Supporting Information

Chen et al. 10.1073/pnas.1112928108

SI Materials and Methods

Cultivation of *Methylocella silvestris*. *M. silvestris* was grown at 25 °C in either a 4-L fermentor or 125-mL serum vials using diluted mineral salt medium as described previously (1). The following compounds were used as sole carbon and nitrogen sources, trimethylamine hydrochloride (10 mM), dimethylamine hydrochloride (10 mM), monomethylamine hydrochloride (10 mM), trimethylamine *N*-oxide (TMAO, 10 mM), and methanol (10 mM) plus ammonium chloride (2 mM). For enzyme assays, cells were harvested from the fermentor at late exponential phase, resuspended in 10 mM 1,4-piperazine diethanesulfonic acid (Pipes) buffer (pH 7.6), and then stored at –80 °C before assays were carried out. To test if the trimethylamine monooxygenase (*tmm*) mutant could grow on methylated amines, all growth experiments were set up in triplicate using 120-mL serum vials containing 20 mL medium with an inoculum size of 10%. The serum vials were incubated at 25 °C in a shaker (150 rpm, Sanyo-Gallenkamp Inc).

Cultivation of *Ruegeria pomeroyi* DSS-3 and *Roseovarius* sp. 217. *Ruegeria pomeroyi* DSS-3 and *Roseovarius* sp. 217 were cultivated in shake flasks in triplicate using a defined medium containing artificial sea salts from Sigma-Aldrich (S9883) 40 $g{\cdot}L^{-1}$, sodium phosphate 0.2 mM (pH 8.0), Hepes 10 mM (pH 8.0), $FeCl_3$ 50 μM, and malate (10 mM). The vitamins were added as described previously (2). The nitrogen sources used were trimethylamine (2.0 mM) or ammonium chloride (2.0 mM). Negative controls, without added N compounds, were also set up to evaluate contamination of N in the chemicals used for growth. Samples were taken at various time points to determine the optical density at 540 nm. Subsets of samples were taken at late exponential phase, and cell-free crude extracts were prepared. These were then used to determine the NADPH-dependent Tmm activities using the assay as described below.

Quantitative Comparative Proteomics. A total of 700 μg of soluble protein extract from MMA, TMA, and methanol grown *M. silvestris* cells were resuspended in 1 mL of 0.1% RapiGest (Waters Corporation) and concentrated using a 5-kDa cutoff spin column. The solution was then heated at 80 °C for 15 min, reduced with 100 mM DTT at 60 °C for 15 min, alkylated in the dark with 200 mM iodoacetamide at ambient temperature for 30 min, and digested with 1:50 (wt/wt) sequencing-grade trypsin (Promega) at 37 °C overnight. RapiGest was hydrolyzed by the addition of 2 μL of 15 M HCl, vortexed, and centrifuged in a 0.22-μm Nylon membrane filter (Corning Inc.) to remove particulate matter. Each sample was diluted 1:1 with 100 fmol/μL rabbit glycogen phosphorylase B standard tryptic digest in 0.1% (vol/vol) formic acid, to give a final protein concentration of 500 ng/μL and an internal standard concentration of 50 fmol/μL. Nanoscale liquid chromatography (LC) separations of tryptic peptides were performed with a nanoACQUITY ultrapressure liquid chromatograph system (Waters Corporation) and mass analyzed on a Synapt HDMS (Waters Corporation) in a data-independent manner (MS^E). Details of LC and mass spectrometer configurations are described elsewhere (3).

Raw data files were processed using ProteinLynx Global Server v.2.4 and searched against a *M. silvestris* database. The sequence for rabbit phosphorylase B and contaminants from the common Repository of Adventitious Proteins proteins were added to the database, along with randomized sequences for each database entry. A fixed modification of carbamidomethyl-C was specified, in addition to variable modifications acetyl N terminus, deamidation N, deamidation Q, and oxidation M. Automatic settings for mass accuracy were used, and one missed tryptic cleavage site was allowed. The protein identification criteria included the detection of at least three fragment ions per peptide, a minimum of seven total product ion matches per protein, a minimum of one peptide determined per protein, and identification of the protein in at least two of the four technical replicates.

A label-free method was used to allow simultaneous identification and quantification of proteins in a LC-MS workflow. This method is based on the relationship between peptide peak area and intensity observed by electrospray ionization MS and protein concentration (4). With the use of an internal standard, this relationship was used to calculate a universal response factor. Absolute quantification of a protein was determined by the application of the universal response factor to its corresponding peptides.

Enzyme Assays and Kinetics. For cell-free crude extract, Tmm and DMA monooxygenase activities were measured by following the decrease in absorbance at 340 nm of substrate-dependent oxidation of NADPH (Sigma-Aldrich). The following concentrations of substrates were used: TMA or DMA (1 mM) and NADPH (0.1 mM). TMAO demethylase activity was measured by quantifying the substrate-dependent production of formaldehyde using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald reagent; Sigma) as described previously (1).

Overexpressed TMA monooxygenases were purified using a His-tag protein purification kit as described in the manufacturer's protocol (Merck), except that the column was washed using a wash buffer containing 80 mM imidazole before elution to remove trace protein contaminants. Kinetic assays were performed in triplicate at 22 °C on a UV-visible spectrophotometer. A 1-mL mixture contained 0.8 mg purified enzyme, 80 mM Pipes (pH 7.6), and 0.25 mM NADPH. The reaction was initiated by adding substrate, and the decrease in absorbance at 340 nm was recorded continuously for 3 min. All substrates were dissolved in water except *N,N*-dimethylaniline, which was dissolved in ethanol.

1. Chen Y, et al. (2010) γ-Glutamylmethylamide is an essential intermediate in the metabolism of methylamine by *Methylocella silvestris*. *Appl Environ Microbiol* 76: 4530–4537.
2. Kanagawa T, Dazai M, Fukuoka S (1982) Degradation of *O, O*-dimethyl phosphorodithioate by *Thiobacillus thioparus* TK-1 and *Pseudomonas* AK-2. *Agric Biol Chem* 46:2571–2578.
3. Patel VJ, et al. (2009) A comparison of labeling and label-free mass spectrometry-based proteomics approaches. *J Proteome Res* 8:3752–3759.
4. Silva JC, Gorenstein MV, Li GZ, Vissers JP, Geromanos SJ (2006) Absolute quantification of proteins by LCMSE: A virtue of parallel MS acquisition. *Mol Cell Proteomics* 5: 144–156.

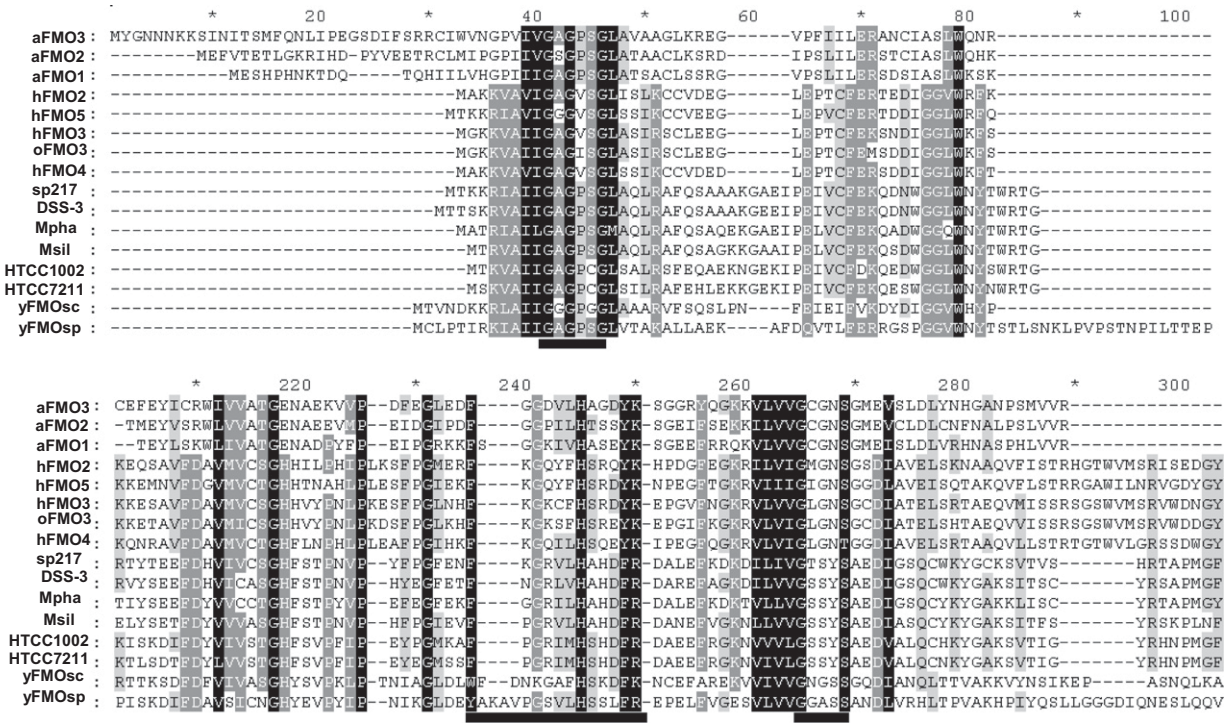

Fig. S1. Alignment of the sequences of *Methylocella silvestris* FMO (Msil), *Methylopha* sp. SK1 FMO (Mpha), *Homo sapiens* FMOs (hFMO2, hFMO3, hFMO4, hFMO5), *Oryctolagus cuniculus* FMO3 (oFMO3), *Arabidopsis thaliana* FMOs (aFMO1, aFMO2, aFMO3), *Saccharomyces cerevisiae* FMO (yFMOsc), *Schizosaccharomyces pombe* FMO (yFMOsp), and FMOs from *Roseovarius* sp. 217 (sp217), *Ruegeria pomeroyi* DSS-3 (DSS-3), and *Pelagibacter ubique* HTCC1002 and HTCC7211. The black bars indicate the FAD binding domain, the fingerprint sequence for FMOs, and the NADP binding domain, respectively. The alignment was performed with ClustalX and displayed with Genedoc.

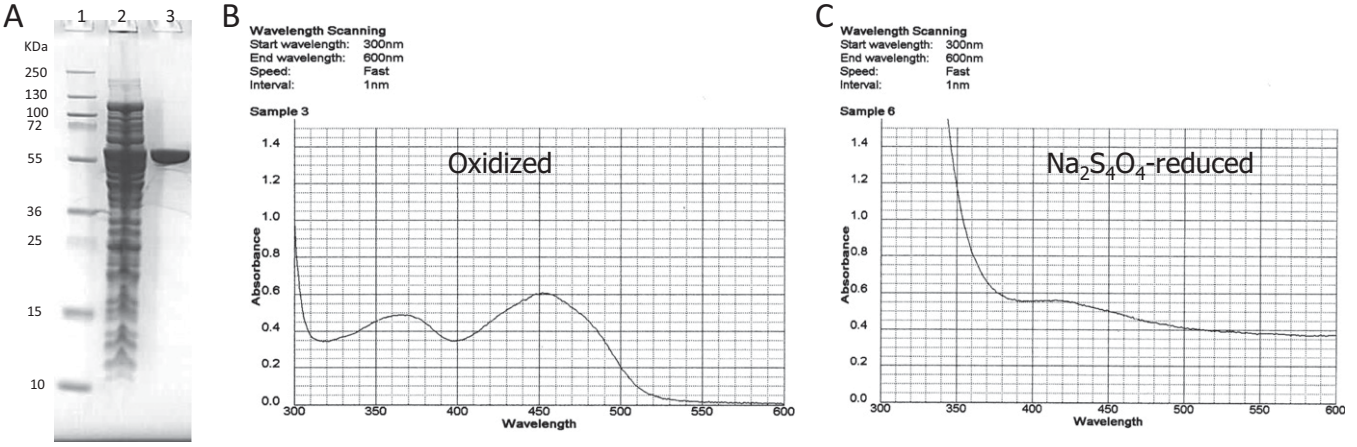

Fig. S2. (*A*) One-dimensional gel electrophoresis of purified Tmm of *Methylocella silvestris* from recombinant *E. coli*. Lane 1, protein marker; lane 2, cell-free extract of *E. coli* BLR(DE3) [pET28a-*tmm*] induced by isopropyl β-D-1-thiogalactopyranoside; lane 3, purified Tmm from recombinant *E. coli*. Absorbance spectra of oxidized (*B*) and reduced (*C*) form of the recombinant Tmm (8 mg·mL^{-1}).

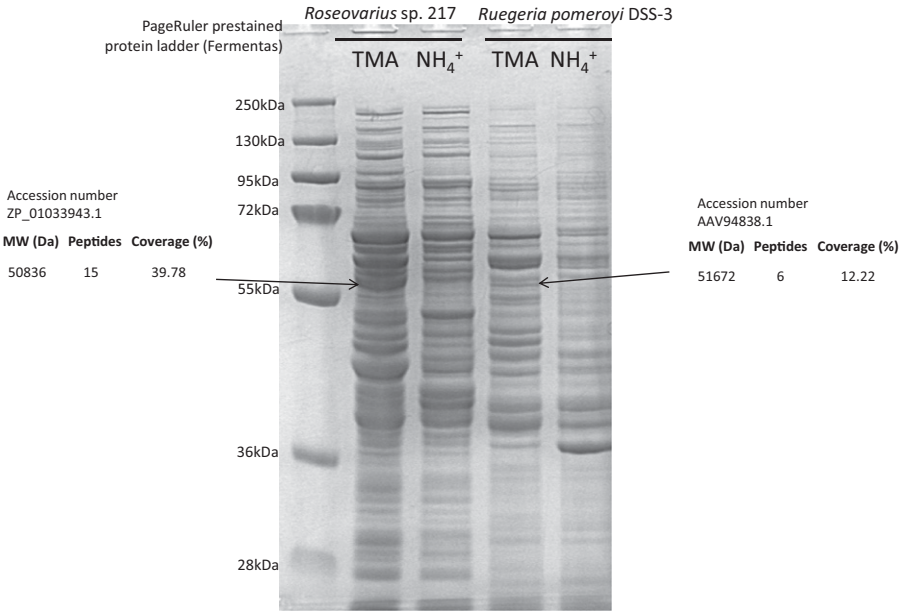

Fig. S3. One-dimensional gel electrophoresis of crude extract of TMA-grown and ammonium-grown *Roseovarius* sp. 217 and *Ruegeria pomeroyi* DSS-3, respectively. The expression of Tmm homologs in TMA cultures was confirmed by MALDI/MS analyses.

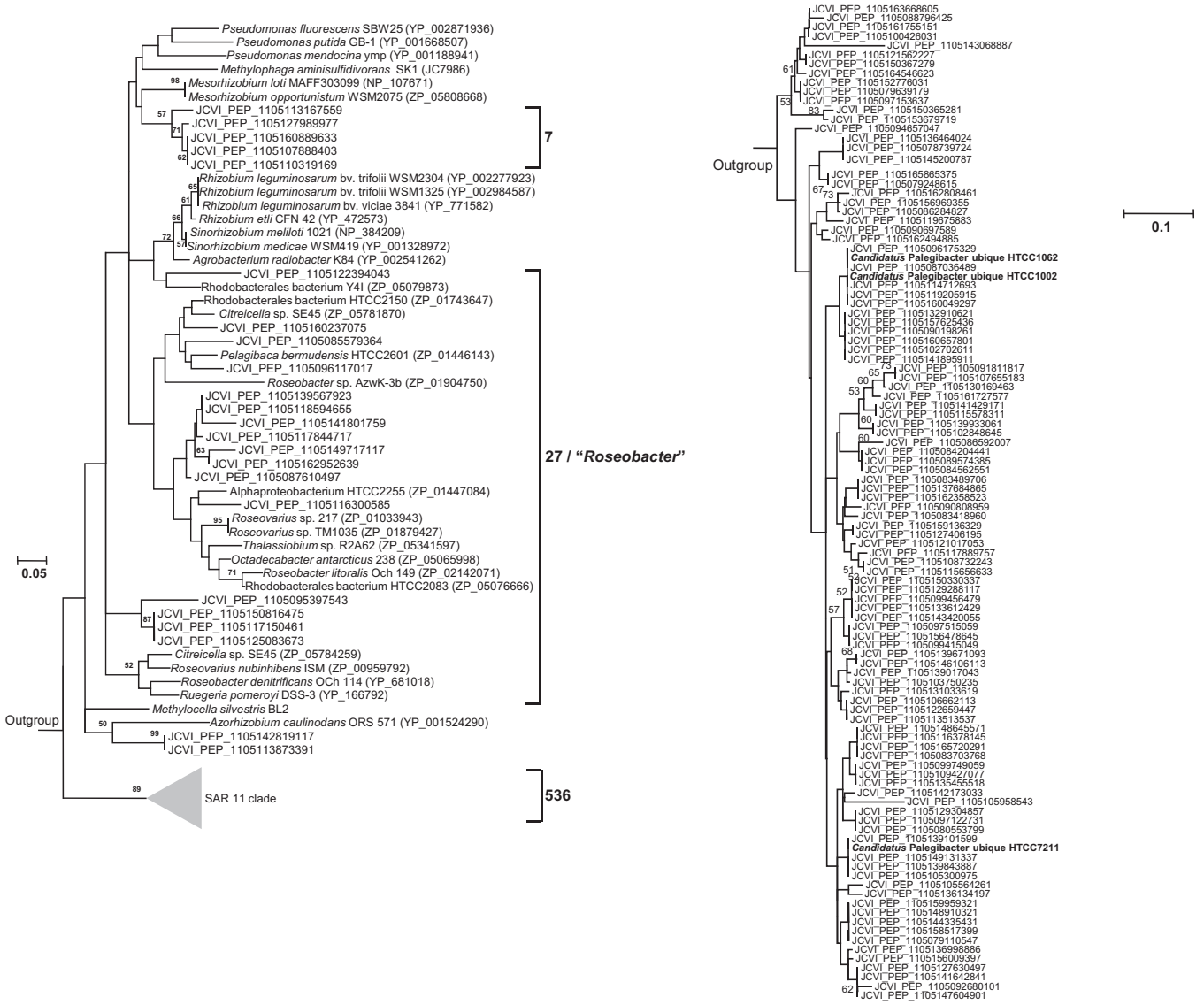

Fig. S4. A phylogenetic tree showing the Tmm homologs from marine *Roseobacter* clade bacteria (*A*) and marine SAR11 clade bacteria (*B*), respectively.

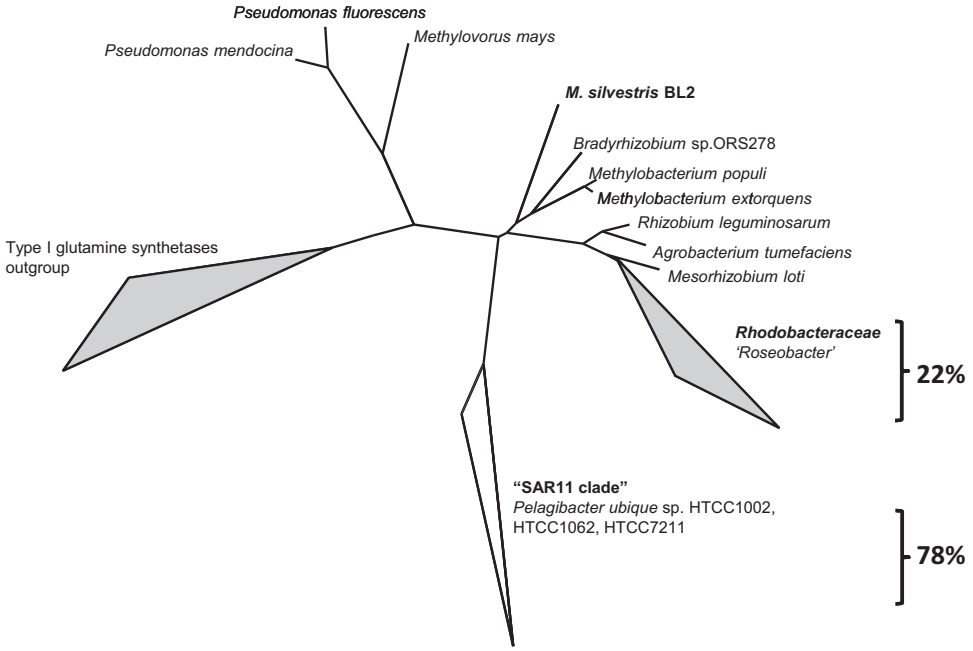

Fig. S5. A phylogenetic tree showing the GmaS homologs from sequenced bacterial genomes and environmental sequences retrieved from the GOS data set.

Table S1. Oligonucleotides used in this study

Target	Primer sequences (5′–3′)		Product size, bp	Annealing temperature, °C
	Forward primer	Reverse primer		
Msil3604	CGCCAGCCAATGCTATAAAT	ATTGTCCTGCCAGAAAATGC	286	55
Msil3604/3605	GCGCCAGAATTCAAGTCATT	GTCGGACTGTTTTTCGAAGC	305	55
Msil3603/3604	GTGGGAGGAAGACAAGGTCA	CCCCCTCGAGATTTGTAATG	360	55
Construction of *tmm*	CCCTCTTGTTCTTCCCCTTC	TTGACCGTCACCGTAAACAA	888	54
mutant of *M. silvestris*	CGCCAGCCAATGCTATAAAT	GCGATATCCCATGATGCTCT	601	54
Confirmation of *tmm::kan*	ATCGCGCTACTTCGAGCAT	GAGCCTTTTCAATGCCTGTC	2,077 (WT)	53
mutant of *M. silvestris*			3,054 (mutant)	53
tmm of *M. silvestris*	CATATGACTCGTGTTGCAATTA TTGGC	AAGCTTTTATTCCGCGGCGGC TTCCG	1356	54
tmm of *Roseovarius* sp. 217	CATATGACCAAAAAACGAAT TGCGATC	AAGCTTTTAGTTCTGGAGGTA AACTTCGAG	1338	54
tmm of *R. pomeroyi* DSS-3	CATATGACCACCAGCAAACGTG TGGC	AAGCTTTTAGTTGCGCAGATA CGCCTCCATGC	1353	54

Table S2. Presence of *tmm* homologs and growth on TMA in *Roseobacter* clade bacteria

	Organism	*tmm*[†]	TMA[‡]
1	*Rhodobacterales* bacterium HTCC2150	+	NT
2	*Maritimibacter alkaliphilus* HTCC2654	—	NT
3*	*Citreicella* sp. SE45	+	+
4*	*Dinoroseobacter shibae* DFL 12	—	—
5	*Jannaschia* sp. CCS1	—	NT
6	*Labrenzia aggregata* IAM 12614	—	NT
7	*Labrenzia alexandrii* DFL-11	—	NT
8	*Loktanella vestfoldensis* SKA53	—	NT
9	*Oceanibulbus indolifex* HEL-45	—	NT
10*	*Oceanicola batsensis* HTCC2597	—	—
11	*Oceanicola granulosus* HTCC2516	—	NT
12	*Octadecabacter antarcticus* 238	+	NT
13	*Octadecabacter antarcticus* 307	—	NT
14	*Phaeobacter gallaeciensis* 2.10	—	NT
15	*Phaeobacter gallaeciensis* BS107	—	NT
16	*Rhodobacteraceae* bacterium KLH11	—	NT
17*	*Roseobacter denitrificans* OCh 114	+	+
18*	*Roseobacter litoralis* Och 149	+	+
19	*Roseobacter* sp. AzwK-3b	+	NT
20	*Roseobacter* sp. CCS2	—	NT
21	*Roseobacter* sp. GAI101	—	NT
22	*Roseobacter* sp. MED193	—	NT
23*	*Roseobacter* sp. SK209-2-6	—	—
24*	*Roseovarius nubinhibens* ISM	+	+
25*	*Roseovarius* sp. 217	+	+
26	*Roseovarius* sp. HTCC2601	+	NT
27*	*Roseovarius* sp. TM1035	+	+
28	*Ruegeria lacuscaerulensis* ITI-1157	—	NT
29*	*Ruegeria pomeroyi* DSS-3	+	+
30	*Ruegeria* sp. R11	—	NT
31	*Ruegeria* sp. TM1040	—	NT
32	*Silicibacter* sp. TrichCH4B	—	NT
33*	*Sagittula stellata* E-37	—	—
34	*Sulfitobacter* sp. EE-36	—	NT
35	*Sulfitobacter* sp. NAS-14.1	—	NT
36	*Thalassobium* sp. R2A62	+	NT
37	*Rhodobacterales* bacterium HTCC2083	+	NT
38	*Rhodobacterales* bacterium Y4I	+	NT
39	*Rhodobacterales* sp. HTCC2255	+	NT

*Strains that have been tested for their growth on TMA in this study.
[†]Presence (+) or absence (–) of *tmm* genes in the genome sequences.
[‡]Growth test on TMA as sole nitrogen source. NT, not tested.

Table S3. Steady-state kinetic assays on purified Tmm from recombinant *E. coli*

	Substrate	K_m, μM	V_{max}, nmol·min^{-1}·mg^{-1}
Methylocella silvestris BL2	TMA	9.4 ± 2.1	29.4 ± 3.2
	DMA	89.7 ± 13.2	6.9 ± 0.1
	DMS	10.3 ± 0.7	34.6 ± 0.2
	DMSO	3,575 ± 151	4.8 ± 1.5
Ruegeria pomeroyi DSS-3	TMA	20.8 ± 2.9	267.7 ± 52.2
	DMA	1,119.7 ± 55.3	83.7 ± 4.0
	DMS	97.3 ± 8.8	374.5 ± 83.2
	DMSO	16,424.5 ± 1,033.2	70.4 ± 10.5
Roseovarius sp. 217	TMA	21.6 ± 1.9	1,133.6 ± 58.6
	DMA	864.2 ± 35.3	358.0 ± 12.3
	DMS	25.7 ± 4.1	577.4 ± 75.7
	DMSO	16,340.8 ± 1155.2	179.4 ± 41.2
Pelagibacter ubique HTCC7211	TMA	28.5 ± 4.4	67.3 ± 3.2
	DMA	306.1 ± 51.3	41.4 ± 2.7
	DMS	26.4 ± 7.2	97.2 ± 6.9
	DMSO	7,456.0 ± 907.8	41.3 ± 4.7
Pelagibacter ubique HTCC1002	TMA	27.5 ± 4.2	70.8 ± 7.7
	DMA	1,237.7 ± 98.5	41.2 ± 5.6
	DMS	33.2 ± 5.6	50.8 ± 5.0
	DMSO	19,334.3 ± 1,870.4	29.9 ± 8.9

DMA, dimethylamine; DMS, dimethylsulfide; DMSO, dimethyl sulfoxide; TMA, trimethylamine.

Dataset S1. Polypeptides detected in each grown culture of *Methylocella silvestris* BL2

Dataset S1 (XLSX)

The numbers show the relative abundance (in percentages) of each polypeptide in the soluble proteome for each growth condition.