environmental microbiology reports

Environmental Microbiology Reports (2011) **3**(1), 91–100 doi:10.1111/j.1758-2229.2010.00192.x

A novel family of functional operons encoding methane/ammonia monooxygenase-related proteins in gammaproteobacterial methanotrophs

Patricia L. Tavormina,[1]* Victoria J. Orphan,[1] Marina G. Kalyuzhnaya,[2] Mike S. M. Jetten[3] and Martin G. Klotz[4]**

[1]*Division of Geological and Planetary Sciences, California Institute of Technology, 1200 E. California Blvd, Pasadena, CA 91125, USA.*
[2]*Department of Microbiology, University of Washington, Box 355014, Seattle, WA 98115, USA.*
[3]*Department of Microbiology, Radboud University Nijmegen, 6525 AJ Nijmegen, The Netherlands.*
[4]*Department of Biology, University of Louisville, 139 Life Sciences Building, Louisville, KY 40218, USA.*

Summary

Genomes of alphaproteobacterial and verrucomicrobial methane-oxidizing bacteria (MOB) encode sequence-divergent copies of particulate methane monooxygenase [pMMO = (PmoABC); *pmoCAB*]. In contrast, sequenced gammaproteobacterial MOB (Gamma-MOB) genomes contain single or multiple near-identical copies of *pmoCAB* operons. In betaproteobacterial ammonia-oxidizing bacteria (Beta-AOB), near-identical *amoCAB* operons encode ammonia monooxygenase (AMO), a homologue of pMMO. Here, we report that Gamma-MOB in the genera *Methylomonas, Methylobacter* and *Methylomicrobium* also encode a sequence-divergent particulate monooxygenase (pXMO). Whereas all known genes encoding pMMO or AMO cluster in the order 'CAB', the genes encoding pXMO are uniquely organized in the non-canonical form *'pxmABC.'* Steady state *pxm* mRNA was detected in cultures of *Methylomonas* sp. as well as in freshwater creek sediment samples, demonstrating that *pxm* genes are expressed in culture and *in situ*. Inclusion of PxmA and PxmB proteins in phylogenetic analyses of the Pmo/Amo protein superfamilies created trifurcated trees with three major clades: (i) Pmo of Alpha- and Gamma-MOB and Amo of Gamma-AOB; (ii) Amo of Beta-AOB, Pmo of putative ethane-oxidizing Gamma-MOB and Pxm of Gamma-MOB; and (iii) verrucomicrobial Pmo and Amo of ammonia-oxidizing *Archaea*. These data support but do not prove the hypothesis that oxygen-dependent methane and ammonia monooxygenases evolved from a substrate-promiscuous ancestor after horizontal transfer while being integrated into the catabolic contexts of their extant hosts.

Received 20 February, 2010; accepted 1 June, 2010. For correspondence. *E-mail pattytav@gps.caltech.edu; **E-mail martin.klotz@louisville.edu; Tel. (+1) 502 852 7779; Fax (+1) 502 852 0725.

© 2010 Society for Applied Microbiology and Blackwell Publishing Ltd

Introduction

Copper-containing membrane-bound monooxygenases (CuMMOs) comprise a diverse family of enzymes that facilitate the oxidation of methane (catalysed by particulate methane monooxygenase, pMMO), ammonia (ammonia monooxygenase, AMO) and potentially additional substrates including ethane (Nakamura *et al.* BAH22833, BAH22839). Additionally, non-specific substrate catabolism (with relatively high K_m values) has been observed in some members of this family, suggesting the possibility of substrate promiscuity. CuMMOs are heterotrimeric enzymes that occur in *Alpha-*, *Beta-* and *Gammaproteobacteria*, *Verrucomicrobia* and *Crenarchaea*. Genes encoding CuMMOs in bacteria are canonically organized in gene clusters with the gene order C-A-B; however, the *amoA*, *amoB* and *amoC* genes are not clustered in all archaea and do not follow the C-A-B order (Könneke *et al.*, 2005; Nicol and Schleper, 2006; Walker *et al.*, 2010 and references therein).

The ability to metabolize methane aerobically as a carbon and energy source has been identified in a variety of bacterial taxa, including members of the *Gammaproteobacteria* (type I methanotrophs and *Crenothrix*), *Alphaproteobacteria* (type II methanotrophs) and *Verrucomicrobiales* (Hanson and Hanson, 1996; Stoecker *et al.*, 2006; Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008; Op den Camp *et al.*, 2009). Most aerobic methane-oxidizing bacteria (MOB) encode pMMO (*pmoCAB*), a member of the CuMMO family (Balasubramanian and Rosenzweig, 2007) that facilitates the first step in aerobic methane oxidation (reviewed in Hanson and Hanson, 1996; Trotsenko and Murrell, 2008). Analysis of genome sequences from proteobacterial and

verrucomicrobial MOB has revealed that *pmo* operons are often present in multiple copies within individual genomes (Stolyar *et al*., 2001; Dunfield *et al*., 2002; 2007; Ricke *et al*., 2004; Ward *et al*., 2004; Op den Camp *et al*., 2009). These multiple copies occur as either near sequence-identical copies that are thought to have arisen through gene duplication events, or as divergent copies whose evolutionary origins are speculative. In the case of substantially sequence-divergent copies, the potential for alternative physiological function under different environmental conditions exists (Yimga *et al*., 2003; Dunfield *et al*., 2007). For example, in *Methylocystis* strain SC2, the pMMO encoded by *pmoCAB2* oxidizes methane at a lower apparent K_m than that encoded by *pmoCAB1*, potentially influencing the distribution of *Methylocystis* in the environment (Baani and Liesack, 2008). pMMO is a homologue of ammonia monooxygenase (AMO, *amo*) (Klotz and Norton, 1998; Norton *et al*., 2002), another CuMMO that oxidizes ammonia to hydroxylamine in obligate aerobic ammonia-oxidizing bacteria (AOB) (Arp *et al*., 2002). The sequences of multiple *amo* operon copies in individual betaproteobacterial AOB genomes are nearly identical (Arp *et al*., 2007; Stein *et al*., 2007; Norton *et al*., 2008). In all bacterial operons encoding CuMMOs, the genes are organized in the canonical form *CAB*. Additionally, all *amo* and *pmo* homologues have diversified holophyletically in individual strains (where they evolved as orthologues under biased AT/GC pressure) rather than by horizontal transfer (Klotz and Norton, 1998).

Here, we report the identification of a gene cluster (*pxm*) in select Gamma-MOB that encodes the three subunit proteins typical of CuMMOs but whose substrate specificity is still elusive (pXMO). The *pxm* operon sequences are substantially divergent from all previously characterized gammaproteobacterial *pmo* and *amo* genes. In contrast to all other *pmoCAB* and *amoCAB* operons, *pxm* genes from *Methylomonas* sp. strains LW13 and S1 are uniquely organized as an operon in the non-canonical form *pxmABC*. Messenger RNA of *pxmA* was detected in both pure cultures and in freshwater creek sediments, indicating that expression of the *pxm* genes is of functional environmental relevance. Our analysis also revealed the environmental distribution of a clade of putatively ethane-oxidizing *Gammaproteobacteria*.

Results and discussion

A primer pair previously developed for use in marine settings [*pmoA*189f/*novelmoA*634r (Tavormina *et al*., 2008)] was found to amplify a ~500-bp product from *Methylomonas* sp. strain LW13. Sequencing this product revealed characteristics of a *pmoA*-like gene significantly divergent from *Methylomonas* sp. strain LW13 *pmoA* reported earlier (AF150793). Using a combination of inverse and nested PCRs with targeted degenerate primers (Table S1), about 4 kb of contiguous nucleic acid sequence from *Methylomonas* sp. strain LW13 was recovered, including a putative promoter and three open reading frames (ORFs) termed *pxmA, pxmB* and *pxmC* (Fig. S1A). The gene designation *pxm* was chosen because the gammaproteobacterial PxmA and PxmB proteins grouped phylogenetically in a clade together with Amo proteins from Beta-AOB, 'unusual' (Stoecker *et al*., 2006) Pmo proteins from a *Crenothrix* enrichment and a cohort of putative ethane-oxidizing MOB. This *pxm*-containing clade grouped separately from all other PmoA and PmoB proteins of identified obligate aerobic methanotrophs in the respective trees of the subunit A and B protein superfamilies (Fig. 1). Both clades including known functional proteobacterial Pmo and Amo proteins are separated from the Pmo proteins of *Verrucomicrobia* and the Amo proteins of the *Crenarchaea* by a single trifurcation point.

A primer pair targeting the *Methylomonas* sp. strain LW13 *pxm* gene cluster successfully amplified a 3.5-kb fragment which contained a near-full-length operon of three genes *pxmA*, *pxmB* and *pxmC* (Table S1; EU722432). Most strikingly, the organization of *pxm* genes in *Methylomonas* sp. strain LW13 was in the non-canonical order 'ABC' and thus different from all previously identified *pmoCAB* gene clusters (e.g. AB253367, AJ584611, L40804).

To characterize the distribution of this novel *pxm* operon among the Gamma-MOB, we screened 6 additional cultured Gamma-MOB and two Alpha-MOB, as well as two genome-sequenced AOB as controls, employing primer set *pmoA189f* and *pmoA*634r (Table 1, Table S1). Four Gamma-MOB representing three different genera (*Methylomonas methanica* strain S1*, Methylomonas* sp. strain LW13*, Methylomicrobium album* strain BG8*,* and *Methylobacter marinus* strain A45) contained the *pxmA* gene (Table 1); a near-full-length *pxmABC* operon sequence (3.5 kb) was also recovered from *Methylomonas* sp. strain S1 (Accession No. EU722433).

The protein sequences deduced from *pxmA, pxmB* and *pxmC* were aligned using ClustalX version 1.83 (Thompson *et al*., 1997) with respective AMO and pMMO subunit protein sequences from Alpha- and Gamma-MOB as well as Gamma- and Beta-AOB [retrieved from public databases and embargo genome projects using the BLAST algorithm (Altschul *et al*., 1997)]. Based on these alignments, distance neighbour-joining trees were constructed with the BioNJ function in PAUP* version 4.10b (Swofford, 1999), which were used as guide trees for manual refinement of the ClustalX alignments. While the predicted PxmA, PxmB and PxmC proteins were ~53% identical and

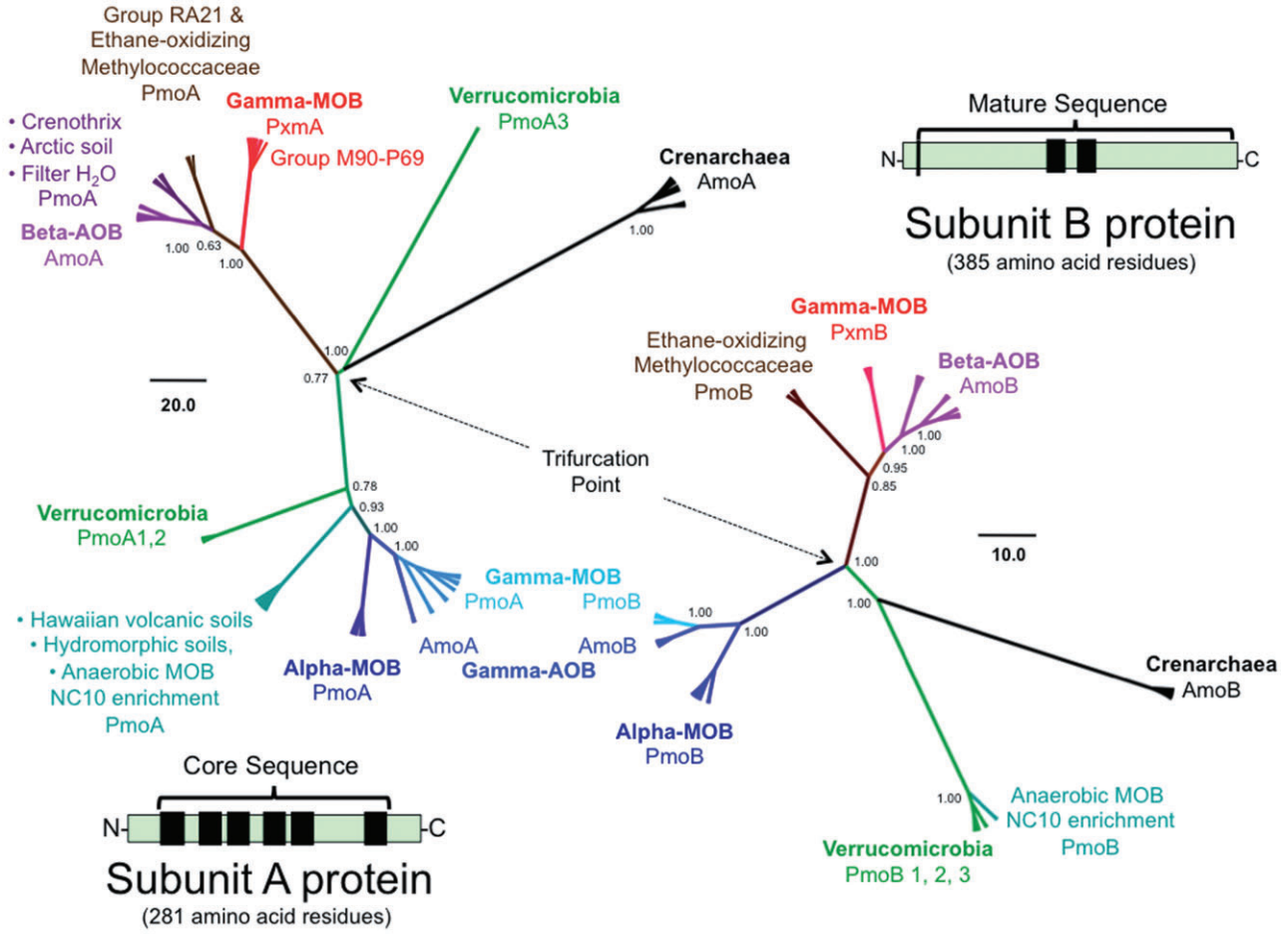

Fig. 1. Unrooted phylogenetic consensus trees constructed after Bayesian inference of phylogeny from the ClustalX alignments of 127 PxmA, PmoA and AmoA and 51 PxmB, PmoB and AmoB protein sequences. As indicated by the insets, the N- and C-terminal sequences outside the first and sixths transmembrane-spanning domains of the A proteins (leaving the 'core sequence') and the N-terminal signal sequences of the B proteins were eliminated from the final alignments that were subjected to a Bayesian inference of phylogeny using the BEAST package [BEAUti v1.5.3, BEAST v1.5.3, TreeAnnotator v1.5.3, FigTree v.1.3 (Drummond and Rambaut, 2007)]. By utilizing unique sites, tree likelihoods (ignoring ambiguities) were determined for each alignment by creating a Monte-Carlo Markov Chain (10 000 000 generations) in three independent runs. The searches were conducted assuming an equal or a gamma distribution of rates across sites, sampling every 1000th generation and using the WAG empirical amino acid substitution model (Whelan and Goldman, 2001). Unrooted 50% majority rule consensus phylograms were displayed as star trees, for which posterior probability values of the main nodes are shown. The mean branch lengths are characterized by a scale bar indicating the evolutionary distance (changes per amino acid position). The clades are annotated with terms indicating cohorts of source organisms. Protein sequence accession numbers for all protein sequences in the alignments are displayed together with information of the source organism in the detailed Fig. S1A and B.

~73% similar in sequence to corresponding pMMO and AMO subunit proteins, more than 90% of invariant amino acid residues in pMMO/AMO alignments as well as all predicted membrane-spanning segments were conserved in *pxm* sequences, suggesting membrane localization (data not shown). Alignments of PxmA, PmoA and AmoA protein core sequences and of PxmB, PmoB and AmoB mature protein sequences were used for the inference of phylogeny employing distance- and character-based tree-searching methods. Representative phylogenetic trees were built from the refined ClustalX alignments of 139 PxmA, PmoA and AmoA and 51 PxmB, PmoB and AmoB protein sequences, and subjected to a Bayesian inference of phylogeny (Fig. 1 and Fig. S1). The phylogenetic trees reveal that the PxmA and PxmB proteins form monophyletic clades in the AmoA/PmoA and PmoB/AmoB protein superfamilies respectively. The Pxm proteins did not group in a clade with Pmo proteins from aerobic obligate alpha- and gammaproteobacterial methanotrophs or AmoA proteins from Gamma-AOB; instead, the Pxm proteins grouped in a clade with Amo proteins of Beta-AOB (Fig. 1 and Fig. S1). Inclusion of the Pxm proteins in the analyses created a trifurcation point in the trees of the Amo/Pmo subunit protein superfamilies thereby creating three major subclades; For the subunit A protein: (i) PmoA from Alpha- and Gamma-MOB, AmoA from Gamma-AOB,

Table 1. Strains used in this study.

Strain	Phylogenetic classification	Function	*pxmA*[a]	*pxmABC*[b]
Methylomonas sp. strain LW13	*Gammaproteobacteria*	MOB	+	+
Methylomonas methanica strain S1	*Gammaproteobacteria*	MOB	+	+
Methylomicrobium album strain BG8	*Gammaproteobacteria*	MOB	+	–
Methylomicrobium kenyense	*Gammaproteobacteria*	MOB	–	–
Methylomicrobium alcaliphilum strain 2OZ[c]	*Gammaproteobacteria*	MOB	–	–
Methylobacter marinus strain A45	*Gammaproteobacteria*	MOB	+	–
Methylosarcina lacus strain LW14	*Gammaproteobacteria*	MOB	–	–
Methylocystis parvus strain OBBP	*Alphaproteobacteria*	MOB	–	–
Methylosinus trichosporium strain OB3b[c]	*Alphaproteobacteria*	MOB	–	–
Nitrosomonas europeaea strain ATCC 19718[c]	*Betaproteobacteria*	AOB	–	–
Nitrosococcus oceani strain ATCC 19707[c]	*Gammaproteobacteria*	AOB	–	–

a. Detection of *pxmA* using primers *pmoA*189f and *pxm*A634r; distribution corroborated with primers *pxmA*230f and *pxmA*732r.
b. Detection of near-full-length *pxm* operon using LW13-targeted primers LTTR842r and LW13*pxmC*420r.
c. Genome sequence verifies the absence of *pxm* genes in these strains.

PmoA1 and 2 from *Verrucomicrobia*, and the PmoA from anerobic MOB, *Methylomirabilis oxyfera*, in the NC10 phylotype (Ettwig *et al*., 2010); (ii) PxmA of Gamma-MOB [including clade M90-P69 (Stoecker *et al*., 2006)], the AmoA of Beta-AOB, the unusual PmoA from *Crenothrix*, and the PmoA from ethane-oxidizing *Methylococcaceae* [including clade RA21 (Stoecker *et al*., 2006)]; and (iii) the verrucomicrobial PmoA3 and the AmoA from ammonia-oxidizing *Crenarchaea*. Interestingly, in the tree showing the phylogenetic relationships of the B subunit proteins, all three verrucomicrobial B subunit proteins and the PmoB from *Methylomirabilis oxyfera* in the NC10 phylotype group in the clade with crenarchaeal AmoB proteins (Fig 1 and Fig. S1). This difference between the two subunit protein trees may be a reflection of different functional pressures on subunit protein evolution or the result of an analysis with unevenly populated clades or both, which can be tested once more B subunit protein sequences become available.

To date, only near sequence-identical operon copies have been described in gammaproteobacterial MOB. Although the genome of the Gamma-MOB *Methylococcus capsulatus* strain Bath encodes a sequence-divergent (94% similarity to *pmoC*1 and 2) copy of an essential *pmoC*-like gene singleton in addition to two near-sequence-identical *pmoCAB* operons (Stolyar *et al*., 2001), the genome of the gamma-MOB *Methylomicrobium alcaliphilum* strain 20Z, for instance, contains only one *pmoCAB* gene cluster (M.G. Kalyuzhnaya, unpubl. results). Genomes of sequenced Gamma-AOB also contain only one *amoCAB* gene cluster and no additional non-operonal *amoC* genes (Alzerreca *et al*., 1999; Klotz *et al*., 2006; Arp *et al*., 2007; Genbank CP001798). Similarly, near sequence-identical operon copies, as well as singleton copies of *amoC*, have been found in Beta-AOB (Arp *et al*., 2007; Norton *et al*., 2008). In contrast, significantly sequence-divergent copies of *pmo* operons have been reported for individual genomes of alphaproteobacterial and verrucomicrobial MOB (Dunfield *et al*., 2002; Ricke *et al*., 2004; Op den Camp *et al*., 2009). Even though the deduced protein sequences of sequence-divergent *pmoA* genes in Alpha-MOB group within different sub-clades, all alphaproteobacterial PmoA proteins form a single clade in the phylogenetic tree (Fig. 1 and Fig. S1). Importantly, all reported *amo* and *pmo* operons from bacterial AOB and MOB have a prototypical 'C-A-B' gene order (Arp *et al*., 2007; Op den Camp *et al*., 2009). In contrast, the order of genes in the *pxm* operon is 'A-B-C.' This novelty suggests either a different origin for the additional gene clusters relative to prototypical bacterial *amo* and *pmo* operons, or markedly different selective pressures such as resistance to or absence of the rectification mechanisms that have been implicated in maintaining identical mutational bias and gene order in AOB and MOB (Klotz and Norton, 1998), or both. Interestingly, *amo* genes in the *Crenarchaea* are also not in the canonical order 'C-A-B' (Könneke *et al*., 2005; Hallam *et al*., 2006; Leininger *et al*., 2006; Nicol and Schleper, 2006; Prosser and Nicol, 2008; Walker *et al*., 2010). Because *pxm* and crenarchaeal *amo* genes are only distantly related (Fig. 1 and Fig. S1), reconstruction of the evolutionary scenario will need more genome sequence information from archaeal ammonia oxidizers.

Inclusion of the *pxmA* sequences in AT/GC bias analysis suggests that *pxmA* genes, like all other *amoA* and *pmoA* genes (Klotz and Norton, 1998), evolved holophyletically in individual strains under similar biased AT/GC pressure rather than by frequent and recent horizontal transfer (Fig. 2). This leaves the possibility of a different mechanism of operon formation as compared to the formation of prototypical *amo* and *pmo* operons, a possibility which can be investigated in more detail by comparative sequence analysis of genomes from Gamma-MOB with and without *pxm* genes. Comparison of the *Methylococcus capsulatus* strain Bath and *Methylomicrobium alcaliphilum* 20Z genomes [lacking *pxm* (Ward *et al*., 2004); M.G. Kalyuzhnaya, unpublished) with *pxm*-

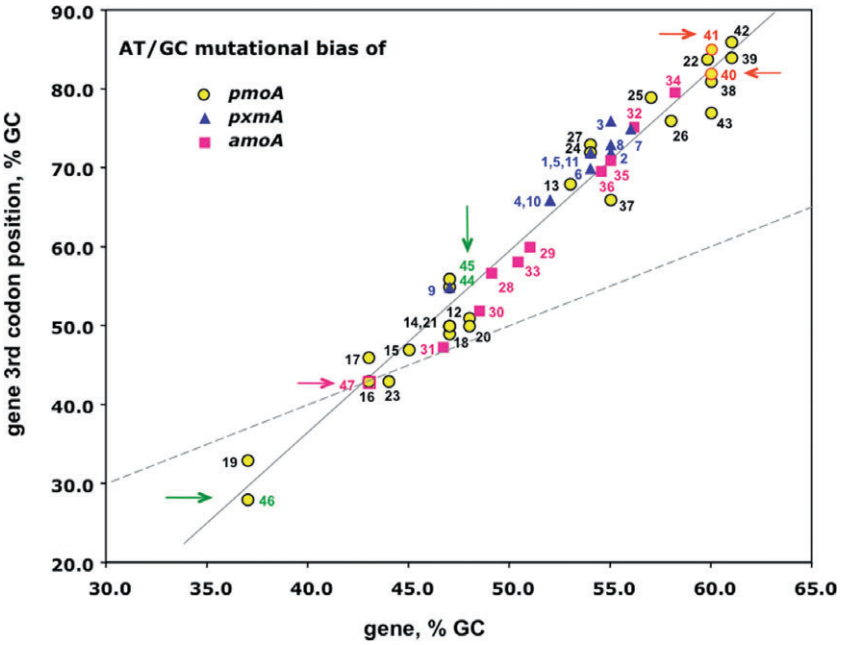

Fig. 2. AT/GC mutational bias analysis using the correlation of GC content of the complete *amo*, *pmo* or *pxm* genes and the third codon positions of these genes. The symbol for each individual gene copy is numbered. Circles, triangles and squares represent *pmoA*, *pxmA* and *amoA* genes respectively. The straight full line was determined by linear regression (slope: 2.3); the stippled line indicates the theoretical position for the case that gene and third codon GC contents were identical. The green, red and blue arrows highlight data points 40, 41 and 44 to 47 and are discussed in the text. The numbers refer to the following genes and organisms ('CC' stands for 'Conejo Clone'): 1 – *Methylomonas* sp. strain LW13 *pxmA*; 2 – *Methylomonas methanica* strain S1 *pxmA*; 3 – CC_A12 *pxmA*; 4 – CC_B2 *pxmA*; 5 – CC_B10 *pxmA*; 6 – CC_C4 *pxmA*; 7 – CC_E12 *pxmA*; 8 – CC_F1 *pxmA*; 9 – CC_G9 *pxmA*; 10 – CC_H2 *pxmA*; 11 – CC_H8 *pxmA*; 12 – *Methylomonas* sp. strain LW13 *pmoA*; 13 – *Methylomonas methanica* strain S1 *pmoA*; 14 – CC_A6 *pmoA*; 15 – CC_B1 *pmoA*; 16 – CC_B5 *pmoA*; 17 – CC_B10 *pmoA*; 18 – CC_F8 *pmoA*; 19 – *Methylococcaceae* bacterium isolate SF-BR *pmoA*; 20 – *Methylococcaceae* bacterium isolate T2 –1 *pmoA*; 21 – *Methylococcaceae* bacterium isolate IT-4 *pmoA*; 22 –*Methylococcus capsulatus* strain Bath *pmoA*; 23 – *Methylomicrobium japanense pmoA*; 24 – *Methylocaldum* sp. isolate T-025 *pmoA*; 25 – *Methylohalobius* sp. isolate IT-9 *pmoA*; 26 – *Methylomicrobium album* strain BG8 *pmoA*; 27 – *Methylomicrobium* sp. strain A45 *pmoA*; 28 – *Nitrosococcus oceani* strain ATCC 19707 *amoA*; 29 – *Nitrosococcus halophilus* strain Nc4 *amoA*; 30 – *Nitrosomonas europaea* strain ATCC 19718 *amoA*; 31 – *Nitrosmonas eutropha* strain C91 *amoA*; 32 – *Nitrosovibrio tenuis* strain Nv-12 *amoA*; 33 – *Nitrosospira multiformis* strain ATCC 25196 *amoA*; 34 – *Nitrosospira briensis* strain C-128 *amoA*; 35 – *Nitrosospira* sp. strain N39 *amoA*; 36 – *Nitrosospira* sp. strain NpAV *amoA*; 37 – *Crenothrix polyspora* strain 13 *pmoA*; 38 – *Methylocystis* sp. strain GSC357 *pmoA*; 39 – *Methylocystis* sp. strain M *pmoA*; 40 – *Methylocystis* sp. strain SC2 *pmoA1*; 41 – *Methylocystis* sp. strain SC2 *pmoA2*; 42 *Methylosinus trichosporium* strain OB3b *pmoA*; 43 – *Methylocapsa acidiphila* strain B2 *pmoA*; 44 – *Methylacidiphilum infernorum* strain V4 *pmoA1*; 45 – *M. infernorum pmoA2*; 46 – *M. infernorum pmoA3*; 47 – *Nitrosopumilus maritimus* strain SMC1 *amoA*.

containing genomes such as *Methylomonas* sp. strain LW13 and *M. album* strain BG8 will be possible shortly (whole genome sequencing projects for these MOB are presently in progress).

Secondary structure analysis of the putative initial transcribed region of *pxm* from *Methylomonas* sp. strain LW13 using the RNAfold webserver (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) predicted two alternative stem loop configurations (Fig. S2; DG = –25.2 and –44.4). The longer putative stem loop is predicted to co-opt a ribosomal binding site and is followed by a U-rich region, suggesting a role of the longer hairpin in transcription termination. In analogy to an attenuation scenario, a faster formation and stabilization of the shorter hairpin could preclude formation of the terminator structure and allow transcription of the *pxm* gene cluster (Fig. S2). In context with the novel organization of the *pxm* genes (Fig. S2), these genetic features indicate that the *pxm* genes maybe regulated differently compared with transcription of the *pmo* and *amo* gene clusters, which constitute several overlapping transcriptional units that are differentially regulated (Nielsen *et al*., 1997; El Sheikh and Klotz, 2008; El Sheikh *et al*., 2008).

The finding that sequence-divergent homologues of *pmo* operons co-exist in all classes (but not all strains) of known bacterial methanotrophs may suggest an ecophysiological significance of this inventory for niche differentiation. Because these *pxm* operons have been retained in several Gamma-MOB, it is conceivable that their expression products impact methanotroph fitness by conferring broader physiological capabilities including the utilization of other substrates or detoxification, thereby potentially influencing the distribution of these organisms *in situ*. Primers designed to survey this genetic unit *in situ*

(*pxmA*230f/*pxmA*732r, Table S1) amplified products from two freshwater environmental samples (peat bog and creek sediment), but not from marine or compost samples (not shown). Using reagents and methods described previously (Tavormina *et al*., 2008), clone libraries were constructed for *pmoA* and *pxmA* products amplified from one of these freshwater environmental samples (Conejo Creek sediment, 0–3 cm depth; longitude – 118.87N, latitude 34.216W; Ventura County, CA). Both *pmoA* and *pxmA* libraries contained sequences related to *pmoA* and *pxmA* sequences from *Methylomonas* sp. strain LW13 (Fig. S1), verifying environmental distribution of both gene clusters.

Preservation of conserved residues and structural motifs in the deduced Pxm protein sequences as well as the conserved organization of *pxm* genes are likely the result of functional pressure and suggest that pXMO is functional. To test this hypothesis at the level of gene expression, transcription of both *pxmA* and *pmoA* was assessed in *Methylomonas* sp. strains LW13 and S1 cultures, as well as in an environmental sample from Conejo Creek sediment (Fig. 3). Primers specific to *Methylomonas pxmA* and *pmoA* were developed for use in real-time fluorescent PCR (qPCR) (Table S1) and determined via standard curve analysis over 6 orders of magnitude to have priming efficiencies > 95%. Melt profiles for *pmoA* and *pxmA* amplicons (80.7°C and 83.7°C respectively) revealed well-defined melting temperatures that were discriminatory for each target, indicating that the amplifications generated single products with no significant cross-recognition between targeted sites. Serial dilutions of cDNA were subjected to qPCR to determine relative steady-state transcript levels of *pxmA* and *pmoA* as indicated by cycle threshold (Ct). Cycle threshold was undetermined (> 40 cycles) in control samples (see for example 'no RT' in Fig. S3), indicating that genomic DNA contamination was not detected in DNase-treated RNA preparations.

For *Methylomonas* sp. strains LW13 and S1 grown under standard laboratory conditions (NMS + 50% methane headspace), threshold for amplification from *pxmA* in cDNA samples was 28.50 cycles ($n = 4$, SD = 1.23) demonstrating the presence of steady state *pxmA* mRNA in cultured cells (not shown). Cycle threshold for amplification of *pxmA* cDNA from total community RNA isolated from creek sediment was 36.61 ($n = 3$, SD = 0.87), indicating that *pxmA* was transcribed in environmental samples (see for example 'RT' in Fig. S3). Steady state *pxmA* mRNA was detected albeit at levels approximately 2–3 orders of magnitude below those of *pmoA* transcript levels under all conditions tested (Fig. 3).

While detection of *pxmA* transcripts indicated gene expression under all conditions analysed, a function for pXMO remains to be determined. It is possible that the

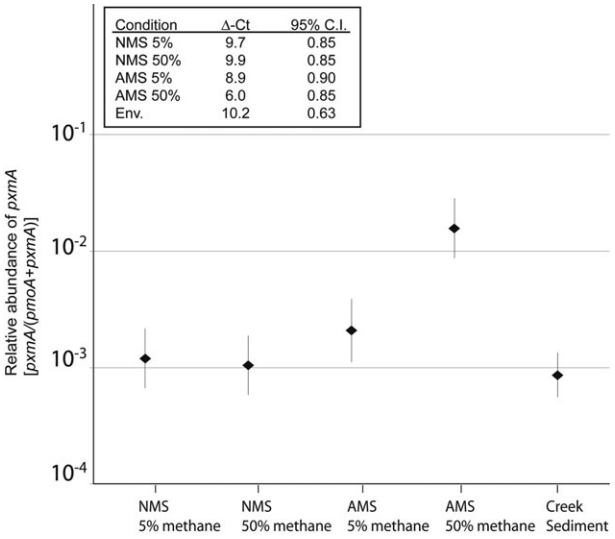

Fig. 3. Relative abundance of *pxmA* compared with *pmoA* in *Methylomonas* sp. strain LW13. Relative abundance was calculated as the difference in the number of qPCR cycles needed to reach threshold (Ct) for *pxmA* and *pmoA*. Based on three biological replicate cultures grown on NMS media (5% and 50% methane headspace each), detection of *pxmA* transcripts required 9–10 additional cycles compared with *pmoA* transcripts. When grown on AMS medium (50% methane headspace), detection of *pxmA* required 6 additional cycles. This corresponds to an approximate 10-fold relative increase in abundance of *pxmA* compared with *pmoA* steady state mRNA when compared with growth on NMS medium. The non-significant difference in *pxmA* vs. *pmoA* abundance when comparing NMS-grown cultures with AMS-grown culture with only 5% methane in the headspace is likely due to competitive inhibition of pMMO by ammonia, which leads to starvation and thus less overall transcriptional activity. Similar trends were observed for *Methylomonas methanica* strain S1 (not shown). In an environmental sample demonstrated to harbour *Methylomonas* strains, detection of *pxmA* transcripts required 10 additional cycles compared with *pmoA* transcripts, similar to the difference seen under routine laboratory growth conditions for *Methylomonas* strains.

Duplicate *Methylomonas* cultures were grown at 28°C with 5% or 50% methane headspace, on nitrate mineral salts (NMS) or ammonium mineral salts (AMS) medium (Whittenbury *et al*., 1970). Cells in exponential phase (OD 0.6–0.7) were harvested by addition of stop solution (95% ethanol/5% phenol; 1/10 volume) followed by centrifugation (4500 *g* for 15′ at 4°C). The cell pellet was resuspended in approximately 1 ml of RNA*later* (Applied Biosystems/Ambion, Austin, TX) and stored at −80°C. Separately, ~5 g of freshwater creek sediment (Conejo Creek, 0–3 cm) was sampled into ~20 ml RNA*later* and stored at −80°C. To extract total RNA, sediment was pelleted and incubated with 5 ml lysozyme solution (1 mg ml^{-1} in Tris-EDTA pH 8.0) for 10′. Solution RLT [7.5 ml, RNeasy kit (Qiagen, Valencia, CA)] was added to lyse cells and stabilize RNA; solids were precipitated by centrifugation (4500 *g* for 15′ at 4°C). The cell lysate was layered onto a 4.7 M CsCl (pH 5.0) cushion and spun overnight (35 000 r.p.m., 16 h, 20°C). The supernatant was decanted and the nucleic acid pellet resuspended in 100 μl TE buffer (Tris-HCl/EDTA, pH 8.0). Total RNA from this suspension as well as from cultured cell pellets was isolated using the RNeasy kit with an additional DNase treatment to remove contaminating genomic DNA (RNase-free DNase set; Qiagen, Valencia, CA, USA), and 0.4 μg of purified RNA were incubated without ('no RT') or with ('RT') reverse transcriptase to generate cDNA using the High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA).

primary substrate of pXMO is a compound other than methane or ammonia. For example, it has been shown that growth of *M. album* strain BG8 on methanol is enhanced by chloromethane (Han and Semrau, 2000). A role of pXMO in ammonia oxidation is a possibility. As seen in the case of *Crenothrix*, a rather 'unusual' Gamma-MOB (Stoecker *et al*., 2006), Pxm proteins group phylogenetically with proteins not linked to methanotrophy but implicated in ammonia and ethane oxidation (Fig. 1). Since gammaproteobacterial Amo proteins are for instance more closely related to gammaproteobacterial Pmo proteins than to betaproteobacterial Amo proteins (Norton *et al*., 2002), albeit nearly equally substrate-specific for methane and ammonia (Lontoh *et al*., 2000), which correlates with their fairly low affinity for ammonia (Martens-Habbena *et al*., 2009), the reasons for why one CuMMO oxidizes ammonia and another one oxidizes methane is likely a mixture of catalytic properties (i.e. as determined by specific amino acid residues; Norton *et al*., 2002; Casciotti *et al*., 2003; Stoecker *et al*., 2006; Ettwig *et al*., 2010) and the metabolic context. The Gamma-AOB *Nitrosococcus oceani*, for instance, can oxidize methane but lacks an efficient formaldehyde utilization and detoxification system in order to make a living on methane oxidation (Klotz *et al*., 2006). Hence its physiological substrate is ammonia and not methane. Likewise, co-oxidation of ammonia by MOB does not contribute to reduction of the quinone pool and does not support their growth (DiSpirito *et al*., 2005; Trotsenko and Murrell, 2008). Furthermore, *pxm* gene clusters have been detected only in a subgroup of Gamma-MOB that use functional pMMO to support catabolism and growth. This alone may preclude any inference of the substrate based on data not obtained with physiological experiments and leaves credible room for our hypothesis that pXMO may have evolved for catabolism of methane, ammonia or a different substrate, or for detoxification purposes. Furthermore, we identified a *pxm* gene cluster in *M. methanica*, a Gamma-MOB incapable of ammonia oxidation and nitrite formation, whereas the *pxm* gene-positive *M. album strain* BG8 does nitrify (Nyerges and Stein, 2009). While Fig. 3 supports the hypothesis that exposure to ammonia increases relative transcript abundance of *pxmA* compared with *pmoA* and thus suggests involvement of pXMO in ammonia oxidation, physiological experiments with wild-type and knockout mutant cells testing a broad range of substrates are necessary to determine the functional role of pXMO in Gamma-MOB.

Taken together, the discovery of the *pxm* operon in diverse aerobic obligate Gamma-MOB, and the novel 'A-B-C' organization of its member genes whose transcriptional regulation is also likely different from that of *pmo* and *amo* operons, significantly extends our understanding of the evolutionary relationships among members of the CuMMO superfamily. Inclusion of the gammaproteobacterial Pxm proteins in phylogenetic analyses of Pmo/Amo/Pxm subunit proteins generated trifurcated trees that clearly delineate three major clades (Fig. 1 and Fig. S1). The subclade comprised of PmoA1 and 2 proteins from obligate aerobic *Verrucomicrobia* and diverse environmental isolates also included the PmoA protein of the recently discovered nitrite-dependent methanotroph *Methylomirabilis oxifera* (Ettwig *et al*., 2010), that is a member of a new deep-branching bacterial phylum (Raghoebarsing *et al*., 2006; Ettwig *et al*., 2008; 2009), suggesting that this obligate anaerobic bacterium that produces the molecular oxygen it needs for monooxygenation of methane most likely acquired, rather than invented, pMMO for methanotrophy and putative ammonia catabolism. In context with the small subunit ribosomal RNA tree focusing on methane monooxygenase-positive taxa (Op den Camp *et al*., 2009), the PmoA tree in Fig. 1 also suggests that the ancestral oxygen-dependent monooxygenase was likely not invented in the common ancestor of the *Verrucomicrobia* and *Proteobacteria* phyla as such a scenario would have involved a large number of deletion events during evolutionary time. Unlike the sequence-divergent *pmoA* and *pxmA* genes in Gamma-MOB, the two sequence-divergent *pmoA* genes in Alpha-MOB have identical GC content (60%) and only slightly different GC bias (red arrows in Fig. 2). While the *pmoA1* and *pmoA2* genes have a similar GC content as the verrucomicrobial genome (47% versus 45%), the verrucomicrobial *pmoA3* genes consist of only 37% GC (green arrows in Fig. 2). In context, the *amoA* gene of the nitrifying Crenarchaeaon, *Nitrosopumilus maritimus*, has a GC content that is higher (43%; magenta arrow in Fig. 2) than the genome's (34%) (Walker *et al*., 2010). While it is presently not clear how these GC skewed islands are maintained in both respective genomes, it appears that the ancestors of verrucomicrobial *pmoA3* genes could be the source of the *amoA* genes in extant ammonia-oxidizing archaea, a concept supported also by the phylogenetic positions of *pmoB3* and *pmoC3* genes in respective phylogenetic trees (Figs S2 and S3 in Op den Camp *et al*., 2009 and see Fig. 1 and Fig. S1).

Methane and ammonia monooxygenase likely evolved before oxygen concentrations were sufficient to support an oxygen-dependent high-throughput machine that produces highly toxic intermediates (methanol/formaldehyde and hydroxylamine respectively) suggesting that they must have evolved after respective detoxification mechanisms were in place (Klotz and Stein, 2010). Interestingly, *pmo/amo* genes are absent from all sequenced genomes of *Planctomycetes*, *Epsilon-* and *Deltaproteobacteria* but present in *Alpha-*, *Beta-* and *Gammaproteobacteria*, whereas genomes of all proteobacterial classes and the

Brocadiaceae family of the *Planctomycetes* encode respective detox systems (Klotz *et al*., 2008; Klotz and Stein, 2010). These detox systems have likely evolved in anaerobic ancestors of *Planctomycetes* and *Proteobacteria* (Klotz and Stein, 2008; 2010; Klotz *et al*., 2008) suggesting that the genes encoding ancestral oxygen-independent, substrate promiscuous monooxygenase may have evolved first in anaerobic *Proteobacteria*. Subsequent transfer of its encoding genes into the anaerobic ancestors of verrucomicrobial methanotrophs likely occurred before the big oxygenation event, a process long ago enough to have its molecular traces erased in extant bacteria.

Acknowledgements

We thank Dan Arp for providing genomic DNA from Nitrosomonas europaea, and Burt Thomas for peat bog samples. We also thank three anonymous reviewers for critical reading of this manuscript. Funding for this project was provided by the Gordon and Betty Moore Foundation, and from National Aeronautics Space Administration grant NNF06GB34G (VJO), UW Royalty Research Fund grant 4064 and NSF Grant MCB-0604269 (M.G. Kalyuzhnaya), ERC Advanced Grant 232937 (MSMJ) and the Office of the EVP Research of UofL (M.G. Klotz).

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zheng, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST – a new generation of protein database search programs. *Nucleic Acids Res* **25:** 3389–3402.

Alzerreca, J.J., Norton, J.M., and Klotz, M.G. (1999) The *amo* operon in marine, ammonia-oxidizing *Gammaproteobacteria*. *FEMS Microbiol Lett* **180:** 21–29.

Arp, D.J., Sayavedra-Soto, L.A., and Hommes, N.G. (2002) Molecular biology and biochemistry of ammonia oxidation by *Nitrosomonas europaea*. *Arch Microbiol* **178:** 250–255.

Arp, D.J., Chain, P.S.G., and Klotz, M.G. (2007) The impact of genome analyses on our understanding of ammonia-oxidizing bacteria. *Annu Rev Microbiol* **61:** 21–58.

Baani, M., and Liesack, W. (2008) Two isozymes of particulate methane monooxygenase with different methane oxidation kinetics are found in *Methylocystis* sp. strain SC2. *Proc Natl Acad Sci USA* **105:** 10203–10208.

Balasubramanian, R., and Rosenzweig, A.C. (2007) Structural and mechanistic Insights into methane oxidation by particulate methane monooxygenase. *Acc Chem Res* **40:** 573–580.

Casciotti, K., Sigman, D., and Ward, B. (2003) Linking diversity and stable isotope fractionation in ammonia-oxidizing bacteria. *Geomicrobiol J* **20:** 335–353.

DiSpirito, A.A., Kunz, R.C., Choi, D.-W., and Zahn, J.A. (2005) Respiration in methanotrophs. In *Respiration in Archaea and Bacteria: Diversity of Procaryotic Respiratory Systems*. Zannoni, D. (ed.). Dordrecht, The Netherlands: Springer, pp. 149–168.

Drummond, A., and Rambaut, A. (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* **7:** 214.

Dunfield, P.F., Yimga, M.T., Dedysh, S.N., Berger, U., Liesack, W., and Heyer, J. (2002) Isolation of a *Methylocystis* strain containing a novel *pmoA*-like gene. *FEMS Microbiol Ecol* **41:** 17–26.

Dunfield, P.F., Yuryev, A., Senin, P., Smirnova, A.V., Stott, M.B., Hou, S., *et al*. (2007) Methane oxidation by an extremely acidophilic bacterium of the phylum *Verrucomicrobia*. *Nature* **450:** 879–882.

El Sheikh, A.F., and Klotz, M.G. (2008) Ammonia-dependent differential regulation of the gene cluster that encodes ammonia monooxygenase in *Nitrosococcus oceani* ATCC 19707. *Environ Microbiol* **10:** 3026–3035.

El Sheikh, A.F., Poret-Peterson, A.T., and Klotz, M.G. (2008) Characterization of two new genes, *amoR* and *amoD*, in the *amo* operon of the marine ammonia oxidizer *Nitrosococcus oceani* ATCC 19707. *Appl Environ Microbiol* **74:** 312–318.

Ettwig, K.F., Shima, S., van de Pas-Schoonen, K.T., Kahnt, J., Medema, M.H., Op den Camp, H.J.M., *et al*. (2008) Denitrifying bacteria anaerobically oxidize methane in the absence of *Archaea*. *Environ Microbiol* **10:** 3164–3173.

Ettwig, K.F., van Alen, T., van de Pas-Schoonen, K.T., Jetten, M.S.M., and Strous, M. (2009) Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 phylum. *Appl Environ Microbiol* **75:** 3656–3662.

Ettwig, K.F., Butler, M.K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M.M.M., *et al*. (2010) Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* **464:** 543–548.

Hallam, S.J., Mincer, T.J., Schleper, C., Preston, C.M., Roberts, K., Richardson, P.M., and DeLong, E.F. (2006) Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine Crenarchaeota. *PLoS Biol* **4:** e95.

Han, J.I., and Semrau, J.D. (2000) Chloromethane stimulates growth of *Methylomicrobium album* BG8 on methanol. *FEMS Microbiol Lett* **1877:** 77–81.

Hanson, R.S., and Hanson, T.E. (1996) Methanotrophic Bacteria. *Microbiol Rev* **60:** 439–471.

Islam, T., Jensen, S., Reigstad, L.J., Larsen, O., and Birkeland, N.-K. (2008) Methane oxidation at 55C and pH 2 by a thermoacidophilic bacterium belonging to the *Verrucomicrobia* phylum. *Proc Natl Acad Sci USA* **105:** 300–304.

Klotz, M.G., and Norton, J.M. (1998) Multiple copies of ammonia monooxygenase (*amo*) operons have evolved under biased AT/GC mutational pressure in ammonia-oxidizing autotrophic bacteria. *FEMS Microbiol Lett* **168:** 303–311.

Klotz, M.G., and Stein, L.Y. (2008) Nitrifier genomics and evolution of the N-cycle. *FEMS Microbiol Lett* **278:** 146–156.

Klotz, M.G., and Stein, L.Y. (2010) Genomics of ammonia-oxidizing bacteria and insights to their evolution. In *Nitrification*. Ward, B.B., Arp, D.J., and Klotz, M.G. (eds). Washington, DC, USA: ASM Press (in press).

Klotz, M.G., Arp, D.J., Chain, P.S.G., El-Sheikh, A.F., Hauser, L.J., Hommes, N.G., *et al*. (2006) Complete genome sequence of the marine, chemolithoautotrophic, ammonia-

oxidizing bacterium *Nitrosococcus oceani* ATCC 19707. *Appl Environ Microbiol* **72:** 6299–6315.
Klotz, M.G., Schmid, M.C., Strous, M., Op den Camp, H.J.M., Jetten, M.S.M., and Hooper, A.B. (2008) Evolution of an octaheme cytochrome *c* protein family that is key to aerobic and anaerobic ammonia oxidation by bacteria. *Environ Microbiol* **10:** 3150–3163.
Könneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B., and Stahl, D.A. (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* **437:** 543–546.
Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W., *et al*. (2006) *Archaea* predominate among ammonia-oxidizing prokaryotes in soils. *Nature* **442:** 806.
Lontoh, S., DiSpirito, A.A., Krema, C.L., Whittaker, M.R., Hooper, A.B., and Semrau, J.D. (2000) Differential inhibition in vivo of ammonia monooxygenase, soluble methane monooxygenase and membrane-associated methane monooxygenase by phenylacetylene. *Environ Microbiol* **2:** 485–494.
Martens-Habbena, W., Berube, P.M., Urakawa, H., de la Torre, J.R., and Stahl, D.A. (2009) Ammonia oxidation kinetics determine niche separation of nitrifying *Archaea* and Bacteria. *Nature* **461:** 976–979.
Nicol, G.W., and Schleper, C. (2006) Ammonia-oxidising crenarchaeota: important players in the nitrogen cycle? *Trends Microbiol* **14:** 207.
Nielsen, A.K., Gerdes, K., and Murrell, J.C. (1997) Copper-dependent reciprocal transcriptional regulation of methane monooxygenase genes in *Methylococcus capsulatus* and *Methylosinus trichosporium*. *Mol Microbiol* **25:** 399–409.
Norton, J.M., Alzerreca, J.J., Suwa, Y., and Klotz, M.G. (2002) Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. *Arch Microbiol* **177:** 139–149.
Norton, J.M., Klotz, M.G., Stein, L.Y., Arp, D.J., Bottomley, P.J., Chain, P.S.G., *et al*. (2008) Complete genome sequence of *Nitrosospira multiformis*, an ammonia-oxidizing bacterium from the soil environment. *Appl Environ Microbiol* **74:** 3559–3572.
Nyerges, G., and Stein, L.Y. (2009) Ammonia co-metabolism and product inhibition vary considerably among species of methanotrophic bacteria. *FEMS Microbiol Lett* **297:** 131–136.
Op den Camp, H.J., Islam, T., Stott, M.B., Harhangi, H.R., Hynes, A., Schouten, S., *et al*. (2009) Environmental, genomic, and taxonomic perspectives on methanotrophic *Verrucomicrobia*. *Environ Microbiol Rep* **1:** 293–306.
Pol, A., Heijmans, K., Harhangi, H.R., Tedesco, D., Jetten, M.S.M., and Op den Camp, H.J.M. (2007) Methanotrophy below pH 1 by a new *Verrucomicrobia* species. *Nature* **450:** 874–878.
Prosser, J.I., and Nicol, G.W. (2008) Relative contributions of archaea and bacteria to aerobic ammonia oxidation in the environment. *Environ Microbiol* **10:** 2931–2941.
Raghoebarsing, A.A., Pol, A., van de Pas-Schoonen, K.T., Smolders, A.J.P., Ettwig, K.F., Rijpstra, W.I.C., *et al*. (2006) A microbial consortium couples anaerobic methane oxidation to denitrification. *Nature* **440:** 918–921.
Ricke, P., Erkel, C., Kube, M., Reinhardt, R., and Liesack, W. (2004) Comparative analysis of the conventional and novel *pmo* (particulate methane monooxygenase) operons from *Methylocystis* strain SC2. *Appl Environ Microbiol* **70:** 3055–3063.
Stein, L.Y., Arp, D.J., Berube, P.M., Chain, P.S.G., Hauser, L.J., Jetten, M.S.M., *et al*. (2007) Whole-genome analysis of the ammonia-oxidizing bacterium, *Nitrosomonas eutropha* C91: implications for niche adaptation. *Environ Microbiol* **9:** 1–15.
Stoecker, K., Bendinger, B., Schöning, B., Nielsen, P.H., Nielsen, J.L., Baranyi, C., *et al*. (2006) Cohn's *Crenothrix* is a filamentous methane oxidizer with an unusual methane monooxygenase. *Proc Natl Acad Sci USA* **103:** 2363–2367.
Stolyar, S., Franke, M., and Lidstrom, M.E. (2001) Expression of individual copies of *Methylococcus capsulatus* Bath particular methane monooxygenase. *J Bacteriol* **183:** 1810–1812.
Swofford, D.L. (1999) *PAUP (Phylogenetic Analysis Using Parsimony)**. Sunderland, MA, USA: Sinauer Associates.
Tavormina, P.L., Ussler, W., III, and Orphan, V.J. (2008) Planktonic and Sediment-Associated Aerobic Methanotrophs in Two Seep Systems along the North American Margin. *Appl Environ Microbiol* **74:** 3985–3995.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **24:** 4876–4882.
Trotsenko, Y.A., and Murrell, J.C. (2008) Metabolic aspects of aerobic obligate methanotrophy. *Adv Appl Microbiol* **63:** 183–229.
Walker, C.B., de la Torre, J.R., Urakawa, H., Klotz, M.G., Manning, G., Pinel, N., *et al*. (2010) The genome of *Nitrosopumilus maritimus* reveals a close functional relationship to the globally distributed marine Crenarchaeota. *Proc Natl Acad Sci USA* **107:** 8818–8823.
Ward, N., Larsen, O., Sakwa, J., Bruseth, L., Khouri, H., Durkin, A.S., *et al*. (2004) Genomic insights into methanotrophy: the complete genome sequence of *Methylococcus capsulatus* (Bath). *PLoS Biol* **2:** e303.
Whelan, S., and Goldman, N. (2001) A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol Biol Evol* **18:** 691–699.
Whittenbury, R., Phillips, K.C., and Wilkinson, J.F. (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. *J Gen Microbiol* **61:** 205–218.
Yimga, M.T., Dunfield, P.F., Ricke, P., Heyer, J., and Liesack, W. (2003) Wide distribution of a novel *pmoA*-like gene copy among Type II methanotrophs, and Its expression in *Methylocystis* strain SC2. *Appl Environ Microbiol* **69:** 5593–5602.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Unrooted phylogenetic consensus trees constructed after Bayesian inference of phylogeny from the ClustalX alignments of 127 PxmA, PmoA and AmoA and 51 PxmB, PmoB and AmoB protein sequences subjected to a Bayesian

inference of phylogeny using the BEAST package [BEAUti v1.5.3, BEAST v1.5.3, TreeAnnotator v1.5.3, FigTree v.1.3 (Drummond and Rambaut, 2007)] as described in Fig. 1 (Monte-Carlo Markov Chain with 10 000 000 generations, WAG substitution model). Unrooted 50% majority rule consensus phylograms (panel A, A protein alignments; panel B, B protein alignments) were constructed and mean branch lengths (posterior probability values of all observed clades < 1.0 are indicated) are characterized by a scale bar indicating the evolutionary distance (changes per amino acid position). Protein sequence accession numbers for all protein sequences in the alignments were included into the branch labels together with information of the source organism. The trifurcation point identified by the star topology of the tree shown in Fig. 1 is indicated.

Fig. S2. Operon structure of pxmABC genes in Methylomonas sp. strain LW13. A. Retrieved contiguous sequence contains pxm genes in the non-canonical order A-B-C as indicated. An open reading frame (orf1) encoding a protein related to LysR type transcription regulators is divergently transcribed upstream of pxm coding sequence. Dashed lines indicate partial ORFs. Approximate locations of PCR primers (given in Table S1) are indicated by arrows. B. The putative pxm initial transcribed region is predicted to contain two alternative hairpins. The longer hairpin contains features suggesting a transcriptional regulatory role.

Fig. S3. Endpoint analysis of Reverse-Transcription real-time PCR for detection of pxmA and pmoA transcripts in creek sediment samples. PCR-amplification was performed using three serial dilution samples (lanes 2, 3 and 4) of cDNA generated from DNase-treated RNA obtained from creek sediments ('RT') as described in Fig. 3 and three serial dilution samples (lanes 5, 6 and 7) of the DNase-treated RNA incubated with reverse transcription buffer omitting reverse transcriptase enzyme ('no-RT'). 'no-RT' samples were used as controls to demonstrate absence of contamination by genomic DNA in the cDNA. Lane 1 contains 4 ul of the size standard (Low range ladder, Fisher Scientific, Pittsburgh, PA) to estimate size and intensity of the obtained amplicons. Cycle threshold determined during real time PCR is indicated. The melting temperature (T_m) indicates that the pxmA and pmoA amplicons are distinct species.

Table S1. Primers and conditions used in this study.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.