Identification and characterization of trimethylamine *N*-oxide (TMAO) demethylase and TMAO permease in *Methylocella silvestris* BL2

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Running title: TMAO degradation by *Methylocella silvestris*

Abstract

Methylocella silvestris, an alphaproteobacterium isolated from a forest soil, can grow on trimethylamine *N*-oxide (TMAO) as a sole nitrogen source, however, the molecular and biochemical mechanisms underpinning its growth remain unknown. Marker-exchange mutagenesis enabled the identification of several genes involved in TMAO metabolism, including *Msil_3606*, a permease of the amino acids-polyamine (APC) superfamily, and *Msil_3603*, consisting a N-terminal domain of unknown function (DUF1989) and a C-terminal tetrahydrofolate-binding domain. Null mutants of *Msil_3603* and *Msil_3606* can no longer grow on TMAO. Purified Msil_3603 from recombinant *Escherichia coli* can convert TMAO to dimethylamine and formaldehyde (1 TMAO → 1 dimethylamine + 1 formaldehyde), confirming that it encodes a *bona fide* TMAO demethylase (Tdm). Tdm of *M. silvestris* and eukaryotic TMAO demethylases have no sequence homology and contrasting characteristics. Recombinant Tdm of *M. silvestris* appears to be hexameric, has a high affinity for TMAO (*K*m= 3.3 mM; Vmax= 21.7 nmol min^{-1} mg^{-1}) and only catalyses demethylation of TMAO and a structural homolog, dimethyldodecylamine *N*-oxide. Our study has contributed to the understanding of the genetic and biochemical mechanisms for TMAO degradation in *M. silvestris*.

Keywords, *M. silvestris*/ TMAO degradation/ TMAO demethylase/ TMAO permease

Introduction

In recent years, the cycling of methylated amines (MAs) in the terrestrial environment has attracted great attention (Ge et al., 2011). MAs, together with other aliphatic amines, have been identified as one of the important components of trace gases in the atmosphere, contributing to the growth of the so-called secondary organic aerosols and likely leading to the formation of cloud condensation nuclei (Cape et al., 2011; Ge et al., 2011). Since MAs are basic, they also play a role in neutralizing atmospheric acidity caused by organic and inorganic acids, including sulphuric acid, nitric acid and formic acid (Murphy et al., 2007). MAs are produced both biologically and abiotically. Abiotic sources of MAs include biomass burning and emissions from vehicle exhaust (Ge et al., 2011). The annual flux of MAs into the atmosphere is estimated to be in the order of 285 ± 78 Gg globally, a large proportion of which originates from animal husbandry and biomass burning although other anthropogenic activities such as agriculture also play a role (Ge et al., 2011). The presence of such large quantities of MAs in the atmosphere can significantly affect human well-being. For example, MAs can be precursors for carcinogens such as *N*-nitrosodimethylamine, causing concerns for public health (Mitch et al., 2003). Being an important component of organic nitrogen in the atmosphere, the cycling of MAs between land and oceans can also affect global biogeochemical cycles of nitrogen through atmospheric deposition (Cape et al., 2011). Therefore, understanding the sources and sinks of MAs in the environment will contribute to

58 a better assessment of the MA cycle and subsequent impact on public health and ecosystem
59 function.
60
61 Several processes contribute to biological MA production in the terrestrial environment,
62 including degradation of herbicides and pesticides (Bhadbhade et al., 2002; Kamanavalli and
63 Ninnekar, 2000; Topp et al., 1993), protein putrefaction (Kamiya and Ose, 1984), anaerobic
64 microbial respiration (Barrett and Kwan, 1985), as well as degradation of quaternary amines
65 (King, 1988). Quaternary amines such as choline and carnitine are significant components of
66 eukaryotic cells and are released to the environment due to normal cell turnover and
67 programmed cell death. In agricultural and forest soils, MAs co-exist with quaternary amines,
68 which represent a major pool of dissolved organic nitrogen, suggesting that quaternary
69 amines are likely to be important MA precursors in these soils (Warren, 2013a, b; Yu et al.,
70 2002).
71
72 It is known that many soil bacteria can sequester MAs from their environment as their carbon
73 and nitrogen source (Anthony, 1982). *Methylocella silvestris* BL2, a facultative one-carbon
74 utilizing alphaproteobacterium isolated from a forest soil in Germany, can utilise MAs,
75 including tri-, di- and mono-methylamine (TMA, DMA and MMA, respectively), as carbon,
76 nitrogen and energy sources (Chen et al., 2010a; Chen et al., 2010b; Dunfield et al., 2003). *M.*

77 *silvestris* BL2 employs an indirect pathway involving trimethylamine *N*-oxide (TMAO) as
78 the key intermediate for the degradation of TMA to ammonium and formaldehyde (Chen et
79 al., 2011). We have previously identified the enzymes responsible for MMA degradation in
80 this bacterium through γ-glutamylmethylamide (GMA) and *N*-methylglutamate (NMG) and
81 the enzyme TMA monooxygenase responsible for the initial oxidation of TMA to TMAO
82 (Chen et al., 2011; Chen et al., 2010b). It is hypothesized that TMAO can be further
83 converted to MMA though a demethylation and an oxidation step, however, the genetics and
84 biochemistry underpinning TMAO catabolism in this bacterium remains to be established.
85 TMAO not only occurs in the natural environment but also is widely used as important
86 industrial solvent (Yancey, 2005). Studying the catalytic mechanisms of TMAO degradation
87 by microorganisms and its subsequent conversion to methylated amines, such as DMA, will
88 advance our understanding of the impact of TMAO release into the environment. In this
89 study, we report the genetic and biochemical mechanisms underpinning aerobic TMAO
90 catabolism in this bacterium, which involves a TMAO demethylase encoded by a DUF1989-
91 containing protein, a novel membrane permease for TMAO and genes likely encoding the
92 DMA monooxygenase subunits.
93

Results

Genes of Msil_3603 to Msil_3609 are involved in methylated amine metabolism in *Methylocella silvestris*

We have previously identified the gene (*Msil_3604*) encoding TMA monooxygenase (Tmm), the first enzyme in the TMA oxidation pathway in this bacterium (Chen et al., 2011). Comparative proteomics data have shown that peptides encoded by the genes in the neighbourhood of *tmm* (**Figure 1**) were also induced in the presence of TMA, suggesting a role in TMA oxidation (Chen et al., 2011). In order to establish the role of the neighbouring gene in TMA metabolism, we generated several marker-exchange mutants in this bacterium (**Table 1**). The mutants were then cultivated in a defined medium using TMA, TMAO, DMA or MMA as the sole nitrogen source in order to establish their growth phenotype on MAs.

As we have predicted previously (Chen et al., 2011), the *tmm* mutant *(*Δ*Msil_3604*) could grow on TMAO, DMA and MMA, but not TMA (**Table 1**), confirming that it is only involved in the first step of TMA oxidation. Two mutants, Δ*Msil_3608* and Δ*Msil_3609*, could only grow on MMA (**Table 1**), suggesting that they are likely to encode subunits of the DMA monooxygenase (Dmm), which is the immediate upstream step for the oxidation of DMA to MMA. The gene *Msil_3607* is only 585 bp long and a mutant of *Msil_3607* is therefore not constructed in this study. However, Dmm has been previously purified from

113 *Aminobacter aminovorans* and it is known to consist of 3 subunits (Alberta & Dawson,
114 1987). We therefore hypothesize that *Msil_3607-Msil_3609* may encode a Dmm in *M.*
115 *silvestris*.
116
117 The mutant Δ*Msil_3603* can utilize DMA and MMA, but not TMA or TMAO (**Table 1**).
118 Msil_3603 is annotated as a glycine cleavage T protein (aminomethyl transferase) in the
119 Genbank and Uniprot databases. It is composed of two domains, an uncharacterized N
120 terminal domain (DUF1989) and a conserved THF-binding C terminal domain (Gcv_T),
121 which is found in several very well characterized THF-dependent enzymes, such as glycine
122 cleavage T protein (Okamura-Ikeda et al., 2005) and dimethylsulfoniopropionate
123 demethylase (Schuller et al., 2012). Phylogenetic analysis of the THF-binding domain
124 revealed that Msil_3603 formed a unique cluster in the family (**Figure 2A**). Other sequences
125 clustered within this clade include representatives that are known to metabolise MAs
126 (Kalyuzhnaya et al., 2006; Lidbury et al., 2014). The N terminus of Msil_3603 is an
127 uncharacterized domain (DUF1989) with no known function. Phylogenetic analysis of the
128 proteins of the DUF1989 superfamily showed the presence of four distinct clusters (**Figure**
129 **2B**), none of which has been assigned function experimentally. DUF1989 in Msil_3603
130 shows modest sequence similarity (<30%) to urea-carboxylase associated proteins, whose
131 functions in urea catabolism are not yet known (Kanamori et al., 2004). We hypothesized that

the ORF *Msil_3603* may encode the TMAO demethylase (E.C. 4.1.2.32) in this bacterium and this was tested further as shown below.

The ORF *Msil_3606* encodes a membrane protein, consisting of 12 transmembrane helices. It is annotated as a putative transmembrane amino acid transporter protein in the Genbank and Uniprot databases. Our phylogenetic analyses suggest that Msil_3606 belongs to the amino acid-polyamine membrane transporter superfamily (APC family) (**Figure 3**). The APC family currently consists of 14 clades, 12 of which have been functionally assigned (Saier MH, 2000). Msil_3606, together with sequences from known MA utilizers (*e.g. Methyloversatilis*) formed a distinct clade independent of the currently known APC family members. Marker-exchange mutagenesis experiments showed that the mutant (Δ*Msil_3606*) can grow on TMA, DMA and MMA, but not TMAO (**Table 1**), suggesting that it encodes a functional TMAO transporter. The role of *Msil_3605* in MA oxidation was not very clear. Msil_3605 has a single THF-binding domain (Gcv_T), which shows 32% identity to the C terminal Gcv_T domain in Msil_3603. The *ΔMsil_3605* mutant was able to grow on MMA, however its growth on TMA, TMAO and DMA was much slower compared to those of the wild type (**Table 1**).

_Msil_3603_ and _Msil_3606_ are required for _Methylocella silvestris_ to grow on TMAO

In order to establish if *Msil_3603* and *Msil_3606* are indeed specifically required for TMAO metabolism, we further quantified TMAO concentrations by ion-exchange chromatography in the culture medium in the wild type and the mutants of *M. silvestris*. We used succinate as the sole carbon source instead of methanol because methanol can damage the ion-exchange chromatography column used in this study. As shown in **Figure 4**, the wild type strain could grow on TMAO plus succinate and TMAO was completely depleted within 10 days (detection limit, 5 μM). However, the growth rates of the mutants (Δ*Msil*_3603, Δ*Msil_3606*) on TMAO as a sole nitrogen source were significantly reduced compare to that of the wild type, and TMAO concentrations in the medium remained unchanged throughout the experiment. Therefore, the data indicate that *Msil_3606* encodes a transporter required for TMAO uptake in *M. silvestris*, which we designated as TmoP.

Msil_3603 **encodes a bacterial TMAO demethylase**

We cloned the gene *Msil_3603* from *M. silvestris* into an *Escherichia coli* host, overexpressed and further purified this protein with 6×His tag at its N-terminus by nickel (Ni^{2+}) affinity chromatography in order to establish whether it is a *bona fide* TMAO demethylase (Tdm). Eukaryotic Tdm has been purified previously (Kimura et al., 2000; Parkin and Hultin, 1986; Fu et al., 2006; Takeuchi et al., 2003), however, its microbial counterpart has only been partially purified from *Aminobacter aminovorans* and *Bacillus* sp.

PM6 (Large PJ, 1971; Myers & Zatman, 1971). The two products of TMAO demethylation are DMA and formaldehyde, and these two compounds were indeed detected when the purified protein was presented with TMAO (**Figure 5**). The stoichiometry of TMAO demethylation is determined to be 1 TMAO → 1 DMA + 1 HCHO.

Characterization of Tdm of *M. silvestris* from recombinant *E. coli*.

The purified Tdm protein from recombinant *Escherichia coli* had a molecular weight of ~80 kDa under denaturing conditions (**Figure 6A**), in good agreement with the calculated value from its amino acid sequence (82,547 Da). Its native molecular weight was estimated by two complementary methods, native gel electrophoresis and analytical ultracentrifugation, both of which suggested that the native Tdm was likely to be hexameric (**Figure 6 B, C**). The purified protein has an optimum pH at ~ 6.0 (**Figure S1**) and had no recognizable absorbance peak under UV-visible light (220 nm – 600 nm) besides the peak at 280 nm (**Figure S2**). Under optimum conditions, V_{max} and K_m of the recombinant Tdm were determined to be 21.7 ± 0.74 nmol min^{-1} mg^{-1} and 3.3 ± 0.64 mM, respectively by the Eadie-Hofstee plot (**Figure 6D**). Its K_m value of Tdm of *M. silvestris* is in good agreement to that of *Aminobacter aminovorans* (2 mM) and *Bacillus* sp. (2.85 mM), respectively (Large PJ, 1971; Myers & Zatman, 1971). The recombinant Tdm enzyme is specific for TMAO, among the compounds tested, it only showed ~ 50% activity to dimethyldodecylamine *N*-oxide (**Figure 7**). In

189 contrast to the eukaryotic counterparts (Parkin and Hultin, 1986), no enhancement of activity
190 was found with additional ferrous iron or cysteine added to the *in vitro* enzyme assays
191 (**Figure S3**).
192

Discussion

In this study we report the discovery of a membrane transporter required for the uptake of TMAO and biochemical characterization a microbial TMAO demethylase (Tdm) of *Methylocella silvestris*. In addition, we also identified additional genes, likely encoding a DMA monooxygenase, required for metabolism of DMA, which is an intermediate of TMA and TMAO metabolism. The presence of a specific transporter required for TMAO suggest that it can be taken up by *Methylocella silvestris* from the environment (Anthony, 1982; Chen et al., 2011). Although it is clear that TMAO can be used as a ubiquitous osmolyte by a range of marine biota (Gibb and Hatton, 2004), the environmental sources of TMAO in soils and other terrestrial habitats are less clear. TMAO is a central metabolite involved in lipid metabolism in mammals and significant concentrations of TMAO have been detected in urine and other body fluids of humans (Zhang et al., 1992), rats (Smith et al., 1994) and dogs (Richards et al., 2013). It is therefore possible that the presence of TMAO in terrestrial environments, including soils, is the result of excretion from wild animals. However, it is also likely that TMAO is leaked out from microorganisms during the oxidation of TMA by microbial TMA monooxygenases (Chen et al., 2011). Recent studies have shown that in agricultural and forest soils, precursors of TMA such as quaternary amines represent a major pool of dissolved organic nitrogen (Warren, 2013a, b). Microbial oxidation of TMA in the soils may represent yet another source of TMAO in the environment.

The only known microbial TMAO transporter in the literature is an ATP-dependent active transporter of the ABC superfamily found in *Aminobacter aminovorans* (Raymond and Plopper, 2002) and *Ruegeria pomeroyi* (Lidbury et al., 2014). Our study indicates that another type of microbial transporter for TMAO is present. This newly identified TMAO permease (TmoP) of *Methylocella silvestris* belongs to the APC superfamily but forms a distinct cluster (**Figure 3**). APC transporters are membrane permeases co-transporting another solute, acting as either a symporter or an antiporter (Saier, 2000). It is not clear whether TmoP acts as a symporter or an antiporter and the co-transporting solute remains to be established. It is interesting to note that TmoP homologues are also found in some methanogenic Archaea, *e.g. Methanosarcina acetivorans*, *Methanosarcina mazei* (**Figure 3**) but it remains unclear whether TMAO can be directly used as a substrate for methanogenesis.

Microbial Tdm has been partially purified previously (Large, 1971; Myers and Zatman, 1971), and the gene encoding microbial Tdm has been identified very recently (Lidbury et al., 2014). Tdm from marine eukaryotes has also been purified, including the Alaskan Pollock (*Theragra chalcogramma*, Kimura et al., 2000), the red hake (*Urophycis chuss*, Parkin and Hultin, 1986) and the Humboldt squid (*Dosidicus gigas*, Fu et al., 2006). Tdm sequences from bacteria and eukaryotes (Takeuchi et al., 2003) have no sequence homology and have

231 contrasting characteristics. For example, purified Tdm from *Dosidicus gigas* and *Theragra*
232 *chalcogramma* have much smaller molecular mass, being 17.5 kDa and 25 kDa respectively.
233 Their K_m values for TMAO (30 mM for *T. chalcogramma* and 26.2 mM for *D. gigas*) are
234 significantly higher than those of the microbial Tdm (2 - 4 mM). Eukaryotic Tdm requires
235 ferrous ion as an essential metal for activity whereas it has no obvious impact on microbial
236 Tdm in *in vitro* assays. Tdm in bacteria and eukaryotes represent another example of
237 convergent evolution where two forms of Tdm have evolved independently to catalyse the
238 same biochemical reaction.
239
240 Another important finding from this study is the functional assignment of the DUF1989
241 domain as the N terminus of the microbial Tdm. Proteins having domains of unknown
242 functions (DUF) currently represent more than a quarter of sequence entries in public
243 databases such as Pfam (Punta et al., 2012). Functional annotation of DUFs remains a great
244 challenge for the scientific community since they not only present a major knowledge gap
245 between protein structure and functional relationship but also prevent the complete
246 understanding of cellular functions from completed genomes (Galperin and Koonin, 2010).
247 Our phylogenetic analyses of DUF1989 representatives (1044 entries in Pfam in total)
248 suggest the presence of at least four major clades, two of which are proteins associated with
249 the urea carboxylase gene cluster (Kanamori et al., 2004). However, the functions of the two

DUF1989-containing proteins associated to this enzyme in microbial genomes remain unknown and warrant further experimental characterization.

The C-terminus of *M. silvestris* Tdm contains a highly conserved THF-binding domain, which is found in several enzymes catalysing the release of a formaldehyde molecule. Phylogenetic analyses of the THF-binding domain separate the sequences into five major clusters (**Figure 2A**), three of which have been characterized previously, including the T protein of the glycine cleavage system, dimethylglycine and sarcosine dehydrogenase and dimethylsulfoniopropionate demethylase. The THF-binding domain of Tdm falls in to one of the previously recognized, but so far uncharacterized clades (Sun et al., 2011; Reisch et al., 2008). Comparative genomic analyses of the other group of THF-binding domain protein, represented by Msil_3605, revealed that they are located in the neighbourhood of the putative DMA monooxygenases (Dmm) in other methylamine-utilizers (**Figure S4**), suggesting a role in DMA oxidation.

Based on the present and previous studies (Chen et al., 2011; Chen et al., 2010b), we have proposed a model of methylamine metabolism in *Methylocella silvestris* BL2 (**Figure 8**). TMA is likely to be transported into the cell via a yet unidentified transporter and is subsequently oxidised to release formaldehyde and ammonium. Previous genome analysis

only identified the GS/GOGAT as the pathway for ammonium assimilation in this bacterium (Chen et al., 2010a). Formaldehyde released from MA oxidation can be either incorporated into biomass through the serine cycle or subjected to oxidation to CO_2 for generating energy and reducing equivalent. Because *M. silvestris* can grow on DMA and MMA, it is therefore likely that specific membrane transporters for these compounds are present in its genome. This study has suggested that the genes *Msil_3607- Msil_3609* are likely to encode the Dmm whose activity has been confirmed previously in this bacterium (Chen et al., 2011), and the knockout mutants can no longer grow on DMA. Dmm has previously been purified from *Aminobacter aminovorans* and shown to contain three subunits consisting of 24, 36 and 42 kDa respectively (Alberta and Dawson, 1987), which are in a good agreement with the predicted molecular mass of Msil_3607-Msil_3609, respectively. However, we are unable to demonstrate the Dmm activity heterologously in *E. coli* due to poor expression of the second and the third subunit (data not shown). Currently work is underway in the laboratory to improve the heterologous expression system in order to test this hypothesis. The role of the THF-containing ORF *Msil_3605* in this pathway is not clear. The mutant had reduced growth rates when grown on DMA and TMAO, and we thus postulate that it may encode a subunit, which can be loosely associated with Dmm but facilitate the conjugation of formaldehyde released from TMAO demethylation, which may help to offset the toxicity effect of formaldehyde accumulation in the cell.

To conclude, we have identified the genes and encoding enzymes responsible for the uptake and catabolism of TMAO in *Methylocella silvestris*. The newly identified Tdm and TmoP proteins have not only furthered our understanding of TMA/TMAO degradation in this soil bacterium, but also expanded our knowledge in microbial cycling of MAs in terrestrial environments, functional assignment of the DUF1989 family and the expanding functions encoded in the APC superfamily.

Materials and methods

Growth of *Methylocella silvestris* and mutants

M. silvestris was grown at 25 °C under natural light conditions in 125-ml serum vials containing 20 ml diluted mineral salt medium (DNMS) with an inoculum size of 10% as described previously (Chen et al., 2010b). MAs (final concentration 1.5 mM), *i.e.* TMA, TMAO, DMA and MMA, were used as the nitrogen source. Either methanol (10 mM) or succinate (5 mM) was used as the carbon source. Concentrations of MAs in the media were determined by ion-exchange liquid chromatography. The ion chromatography system used consisted of a Metrohm 881 Compact IC Pro (Metrohm, UK) with a Metrosep C4/250 column. The eluent solution contained HNO_3 (1.5 mM), 2, 6-pyridinedicarboxylic acid (0.7 mM) and acetone (5%, v/v). All solutions were prepared gravimetrically using Milli-Q water (Millipore, USA).

Construction of marker-exchange mutants in *Methylocella silvestris*

Mutants of *M. silvestris* were constructed as described previously (Chen et al., 2010b). Briefly, a downstream region and an upstream region of the target gene were amplified by PCR and sub-cloned into the pGEM-T vector (Promega) together with a kanamycin (*kan*) gene cassette amplified from the plasmid pCM184 (Marx & Lidstrom, 2002), which was inserted between the two regions (primers used are listed in **Table S1**). The downstream and

315 upstream regions together with the *kan* gene cassette were then released from the resulting
316 plasmid and transformed into *M. silvestris* competent cells *via* electroporation as described
317 previously (Chen et al., 2010b). Mutants were selected on the solid DNMS medium
318 containing kanamycin (25 μg ml^{-1}), which were then screened by diagnostic PCR and
319 subsequent sequencing.
320
321 **Cloning and heterologous expression of *tdm* in *Escherichia coli***
322 Plasmids and strains used for cloning and overexpression of *tdm* in *E. coli* are listed in **Table**
323 **2**. Briefly, the *tdm* gene of *M. silvestris* (*Msil_3603*) was amplified by PCR and sub-cloned
324 into the pGEM-T vector (Promega), which was then excised using the *Nde*I/*Bam*HI sites and
325 ligated into the expression vector pET28a (Merck Biosciences). The resulting plasmid was
326 sequenced prior to being transformed into the expression host *E. coli* BLR(DE3) pLysS
327 (Merck Biosciences).
328
329 **Protein purification and enzymatic assays**
330 *E. coli* cells containing the *tdm* gene in pET28a were grown in 250 ml Luria broth (LB) with
331 25 μg ml^{-1} kanamycin at 37 °C with agitation at 250 $r \cdot min^{-1}$. When the cell density (OD_{600})
332 reached 0.4 - 0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final
333 concentration of 0.2 mM to induce Tdm expression and the cultures were then shifted to 25

°C for 18 h before harvesting the cells by centrifugation (6,000×g, 10 min). Cells were stored at -20 °C prior to cell lysis by passing three times through a chilled French press (American Instrument Co.) at 110 megapascals. The lysates were centrifuged at 100,000×g for 40 min, and the supernatants were saved as cell extracts for the purification of Tdm. Overexpressed Tdm was purified using a His-tag protein purification kit according to the manufacturer's instructions (Novagen) and eluted using an elution buffer, containing 1M imidazole, 0.5 M NaCl and 20 mM Tris-HCl (pH 7.9). Removal of the 6×His tag at the N-terminus of recombinant Tdm was carried out using thrombin (GE, UK) according to manufacture's instruction. One mg purified recombinant Tdm was incubated with 10 units thrombin at 4 °C overnight (16 hrs). The 6×His tag was efficiently removed as determined by sodium dodecyl sulfate (SDS) -polyacrylamide gel electrophoresis (PAGE) and the inability to bind to the nickel affinity column. Removal of the 6×His tag did not change the optimum pH and pH profile of the recombinant Tdm (Figure S1), however, incubation at 4 °C led to 22% reduction in enzyme activity. Therefore, throughout the experiments, the 6×His-tagged Tdm were used.

Several buffers with a range of pH were first compared in order to determine a suitable buffering system and the optimum pH for the purified Tdm. The data presented in **Figure S1** demonstrated that Tdm had highest activity at pH 6.0 in 10 mM MES (2-(*N*-morpholino)

353 ethanesulfonic acid) buffer, which was then chosen for the following experiments. For
354 enzymatic activity assays, either formaldehyde or DMA production from TMAO was
355 quantified. DMA quantification was carried out using the ion-exchange chromatography as
356 described above and formaldehyde was quantified using the Purpald reagent (4-amino-3-
357 hydrazino-5-mercapto-1,2,4-triazole, Sigma) (Quesenberry et al., 1996). Enzyme assays were
358 carried out at room temperature (~ 22 °C), containing 2.5 μg of purified Tdm in 50 μl of 10
359 mM MES buffer. The reactions were initiated by adding TMAO into the mixture (final
360 concentration 10 mM) and incubated for 10 min which was within the linear phase as
361 assessed by formaldehyde release. Measurement of formaldehyde was performed by mixing
362 10 μl of the sample with 25 μl of 0.2% (w/v) Purpald reagent and 215 μl of Milli-Q water in a
363 96-well micro-plate. The Purpald solution was freshly prepared by dissolving in 1 M NaOH.
364 Absorbance at 540 nm was determined after 20 min incubation at room temperature using a
365 Bio-Rad iMark micro-plate reader. Calibration curves were prepared with formaldehyde of
366 analytical grade purity (Thermo Scientific) from 20 μM to 180 μM (final concentration).
367 Steady-state kinetics were performed in triplicate. To determine the substrate specificity of
368 Tdm, the compounds were purchased from Sigma-Aldrich. The assays were performed in
369 triplicate and the compounds sued were at a final concentration of 10 mM. To determine the
370 stoichiometry of TMAO demethylation by Tdm, the enzyme reaction was initiated by adding
371 TMAO at 2-8 mM and DMA and formaldehyde production was quantified after 60 min when

372 TMAO was completely consumed.

373

374 Protein concentrations were determined using a protein assay kit (Bio-Rad). One-dimensional
375 protein analyses were carried out using a Bio-Rad precast SDS/PAGE gel (12.5%, w/v) and
376 stained with Fast Blue gel staining reagent (Expedeon, UK). Bands of interest were excised,
377 digested with trypsin, and analyzed to confirm their identity using the matrix-assisted laser
378 desorption ionization–mass spectrometry (MALDI-MS) and tandem mass spectrometry at the
379 Mass Spectrometry and Proteomics Facility Laboratory, School of Life Sciences, University
380 of Warwick.

381

382 **Bioinformatics**

383 Homologous proteins were identified using the BLASTp program using the Msil_3603 and
384 Msil_3606 sequences of *M. silvestris* as the query. Protein sequences were aligned, end-
385 trimmed and analyzed using the MEGA5 package (Tamura et al., 2011). All phylogenetic
386 trees were constructed using the minimum evolution method (default settings) with 1,000
387 bootstrap replicates. Accession numbers from the Uniprot database for all sequences used in
388 phylogenetic analyses are listed in **Tables S2, S3 and S4** for tetrahydrofolate (THF)-binding
389 domains, DUF1989 domains and the amino acids-polyamine (APC) superfamily members,
390 respectively. Analysis of conserved domains in protein was carried out using Pfam (release

27.0, Punta et al., 2012).

Analytical ultracentrifugation

Purified Tdm from recombinant *E. coli* was exhaustively dialyzed against 50 mM sodium phosphate (pH 7.0) containing 100 mM NaCl, 1 mM D, L-dithiothreitol. Tdm protein samples were centrifuged at 30,000 $r \cdot min^{-1}$ at 4 $^{\circ}C$ for 16 h in an eight-cell An-50 Ti rotor in a Beckman XLI analytical ultracentrifuge (Beckman). Migration of the protein during centrifugation was monitored by measuring the distribution of absorbance at 280 nm across the sample in the centrepiece at 120 consecutive time points. Molecular masses were calculated by the SEDFIT package using a c(s) model (Dam et al., 2005; Schuck et al., 2000). Protein partial specific volumes, buffer viscosities and densities were all calculated using SEDNTERP (http://sednterp.unh.edu/).

Native polyacrylamide gel electrophoresis

Native (non-denaturing) polyacrylamide gel electrophoresis was performed at a constant voltage of 150 V using an Invitrogen electrophoresis system on a NuPAGE® Novex® 3–8% Tris-Acetate (w/v) polyacrylamide gel. The gels were stained with the Fast Blue reagent (Expedeon, UK).

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532 **Figure legends**

533 **Figure 1** The genomic neighbourhood of trimethylamine (TMA) monooxygenase (*tmm*) in
534 *Methylocella silvestris* BL2 and their putative functions. Conserved domains (highlighted in
535 black) in Msil_3603 and Msil_3605 were identified using the conserved domain database
536 (Marchler-Bauer et al., 2013). DMA, dimethylamine; TMAO, trimethylamine *N*-oxide; DUF,
537 domain of unknown function.

538

Figure 2 Phylogenetic analyses of the tetrahydrofolate (THF)-binding domain (~ 358 amino acids) (**A**) and the DUF1989 domain (~ 197 amino acids) (**B**) of the putative trimethylamine *N*-oxide (TMAO) demethylase (Tdm, encoded by *Msil_3603*). Bootstrap values (1,000 replicates) great than 90% are shown in percentage for each node. Accession numbers are from the UniProt database. The bar represents 1 substitution per 10 amino acids in the aligned sequences. DUF: domain of unknown function.

Figure 3 Phylogenetic analysis of the putative trimethylamine *N*-oxide (TMAO) permease (TmoP) in the amino acid/polyamine/organocation (APC) superfamily (~ 375 amino acids). Bootstrap values (1,000 replicates) greater than 90% are shown for each node. Accession numbers are from the UniProt database. The bar represents 2 substitutions per 10 amino acids in the aligned sequences.

Figure 4 Growth of *Methylocella silvestris* of wild type (**A**), the Δ*Msil_3603* mutant (**B**) and the Δ*Msil_3606* mutant (**C**) on TMAO (—○—) or nitrate (—●—) as the sole nitrogen source. Quantification of trimethylamine *N*-oxide (TMAO) consumption during growth of the wild type (—△—), Δ*Msil_3603* mutant (—○—) and the Δ*Msil_3606* mutant (—□—) (**D**). Nitrate was used as a sole nitrogen source as positive controls. Error bars indicate standard deviations of experiments run in triplicate.

Figure 5 (A) Stoichiometry of trimethylamine *N*-oxide (TMAO) demethylation by Tdm. Black and grey bars represent formaldehyde and dimethylamine (DMA) concentrations, respectively. (B) Ratio of dimethylamine to formaldehyde resulted from TMAO degradation by Tdm. Error bars indicate standard deviations of triplicate experiments.

Figure 6 Estimation of molecular weight of purified Tdm by denaturing (**A**) and native (**B**) gel electrophoresis and analytical ultracentrifugation (**C**). Mf means the molar mass taking into account the current best-fit frictional ratio f/f0. Steady-state kinetic parameters of Tdm by the Eadie-Hofstee plot (**D**). Error bars indicate standard deviations of experiments run in triplicate.

Figure 7 Relative activity of Tdm to selected structure homologs of trimethylamine *N*-oxide (TMAO). Error bars indicate standard deviations of experiments run in triplicate.

Figure 8 Proposed model of trimethylamine *N*-oxide (TMAO) transport and metabolism in *Methylocella silvestris*. TMAO is either directly imported through the TMAO permease (TmoP) or resulted from the oxidation of trimethylamine (TMA) by TMA monooxygenase (Tmm). A membrane transporter for TMA in this bacterium is yet to be discovered. TMAO

577 degradation by Tdm yields dimethyalmine (DMA) and formaldehyde (HCHO), which is
578 likely to be conjugated to tetrahydrofolate (THF). DMA is degraded by DMA
579 monooxygenase (Dmm) to monomethylamine (MMA) and formaldehyde which is likely
580 conjugated to THF by the protein encoded by *Msil_3605*. MMA is converted to ammonium
581 through the γ-glutamylmethylamide/*N*-methylglutamate pathway, involving γ-
582 glutamylmethylamide synthetase (*Msil_2635*), *N*-methylglutamate synthase (*Msil_2632-*
583 *Msil_2634*) and *N*-methylglutamate dehydrogenase (*Msil_2636-Msil_2639*) (Chen et al.,
584 2010b). Ammonium is assimilated by *M. silvestris* as a nitrogen source through the glutamine
585 synthetase (GS)/ glutamate synthase (GOGAT) pathway (Chen et al., 2010a).

Table 1 Growth of wild type and mutants of *Methylocella silvestris* on methylated amines*

Substrate	Wild type (h^{-1})	Δ*Msil_3603* (h^{-1})	Δ*tmm* (Δ*Msil_3604*) (h^{-1})	Δ*Msil_3605* (h^{-1})	Δ*Msil_3606* (h^{-1})	Δ*Msil_3608* (h^{-1})	Δ*Msil_3609* (h^{-1})
TMA	0.030 ± 0.002	-	-	0.010 ± 0.001	0.025 ± 0.001	-	-
TMAO	0.045 ± 0.000	-	0.045 ± 0.003	0.010 ± 0.003	-	-	-
DMA	0.032 ± 0.002	0.027 ± 0.004	0.040 ± 0.006	0.024 ± 0.004	0.024 ± 0.001	-	-
MMA	0.031 ± 0.001	0.034 ± 0.005	0.047 ± 0.003	0.029 ± 0.000	0.025 ± 0.001	0.027 ± 0.003	0.027 ± 0.001

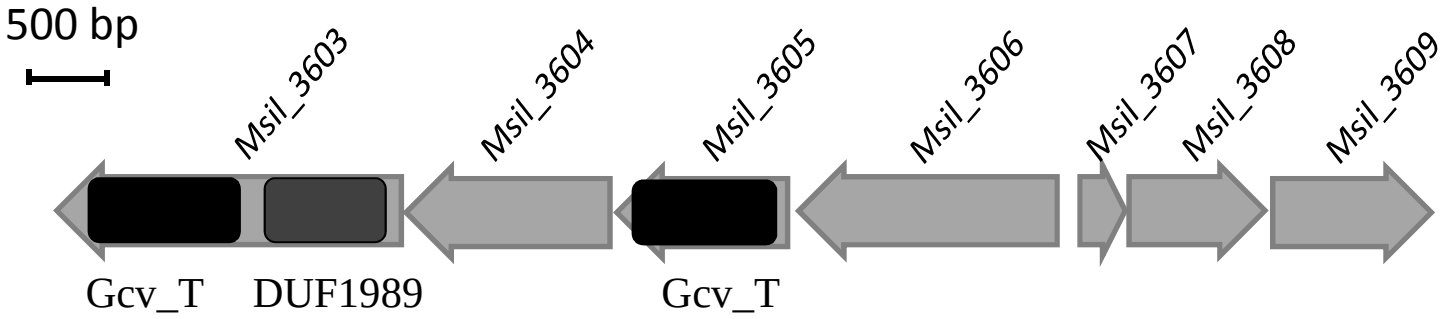
*, Methanol was used as the carbon source and methylated amines were used as the sole nitrogen source.

-, No growth. Values presented are average ± standard deviations of experiments run in triplicates.

Table 2 List of bacteria and plasmids used in this study

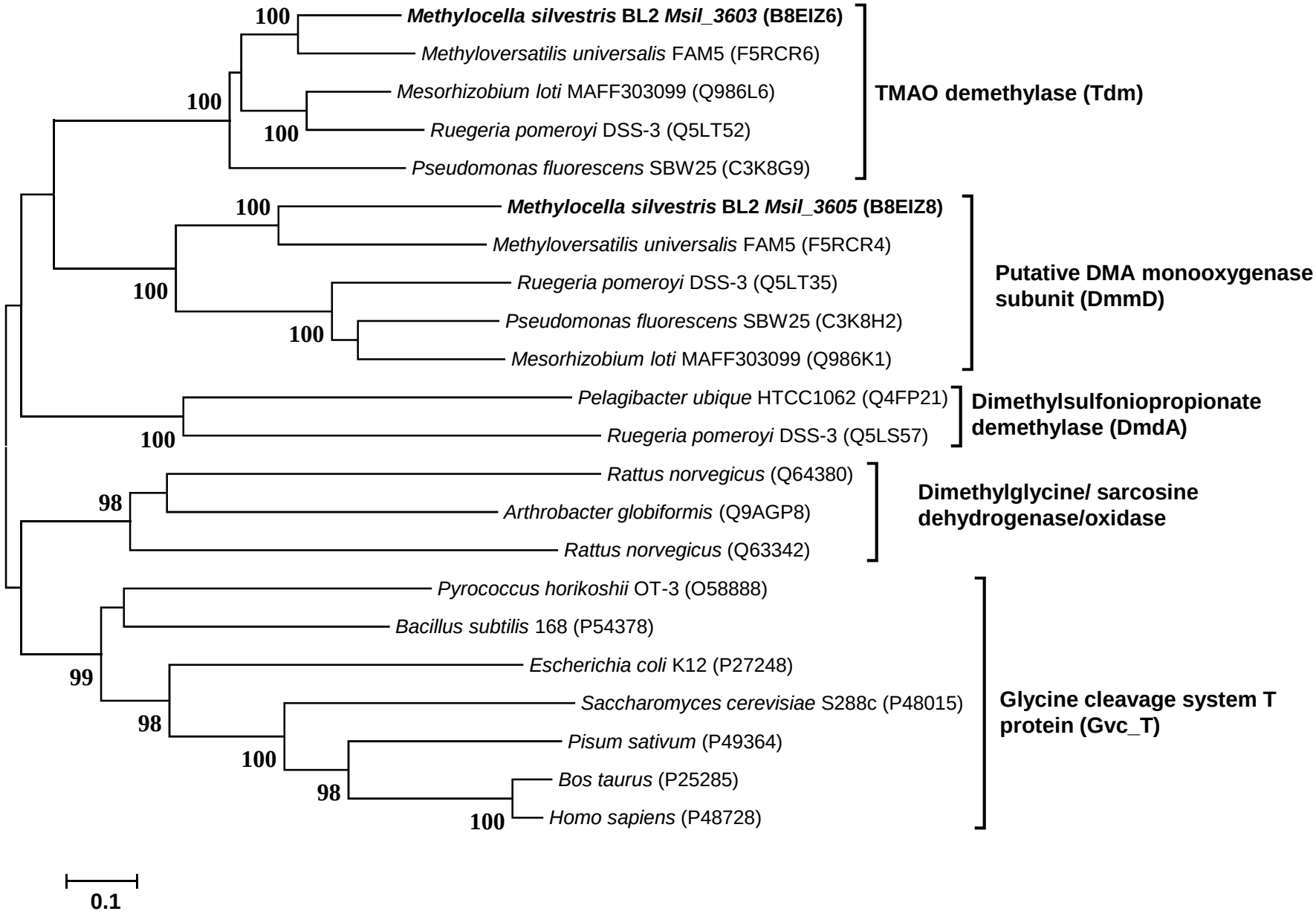
Strains/plasmids	Relevant features	References
***Methylocella silvestris* BL2**	Wild type, isolated from a forest soil in Germany	Dunfield et al., 2003; Chen et al., 2010a
Δ*Msil_3603*	TMAO demethylase (Tdm) mutant	This study
Δ*tmm* (Δ*Msil_3604*)	TMA monooxygenase (Tmm) mutant *(*Δ*Msil_3604*), with *kan* insertion	Chen et al., 2011
Δ*Msil_3605*	Mutant of the gene *Msil_3605* which encodes a tetrahydrofolate-binding domain	This study
Δ*Msil_3606*	TMAO permease (TmoP) mutant	This study
Δ*Msil_3608*	DMA monooxygenase β subunit mutant	This study
Δ*Msil_3609*	DMA monooxygenase γ subunit mutant	This study
Escherichia coli		
BLR(DE3)pLysS	Host for heterologous protein overexpression	Novagen
JM109	General cloning	Promega
Plasmids		
pGEM-T	Cloning vector	Promega
pCM184	Source of the kanamycin-resistant gene cassette (*kan*)	Marx and Lidstrom, 2002
pET28a	Expression vector in *E. coli* BLR(DE3)pLysS	Novagen
pET28a-3603	*Msil_3603* cloned into pET28a under the *Nde*I/*Bam*HI sites	This study
pET28a-3607/9	*Msil_3607 to Msil_3609* cloned into pET28a under the *Nde*I/*Hind*III sites	This study

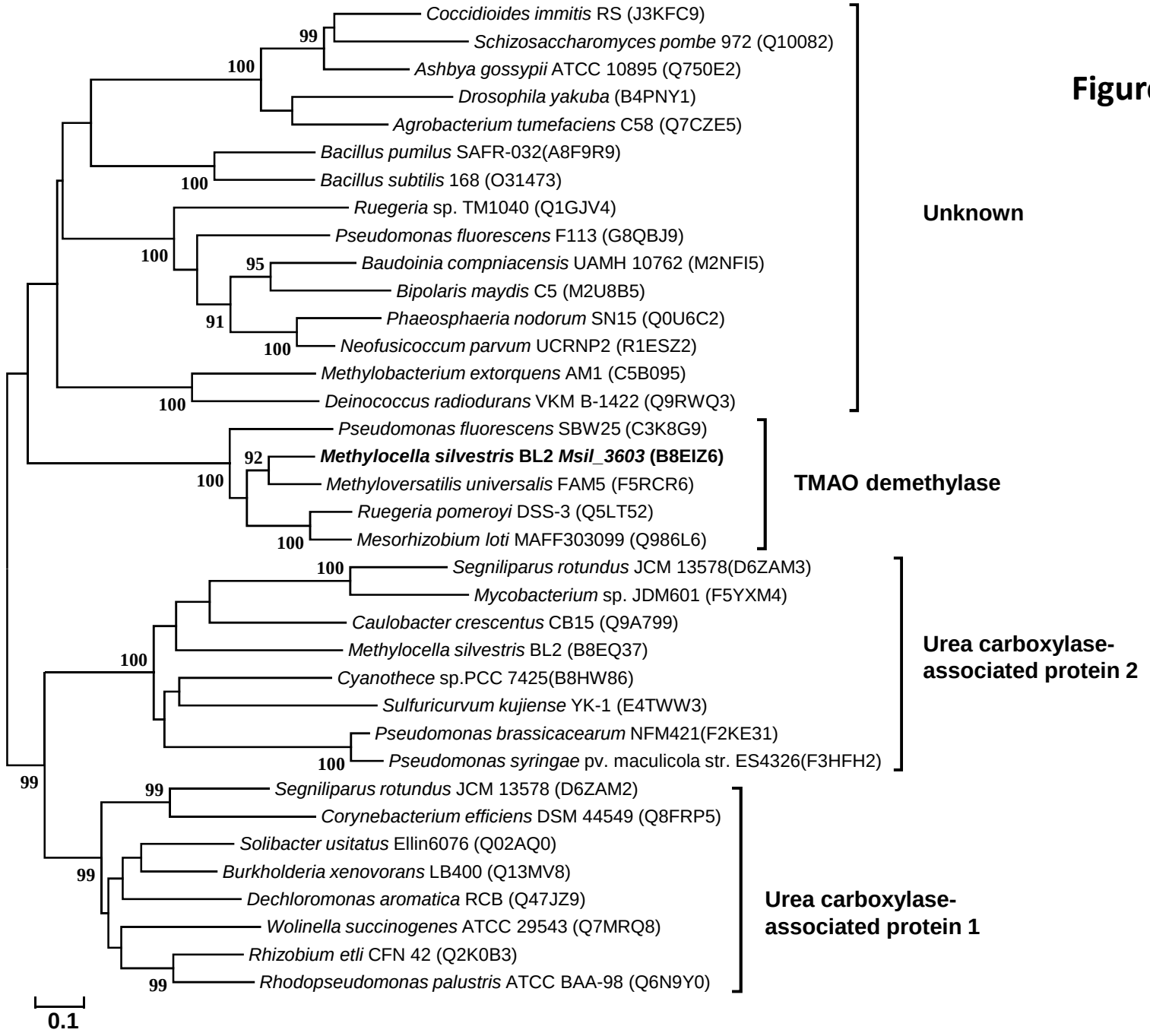
Figure 1



ORF	Length (AA)	Gene name	Annotation	Function	Reference
Msil_3603	761	*tdm*	Glycine cleavage system T protein, containing a N-terminal domain of unknown function (DUF1989) and a C-terminal tetrahydrofolate-binding domain (Gcv_T)	TMAO demethylase (Tdm)	This study
Msil_3604	451	*tmm*	A flavin-containing monooxygenase	TMA monooxygenase (Tmm)	Chen *et al*., 2011
Msil_3605	378	*dmmD*	Aminomethyltransferase, containing a tetrahydrofolate-binding domain (Gcv_T)	DMA monooxygenase δ subunit?	This study
Msil_3606	571	*tmoP*	Putative transmembrane amino acid transporter protein	TMAO permease (TmoP)	This study
Msil_3607	195	*dmmA*	Hypothetical protein	DMA monooxygenase subunit α	This study
Msil_3608	317	*dmmB*	Putative NADPH-flavodoxin reductase	DMA monooxygenase subunit β	This study
Msil_3609	351	*dmmC*	Protein of unknown function (DUF3445)	DMA monooxygenase subunit γ	This study

Figure 2A


100
Methylocella silvestris BL2 Msil_3603 (B8EIZ6)
Methyloversatilis universalis FAM5 (F5RCR6)
100
Mesorhizobium loti MAFF303099 (Q986L6)
100
Ruegeria pomeroyi DSS-3 (Q5LT52)
Pseudomonas fluorescens SBW25 (C3K8G9)
TMAO demethylase (Tdm)
100
Methylocella silvestris BL2 Msil_3605 (B8EIZ8)
Methyloversatilis universalis FAM5 (F5RCR4)
100
Ruegeria pomeroyi DSS-3 (Q5LT35)
Pseudomonas fluorescens SBW25 (C3K8H2)
100
Mesorhizobium loti MAFF303099 (Q986K1)
Putative DMA monooxygenase subunit (DmmD)
Pelagibacter ubique HTCC1062 (Q4FP21)
100
Ruegeria pomeroyi DSS-3 (Q5LS57)
Dimethylsulfoniopropionate demethylase (DmdA)
Rattus norvegicus (Q64380)
98
Arthrobacter globiformis (Q9AGP8)
Rattus norvegicus (Q63342)
Dimethylglycine/ sarcosine dehydrogenase/oxidase
Pyrococcus horikoshii OT-3 (O58888)
Bacillus subtilis 168 (P54378)
99
Escherichia coli K12 (P27248)
98
Saccharomyces cerevisiae S288c (P48015)
100
Pisum sativum (P49364)
98
Bos taurus (P25285)
100
Homo sapiens (P48728)
Glycine cleavage system T protein (Gvc_T)
0.1


Coccidioides immitis RS (J3KFC9)
Schizosaccharomyces pombe 972 (Q10082)
Ashbya gossypii ATCC 10895 (Q750E2)
Drosophila yakuba (B4PNY1)
Agrobacterium tumefaciens C58 (Q7CZE5)
Bacillus pumilus SAFR-032(A8F9R9)
Bacillus subtilis 168 (O31473)
Ruegeria sp. TM1040 (Q1GJV4)
Pseudomonas fluorescens F113 (G8QBJ9)
Baudoinia compniacensis UAMH 10762 (M2NFI5)
Bipolaris maydis C5 (M2U8B5)
Phaeosphaeria nodorum SN15 (Q0U6C2)
Neofusicoccum parvum UCRNP2 (R1ESZ2)
Methylobacterium extorquens AM1 (C5B095)
Deinococcus radiodurans VKM B-1422 (Q9RWQ3)
Unknown
Pseudomonas fluorescens SBW25 (C3K8G9)
Methylocella silvestris BL2 Msil_3603 (B8EIZ6)
Methyloversatilis universalis FAM5 (F5RCR6)
Ruegeria pomeroyi DSS-3 (Q5LT52)
Mesorhizobium loti MAFF303099 (Q986L6)
TMAO demethylase
Segniliparus rotundus JCM 13578(D6ZAM3)
Mycobacterium sp. JDM601 (F5YXM4)
Caulobacter crescentus CB15 (Q9A799)
Methylocella silvestris BL2 (B8EQ37)
Cyanothece sp.PCC 7425(B8HW86)
Sulfuricurvum kujiense YK-1 (E4TWW3)
Pseudomonas brassicacearum NFM421(F2KE31)
Pseudomonas syringae pv. maculicola str. ES4326(F3HFH2)
Urea carboxylase-associated protein 2
Segniliparus rotundus JCM 13578 (D6ZAM2)
Corynebacterium efficiens DSM 44549 (Q8FRP5)
Solibacter usitatus Ellin6076 (Q02AQ0)
Burkholderia xenovorans LB400 (Q13MV8)
Dechloromonas aromatica RCB (Q47JZ9)
Wolinella succinogenes ATCC 29543 (Q7MRQ8)
Rhizobium etli CFN 42 (Q2K0B3)
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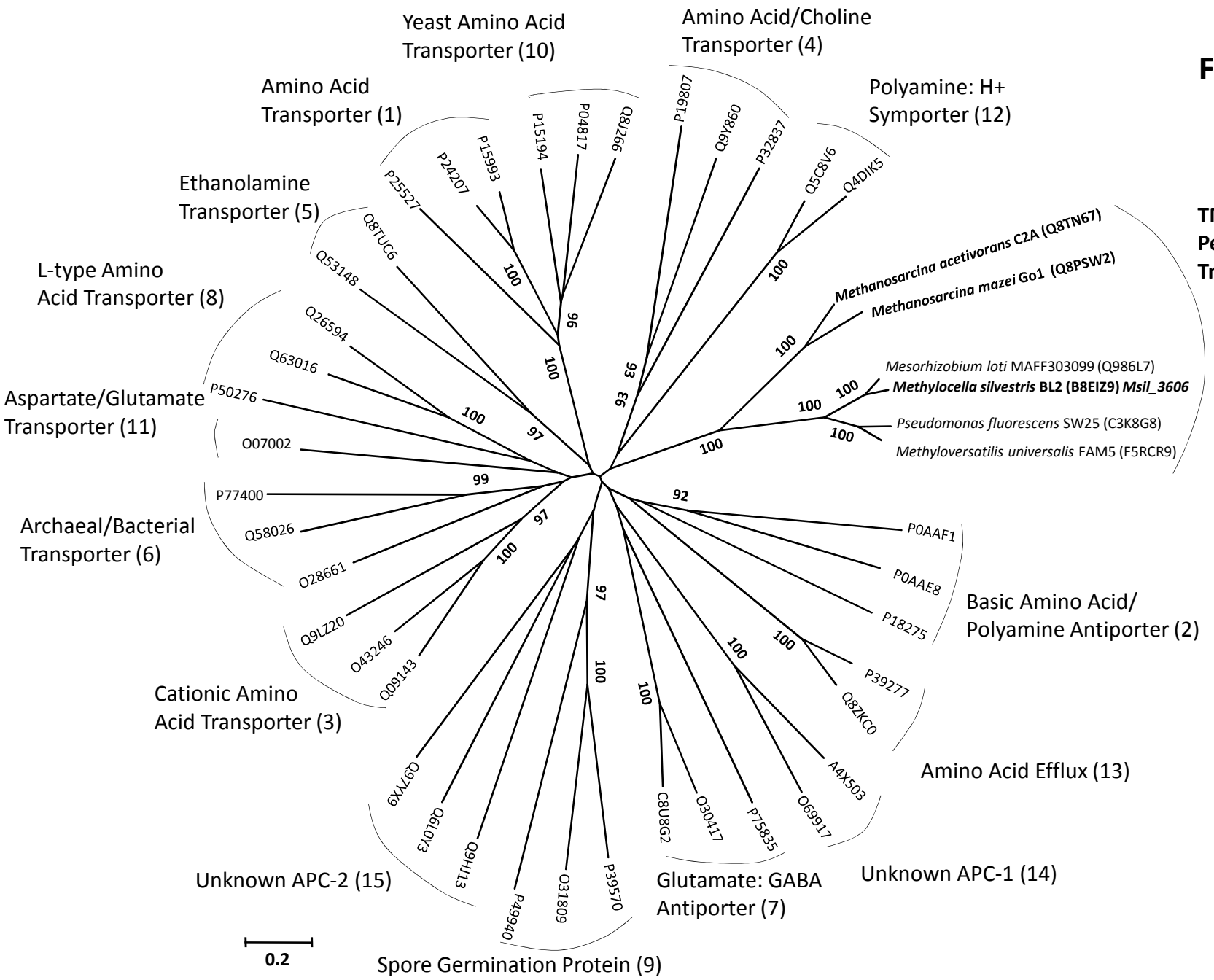
Urea carboxylase-associated protein 1
0.1

Figure 2B

Figure 3


Yeast Amino Acid Transporter (10)
Amino Acid/Choline Transporter (4)
Amino Acid Transporter (1)
Polyamine: H+ Symporter (12)
Ethanolamine Transporter (5)
TMAO Permease, TmoP (16)
L-type Amino Acid Transporter (8)
Methanosarcina acetivorans C2A (Q8TN67)
Methanosarcina mazei Go1 (Q8PSW2)
Mesorhizobium loti MAFF303099 (Q986L7)
Methylocella silvestris BL2 (B8EIZ9) Msil_3606
Pseudomonas fluorescens SW25 (C3K8G8)
Methyloversatilis universalis FAM5 (F5RCR9)
Aspartate/Glutamate Transporter (11)
Archaeal/Bacterial Transporter (6)
Basic Amino Acid/ Polyamine Antiporter (2)
Cationic Amino Acid Transporter (3)
Amino Acid Efflux (13)
Unknown APC-2 (15)
Glutamate: GABA Antiporter (7)
Unknown APC-1 (14)
Spore Germination Protein (9)
0.2
P15194
P04817
Q8Z266
P19807
Q9Y860
P32837
Q5C8V6
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P15993
P24207
P25527
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Figure 7

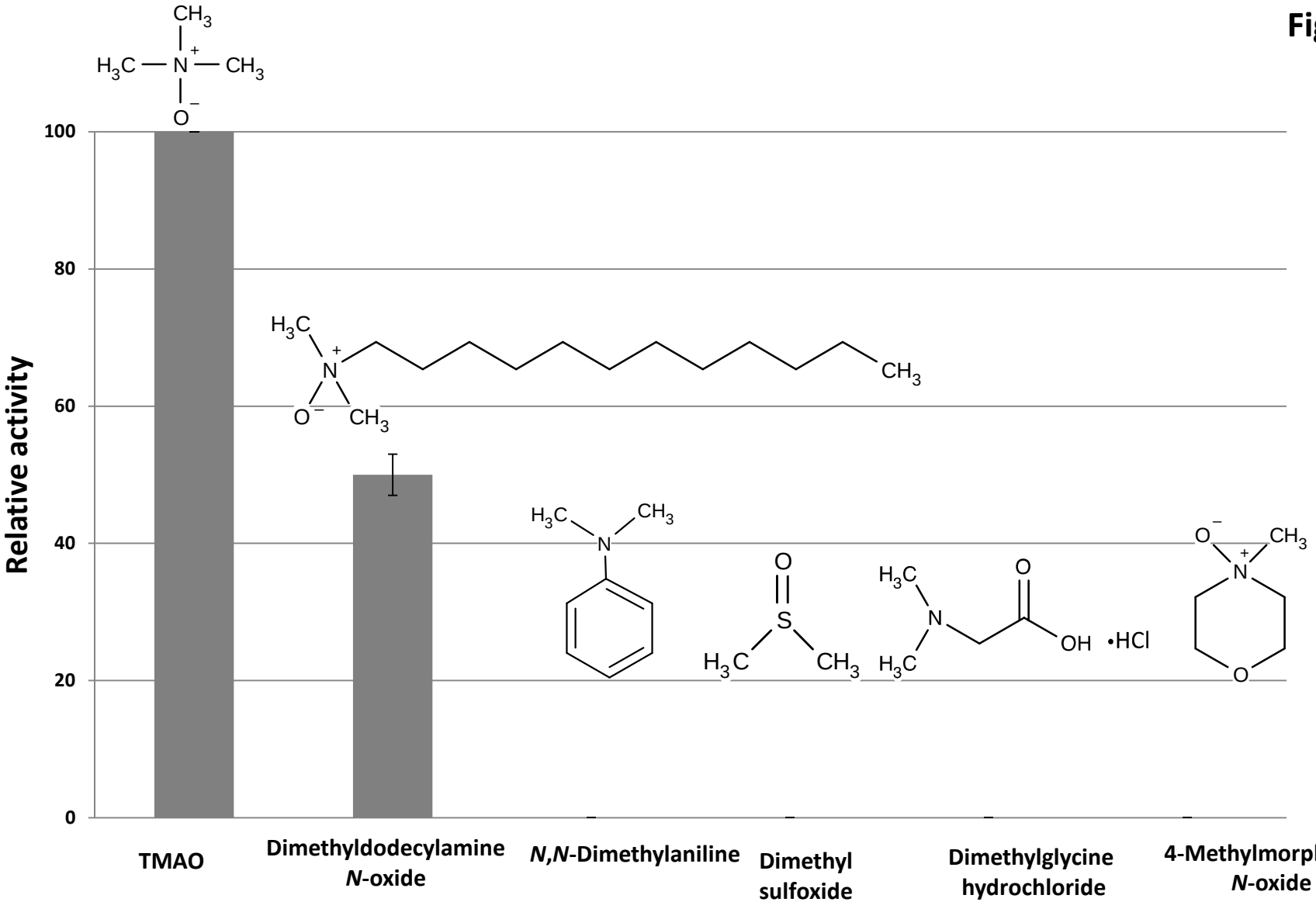

Relative activity
100
80
60
40
20
0
TMAO
Dimethyldodecylamine N-oxide
N,N-Dimethylaniline
Dimethyl sulfoxide
Dimethylglycine hydrochloride
4-Methylmorpholine N-oxide
•HCl

Figure 5

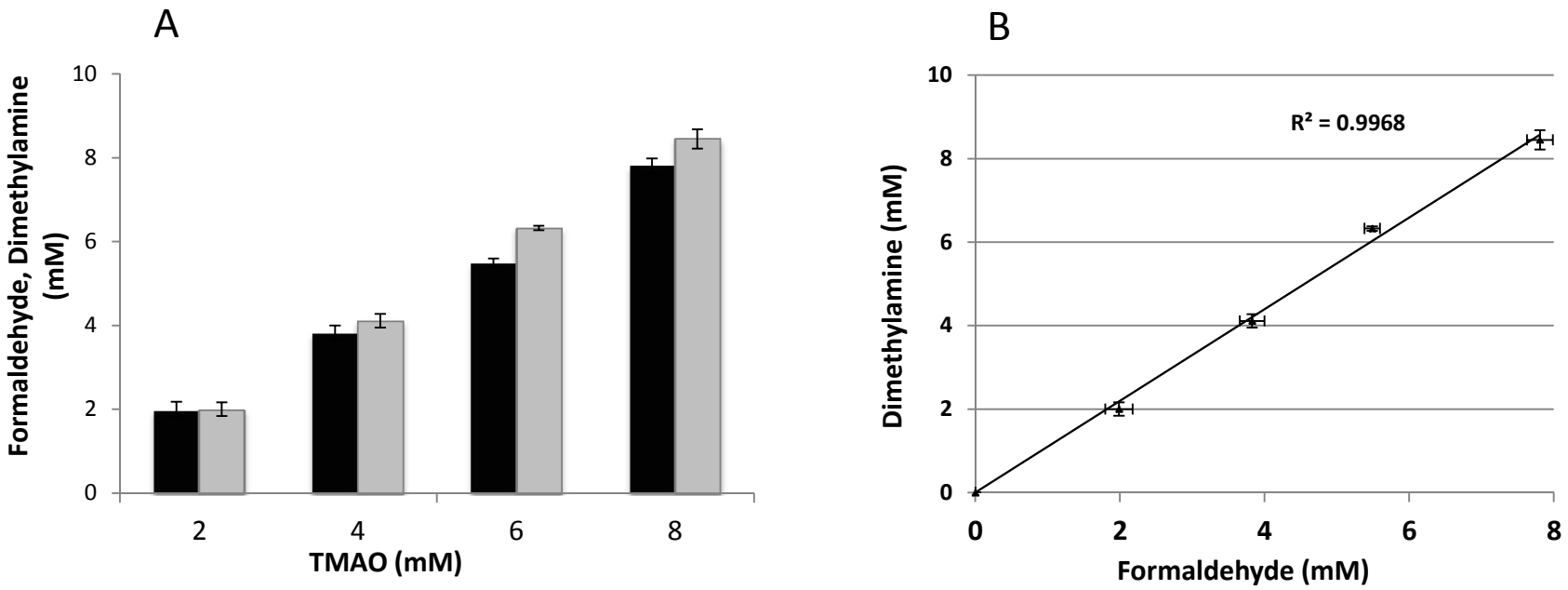

A
Formaldehyde, Dimethylamine (mM)
10
8
6
4
2
0
2
4
6
8
TMAO (mM)
B
Dimethylamine (mM)
R² = 0.9968
10
8
6
4
2
0
0
2
4
6
8
Formaldehyde (mM)

Figure 6

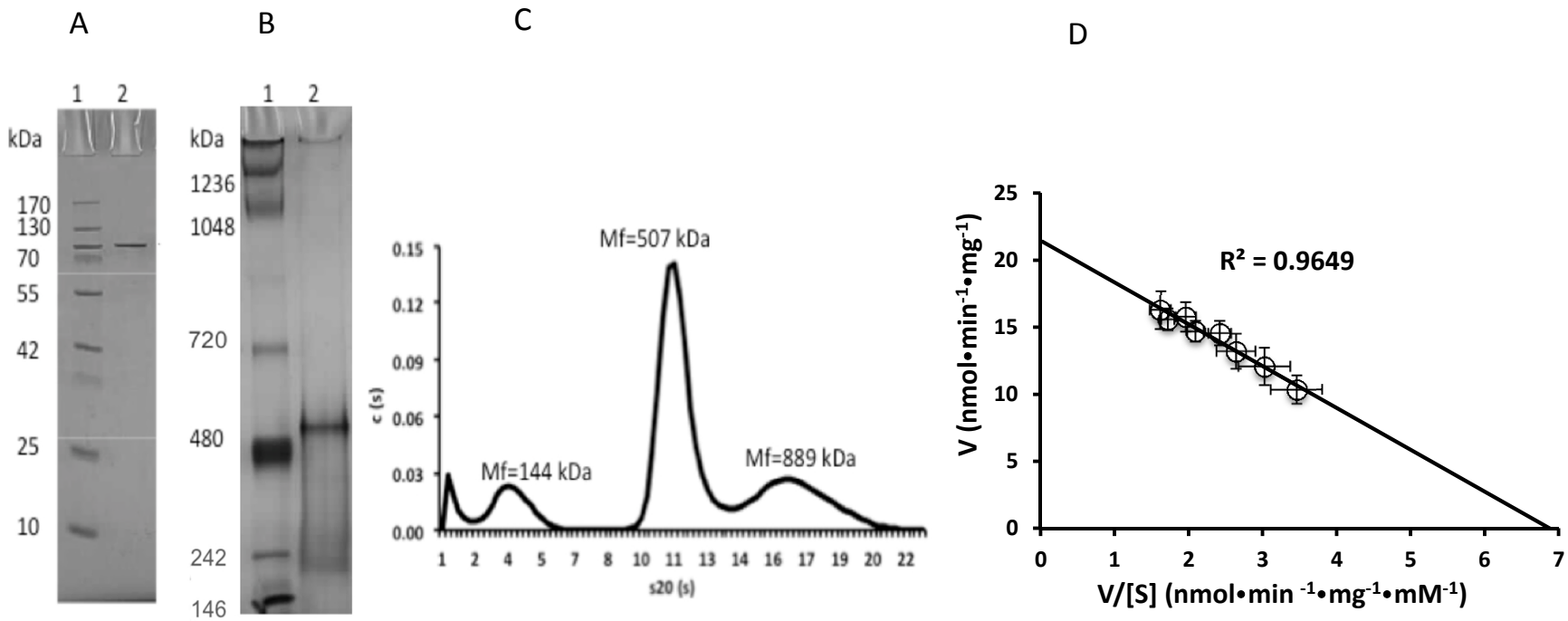
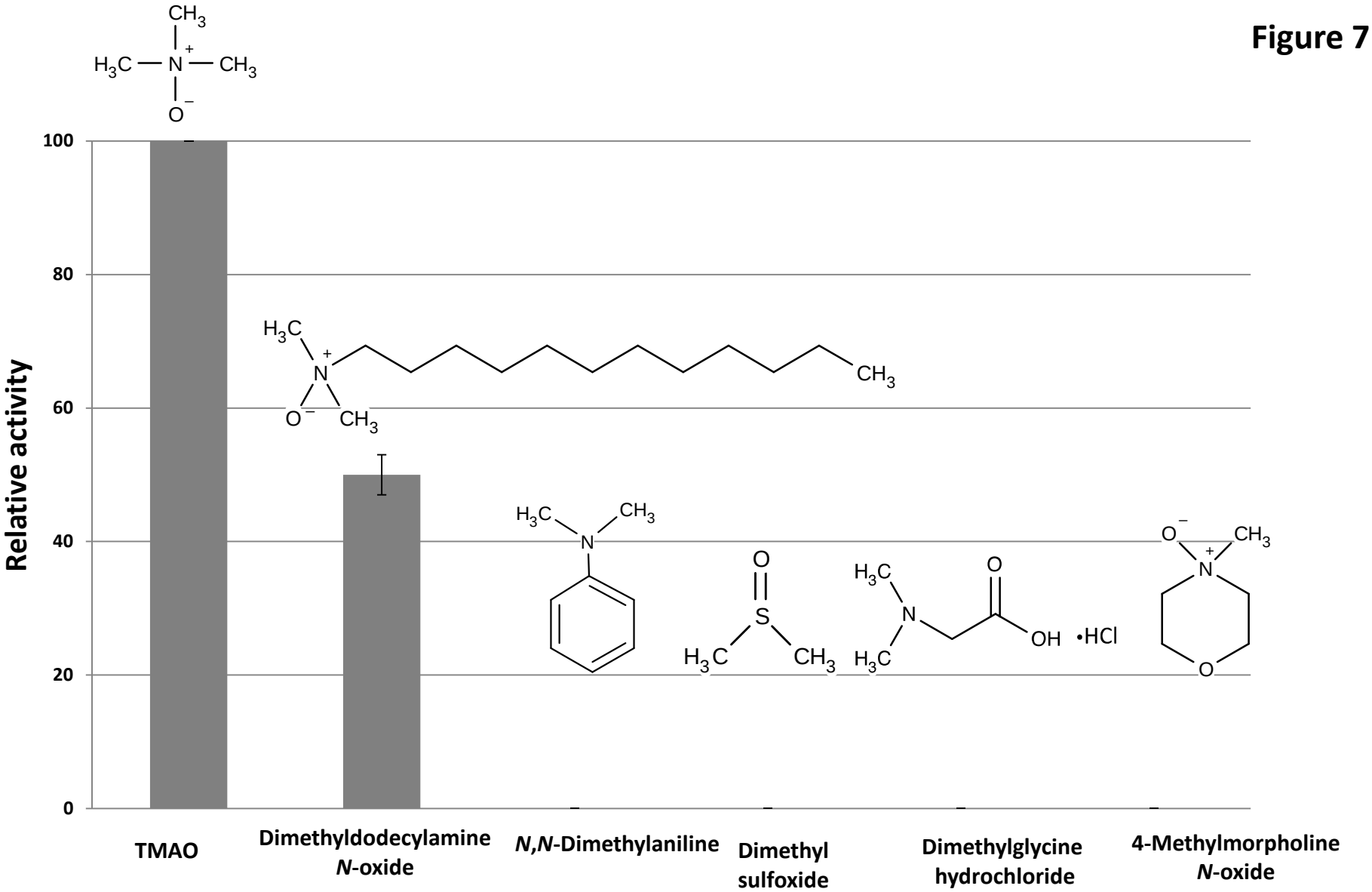
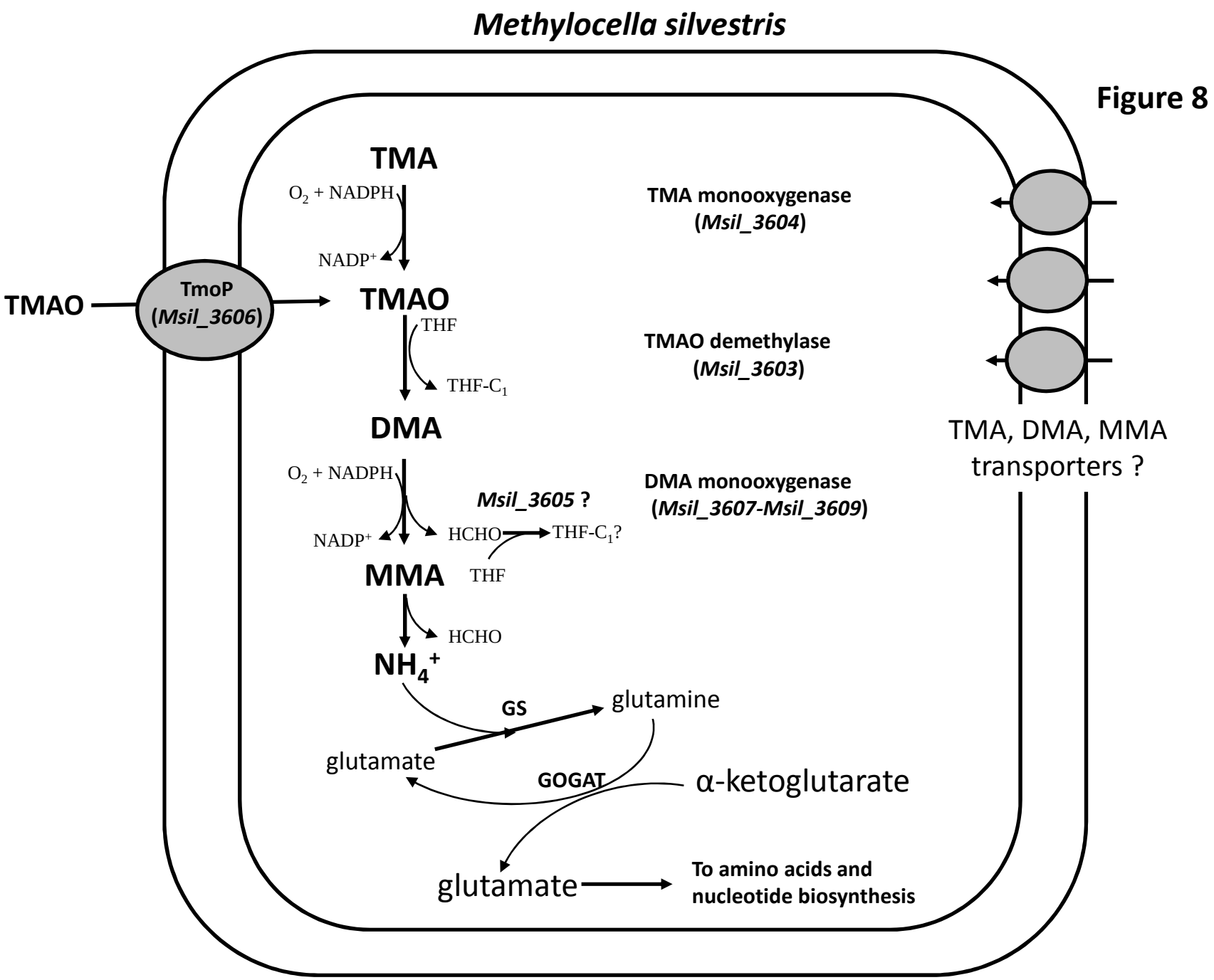

A
B
C
D
1
2
kDa
170
130
70
55
42
25
10
1236
1048
720
480
242
146
Mf=507 kDa
Mf=144 kDa
Mf=889 kDa
c (s)
s20 (s)
R² = 0.9649
V (nmol•min-1•mg-1)
V/[S] (nmol•min -1•mg-1•mM-1)

Figure 7


Relative activity
100
80
60
40
20
0
TMAO
Dimethyldodecylamine N-oxide
N,N-Dimethylaniline
Dimethyl sulfoxide
Dimethylglycine hydrochloride
4-Methylmorpholine N-oxide
•HCl


Methylocella silvestris
Figure 8
TMA
O2 + NADPH
NADP+
TMA monooxygenase
(Msil_3604)
TMAO
TmoP
(Msil_3606)
TMAO
THF
THF-C1
TMAO demethylase
(Msil_3603)
DMA
O2 + NADPH
Msil_3605 ?
NADP+
HCHO
THF-C1?
THF
DMA monooxygenase
(Msil_3607-Msil_3609)
MMA
HCHO
NH4+
GS
glutamine
glutamate
GOGAT
α-ketoglutarate
glutamate
To amino acids and
nucleotide biosynthesis
TMA, DMA, MMA
transporters ?