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Research Letter

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The origin of aerobic methanotrophy within the Proteobacteria

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One sentence summary: Methanotrophy likely arose via lateral gene transfer of methane monooxygenase to methylotrophs that had multiple forms of the MeDH and a copper uptake system.

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ABSTRACT

Aerobic methanotrophs play critical roles in the global carbon cycle, but despite their environmental ubiquity, they are phylogenetically restricted. Via bioinformatic analyses, it is shown that methanotrophy likely arose from methylotrophy from the lateral gene transfer of either of the two known forms of methane monooxygenase (particulate and soluble methane monooxygenases). Moreover, it appears that both known forms of pyrroloquinoline quinone-dependent methanol dehydrogenase (MeDH) found in methanotrophs—the calcium-containing Mxa-MeDH and the rare earth element-containing Xox-MeDH—were likely encoded in the genomes before the acquisition of the methane monooxygenases (MMOs), but that some methanotrophs subsequently received an additional copy of Xox-MeDH-encoding genes via lateral gene transfer. Further, data are presented that indicate the evolution of methanotrophy from methylotrophy not only required lateral transfer of genes encoding for methane monooxygenases, but also likely the pre-existence of a means of collecting copper. Given the emerging interest in valorizing methane via biological platforms, it is recommended that future strategies for heterologous expression of methane monooxygenase for conversion of methane to methanol also include cloning of genes encoding mechanism(s) of copper uptake, especially for expression of particulate methane monooxygenase.

Keywords: methanotrophy; copper uptake; evolution; methanol dehydrogenase; methane monooxygenase

INTRODUCTION

Methanotrophs are a remarkable group of microbes that can utilize methane as their sole energy source. Both aerobic and anaerobic methanotrophs have been characterized, the latter in both Bacteria and Archaea. Although methanotrophy is commonly found in diverse environments ranging from peat bogs, rice paddies, forest, agricultural and urban soils (amongst many other locations; (Semrau, DiSpirito and Yoon 2010)), they are restricted to handful of phylogenetic groups, especially the aerobic methanotrophs. That is, methanotrophs coupling methane oxidation to O_2 reduction are only found within four groups—the Verrucomicrobia and NC10 phyla (the latter actually uses nitrite as the terminal electron acceptor, but creates di-oxygen from nitric oxide that is required for methane oxidation), as well as two classes of Proteobacteria: the *Alphaproteobacteria* and *Gammaproteobacteria* (Op den Camp *et al.* 2009; Ettwig *et al.* 2010; Semrau, DiSpirito and Yoon 2010).

All aerobic methanotrophs utilize methane monooxygenase to convert methane to methanol, which is then further oxidized to formaldehyde for carbon assimilation or completely to carbon dioxide for generation of reducing equivalents (Semrau

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et al. 2018). There are two forms of MMO, a membrane-bound or particulate form (pMMO) found in most (but not all) aerobic methanotrophs as well as a cytoplasmic or soluble form (sMMO) found in some aerobic methanotrophs. A handful of aerobic methanotrophs have both forms of MMO, although most have either one or the other (Table S1, Supporting Information). It should be noted that many methanotrophs have multiple operons of genes encoding for pMMO (*pmoCAB*) but methanotrophs capable of expressing sMMO have only been found to have one operon of these genes (*mmoXYBZDC*; Table S1, Supporting Information).

It is well-known that pMMO requires copper for its activity, and methanotrophs have multiple mechanisms for copper collection (Kim et al. 2004; Helland et al. 2008; Krentz et al. 2010; Fru et al. 2011; El Ghazouani et al. 2012; Ve et al. 2012; Gu et al. 2016; Gu, Ul Haque and Semrau 2017; Semrau et al. 2018). Further, for those methanotrophs that can express both sMMO and pMMO, expression is strongly regulated by the availability of copper. Specifically, sMMO expression is only observed in the absence of copper, while pMMO expression increases with increasing copper, i.e. the canonical 'copper-switch' (Stanley et al. 1983; Semrau et al. 2013).

In addition to pMMO and sMMO, several *Gammaproteobacteria* methanotrophs (but few *Alphaproteobacteria* methanotrophs) appear to have genes for a divergent form of pMMO, called pXMO (Tavormina et al. 2011). Expression of *pxm* genes, however, has been reported to be two to three orders of magnitude lower than *pmo* genes (Tavormina et al. 2011; Kits, Klotz and Stein 2015). Further, evidence for translation of *pxm* transcripts and activity of pXMO is lacking. As such, its importance in methanotrophic physiology is largely unknown, although it may have some role in responding to oxygen limitation and/or increasing growth substrate range (Tavormina et al. 2011; Kits, Klotz and Stein 2015).

The origin of methanotrophy has been a subject of some debate, but it is believed that aerobic methanotrophs derived from methylotrophs (i.e. methanol-utilizing microbes), since a critical constraint is the effective handling of the initial product of methane oxidation, i.e. methanol (Tamas et al. 2014; Osborne and Haritos 2018). Methanol oxidation is critical to prevent methanol from accumulating to toxic levels, to allow carbon assimilation from downstream products (formaldehyde) and to regenerate reducing equivalents consumed in methane oxidation (Im et al. 2011; Farhan Ul Haque et al. 2017; Semrau et al. 2018). This conclusion was supported by phylogenetic study of genes encoding methylotrophy and methanotrophy in the *Alphaproteobacteria*, which indicated that methanotrophic groups are nested within a much larger lineage of bacteria sharing vertical inheritance of methylotrophy genes (Tamas et al. 2014). Subsequent bioinformatic interrogation demonstrated that genes encoding pMMO (*pmoCAB*) in most proteobacterial methanotrophs have significantly different compositional biases than their host genomes, suggesting that these genes were obtained via recent lateral gene transfer (LGT) events (Khadka et al. 2018;). That is, methane oxidation may have occurred as the result of LGT of ammonia monooxygenase from nitrifying bacteria to methylotrophs (Khadka et al. 2018). Indeed, it has been reported that when genes encoding ammonia monooxygenase are inserted in the methylotroph *Methylobacterium extorquens* AM1, the microbe can subsequently grow on methane (Crossman et al. 1997). It has also been concluded that sMMO was also the result of a LGT in at least some *Alphaproteobacteria* methanotrophs (Tamas et al. 2014).

Interestingly, not only are there multiple forms of MMO, most aerobic methanotrophs (as well as many methylotrophs) have two forms of a pyrroloquinoline quinone (PQQ)-dependent methanol dehydrogenase (MeDH) responsible for oxidation of methanol. One form, 'Mxa-MeDH', contains calcium, while another form, 'Xox-MeDH', contains a rare earth element (REE), e.g. lanthanum or cerium (Keltjens et al. 2014; Pol et al. 2014; Wu et al. 2015; Farhan Ul Haque et al. 2016). Similar to the 'copper-switch', an 'REE-switch' is also found in methanotrophs where relative expression of MeDHs is controlled by the availability of REEs (Farhan et al. 2015; Chu, Beck and Lidstrom 2016; Vu et al. 2016; Gu and Semrau 2017). Many methanotrophs have multiple copies of genes encoding Xox-MeDH (*xoxFJ*), while only one copy is found for Mxa-MeDH in methanotroph genomes (*mxaFJGI*, Table S1, Supporting Information).

Given that methanotrophy possibly arose via transfer of MMO to methylotrophs, we chose to bioinformatically examine genes involved in methanol oxidation as well as genes involved in copper uptake. This is particularly relevant as methylotrophy is much more wide-spread than methanotrophy, suggesting that some methylotrophs lack essential cell machinery to be effectively transformed into methanotrophs through LGT of genes encoding MMO.

MATERIALS AND METHODS

Genome sequences

All genomes used in this study were acquired from MicroScope or NCBI databases. A total of 39 methanotroph genomes from various taxonomic families/phyla were considered in this study—*Methylocystaceae* (9), *Beijerinckiaceae* (4), *Methylococcaceae* (20), *Methylothermaceae* (1), '*Methyloacidiphilaceae*' (4) and NC10 (1).

Evidence for LGT

Nucleotide bias between specific genes and the rest of the genome to detect possible LGT was calculated using two different programs—Alien Hunter and CodonW—as described earlier (Khadka et al. 2018). Briefly, these packages determine the Kullback-Leibler divergence statistic (D_{KL}) for different DNA regions as compared to the overall genome using different criteria. Alien Hunter considers combined 2-mers through 8-mers for both specific regions and the genome using a 2500 bp window. CodonW considers codon bias between specific regions and the genome. For Alien Hunter, complete genomes in FASTA format were used as input (Vernikos and Parkhill 2006). The D_{KL} values of operons (regions) of interest were extracted from the resulting output file to determine whether the operon fell in a region of potential LGT. For CodonW, the coding sequence of each genome was extracted from GenBank (.gbk) files using FeatureExtract 1.2 L Server (Wernersson 2005). The coding sequence of DNA regions and genomes were then uploaded to the online tool, CodonW Galaxy v1.4.4 (Peden 1999). The frequency of all 64 codons was then counted for regions and genomes to calculate D_{KL} values. DNA regions considered here were: *pmoCAB* (for pMMO); *mmoXYBZDC* (for sMMO), *mxaFJGI* (for Mxa-MeDH), *xoxFJ* (for Xox-MeDH), *sga* (encoding serine-glyoxylate aminotransaminase), *fdhBA* and *fdsBA* (encoding the tungsten and molybdenum dependent forms of formate dehydrogenase, respectively), as well as genes for three different identified copper uptake systems in methanotrophs, i.e. (1) *mbnABCM* (encoding methanobactin; (Semrau et al. 2013)), (2) *mopE* + MCA 2590 (encoding Methylococcus outer membrane protein (MopE) and its

surface associated cytochrome c peroxidase; (Helland et al. 2008; Ve et al. 2012) as well as close analogs corAB (Berson and Lidstrom 1997; Johnson et al. 2014) and; (3) copCD (encoding a periplasmic copper binding protein and its partner inner membrane transport protein; (Arnesano et al. 2003; Gu, Ul Haque and Semrau 2017)).

Phylogenetic analysis of operons and genes

The alignments of genes and concatenated genes were constructed using T-COFFEE (Notredame Higgins and Heringa 2000). Bayesian and maximum likelihood phylogenies were generated based on these alignments using PhyML 3.1 and BEAST 1.10.4, respectively (Huelsenbeck et al. 2001; Guindon et al. 2010). The resulting Bayesian and maximum likelihood phylogenies were evaluated using 10^7 generations discarding a burn-in of 25% and 100 bootstrap replicates, respectively. The phylogenetic trees were visualized using ggtree 1.14.4 (Yu et al. 2017).

RESULTS

Evidence of LGT of methane monooxygenase

The different software packages used to detect LGT events—Alien Hunter and CodonW—gave similar trends for assessed genes (Fig. 1), but in general, greater divergence values were found using Alien Hunter than CodonW, likely due to its greater sensitivity as it uses variable order motif distributions. It should be noted that Alien Hunter does not provide numerical divergence values below a critical threshold calculated based on these distributions. These are reported as '0' in Fig. 1.

As previously shown (Khadka et al. 2018), the pmo operon (pmoCAB) appears to be the result of a relatively recent LGT event in most methanotrophic groups as the nucleotide composition of this operon is significantly divergent from the genome composition of the host microbe (Fig. 1; Tables S2 and S3, Supporting Information). Bayesian phylogenetic trees based either on 16S rRNA gene or concatenated pmoCAB gene sequences (Fig. 2 and 3, respectively), however, were similar. It should be noted that D_{KL} values were not calculated for pxm sequences, nor are they included in the phylogenetic trees, since only a fraction of characterized methanotrophs have been found to have these genes in their genomes (Table S1, Supporting Information) and their importance or function in methanotrophic physiology is largely unknown. Maximum-likelihood phylogenetic trees based on either 16S rRNA or pmoCAB gene sequences (Fig. S1 and S2, Supporting Information) exhibited similar profiles as Bayesian trees.

Evidence for LGT of the mmo operon (mmoXYBZDC), however, is not as robust as observed for pmo genes. That is, D_{KL} values as predicted using Alien Hunter suggest that these genes may have been incorporated into the genomes of the two considered Biejerinckiaceae species, as well as some Methylococcaceae and Methylocystaceae species via LGT (Fig. 1 and Table S2, Supporting Information). Many species in Methylococcaceae, however, had D_{KL} values for mmo genes below the detection threshold of Alien Hunter, unlike what was found for pmoCAB in these same microorganisms. D_{KL} values for mmo genes as determined using CodonW were low for most methanotrophs, and lower than those for pmoCAB in those strains that can express both forms (Table S3, Supporting Information). It should be noted that relatively few methanotrophs have been found to possess mmo genes, limiting our ability to be as thorough in our analyses as can be done for pmoCAB. As a result, construction of a phylogenetic tree based on the concatenated mmoXYBZDC sequence provides limited additional information as to the origin of sMMO in methanotrophy (Fig. S3, Supporting Information).

Evidence of LGT of MeDH

Earlier evidence suggested that different methanotrophic groups arose via ancestral methylotrophs procuring MMO genes via LGT (Tamas et al. 2014; Khadka et al. 2018; Osborne and Haritos 2018). To examine this hypothesis, we calculated the D_{KL} values for operons encoding components of the PQQ-dependent Mxa-MeDH and Xox-MeDH. As shown in Fig. 1 the composition of genes involved in formation/activity of Mxa-MeDH—mxaFJGI—were generally indistinguishable from the host genome for most known families of methanotrophs (it should be noted that Verrucomicrobia methanotrophs lack these genes). Relatively high D_{KL} values were calculated for this DNA region for members of the Beijerinckiaceae using Alien Hunter, i.e. Methylocapsa species (Table S2, Supporting Information), but these were lower than values calculated for pmoCAB. A Bayesian phylogenetic tree based on the concatenated mxaFJGI sequence (Fig. 4) is similar to the 16S rRNA phylogenetic analysis (Fig. 2), and comparable results were found for maximum likelihood phylogenies (Fig. S1 and S4, Supporting Information). Collectively these data indicate that in most cases the genes encoding Mxa-MeDH are more ancestral than the genes encoding MMOs, i.e. that they were present in the genomes before the MMO genes were obtained via LGT event(s).

Interestingly, there is evidence that the alternative PQQ-dependent MeDH—Xox-MeDH—that contains a REE may have been incorporated into methanotrophic genomes via LGT. First, genes with high levels of duplication are often indicative of LGT (Wellner, Lurie and Gophna 2007), and multiple operons of xox genes (as well as pmo genes) are commonly found in methanotrophs. However, mxa genes do not exist in multiple operons for any examined methanotroph (Table S1, Supporting Information). Second, high D_{KL} values were calculated for some copies of xoxFJ in several methanotrophs using both Alien Hunter and CodonW, and these were higher than those calculated for mxaFJGI (Fig. 1, Tables S2 and S3, Supporting Information). Third, copies of xoxFJ in many different methanotrophs are placed differently in Bayesian and maximum likelihood trees based on xoxFJ as compared to 16S rRNA phylogeny, e.g. Methylobacter and Methylocystis species, Verrucomicrobia methanotrophs, as well as Methyloferula stellata, Methylosinus trichosporium OB3b and 'Methylomirabilis oxyfera' (Fig. 2, 5, S1 and S5, Supporting Information).

Despite evidence for LGT of some xox genes, the data suggest that genes encoding Xox-MeDH in methanotrophic genomes were present before the acquisition of MMO. First, the D_{KL} values for xoxFJ in many species were (as found for mxaFJGI) below the detection threshold of Alien Hunter, and CodonW agreed with these results (Tables S2 and S3, Supporting Information). Second, although divergence is evident when comparing phylogenetic trees based on 16S rRNA and xoxFJ, there are also some intriguing similarities. That is, the phylogenetic grouping of xoxFJ for the Methylococcaceae, Methylocystaceae and Beijerinckaceae at the top of Fig. 5 is similar to that observed in 16S rRNA phylogeny (Fig. 2) and almost all (28/30) of these xoxFJ sequences had D_{KL} values below the threshold of Alien Hunter (as noted in Fig. 5). The other groupings

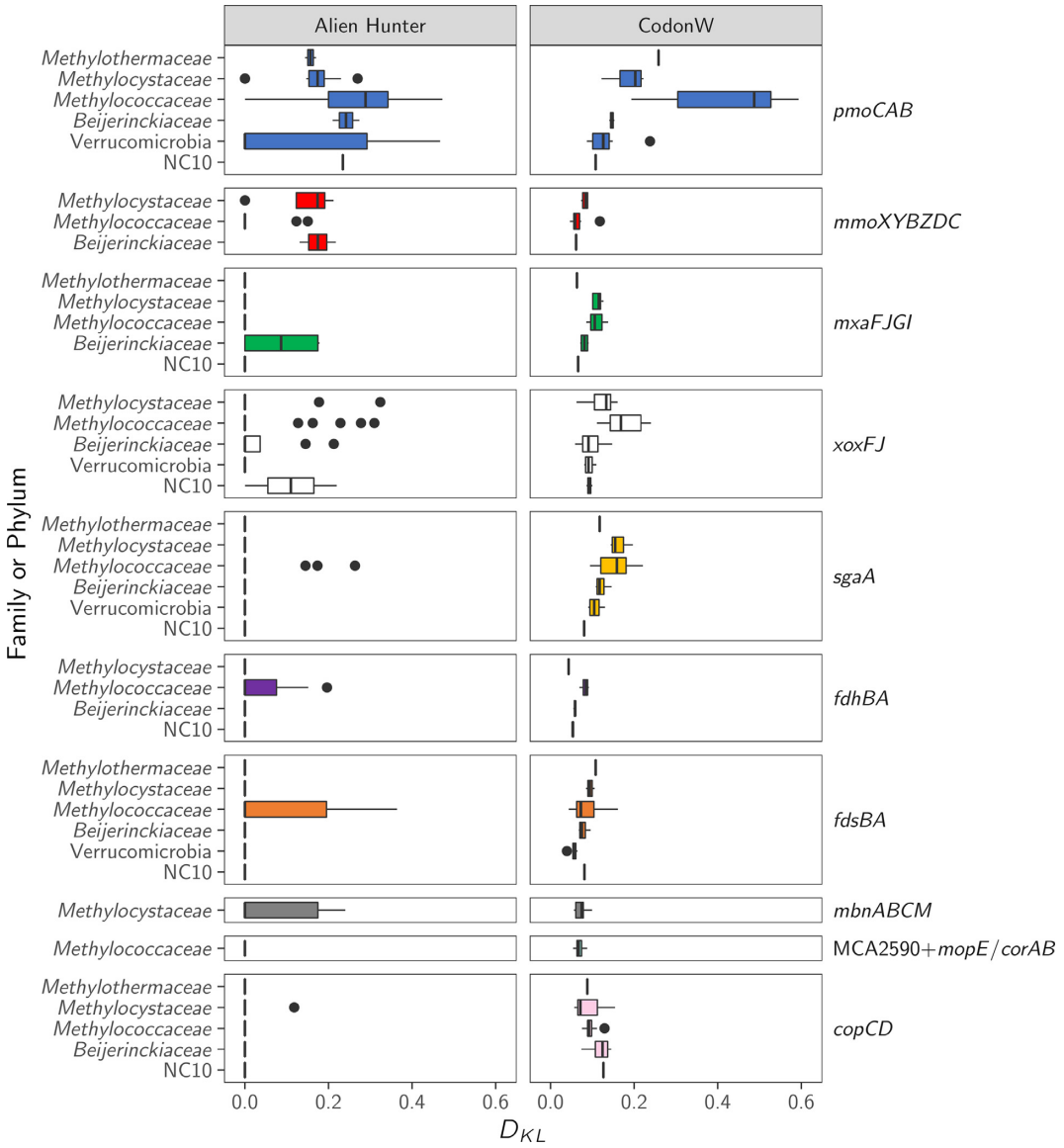

Figure 1. Nucleotide compositional biases (D_{KL}) of key gene clusters involved in methane oxidation (*pmoCAB* and *mmoXYBZDC*), methanol oxidation (*mxaFJGI* and *xoxFJ*), carbon assimilation (*sgaA*), formate oxidation (*fdhBA* and *fdsBA*) and copper uptake (*mbnABCM*, *corAB*, *mopE*/MCA2590 and *copCD*) in methanotrophs compared to their respective host genomes. The box indicates the interquartile range between 25th and 75th percentile, and the solid line indicates the 50th percentile. Outliers determined by the Tukey method are marked by dots.

at the bottom of Fig. 5 appear to have been influenced by several LGT events, and many of these copies exhibit significant D_{KL} values (based on Alien Hunter). Thus, it appears that both forms of MeDH were ancestral, but some genes for XoxMeDH were subsequently laterally transferred across multiple species.

Evidence of LGT of additional genes involved in carbon oxidation and assimilation

Previously, phylogenetic analyses focusing on *Alphaproteobacteria* methanotrophs indicated that a set of conserved genes encoding for one-carbon oxidation and assimilation were vertically inherited in these microbes. (Tamas *et al.* 2014). We extended these analyses to examine the compositional divergence and phylogenetic relationships of *sga* (encoding serine-glyoxylate aminotransaminase), *fdh* and *fds* (encoding the tungsten and molybdenum dependent forms of formate dehydrogenase, respectively) for other phylogenetic groups of methanotrophs. As shown in Fig. 1 and Tables S2 and S3 (Supporting Information), little sequence divergence of these genes was calculated using either Alien Hunter or CodonW for any family/phylum of methanotrophs. Further, phylogenetic trees based on these sequences (Fig. S6–S9, Supporting Information) were similar to 16S rRNA phylogenetic analysis (Fig. 2 and S1). Such data indicate that not only were multiple forms of MeDH likely vertically inherited, so were other aspects of C1 metabolism, i.e. methanotrophs likely arose from methylotrophs.

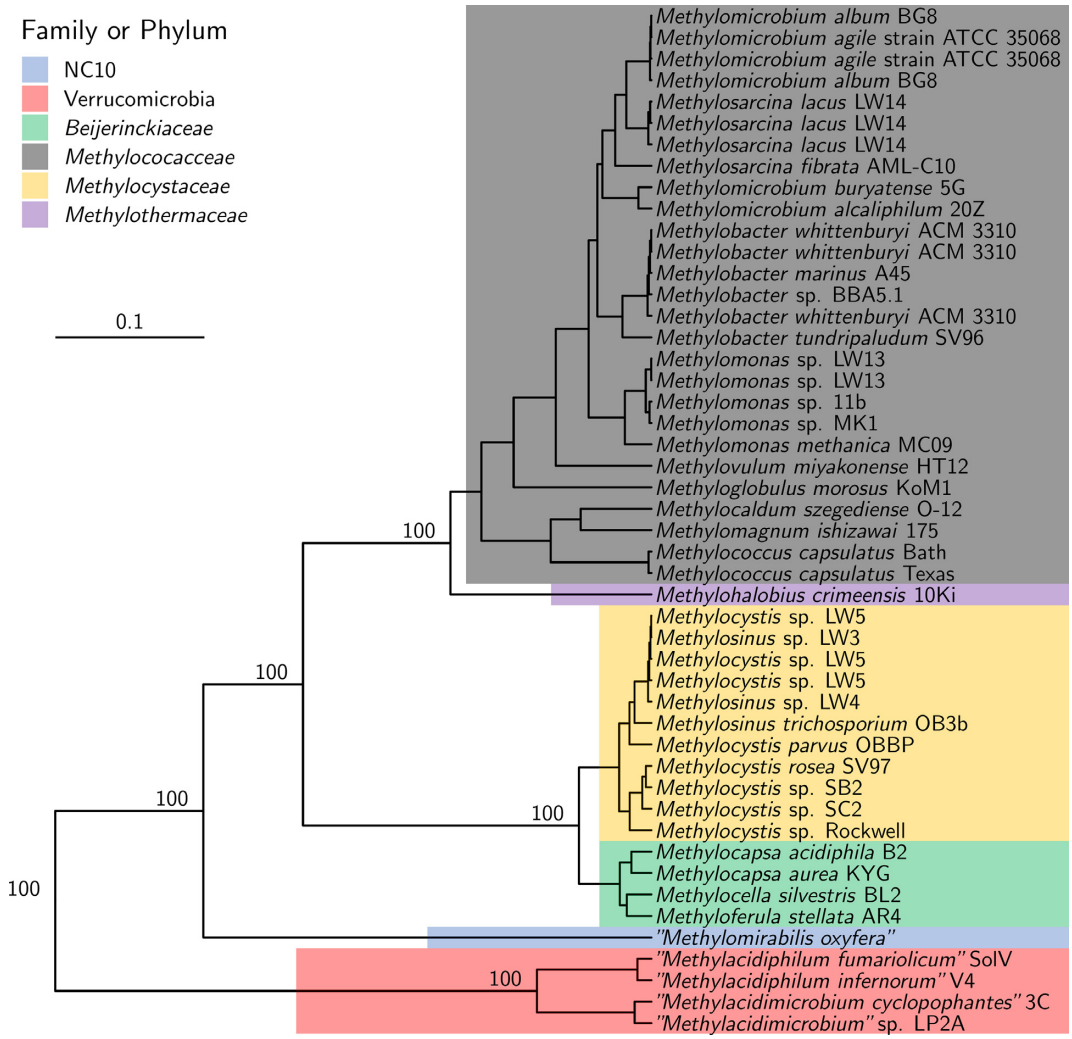

Figure 2. Bayesian 16S rRNA gene based phylogeny of aerobic methanotrophs. The tree was constructed using the general time-reversible model with invariant sites and four distinct gamma categories (GTR + I + G) under a strict clock with a minimum nucleotide sequence length of 1132. Node values indicate posterior probabilities based on 10 000 000 iterations with a burn-in of 25%. The scale bar represents 0.1 changes per nucleotide position.

Origin of copper uptake systems in methanotrophs

The existence of copper uptake systems in methanotrophs was screened by conducting BLAST searches for genes encoding the copper-binding peptides methanobactin, MopE, CorA and CopCD (Table S1, Supporting Information). Genes encoding for methanobactin (*mbnABCM*) were observed only in methanotrophs from the *Methylocystaceae*, while MopE and CorA (both with their surface associated cytochrome *c* peroxidases) were found only in methanotrophs affiliated with *Methylococcaceae*. Evidence of CopCD was found in most methanotrophs, with Verrucomicrobia methanotrophs being the only exception. Very little compositional divergence for *mopE* and its surface associated cytochrome *c* peroxidase were found, nor for *corA* and its surface associated cytochrome *c* peroxidase (Fig. 1). These data suggest these genes did not arise from a recent LGT, and were present prior to the acquisition of MMO. Methanobactin genes, however, showed a greater range of D_{KL} values, with some methanotrophs having D_{KL} values below the threshold of Alien Hunter indicating an LGT being unlikely, while others had values greater than 0.2, suggestive of an LGT. Finally, compositional divergence of *copCD* indicated that this copper uptake system was not the result of an LGT event for most methanotrophs. For those methanotrophs with multiple copies of *copCD* (of the *Methylocystaceae* and *Biejeirinckaceae* families), although Alien Hunter typically calculated divergence values below threshold for both copies, CodonW indicated that one copy had D_{KL} values two-three times greater than the other. These data suggest that the second copy of *copCD* in these species may have arisen as a result of LGT, and Bayesian and Maximum likelihood phylogenetic trees based on *copCD* (Fig. 6 and S10, Supporting Information) support this finding.

DISCUSSION

It has been speculated, based on the complexity hypothesis (Jain, Rivera and Lake 1999), that methanotrophy arose from LGT of MMO-encoding genes to methylotrophs (Tamas *et al.* 2014). If true, one would expect to find greater compositional divergence of MMO-encoding genes than of genes involved in further carbon oxidation or carbon assimilation. Indeed, all tested genes

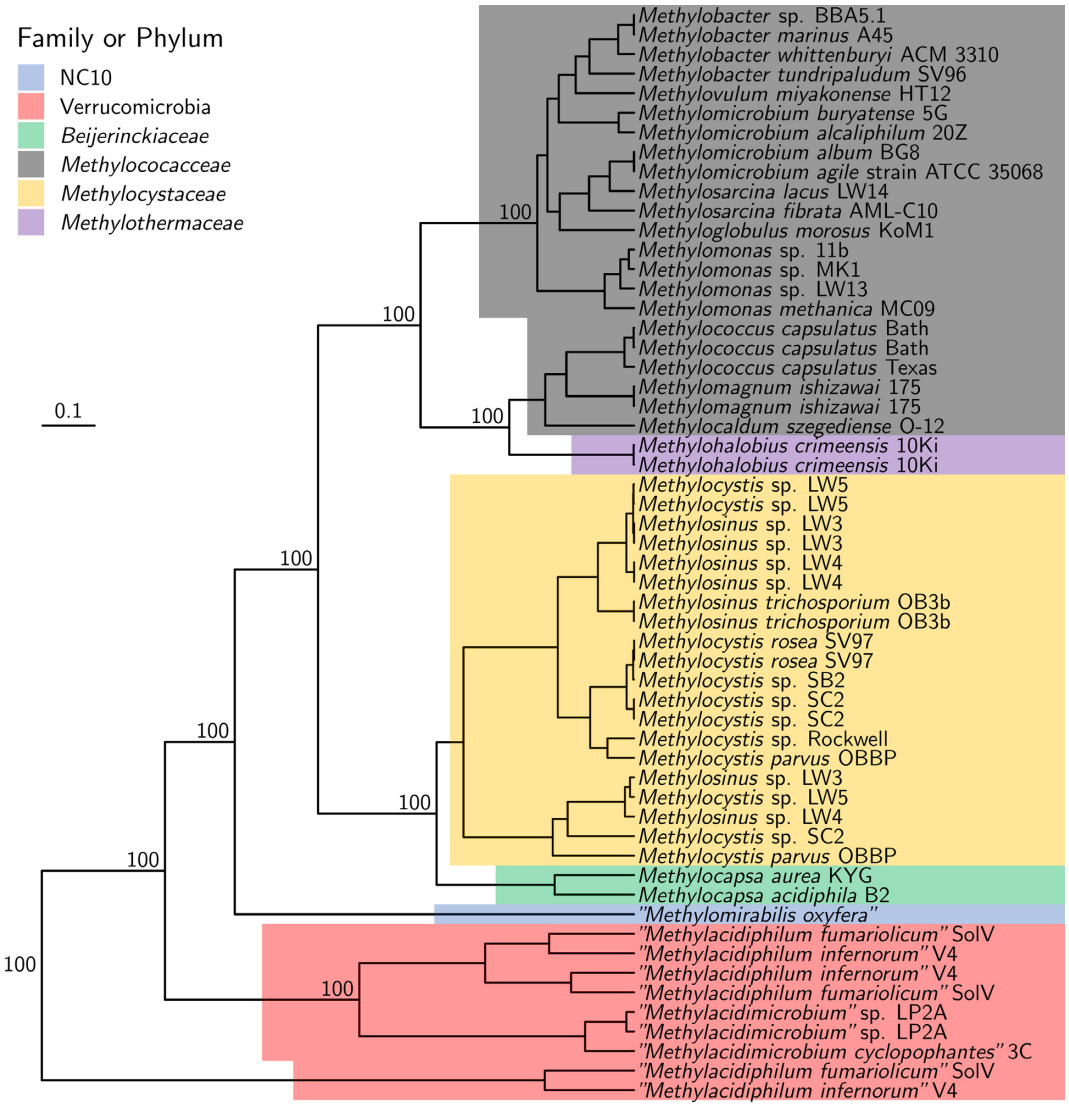

Figure 3. Bayesian *pmoCAB* based phylogeny of aerobic methanotrophs. The tree was constructed using the general time-reversible model with invariant sites and four distinct gamma categories (GTR + I + G) under a strict clock with a minimum nucleotide sequence length of 2085. Node values indicate posterior probabilities based on 10 000 000 iterations with a burn-in of 25%. The scale bar represents 0.1 changes per nucleotide position.

for MeDH, formate dehydrogenase and serine-glyoxylate aminotransaminase had low D_{KL} values as well as predicted phylogenies similar to that based on 16S rRNA gene sequences, suggesting that these genes were indeed present in the respective genomes before LGT acquisition of MMO.

Compositional and phylogenetic analyses of genes encoding Xox-MeDH indicate that this form, although likely ancestral, was more easily shared via LGT. That is, for those methanotrophs with multiple copies of *xoxFJ*, often one copy had a nucleotide composition indistinguishable from the host genome, while a duplicate copy was appreciably different (Fig. 1, Tables S2 and S3, Supporting Information). At this level of analysis, we cannot state with any certainty why one form of MeDH may be more amenable to LGT than another. One possibility may be that as REEs are strong Lewis acids (Lim and Franklin 2004), MeDH with REEs are catalytically superior to calcium-containing MeDH (Keltjens *et al.* 2014). As a result, competition pressure may promote the acquisition of *xox* genes via LGT. Interestingly, it has been shown that methanotrophs expressing Xox-MeDH do not excrete methanol, but do when expressing Mxa-MeDH (Krause *et al.* 2017). Thus acquisition/expression of Xox-MeDH may provide methanotrophs with a competitive growth advantage by limiting the loss of methanol.

The evidence that some methanotrophs have evolved via LGT of pMMO-encoding genes (and possibly sMMO-encoding genes) to methylotrophs raises the intriguing question of why aerobic methanotrophs have limited phylogenetic diversity, being largely restricted to the *Alpha-* and *Gammaproteobacteria*. That is, aerobic methylotrophs have been found in the *Alpha-*, *Beta-*, and *Gammaproteobacteria* as well as in the *Actinobacteria*, *Firmicutes*, and in some eukaryotes (Kolb 2009). Why then are there no identified methanotrophs in these additional classes/phyla/domain? This question cannot be unequivocally answered at this time, but it may be that such metabolisms exist in these phylogenetic groups, but have yet to be discovered.

Alternatively, it may be that the ability of a methylotroph to become a methanotroph requires more than LGT of MMO genes. Specifically, it is well-known that copper strongly affects the expression and activity of both sMMO and pMMO (Green,

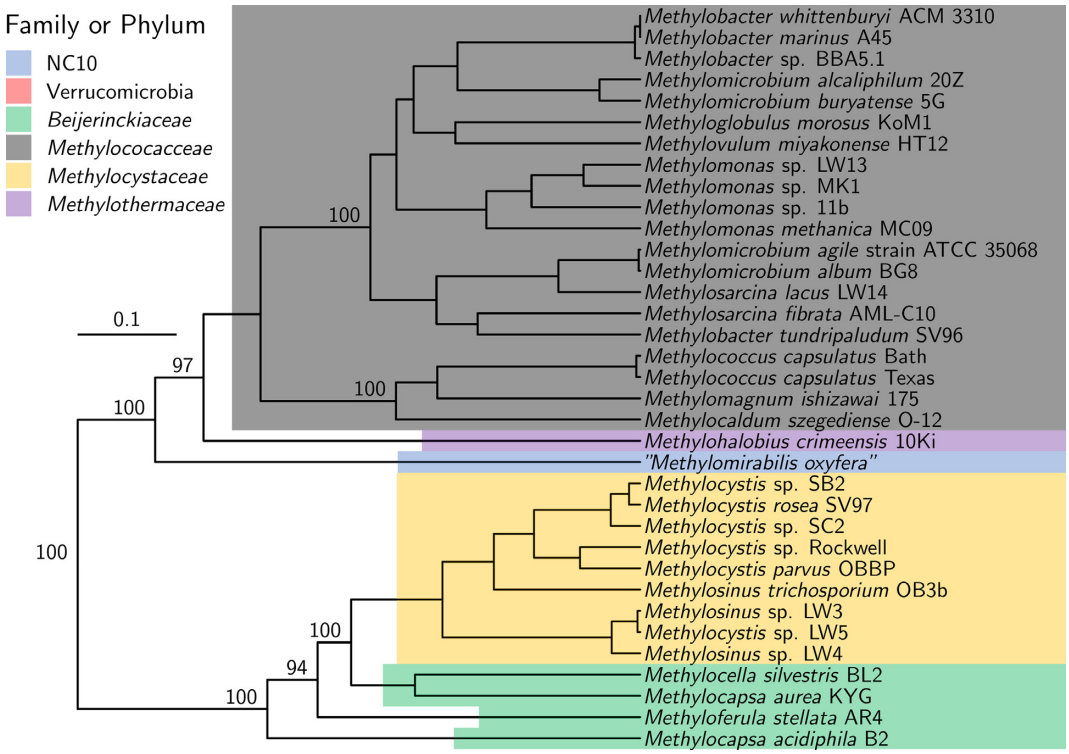

Figure 4. Bayesian *mxaFJGI* based phylogeny of aerobic methanotrophs. The tree was constructed using the general time-reversible model with invariant sites and four distinct gamma categories (GTR + I + G) under a strict clock with a minimum nucleotide sequence length of 3144. Node values indicate posterior probabilities based on 10 000 000 iterations with a burn-in of 25%. The scale bar represents 0.1 changes per nucleotide position.

Prior and Dalton 1985; Prior and Dalton 1985a, b; Leak and Dalton 1986; Semrau, DiSpirito and Yoon 2010; Semrau *et al.* 2013; Semrau *et al.* 2018), suggesting that conversion from a methylotrophic to a methanotrophic lifestyle not only requires incorporation of MMO genes, but also the means to sense, collect, and respond to copper. If this were true, one would expect that copper likely played a key role in the physiology of the methanotrophic ancestor. In such a case, genes other than those involved in methane oxidation would be expected to be regulated by copper in methanotrophs. Indeed, it has been shown that varying copper not only affects expression of genes encoding polypeptides of sMMO and pMMO in *Methylosinus trichosporium* OB3b, but also *mxaF* (encoding the large subunit of MxaMeDH) and genes involved in cell synthesis and transcriptional regulation (Farhan *et al.* 2015; Farhan Ul Haque *et al.* 2017; Gu and Semrau 2017). Further, copper affects the formation of intracytoplasmic membranes in *Methylococcus capsulatus* Bath (Choi *et al.* 2003), as well as the expression of genes involved in energy metabolism, cell synthesis, transcriptional regulation, and electron transport (Larsen and Karlsen 2016).

If copper uptake were required for the evolution of methanotrophy from methylotrophy, it is reasonable then to expect that all known aerobic methanotrophs would have identified copper uptake systems. Methanotrophs have been shown to have multiple mechanisms for copper uptake, i.e. methanobactin (found in the *Methylocystaceae*), MopE/CorA (found in the *Methylococcaceae*) and CopCD (found in the *Methylocystaceae*, *Methylococcaceae*, *Methylothermaceae, Beijerinckaceae* and NC10). If one assumes that the ability to collect copper is a requirement for the evolution of methanotrophy from methylotrophy, then it is reasonable to expect that these genes would either be predicted to be part of the ancestral genome or have been incorporated into methanotrophic genomes in the same time frame as MMO genes. Of these copper uptake systems, *copCD* was present in all but the Verrucomicrobia methanotrophs, and at least one copy of *copCD* in each methanotroph examined had low divergence values. Genes encoding for MopE/CorA also had low D_{KL} values, suggesting that this copper uptake system was present in the genome of the ancestor of *Methylococcaceae* methanotrophs prior to LGT of MMO. On the other hand, methanobactin appears to be ancestral for some methanotrophs of the *Alphaproteobacteria*, and a product of LGT for others. What is also notable is that representative methylotrophs do not have genes for methanobactin, MopE or CorA, although some appear to have *copCD* (Table S4, Supporting Information). Collectively the presence of multiple copper uptake systems in methanotrophs juxtaposed with the absence of copper-uptake systems in many methylotrophs provides circumstantial evidence that the evolution of methanotrophy from methylotrophy not only required LGT of MMO genes, but also the means to sequester copper.

It should be noted, however, that none of these copper uptake systems were found in Verrucomicrobia methanotrophs, although it also appears that MMO genes in these methanotrophs were the result of a LGT. At this time, it is unknown why these methanotrophs lack known copper uptake systems, but it may be due to copper availability not being an issue in the conditions these microbes grow. That is, in extremely low pH and metal-rich geothermal environments favored by these microbes (Op den Camp *et al.* 2009), metal availability is high as metal solubility increases with increasing H^+ concentrations. Further, at this time, it is unclear why *Methylocella* and *Methyloferula* species that cannot express pMMO have the CopCD copper uptake system as they would appear to have little need for collecting copper for pMMO activity, and copper uptake can inhibit sMMO

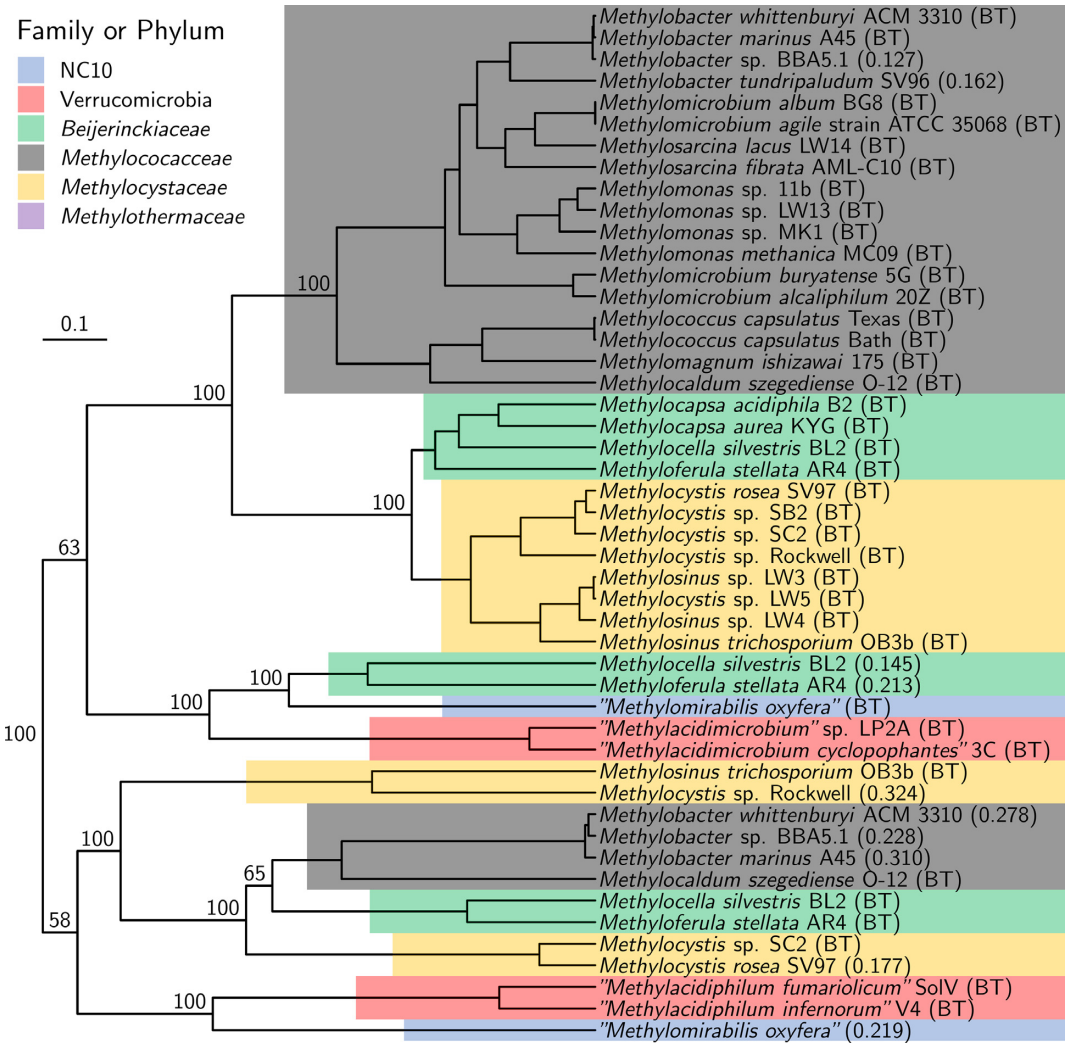

Figure 5. Bayesian *xoxFJ* based phylogeny of aerobic methanotrophs. The tree was constructed using the general time-reversible model with invariant sites and four distinct gamma categories (GTR + I + G) under a strict clock with a minimum nucleotide sequence length of 2604. Node values indicate posterior probabilities based on 10 000 000 iterations with a burn-in of 25%. The scale bar represents 0.1 changes per nucleotide position. Alien Hunter D_{KL} values are provided in parentheses. BT indicates below threshold.

expression. It has been suggested earlier that these methanotrophs likely once had *pmo* genes, but subsequently lost them (Tamas *et al.* 2014). In this case, the presence of *copCD* may be an evolutionary artefact. In addition, it may be that *copCD* expression is low in these strains, limiting copper uptake. We stress, however, that such statements should be considered speculative as no transcriptomic data have been published for these methanotrophs.

It is likely that aerobic methanotrophs evolved from methylotrophs via LGT of MMO genes, and that this has occurred several times independently (Khadka *et al.* 2018; Osborne and Haritos 2018). Here we present evidence that genes involved in carbon oxidation/carbon assimilation were present in the genomes of the receptor organisms before these LGT events, although additional copies of Xox-MeDH-encoding genes were later acquired by many methanotrophs via LGT. Transformation of an aerobic methylotroph to a methanotroph, however, not only required LGT of genes encoding for MMOs, but also the presence of a copper-uptake system(s), particularly for mesophilic aerobic methanotrophs. One or more copper uptake systems appear to have been encoded in the genomes of the receptor organisms prior to LGT of MMO-encoding genes.

There has been great interest in engineering methanotrophy in other microbes to valorize methane, currently a relatively inexpensive carbon source, into precursors of bioplastics and biofuels (Khmelenina *et al.* 2015; Strong, Xie and Clarke 2015; Strong *et al.* 2016). To date only limited success has been reported for the heterologous expression of methane monooxygenases in non-methylotrophs (Jahng and Wood 1994; Jahng *et al.* 1996; Sun and Wood 1996), with expression being difficult to maintain. It is recommended that future efforts to engineer methanotrophy in foreign hosts also consider incorporating a copper-uptake system to ensure sufficient quantities of copper are available for optimal expression/activity, particularly of the pMMO. In such an event, net copper uptake must be carefully controlled, however, to ensure that copper does not build up to toxic levels i.e. due to copper's high redox activity and binding to iron-sulfur proteins (Semrau *et al.* 2018). Strategies to regulate copper toxicity could include incorporation of copper efflux systems, e.g. *cusA*, and/or copper storage proteins found in methanotrophs (Vita *et al.* 2015; Vita *et al.* 2016; Gu and Semrau 2017). Including

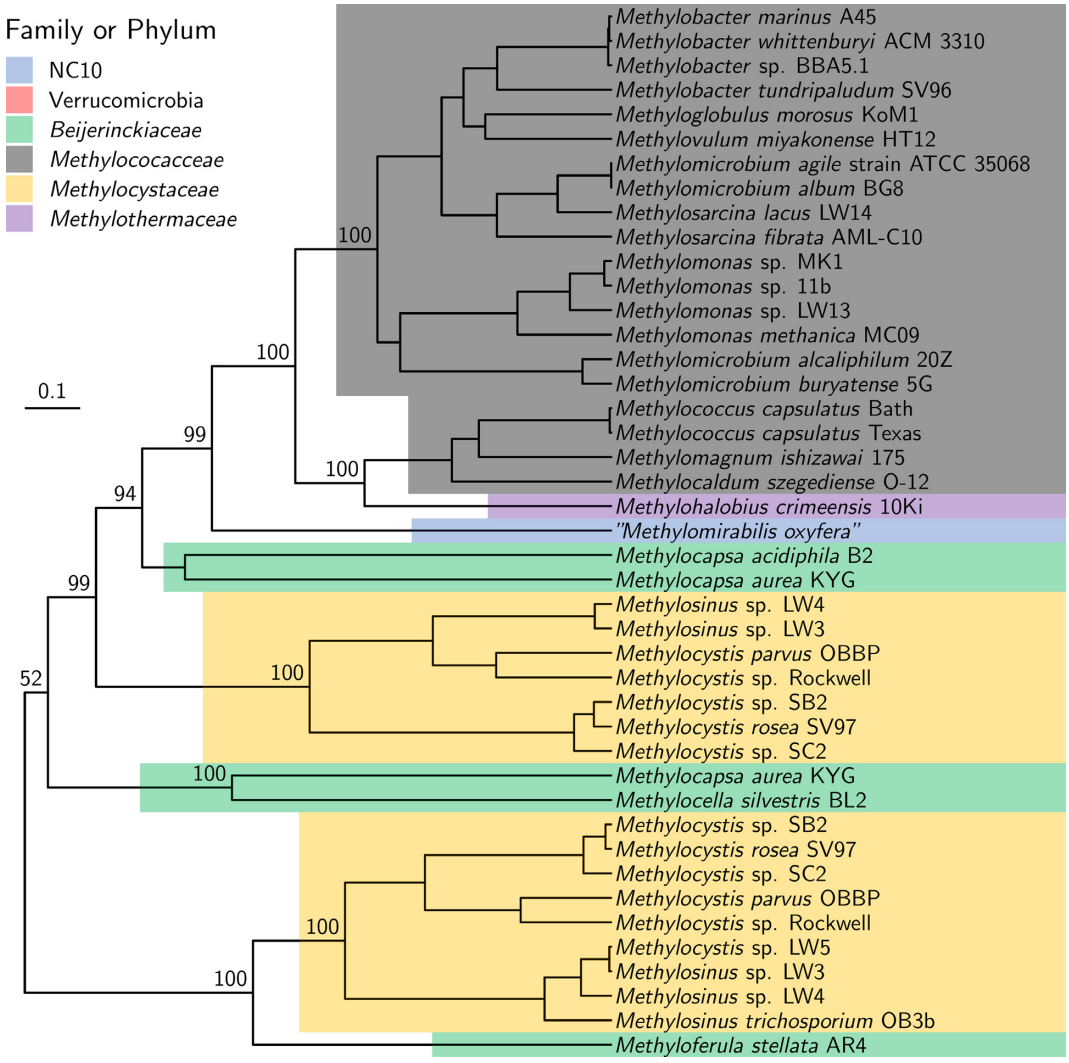

Figure 6. Bayesian *copCD* based phylogeny of aerobic methanotrophs. The tree was constructed using the general time-reversible model with invariant sites and four distinct gamma categories (GTR + I + G) under a strict clock with a minimum nucleotide sequence length of 1351. Node values indicate posterior probabilities based on 10 000 000 iterations with a burn-in of 25%. The scale bar represents 0.1 changes per nucleotide position.

such systems could also help optimize heterologous expression of sMMO.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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Conflict of interest. None declared.

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