchaeota* (Fig. S3). The dominance of this OTU in all samples suggests that the presence of hydrocarbons did not have a large impact, although deeper sequencing of these samples could possibly show an impact on less abundant members of the community. Archaea have not been shown to play a significant role in hydrocarbon degradation in aerobic marine environments, and the effect of crude oil on archaea is not well understood. Some evidence suggests that the presence of oil has a strong negative influence on archaea, whereas other studies see little effect (31). That archaea do not bloom in response to a sudden input of oil is consistent with their hypothesized ecology (32).

In addition to deep water plume samples, we analyzed water samples from five sites with surface oiling. The amount of oil at these sites varied: three sites (WS6-S, WS47-S, and WS73-S) had an oil sheen, whereas the other two contained a thicker coating of oil. The three sheen samples were dominated by *Cyanobacteria* and *Alphaproteobacteria* (SAR11 clade, *Rhodobacterales*, and *Rhodospirillales*), with just 15% of sequences affiliated with possible hydrocarbon degraders from the *Aalteromonadales* and *Oce-*

anospirillales (Fig. 1). *Gammaproteobacteria* were more abundant in the more heavily oiled samples, accounting for 100% of sequences in WS85-S (93% *Pseudoalteromonas*) and 48% of sequences in WSV-S (*Pseudomonas*, *Vibrio*, *Acinetobacter*, and *Alteromonas*). No DWH *Oceanospirillales* were detected in these samples, and *Colwellia* and *Cycloclasticus* accounted for less than 5% of sequences in each sample.

Role of Temperature. The difference between surface slick and deep water plume communities suggests that temperature may have played a significant role in determining which members of the microbial community responded to the *Deepwater Horizon* spill. Most cultivated strains of *Colwellia* are psychrophiles (33), and one of the cultivated organisms most closely related to the DWH *Oceanospirillales*, *Oleispira antarctica*, is also psychrophilic (34). *Cycloclasticus* has been shown to be abundant in oil-degrading microcosms at both 4 °C and 20 °C (14). Although no cultivated strain of *Colwellia* has been shown to oxidize hydrocarbons and it is not commonly observed in oil degradation studies, several studies have detected *Colwellia* in oil-contaminated ice cores or sediments incubated at low temperatures (35, 36). To determine the impact of temperature on oil-degrading communities, we added crude oil to seawater collected from a deep water sample on the September cruise and incubated in triplicate at 4 °C and room temperature (~20 °C) for 10 d. In 16S rRNA gene clone libraries from these samples, *Colwellia* accounted for 87% of total clones when incubated at 4 °C, but only 5% at room temperature (Fig. 2). *Alphaproteobacteria*, predominantly *Rhodobacteraceae* closely related to those abundant in plume samples from September (18), accounted for 93% of clones at room temperature, but 13% at 4 °C. Neither *Cycloclasticus* nor DWH *Oceanospirillales* were detected at either temperature.

SIP. To identify the bacteria consuming specific hydrocarbon compounds abundant in the deep plume, we used SIP with ^{13}C -labeled methane, ethane, propane, or benzene. Seawater from site P222 from the September cruise was incubated for 10 d at 6 °C, and the ^{13}C -labeled substrates were converted to ^{13}C -labeled dissolved inorganic carbon (DIC; Fig. 3A). ^{13}C -labeled (i.e., “heavy”) and unlabeled (i.e., “light”) DNA were then separated by CsCl density gradient centrifugation. 16S rRNA gene clone libraries from the heavy and light DNA and T-RFLP analysis of the unfractionated DNA and selected gradient fractions showed that the addition of these substrates led to changes in the microbial community (Fig. 3B and Fig. S1B). *Colwellia* sequences were not detected in the initial sample and were detected in only one of the 11 other September samples, but were the most abundant group in the heavy DNA from the ethane, propane, and benzene incubations (45–72% of sequences) and were also abundant in the unlabeled DNA from all

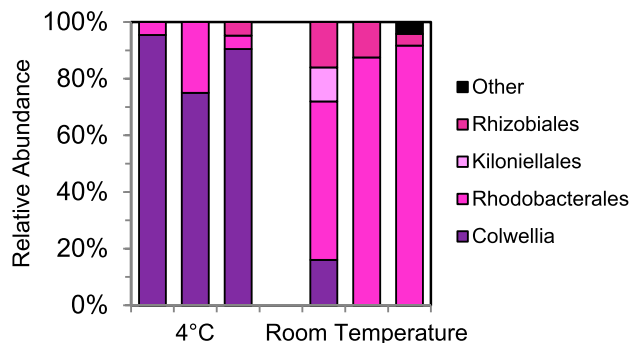


Fig. 2. Relative abundances of 16S rRNA genes in clone libraries from crude oil enrichment cultures incubated at 4 °C and room temperature (~20 °C). $n = 20$ –24 sequences per sample, three samples for each temperature.

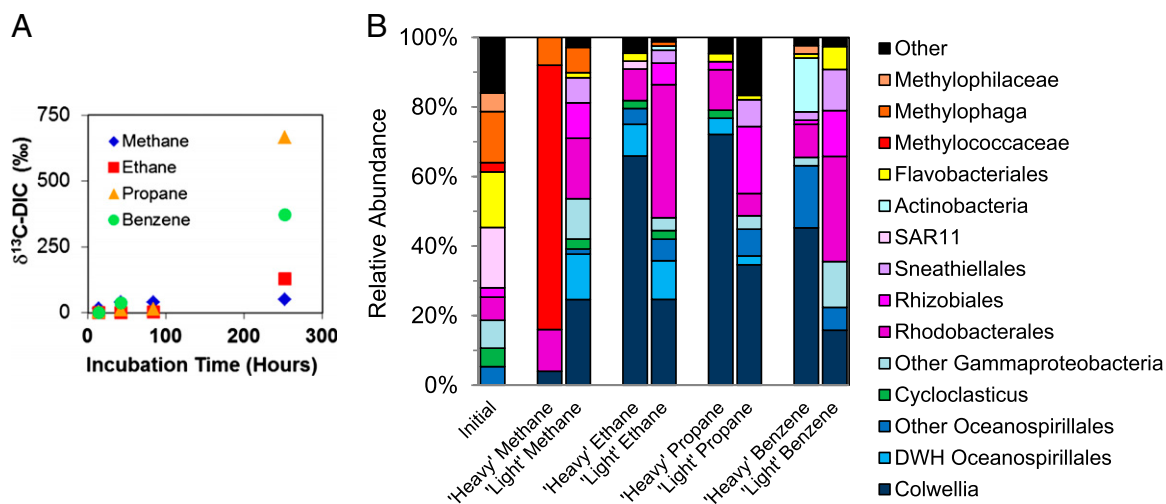


Fig. 3. (A) Conversion of ¹³C methane, ethane, propane, and benzene to [¹³C]DIC during SIP incubations of seawater at 6 °C. (B) Relative abundances in 16S rRNA gene clone libraries from heavy (i.e., ¹³C-labeled) and light (i.e., unlabeled) DNA from SIP incubations with ¹³C methane, ethane, propane, and benzene, and the initial seawater (sample P222). *n* = 75 (initial), *n* = 25 (heavy methane), *n* = 69 (light methane), *n* = 44 (heavy methane), *n* = 81 (light methane), *n* = 43 (heavy methane), *n* = 78 (light methane), *n* = 84 (heavy methane), and *n* = 76 (light benzene).

four incubations. The DWH *Oceanospirillales* were also not detected in any of the September samples, but accounted for 3% to 13% of sequences in the heavy and light DNA from the ethane and propane incubations and light DNA from the methane incubation. DWH *Oceanospirillales* were not detected in the heavy or light DNA from the benzene incubations, whereas *Actinobacteria* accounted for 15% of sequences in the benzene heavy DNA clone libraries, but were not detected in any of the others. Heavy benzene fractions and light ethane and propane fractions contained a number of other *Oceanospirillales* sequences affiliated with the genus *Neptunomonas*, closely related to the aromatic hydrocarbon degrader *Neptunomonas naphthovorans* (37) and symbionts of bone-eating *Osedax* species (38).

Despite the abundance of methylotrophs in the initial seawater (23% of sequences), methane was converted to [¹³C]DIC much more slowly than the other substrates (Fig. 3A). This is consistent with the low methane oxidation rates measured in September and supports the hypothesis that the methylotrophs in the September samples were metabolically inactive remnants of a bloom that had peaked earlier in the summer (18). The majority of methylotrophs in the initial sample were affiliated with *Methylophaga* or *Methylophilaceae*, which are likely secondary consumers of C1 compounds after *Methylococcaeae* oxidize methane to methanol. These processes appear to be tightly coupled, but presumably require *Methylococcaeae* to respond initially (6, 39). *Methylococcaeae* accounted for only 3% of sequences in the initial sample, and they appear to bloom more slowly in response to natural gas inputs than ethane and propane oxidizers, just as observed earlier in the summer (2). The low DNA concentrations in the heaviest fractions necessitated the use of DNA from a slightly less dense fraction for the heavy DNA clone library, but 84% of sequences in this fraction were affiliated with the *Methylococcaeae* or *Methylophaga*, compared with just 7% in the light fraction. These sequences, along with *Colwellia*, DWH *Oceanospirillales*, and *Actinobacteria*, were closely related to the sequences observed in the environmental samples (Fig. 4).

Although SIP experiments typically try to mimic in situ conditions and use low concentrations of substrate to avoid microbial community changes over the course of the incubation, our goal was to replicate the bloom conditions caused by hydrocarbon inputs following the well blowout. *Colwellia* was able to respond rapidly, becoming abundant in heavy and light DNA. To become so abundant in the heavy DNA, *Colwellia* had to consume ¹³C-

labeled ethane, propane, or benzene, and then use that ¹³C in the production of new DNA, a process which is typically associated with cell division (39). The *Colwellia* sequences in the unlabeled DNA were from organisms that were either consuming the ¹³C substrate but had not yet synthesized a sufficient amount of DNA with that labeled carbon or from organisms consuming an alternate carbon source. Given that the cultivated strains of *Colwellia* are capable of degrading many different carbon sources (33, 40), either explanation is possible. It is also important to note that we cannot exclude the possibility of cross-feeding (25, 39), whereby one organism is responsible for the initial oxidation step (e.g., ethane to ethanol) and a different organism then consumes this ¹³C-labeled intermediate (e.g., ethanol) and incorporates it into ¹³C biomass. However, given the dominance of *Colwellia* in the SIP samples and the high rates of ethane and propane oxidation in June, when *Colwellia* sequences accounted for approximately 70% of sequences in plume samples (2), it is likely that *Colwellia* was responsible for the bulk of ethane and propane oxidation in situ, although the SIP results show the DWH *Oceanospirillales* may also have played a role.

Conclusions

The microbial community response to the *Deepwater Horizon* oil spill was distinct from that observed in previous spills or mesocosm studies. Deep water plume communities were dominated by just three groups of *Gammaproteobacteria* in May and June, none of which were abundant in surface oil samples. Two of these groups, the DWH *Oceanospirillales* and *Colwellia*, are related to known psychrophiles, and *Colwellia* was much more abundant in crude oil enrichments at 4 °C than at room temperature, suggesting that the temperature played a significant role. The abundance of natural gas in the *Deepwater Horizon* spill seemingly provided an advantage to *Colwellia* and the DWH *Oceanospirillales*, as both these groups became more abundant with the addition of ethane or propane alone. *Colwellia* was likely responsible for the majority of ethane and propane oxidation, but may also have been involved with the degradation of higher molecular weight hydrocarbons.

Methods

Study Site and Sample Collection. Samples were collected on three cruises: *R/V Walton Smith* (May 26 to June 5, 2010), *R/V Cape Hatteras* (June 11–21, 2010), and National Oceanic and Atmospheric Administration ship *Pisces* (September

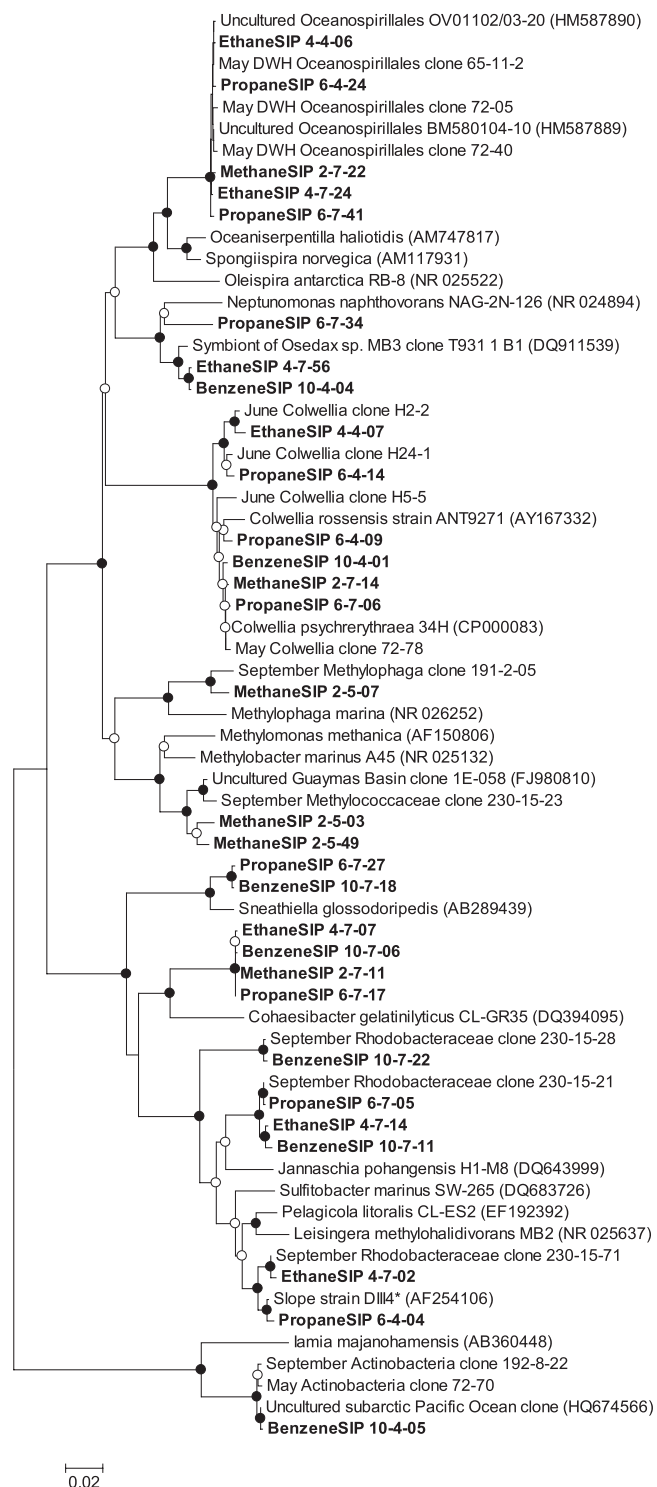


Fig. 4. Neighbor-joining tree showing phylogenetic relationships between bacterial 16S rRNA gene sequences from the major groups of *Gammaproteobacteria*, *Alphaproteobacteria*, and *Actinobacteria* in SIP samples (bold), environmental samples from May, June, or September, and reference sequences (GenBank accession numbers in parentheses). Filled circles indicate nodes with bootstrap values greater than 90% (1,000 replicates); open circles indicate bootstrap values greater than 50%.

7–17, 2010). Sample locations are shown on the map in Fig. S4 and listed with additional details in Table S1. Dissolved oxygen concentrations were measured with the SBE-43 dissolved oxygen sensors on the ships' conductivity/

temperature/depth (CTD) rosettes and on select samples by Winkler titration, as described previously (2). A 10th-degree polynomial was fit to the dissolved oxygen-depth profiles to determine a background oxygen concentration, as described previously (18). Oxygen anomalies were calculated as the difference between the measured dissolved oxygen concentration and the background concentration for a given depth. For the *R/V Walton Smith* cruise, oxygen anomalies were calculated with the publically available CTD profile data (41). Fluorescence was measured with a WETLabs ECO FL CDOM fluorometer (excitation of 370 nm, emission of 460 nm) on the *R/V Walton Smith* and *R/V Cape Hatteras* and with a Chelsea Technologies UV AquaTracka (excitation of 239 nm, emission of 360 nm) on the *Pisces*. Deep water samples were collected in Niskin bottles on the ships' CTD rosettes. One liter of seawater was filtered onto a 0.2- μ m Sterivex filter (Millipore) which was stored frozen until extraction. Surface samples were collected only on the May cruise, by using a plastic bucket. An oil/water mixture was collected from the surface of the bucket sample into a 125-mL glass jar and stored frozen for several months. Samples were then thawed and filtered onto 0.2- μ m Sterivex filters (Millipore); DNA was extracted from the filters as described later.

16S rRNA Clone Libraries. DNA was extracted from filters with the FastDNA SPIN kit for soil (MP Biomedicals). Bacterial sequences were amplified with the primers 27F and 1392R, as previously described (6), and archaeal sequences were amplified with 21F and 958R (30). For each sample, duplicate PCR reactions were performed, then pooled and cleaned with the Wizard SV Gel and PCR Clean-Up kit (Promega). PCR products were cloned with the PCR Cloning Kit (Qiagen), and randomly selected clones were selected for sequencing at the University of California, Berkeley, DNA Sequencing Facility. Sequences were edited and assembled with Sequencher (Gene Codes) and screened for chimeras with Bellerophon (42) and Pintail (43). Additional chimeras were detected by comparison with closely related sequences via BLAST searches (44). In plume samples with two or three dominant sequences, as many as one third of sequences were found to be chimeric; all suspected chimeras were omitted from subsequent analysis. Sequences were assigned to operational taxonomic units (OTUs) with MOTHUR (45), and each sequence was also identified using the RDP Classifier tool (46). For bacteria, OTU clustering at the 3% difference threshold corresponded with the RDP taxonomy at the family or genus level, with the exception of the SAR 406 clade. Representative members of relevant OTUs were aligned with ClustalW, and MEGA4 (47) was used to construct neighbor-joining phylogenetic trees (maximum composite likelihood method, 1,000 bootstrap replicates). At least one sequence from each OTU was submitted to GenBank under the accession numbers JN018421 to JN018515 (SIP incubations), JN018516 to JN018646 (archaea), JN018647 to JN018743 (surface samples), and JN018744 to JN019023 (deep water samples).

Enrichment Cultures. Seawater was collected on the September cruise and stored at 4 °C for 3 mo before starting enrichment cultures. Crude oil (0.5 mL) was added to 100 mL seawater and 50 mL artificial seawater in a 250-mL sterile glass serum bottle, sealed with a Teflon-coated butyl rubber stopper and an aluminum crimp seal. The artificial seawater contained, per liter, 20 g NaCl, 3 g $MgCl_2 \cdot 6 H_2O$, 4 g Na_2SO_4 , 0.15 g $CaCl_2 \cdot 2 H_2O$, 0.5 g KCl, 0.2 g $NaHCO_3$, 0.85 g $NaNO_3$, and 20 mL 0.5 M phosphate buffer (pH 7.4). Three bottles were incubated at 4 °C and three at room temperature (~20 °C) for 10 d. Each bottle was filtered onto a 0.2- μ m Sterivex filter (Millipore); DNA was extracted and clone libraries were constructed as described earlier.

SIP. Immediately after collection, seawater from sample P222 was incubated with 99+% ^{13}C methane, ethane, propane, or benzene (Isotec/Sigma Aldrich), as well as ^{12}C (natural abundance of ^{13}C , ~1.1%) controls for methane, ethane, and propane (Airgas). For each incubation, 200 mL seawater was added to a 250-mL sterile glass serum bottle, sealed with a Teflon-coated butyl rubber stopper and an aluminum crimp seal. Five milliliters of methane, ethane, or propane, or 70 μ mol benzene was added and samples were incubated at 6 °C for 10 d. Ten milliliters of water was removed for [$\delta^{13}C$]DIC analysis, and the remainder was filtered onto a 0.2- μ m Sterivex filter (Millipore) and stored frozen until DNA extraction. Replicate bottles for each substrate were also harvested at 2 d and 3.5 d for [$\delta^{13}C$]DIC measurements; DNA from these samples was not analyzed as a result of insufficient ^{13}C uptake. Analysis of [$\delta^{13}C$]DIC was performed as described previously (2), and DNA extraction as described earlier. ^{12}C and ^{13}C DNA were separated by CsCl density gradient centrifugation as described previously (6) and separated into 10 fractions. 16S rRNA gene clone libraries were constructed from DNA from one heavy fraction (1.74 g mL $^{-1}$ for ethane, propane, and benzene; 1.72 g mL $^{-1}$ for methane) and one light fraction (1.69 g mL $^{-1}$) from each ^{13}C substrate. There was insufficient DNA for analysis in the heavy fractions from the ^{12}C controls. Cloning and sequence analysis were performed as described earlier.

T-RFLP. T-RFLP analysis was performed on selected samples from the SIP experiment and the September cruise. DNA was amplified as described earlier, but with a 6-carboxyfluorescein-labeled forward primer. Cleaned PCR product (100 ng) was digested with *MspI*, and samples were analyzed with T-REX (48), as described previously (6). Terminal restriction fragments (T-RFs) were identified by *in silico* digestion of all clone library sequences from each batch of samples (SIP and September samples were analyzed separately). Some T-RFs could be assigned to multiple groups (there was significant overlap between the methanotrophs, methylotrophs, and some other *Gammaproteobacteria*, and it was not possible to distinguish the *Deltaproteobacteria*, *Actinobacteria*, and SAR406 clade from each other, but

the *Flavobacteria*, *Alphaproteobacteria*, and *Gammaproteobacteria* were readily differentiated.

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