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Novel hydrocarbon monooxygenase genes in the metatranscriptome of a natural deep-sea hydrocarbon plume

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Summary

Particulate membrane-associated hydrocarbon monooxygenases (pHMOs) are critical components of the aerobic degradation pathway for low molecular weight hydrocarbons, including the potent greenhouse gas methane. Here, we analysed pHMO gene diversity in metagenomes and metatranscriptomes of hydrocarbon-rich hydrothermal plumes in the Guaymas Basin (GB) and nearby background waters in the deep Gulf of California. Seven distinct phylogenetic groups of pHMO were present and transcriptionally active in both plume and background waters, including several that are undetectable with currently available polymerase chain reaction (PCR) primers. The seven groups of pHMOs included those related to a putative ethane oxidizing *Methylococcaceae*-like group, a group of the SAR324 *Deltaproteobacteria*, three deep-sea clades (Deep sea-1/symbiont-like, Deep sea-2/PS-80 and Deep sea-3/OPU3) within gammaproteobacterial methanotrophs, one clade related to Group Z and one unknown group. Differential abundance of pHMO gene transcripts in plume and background suggests niche differentiation between groups. Corresponding 16S rRNA genes reflected similar phylogenetic and transcriptomic abundance trends. The novelty of transcriptionally active pHMOs we recovered from a hydrocarbon-rich hydrothermal plume suggests there are significant gaps in our knowledge of the diversity and function of these enzymes in the environment.

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Introduction

The copper-containing membrane-bound monooxygenases (CuMMO) are a large family of environmentally important enzymes that mediate the biogeochemical cycling of carbon and nitrogen. The most notable of these are particulate methane monooxygenase (pMMO) and ammonia monooxygenase (AMO). While CuMMOs are widely distributed across phyla *Alpha, Beta and Gammaproteobacteria* (Hanson and Hanson, 1996; Dunfield *et al.*, 2002; Stoecker *et al.*, 2006; Arp *et al.*, 2007; Baani and Liesack, 2008; Chen *et al.*, 2010), *Verrucomicrobia* (Dunfield *et al.*, 2007; Pol *et al.*, 2007), NC10 (Ettwig *et al.*, 2010) and *Thaumarchaea* (Könneke *et al.*, 2005; Tourna *et al.*, 2011), their functions are generally restricted to oxidation of methane (catalysed by pMMO) and ammonia (catalysed by AMO). However, the recent discovery of novel membrane-associated, copper containing, hydrocarbon monooxygenases (pHMOs) expands the known functional diversity of the CuMMO protein family (Sayavedra-Soto *et al.*, 2011; Coleman *et al.*, 2012). Redmond and colleagues (2010) found that ^{13}C-labeled ethane was incorporated into biomass [(deoxyribonucleic acid) DNA] by a group of *Methylococcaceae* with a novel pMMO, suggesting these enzymes may be involved in ethane utilization. Recognition of divergent CuMMO-like coding genes in the genomes of bacterial isolates further highlights the major challenges in relating CuMMO diversity and function (Balasubramanian *et al.*, 2010; Sayavedra-Soto *et al.*, 2011; Tavormina *et al.*, 2011; Coleman *et al.*, 2012; Mason *et al.*, 2012).

Evidence of substrate diversity of the CuMMO was first identified in two butane-oxidizing *Actinobacteria*, *Nocardioides* sp. CF8 and *Mycobacterium* sp. JOB5 (Hamamura *et al.*, 1999; 2001; Hamamura and Arp, 2000). Subsequently, expression of genes encoding a three-component membrane-associated monooxygenase with sequence homology to CuMMO correlated with butane utilization by *Nocardioides* sp. CF8 (Sayavedra-Soto *et al.*, 2011). Similarly, novel CuMMOs capable of oxidizing C_2–C_4 alkanes were identified in two strains of *Mycobacteria*, which like *Nocardioides*, are also members of the *Actinomycetales* (Coleman *et al.*, 2011;

2012). Furthermore, novel pMMO-like sequences have been identified in recently sequenced genomes, including gammaproteobacterial methanotrophs (Tavormina *et al*., 2011), two marine ethylene-assimilating *Haliea* spp. (Suzuki *et al*., 2012), *Nocardioides* Broad-1 (ZP_08197402) and SAR324 *Deltaproteobacteria* (Swan *et al*., 2011). Thus, it is apparent that CuMMOs can oxidize not only ammonia or methane but also short chain hydrocarbons, suggesting the sequence divergence within this protein family may reflect novel biochemical and physiological roles within the cell.

The role of pMMO/AMO enzymes in methane and ammonia oxidation has been well established, and genes encoding subunit A (PmoA/AmoA) are commonly used as functional biomarkers for surveying aerobic methane and ammonia oxidizers in nature (Hanson and Hanson, 1996; Costello and Lidstrom, 1999; Norton *et al*., 2002; Inagaki *et al*., 2004; Francis *et al*., 2005; McDonald *et al*., 2008; Chistoserdova, 2011; Lüke and Frenzel, 2011). However, little is known of the distribution and diversity of CuMMOs responsible for the oxidation of short chain hydrocarbons especially in hydrocarbon-rich environments, which are globally distributed in deep sea, including natural methane and hydrocarbon seeps (Milkov *et al*., 2003; Campbell, 2006), hydrothermal vents (Beaulieu, 2010) and oil spills such as the Deepwater Horizon (Valentine *et al*., 2010). These hydrocarbon seeps substantially contribute to the deep-sea carbon pool and have been postulated to maintain populations of low molecular weight hydrocarbon-degrading bacteria (Baelum *et al*., 2012; Muyzer and van der Kraan, 2008; Mason *et al*., 2012; Valentine, 2010; Valentine, 2011).

Guaymas Basin (GB) is a sediment-hosted hydrothermal system within a semi-enclosed basin in the central Gulf of California. Guaymas Basin hydrothermal plumes are chemically distinct from typical mid-ocean ridges because hydrothermal fluids interact with sediments as they ascend to the seafloor (Vondamm *et al*., 1985). These plumes are enriched in potential microbial energy sources, such as ammonium, methane and other gaseous hydrocarbons (Welhan and Lupton, 1987; Lam, 2004; Dick *et al*., 2009; Dick and Tebo, 2010). It has been estimated that methane concentration could reach up to 54 mM in vent fluids and more than 31.6 μM in the plumes, while C_2–C_4 alkanes concentrations range from 1.4 μM (n-C_4H_{10}) to 444 μM (C_2H_6) in the fluids (Welhan and Lupton, 1987; Lam, 2004; McCollom, 2008). Previous studies of microbial methane oxidation at GB have focused primarily on warm, anoxic sediments (Teske *et al*., 2002; Biddle *et al*., 2011), however methanotrophy is also a dominant metabolism in the cold, oxic water column (Lesniewski *et al*., 2012). Here, we present a detailed analysis of pHMOs in the GB metagenomes and metatranscriptomes and find that seven different phylogenetically diverse pHMOs are simultaneously transcriptionally active in both of hydrothermal plume and background waters.

Results and discussion

Presence of pHMO genes in metagenomic and metatranscriptomic datasets

To investigate the diversity of active membrane-associated pHMOs in natural deep-sea hydrocarbon plumes, we analysed shotgun sequenced community DNA and complementary DNA (cDNA) from four hydrothermal plume samples and two background samples retrieved from the deep Gulf of California on three different cruises (Table S1). A total of 102 631 pHMO related DNA and cDNA sequence reads and up to 40 unique contigs were identified in metagenomes and metatranscriptomes of GB hydrothermal plumes and background samples. In each instance, there was a greater percentage of total metagenome derived reads belonging to genes encoding A, B and C subunits of pHMOs in the plumes than in background (Table 1), whereas only the A and B subunits had higher percentages of cDNA reads in plumes compared to background (Table 1). An extra gene copy of *pmoC* is present in the genome of *Methylococcus capsulatus* (Bath) (Stolyar *et al*., 1999), but the near-stoichiometric recovery of genes for the three subunits suggests this not the case for GB populations. Our data are more consistent with differential expression of genes for different pHMO subunits as has been observed in a verrucomicrobial methanotroph (Erikstad *et al*., 2012). Interestingly, a divergent C subunit of AMO in *Nitrosomonas europaea* (homologous to *pmoC*) has been implicated in stress response (Berube and Stahl, 2012). By analogy, the disproportionate subunit C expression we observe (especially in background, where substrate concentrations are lower) may reflect an equivalent stress response, albeit by differential expression rather than extra gene copies. No genes encoding soluble methane monooxygenase (sMMO) or soluble butane monooxygenase (sBMO) were identified in any of the GB metagenomic and metatrascriptomic datasets. This suggests that gaseous hydrocarbon oxidation in the deep Gulf of California, including hydrocarbon-enriched hydrothermal plumes, is dominated by pHMO-mediated oxidation.

Phylogenetic analysis of pHMOs and comparison to 16S rRNA phylogeny

Phylogenetic analyses of genes for pHMO subunit A revealed seven distinct clusters, including pMMOs of

Table 1. Abundance of DNA and cDNA sequence reads (total 102 631 reads) and contigs containing pHMO subunits (A, B, C) in metagenomes and metatranscriptomes of Guaymas Basin hydrothermal plume and background samples. Shown are pHMO-containing reads (as a percentage of total reads for each sample) and contigs (raw number) for each sample.

Subunits	454 metagenomic reads (per cent of total reads)[a]		454 metatranscriptomic reads (per cent of total reads)[a]		Illumina metatranscriptomic reads (per cent of total reads)[a]		454 assembled contigs		Illumina assembled contigs		Unique phylogenetic groups
	Plume	Background	Plume	Background	Plume	Background	Metagenome	Metatranscriptome	Plume	Background	
A	2.94×10^{-3}	1.80×10^{-3}	9.05×10^{-3}	7.34×10^{-3}	9.56×10^{-3}	2.88×10^{-3}	8	22	40	14	6
B	5.49×10^{-3}	2.48×10^{-3}	14.5×10^{-3}	10.5×10^{-3}	7.64×10^{-3}	3.65×10^{-3}	10	19	18	14	5
C	4.93×10^{-3}	2.36×10^{-3}	27.8×10^{-3}	33.5×10^{-3}	10.5×10^{-3}	11.4×10^{-3}	9	32	35	16	6

a. Average abundance from libraries of two plumes and two backgrounds (Lesniewski *et al.*, 2012).

Deep sea-1/symbiont-like, Deep sea-2/PS-80 and Deep sea-3/OPU3 groups (Lüke and Frenzel, 2011), pHMOs affiliated with putative ethane oxidizing *Methylococcaceae*-like bacteria (Redmond *et al.*, 2010), a group closely related to SAR324 *Deltaproteobacteria* (Swan *et al.*, 2011), and two groups, Group Z (Tavormina *et al.*, 2010) and Unknown group, of unknown function (Fig. 1A). Similar phylogenetic groups and relationships of pHMOs were also obtained from respective B and C subunits (Figs S1 and S2) and from related 16S ribosomal ribonucleic acid (rRNA) genes (Fig. 1B). Thus, at least seven distinct phylogenetic pHMO groups are present and transcriptionally active in the GB hydrothermal plume. Five of the groups are related to previously reported sequences that have been implicated in the oxidation of C_1 to C_4 alkanes. Comparative analysis of active sites/metal centres strongly support these pHMOs belong to the CuMMO superfamily, sharing relatively conserved amino acids at the Cu-binding sites (Fig. S3). Although Mason and colleagues (2012) also noted a diversity of *pmo* sequences expressed in plumes of the Deepwater Horizon oil spill, we believe that our study is the first detailed report of concurrent abundant transcription of multiple phylogenetically distinct pHMO genes in a single environment.

Three groups of pHMO sequences (Deep sea-1/2/3) share high amino acid sequence identity with environmental sequences but have relatively low sequence identity to those from pure cultures (Table S2). All three of these pMMOs belong to gammaproteobacterial methanotrophs, consistent with the dominance of gammaproteobacterial methanotrophs in hydrothermal systems (Elsaied *et al.*, 2004; Wasmund *et al.*, 2009; Crépeau *et al.*, 2011). Interestingly, the Deep sea-2/PS-80 and Deep sea-3/OPU3 pMMOs share high similarity (95–100% amino acid sequence identity) to sequences retrieved from the Deepwater Horizon oil spill in the Gulf of Mexico (Kessler *et al.*, 2011), the Santa Monica Basin methane seep (Tavormina *et al.*, 2008; 2010; Redmond *et al.*, 2010) and the North Fiji hydrothermal vent field (Tavormina *et al.*, 2010), indicating that these genes are widely distributed in pelagic water column environments. One pMMO group, referred to here as 'Deep sea-1/symbiont-like', is most closely related to sequences from the water column of the Eastern Pacific Ocean oxygen minimum zone (OMZ) (Hayashi *et al.*, 2007), sediments from a methane seep (Redmond *et al.*, 2010), and endosymbionts of the hydrothermal vent mussel *Bathymodiolus azoricus* (Spiridonova *et al.*, 2006). To date, the Deep sea-1/symbiont-like pMMO clone has only been reported once from water column samples (282 m and 497 m below surface; referred to as OPU2 in their study) by Hayashi and colleagues (2007). Thus, our result is the first demonstration of the significant presence and

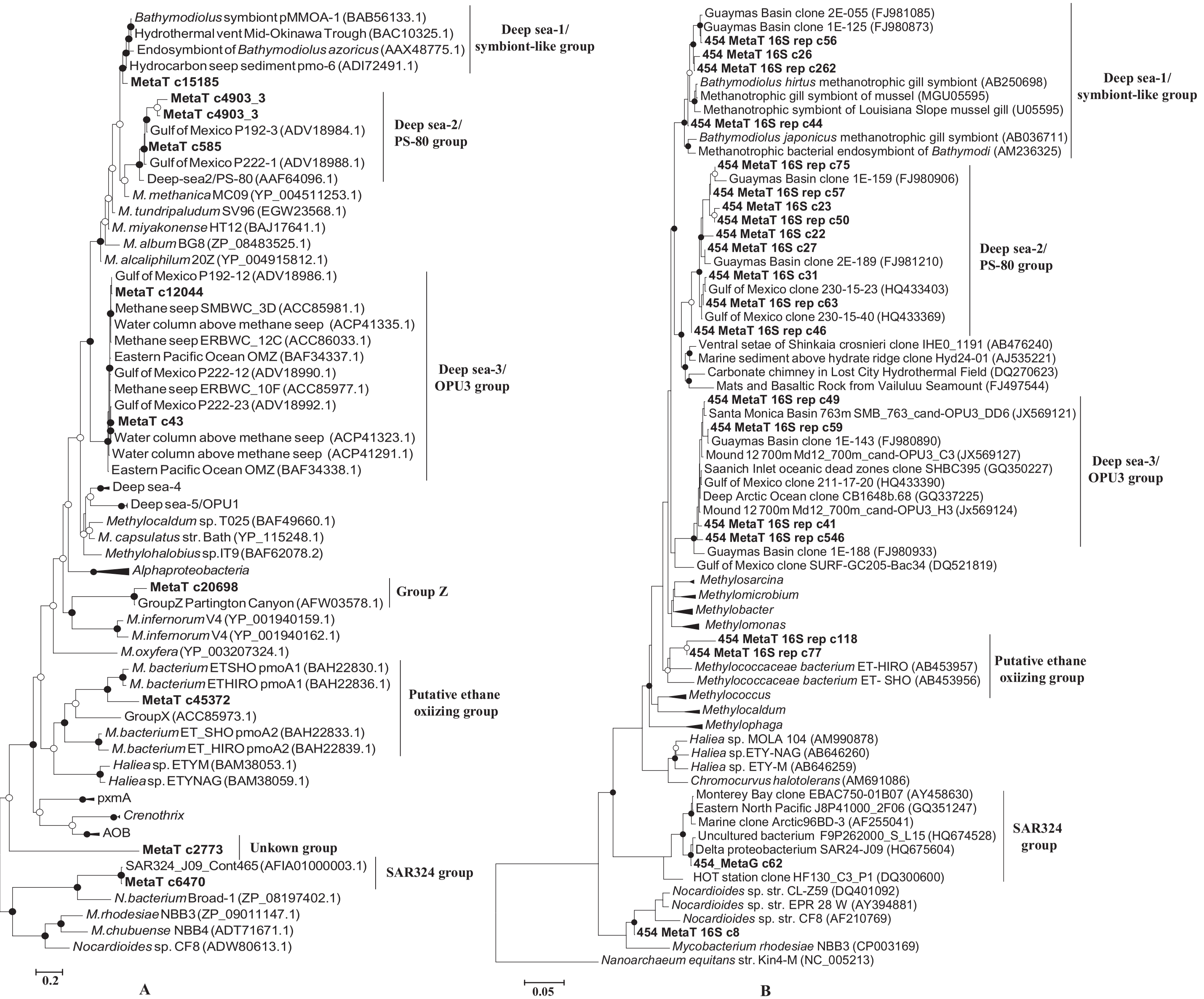

Fig. 1. Phylogenetic relationship of pHMOs based on subunit A (A) and comparison with the 16S rRNA phylogeny (B). The trees were constructed by Maximum Likelihood (ML) method with 1000 bootstraps to evaluate tree topology. Black circles at branch nodes bootstrap values larger than 70, while white circles represent bootstrap values between 50 and 70.

transcriptional activity of Deep sea-1/symbiont-like pMMOs in the deep water column (see below).

Two novel groups of GB pHMO sequences were phylogenetically affiliated with proteins for oxidation of C_2–C_4 alkanes. First, the putative ethane-oxidizing cluster has relatively low similarity to any pMMO from methanotrophs (< 50%) but up to 70.2% sequence identity to pHMOs from two putative ethane-oxidizing *Methylococcaceae*-like species (Table S2). 16S rRNA genes with high sequence identity (94%–96%) to these of two putative ethane-oxidizing *Methylococcaceae*-like species were also recovered from metatranscriptome (Fig. 1B), indicating the presence of transcriptionally active putative ethane-oxidizing *Methylococcaceae*-like bacteria. Second, the putative C_2–C_4 alkane-oxidizing group contains novel GB pHMO sequences that share 93.6% to 98.2% protein sequence identity to the three subunits of pHMO recently identified in the single-cell amplified genome of SAR324_J09 (Swan *et al.*, 2011) (Table S2 and Fig. 1A). SAR324 is an ubiquitous group of uncultured *Deltaproteobacteria* recently proposed to be capable of C_1 metabolism based on the presence of the pHMO operon in the genome of SAR324_J09 (Swan *et al.*, 2011). SAR324 is one of the most abundant microbial groups in the GB plumes (Dick and Tebo, 2010; Lesniewski *et al.*, 2012), and its 16S rRNA genes show high similarity (99%) to SAR324_J09 (Fig. 1B). Our phylogenetic analysis shows that both pHMOs from SAR324_J09 and those recovered from GB are most closely related to those of *Actinomycetes, Mycobacterium chubuense* NNB4 and *Nocardioides* sp. CF8 (Fig. 1A), which are capable of C_2–C_4 alkane degradation (Sayavedra-Soto *et al.*, 2011; Coleman *et al.*, 2012). Furthermore, our parallel investigation on the metagenome and metatranscriptome of GB-SAR324 indicates that this group also contains and expresses the necessary metabolic pathways for C_2–C_4 alcohol utilization (Sheik *et al.*, 2013), suggesting that the GB population utilizes C_2–C_4 alkanes rather than methane. However, it should be noted that the sequence similarity between SAR324 pHMO and these of C_2–C_4 alkane-oxidizing bacteria (*Mycobacterium chubuense* NNB4 and *Nocardioides* sp. CF) is relatively low (< 43%), thus further experimental data are needed to confirm the actual substrate specificity of these enzymes. Nevertheless, our results constitute the first environmental detection of high transcription levels (see below) for this group of enzymes, further emphasizing the previously unrecognized role of SAR324 cluster in the oxidation of hydrocarbons in the oceans.

Given the novelty of pHMOs at GB, we compared our sequences to PCR primers commonly used for pHMO detection. The Deep sea-3/OPU3 pHMOs are perfect matches for pmoA189f-pmoAwc377r and pmoC346f-pmoAwc377r (Costello and Lidstrom, 1999; Tavormina *et al.*, 2008; 2010), and the Deep sea-1/symbiont-like and Deep sea-2/PS-80 pHMOs are perfect matches for primer set pmoA189f-mb661r (Costello and Lidstrom, 1999). However, no combination of currently available primer sets perfectly matches the GB pHMO sequences related to putative ethane oxidizing and SAR324 groups. This result suggests that much of the natural community diversity may be missed using a traditional PCR-based approach and highlights the utility of random shotgun sequencing in surveying natural communities (Gilbert *et al.*, 2008; Gilbert and Dupont, 2011).

Another dimension of pHMO diversity, the microdiversity within populations, was revealed by the deep sequence coverage provided by Illumina metatranscriptomic sequencing. Two metatranscriptomic contigs (contig_43 and contig_585) related to Deep sea-2/PS-80 and Deep sea-3/OPU3 pMMOs displayed variable sequences for each group within the deep-sea populations respectively. These variable sequences were on average 99.2% similar to each other at the nucleotide level. The variable nucleotide positions were observed in the *pmoC* and *pmoB* for contig_43 and *pmoA* and *pmoB* in contig_585 (Fig. S4). These nucleotide substitutions resulted in changes in amino acid sequences but did not occur at metal centre sites that are critical for function (Lieberman and Rosenzweig, 2005; Balasubramanian *et al.*, 2010) (Fig. S3). Since genomic context for each of these variable sequences is unavailable, it is impossible to determine if they belong to different species/strains or are gene duplicates within a single genome (Ward *et al.*, 2004; Hou *et al.*, 2008; Boden *et al.*, 2011; Stein *et al.*, 2011; Svenning *et al.*, 2011). Regardless, the presence of closely related sequence types within each pMMO group further underscores the multidimensional diversity of pHMOs in the GB hydrothermal system.

Transcriptional activity and distribution of different groups

To assess the transcriptional activity and distribution of pHMOs, we mapped cDNA reads to the different pHMO groups in plume and background samples. pHMO-coding genes from Deep sea1/symbiont-like, Deep sea-2/PS-80 and putative C_2–C_4 alkane-oxidizing (SAR324) group account for a greater portion of metatranscriptomic reads in plume than in background (Fig. 2 and Fig. S5). In contrast, Deep sea-3/OPU3 and putative ethane oxidizing groups have similar or even higher relative abundance of transcripts in background samples. This suggests that pHMOs of Deep sea-2/PS-80, Deep sea1/symbiont-like and SAR324 group are more responsive to increased concentrations of hydrocarbons in plumes, whereas the Deep sea-3/OPU3 and putative ethane oxidizing groups

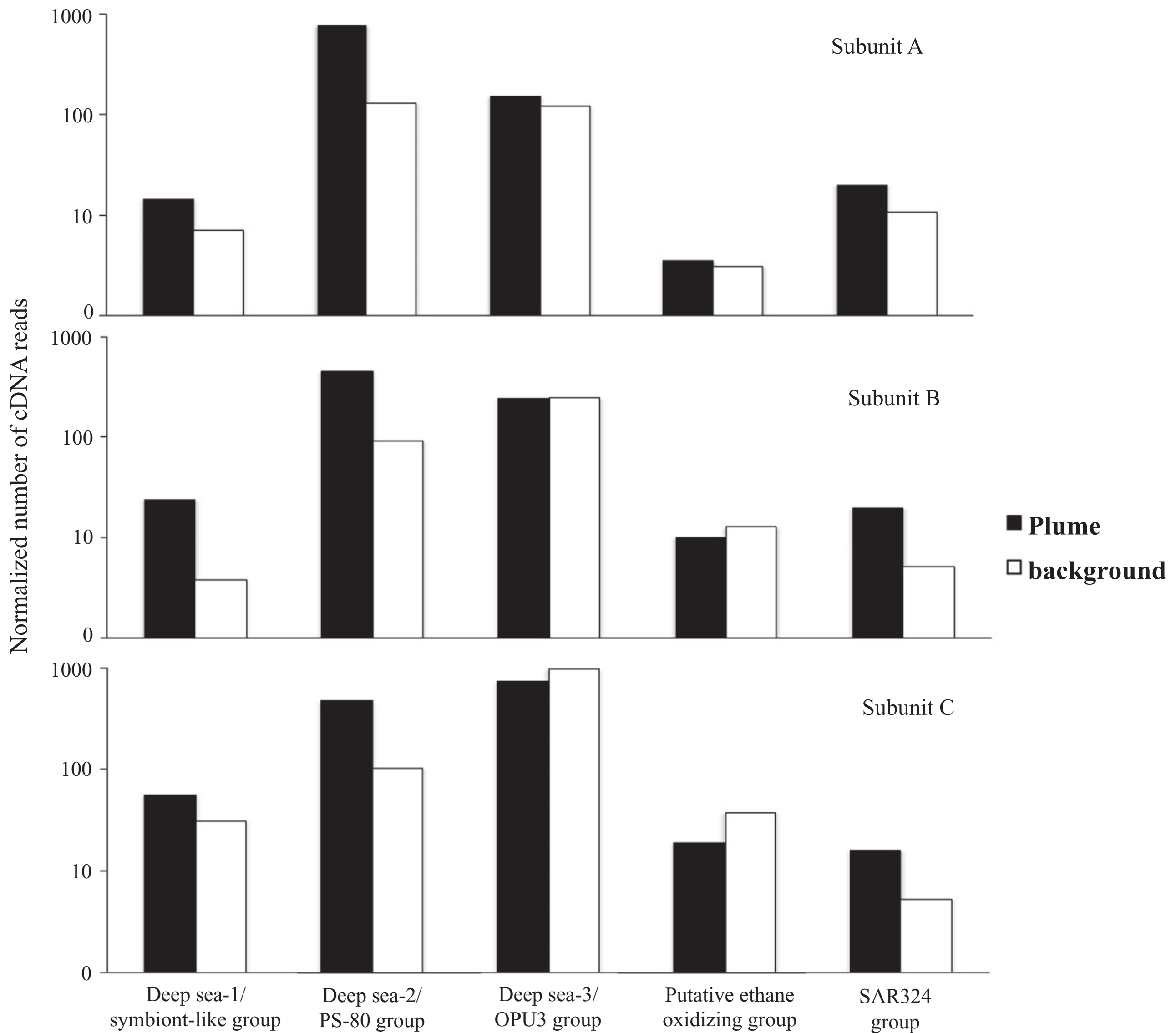

Fig. 2. Relative abundance of transcripts (Illumina) for genes encoding three subunits of different groups of pHMOs from plume and background samples. Transcript abundance is normalized to the length of gene and total number of transcript reads from each sample.

may have higher affinity for low substrate concentrations. Consistent with this conclusion, lack of correlation of Deep sea-3/OPU3 abundance with methane has been previously interpreted as evidence for adaptation of this group to low methane concentration (Tavormina *et al.*, 2013). Interestingly, OPU1 and Deep sea-3/OPU3 were previously observed to largely occur together (Tavormina *et al.*, 2008; 2010; 2013), but OPU1 is conspicuously missing from the GB dataset. Differences in the relative abundance of these two groups correlate with reduced O_2 concentrations and depth within the Costa Rica OMZ (Tavormina *et al.*, 2013), where OPU3 is more abundant in the low O_2 zone (~ 5.80 μM O_2) at 200–650 m, while OPU1 dominates in deeper water (below ~ 700 m) with higher O_2 concentration (~ 17.8 μM O_2). Our samples all have higher O_2 concentrations (26–28 μM O_2 in GB, 46 μM O_2 in Carmen Basin), so other environmental factors might also be involved in controlling the distribution of OPU1 and OPU3.

Similar trends of transcript abundance were apparent in 16S rRNA genes, where transcripts from Deep sea-2/PS-80 and Deep sea-1/symbiont-like methanotrophs are much more abundant in plume than background (Fig. S6). Methane concentrations in buoyant and neutrally buoyant GB plumes can reach up to 31.6 and 11.2 μM respectively, while concentrations in the overlying water are only

16–65 nM (Lam, 2004). Differential abundance of transcripts from 16S rRNA and corresponding pHMO genes between the background and the plume may be indicative of niche differentiation between different groups. Furthermore, it should also be noted that all of the methanotrophic genes and groups we report here to be operating under dysoxic conditions and a relatively narrow range of cold temperatures are distinct from those of hydrothermal sediments at GB, where anaerobic methane oxidation is carried out by archaea under anoxia and a much higher and wider temperature range (15 to 95°C) (Teske *et al*., 2002; Biddle *et al*., 2011).

To assess the broader distribution of these novel pHMOs, we used subunit A sequences of each group to query data from diverse marine environments (Tables S3 and S4). We found that *pmoA* sequences from the Deep sea-1/symbiont-like group are often present in association with hydrothermal vent fauna, mats and deposits (Elsaied *et al*., 2004; Spiridonova *et al*., 2006; Duperron *et al*., 2007; Zbinden *et al*., 2008; Crépeau *et al*., 2011; Raggi *et al*., 2012) or sediments of methane seeps (Yan *et al*., 2006; Redmond *et al*., 2010) but only rarely in the water column. In contrast, *pmoA* related to Deep sea-2/PS-80 and Deep sea-3/OPU3-like groups are widely distributed in diverse environments, including sediments and water column of methane seeps (Inagaki *et al*., 2004; Tavormina *et al*., 2008; 2010; Reed *et al*., 2009; Wasmund *et al*., 2009; Redmond *et al*., 2010; Havelsrud *et al*., 2011), hydrothermal vent systems (Nercessian *et al*., 2005; Reed *et al*., 2009; Tavormina *et al*., 2010; Crépeau *et al*., 2011) and other pelagic marine environments (Hayashi *et al*., 2007; Tavormina *et al*., 2010; 2013). We identified sequences (> 60% amino acid similarity) similar to pHMO of putative ethane oxidizing bacteria and putative C_2–C_4 alkane-oxidizing bacteria (SAR324) in metatranscriptomes of water column samples from the Deepwater Horizon at Gulf of Mexico (Table S4), supporting the potential participation of these enzymes in hydrocarbon oxidation. In addition, related sequences of the SAR324 pHMO were also identified from OMZs of Eastern Tropical South Pacific Ocean and Eastern Subtropical North Pacific Ocean, as well as water column samples from two profiles of HOT station at 500 m (Table S4), indicating a broad distribution of SAR324-like pHMOs in diverse marine environments. Furthermore, related sequences to the two unknown functional pHMO groups (Group Z and Unknown group) were recovered from deep-sea hydrocarbon-enriched environments, such as Partington Canyon (Tavormina *et al*., 2010) and the Deepwater Horizon oil spill in Gulf of Mexico (Tables S3 and S4). Future studies are required to definitively link this genetic potential to actual quantitative contributions and rates of hydrocarbon oxidation in the deep sea.

Conclusions

In this study, we found an array of diverse and novel pHMO genes in the community genomes and transcriptomes of a natural deep-sea hydrocarbon-rich hydrothermal plume and surrounding background waters in the Gulf of California. In addition to genes of known methanotrophs that are widely distributed yet uncultivated, these pHMOs affiliate with recently discovered putative C_2–C_4 alkane-oxidizing enzymes that have not previously been described in any detail in marine systems. Particularly intriguing is the potential for a significant yet previously unrecognized role of versatile SAR324 bacteria in the oxidation of hydrocarbons in the marine environment. More broadly, these results underscore the power of metagenomics and metatranscriptomics for identification of genetic and functional diversity present in natural microbial communities. In practical terms, the prevalence of novel pHMO genes that are undetectable by current primers highlights the persisting limitations of traditional PCR-based approaches for tackling the staggering diversity of natural microbial communities. While lack of cultured representatives continues to hinder our understanding of the biochemical and physiological underpinnings of this diversity, cultivation-independent approaches can elucidate the ecology of these organisms by defining how they vary in space and time as a function of environmental parameters.

Although the diversity of pHMOs is increasingly well recognized, this study is the first to demonstrate simultaneous transcriptional activity of multiple phylogenetically distinct pHMOs rather than spatial and/or temporal separation of activity. This co-occurrence of multiple transcriptionally active pHMO genes and variants within deep-sea microbial communities and populations likely reflects intense competition and suggests extensive resource partitioning during microbially mediated alkane oxidation. Given the ubiquity of alkanes (van Beilen *et al*., 2003; van Beilen and Funhoff, 2007) and the environmental costs of oil spills, future studies must address these dynamics and the relationship between pHMO diversity and function in natural environments if we are to understand and predict the fate of hydrocarbons released into the oceans.

Experimental procedures

Sample collection and extraction of nucleic acids. Samples were collected in 2004 and 2005 aboard the R/V New Horizon as described previously (Dick *et al*., 2009; Dick and Tebo, 2010). Briefly, samples were collected in Niskin bottles by CTD-rosette and kept under *in situ* conditions (cold and dark) until processing. Immediately upon shipboard retrieval, samples were pressure filtered with N_2 gas onto 0.2 μm pore size polycarbonate filters, which were immediately preserved in RNAlater as instructed by the manufacturer (Ambion). DNA was extracted from filters as described previously (Dick and

Tebo, 2010). RNA extraction and random RNA amplification and cDNA synthesis were performed as described previously (Frias-Lopez *et al*., 2008; Shi *et al*., 2011). Details of the location, sampling date, environmental conditions and concentration of nucleic acids of each sample are provided in Table S1.

DNA sequencing, de novo assembly and annotation. Purified total genomic DNA and cDNA (no rRNA removal was conducted) were used to prepare DNA libraries for 454 Titanium pyrosequencing at the Dedicated Marine Microbiology DNA Sequencing Pipeline at Pennsylvania State University according to standard protocols (454 Life Sciences, Roche). In addition, samples plume-4 (GD6) and background-1 (GD7) were used for metatranscriptomic sequencing by Illumina HiSeq 2000 (Illumina) at the University of Michigan DNA Sequencing Core. DNA and cDNA sequence reads obtained from 454 and Illumina sequencing were separately *de novo* assembled by different strategies. The read sequences obtained from 454 Titanium pyrosequencing were trimmed and quality filtered with the Mothur software package (Schloss *et al*., 2009, http://www.mothur.org). The trimmed and quality-filtered short reads were utilized for *de novo* assembly as described in detail previously (Baker *et al*., 2012; Lesniewski *et al*., 2012). For Illumina datasets, cDNA reads were first de-replicated by removing identical reads, then cDNA reads were quality trimmed by Sickle (quality score > 30, https://github.com/najoshi/sickle). The de-replication and trimming processes reduced the number cDNA reads from 206 to 45 million in the plume and 245 to 130 million in the background sample. These de-replicated and trimmed cDNA datasets were then assembled by Velvet (1.2.01) and subsequently processed using the transcriptomic assembler Oases 0.2.04 (Zerbino and Birney, 2008; Schulz *et al*., 2012; Baker *et al*., 2013, http://www.ebi.ac.uk/~zerbino/oases/). All contigs were submitted to the DOE Joint Genome Institute (JGI) Integrated Microbial Genomes website (https://merced.jgi-psf.org/cgi-bin/mer/main.cgi) for gene calling and annotation.

Identification and phylogenetic analysis of pHMO and 16S rRNA genes. Hydrocarbon monooxygenase gene sequences in GB metagenomes and metatranscriptomes were identified using BLASTX searches (bit score > 50) against methane/ammonia monooxygenase sequences in National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and Functional Gene Database (http://fungene.cme.msu.edu). To confirm that they were indeed most closely related to genes encoding pHMO, candidate putative pHMO genes were then used as queries against the non-redundant database at NCBI, as well as against custom databases of manually identified and curated genes. For sequences that passed this test, affiliations with contigs were determined, and respective contigs were analysed. Each pHMO-related contig was manually analysed via BLASTP analysis of predicted proteins against the non-redundant database to determine degree of similarity to known sequences. Full length of ammonia/methane monooxygenase subunit A open reading frames (ORFs) were extracted from the operons with BLASTX and translated into amino acids, then aligned to known related ammonia/methane monooxygenase subunit A sequences using Clustal/omega/1.1.0 (Sievers *et al*., 2011, http://www.clustal.org/omega/). A high-quality alignment region consisting of 221 amino acids was selected using TrimAl (Capella-Gutierrez *et al*., 2009). The PhyML mixture was used to generate a maximum likelihood mixture model tree using linkage disequilibrium (LD) model after testing of aligned sequences using ProTest (Abascal *et al*., 2005). Partial length ammonia/methane monooxygenase subunit A sequences were aligned with 34 full length sequences and placed on the tree with the maximum likelihood placement algorithm in RAxML (Stamatakis, 2006; Berger *et al*., 2011). The tree of ammonia/methane monooxygenase subunit B and C was constructed using the same method. To search for 16S rRNA gene sequences from the pHMOs bacterial hosts, cDNA reads related to methanotrophs, SAR324 cluster and *Actinomycetales* from 454 metatranscriptomic dataset were identified by BLASTN (sequence identity > 99 %), then these 16S rRNA related short reads were *de novo* assembled for longer 16S rRNA contigs using MIRA with the default parameters (Chevreux *et al*., 2004). Contigs longer than 400 bps were aligned with reference sequences via the Greengenes Nearest Alignment Space Termination (NAST) Alignment Tool (DeSantis *et al*., 2006), and the phylogenetic tree was constructed in ARB software with Maximum Likelihood method after the chimera checking (Ludwig *et al*., 2004).

Analysis of pHMO operon structure and population variability. Hydrocarbon monooxygenase operon structure was analysed on sequence similarity of three subunits (A, B and C). Identity values for protein sequence of pHMO were calculated by LALIGN (Huang and Miller, 1991, http://embnet.vital-it.ch/software/LALIGN_form.html). To identify single-nucleotide polymorphisms (SNPs), two contigs with complete pMMO operons from the metatranscriptome assembly were realigned with the original Illumina short reads datasets by Bowtie (Langmead *et al*., 2009) using paired-end model and zero mismatches, and then visualized by Integrated Genome Viewer (Robinson *et al*., 2011) to confirm the SNP positions.

Transcriptional activity and distribution of pHMOs. To investigate the transcriptional activity and distribution of different pHMO groups, we recruited cDNA reads to representative genes of different pHMO groups via BLAST. Following the method described previously (Lesniewski *et al*., 2012), the BLAST hit with highest bit score for each query was used to calculate the number of reads recruited to each subject per dataset. In cases where short read sequences hit multiple subjects with equal bit score, scores were weighted based on number of subjects hit [score = 1/(number of subjects)]. These scores were then normalized to the length of each gene fragment and total number of sequences in each database. To avoid false hits to pHMO sequences from AMO sequences, which are abundant in GB metatranscriptomic datasets (Baker *et al*., 2012), we constructed a database including both pHMO and AMO-coding gene sequences and discarded sequences with best hits to AMO. Sequences related to pHMOs with BLAST bit score > 50 (subunit A) and > 65 (subunits B and C) were assigned as pHMOs. Relative abundance of 16S rRNA of pHMOs-related bacteria was also calculated using the same normalization procedure with the BLAST bit score > 50 and percentage identity > 99%. To

further understand the presence and distribution of these pHMOs in other marine environments, we also searched NCBI and Integrated Microbial Genomes (IMG) databases for sequences similar to PmoA.

Data deposition. This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession AJXC00000000. The version described in this paper is the first version, AJXC01000000. Transcript sequences are available at the NCBI sequence read archive under the accession SRP007999. Annotated gene and protein sequences are available from DOE JGI-IMG under Taxon Object ID: 2061766003.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Phylogenetic relationship of pHMOs based on subunit B.

Fig. S2. Phylogenetic relationship of pHMOs based on subunit C.

Fig. S3. Comparison of the metal-binding sites for pHMOs identified in this study and other representative CuMMOs. The + indicates the carboxylate side-chain residues postulated to coordinate a di-iron center; # indicates the variable metal site of subunits A and C; & indicates the ligands to mononuclear copper; ***** indicates the subunit B dinuclear copper site. Colored shading indicates putative substrate of each enzyme: light blue is methane, dark blue is ethylene, pink and green are ethane and C_2-C_4 alkanes, respectively. *[1]*these sequences are incomplete; dashes shows missing data.

Fig. S4. Sequence variation of Deep sea-2/PS-80 and Deep sea-3/OPU3 pMMO operons and corresponding variation in their amino acid sequences.

Fig. S5. Normalized transcript abundance for 16S rRNA sequences of different pHMO groups in plume and background samples. The normalized numbers were divided to the total number of transcript reads from plume and background samples.

Table S1. Summary of samples and sequencing in this study, after Lesniewski *et al*. 2012.

Table S2. Sequence identities (amino acid %) of three subunits of pHMO identified in this study to CuMMOs contained in type strains.

Table S3. Distribution of different pHMO groups in diverse pelagic marine environments based on NCBI clone sequence survey.

Table S4. Distribution of different pHMO groups in diverse marine environments based on metagenomes and metatranscriptomes survey.