

Bacterial flavin-containing monooxygenase is trimethylamine monooxygenase

Yin Chen¹, Nisha A. Patel, Andrew Crombie, James H. Scrivens, and J. Colin Murrell¹

School of Life Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom

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Flavin-containing monooxygenases (FMOs) are one of the most important monooxygenase systems in Eukaryotes and have many important physiological functions. FMOs have also been found in bacteria; however, their physiological function is not known. Here, we report the identification and characterization of trimethylamine (TMA) monooxygenase, termed Tmm, from *Methylocella silvestris*, using a combination of proteomic, biochemical, and genetic approaches. This bacterial FMO contains the FMO sequence motif (FXGXXHXHXXFY) and typical flavin adenine dinucleotide and nicotinamide adenine dinucleotide phosphate-binding domains. The enzyme was highly expressed in TMA-grown *M. silvestris* and absent during growth on methanol. The gene, *tmm*, was expressed in *Escherichia coli*, and the purified recombinant protein had high Tmm activity. Mutagenesis of this gene abolished the ability of *M. silvestris* to grow on TMA as a sole carbon and energy source. Close homologs of *tmm* occur in many *Alphaproteobacteria*, in particular *Rhodobacteraceae* (marine *Roseobacter* clade, MRC) and the marine SAR11 clade (*Pelagibacter ubique*). We show that the ability of MRC to use TMA as a sole carbon and/or nitrogen source is directly linked to the presence of *tmm* in the genomes, and purified Tmm of MRC and SAR11 from recombinant *E. coli* showed Tmm activities. The *tmm* gene is highly abundant in the metagenomes of the Global Ocean Sampling expedition, and we estimate that 20% of the bacteria in the surface ocean contain *tmm*. Taken together, our results suggest that Tmm, a bacterial FMO, plays an important yet overlooked role in the global carbon and nitrogen cycles.

Flavin-containing monooxygenases (FMOs) catalyze the NADPH-dependent N- or S- oxygenation of heteroatom-containing compounds (1). According to the definition by van Berkel et al. (2), FMOs belong to class B flavoprotein monooxygenases, together with Baeyer–Villiger monooxygenases (BVMO) and N-hydroxylating monooxygenases (NMO). This class of flavoprotein monooxygenases has characteristic conserved sequence motifs and contains enzyme-bound flavin adenine dinucleotide (FAD) (2, 3). FMOs in eukaryotes have been well studied, and diverse functions have been assigned to this group of enzymes (1, 4). The best known example of eukaryotic FMO is probably from mammals, where the predominant physiological function appears to be detoxification of a vast spectrum of xenobiotics, including trimethylamine (TMA), found in the diet of mammals. It has been shown that mutations in human FMO (isoform 3) cause the inheritable disease known as trimethylaminuria (fish odor syndrome) caused by the inability of the body to oxidize TMA to its nonodor-oxygenated form trimethylamine N-oxide (TMAO), the TMA subsequently causing malodor in urine and the breath (5). FMOs have also been found in plants and fungi. Plant FMOs are involved in the biosynthesis of the plant-growth hormone auxin (6, 7) and in pathogen defense (8–10). In fungi, FMOs are vital for the functional expression of proteins that contain disulfide bonds by controlling the redox potential within the endoplasmic reticulum (11–13). FMOs are also present in bacteria (14), and recently, the presence of FMO has been observed in many bacterial genomes. However, thus far, the physiological roles of bacterial FMOs are unknown.

We hypothesized that because eukaryotic FMOs oxidize TMA to TMAO, bacterial FMOs will be involved in TMA metabolism

by bacteria. An enzyme called TMA monooxygenase (termed hereafter Tmm) was suggested by Large et al. (15) in the 1970s to explain the ability of *Aminobacter aminovorans* (previously known as *Pseudomonas aminovorans*), which lacked known pathways for TMA utilization (16), to grow on TMA. In this bacterium, TMA is converted to TMAO by this enzyme, and TMAO is then further converted to formaldehyde [assimilated as the carbon (C) source] and ammonium [assimilated as the nitrogen (N) source] via the intermediate monomethylamine (MMA) (15–17). In other bacteria, such as *Pseudomonas* strain P, this enzyme is involved in utilization of TMA as a sole N source (17). However, the gene encoding Tmm has never been identified.

Methylated amine compounds, including TMA, are ubiquitous in the environment—for example, as end products of protein putrefaction (18). In the marine environment, methylated amines are released as a result of degradation of quaternary amine osmoregulators, such as glycine betaine, which are used by marine organisms to counteract water stress (19–21). Once released into the environment, methylated amines can be used by microorganisms as a C or N source. In fact, in the oceans, methylated amines represent a significant pool of C and N, and standing concentrations up to hundreds of nanomolar and micromolar have been reported in the water column (22, 23) and sediment pore water (24, 25), respectively. In addition to being involved in biogeochemical cycles of C and N, recent studies also suggest that methylated amines have the potential to affect global climate, being precursors of aerosol formation in the upper atmosphere (26–28).

We report here identification and characterization of Tmm (15, 17, 29) using a combination of genetic, proteomic, and bioinformatic approaches. The corresponding gene is particularly abundant in marine *Roseobacter* clade (MRC) and the SAR11 clade, two of the most abundant marine bacteria in the surface ocean (30–33), as well as in bacterial metagenomic sequences in the Global Ocean Sampling expedition (34). Our results suggest that this overlooked enzyme may play an important role in the global C and N cycles.

Results

Identifying the Function of a Bacterial FMO in *M. silvestris* as Tmm.

M. silvestris can grow on TMA as a sole C and N source. We detected inducible activities of three enzymes, Tmm (20.4 ± 1.9 nmol·min⁻¹·mg⁻¹), TMAO demethylase (71.2 ± 16.3 nmol·min⁻¹·mg⁻¹), and dimethylamine (DMA) monooxygenase (21.6 ± 0.7 nmol·min⁻¹·mg⁻¹), in cell-free extracts of this bacterium when it was grown on methylated amines. Because the genes encoding these enzymes have not yet been identified, we carried out comparative proteomic analyses using soluble protein fractions from methanol-, MMA-, and

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¹To whom correspondence may be addressed. E-mail: chen98@gmail.com or j.c.murrell@warwick.ac.uk.

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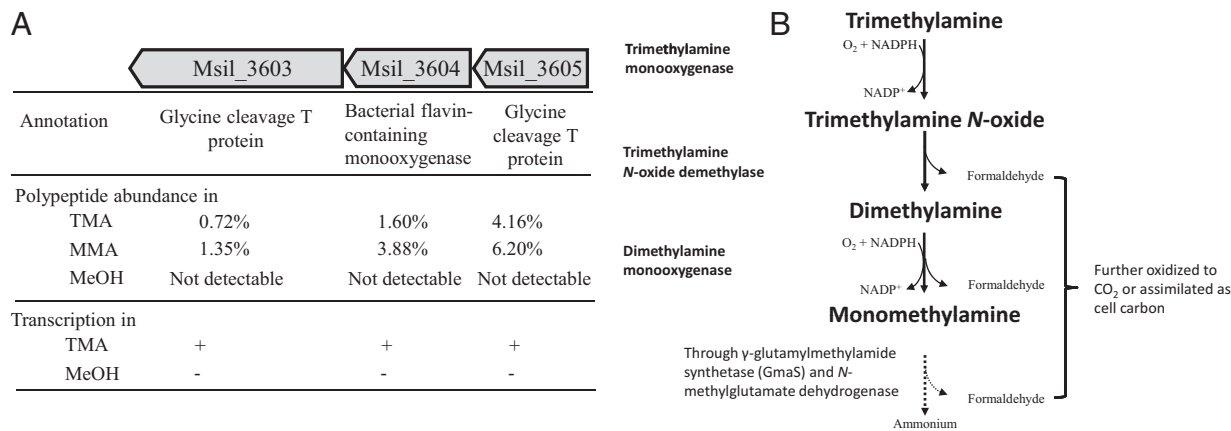


Fig. 1. (A) A summary of comparative proteomics and transcriptional analyses of the three-gene cluster containing a bacterial FMO in *M. silvestris*. The function was based on the annotation from the genome sequence using BLASTP search; the abundance of each of the polypeptides is shown in percentages of the total soluble proteome in each condition, and the presence (+) or absence (–) of transcription of each gene was confirmed by RT-PCR. MeOH, methanol; MMA, monomethylamine; TMA, trimethylamine. (B) The proposed pathway for TMA oxidation involving Tmm.

TMA-grown cultures. Quantitative proteomics data showed that polypeptides encoded by two gene clusters, orf Msil_2632-Msil_2639 and orf Msil_3603-Msil_3605 (Fig. 1A), were particularly abundant in cell-free extracts when *M. silvestris* was grown on TMA, whereas they were not detectable in cell-free extracts from methanol-grown cultures (a full list of polypeptides detected in each sample is shown in Dataset S1). We have previously shown that the former gene cluster was involved in MMA metabolism in *M. silvestris*, which encodes enzymes of the γ -glutamylmethylamide and *N*-methylglutamate pathway, converting MMA to formaldehyde (which is used as C and an energy source) and ammonium (which is used as an N source; Fig. 1B) (35).

The second gene cluster (Fig. 1A) encodes two proteins that are annotated as glycine cleavage T protein and a bacterial-type FMO (Msil_3604), which shows significant similarity to mammalian FMOs. RT-PCR confirmed that transcription of the three genes of this second gene cluster was induced by TMA. These three genes were cotranscribed as an operon, because primers targeting intergenic regions gave RT-PCR products of the expected sizes (primers used are listed in Table S1). We hypothesized that orf Msil_3603-Msil_3605 encodes the three enzymes that metabolize TMA to MMA (Fig. 1B), a pathway for TMA oxidation proposed by Large et al. in the 1970s (15, 17).

Sequence alignments with FMOs from mammals, plant, and fungi identified conserved domains in Msil_3604 (Fig. S1). Msil_3604 contains the three key characteristic sequence motifs of FMOs: the FAD-binding domain (GXGXXG), the FMO-identifying motif (FXGXXXHXXX/Y) (3), and an NADPH-

binding domain (GXSSXA). Phylogenetic analyses of Msil_3604 and known FMOs from mammals, plant, and fungi revealed that this bacterial FMO clustered within the known FMO family (Fig. 2).

To identify the function of Msil_3604, we cloned this gene from *M. silvestris* into pET28a, overexpressed it in *Escherichia coli*, and purified the resulting polypeptide by affinity chromatography (Fig. S2). Steady-state kinetic assays, performed at fixed NADPH concentrations with typical FMO substrates, showed Michaelis–Menten behavior (Table 1). As previously noted for other FMOs, FMO from *M. silvestris* had a broad substrate specificity, oxidizing many nitrogen- and sulfur-containing molecules such as glutathione and cysteine were not oxidized by this bacterial FMO. Interestingly, this enzyme can also oxidize dimethylsulfide (DMS), and the K_m value is similar to that of TMA. Of all of the substrates tested, this enzyme has the highest affinity for TMA, and it is therefore likely to encode the Tmm that is involved in TMA oxidation in this bacterium (Fig. 1B). To further confirm its role in TMA oxidation in *M. silvestris*, a mutant of Msil_3604 was constructed by marker-exchange mutagenesis. The growth rates of the mutant and wild-type strain of *M. silvestris* on various methylamine compounds are presented in Table 2. Although the wild-type strain grows on TMA as a sole C and N source, the mutant could no longer grow on TMA. Overall, our data indicate that this bacterial type FMO encodes the so-called Tmm in bacteria and is essential for bacterial TMA oxidation.

Tmm in Sequenced Bacterial Genomes. The sequence of *M. silvestris* Tmm was used to search microbial genome databases. No archaeal genomes contain Tmm homologs. However, homologs were found in the genomes of some *Gammaproteobacteria*, including *Methylophaga* sp. SK1, *Pseudomonas putida* GB-1, *P. fluorescens* SBW25, and *P. mendocina* ymp. *Methylophaga* sp. SK1 and *P. mendocina* are known to use TMA as a sole C and N

Table 1. Steady-state kinetic assays on purified Tmm of *M. silvestris* from recombinant *E. coli*

Substrate	K_m , μ M	V_{max} , nmol·min ⁻¹ ·mg ⁻¹
TMA	9.4 ± 2.1	29.4 ± 3.2
DMA	89.7 ± 13.2	6.9 ± 0.1
MMA	—	—
DMSO	3,575 ± 151	4.8 ± 1.5
DMS	10.3 ± 0.7	34.6 ± 0.2
Cysteamine	3,139 ± 534	76.2 ± 9.1
Methimazole	28.2 ± 5.1	14.4 ± 0.8
Dimethylaniline	35.7 ± 2.5	29.2 ± 0.9
Glutathione	—	—
Cysteine	—	—

—, substrate not oxidized.

Table 2. Specific growth rates of wild-type and *tmm* mutant of *Methylophaga silvestris* grown on methylated amines

Growth substrate	Wild type, h ⁻¹	Mutant, h ⁻¹
TMA	0.034 ± 0.003	—
DMA	—	0.039 ± 0.003
TMA + DMA	0.042 ± 0.008	0.037 ± 0.002
MMA	0.029 ± 0.003	0.029 ± 0.001

—, no growth.

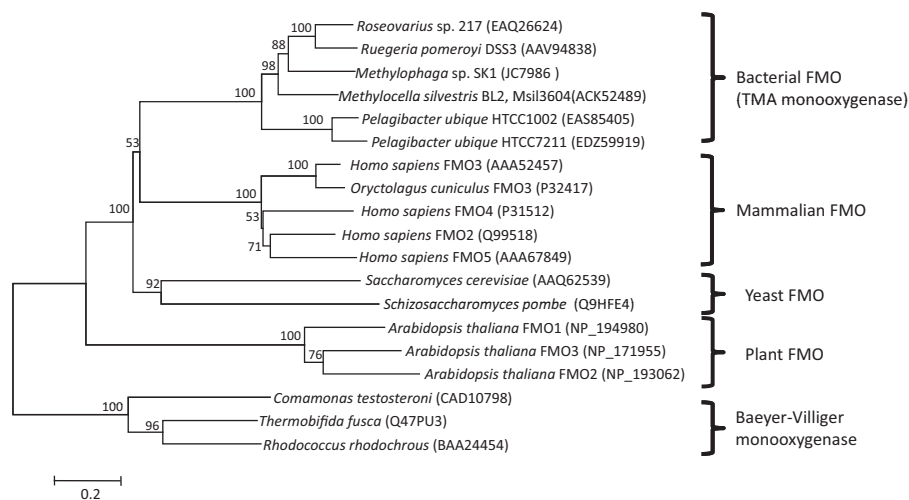


Fig. 2. A neighbor-joining phylogenetic tree showing the relationship of Tmm to other FMOs. The tree was drawn using the MEGA4 (50) based on an alignment of ~450 amino acids of FMOs. Baeyer-Villiger monooxygenases were used as an outgroup. Bootstrap values of 100 replicates are shown.

source (14, 36). Tmm homologs were also identified in the genomes of some terrestrial *Alphaproteobacteria*, including *Agrobacterium*, *Azorhizobium*, and *Mesorhizobium*. Interestingly, Tmm homologs are also found in many marine *Alphaproteobacteria*, including *Rhodobacteraceae* (also known as MRC) and three genomes of the SAR11 clade (*Pelagibacter ubique* sp. HTCC1002, HTCC1062, and HTCC7211), indicating a key role of TMA oxidation in marine C/N cycles.

A search of MRC genome sequences revealed that 15 of the 39 sequenced MRC genomes contain Tmm homologs (Table S2). All of these Tmm homologs have FAD and NADPH binding sites and the FMO sequence motif (FXGXXXHXXXX/Y; Fig. S1), and are clustered within the bacterial FMOs (Fig. 2). We tested 11 of these 39 MRC strains for their ability to use TMA as a C and/or N source. Seven strains containing Tmm homologs in their genomes grew on TMA as a sole N source (Table S2). Of these, three strains (*Roseovarius* sp. 217, *Roseovarius* sp. TM1035, and *Roseovarius nubinhibens* ISM) can also use TMA as a sole C and energy source. Four other strains tested, *Dinoroseobacter shibae*, *Oceanicola batsensis*, *Roseobacter* sp. SK209-2-6, and *Sagittula stellata*, which lack *tmm* in their genomes, did not grow on TMA as either a C or N source. Representative growth curves of *Ruegeria pomeroyi* DSS-3 and *Roseovarius* sp. 217 growing on TMA are shown in Fig. 3. Inducible Tmm activities were detected in the supernatant of TMA-grown cultures, and the expression of Tmm in TMA-grown cultures was confirmed by mass spectrometry (Fig. S3). To further prove the function of these Tmm homologs, we overexpressed *tmm* from *Roseovarius* sp. 217 and *Ruegeria pomeroyi*

DSS-3 in *E. coli* and purified them; they both oxidize TMA (Table 3).

The three genomes of members of the SAR11 clade also contain Tmm homologs. Due to the lack of available isolates to test their growth on TMA, we chemically synthesized two *tmm* genes (sp. HTCC1002 and HTCC7211), overexpressed these genes in *E. coli*, and further purified the recombinant proteins. Kinetics analyses demonstrated that these Tmm homologs can also oxidize TMA (Table 3).

Tmm Is Abundant in the Marine Bacterial Metagenome. The wide occurrence of Tmm homologs in the MRC and the SAR11 clade bacteria suggested that Tmm may play an important role in the marine C and N cycles. We therefore estimated the abundance of Tmm homologs in the Global Ocean Sampling (GOS) expeditions database (34). This database contains ~6.1 million deduced gene products from bacteria sampled directly from surface waters at various sites in the northwest Atlantic Ocean, the Sargasso Sea, and the eastern Pacific Ocean. Using a relatively stringent BLASTP search ($<e^{-80}$), we identified 570 Tmm homologs (Table 4). These homologs occur at nearly all GOS sites, except for two nonmarine sites (Lake Gatun and Punta Cormorant) and one marine site that was sampled with larger-sized fraction filters (Dirty Rock Cocos Island) (34). The retrieved Tmm homologs from the GOS dataset all contain the FMO sequence motif. The abundance of Tmm homologs is comparable to other enzymes carrying out key environmental functions in the marine environment, such as dimethylsulfoniopropionate-dependent demethylase (DmdA), which is involved in dimethylsulfoniopropionate (DMSP) catabolism (37) and is

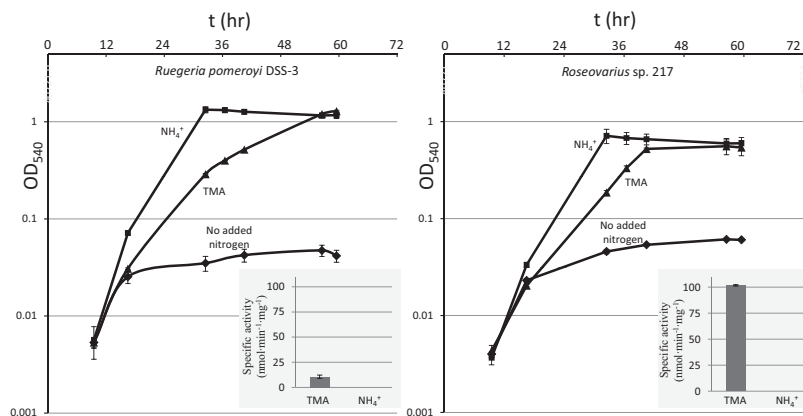


Fig. 3. Representative growth curves of *Ruegeria pomeroyi* DSS-3 and *Roseovarius* sp. 217 growing on TMA or ammonium as the sole nitrogen source. Controls were set up with no added nitrogen. (Insets) Activity of Tmm from the supernatant of the crude extract of *R. pomeroyi* DSS-3 and *Roseovarius* sp. 217 cultures, respectively. No enzyme activities were detected when ammonium was used as the sole nitrogen source. Error bars indicate SD of each experiment ($n = 3$).

Table 3. Kinetic parameters with TMA for purified recombinant Tmm enzymes

Substrate	K_m , μM	V_{max} , $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$
<i>Roseovarius</i> sp. 217	21.6 ± 1.9	$1,133.6 \pm 58.6$
<i>Ruegeria pomeroyi</i> D5S-3	20.8 ± 2.9	267.7 ± 52.2
<i>Pelagibacter ubique</i> HTCC1002	27.5 ± 4.2	70.8 ± 7.7
<i>Pelagibacter ubique</i> HTCC7211	28.5 ± 4.4	67.3 ± 3.2

about 10× more frequent than DddQ, DddP, and DddL, which are involved in DMSP-dependent DMS production (38). On average, we estimate that ~20% of the microbial genomes in the surface ocean contain Tmm homologs (Table 4). Phylogenetic analysis indicated that the Tmm homologs from the GOS database could be placed into three groups (Fig. 4), the majority of which were closely related to Tmm homologs in SAR11 clade bacteria (94.1%) and the MRC bacteria (4.7%), respectively (Fig. S4). The predominance of the MRC and the SAR11 clade bacteria in marine TMA metabolism is supported by the analysis of γ -glutamylmethylamide synthetase (GmaS), an enzyme in-

involved in a subsequent step in the pathway of TMA metabolism (Fig. 1B) (35, 39). GmaS homologs are also found at nearly all GOS sites and are as abundant as the Tmm enzyme (Table 4). Phylogenetic analysis also indicated the sequences retrieved from the GOS dataset are from either the SAR11 clade (78%) or the MRC (22%; Fig. S5).

Discussion

In this study we have identified the gene encoding Tmm (29, 40). The wide occurrence of Tmm homologs in the MRC and the SAR11 clade bacteria indicates that methylated amine compounds may play an important role in marine C and N cycles. These two groups of marine bacteria are particularly abundant in the surface oceans, and previous studies have shown that they play a key role in marine carbon and sulfur cycles (30–33, 41, 42). Although few studies have measured the concentrations of methylated amines in the oceans, existing data suggest that these compounds represent a significant pool of C and N in the marine water column (22, 23) and in marine and coastal sediments (24, 25). We tested representatives of the MRC bacteria, and the ability to use TMA is directly correlated to the presence of Tmm

Table 4. Distribution of Tmm homologs at different sampling sites in the Global Ocean Sampling expedition metagenomic data set

Sample no.	Sampling site	Description	Tmm*	GmaS*	DddP*	DddD*	DddL*	DmdA*	RecA*	% genomes with Tmm [†]	% genomes with GmaS [†]
2	Gulf of Maine	Coastal	12	16	1	2	0	21	35	29	39
3	Brown's Bank-Gulf of Maine	Coastal	13	11	0	0	0	39	52	24	21
4	Outside Halifax Nova Scotia	Coastal	30	21	0	0	0	34	40	55	38
5	Bedford Basin Nova Scotia	Embayment	7	7	2	3	0	26	34	18	18
6	Bay of Fundy Nova Scotia	Estuary	23	25	0	0	0	50	80	39	42
7	Northern Gulf of Maine	Coastal	8	14	2	0	0	20	53	13	23
8	Newport Harbor	Coastal	2	7	4	0	0	9	43	3	12
9	Block Island	Coastal	15	15	0	0	0	28	33	39	39
10	Cape May	Coastal	18	19	0	0	0	36	63	27	29
11	Delaware Bay	Estuary	5	2	0	0	0	7	43	10	3
12	Chesapeake Bay	Estuary	5	4	0	0	0	17	39	10	8
13	Off Nag's Head	Coastal	7	9	1	0	0	17	43	18	24
14	South of Charleston	Coastal	18	14	0	0	0	42	67	29	22
15	Off KeyWest	Coastal	5	15	2	0	0	35	51	8	26
16	Gulf of Mexico	Coastal	14	7	1	0	0	41	50	26	13
17	Yucatan Channel	Open ocean	9	13	1	0	0	32	61	15	23
18	Rosario Bank	Open ocean	9	13	3	0	0	43	83	14	20
19	Northeast of Colon	Coastal	6	16	2	0	0	33	72	10	27
20	Lake Gatun	Fresh water	0	1	0	0	0	3	61	0	1
21	Gulf of Panama	Coastal	5	14	4	0	0	31	53	10	27
22	250 miles from Panama City	Open ocean	12	16	1	0	0	35	50	22	28
23	30 miles from Cocos	Open ocean	11	16	0	0	0	35	54	18	26
25	Dirty Rock Cocos Island	Fringing reef	0	1	1	0	0	5	13	0	6
26	134 miles NE of Galapagos	Open ocean	7	11	1	0	0	41	55	13	20
27	Devil's Crown	Coastal	16	13	4	0	0	41	66	26	20
28	Coastal Floreana	Coastal	10	12	2	0	0	48	70	16	20
29	North James Bay	Coastal	9	24	2	0	0	46	50	20	52
30	Warm Seep Roca Redunda	Warm seep	19	17	1	0	0	52	55	36	33
31	Upwelling Fernandina Island	Coastal	26	27	1	0	0	57	61	46	49
32	Mangrove on Isabella	Mangrove	8	5	1	1	0	16	43	20	14
33	Punta Cormorant	Hypersaline	0	2	4	0	6	2	43	0	4
34	North Seamore Island	Coastal	9	17	2	0	0	42	51	17	32
35	Wolf Island	Coastal	11	13	2	0	0	38	51	21	26
36	Cabo Marshall	Coastal	14	19	0	0	0	45	54	24	33
37	Equatorial TAO	Open ocean	14	6	5	0	0	48	38	30	13
47	201 miles from French Polynesia	Open ocean	9	18	5	0	0	48	62	15	29
51	Rangiora Atoll	Coral reef	12	14	2	0	0	28	53	25	29
		Average	11	13	1.5	0.2	0.2	32	52	20	23

*The sampling sites are as detailed in Howard et al. (37). Numbers of Tmm and GmaS homologs, per 100,000 reads, with BLASTP values of $<e^{-80}$. Values of DddD, DddL, DmdA, and RecA were from Howard et al. (37), and values of DddP were from Todd et al. (38).

[†]Frequencies of Tmm- and GmaS-containing strains, expressed as genome equivalents, as described in Howard et al. (37).

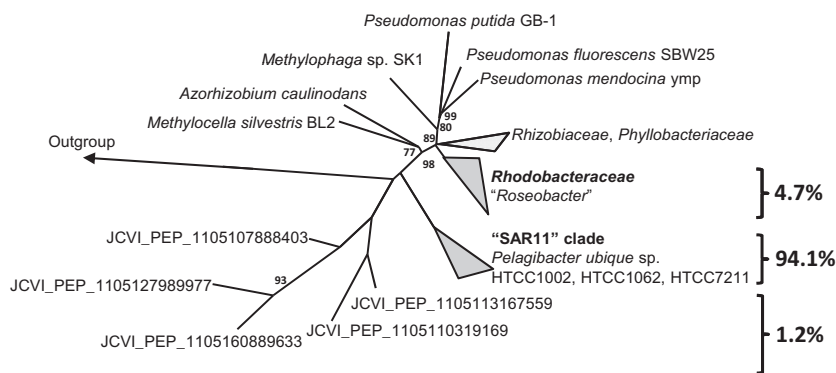


Fig. 4. An unrooted tree showing Tmm homologs retrieved from sequenced bacterial genomes and the Global Ocean Sampling expedition data set. The neighbor-joining tree was constructed using sequences retrieved from sequenced bacterial genomes (~450 amino acids). Environmental sequences were added by parsimony. Bootstrap values were calculated based on 100 replicates. *Homo sapiens* FMO3 was used as the outgroup.

in their genomes (Table S2). The purified Tmm enzyme of MRC and SAR11 bacteria all oxidize TMA (Table 3), indicating that this enzyme is likely to contribute to the removal of methylated amines in the marine environment. Further evidence comes from analysis of the GOS data set, where it is estimated that, on average, 20% of the bacteria in the surface ocean contain Tmm, the majority of which originated from the SAR11 clade bacteria (Table 4). Indeed, previous metaproteome analysis of bacteria collected from surface seawater has identified Tmm polypeptides from SAR11 clade bacteria (43). Therefore, it is tempting to speculate that the MRC and the SAR11 clade bacteria may play key roles in sequestering N from methylated amines; this may therefore give them a selective advantage in the oceans over microorganisms unable to use such compounds, where they face severe competition for N.

The purified Tmm has broad substrate specificity and can also oxidize DMA, although the affinity for DMA is lower than for TMA (Table 1 and Table S3). This ability to oxidize secondary amines has been shown previously with purified TMA monooxygenase from *A. aminovorans*, and it has been shown that oxidizing DMA has no physiological function (40). Nonspecific oxidation of DMA produces formamide, which is toxic (40), and this may have inhibited the growth of the wild-type *M. silvestris* on DMA alone (Table 2). This finding is supported by the fact that wild-type *M. silvestris* can grow on TMA in the presence of DMA (Table 2), because TMA is the preferred substrate of Tmm. Although Tmm from *M. silvestris* can oxidize DMA, its function as a DMA monooxygenase can be ruled out because FMOs are not sensitive to carbon monoxide, a characteristic of secondary amine monooxygenases (44). In fact, DMA monooxygenase would release formaldehyde from the oxidation of DMA (Fig. 1B), and the presence of tetrahydrofolate-binding domains in orfs Msil_3603 and Msil_3605 suggests that one of these genes may encode the DMA monooxygenase (the other is likely to encode TMAO demethylase, which also releases formaldehyde).

The ability of bacterial FMOs to oxidize DMS and DMSO is surprising. DMSO was initially used as a solvent to dissolve some of the substrates. It was noticed previously that the FMO from *Methylophaga* sp. SK1 also oxidized DMSO (45). The ability of bacterial FMOs to oxidize DMS may be of special interest. It has been previously noted that there is an uncharacterized route for oxidation of DMS to DMSO by heterotrophic bacteria, such as *Delftia acidovorans* (46). In this bacterium, the oxidation of DMS to DMSO was dependent on NADPH, which could not be replaced by NADH. A search of the genome of *D. acidovorans* sp. SPH-1 does indeed reveal FMO-like protein-encoding genes. Because FMO of the MRC and the SAR 11 clade bacteria can also oxidize DMS, they may also represent a previously uncharacterized sink of DMS from the marine environment. Whether FMO is responsible for this uncharacterized DMS conversion in these bacteria, and to what extent they reduce DMS emission from the marine environment, certainly warrants further investigation.

To conclude, our results reveal the function of bacterial FMOs as an enzyme involved in bacterial TMA metabolism, Tmm. The wide occurrence of this enzyme in marine bacteria, together with its high abundance in the surface ocean, points to a key role for methylated amines in marine C and N cycling.

Materials and Methods

Cultivation of *Methylocella silvestris*. *M. silvestris* was grown at 25 °C either in a 4-L fermentor or in 125-mL serum vials using diluted mineral salt medium as described previously (35). To test if the trimethylamine monooxygenase (*tmm*) mutant could grow on methylated amines, growth experiments were set up in triplicate using 120-mL serum vials, containing 20 mL medium with an inoculum size of 10%. Details of growth conditions and reagents are described in *SI Materials and Methods*.

Quantitative Comparative Proteomics. A total of 700 μ g of soluble protein extract from MMA, TMA, and methanol-grown *M. silvestris* cells were used for proteomic analyses (47). The details of the method are shown in *SI Materials and Methods*.

Marker-Exchange Mutagenesis of *tmm* in *M. silvestris*. To construct a *tmm* mutant of *M. silvestris*, a downstream region (containing a KpnI site) and an upstream region (containing an MluI site) of the target were amplified by PCR and subcloned into pGEM-T vector (Promega) together with a kanamycin gene cassette (amplified from pCM184) inserted between the two regions (primers used are listed in Table S1). The resulting plasmid was then cut with KpnI and MluI, and the 2.6-kb fragment containing the kanamycin gene cassette was electroporated into *M. silvestris* as described previously (35).

Overexpression of *tmm* in *E. coli*. The *tmm* genes from *M. silvestris*, *Roseovarius* sp. 217, and *Ruegeria pomeroyi* D5S-3 were amplified by PCR (primers used are listed in Table S1) and subcloned into pGEM-T vector (Promega), which were then excised using NdeI/HindIII and ligated into the expression vector pET28a (Merck Biosciences). The *tmm* homologs in the *Pelagibacter ubique* genomes (strain HTCC1002 and HTCC7211) were synthesized commercially with *E. coli* codon usage (GenScript Corporation). The synthesized genes were inserted into the expression vector pET28a using the NdeI/HindIII restriction sites. The resulting plasmids were then transformed into the expression host *E. coli* BLR(DE3) pLysS (Merck Biosciences). To overexpress TMA monooxygenases, *E. coli* cells were grown at 37 °C to an OD₅₄₀ of 0.6, and isopropyl β -D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.1 mM.

Enzyme Assays and Kinetics. Cells were broken for protein analyses and enzyme assays by passing three times through a French pressure cell (American Instrument Co.) at 110 megapascals. Cell debris was removed by centrifugation at 6,000 \times g for 15 min. One-dimensional protein analyses were carried out using a precast NuPAGE Bis-Tris gel (10%; Invitrogen). All enzyme assays were carried out in triplicate in 80 mM Pipes buffer (pH 7.6) at room temperature (22 °C) unless otherwise stated. Details are shown in *SI Materials and Methods*.

Cultivation of MRC Bacteria on Methylamines. Growth tests were carried out using a defined medium in triplicate, containing artificial sea salts from Sigma-Aldrich (S9883) 40 g L⁻¹, sodium phosphate 0.2 mM (pH 8.0), Hepes 10 mM (pH 8.0), methylated amines 2 mM, FeCl₃ 50 μ M, and a mixture of C

sources containing glucose (5.6 mM), fructose (5.6 mM), succinate (8.5 mM), pyruvate (11.4 mM), glycerol (10.9 mM), and acetate (17 mM). The vitamins were added as described in *SI Materials and Methods*. Positive growth controls with ammonium chloride (1.0 mM) and negative controls with no added N compounds were also set up. To test if these isolates could use TMA as a sole C source, the above medium was used, except that the carbon mixture was omitted and TMA was added to a final concentration of 5 mM.

Identification of Tmm and GmaS Homologs in the GOS Metagenome. Tmm sequence of *M. silvestris* was used as the query sequence for a BLASTP search of the GOS peptides at CAMERA [<https://portal.camera.calit2.net/gridsphere/gridsphere?cid=>]; GOS: all ORF peptides (P) database, e⁻⁸⁰, and resulted in 568 unique sequences. An additional search using the same settings was performed using the FMO of *Methylophaga* sp. SK1 (JC7986) as the query, and two additional sequences were retrieved. These 570 sequences were further grouped into 127 unique groups (identity >90% within each group) using the CD-HIT program (48). Representative sequences from each group

were aligned using the ARB program (49), and each sequence was manually checked for the FMO sequence motif. To estimate the frequency of Tmm-containing cells, the numbers of Tmm homologs were normalized against the average numbers of the six essential single-copy genes (*atpD*, *rpoB*, *gyrB*, *hsp70*, *tufA*, and *recA*) as described previously (37). The identification of GmaS homologs were estimated as described, except that the GmaS of *M. silvestris* (ACK51558) was used as the query, which resulted in 682 unique sequences. Additional queries using the GmaS sequences of *M. universalis* FAM5 (ADH10360), *M. mays* (BAF99006), and *A. tumefaciens* C58 (AAK89209) did not yield additional new sequences. These 682 sequences were further grouped by CD-HIT and analyzed by ARB.

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