

ORIGINAL ARTICLE

Hydrocarbon monooxygenase in *Mycobacterium*: recombinant expression of a member of the ammonia monooxygenase superfamily

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The copper membrane monooxygenases (CuMMOs) are an important group of enzymes in environmental science and biotechnology. Areas of relevance include the development of green chemistry for sustainable exploitation of methane (CH₄) reserves, remediation of chlorinated hydrocarbon contamination and monitoring human impact in the biogeochemical cycles of CH₄ and nitrogen. Challenges for all these applications are that many aspects of the ecology, physiology and structure–function relationships in the CuMMOs are inadequately understood. Here, we describe genetic and physiological characterization of a novel member of the CuMMO family that has an unusual physiological substrate range (C₂–C₄ alkanes) and a distinctive bacterial host (*Mycobacterium*). The Mycobacterial CuMMO genes (designated *hmoCAB*) were amenable to heterologous expression in *M. smegmatis*—this is the first example of recombinant expression of a complete and highly active CuMMO enzyme. The apparent specific activity of recombinant cells containing *hmoCAB* ranged from 2 to 3 nmol min⁻¹ per mg protein on ethane, propane and butane as substrates, and the recombinants could also attack ethene, *cis*-dichloroethene and 1,2-dichloroethane. No detectable activity of recombinants or wild-type strains was seen with methane. The specific inhibitor allylthiourea strongly inhibited growth of wild-type cells on C₂–C₄ alkanes, and omission of copper from the medium had a similar effect, confirming the physiological role of the CuMMO for growth on alkanes. The hydrocarbon monooxygenase provides a new model for studying this important enzyme family, and the recombinant expression system will enable biochemical and molecular biological experiments (for example, site-directed mutagenesis) that were previously not possible.

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Introduction

Enzymes in the ammonia monooxygenase superfamily (here termed copper-containing membrane monooxygenases—CuMMOs) are of high biogeochemical and chemical importance (Bedard and Knowles, 1989; Hakemian and Rosenzweig, 2007). Known CuMMOs are restricted to methanotrophs and autotrophic ammonia-oxidizers, nearly all of which grow solely on C₁ compounds (Table 1) (Konneke *et al.*, 2005; Stoecker *et al.*, 2006; Dunfield *et al.*, 2007; Baani and Liesack, 2008; Martens-Habbena *et al.*, 2009; Ettwig *et al.*, 2010; Semrau *et al.*, 2010). The resulting ‘restricted role’ paradigm has implications for applications involving CuMMOs.

The unusually tight correlation between a single enzyme family and globally important biogeochemical processes such as methane and nitrous oxide flux (Conrad, 1996) suggests CuMMO genes are extremely useful markers of biological feedbacks in global climate change (Singh *et al.*, 2010). However, the distinctive chemistry of CuMMOs and physiological specialization of their native hosts creates major challenges for biochemical characterization of the enzymes (Balasubramanian *et al.*, 2010).

PCR-based environmental surveys have made great progress in identifying the ecological distribution and relevance of CuMMO-containing organisms. Such surveys have repeatedly shown CuMMO sequences are correlated to parameters, such as gas flux, land use and climatic conditions (Menyailo *et al.*, 2008; Avrahami and Bohannan, 2009). However, they have also revealed novel diversity not represented in cultured organisms (Holmes *et al.*, 1999; Leininger *et al.*, 2006; Kip *et al.*, 2010; Luke *et al.*, 2010). These novel environmental sequences pose a challenge for biogeochemical interpretation

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Table 1 Attributes of CuMMO-containing organisms represented in culture

Systematic group	Selected examples	CuMMOs ^a	Other MOs	Comments ^b
<i>γ</i> -Proteobacteria methanotrophs	<i>Methylococcus capsulatus</i>	pMMO (homoalleles)	sMMO	'Obligate' methylotroph, facultatively dependent on CuMMO
	<i>Methylobacter album</i>	PMMO, pXMO (heteroalleles)		'Obligate' methylotroph, dependent on CuMMO. The CuMMO homolog pxmA is of unknown function
	<i>Candidatus</i> Crenothrix polyspora	pMMO		Not in pure culture, data from enriched culture analysis
<i>α</i> -Proteobacteria methanotrophs	<i>Ms. trichosporium</i> (Methylocystaceae)	pMMO (heteroalleles)	sMMO	'Obligate' methylotroph, facultatively dependent on CuMMO
	<i>Methylocystis</i> sp. SC2 (Methylocystaceae)	pMMO (heteroalleles)		'Obligate' methylotroph, dependent on CuMMO. CuMMO alleles differ in methane affinity
	<i>Methylocapsa</i> <i>acidiphila</i> (Beijerinckiaceae)	pMMO		'Obligate' methylotroph, dependent on CuMMO
	<i>Methylocella silvestris</i> ^c (Beijerinckiaceae)	none	sMMO	Restricted facultative methylotroph, no CuMMO
<i>Verrucomicrobiales</i> methanotrophs	<i>Ma. Inferorum</i> (Verrucomicrobiales)	pMMO (heteroalleles)		'Obligate' methylotroph, extreme acidophile. Both CuMMO alleles lack ligands for the binuclear copper center. At least one of the two CuMMOs is inferred to be a pMMO
Candidate division NC10 Anaerobic methanotrophs	<i>Candidatus</i> <i>Methyloirabilis</i> oxyfera	pMMO		Not in pure culture, data from enriched culture analysis
<i>β</i> -Proteobacteria AOB	<i>Nitrosomonas europaea</i>	AMO (homoalleles)		Lithoautotroph, dependent on CuMMO
<i>γ</i> -Proteobacteria AOB	<i>Nitrosococcus oceanus</i>	AMO		Lithoautotroph, dependent on CuMMO
<i>Thaumarchaeota</i> AOA	<i>Nitrosopumilus maritima</i>	AAMO		Lithoautotroph, dependent on CuMMO ^d
<i>Actinobacteria</i> Small alkane degraders	<i>Mycobacterium</i> NBB4	HMO	Eight	Heterotrophs with wide substrate range. Facultative CuMMO metabolism ^e
	<i>Mycobacterium</i> NBB3	PBMO	Two	
	<i>Nocardioides</i> CF8	HMO	?	

Abbreviations: AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria; CuMMO, copper membrane monooxygenase.

^aThe number of copies and degree of relatedness of CuMMO alleles is complex and may vary within strains of a species. The term heteroalleles indicates copies proven, or thought, to encode functionally different enzymes exist.

^bThe term 'obligate' refers to practical laboratory growth on exogenously supplied substrates.

^cIncluded for comparative purposes—one of only two known methanotrophs that do not contain a CuMMO.

^dAAMO—Archaeal ammonia monooxygenase. Very divergent with distinct operon structure.

^eThe propane degrader *Mycobacterium* JOB5 almost certainly contains a CuMMO. A CuMMO is present in the genome of the uncharacterized organism *Nocardioides* Broad-1 (gb:ADVI01000043).

since our current understanding of structure–function relationships in CuMMOs is too limited to allow us to predict their functions (Singh *et al.*, 2010). This limitation largely reflects the inability to efficiently produce recombinant enzymes or genetically manipulate wild-type strains. These challenges are widely attributed as consequences of both the innate enzyme properties (for example, membrane localization and distinctive metal centers), and the specialized metabolism and genetic intractability of the host bacteria (Semrau *et al.*, 2010). Characterizing the CuMMOs of uncultured organisms is essential to allow functional interpretation of environmental data sets, and also offers the potential to identify more experimentally tractable model organisms.

It has long been postulated that CuMMOs may have roles outside of methanotrophy and ammonia oxidation. The first evidence in this regard came from butane-oxidizing bacteria. Two strains (*Nocardioides* CF8 and *Mycobacterium* JOB5) contained a butane monooxygenase activity with similar

acetylene and allylthiourea (ATU) inhibition characteristics to the pattern seen for particulate methane monooxygenase (pMMO) and ammonia monooxygenase (AMO) (Hamamura *et al.*, 1999, 2001). In both strains, ATU inhibited butane growth and ¹⁴C-labelled acetylene inhibited growth and resulted in labelling of a polypeptide corresponding to the size of a CuMMO subunit. Very recently, genes coding for a three-component monooxygenase with sequence homology to CuMMOs were reported in the draft genome sequence of *Nocardioides* CF8, and were transcribed in the presence of butane (Sayavedra-Soto *et al.*, 2011). CuMMO sequences have also been linked to ethane-degrading organisms (Genbank accession number: BAH22836). These significant observations suggest that the paradigm of obligate association with physiologically specialized organisms needs revision.

Mycobacterium strain NBB4 was isolated from estuarine sediment by enrichment on ethene as sole carbon source (Coleman *et al.*, 2006). This strain utilizes a broad spectrum of hydrocarbons (C₂ to C₁₆

alkanes, C₂–C₄ alkenes, but not methane) and contains a wide diversity of monooxygenase genes, including four different soluble di-iron monooxygenases (SDIMOs), three cytochrome p-450's and an *alkB* homolog (Coleman *et al.*, 2011). We previously proved that growth on ethene was linked to one of the SDIMOs (EtnABCD), but we could not identify the monooxygenase (MO) responsible for oxidation of gaseous alkanes. Here, we show that the 'missing' gaseous alkane MO of strain NBB4 is a CuMMO, and amenable to recombinant expression in *Mycobacterium smegmatis*, thus providing a new model system for the study of this important enzyme family.

Materials and methods

Media, bacterial strains and culture conditions

Mycobacterium strains were grown aerobically at 30 °C, using a minimal medium described previously (Coleman *et al.*, 2006), while *Escherichia coli* (*E. coli*) strains were grown aerobically at 37 °C in LB medium. Broth media were shaken at 200 r.p.m. *Mycobacterium rhodesiae* NBB3 and *Mycobacterium chubuense* NBB4 were isolated in a previous study (Coleman *et al.*, 2006) by enrichment culture on ethene. *E. coli* EPI300 (*F*[−]*mcrA* Δ (*mrr-hsdRMS-mcrBC*) Φ 80*lacZ* Δ M15 Δ *lacX74 recA1 endA1 araD139* Δ (*ara, leu*)7697 *galU galK* λ -*rpsL* (*Str*^R) *nupG trfA dhfr*) (Epicentre Biotechnologies, Madison, WI, USA), *E. coli* TOP10 (*F*[−]*mcrA* Δ (*mrr-hsdRMS-mcrBC*) Φ 80*lacZ* Δ M15 Δ *lacX74 recA1 araD139* Δ (*ara leu*) 7697 *galU galK rpsL* (*Str*R) *endA1 nupG*) (Invitrogen, Carlsbad, CA, USA) and *Mycobacterium smegmatis* mc²-155 (Snapper *et al.*, 1990) were used as cloning hosts. Where required for plasmid maintenance, kanamycin (Km) was added at 50 μ g ml^{−1} for *E. coli* and 20 μ g ml^{−1} for *Mycobacterium*.

Analytical techniques

Alkanes and chlorinated aliphatic compounds were analyzed by gas chromatography of headspace gas samples (250 μ l) using an HP 5890 series II machine (Hewlett-Packard, Wilmington, DE, USA) with a PLOT-Q column, using helium as the carrier gas and flame ionization detection. Quantification was done via an external standard method using a five-point standard curve, with each injection performed in triplicate. Peak areas were converted using the standard curve to amounts expressed as μ moles per bottle. Protein was quantified by an ultraviolet absorption method (Kalb and Bernlohr, 1977) following hot alkaline lysis of the cells (Coleman *et al.*, 2002a). Chloride analysis was done via a colorimetric assay (Coleman *et al.*, 2002b).

Epoxides were detected using a nitrobenzylpyridine assay based on the protocol of Guengerich *et al.* (1979), as follows. A 2-ml glass vial containing 500 μ l of 4-(4-nitrobenzyl)pyridine (100 mM in ethylene glycol) was placed inside a 16 ml bottle

containing ethene-grown NBB4 cells (4 ml) at an OD₆₀₀ of 13 ± 0.5 (0.86 mg protein ml^{−1}). The outer bottle was crimp-sealed with a Teflon-faced butyl rubber stopper, while the inner vial was left open. Substrate (ethene) was added as 10% v/v gas in the headspace, and the bottles incubated at 30 °C with shaking at 200 r.p.m for 24 h. The bottle containing 4-(4-nitrobenzyl)pyridine reagent was removed, and heat-treated (80 °C, 1 h) to enhance conjugate formation, then triethylamine solution (0.5 ml, 1:1 v/v in acetone) was added, and the absorbance at 550 nm was measured immediately. The absorbance was converted to nmol epoxide using the extinction coefficient for the epoxyethane:4-(4-nitrobenzyl)pyridine conjugate (2100 M cm^{−1}) (Morrill *et al.*, 1981).

Effect of inhibitors on growing cultures

The effect of the inhibitors ATU and nitrapyrin was studied by monitoring the change in optical density at 600 nm (OD₆₀₀) over time in NBB4 cultures growing on C₂–C₄ alkanes (10% of headspace), ethene (10% of headspace), potassium acetate (20 mM) or glucose (20 mM) in the presence or absence of ATU (25 μ M) or nitrapyrin (25 μ M), which were added to the medium before inoculation. The effect of copper on growth was studied by comparing growth (as OD₆₀₀) on the same carbon sources described above in regular minimal medium (0.8 μ M Cu²⁺) or copper-free medium, which was prepared in nitric-acid washed glassware with high purity Milli-Q water (Millipore, Billerica, MA, USA).

Construction, screening and sequencing of fosmid libraries

High molecular weight genomic DNA from strain NBB4 was extracted and purified by a previously described method (Coleman *et al.*, 2002a), then a fosmid library using the pCC1FOS vector was prepared according to the manufacturer's instructions (Epicentre Biotechnologies). Clones were screened for the presence of SDIMOs by PCR as described previously (Coleman *et al.*, 2011), and one clone containing an SDIMO similar to soluble methane monooxygenase was sequenced on both strands by a primer-walking strategy using the Sanger sequencing method at the Australian Genome Research Facility (Westmead node). The sequences were assembled and visualized using the Vector NTI software (Invitrogen). Gene functions were inferred from BLAST searches with the inferred protein sequences against the Genbank and conserved domain databases.

Phylogenetic analysis

Searches of public databases were conducted using BLASTN and BLASTP for *hmoCAB* and HmoCAB, respectively. In environmental sequence databases and the non-redundant databases, the only hits of >50% identity for any of the three genes or their inferred proteins were recently deposited, unpub-

lished Actinobacterial genome sequences, *Nocardioide*s CF8, and *Nocardioide*s Broad-1. For construction of phylogenetic trees a representative selection of sequences homologous to PmoA, encompassing all major groups identified in four recent studies was used (Ettwig *et al.*, 2010; Kip *et al.*, 2010; Luke *et al.*, 2010; Tavormina *et al.*, 2010). An ungapped alignment corresponding to the region between Lys59 and Thr207 of *Mc. capsulatus* Bath PmoA was used for analyses.

Both protein and nucleotide sequences were used for construction of trees using programs available in the Phylyp and Geneious packages (<http://www.geneious.com>). Many CuMMO-containing organisms contain multiple non-orthologous alleles, so this dendrogram does not reflect the phylogenetic relationships of the parent bacterial strains. The isolated phylogenetic position of the Hmo proteins was seen with all methods of analysis, and for all Hmo subunits (trees derived from HmoB and HmoC analysis were not shown here because no sequence data are available for these subunits in most environmental sequence groups). The tree shown in Figure 2 is a distance matrix tree of HmoA homologs constructed using Protdist (JTT matrix) and Fitch, with the *Nitrosopumilis mirabilis* AmoA sequence (ABY89319) used as an outgroup. Note that this outgroup sequence cannot be unambiguously aligned over the full length of the alignment and accordingly the position of this sequence is shown as a dimensionless line. Note that the Proline-169 residue of the *M. oxyfera* sequence (CBE69519) has no homologous position in all other bacterial sequences—this site was deleted from the alignment.

Protein accession numbers for sequences included in the tree shown in Figure 2 are: M84-P22 group (1_ABC86668, 2_ACD68272, 3_ACD68273, 4_ACM46919, 5_ACD68292, 6_CAI30606); *Candidatus* *Methylomirabilis oxyfera* (7_CBE69519); Verrucomicrobia PmoA1/PmoA2 group (8_ACK55191, 9_ABX56601, 10_ACK55192, 11_ABX56604); *alpha-Proteobacteria* methanotrophs group (12_AAD47927, 13_AAV67717, 14_AAL86710, 15_CAC45630, 16_CAD24093, 17_CAI30620, 18_CAD30373, 19_CAD30371, 20_CAD30381, 21_CAD24094, 22_CAD24092); *gammaProteobacteria* methanotroph PmoA group (23_ACS72359, 24_CAE22498, 25_AAG13081, 26_AAC61804, 27_AAX48776, 28_AAG13080, 29_AAL09399, 30_AAF08215, 31_AAA87218, 32_AAX37294, 33_AAB49821, 34_ADM12710, 35_AAC04380); *gammaProteobacteria* AOB group (36_AAV67698, 37_AAV67707, 38_AAV67689, 39_AAC25091); TUSC group (40_AAZ06193, 41_AAZ06151, 42_CAC69466); *gammaProteobacteria* ET-HIRO (43_BAH22836, 44_ACC85973); RA21 group (45_CAC84847, 46_ACJ54056, 47_AAL87425, 48_AAD47928); gp23 clone 49_AAK51669; MR1 group (50_ACS94422, 51_ACM46913, 52_AAF19026); *gammaProteobacteria* methanotroph PxmA group (53_ABL85665, 54_ACE95888, 55_ACJ53965, 56_ACE95881, 57_ACE95890); M84-P105 group (58_CBH32423, 59_CAC69467, 60_CAC

69468, 61_ACJ53974, 62_ACJ54003, 63_CAZ15776, 64_AAF64113); *betaProteobacteria* AOB group (65_AAA66194, 66_CAA62336, 67_AAB38710, 68_AAC25057); P13.9 group (69_ABU45542, 70_AAL87436, 71_ACM46931, 72_ACM46953); *Candidatus* *Crenothrix* PmoA (73_ABC59825; 74_ABC59822; 75_ABC59827); Verrucomicrobia PmoA3 group (76_ACK55193, 77_ABX56597); Actinobacterial group (NBB4_ADT71671; NBB3_draft genome; CF8_ADW80613; Broad_EGD43187).

Cloning of hmoCAB genes into pMycoFos to make pHMO
A newly developed *Mycobacterium-E. coli* shuttle fosmid vector (pMycoFos, 12.5 kb, Km^R) was used to clone and express the *hmoCAB* genes (Figure 5). Full details of the construction and evaluation of this vector are reported elsewhere (Ly and Coleman, 2011), but in brief, this vector combines the *E. coli* replication and stabilization functions of the pCC1FOS fosmid (Epicentre Biotechnologies) (Wild *et al.*, 2002) with the *Mycobacterium* replicon and acetamidase promoter regions from the pJAM2 shuttle vector (Triccas *et al.*, 1998)—this allows control of the copy number in *E. coli* via arabinose induction, and expression of cloned genes in *Mycobacterium* via acetamide induction.

The *hmoCAB* genes of NBB4 were amplified from genomic DNA using primers NVC307 (5'-TTTATTTAAATCTGCCAGCTAACAAATCTCG-3') and NVC308 (5'-TTTATTTAAATCTATTGGGTTCCGTATTCAGG-3') containing *Swa*I sites (underlined). The resulting PCR product (3.2 kb) was digested with *Swa*I, ligated with similarly digested pMycoFos vector, and transformed into chemically competent *E. coli* EPI300 cells (Epicentre Biotechnologies) by heat shock. After recovery and plating on LB-Km agar, positive clones containing *hmoCAB* were detected by PCR using primers HEO3 (5'-CAGCCAGGATAAAAGTCTCGGGC-3') (binds in *hmoA*) and NW211 (5'-GCTCATAACACCCCTTGTATTACTG-3') (binds in Km^R gene of vector), which yields a 2.2 kb product if the insert DNA had ligated correctly into pMycoFos. One positive clone (designated pHMO) was retained for further work, and its structure confirmed by sequencing the ligation junctions, and by restriction digests using *Swa*I, *Eco*RI, *Bam*HI, *Hind*III and *Kpn*I. The pHMO plasmid was transformed into electrocompetent cells of strain mc²-155 by electroporation (Bio-Rad GenePulser, Hercules, CA, USA; 800 ohms, 2.5 µF, 2.5 kV). After recovery in 1 ml LB broth for 4 h at 30 °C, transformants were plated on LB-Km agar and incubated at 30 °C for 7 days. All subsequent activity assays were done with freshly transformed cells of mc²-155(pHMO) (that is, not from glycerol stocks).

Activity assays with resting cell suspensions

The activity of the cloned *hmoCAB* genes was quantified in resting cell suspensions using gas chromatography-flame ionization detection (alkanes,

chlorinated aliphatics), colorimetric analysis of epoxides (ethene) and/or chloride analysis (chlorinated aliphatics). Cells of mc²-155(pHMO) were grown in 30 ml minimal medium supplemented with glucose (20 mM), Tween-80 (0.05 % v/v), acetamide (0.2 % v/v) and Km (20 µg ml⁻¹) to an OD₆₀₀ of 1.0. The cells were washed twice in potassium phosphate buffer (20 mM K₂HPO₄, pH 7.0) containing Tween-80 then resuspended in 4 ml potassium phosphate buffer containing 20 mM glucose and Tween-80, and transferred to a gas-tight 16 ml serum bottle, and crimp-sealed with a Teflon-faced butyl rubber stopper, and aluminum crimp seal cap. Glucose was added to the cell suspensions as preliminary tests (not shown) indicated that higher activity could be maintained in the resting cells when they were provided with a supplemental energy source, but it should be noted that cells could not grow under the conditions of this assay because they lacked a nitrogen source and trace metals.

Test substrate (2 µmol methane, ethane, ethene, propane, butane, cis-dichloroethene or 1,2-dichloroethane) was added either as neat gas (alkanes) or from a 20 mM aqueous stock solution (chlorinated compounds) and the suspensions were incubated at 30 °C with shaking at 200 r.p.m. In the case of alkanes and chlorinated aliphatics, headspace samples (250 µl) were taken at intervals for gas chromatography–flame ionization detection analysis. For alkanes, degradation rates were calculated from the initial linear part of the substrate depletion curves, and then the rates were converted to apparent specific activities, expressed as nmol substrate consumed min⁻¹ per mg protein. For chlorinated aliphatics, rates were not measured, but instead, an end-point assay was used where the amount of substrate consumed and the amount of chloride released were calculated after overnight incubation (16 h). In the case of ethene, many identical bottles were set up and one was destroyed at each time point for 4-(4-nitrobenzyl)pyridine assay, as described above, and the activity was expressed as nmol epoxide formed min⁻¹ per mg protein.

All activity assays included controls consisting of mc²-155 cells carrying the vector only, with no CuMMO gene insert. All experiments were repeated at least three times, and the average apparent specific activity was calculated.

Results and Discussion

Detection of copper monooxygenase genes in *Mycobacterium* NBB4

Our initial interest in strain NBB4 concerned its unusual complement of SDIMOs. A fosmid library of NBB4 genomic DNA was screened for clones that contained monooxygenase genes as previously described (Coleman *et al.*, 2011). Additional sequencing of one SDIMO-containing fosmid led to the identification of a CuMMO gene cluster with

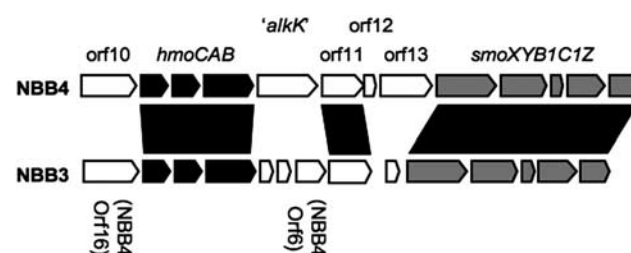


Figure 1 Schematic diagram of the genomic context of *hmoCAB* genes in strains NBB4 and NBB3. Details of NBB4 gene annotations are in Supplementary Table S1. Regions of synteny with strain NBB3 are indicated by shaded blocks. The two indicated NBB3 predicted genes show homology to NBB4 genes outside the displayed region (Supplementary data).

homology to pMMO and AMO (each predicted protein 30–40% identity to known bacterial pMMO or AMO proteins). The full sequence of this fosmid insert (35 662 bp) has been deposited in GenBank with accession number GU174751. The CuMMO genes are henceforth referred to as *hmoCAB* (hydrocarbon monooxygenase); the gene names chosen correspond to the subunits of pMMO and AMO, because the arrangement of the *hmoCAB* subunits was identical to that of *amoCAB* and *pmoCAB*. A schematic diagram of the *hmoCAB* genomic region of strain NBB4 and a similar gene cluster observed in the draft genome of the closely related alkene/alkane-degrading *Mycobacterium rhodesiae* NBB3 are shown in Figure 1. A detailed annotation of the strain NBB4 region is shown in Supplementary Table 1. Surprisingly, despite the very close phylogenetic relationship of the two *Mycobacterium* strains (NBB3 and NBB4) the level of homology between their CuMMO genes is only moderate (61%, 68%, 52% amino acid identity in HmoC, HmoA, HmoB, respectively).

Two lines of evidence led us to postulate that *hmoCAB* and related enzymes are involved in alkane metabolism. First, biochemical evidence for the presence of CuMMOs in *Actinobacteria* with a physiological role in butane degradation had previously been reported in *Nocardioides* CF8 and *Mycobacterium* JOB5 (Hamamura *et al.*, 1999, 2001). Very recently, the presence of CuMMO genes in strain CF8 was confirmed in the draft genome sequence (Sayavedra-Soto *et al.*, 2011). We also note that CuMMO homologs are present in the draft sequence of a presumed *Nocardioides* organism (Broad-1) serendipitously obtained during sequencing of the fungus *Coccidioides* (gb:EGD43186). Second, in our NBB4 fosmid sequence, genes encoding alcohol dehydrogenase and acyl-CoA ligase homologs were found in close proximity to *hmoCAB*—these potentially perform the second and third steps in an alkane assimilation pathway. A range of bioinformatic and physiological experiments were conducted to test the hypothesis that, with respect to previously known CuMMOs, the

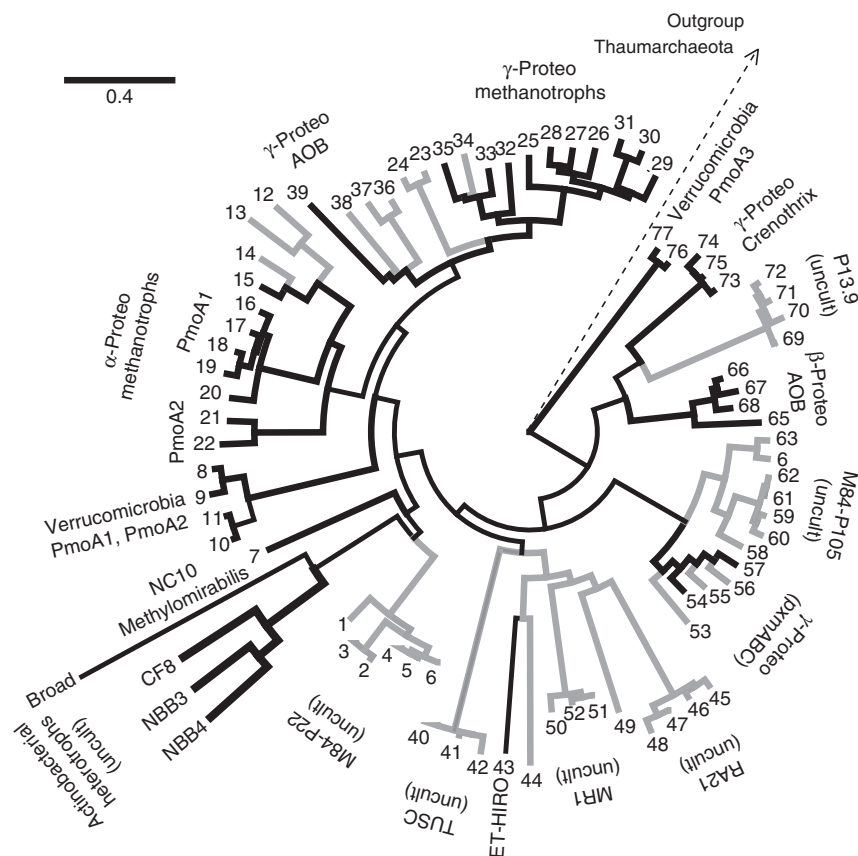


Figure 2 Relationship of the NBB4 HmoCAB enzyme to other Bacterial CuMMOs based on PmoA homologs. Tree was constructed from an ungapped alignment of 149 residues. Numbers at each tip refer to the sequence used for tree construction. Accession numbers and details of tree construction are given in the Materials and methods section. Environmental sequences are shown in grey. Branches leading to a sequence derived from an organism of known physiology and phylogenetic affiliation are shown in bold. These include: Alkane-degrading heterotrophs, Proteobacterial methanotrophs, Proteobacterial ammonia oxidizers, Verrucomicrobial methanotrophs and the candidate division NC10 anaerobic methanotroph. The disjunct distribution of sequences from the same phylogenetic groups highlights that CuMMO sequences are not all orthologous.

Actinobacterial enzymes comprise a new subgroup with a distinct physiological role.

The HmoCAB enzymes of NBB4 and NBB3 were shown to have a deep-branching position within the bacterial radiation of CuMMOs (Figure 2). Together with the CuMMO homologs of strain CF8 and Broad-1 they represent a group with a distinct evolutionary history that we refer to as the Actinobacterial copper monooxygenases. Comparative sequence analysis suggests these enzymes all share the key structural attributes of the CuMMOs. Structures of the pMMO of *Ms. trichosporium* and *Mc. capsulatus* have identified two metal centers whose ligands are strongly conserved in other CuMMOs (Figure 3). A metal center in PmoB has been shown to be a dinuclear copper site coordinated by three His residues and has been implicated in methane oxidation (Balasubramanian *et al.*, 2010). All the ligands of this copper site are conserved in HmoCAB, and the surrounding region also shows other conserved residues (Figure 3). The nature and role of the metal center in PmoA/PmoC is more controversial. The three residues identified as

metal ligands in the pMMO structures of *Mc. capsulatus* (Lieberman and Rosenzweig 2005) and *Ms. trichosporium* (Hakemian *et al.*, 2008) are all conserved in HmoCAB. However, the nature of the metal varies (zinc or copper), and it has also been postulated that in pMMOs this site could be a di-iron site with nearby carboxylate residues also involved in metal co-ordination (Semrau *et al.*, 2010). An interesting feature of the predicted HmoCAB proteins is that an aspartate residue postulated to be important in the PmoA/PmoC site is not conserved in HmoCAB, a trait it shares with the AMOs.

Growth experiments indicate a CuMMO is required for growth on small alkanes

Available sequence data indicate the strain NBB4 genome has at least nine distinct monooxygenases, all of which could conceivably encompass small alkanes within their substrate range (Coleman *et al.*, 2011). Several lines of evidence argue that the eight iron-containing enzymes are not involved in

Binuclear copper site of PmoB		PmoB	
Ligands		*	* *
AAU92191 <i>Mc. capsulatus</i>	SAHGEKSQ..PGDWHVHTMM		
AAF37894 <i>Ms. trichosporium</i>	EAHGEKSQ..AGRWHVHAQI		
AAA66195 <i>Nm. europaea</i>	AAHGERSQ..PGRHMHAML		
ADT71672 <i>Mycobacterium</i> NBB4	SAHGEQSQ..PGTWHVHPAM		
<i>Mycobacterium</i> NBB3	GAHGEEGQ..PGTYHIHPTF		
ADW80614 <i>Nocardioides</i> CF8	SAHGEESQ..PGTWHVHPGF		
EGD43186 <i>Nocardioides</i> Broad-1	SAHGETAQ..VGHWHVHPIF		
CBE69517 <i>Cand. Mm.oxifera_Twente</i>	WAHGERSQ..TGTYHVHPMV		
ACE95891 <i>Methylomonas</i> LW13 PxmB	QAHGKAL..PGHYHIHPPF		
ABY89142 <i>Np. maritimus</i>	DAHGVQAQ..AGVYHVHTQL		
ABU88156 <i>Ma. inferorum</i> PmoB1	SGIGERAQ..PGTWVQVVM		
ABU88153 <i>Ma. inferorum</i> PmoB3	QMGAKSQ..PGIFPMGPML		

Metal sites of PmoA/PmoC		PmoA		PmoC	
Ligands		#	#	#	++ #
AAU92182 <i>Mc. capsulatus</i>	GTPEYIRM			DGTWHQTVIRDTEFTPSH	
AAA87220 <i>Ms. trichosporium</i>	SMPEYIRM			DGTWHMTVIRDTEFTPSH	
AAA66194 <i>Nm. europaea</i>	GTPEYVRH			DASWHQVVIIRDTEFTPSH	
ADT71671 <i>Mycobacterium</i> NBB4	QTPEYLRI			DATWHQILTRDTAFTPSH	
<i>Mycobacterium</i> NBB3	QTPEYLRI			DAAWHQVVIRDTEFTPSH	
ADW80613 <i>Nocardioides</i> CF8	QTPEYLRI			DASWHQVTVIRDTEFTPSH	
EGD43187 <i>Nocardioides</i> Broad-1	QTPEYLRLM			DGVWHQTAVRDTEFTPSH	
CBE69519 <i>Cand. Mm.oxifera_Twente</i>	ATPEYIRI			DGTWHQTVIRDTEFTPSH	
ACE95890 <i>Methylomonas</i> LW13 PxmA	GMPEYLRI			DNSWHQVAIRDTEFTASH	
ABY89139 <i>Np. maritimus</i>	t1ppymtp			DGSLHQTSIRDTEFTPSH	
ABU88156 <i>Ma. inferorum</i> Pmo1	STPEYIRM			DGTWHQTVIRDTEFTPSH	
ABU88155 <i>Ma. inferorum</i> Pmo3	AIPEYLRLM			DGVWHSSMIRDTEFTPSH	

Figure 3 Conservation of the metal ligand-binding sites of pMMO in HmoCAB and other CuMMOs. Accession numbers for PmoB and PmoA proteins are given where available. Isologous sites to ligands of the metal centers identified in the published structures of pMMO are indicated as follows: The PmoB dinuclear copper site is shown with an asterisk (*); the PmoA/PmoC variable metal site with a hash (#) and; the carboxylate side-chain residues postulated to co-ordinate a di-iron center at the variable metal site are marked with a plus (+). Note: the *Np. maritimus* PmoA sequence is shown in lower case because the homology of this protein is not established.

initiating growth on gaseous hydrocarbons. A copper requirement is a distinctive feature of the CuMMO-dependent autotrophic ammonia oxidizers and methanotrophs (Nielsen *et al.*, 1997; Lieberman and Rosenzweig, 2005), therefore we tested the effect of copper-free media vs standard media (0.8 μM Cu^{2+}) on the growth substrate range of strain NBB4. In copper-free media, growth on gaseous alkanes (ethane, propane and butane) was strongly limited, but growth on other carbon sources, including alkenes, organic acids and glucose, was unaffected (Figure 4). This indicates a specific physiological requirement for copper for growth on alkanes as sole carbon source, and that this is likely for enzymes involved in the initial degradative steps.

ATU is a mechanistic inhibitor of CuMMO-dependent growth of methanotrophs and autotrophic ammonia oxidizers (Bedard and Knowles 1989; McCarty, 1999). When ATU was added to normal (copper-containing) media, strong growth inhibition was seen for ethane, propane and butane, but not for growth on acetate or ethene (Figure 1). This indicates it is unlikely any of the known alternate MOs in strain NBB4 are essential for initiating growth on alkanes, since there is no precedent for ATU inhibition of an iron-containing MO, or a copper requirement for iron MO-mediated

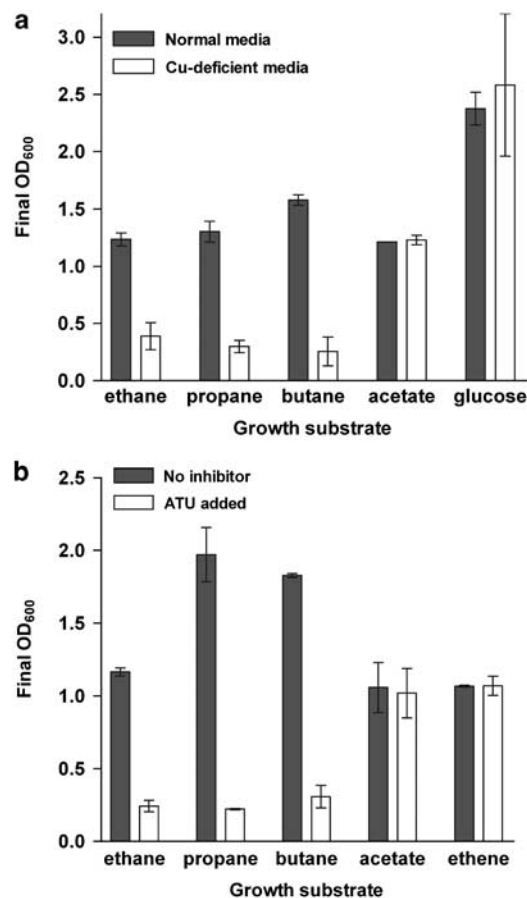


Figure 4 Copper requirement and ATU sensitivity of *Mycobacterium* strain NBB4 for growth on different substrates. (a) Final growth yield (96 h) in minimal medium with 0.8 μM Cu^{2+} or in the absence of added copper. (b) Final growth yield (96 h) in minimal medium in the presence and absence of 25 μM ATU. For both (a) and (b), data are the average of three replicate experiments, and error bars indicate 1 s.d.

growth on hydrocarbons. Experimental data showing a lack of ATU sensitivity exist for the sMMO of *Methylosinus trichosporium* and the butane monoxygenase of *Thauera butanivorans* (Nielsen *et al.*, 1997; Hamamura *et al.*, 2001), and in strain NBB4, SDIMO-mediated growth on ethene was unaffected by ATU. Further indirect support for lack of involvement of iron-MOs comes from the apparent absence of any iron MO from the proteome of NBB4 cells grown on C_2 – C_4 alkanes (Coleman *et al.*, 2011).

Heterologous expression proves HmoCAB is an alkane monoxygenase

We used a heterologous expression approach to link the *hmoCAB* genes to metabolism of particular substrates. CuMMO genes are notoriously difficult to clone in heterologous hosts (Semrau *et al.*, 1995; Balasubramanian *et al.*, 2010) and our initial attempts using standard *E. coli* vectors or *E. coli*–*Mycobacterium* shuttle vectors were unsuccessful (data not shown). To overcome these difficulties, we developed a novel fosmid-based shuttle

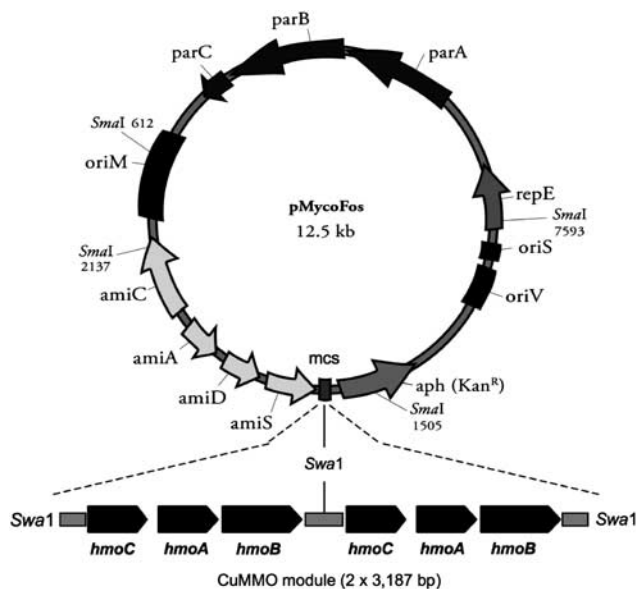


Figure 5 Structure of pHMO construct. The backbone of the construct is the pMycoFos vector, full details of which are described elsewhere (Ly and Coleman, 2011). Two copies of the *hmoCAB* gene cluster (3 187 bp) were cloned into pMycoFos to create pHMO (18 908 bp). The structure of pHMO has been validated by sequencing of the insert-vector junctions and by restriction mapping.

vector, with copy number and expression control in *E. coli* and acetamide-inducible expression of cloned genes in *Mycobacterium*—this enabled the stable cloning of the *hmoCAB* operon of strain NBB4 yielding the plasmid pHMO (Figure 5). Initial characterization of pHMO (sequencing of the junctions between insert and vector) confirmed that the orientation and boundaries of the cloned PCR product were correct. This construct was used to perform the physiological tests described below. Subsequent analysis of the plasmid structure confirmed that no errors had been introduced into the sequence of *hmoCAB* during the PCR-mediated cloning but also indicated that two complete copies of the *hmoCAB* gene clusters had been cloned, with the boundary being the *SwaI* site of the PCR primers. We have subsequently generated a single copy version and confirmed its activity (data not shown).

No alkane monooxygenase activity was seen in cells of *E. coli* EPI300(pHMO), but transfer of pHMO into *Mycobacterium smegmatis* mc²-155 yielded activity. Gas chromatography assays of substrate depletion (Figure 6) indicated that resting cell suspensions had apparent specific activities of 2.1, 2.9 and 2.2 nmol min⁻¹ per mg protein for ethane, propane and butane, respectively. These activities were 10–20-fold lower than those of wild-type NBB4 cells grown on the short chain alkanes as sole carbon source (Coleman *et al.*, 2011). No activity of mc²-155(pHMO) cells was observed toward methane, and no activity was seen in mc²-155(pMycoFos) cells lacking *hmoCAB*. This latter control is important because the *M. smegmatis* mc²-155

genome encodes a putative endogenous aromatic/alkane monooxygenase (Furuya *et al.*, 2011), however, we have not seen evidence of this activity, possibly due to a point mutation in our lineage of strain mc²-155 (unpublished data). There are only two previous reports of successful heterologous expression of CuMMO proteins, but in one case (pMMO expression in *Rhodococcus erythropolis*) the level of activity was very low (~0.2 nmol min⁻¹ per mg protein) (Gou *et al.*, 2006), and in the other case (expression of the N-terminal domain of PmoB in *E. coli*), only a soluble fragment was expressed (Balasubramanian *et al.*, 2010).

HmoCAB also oxidizes ethene to epoxyethane and dechlorinates pollutants

Cells of mc²-155(pHMO) also displayed activity with ethene and chlorinated aliphatic compounds. Production of epoxyethane from ethene was detected using a colorimetric assay (Figure 6), and an activity of 3.2 nmol min⁻¹ per mg protein was calculated based on the rate of epoxide formation, which agrees well with the rates of alkane oxidation observed above. There is an interesting parallel here with the dual-MMO methanotrophs. We have previously demonstrated that growth of NBB4 on ethene is associated with the SDIMO enzyme EtnABCD, as is the case in other ethenotrophic bacteria (Mattes *et al.*, 2010; Coleman *et al.*, 2011). The observation that HmoCAB is also capable of ethene oxidation indicates that this bacterium has distinct enzymes potentially capable of initiating growth on ethene. In dual MMO methanotrophs, cells switch between growth using an SDIMO (sMMO) and a CuMMO (pMMO) according to the availability of copper. There is no obvious effect of copper on cell growth rate on ethene as sole carbon source, but this possibility requires further investigation (for example, to test if *etnABCD* knockout mutants can still grow on ethene).

Wild-type cells of strain NBB4 attack a wide range of pollutant compounds (Le and Coleman, 2011). Recombinant HMO-expressing cells of mc²-155 also attacked the chlorinated aliphatic compounds cis-dichloroethene and 1,2-dichloroethane (Figure 6), which are common groundwater pollutants (Squillace *et al.*, 1999). We did not calculate specific activities with the chlorinated compounds (an end point assay was used), but we did measure inorganic chloride in these experiments, which revealed chloride release from both cis-dichloroethene and 1,2-dichloroethane, confirming that carbon–chlorine bonds in these molecules were cleaved. The stoichiometry of chloride release indicated that only 0.3–0.5 mol chloride per mol substrate was released, and thus that these substrates were not mineralized. It is notable that in other work with ethane-grown wild-type cells of strain NBB4 (Le and Coleman, 2011), the stoichiometry of chloride release from cis-dichloroethene and 1,2-dichloroethane was different—the

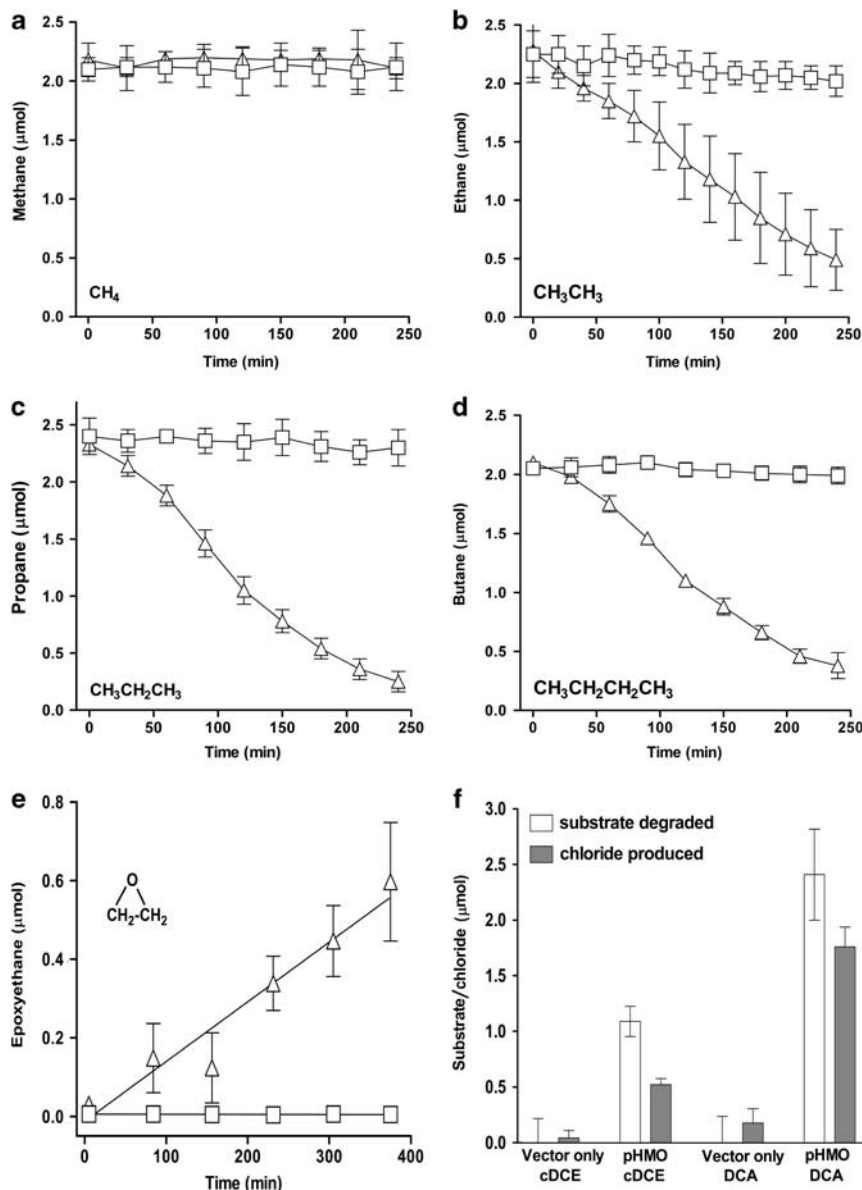


Figure 6 Activity of recombinant HMO toward alkanes, alkenes and chlorinated compounds. Panels (a–d) show substrate depletion for gaseous alkanes over time by resting cells of *M. smegmatis* mc²-155 carrying either pHMO (triangles) or the vector pMycoFos with no insert (squares). For all substrates, data are the average of three independent experiments, and error bars indicate 1 s.d. The data points between 0–180 min were used in each case for calculation of specific activities (linear regressions not shown). Panel (e) shows production of epoxyethane from ethene. Panel (f) shows metabolism of cis-dichloroethene (cDCE) and 1,2-dichloroethane (DCA). Data shown are amount of substrate depleted (white) and amount of chloride released (dark) measured at completion of the timecourse.

reasons for this are unclear, but are likely to include the involvement of additional enzymes from the MO repertoire of strain NBB4. It is evident that part of the chlorinated hydrocarbon degradative capacity of alkane-grown NBB4 cells can be attributed to HMO.

Conclusions

Multiple lines of evidence (comparative sequence analysis, ATU inhibition, copper requirement and recombinant expression) indicate that the HmoCAB enzyme of strain NBB4 is a CuMMO, with C₂–C₄ alkanes as the physiological substrates. Phylogenetic

analyses, along with available physiological data, suggest that HmoCAB of NBB4 is a member of a functionally similar group of enzymes that are present in disparate heterotrophic Actinobacteria, where they enable opportunistic utilization of alkanes. Another member of this group is also likely to be present in the propane oxidizer *Mycobacterium* JOB5 (Hamamura *et al.*, 1997, 1999; Sayavedra-Soto *et al.*, 2011). Two aspects of genome relationships in this group are especially significant; the sequence divergence between these Actinobacterial CuMMOs is large relative to the ribosomal RNA divergence of the parent organisms, and available genome sequence data indicate the genes are not universally

present among closely related bacteria. These are in strong contrast to the Proteobacterial methanotrophs and AOB where the genes encoding pMMO and AMO fit the concept of extended core genes (Lapierre and Gogarten, 2009). That is, they occur in the genomes of nearly all members of an evolutionary group (four separate groups within the Proteobacteria in this case) and are under high selective pressure (slow rates of divergence). The Actinobacterial HMO group are non-essential genes, that fit the concept of character genes (or accessory genes in other definitions). Such genes are variably present within a lineage, evolve more rapidly and are far more prone to horizontal gene transfer. Our data, therefore, demonstrate that the CuMMO paradigm needs to be modified to reflect that members of this family are not always core genes in physiologically specialized organisms.

The Actinobacterial group CuMMOs are almost certainly relevant to short chain hydrocarbon degradation and bioremediation and potentially also significant for heterotrophic nitrification (Hamamura *et al.*, 1999). Genes for the Actinobacterial CuMMO group are not successfully amplified using extant pMMO or AMO primers (Holmes *et al.*, 1995; Rothauwe *et al.*, 1997; Santoro *et al.*, 2010). This situation, where an entire functional group has been overlooked, is reminiscent of the discovery of the ammonia-oxidizing archaea (Konneke *et al.*, 2005) and subsequent demonstration that archaea (not bacteria) are the dominant nitrifiers in many environments (Leininger *et al.*, 2006; Martens-Habbena *et al.*, 2009; Zhang *et al.* 2010). A further consideration for biogeochemical studies is that ecological extrapolations from CuMMO sequences depend on congruence between CuMMO sequence relationships, ribosomal RNA phylogeny and organism physiology (Singh *et al.*, 2010). This congruence is strong for the characterized methanotrophs and ammonia-oxidizers where CuMMOs are core genes, but unlikely to hold true for C₂–C₄ alkane-degraders, because of the diverse MO types that can act on C₂–C₄ alkanes, and the fact that distribution of the CuMMOs in the Actinobacteria lineage appears to be sporadic.

The recombinant expression system we have developed for the HmoCAB holoenzyme opens up new approaches for CuMMO biochemistry (for example, site-directed mutagenesis), and we anticipate it will help resolve questions regarding the catalytic roles of the different protein subunits. Of particular relevance here is that the nature of the metal-containing sites in CuMMOs remains controversial. The PmoA/AmoA subunit was long thought to be the site of catalysis since acetylene, a suicide substrate, binds to this protein (Zahn and DiSpirito, 1996; Gilch *et al.*, 2009). However, when structures of pMMO were solved, two metal sites were revealed (Lieberman and Rosenzweig, 2005; Hakemian and Rosenzweig, 2007) and recently the PmoB site was confirmed as having methane

hydroxylase activity when the PmoB subunit of the *M. capsulatus* pMMO was expressed in soluble form in *E. coli* (Balasubramanian *et al.*, 2010). However, a paradox remains since the ligands for the PmoB site are not conserved across all methanotrophs (Dunfield *et al.*, 2007; Pol *et al.*, 2007), and this experiment does not explain the canonical membrane association of CuMMOs. The availability of a recombinant expression system for the holoenzyme creates new opportunities to probe the nature of the metal centers in this enzyme family. We anticipate it will also be useful for characterizing different CuMMO alleles in organisms containing heterologous copies (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Baani and Liesack, 2008), and for determining the function of environmental CuMMO gene clusters (Dumont *et al.*, 2006).

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