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Hydrocarbon-Degrading Bacteria and the Bacterial Community Response in Gulf of Mexico Beach Sands Impacted by the Deepwater Horizon Oil Spill▽†‡

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A significant portion of oil from the recent Deepwater Horizon (DH) oil spill in the Gulf of Mexico was transported to the shoreline, where it may have severe ecological and economic consequences. The objectives of this study were (i) to identify and characterize predominant oil-degrading taxa that may be used as model hydrocarbon degraders or as microbial indicators of contamination and (ii) to characterize the *in situ* response of indigenous bacterial communities to oil contamination in beach ecosystems. This study was conducted at municipal Pensacola Beach, FL, where chemical analysis revealed weathered oil petroleum hydrocarbon (C_8 to C_{40}) concentrations ranging from 3.1 to 4,500 mg kg^{-1} in beach sands. A total of 24 bacterial strains from 14 genera were isolated from oiled beach sands and confirmed as oil-degrading microorganisms. Isolated bacterial strains were primarily *Gammaproteobacteria*, including representatives of genera with known oil degraders (*Alcanivorax*, *Marinobacter*, *Pseudomonas*, and *Acinetobacter*). Sequence libraries generated from oiled sands revealed phylotypes that showed high sequence identity (up to 99%) to rRNA gene sequences from the oil-degrading bacterial isolates. The abundance of bacterial SSU rRNA gene sequences was ~10-fold higher in oiled (0.44×10^7 to 10.2×10^7 copies g^{-1}) versus clean (0.024×10^7 to 1.4×10^7 copies g^{-1}) sand. Community analysis revealed a distinct response to oil contamination, and SSU rRNA gene abundance derived from the genus *Alcanivorax* showed the largest increase in relative abundance in contaminated samples. We conclude that oil contamination from the DH spill had a profound impact on the abundance and community composition of indigenous bacteria in Gulf beach sands, and our evidence points to members of the *Gammaproteobacteria* (*Alcanivorax*, *Marinobacter*) and *Alphaproteobacteria* (*Rhodobacteraceae*) as key players in oil degradation there.

The blowout of the Deepwater Horizon (DH) drilling rig resulted in the world's largest accidental release of oil into the ocean in recorded history. The equivalent volume of approximately 4.9 million barrels of light crude oil were discharged into the Gulf of Mexico from April to July 2010 (OSAT/NOAA report [56] and oil budget calculator [43]), and the total hydrocarbon discharge was 40% higher if gaseous hydrocarbons are included (34). A large amount of the discharged oil was transported to the surface and reached the shoreline. Although cleanup efforts have remained aggressive, a substantial portion of the oil remains trapped in coastal ecosystems, especially in benthic areas.

Permeable sandy sediments cover large areas of the seafloor in the Gulf of Mexico, including beach ecosystems. Marine sands act as efficient biocatalytic filters that play an important role in the biogeochemical cycles of carbon and nutrients in shallow Gulf waters (11, 19, 20). Marine sands in the Gulf are covered with biofilms of highly diverse microbial communities (30, 53), and the bacterial abundance in sands exceeds that of the overlying seawater by orders of magnitude (7, 35, 38). Enhanced porewater exchange in highly permeable marine sands stimulates microbial metabolism through the delivery of growth substrates and the removal of waste products (7, 15, 28, 29).

Similar to the microbially mediated breakdown of natural organic matter, biodegradation mediated by indigenous microbial communities is the ultimate fate of the majority of oil hydrocarbon that enters the marine environment (4, 42, 59). Hydrocarbon-degrading microorganisms are ubiquitous in the marine environment (27, 76), and biodegradation was shown to be successful in naturally remediating oil contamination associated with several spills that impacted shorelines predominated by permeable marine sediments (8, 46, 66). Although bioremediation field trials were often carried out, there is a paucity of information on the indigenous microbial communities that catalyze oil degradation under *in situ* conditions at spill sites (2). Even less information is available on which members of the microbial community are active in degrading hydrocarbons, and the impacts of various environmental parameters in controlling the activities of indigenous hydrocarbon-degrading microorganisms have not been specifically addressed. Thus, the theoretical basis to understand and predict

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the dynamics of hydrocarbon-degrading microorganisms *in situ* is lacking (27).

Contamination of beach ecosystems by oil has the potential to cause severe environmental and economic consequences in the Gulf region. The risk of accidental oil discharge to the marine environment will remain high for the foreseeable future since increased economic pressure to access new oil reserves in deep marine waters will require less tested technologies (32). Although technologies for oil drilling have advanced rapidly in recent decades, strategies to respond to oil spills and to assess environmental impacts of oil contamination have lagged behind (33). An understanding of the impacts of oil on indigenous microbial communities and identification of oil-degrading microbial groups are prerequisite for directing the management and cleanup of oil-contaminated beach ecosystems. Thus, the objectives of the present study were (i) to identify and interrogate the ecophysiology of predominant oil-degrading taxa that may serve as model organisms and microbial indicators of contamination and (ii) to characterize the *in situ* response of indigenous bacterial communities to oil contamination in Gulf beach sands.

MATERIALS AND METHODS

Site and sample description. This study focused on beach sands collected from Pensacola Beach, FL, in the area of the municipal beach (30°19.57N, 087°10.47W), which was exposed to heavy oil contamination from the Deepwater Horizon (DH) oil spill beginning in the first week of June 2010. Sands in the supratidal zone of the beach where thick buried oil layers were found were dry with no extractable pore water and no dissolved nutrients. Nutrients in the pore water of shallow submerged sand in the region range from 2 to 10 μmol liter^{-1} for dissolved inorganic nitrogen, and from 0.1 to 1 μmol liter^{-1} for dissolved inorganic phosphate and typically are about one order of magnitude higher than the respective nutrient concentrations in the overlying water column (M. Huettel, unpublished data).

Samples were collected on 2 July, 30 July, and 1 September 2010. Sample designations were assigned based on collection order, with OS55 through OS320 samples represented in the present study. During each trip, sediment cores of 50 cm in depth were taken in duplicate from the intertidal and supratidal zones. Cores were aseptically sectioned into 10-cm intervals on site, homogenized, and subsampled into sterile 50-ml conical tubes (cores from 2 July 2010 were labeled OS55 to OS77, cores from 30 July 2010 were labeled OS82 to OS91, and cores from 1 September 2010 were labeled OS291 to OS320). During the latter two sampling trips, a 10-by-1-by-1-m trench was excavated perpendicular to the beach face. Samples were taken along the visible oil layer spanning the length of the trench, and vertically through the oil layer (samples from 30 July 2010 were labeled OS200 to OS244; samples from 1 September 2010 were labeled OS248 to OS290). All samples for nucleic acid extraction were immediately frozen on dry ice and stored at −80°C. For cultivation, samples were immediately transported to the lab on ice, stored at 4°C, and processed within a few days. A second control site, unaffected by oil contamination, was sampled at St. George Island, FL (29°44′88.1″N, 084°42′58.6″W) prior to the DH blowout in April 2010. Cores were sampled in triplicate, and the surface 0- to 5-cm interval was archived as described above for further analysis. Source oil was sampled from the DH wellhead and used for chemical analysis and cultivation. This oil was collected on 20 May 2010 aboard the drillship *Discoverer Enterprise*.

Analysis of oil hydrocarbon composition. Oil contamination was assessed by visual examination in the field according to the following scale: 0 for clean or no visible contamination and 1 for low, 2 for medium, and 3 for high levels of oil contamination. A subset of core samples was ultrasonically extracted and subjected to chemical analysis using established, U.S. Environmental Protection Agency-approved, gas chromatography-mass spectrometry (GC-MS) techniques at TestAmerica Laboratories, Tallahassee, FL. Total petroleum hydrocarbons were determined using the Florida residual petroleum organic method (FL-PRO) in core samples, as well as in source oil from the DH wellhead. In addition, more detailed analysis of oil hydrocarbon composition was conducted for the source oil and oiled beach sands visually assessed as containing medium (2) or high (3) levels of oil contamination. Semivolatile organic compounds (polycyclic aromatic hydrocarbons, aromatics, aliphatics) were determined by GC-MS method SW846 8270C LL.

Enumeration, enrichment, and isolation of oil-degrading bacteria. Oil-degrading bacterial populations were enumerated by the three-tube most-probable-number (MPN) assay using 10-fold serial dilutions of beach sand in growth medium. A minimal, artificial seawater medium was prepared and dispensed according to the method of Widdel and Bak (74). Source oil was sterilized according to the method of Widdel (73) and added as the sole electron donor and carbon source to a final concentration of 0.2% (wt/vol). Under anaerobic conditions, the medium was purged with a 80% N_2–20% CO_2 gas mixture and sealed into Hungate tubes, and nitrate was provided as the sole electron acceptor to 3 mM. For enumeration under aerobic conditions, the medium was prepared aerobically and dispensed into 16-by-150-mm tubes that were loosely capped and continuously shaken at 150 rpm on a shaker table. The tubes were incubated at room temperature for 1 month, and bacterial growth was monitored by culture turbidity and depletion of added oil at regular intervals compared to autoclaved controls. The MPN index was determined from statistical tables published by the American Public Health Association (3).

Strains of oil-degrading bacteria were isolated from the highest positive dilutions of the MPN enrichments, as well as from parallel enrichments conducted in larger volumes of the artificial seawater medium or with filter-sterilized seawater as the enrichment medium. When filtered seawater was used, it was supplemented with vitamin and trace element solutions according to the method of Widdel and Bak (74). In oxic seawater enrichments, the medium was buffered with 10 mM HEPES (pH 7.0) and dispensed into Erlenmeyer flasks, and the flasks were shaken at 150 rpm on a shaker table. Anaerobic seawater enrichments were prepared in serum bottles as described above and sealed with butyl rubber stoppers. Flasks and serum bottles both were inoculated into 10% (wt/vol) with oiled sands, and 0.2% (wt/vol) of sterile source oil served as the sole source carbon and electron donor. Bacterial growth was monitored by measuring the optical density at 600 nm. After four successive transfers to fresh medium, strains were isolated onto Zobell marine agar (HiMedia, India) or artificial seawater medium supplemented with 5% peptone, 1% yeast extract, 10 mM HEPES, and molecular-grade agar (Sigma-Aldrich) as a solidifying agent. Denitrifying bacteria were isolated on agar plates of the artificial seawater medium equilibrated inside a Coy anaerobic chamber with 20 mM nitrate as the sole electron acceptor (22). Repeated streaking on solid medium purified isolated strains, representative pure cultures were again screened in liquid media for oil degradation potential, and frozen stocks were prepared at −80°C in 20% glycerol.

Phenotypic characterization of pure cultures. Representative isolates were further screened for oil degradation capability under aerobic conditions by quantifying the residual oil in liquid cultures using gravimetric and spectrophotometric methods (see further details in the supplemental material).

Initial physiological characterization of representative oil-degrading isolates was conducted using phenotypic MicroArray (PM) analysis. PM tests were performed by Biolog, Inc. (Hayward, CA), according to methods described elsewhere (6, 44; see the supplemental material for further details).

Nucleic acid extraction, DNA fingerprinting, and analysis of SSU rRNA sequences. Total genomic DNA was extracted using a MoBio Powersoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's protocol. Bacterial community structure was initially assessed in DNA extracts by community fingerprinting using the automated ribosomal intergenic spacer analysis (ARISA) method (61). This technique allowed for the rapid comparison of a large number of samples and aided in identifying critical samples for pyrotag sequencing (see the supplemental material for a complete description of the ARISA methods). Total RNA was extracted from sand samples according to the method of Chin et al. (10). Contaminating DNA was removed using DNase I (Ambion, Austin, TX). Reverse transcription reactions for SSU rRNA were performed using GoScript reverse transcriptase (Promega, Madison, WI) and the general bacterial 1492R reverse primer at 0.2 μM (72). Reactions were conducted according to the manufacturer's instructions.

For pyrotag sequencing, ~100 ng of extracted DNA or cDNA was sent to the Research and Testing Laboratory (Lubbock, TX) for bacterial rRNA gene tag-encoded FLX amplicon pyrosequencing. PCR amplification was performed using the primers Gray28F and Gray519r (Table 1) (31), and sequencing reactions utilized a Roche 454 FLX instrument (Roche, Indianapolis, IN) with Titanium reagents, titanium procedures, a one-step PCR, and a mixture of Hot Start and HotStar high-fidelity *Taq* polymerases. After sequencing, all failed sequence reads and low-quality sequence ends and tags were removed, and sequences were depleted of any nonbacterial ribosome sequences and chimeras using custom software (21), as has been described previously (1). Pyrosequences were trimmed to 300 bases, and sequences shorter than 300 bases were removed from further

TABLE 1. Oligonucleotides used in this study

Primer set	Individual primer or probe	Sequence (5′–3′)	Primer location[a]	Annealing temp (°C)	Intended target	Source or reference
PCR, near-full-length SSU rRNA	27F	AGA GTT TGA TCM TGG CTC AG	8–27*	55	Bacteria	71
	1492R	GGT TAC CTT GTT ACG ACT T	1510–1492*			
Pyrosequencing, SSU rRNA	Gray28F	GAG TTT GAT CNT GGC TCA G	9–27*	60	Bacteria	31
	Gray519r	GTN TTA CNG CGG CKG CTG	536–519*			
Quantitative PCR, SSU rRNA	331F	TCC TAC GGG AGG CAG CAG T	340–358*	60	Bacteria	55
	772R	GGA CTA CCA GGG TAT CTA ATC CTG TT	806–781*			
	515R (probe)	CGT ATT ACC GCG GCT GCT GGC AC	537–515*			
Quantitative PCR, SSU rRNA	Alcvx-464F	GAG TAC TTG ACG TTA CCT ACA G	464–485†	60	*Alcanivorax*	This study
	Alcvx-675R	ACC GGA AAT TCC ACC TC	675–659†			This study
	515R (probe)	CGT ATT ACC GCG GCT GCT GGC AC	528–506†			55
ARISA, ITS	S-D-Bact-1522-b-S-20 (1522F)	TGC GGC TGG ATC CCC TCC TT	1522–1541*	50	Bacteria	60
	L-D-Bact-132-a-A-18 (LSU132R)	CCG GGT TTC CCC ATT CGG	132–115‡			

[a] *, according to the small-subunit rRNA (*rrS*) gene of *Escherichia coli* S88 (NC_011742); †, according to the *rrS* gene of *Alcanivorax dieselolei* (AY683537), offset from *E. coli* numbering by seven bases at the start of the rRNA gene; ‡, according to the large-subunit rRNA (*rrL*) gene of *Escherichia coli* S88 (NC_011742).

analysis. Sequence data were submitted to the European Nucleotide Archive Sequence Read Archive under the study accession number ERP000807.

Analysis of bacterial community composition. Sequence analysis was accomplished through the software packages QIIME (9) and Primer6 (Primer-E; Lutton, Ivy Bridge, United Kingdom). Sequences were clustered into operational taxonomic units by 97% sequence identity using UCLUST (17), and reference sequences were picked using scripts in QIIME. Operational taxonomic units (OTU) were classified in QIIME using the RDP classification algorithm set at a 50% confidence rating. Representative sequences were aligned using the PyNAST algorithm, and the alignment was filtered to remove common gaps. A phylogenetic tree was built *de novo* using FastTree (58).

Jack-knifed beta diversity was assessed by iteratively rarefying samples to 1,600 sequences in order to remove the effect of sampling effort, in QIIME. The Bray-Curtis distance was calculated for each rarefied OTU abundance table, and an average pairwise distance and standard deviation were calculated for each pair of samples. The resulting distance matrix was imported into Primer6 and visualized using a nonparametric multidimensional scaling plot (NMDS). The same method was used to generate an NMDS plot of an averaged weighted UniFrac distance matrix and is included in the supplemental material. An analysis of similarity test was performed on the Bray-Curtis distance matrix to determine whether grouping samples by oil presence was significant. Indicator species were identified using a similarity of percentages test in Primer6. Samples were grouped into five types by location (Pensacola and St. George Island), oil presence, and nucleic acid type.

Quantitative molecular analyses for tracking total bacteria and *Alcanivorax*. Quantification of bacterial small subunit rRNA genes (SSU rRNA) was performed according to the method of Nadkarni et al. (Table 1) (55). Primers and probes were ordered from Integrated DNA Technologies (IDT; Coralville, IA), and all had working concentrations of 100 nM in the PCR mastermix. The probe was labeled with a 6-carboxyfluorescein (6-FAM) fluorophore and contained an Iowa Black fluorescent quencher and an internal ZEN quencher (IDT) located nine bases from the 5′ end. All reactions were performed in triplicate and analyzed using an ABI 7900HT Fast real-time PCR system. A new primer set was developed to target SSU rRNA genes from bacteria of the genus *Alcanivorax* (Table 1; see the supplemental material for further details). Briefly, primers were designed using the "probe design" feature of the phylogenetic analysis program package ARB (48) and checked for specificity within ARB and by basic local alignment search tool (BLAST) analyses. These primers were used with the probe developed for domain-level bacterial quantitative PCR (qPCR) analyses under similar PCR conditions. Primers were designed to have similar annealing properties to the general primers described by Nadkarni et al. (55), and the qPCR was performed as described above. For both bacterial and *Alcanivorax*-specific qPCR assays, absolute quantification was performed using a standard curve derived from PCR products of the *Alcanivorax dieselolei* isolate from the present study generated by near-full gene amplification of SSU rRNA genes using the general bacterial primer set 27F and 1492R (41, 72). Standard curves were linear across a six-orders-of-magnitude range (from 2.79×10^8 to 2.79×10^2 copies/reaction), with similar efficiencies (92 to 94%). qPCR data were analyzed using principal component analysis, as implemented within the software package Primer6. Gene abundance values were fourth-root transformed to meet assumptions of normality.

RESULTS

Changes in hydrocarbon composition. Representative samples were selected for hydrocarbon analysis in order to quantify the extent of contamination, to verify our visual observations of oil contamination, and to determine which fractions of oil hydrocarbons in beach sands were depleted in comparison to the source oil collected from the DH wellhead. Chemical analysis revealed petroleum hydrocarbon (C_8 to C_{40}) concentrations ranging from 3.1 to 4,500 mg kg^{-1} in Pensacola Beach sands. All beach sand samples analyzed contained detectable oil hydrocarbon concentrations. At the lower range of contamination detected by chemical analysis (<10 mg kg^{-1}), oil was not observed visually (Table 2).

Two oiled beach sand samples were subjected to detailed chemical analysis (Table 3). In these sand samples considered to be moderately or heavily contaminated by visual observations, total petroleum hydrocarbon concentrations were 1,900 and 4,500 mg kg^{-1}, respectively. Comparison to the source oil from the DH wellhead indicated substantial weathering in that the majority of detectable hydrocarbon compounds that remained resided in the higher-molecular-weight aliphatic (>C_{16}) and aromatic (>C_{35}) fractions (Table 3). When these samples were collected on 1 September, approximately 18 weeks after the onset of the DH spill, detectable polycyclic aromatic hydrocarbons and lighter aliphatics (C_6 to C_{16}) and aromatics (C_8 to C_{21}) were reduced to near the detection limit. Heavier compounds such as larger alkanes (>C_{21}) have been shown to be degraded more slowly than corresponding lighter alkanes (<C_{21}). Thus, the ratio of lighter alkanes (C_6 to C_{16}) to heavier alkanes (C_{16} to C_{35}) was used as a chemical proxy for the biodegradation process (59, 62). The ratio observed in

TABLE 2. Summary comparison of the chemical analysis of total petroleum hydrocarbons, qualitative determination of oil presence, and molecular quantification of bacterial abundance in Pensacola Beach sands collected from 2 July to 2 September 2010

Sample[a]	Total hydrocarbons (mg kg^{-1})	Qualitative oil assessment[b]	Total bacterial SSU rRNA gene copies (g^{-1})	*Alcanivorax* SSU rRNA gene copies (g^{-1})	*% Alcanivorax*
OS-71b	3,600	3	4.44E + 06	3.10E + 06	69.9
OS-67	3.8	0	2.42E + 05	ND[c]	0.0
OS-69	4.2	0	2.45E + 06	1.69E + 04	0.7
OS-82	130	1	1.42E + 08	2.77E + 07	19.4
OS-88a*	680	2	3.38E + 07	4.04E + 06	12.0
OS-88b*		2	4.45E + 07	6.23E + 06	14.0
OS-89a*	320	1	3.99E + 07	3.82E + 06	9.6
OS-89b*		1	5.34E + 07	7.05E + 06	13.2
OS-90a*	7.7	0	1.40E + 07	6.30E + 05	4.5
OS-90b*		0	1.57E + 07	8.90E + 05	5.7
OS-306	3.1	0	8.21E + 05	1.20E + 04	1.5
OS-307	55	0	2.75E + 05	3.06E + 04	11.1
OS-311	3,600	3	1.02E + 08	1.36E + 07	13.3
OS-312	480	1	1.84E + 07	2.68E + 06	14.5

[a] *, replicate beach sand samples collected at the same depth and time. Only single replicates were analyzed for total petroleum hydrocarbons.
[b] O, no visible oil contamination; 1, low-level contamination; 2, medium-level contamination; 3, high-level contamination.
[c] ND, none detected.

TABLE 3. Detailed chemical analysis of hydrocarbon compounds from two oiled sand samples collected on 1 September 2010 from Pensacola Beach compared to source oil sampled on 20 May 2010 from MC block 252 after the DH blowout

Analyte	Hydrocarbon content		
	Moderately oiled (mg of compound/kg of $sand^{-1}$)	Heavily oiled (mg of compound/kg of $sand^{-1}$)	Source oil (mg of compound/kg of oil^{-1})
Polycyclic aromatic hydrocarbons			
1-Methylnaphthalene			1,000
2-Methylnaphthalene			1,600
Anthracene	0.029	0.024	
Benzo[*a*]anthracene	0.037		30
Benzo[*a*]pyrene	0.052		23
Benzo[*b*]fluoranthene	0.075	0.11	26
Benzo[*g,h,i*]perylene	0.032		18
Benzo[*k*]fluoranthene	0.050	0.071	20
Chrysene	0.260	0.62	77
Dibenz(*a,h*)anthracene	0.040		18
Fluoranthene	0.042		25
Fluorene	0.014	0.011	140
Indeno[*1,2,3-cd*]pyrene	0.024		16
Naphthalene			910
Phenanthrene	0.027	0.022	320
Pyrene	0.047		32
Aliphatic hydrocarbons			
C_5 to C_6 aliphatics			5,370
C_6 to C_8 aliphatics	35	24	62,400
C_8 to C_{10} aliphatics	13	10	57,800
C_{12} to C_{16} aliphatics	9.5	14	72,500
C_{16} to C_{35} aliphatics	370	1,900	69,100
Aromatic hydrocarbons			
C_5 to C_7 aromatics			2,590
C_7 to C_8 aromatics			7,010
C_8 to C_{10} aromatics	73	81	20,200
C_{10} to C_{12} aromatics			12,800
C_{12} to C_{16} aromatics	31	6.8	25,100
C_{16} to C_{21} aromatics	72	92	25,600
C_{21} to C_{35} aromatics	280	550	64,200
Total petroleum hydrocarbons (C_8 to C_{40})	1,900	4,500	500,000
Ratio of lighter (C_6 to C_{16}) to heavier (C_{16} to C_{35}) aliphatics	0.16	0.025	2.79

oiled beach sands (0.025 to 0.16) was reduced by 1 to 2 orders of magnitude compared to the ratio observed in the DH source oil (2.79; see Table 3).

Enumeration of oil-degrading bacteria. In the same sand samples for which detailed chemical analysis was performed (Table 3), MPN counts of cultivatable hydrocarbon-degrading bacteria in oil-contaminated sands (2.4×10^{10} cells ml^{-1}) exceeded those from "clean" sands sampled in parallel by 3 to 4 orders of magnitude (2.4×10^6 to 9.3×10^6 cells ml^{-1}).

Enumeration of bacteria with quantitative molecular approaches corroborated cultivation-based evidence. rRNA gene abundances were determined for samples taken from all sampling trips, resulting in a data set of 468 sand samples. Counts of cultivatable hydrocarbon degraders paralleled the molecular quantification of bacterial rRNA gene abundance. In oiled sands that were analyzed for hydrocarbon content, bacterial rRNA gene abundance was ~10 times higher in oiled (0.44×10^7 to 14.2×10^7 copies g^{-1}) versus clean (0.024×10^7 to 1.57×10^7 copies g^{-1}) sand. *Alcanivorax* spp. were not detected by qPCR methods in the clean sands used for MPN enumeration but were detected in Pensacola Beach sands without visible oil contamination. *Alcanivorax* were more abundant in sand samples with visible oil contamination (0.27×10^7 to 2.77×10^7 copies g^{-1}) than in sands without visible oil contamination (0.12×10^5 to 8.9×10^5 copies g^{-1}). Differences in total bacterial and *Alcanivorax* gene abundances between oiled and nonoiled sands are both statistically significant, as assessed by a Student *t* test on log-transformed data ($P < 0.008$).

Principal component analysis was performed to investigate the effect of visible oil contamination and sampling date on bacterial abundances for the larger qPCR data set of 468 samples (Fig. 1). Total bacterial SSU rRNA gene abundance and *Alcanivorax* specific SSU rRNA gene abundance covaried and increased with respect to visible oil contamination (Fig. 1). The percent abundance of *Alcanivorax* did not covary with *Alcanivorax* SSU rRNA gene abundance. The percent abundance of *Alcanivorax* was strongly associated with oil presence in the earliest (2 July) sampling trip and weakly associated with

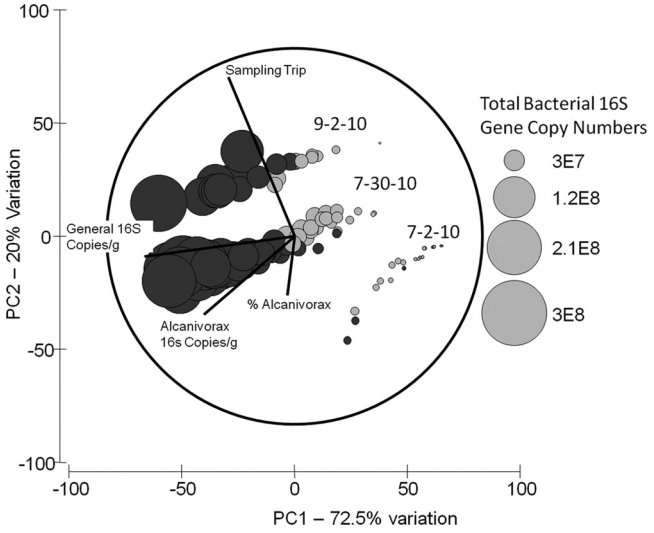

FIG. 1. Principal component analysis of bacterial SSU rRNA gene abundance and days since oil arrival. Dark bubbles represent visibly oil-contaminated samples, whereas light ones indicate visibly clean samples. Bubble size is relative to the number of bacterial SSU rRNA gene copies per gram of sand. Vectors represent variables used to generate the euclidean distance. SSU rRNA abundance data were initially fourth root transformed to meet the assumptions of normality.

oil contamination in the later trips. The first sampling trip was also characterized by a relatively small increase in total bacterial SSU rRNA gene abundance associated with oil contamination compared to later sampling trips. Linear regression analysis of the abundance of overall bacteria in comparison to the abundance of *Alcanivorax* spp. for each sampling trip supported observations from the principal component analysis (Fig. 2). During the July sampling trip, *Alcanivorax* spp. were most abundant and were shown to covary with the abundance of overall bacteria. In subsequent sampling trips, the abundance of *Alcanivorax* declined and did not strongly covary with overall bacterial abundance. Initially, the average percent composition of *Alcanivorax* gene copies comprised ca. 10% of the total bacterial gene copies, which fell to <1% in sampling trips after September 2010.

Enrichment and isolation of oil-degrading bacteria. Bacteria capable of using source oil as the sole carbon and energy source were isolated from oiled beach sands under aerobic and anaerobic conditions (Table 4). Analysis of bacterial SSU rRNA gene sequences revealed that the characterized isolates belong to 14 genera from seven orders and four classes within three phyla (*Proteobacteria*, *Firmicutes*, and *Actinobacteria*). The majority of the isolates were identified as members of four orders (*Oceanospirilalles*, *Alteromonadales*, *Vibrionales*, and *Pseudomonadales*) within the class *Gammaproteobacteria*. A single isolate was identified from the *Alphaproteobacteria*. Two Gram-positive organisms were isolated from the phyla *Firmicutes* and *Actinobacteria*. The SSU rRNA gene sequences of all isolates were highly similar to those retrieved from oiled sands of Pensacola Beach (Fig. 3 and Table 5). In particular, SSU rRNA gene sequences from isolates of the genera *Alcanivorax*, *Marinobacter*, *Pseudoalteromonas*, and *Pseudomonas* shared a high sequence identity (>97%) with recovered sequences from beach sands.

Phenotypic characterization of oil-degrading bacteria. Pure cultures from 5 of the 14 genera (SSU rRNA gene sequences show high sequence identity to those from *Alcanivorax dieselolei*, *Acinetobacter* sp., *Pseudidiomarina maritima*, *Marinobacter*

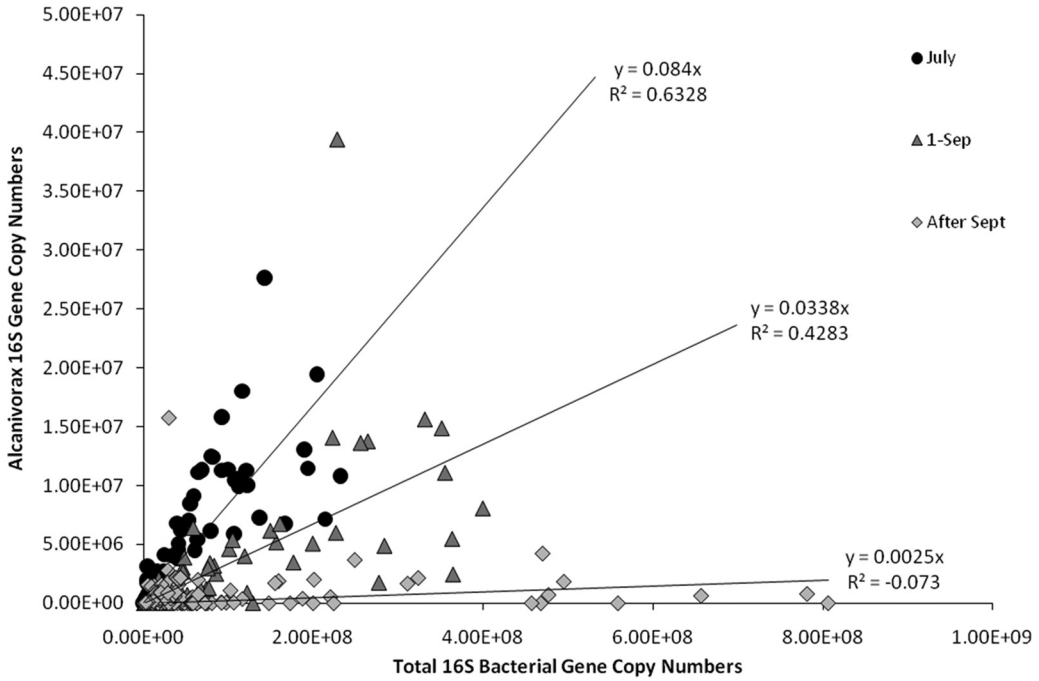

FIG. 2. Linear regression analysis of the abundance of overall bacteria in comparison to the abundance of *Alcanivorax* spp. for each sampling trip, with the abundance determined as SSU rRNA gene copies per gram of sand.

TABLE 4. Genotypic and phenotypic characterization of oil-degrading bacteria isolated from beach sands impacted by the DH oil spill[a]

Closest BLAST match	% Similarity	GenBank accession no.	Enrichment condition	Quantitative test for oil degradation
Pseudomonas pachastrellae	100	EU603457	Anoxic	
Pseudidiomarina maritima	99.9	EU600203	Anoxic	Yes
Marinobacter hydrocarbonoclasticus	99.6	DQ768638	Anoxic	
Shewanella algae	99.2	GQ372877	Anoxic	
Vibrio alginolyticus	97.7	GQ455008	Anoxic	
Pseudomonas stutzeri	99.7	GU396288	Anoxic	Yes
Alcanivorax dieselolei	100	AB453732	Anoxic	
Vibrio hepatarius	98.6	EU834019	Anoxic	Yes
Marinobacter vinifirmus	99.2	FJ161339	Anoxic	
Marinobacter vinifirmus	99.1	FJ161339	Anoxic	
Vibrio sp.	99.9	HM640395	Oxic	
Acinetobacter sp.	100	FJ876296	Oxic	Yes
Pseudoalteromonas sp.	99.8	AY394863	Oxic	
Acinetobacter venetianus	99.8	DQ912805	Oxic	
Bacillus sp.	99.2	HQ588864	Anoxic	
Halomonas shengliensis	99.7	EF121853	Anoxic	
Vibrio hepatarius	99	HM584097	Anoxic	
Vibrio alginolyticus	99.3	GQ455008	Anoxic	
Marinobacter hydrocarbonoclasticus	100	DQ768638	Anoxic	Yes
Labrenzia sp.	99.3	EU440961	Oxic MPN	
Alcanivorax sp.	99.9	AB435642	Oxic MPN	
Microbulbifer sp.	98.7	GQ334398	Oxic MPN	
Microbacterium schleiferi	99.9	EU440992	Oxic MPN	
Marinobacter hydrocarbonoclasticus	99.4	DQ768638	Oxic MPN	

[a] The results are based on comparison of SSU rRNA gene sequences of the isolates to the sequence that shows the highest sequence identity to the isolate. All strains were isolated in a minimal artificial seawater medium with DH source oil as the sole carbon and electron source. The oil degradation activity was quantified for representative isolates using the gravimetric assay described in Materials and Methods. Anoxic enrichment conditions indicate denitrifying enrichments.

hydrocarbonoclasticus, and *Vibrio hepatarius*) were tested for oil consumption under aerobic conditions. Oil degradation capability was confirmed from the quantification of residual oil and concomitant growth as optical density and cell protein (see the supplemental material). Little to no oil was consumed by the *Vibrio* strain in comparison to killed control cultures and minimal growth was observed. In contrast, all of the remaining strains were capable of rapid aerobic growth with source oil as the sole carbon substrate and electron donor. Gravimetric analysis indicated that strains most similar to *A. dieselolei* and by *Acinetobacter* sp. showed the highest oil degradation potential, with 93 and 90% of the chloroform-extractable portion of amended crude oil degraded, respectively, compared to autoclaved and uninoculated control cultures (see Fig. S1 in the supplemental material). The strains most similar to *M. hydrocarbonoclasticus* and *P. maritima* showed moderate amounts of oil degradation, with 36 and 12% of the chloroform-extractable oil fraction removed, respectively. The results from culture experiments in which oil consumption was quantified by spectrophotometry confirmed the above-mentioned results from gravimetric analysis (see Fig. S2 in the supplemental material).

Initial physiological characterization was conducted for five of our isolates (SSU rRNA gene sequences most similar to those from *A. dieselolei*, *Acinetobacter* sp., *M. hydrocarbonclasticus*, *P. maritima*, and *V. hepatarius*) using phenotypic MicroArray (PM) analysis. PM testing included salt tolerance, a wide variety of carbon sources (alcohols, amines, amino acids, carbohydrates, carboxylic acids, esters, fatty acids, and polymers), and inorganic and organic forms of major nutrients (N, P, and S) (see Table S2 in the supplemental material). Consistent with their marine habitat, all strains metabolized in the presence of NaCl concentrations up to 10%. In addition, Tween (polyoxyethylene sorbate) compounds, which contain long-chain alkyl moieties, were metabolized by all strains tested. Utilization of Tween compounds is considered diagnostic of the substrate specificity of marine bacteria toward the use of hydrocarbons as a source of carbon and energy (76). However, contrasts were observed in phenotype between oil-degrading strains that indicate niche specialization in carbon and major nutrient metabolism (see the supplemental material for details). *Vibrio*, *Acinetobacter*, and *Marinobacter* strains all utilized a fairly broad range of carbon substrates. In contrast, the *Alcanivorax* strain utilized relatively few carbon sources among those tested.

Cultivation-independent analysis of bacterial community structure in beach sands. Bacterial community structure was initially assessed in DNA extracts of 178 samples from three field trips by community fingerprinting using the automated ribosomal intergenic spacer analysis (ARISA) method (see the supplemental material for details). Twenty-six samples were chosen for pyrotag sequencing of SSU rRNA amplicons to represent substantial shifts in bacterial community structure. Pyrosequence data were analyzed using a nonmetric multidimensional scaling plot (NMDS) of an averaged Bray-Curtis distance matrix (Fig. 4). The distances reveal a pronounced, uniform response to oil contamination in the total and active bacterial communities. The pattern of sample grouping in ordination space reveals community response to oil presence, with the most heavily oiled samples (dark shading) distinct from clean samples (light shading). The pristine or "clean" samples group together and are most distant to the oiled samples. RNA-based pyrosequence libraries cluster with the corresponding DNA-based library, in most cases, and are also spatially oriented with respect to oil presence.

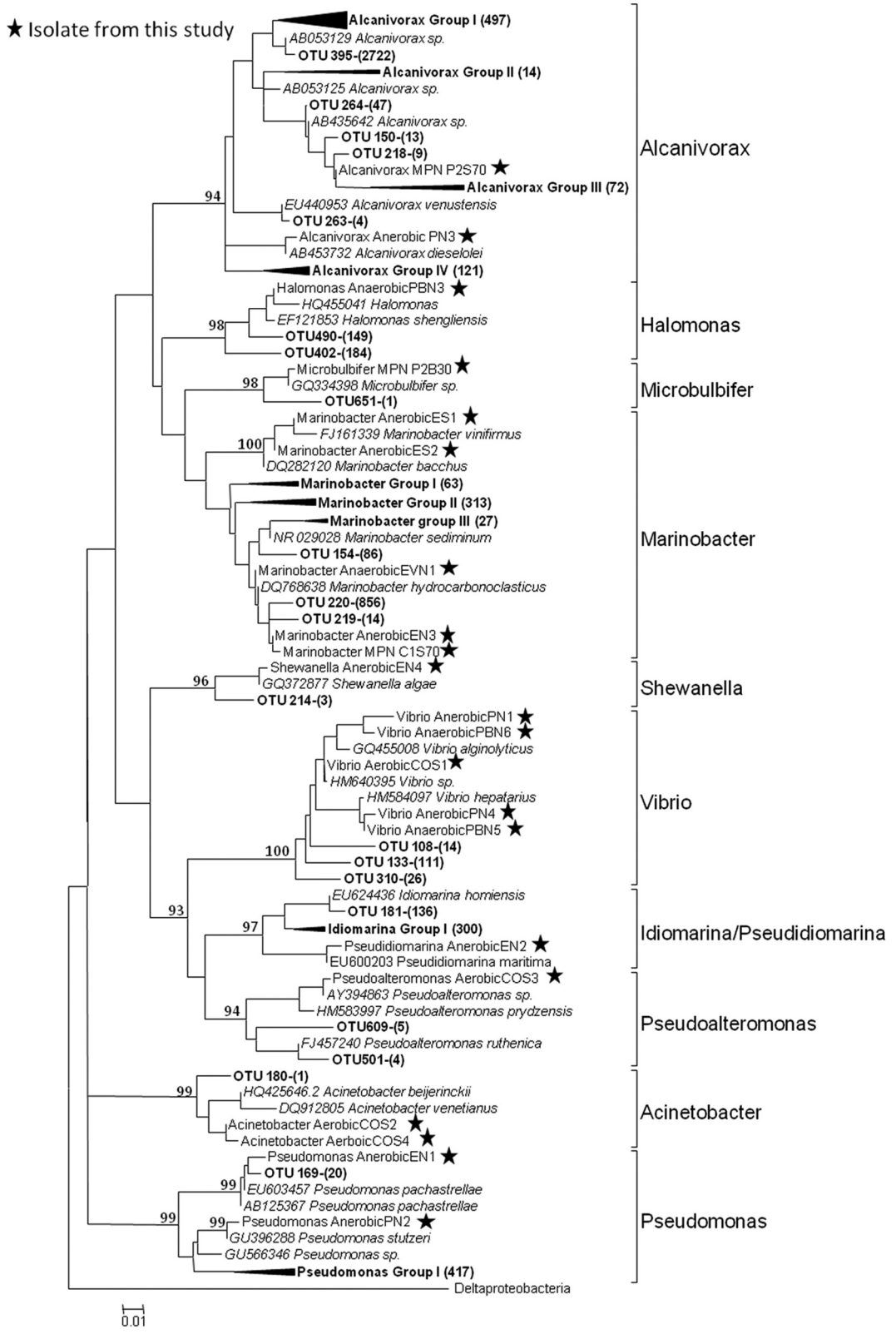

FIG. 3. Phylogenetic comparison of SSU rRNA gene sequences from oil-degrading bacterial isolates (★) and sequences retrieved from oiled Pensacola Beach sands (boldface). Only *Gammaproteobacteria* lineages are included in the analysis. The most similar sequences identified by BLAST are indicated by GenBank accession number. The bootstrapped neighbor-joining phylogenetic tree was generated in MEGA using the maximum composite likelihood model with gamma-distributed rates and pairwise deletion (39). SSU rRNA sequences from oil-degrading isolates, similar environmental OTU from the pyrosequencing data set, and top isolated BLAST hits were aligned by using the GreenGenes NAST aligner (16). Nodes with >70% bootstrapping support, out of 500 replications, are shown.

TABLE 5. Summary of taxa detected in the highest relative abundance in DNA-derived and RNA-derived pyrosequence libraries from beach sands in this study[a]

OTU classification	% Detected				
	Control	DNA PB oiled	RNA PB oiled	DNA PB clean	RNA PB clean
Gammaproteobacteria	28.4	41.5	31.6	32.9	21.9
Oceanospirillales	0.0	19.2	15.2	3.1	3.6
Alcanivorax group I	0.0	**8.9**	**6.4**	1.7	2.6
Alcanivorax group II	0.0	**4.4**	**5.1**	0.6	0.3
Oceanospirillaceae	0.0	**4.3**	**2.0**	0.0	0.1
Alteromonadaceae group I	0.0	0.5	0.8	0.1	0.0
Alteromonadaceae group II	0.0	0.3	0.1	**4.1**	**9.4**
Marinobacter	0.0	**1.6**	**2.9**	0.1	0.1
Xanthomonadales	0.0	2.9	0.3	0.5	0.0
Sinobacteraceae	0.0	2.6	0.3	0.2	0.0
Chromatiales	3.5	4.5	2.2	9.8	3.2
Ectothiorhodospiraceae group I	0.8	1.2	0.7	3.3	1.2
Ectothiorhodospiraceae group II	1.6	0.7	0.1	5.2	1.6
Ectothiorhodospiraceae group III	0.0	0.1	0.0	0.5	0.1
Unclassified	0.0	2.3	1.4	0.6	0.0
Unclassified	1.1	0.0	0.0	0.2	0.2
Pseudomonadales	0.3	0.2	4.7	0.2	0.0
Pseudomonas	0.2	0.0	**4.2**	0.1	0.0
Alphaproteobacteria	6.7	14.5	30.0	3.0	12.1
Rhodobacterales	2.9	5.3	25.6	0.7	7.4
Sulfitobacter	0.2	0.4	**2.9**	0.1	1.4
Hyphomonadaceae	0.0	1.1	0.1	0.0	0.0
Rhodobacteraceae group I	0.0	0.9	1.0	0.0	0.0
Rhodobacteraceae group II	0.0	0.2	1.3	0.1	0.3
Rhodobacteraceae group III	0.0	0.1	**9.7**	0.0	0.0
Rhodobacteraceae group IV	0.1	0.0	1.4	0.0	0.4
Bacteroidetes	10.4	18.1	2.3	14.2	3.5
Muricauda group I	0.0	**3.0**	0.0	0.0	0.0
Muricauda group II	0.0	**2.4**	0.0	0.0	0.0
Robiginitalea	0.1	0.6	0.0	2.1	0.3
Cyanobacteria	2.5	0.0	0.2	0.1	4.6
Synechococcus	1.7	0.0	0.0	0.1	0.4
Firmicutes	0.1	0.3	2.3	0.3	0.1
Bacillus	0.0	0.0	1.3	0.0	0.0

[a] Notable OTU abundances are indicated in boldface. Control, DNA- and RNA-derived libraries from St. George Island sand sampled prior to the oil spill; PB, Pensacola Beach sand.

Nearly all of the groups represented in our culture collection of oil-degrading bacteria were detected in pyrosequence libraries from oiled beach sands (Table 5). *Alcanivorax* spp., in particular, were shown to comprise the most abundant OTU in the pyrosequence libraries. Thus, the relative abundance of *Alcanivorax* OTU is overlaid on the NMDS plot, demonstrating a strong covariation with oil presence (Fig. 4).

As a proxy for the metabolically active bacteria present in beach sands, RNA-based pyrosequence libraries were generated and compared to DNA-based libraries for four samples collected on July 2 and selected to represent a range in visual oil contamination (Fig. 5). RNA- and DNA-based libraries revealed similar patterns in community composition grouped at the class level, especially in the oiled samples. Members of the *Gammaproteobacteria* and *Alphaproteobacteria* were shown to dominate beach sand communities, regardless of oil presence (Fig. 5, Table 5). *Alphaproteobacteria* and *Actinobacteria* show a proportionately higher representation in the RNA-based libraries compared to the DNA-based in all samples. In the most heavily oiled sample, this increase in relative abundance of *Alphaproteobacteria* is due primarily to a single OTU in the family *Rhodobacteraceae* (Table 5).

DISCUSSION

The overall goal of our research is to determine the environmental and ecological controls of hydrocarbon biodegradation under *in situ* conditions in coastal benthic environments impacted by the DH oil spill in the Gulf of Mexico. The oil degradation capacity of microbial populations in marine sedi-

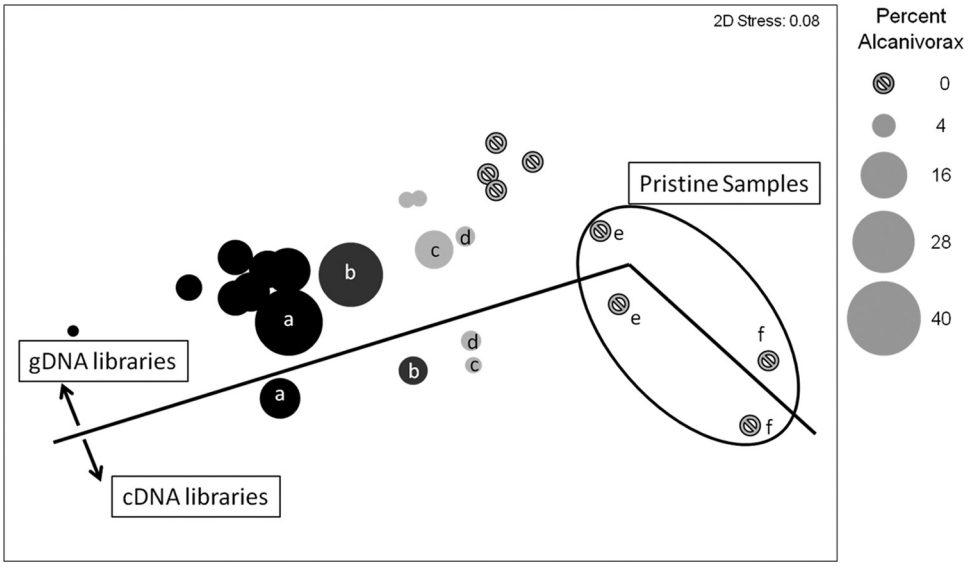

FIG. 4. Multidimensional scaling plot of SSU rRNA pyrosequence libraries derived from oiled Pensacola Beach sands (black), clean Pensacola sands (gray), and pristine sands (gray) from St. George Island. Samples above the black line were obtained from gDNA extracts, while samples below the line were derived from total RNA extracts reverse transcribed using a bacterial SSU rRNA gene primer. Lowercase letters (a to e) indicate pyrosequence libraries derived from the same sand sample. Averaged Bray-Curtis distance is shown. Bubble size is relative to percent abundance of *Alcanivorax*-like OTU as assigned by the RDP classifier.

ments is likely limited by stressors such as anoxia or nutrient starvation, as well as ecological interactions such as mutualistic production and exchange of biosurfactants between bacterial populations (27). Knowledge of bacterial community structure and the response of key microbial players in oil-contaminated environments provide a first glance at metabolic potential and the physiological mechanisms that might drive hydrocarbon degradation.

Response of indigenous bacterial communities to oil contamination in marine sands. Microorganisms with the capacity to degrade hydrocarbons are among the best-studied microbial groups in applied and environmental microbiology. Indeed, more than 200 bacterial, algal, and fungal genera, encompassing over 500 species, have been recognized as capable of hydrocarbon degradation (see reviews by Head et al. [27] and Yakimov et al. [76]). Much progress has been made to deter-

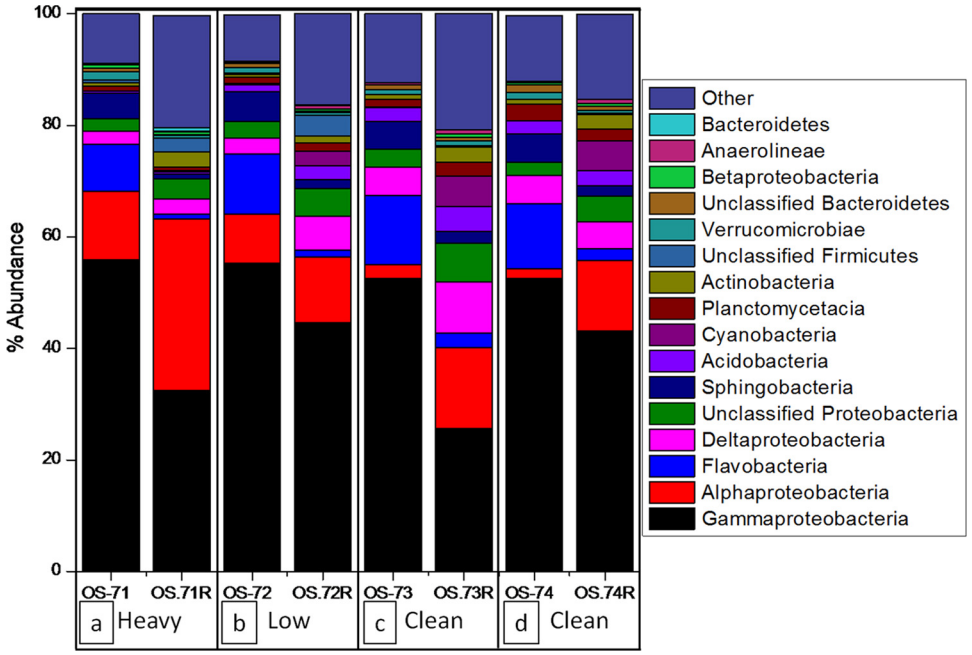

FIG. 5. Phylum- and class-level phylogenetic analysis of RNA and DNA-based bacterial SSU rRNA pyrosequence libraries for Pensacola Beach sand samples collected on 2 July 2010.

mine the response of specific bacterial taxa to oil contamination in marine environments impacted by oil spills (for reviews, see references 4, 5, 23, 27, 42, and 50). However, our ability to understand and predict the dynamics of *in situ* bacterial communities responding to environmental stimuli such as the presence of oil contamination remains in its infancy (27, 60). The majority of previous work on hydrocarbon-degrading bacterial communities in the marine environment has been conducted under enrichment conditions in laboratory microcosms (12, 13, 45, 52, 57, 69, 75). Of the fewer studies that have been conducted in the field, most have focused on biostimulation, for instance the application of nutrients to enhance oil bioremediation (46, 49, 64, 68). Thus, a paucity of information exists on the response of indigenous bacterial communities to oil contamination under *in situ* or natural attenuation conditions (2, 36, 51). The DH oil spill disaster in the Gulf of Mexico has inspired a number of studies that address this knowledge gap, with most of them to date centered on bacterioplankton communities of the deep ocean (26, 37, 70).

In our study, cultivation-based and molecular enumeration of bacterial communities showed a distinct impact of oil on overall bacterial numbers and specifically on the abundance of known oil degraders in Gulf beach sands. Not surprisingly, bacterial abundance increased as oil provides a major source of carbon and electrons in an otherwise nutrient-starved marine environment. Based on an extensive molecular data set from three field campaigns, we show that bacteria in Pensacola Beach sands were on average 2 to 4 orders of magnitude more abundant in the presence of oil contamination (Fig. 2 and 3); and high cultivatable counts as well as RNA-based analyses support the premise that the majority of bacteria in oiled sands are active. These results are consistent with past field research on oiled beaches (2, 46, 49, 63). We further show that the abundance of the known hydrocarbonoclastic group of *Alcanivorax* spp. increased in response to oil contamination, and a comparison of bacterial ribosome abundance in RNA extracts indicated that *Alcanivorax* abundance was proportionately greater within the active bacterial community (data not shown). Few previous studies have applied quantitative molecular approaches to determine the abundance of hydrocarbon degraders *in situ*. In a field bioremediation experiment, Singh et al. (68) reported an elevated abundance of *Alcanivorax* spp. in beach plots amended with oil plus fertilizer but not in plots treated with oil alone.

Oil began to come ashore at Pensacola Beach in early June, and we observed a bloom of *Alcanivorax* spp. and overall bacteria by early July, approximately 4 weeks after the initial oiling event. Maximum abundance was observed in our aerobic bacterial cultures after 1 to 2 weeks of incubation. Thus, our results indicate that the native microbial communities in beach sands respond fairly quickly to oil contamination, metabolizing oil to support growth at a rate which is within a factor of 2 to 4 in comparison to pure cultures. Coupled with our observation that oiled sands were depleted in low-molecular-weight aliphatic and aromatic hydrocarbons, the elevated abundance of hydrocarbon degraders provides an early indication that natural attenuation is a viable strategy for the mitigation of oil contamination in Pensacola Beach sands. The proportion of *Alcanivorax* spp. to the total community was lower at Pensacola Beach in comparison to past studies which used nutrient amendments to stimulate biodegradation. Conditions in subtropical sands (temperature, oxygen supply, and nutrients; see below) appear to favor a broader diversity of hydrocarbon degraders that might render biostimulation unnecessary.

The *in situ* metabolism of oil-degrading bacteria is likely to be limited by a number of environmental parameters, including temperature, the availability of oxygen and major nutrients, oil hydrocarbon content, and weathering or dispersal of the oil (59). Temperatures in this subtropical environment remain optimal for microbial growth throughout the summer, and oxygen profiling indicates that the Pensacola Beach sands remained aerobic throughout the study period with oxygen concentrations at all sampled depth $>90\%$ of sediment surface oxygen concentration. The rapid *in situ* growth we observed after 4 weeks indicates that microbial cells had access to the oil. The majority of our beach sand samples were collected in the supratidal zone and the interstitial pore space of these sands was not saturated with seawater. Therefore, we conclude that the growth of oil-degrading communities was likely limited by desiccation and/or nutrient depletion in the Pensacola Beach sands we studied. Desiccation would exacerbate carbon and nutrient depletion by shutting off the supply of dissolved substrates from inundating tidal waters.

In corroboration of previous work on marine sands in the Gulf of Mexico (30, 53), we observed that Pensacola Beach sands contain highly diverse bacterial communities that are predominated by members of the *Gammaproteobacteria* and *Alphaproteobacteria* (Fig. 5 and Table 5). Bacterial diversity was higher in beach sands in comparison to the bacterioplankton communities that we sampled (see the supplemental material). Shannon indices place the bacterial communities of beach sands (see Table S1 in the supplemental material) as more diverse than bacterioplankton but not as diverse as the bacterial communities of marine muds or soils (54).

Concomitant with changes to bacterial abundance, a pronounced shift in bacterial community structure was observed in Pensacola Beach sands in response to DH oil contamination (Fig. 4). These results emphasize that the embedded oil exerted a strong selective pressure on the sand bacterial community, in concurrence with past research (see reviews by Berthi-Corti and Nachtkamp [5] and Greer [23]). The consensus of studies conducted in oil-contaminated marine environments points to a succession of bacterial populations associated with the early stages of contamination reflecting the initial utilization of various highly degradable hydrocarbon compound classes (aliphatics, aromatics, and polyaromatics [27]). In our study, shifts in community composition in beach sands in response to oil presence were manifested at the strain to family level (Table 5), but little to no change was observed at the class level (Fig. 5). The present study, along with past studies, shows that the *Gammaproteobacteria*, and to a lesser extent the *Alphaproteobacteria*, predominate the bacterial communities of marine sediment ecosystems following exposure to oil hydrocarbons (see reviews by Head et al. [27], Yakimov et al. [76], Berthe-Corti and Nachtkamp [5], and Greer [23]). Among the *Gammaproteobacteria* in the oiled Pensacola Beach sands, members of the *Alcanivorax* genus were by far the most abundant. The *Alcanivorax* genus has been associated with the early stages of hydrocarbon degradation (weeks to months after the spill) and has been shown to utilize saturated hydrocarbons

such as straight-chain and branched alkanes (50, 76). Other hydrocarbon-degrading members of the *Gammaproteobacteria* that were detected in the present study (including *Acinetobacter*, *Marinobacter*, and *Pseudomonas*), although they also tend to be less abundant at the onset of hydrocarbon degradation, are more metabolically versatile than *Alcanivorax* and have been shown to degrade polycyclic aromatic hydrocarbons (PAHs) as well as alkanes.

Evidence from the present study also points to other microbial groups as key players in oil degradation. RNA-based pyrosequence libraries supported the DNA-based results as members of the *Rhodobacteraceae* family of the *Alphaproteobacteria* were among the most abundant phylotypes detected in the presence of oil. A shift toward the *Rhodobacteraceae* and Gram-positive groups may indicate that a succession has begun in Pensacola Beach oiled sands toward microbial groups involved in the degradation of more recalcitrant oil hydrocarbons. In field studies of weathered marine sediments which were depleted in alkanes, members of the *Alphaproteobacteria* and Gram positives were the prevailing groups detected (2, 36), and members of these groups represented in our culture collection (*Labrenzia*, *Bacillus*, and *Microbacterium*) were shown to degrade PAHs in pure culture (40, 67). OTU that showed a high sequence identity to *Sulfitobacter* were the most abundant members of the *Rhodobacteraceae* that we could assign at the genus level in our pyrosequence libraries. *Sulfitobacter* is a sulfite-oxidizing bacterium that has been isolated from a variety of marine environments. Thus, the capabilities of the *Alphaproteobacteria* genera *Labrenzia* and *Sulfitobacter* to degrade specific oil compounds should be further explored.

Isolation, identification, and characterization of model hydrocarbon-degrading bacteria. Evidence from cultivation-based approaches corroborated results from our cultivation-independent molecular-based analyses. We isolated organisms from several well known oil-degrading bacterial genera (*Alcanivorax*, *Marinobacter*, *Pseudomonas*, *Acinetobacter*, *Bacillus*, and *Microbulbifer*) that were detected in abundance in oiled Pensacola Beach sands. All 24 of our isolates were screened initially in minimal media with oil as the sole carbon and electron source, thereby assessing their potential to degrade oil. The majority of strains from oiled Pensacola Beach sands showed high SSU rRNA gene sequence identity to isolates previously cultured from marine or saline habitats that were contaminated with oil hydrocarbons (14, 40, 47, 65, 71). All of these organisms were demonstrated to degrade oil hydrocarbons in pure culture in the present study or by others (14, 40, 47, 65, 71, 76) or were detected in oil-contaminated marine environments (27) (Fig. 3 and Table 5). Bacteria from the genus *Alcanivorax* and other members of the *Gammaproteobacteria* isolated in the present study have been demonstrated to degrade alkanes in pure culture (76). Representatives from at least six of the species we isolated (*Acinetobacter* sp., *Bacillus* sp., *Labrenzia* sp., *Microbacterium* sp., *M. hydrocarbonoclasticus*, *P. pachastrellae*, and *P. stutzeri*) have been linked to PAH degradation in pure culture (27, 40, 67). Although the *Vibrio* species represented in the present study have not yet been shown to degrade oil in pure culture, some vibrios have been found to metabolize hydrocarbons, including PAHs (24).

Our results are in general agreement with previous cultivation studies of these hydrocarbonoclastic taxa. Among the OTU that could be classified to the genus level, *Alcanivorax* and *Marinobacter* groups showed the highest relative abundance among known hydrocarbon degraders in our pyrosequence libraries (Table 5) and were well represented among our isolates (Table 4). *Marinobacter* is a metabolically versatile hydrocarbon-degrading taxon, capable of utilizing a broad range of carbon substrates including aliphatics and PAHs (18, 25). In contrast, the 15 described species of the *Alcanivorax* group are thought to be highly specialized (50), with *A. dieselolei* listed as an "obligate hydrocarbonoclastic bacterium" (OHCB) that is capable of utilizing a very narrow range of carbon substrates (76). Our isolate with 100% sequence identity (across 850 bases) with *A. dieselolei* (AB453732) did show the narrowest range of carbon substrate utilization among the strains we tested (see Results and the supplemental material). The results of the present study and others indicate that the OHCB designation may need to be reconsidered, however, and genome sequencing will be essential to verifying the absence of metabolic pathways. Two different phylotypes of *Alcanivorax* spp. were abundant in our pyrosequence libraries, and previous work has shown that the physiology of *Alcanivorax* is likely to be strain specific (27). As with other functional guilds of bacteria, the taxonomy of hydrocarbon degraders is rapidly evolving. Many species have not been validly described. Of those that have been formally described, the phenotype of most strains has been largely characterized using Biolog testing. Biolog tests are effective for range finding and for testing many strains at once, as we have done here. However, these should be considered as preliminary, and they require verification with traditional physiological screening in pure culture. A further understanding of the ecophysiology of hydrocarbon degraders will be crucial to uncovering the *in situ* controls of oil degradation and to the development of improved mitigation strategies for oil spills. Through the isolation of model organisms, physiological testing of isolates, and genome sequencing, the activity, physiological potential and environmental distribution of hydrocarbon degraders can be confirmed and understood.

Conclusions. The quantitative increase in bacterial gene sequences, the increased relative abundance of known oil-degrading taxa, and the isolation of oil-degrading pure cultures from many of the same taxa confirmed the strong selective response of indigenous bacterial communities in Pensacola Beach sands to the presence of oil from the DH oil spill. We hypothesize that *Alcanivorax* spp. of the *Gammaproteobacteria* are effective microbial indicators or sentinels of the early stages of oil hydrocarbon degradation in Gulf beach sands when more reactive components such as *n*-alkanes abound, whereas members of the *Alphaproteobacteria* (*Labrenzia* and *Rhodobacteraceae*) and Gram-positive groups (*Bacillus* and *Microbacterium*) may be used as sentinels for the later stages of degradation when more recalcitrant oil hydrocarbon compounds such as PAHs predominate. In addition, we provide a number of pure cultures from these groups that may be used as model organisms to investigate the physiological ecology of hydrocarbon degraders in benthic habitats of the Gulf of Mexico. Further study is needed to delineate the specific role for each of these groups in hydrocarbon degradation on contaminated beaches.

ACKNOWLEDGMENTS

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