A mechanism for bacterial transformation of dimethylsulfide to dimethylsulfoxide: a missing link in the marine organic sulfur cycle

Ian Lidbury,[1][†] Eileen Kröber,[1][†] Zhidong Zhang,[2] Yijun Zhu,[1] J. Colin Murrell,[3] Yin Chen[1]* and Hendrik Schäfer[1]**

[1]School of Life Sciences, Gibbet Hill Campus, University of Warwick, Coventry CV4 7AL, UK.
[2]Institute of Microbiology, Xinjiang Academy of Agriculture Sciences, Urumqi 830091, China.
[3]School of Environmental Sciences, University of East Anglia, Norwich Research Park, NR4 7TJ, Norwich, UK.

Summary

The volatile organosulfur compound, dimethylsulfide (DMS), plays an important role in climate regulation and global sulfur biogeochemical cycles. Microbial oxidation of DMS to dimethylsulfoxide (DMSO) represents a major sink of DMS in surface seawater, yet the underlying molecular mechanisms and key microbial taxa involved are not known. Here, we reveal that *Ruegeria pomeroyi*, a model marine heterotrophic bacterium, can oxidize DMS to DMSO using trimethylamine monooxygenase (Tmm). Purified Tmm oxidizes DMS to DMSO at a 1:1 ratio. Mutagenesis of the *tmm* gene in *R. pomeroyi* completely abolished DMS oxidation and subsequent DMSO formation. Expression of Tmm and DMS oxidation in *R. pomeroyi* is methylamine-dependent and regulated at the post-transcriptional level. Considering that Tmm is present in approximately 20% of bacterial cells inhabiting marine surface waters, particularly the marine *Roseobacter* clade and the SAR11 clade, our observations contribute to a mechanistic understanding of biological DMSO production in surface seawater.

Introduction

Approximately 300 Tg of the volatile organosulfur compound, dimethylsulfide (DMS) is produced in the marine environment annually (Curson *et al.*, 2011), making the oceans the primary contributor to DMS in the atmosphere with an estimated flux of $\sim$20.7 Tg year^{-1} (Watts, 2000). DMS production occurs through transformation of the multifunctional metabolite dimethylsulfoniopropionate (DMSP) by a variety of algal and bacterial lyases (Curson *et al.*, 2011; Todd *et al.*, 2012). Interest in the cycling of DMS increased after the proposal that it can lead to the formation of cloud condensation nuclei, increasing cloud albedo and thereby acting as a global coolant (Charlson *et al.*, 1987). Therefore, any process contributing to the removal of DMS from surface seawater will have a direct effect on the attenuation of this flux, which may in turn have significant impacts on the global sulfur cycle, specifically its deposition to the terrestrial biome. DMS loss in surface seawater is mediated through different pathways including photochemical oxidation (Brimblecombe and Shooter, 1986) and biological consumption, which includes its assimilation into biomass, dissimilation to CO_2 and inorganic soluble sulfur compounds, or oxidation to dimethylsulfoxide (DMSO) (Schäfer *et al.*, 2010). Importantly, DMSP, DMS and DMSO are frequently detected in the water column (Hatton *et al.*, 1998; Simó *et al.*, 2000; Vila-Costa *et al.*, 2008) and also sea ice (Asher *et al.*, 2011) at comparable values with concentrations of dissolved DMSO tending to increase towards the end of phytoplankton blooms as cells undergo senescence (Simó *et al.*, 1998; Hatton and Wilson, 2007) when DMSO concentrations can even exceed those of DMSP (Hatton *et al.*, 1998).

In surface seawater, biological consumption of DMS was shown to be a major component of the global sink for DMS, accounting for up to 99% of the DMS produced (Kiene and Bates, 1990). Tracer studies using ^{35}S-DMS suggest that only a small fraction of DMS ($\sim$2–7%) is assimilated as a sulfur source by marine microorganisms whilst the majority of DMS is transformed into either DMSO or sulfate (Zubkov *et al.*, 2001; Vila-Costa *et al.*, 2006; del Valle *et al.*, 2007a), potentially *via* other inorganic sulfur compounds such as thiosulfate and tetrathionate

which are known end-products of DMS catabolism in marine DMS-degrading bacteria (De Zwart *et al.*, 1996; Boden *et al.*, 2012). In marine surface waters, oxidation to DMSO is the major fate of DMS, accounting for ∼70% and 50–70% of the total oxidized DMS in the Sargasso Sea (del Valle *et al.*, 2007a) and in the Ross Sea (del Valle *et al.*, 2009) respectively. Although it has previously been suggested that DMSO production is carried out by phytoplankton (Simó *et al.*, 1998; del Valle *et al.*, 2007b), DMSO production from DMS oxidation can be stimulated by the availability of organic carbon suggesting a role of marine heterotrophic bacteria in DMSO formation. The underpinning molecular and biochemical mechanisms, however, remain poorly understood (González *et al.*, 1999; Green *et al.*, 2011; Hatton *et al.*, 2012).

Marine surface waters are frequently dominated by two groups of heterotrophic bacteria of the *Alphaproteobacteria* phylum, the marine *Roseobacter* clade (MRC) and the SAR11 clade (Morris *et al.*, 2002; Buchan *et al.*, 2005; Giebel *et al.* 2011; Gilbert *et al.*, 2012). The SAR11 clade are oligotrophic microbes that are adapted to low nutrient environments and are found throughout the world's oceans, dominating bacterial communities in the open ocean gyres (Morris *et al.*, 2002; Giovannoni *et al.*, 2005). In contrast, the MRC can account for up to 20% of bacterial cells in coastal waters during periods of elevated primary production (Gonzáles *et al.*, 2000; Buchan *et al.*, 2005; Gilbert *et al.*, 2012). Combined, these two clades are major players in the uptake and metabolism of phytoplankton-derived organic carbon and nitrogen and reduced sulfur compounds in marine surface waters (Miller and Belas, 2004; Alonso and Pernthaler, 2006; Sowell *et al.*, 2008; Sun *et al.*, 2011; Williams *et al.*, 2012; Gifford *et al.*, 2013; Ottesen *et al.*, 2013; Aylward *et al.*, 2015; Lidbury *et al.*, 2015a). The abundance of MRC in surface seawater is often positively correlated with elevated levels of DMSP, DMS and DMSO (Gonzáles *et al.*, 2000; Zubkov *et al.*, 2001; Hatton *et al.*, 2012; Nelson *et al.*, 2014; Cui *et al.*, 2015).

We have previously shown that bacterial trimethylamine monooxygenase (Tmm), which is responsible for the oxidation of trimethylamine (TMA) into trimethylamine *N*-oxide (TMAO), can also oxidize DMS at comparable rates to that of TMA (Chen *et al.*, 2011). Specifically, purified Tmm from both the SAR11 clade and MRC can efficiently oxidize DMS. The gene encoding Tmm occurs in ∼20% of bacterial cells inhabiting the surface waters of the oceans (Chen *et al.*, 2011), and is particularly common in bacteria of the MRC and SAR11 clade. In the Sargasso Sea, a region where biological DMSO production occurs (del Valle *et al.*, 2007a), TMA was rapidly oxidized to CO_2 (Sun *et al.*, 2011) and in the Antarctic Ocean, both concentrations of TMAO and DMSO were significantly higher than either TMA or DMS, suggesting that cooxidation of these compounds was taking place at the time of sampling (Gibb and Hatton, 2004). We, therefore, hypothesized that a significant proportion of the observed DMSO formation in surface seawater is due to the oxidation of DMS by Tmm-containing heterotrophic bacteria. In this study, we describe for the first time DMSO formation in the model marine bacterium, *Ruegeria pomeroyi* DSS-3 (Gonzáles *et al.*, 2003) and demonstrate that methylated amine-dependent DMSO formation is a common trait of the MRC.

Results

Oxidation of DMS to DMSO by *Ruegeria pomeroyi* DSS-3

We previously showed that purified Tmm from selected MRC and SAR11 bacteria can oxidize DMS with an affinity similar to that of its native substrate, TMA (Chen *et al.*, 2011). In order to determine the product of DMS oxidation by Tmm, we purified Tmm of *R. pomeroyi* from a recombinant *E. coli* expressing Tmm and showed that DMS oxidation resulted in the formation of DMSO at a 1:1 ratio (Fig. 1A). Unlike TMAO, which can be further degraded in this bacterium through a TMAO demethylase (Tdm, E.C.4.1.2.32) (Lidbury *et al.*, 2014, Zhu *et al.*, 2014), DMSO is not a substrate for Tdm (Fig. 1B), suggesting that DMSO is the end-product of DMS co-oxidation by Tmm (Fig. 1C).

We then investigated whether DMSO could be formed from DMS oxidation by *R. pomeroyi*. It has previously been reported that *R. pomeroyi* can oxidize DMS (González *et al.*, 1999), however, no significant DMS oxidation was observed when it was grown on glucose and ammonium as the sole carbon and nitrogen source, respectively, (Fig. 2A). Under these growth conditions, Tmm activity was not detectable (Chen *et al.*, 2011). TMA has previously been shown to be both a nitrogen and energy, but not a carbon source for *R. pomeroyi* (Chen *et al.*, 2011; Lidbury *et al.*, 2014). In order to induce Tmm expression, we incubated a *R. pomeroyi* starter culture supplemented with 0.25 mM TMA in addition to glucose and ammonium (Chen *et al.*, 2011) and used this as the inoculum for induction experiments. Subsequently, complete DMS oxidation occurred within 42 h (Fig. 2B). All the DMS (0.8 mM) was transformed into DMSO (0.81 ± 0.03 mM) indicating that *R. pomeroyi* does not further catabolize DMSO under these growth conditions. To further confirm that DMS oxidation was due to the activity of Tmm *in vivo*, we tested the *tmm* knockout mutant (Δ*tmm::Gm*) (Lidbury *et al.*, 2015b), which can no longer utilize TMA, under the same conditions. As expected, no DMS oxidation was detected throughout the growth cycle (Fig. 2C). The essential role of Tmm in DMS oxidation to DMSO was further validated by complementation of the *tmm* knockout mutant with either *tmm* from *R. pomeroyi* or *Roseovarius* sp. 217 (Schäfer *et al.*, 2005). Complementation of the Δ*tmm::Gm* mutant with either *tmm* homolog restored both growth on TMA as
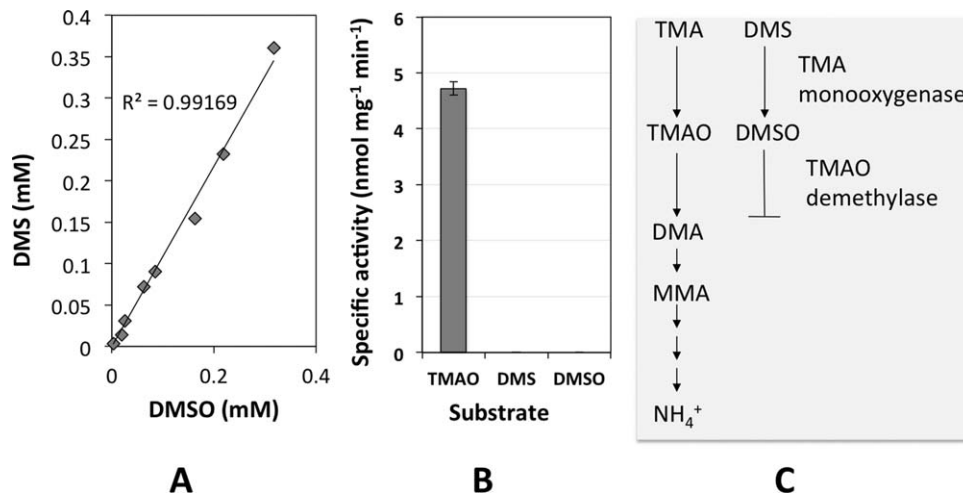
Fig. 1. Co-oxidation of DMS to DMSO by Tmm.
A. Trimethylamine monooxygenase (Tmm) of *R. pomeroyi* DSS-3 oxidizes DMS to DMSO at a 1:1 ratio.
B. The substrate specificity of purified trimethylamine *N*-oxide demethylase (Tdm) of *R. pomeroyi*. Error bars denote s.d. from triplicate reactions.
C. TMA oxidation and subsequent metabolism by Tmm, Tdm and the enzymes involved in the gammaglutamylmethylamide/*N*-methylglutamate pathway (Chen, 2012) results in the release of ammonium as a nitrogen source for this bacterium. However, DMSO, the product from the co-oxidation of DMS by Tmm, appears to be the end product of this pathway because it is not a substrate for Tdm.
TMA, trimethylamine; TMAO, trimethylamine *N*-oxide; DMA, dimethylamine; MMA, monomethylamine, NH_4^+, ammonium.
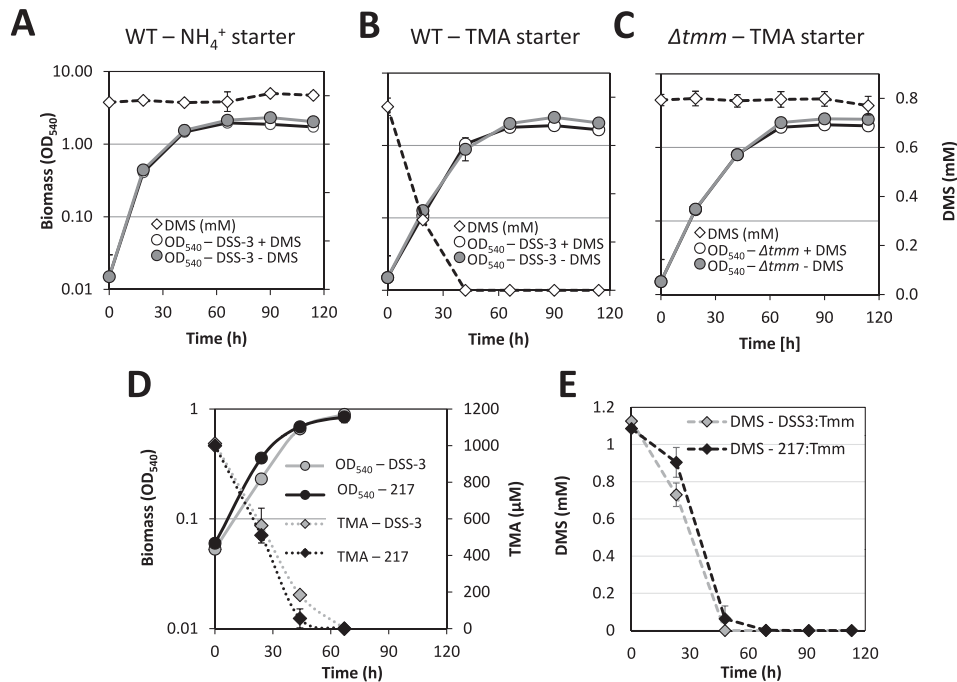
Fig. 2. DMS oxidation in the cultures of *R. pomeroyi* DSS-3 wild type and a *tmm* knockout mutant. (A) No decrease in DMS concentration (white diamonds) was observed in cultures of wild-type cells pre-incubated with glucose and ammonium only. (B) Complete oxidation of DMS (white diamonds) to DMSO occurred when wild-type cells were pre-incubated with TMA (250 μM) as well as glucose and ammonium. (C) DMS oxidation (white diamonds) did not occur in the mutant, Δ*tmm::Gm*, despite pre-incubation with TMA, glucose and ammonium (250 μM). Complementation of the Δ*tmm::Gm* mutant with either native *tmm* of *R. pomeroyi* DSS-3 or *tmm* from *Roseovarius* sp. 217 restored its growth on TMA as a sole nitrogen source (D) and TMA-dependent DMS oxidation (E). No effect on the growth rate or final growth yield was observed for *R. pomeroyi* cultures grown in the presence (white circles) or absence (grey circles) of DMS (0.8 mM). Results are the mean of triplicate cultures. Error bars denote s.d.
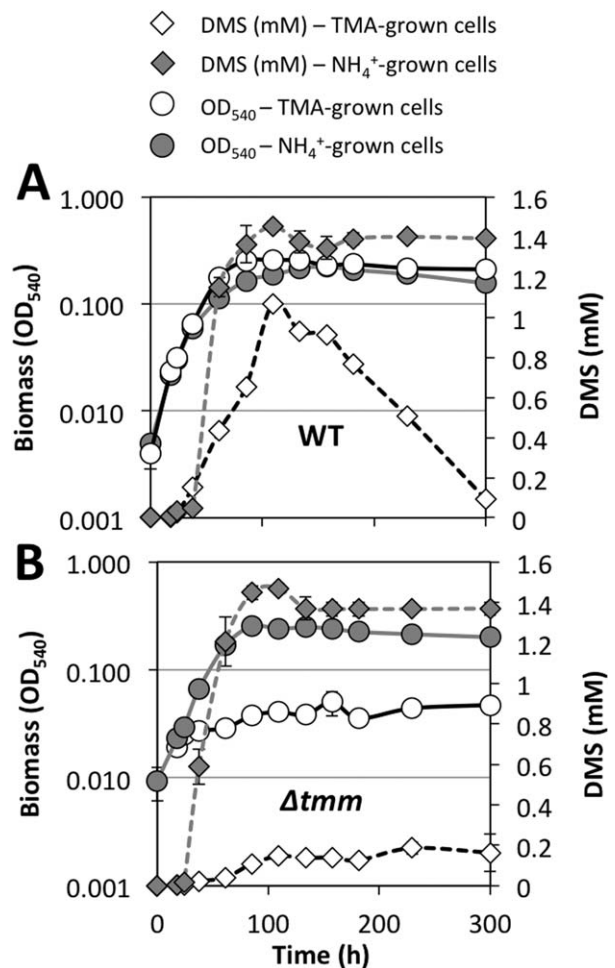
a sole nitrogen source (Fig. 2D) and TMA-dependent DMS oxidation (Fig. 2E). Collectively, our data confirm that DMS can be oxidized to DMSO in *Ruegeria pomeroyi* through Tmm, which can be induced by TMA.

TMA attenuates DMS production during growth of R. pomeroyi *on DMSP*

Next, we investigated whether *R. pomeroyi* can also produce DMSO during growth on DMSP. DMSP is an important osmolyte and a precursor for DMS, which is common in seawater (Kiene *et al.*, 2000). *R. pomeroyi* was isolated using DMSP as the sole carbon source from seawater collected off the coast of Georgia during an algal bloom (González *et al.*, 1999; 2003). *R. pomeroyi* can use DMSP as the sole carbon source and it is known to produce DMS directly from the cleavage of DMSP using various DMSP lyases (Curson *et al.*, 2011; Todd *et al.*, 2012). When the wild type and the Δ*tmm* mutant were grown on ammonium and DMSP (5 mM) as the sole nitrogen and carbon sources, respectively, ~1.4 mM of DMS accumulated in the culture and was not subjected to further degradation (Fig. 3A and B). To determine whether TMA had an impact on the production and subsequent conversion of DMS to DMSO, *R. pomeroyi* was grown on DMSP (5 mM) as the sole carbon source with TMA (0.25 mM) as the sole nitrogen source. The Δ*tmm* mutant failed to grow on TMA as the sole nitrogen source (Fig. 3B); consequently, only 0.2 mM DMS was produced (Fig. 3B). Given the fact that no DMS oxidation occurred when this mutant was grown on glucose and ammonium after pre-incubation with TMA (Fig. 2C) it was not surprising that none of the DMS produced was converted to DMSO. Wild-type TMA-grown cells showed a 27% reduction of maximal DMS concentrations in the headspace during initial growth (within 100 h) compared to that in the ammonium-grown cultures (Fig. 3A). This was followed by near complete oxidation of DMS to DMSO in TMA-grown cells (Fig. 3A). These data therefore suggest that in the presence of TMA, DMSO represents a major metabolic end product of DMSP catabolism in *R. pomeroyi*.

Co-oxidation of DMS can also be induced by the metabolites of TMA degradation in R. pomeroyi

Considering that individual methylated amines species [monomethylamine (MMA), dimethylamine (DMA), TMA or TMAO] rarely exist in isolation in the marine environment (Gibb *et al.*, 1999; Gibb and Hatton, 2004), we investigated whether Tmm activity and hence DMS oxidation occurred in the presence of other methylated amines e.g. MMA, DMA or TMAO. The wild-type and the Δ*tmm* mutant of *R. pomeroyi* were grown on methylated amines which are metabolites of TMA degradation (TMAO, DMA and MMA) as a source of

◇ DMS (mM) – TMA-grown cells
◆ DMS (mM) – NH$_4^+$-grown cells
○ OD$_{540}$ – TMA-grown cells
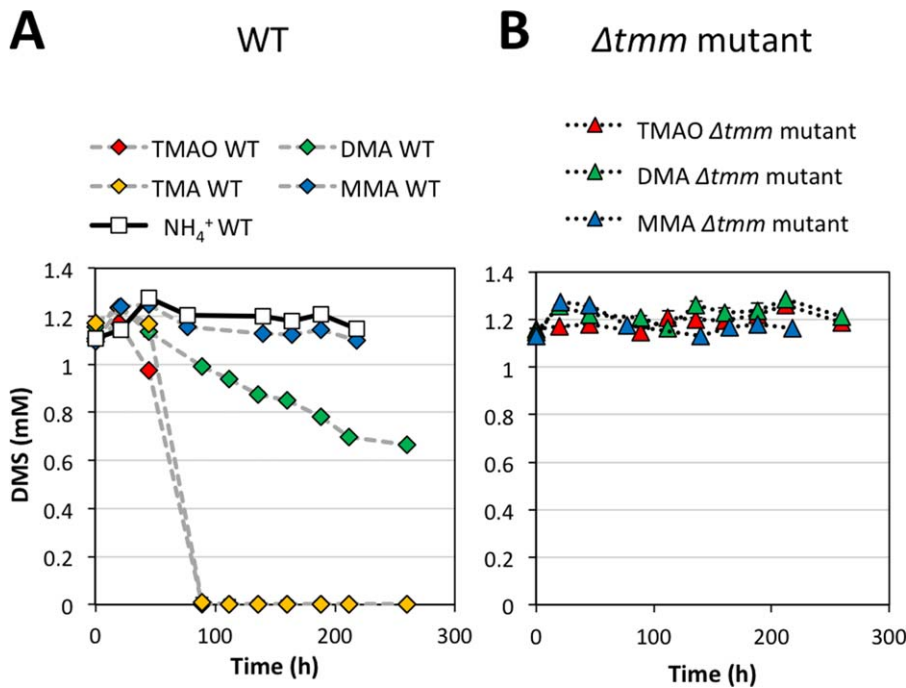● OD$_{540}$ – NH$_4^+$-grown cells

Fig. 3. DMS oxidation to DMSO in *R. pomeroyi* DSS-3 grown on DMSP as the main carbon substrate. Quantification of DMS (diamonds) during growth of *R. pomeroyi* (circles) on either ammonium (grey) or TMA (white) as the sole nitrogen source (0.25 mM) and DMSP (5 mM) as the main carbon substrate. Either wild-type (A) or the mutant, Δ*tmm::Gm* (B), cells were used for incubations. Results are the mean of triplicate cultures. Error bars denote s.d.

nitrogen and DMS consumption was quantified. For the wild-type cells, in addition to TMA, growth on TMAO and DMA as a nitrogen source also led to the oxidation of DMS to DMSO (Fig. 4A). As expected, the Δ*tmm* mutant failed to oxidize DMS despite growth on these metabolites of TMA metabolism (Fig. 4B) further confirming that Tmm is solely responsible for DMS oxidation in *R. pomeroyi*.

To investigate the transcriptional regulation of *tmm*, and hence DMS oxidation to DMSO by Tmm, the sensitivity of the promoter of *tmm* (P$_{tmm}$) in response to methylated compounds was determined using a *lacZ* reporter assay (Todd *et al.*, 2012). A *R. pomeroyi* transconjugant, containing the P$_{tmm}$-*lacZ* fusion plasmid, was grown overnight on a minimal medium containing glucose and ammonium and

A WT

- ◆ TMAO WT
- ◆ TMA WT
- ◆ DMA WT
- ◆ MMA WT
- □ NH$_4^+$ WT

B Δtmm mutant

- ▲ TMAO Δtmm mutant
- ▲ DMA Δtmm mutant
- ▲ MMA Δtmm mutant

Fig. 4. Methylamine-dependent DMS oxidation in *R. pomeroyi* DSS-3. Quantification of DMS during growth of *R. pomeroyi* wild type (A) or the mutant Δtmm::Gm (B) on different methylated amines as a sole nitrogen source. Results are mean of triplicate cultures. Error bars denote s.d.

supplemented with TMA, TMAO, DMA, MMA, DMS or DMSO (0.5 mM) before assaying for the reporter, β–galactosidase activity (Lidbury *et al.*, 2014). Compared with controls with NH$_4^+$, incubation with TMA and DMA led to the induction of the P$_{tmm}$-*lacZ* fusion giving twofold and threefold levels of β–galactosidase activity (Fig. 5A). Strikingly, DMS alone resulted in a ~7-fold induction of the P$_{tmm}$-*lacZ* fusion, however, *R. pomeroyi* does not oxidize DMS without methylated amines in the defined medium (Fig. 4A). RT-PCR assays targeting *tmm* confirmed that the presence of DMS does indeed result in the upregulation of *tmm* transcription (Fig. 5C) despite there being no detectable enzyme activity of Tmm (Fig. 5B). No apparent difference in the expression of the 16S rRNA gene was detected between either the control cultures or those cultures supplemented with either TMA or DMS (Fig. 5C). Furthermore, no detectable Tmm activity was observed in cells grown on TMAO as a nitrogen source, but the presence of both TMAO and DMS resulted in the induction of Tmm activity (Fig. 5B). These results confirm that TMA, DMA, as well as DMS are capable of inducing *tmm* transcription. However this induction of *tmm* at the transcriptional level does not necessarily result in a functional Tmm.

The transcriptional regulator TmoR is required for tmm *transcription, however production of a functional Tmm is regulated post-transcriptionally in* R. pomeroyi

The *lacZ* reporter assays suggested that *tmm* transcription in wild type cells of *R. pomeroyi* is induced by TMA and other methylated amine intermediates, such as DMA. Transcriptional regulation of *tmm* is predicted to be regulated

by a putative GntR repressor encoded by SPO1553, containing a conserved pfam00392 domain, which is found in GntR-like proteins. This putative GntR-like repressor (hereafter named as TmoR) was therefore targeted for mutagenesis, generating the mutant, ΔtmoR::Gm. Results from P$_{tmm}$-*lacZ* reporter assays in the ΔtmoR::Gm mutant (Fig. 5A) demonstrate that *tmm* is constitutively expressed in this mutant, confirming that TmoR functions as a repressor for *tmm*. RT-PCR assays confirmed that *tmm* is constitutively expressed even in the absence of methylated amines or DMS (Fig. 5C). Despite constitutive expression of *tmm* in the ΔtmoR::Gm mutant, this strain still could not oxidize DMS in the absence of methylated amines (Fig. 5D), but could oxidize DMS in their presence. These results, therefore, point to post-transcriptional regulation of Tmm activity, and hence mediation of DMS oxidation to DMSO by methylated amines.

To better understand which methylated amines are responsible for post-transcriptional regulation of Tmm activity, we performed DMS oxidation experiments using the Δtdm::Gm mutant, which is unable to demethylate TMAO to DMA (Lidbury *et al.*, 2014). When grown under similar conditions to the wild-type, i.e. the addition of TMA to the starter culture, no DMS oxidation was observed (Fig. 6A). When the same experiment was performed using the *tdm*-complemented strain, which has a restored ability to demethylate TMAO to DMA (Lidbury *et al.*, 2014), complete oxidation of DMS occurred (Fig. 6A). This suggests that formation of a fully functional Tmm is probably dependent on the presence of DMA. To test this hypothesis, we grew the Δtdm mutant (using a starter culture
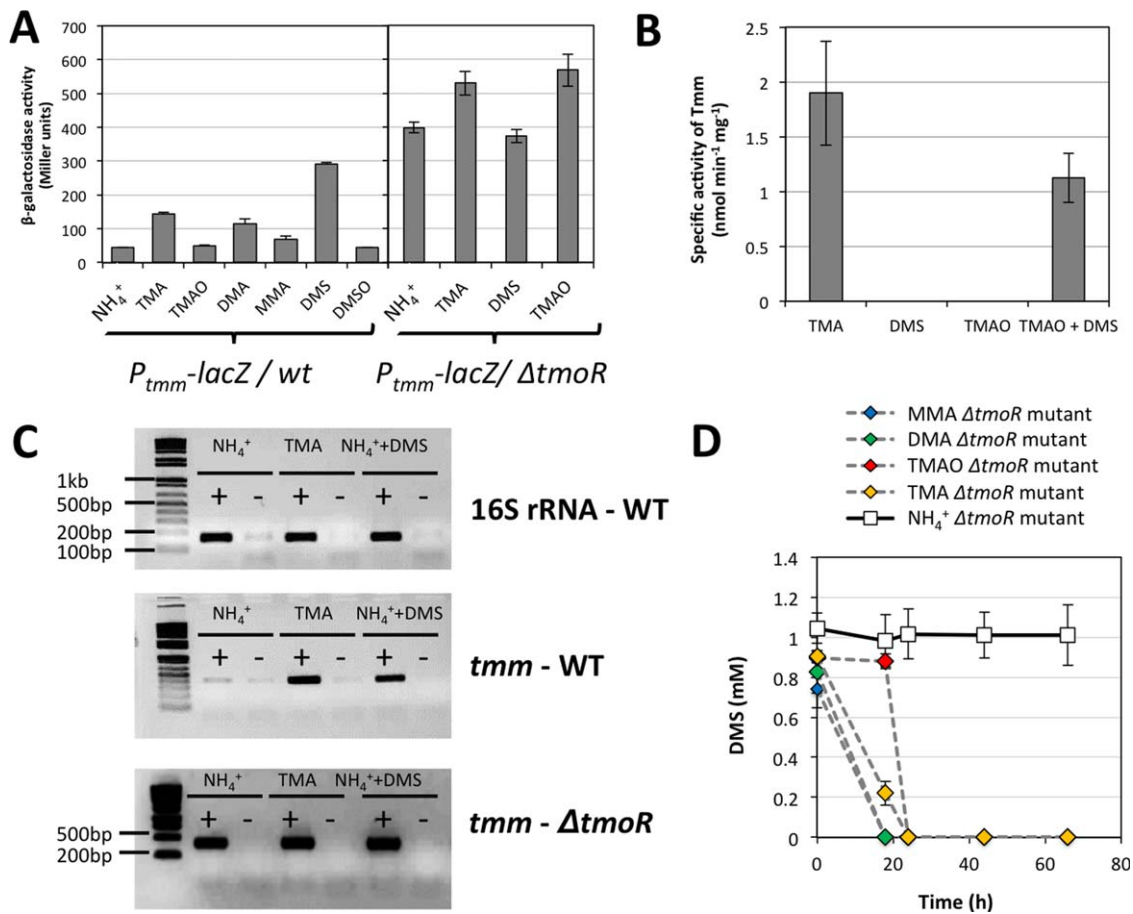
Fig. 5. Transcriptional regulation of *tmm* in *R. pomeroyi*.
A. *lacZ* reporter assays using the promoter of *tmm* (P*tmm*) show that P*tmm* is activated by DMS as well as TMA and that knocking out the repressor gene (*tmoR*) leads to constitutive transcription of P*tmm*.
B. No Tmm activity was detected when *R. pomeroyi* was exposed to DMS without methylated amines present.
C. Reverse transcription-PCR results show that both DMS and TMA led to the up-regulation of transcription of *tmm* (compared to the levels of 16S rRNA transcription) in the wild type strain whereas in the *tmoR* mutant, *tmm* transcription appears to be constitutive. Minus sign indicates negative control without reverse transcriptase.
D. DMS consumption in the Δ*tmoR* mutant in the presence of various methylamines. Results presented in panels A, B and D are mean of triplicate cultures. Error bars denote s.d.

supplemented with TMA to induce *tmm*) using ammonium as a nitrogen source and supplemented it with TMA, TMAO or DMA (0.5 mM). Indeed, the rate of DMS oxidation was significantly greater in the presence of DMA (1.38 mM d^{-1}) compared with that of cultures grown in the presence of either TMA (0.32 mM d^{-1}) or TMAO (0.22 mM d^{-1}) or ammonium alone (0 mM d^{-1}) (Fig. 6B), suggesting that, out of the compounds tested in this study, DMA is mainly responsible for post-transcriptional regulation of Tmm activity.

Methylamine-dependent DMS co-oxidation is a common trait of MRC bacteria

In order to investigate whether methylamine-dependent DMS co-oxidation is unique in *Ruegeria pomeroyi* DSS-3,

we further tested a number of cultivated bacteria of the MRC clade. The data presented in Table 1 showed that several members of the MRC that are capable of utilizing TMA, including *Roseovarius* sp. TM1035, *Roseobacter litoralis* Och149, also displayed methylamine-dependent DMS oxidation. No DMS oxidation occurred unless cultures were supplemented with methylated amines such as TMA and TMAO. Those isolates containing *tmm* also oxidized DMS when grown in a complex medium (1/2 YTSS), probably due to the presence of amines in this complex medium. Furthermore, two new strains, *Phaeobacter* sp. TMAL401 and *Roseobacter* sp. TMAL402, which were isolated during this study on TMA as a sole carbon source from coastal seawater off the coast of Plymouth also showed methylamine-dependent DMS oxidation (Table 1). Together these data suggest that methylamine-dependent
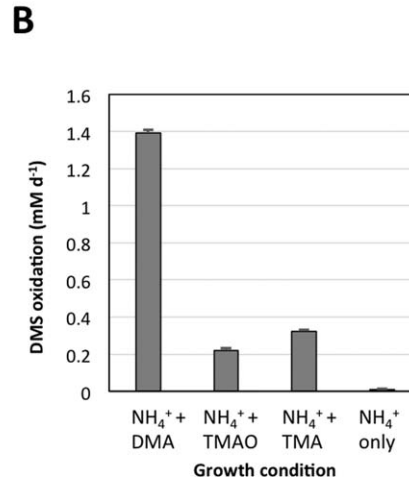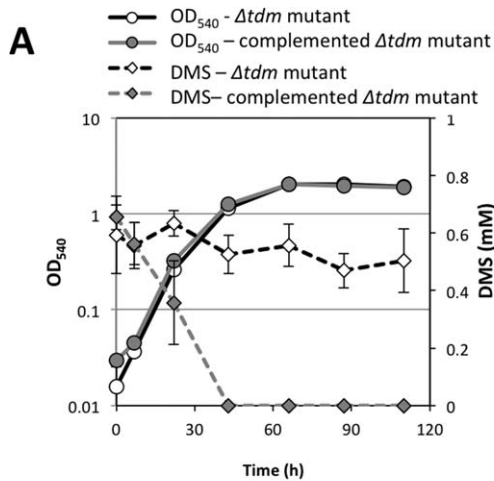
Fig. 6. Methylated amine-dependent DMS oxidation in the *R. pomeroyi* mutant, Δ*tdm::Gm*. The Δ*tdm* mutant (white) and the complemented Δ*tdm* mutant (grey) grown on glucose as a sole carbon and energy source and ammonium as a sole nitrogen source with TMA (0.25 mM) added to the starter culture (A). DMS oxidation rates calculated for the Δ*tdm* mutant when grown on glucose and ammonium and supplemented with either TMA, TMAO or DMA (B). Results are mean of triplicate cultures. Error bars denote s.d.

co-oxidation of DMS may be a ubiquitous trait of TMA-utilising heterotrophic bacteria.

Discussion

Here we present evidence that the oxidation of DMS by Tmm is responsible for DMSO formation in the heterotrophic bacterium, *R. pomeroyi* and other closely-related members of the MRC that harbour the *tmm* gene. The MRC is frequently associated with phytoplankton blooms and often represents a major proportion of the active community involved in the turnover of algal-derived organic matter, including DMSP (González *et al.*, 2000; Vila *et al.*, 2004; Buchan *et al.*, 2005; Alonso and Pernthaler, 2006). Our results reveal that in *R.*

pomeroyi the DMS produced by DMSP lyases (Curson *et al.*, 2011; Todd *et al.*, 2012) during growth on DMSP can be further oxidized to DMSO by Tmm in the presence of methylated amines. We therefore speculate that this molecular mechanism of DMS oxidation likely explains the frequently observed link between increased phytoplankton activity, dissolved organic matter including DMSP, abundance of MRC cells and elevated concentrations of DMSO (Hatton *et al.*, 1998; 1999; 2012; Zubkov *et al.*, 2001; 2004; Hatton and Wilson, 2007; Green *et al.*, 2011). Further research measuring the concentrations of methylated amines during periods of elevated primary production and phytoplankton blooms is required to better understand the role of Tmm-containing heterotrophs in the production of DMSO.

Table 1. A comparison of dimethylsulfide consumption among different members of the MRC when grown on different media.

Strain[1]	*tmm*[2]	NH_4^{+}[3]	TMA[3]	TMAO[3]	½ YTSS
Ruegeria pomeroyi DSS-3 wild type	+	−	+	+	a
Δ*tmm::Gm*	−	−	−	+	−
Δ*tmm::Gm* + DSS-3:*tmm*	+	−	+	NT	NT
Δ*tmm::Gm* + 217:*tmm*	+	−	+	NT	NT
Δ*tmoR::Gm*	+	−	+	+	NT
Δ*tdm::Gm*	+	−	Reduced	Reduced	NT
Δ*tdm::Gm* + DSS-3:*tdm*	+	−	+	+	NT
Roseovarius sp. 217	+	−	+	+	+
Roseovarius sp. TM1035	+	−	+	+	+
Roseovarius nubinhibens ISM	+	−	+	+	+
Roseobacter litoralis Och 149	+	−	+	+	+
Phaeobacter sp. TMAL401	+	a	+	+	+
Roseobacter sp. TMAL402	+	−	+	+	+
Roseobacter sp. MED193	−	−	−	−	−

a. Indicates variable DMS oxidation.
[1]Incubations were performed in triplicate supplemented with 1 mM DMS.
[2]Presence (+) or absence (−) of the *tmm* gene in the sequenced genome.
[3]Defined medium supplemented with different nitrogen sources.
NH_4^{+}, ammonium; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide; ½ YTSS, half strength yeast extract-tryptone-sea salt medium.

It was previously shown that *tmm* is abundant in metagenomes of marine surface waters, due to the occurrence of members of the MRC and the SAR11 clade (Chen *et al.*, 2011). Whether or not SAR11 clade bacteria can perform methylated amine-dependent DMS oxidation remains to be established, however purified recombinant Tmm from strains of the SAR11 clade can oxidize DMS in a similar manner to that of Tmm from MRC (Chen *et al.*, 2011). It is also known that *Candidatus* Pelagibacter ubique HTCC1062, a Tmm-containing representative of the SAR11 clade, can oxidize TMA to stimulate ATP production (Sun *et al.*, 2011). Furthermore, addition of TMA to natural Sargasso Sea water resulted in its complete oxidation (Sun *et al.*, 2011). In oligotrophic waters, such as the Sargasso Sea, SAR11 clade bacteria represent the numerically dominant proportion of the heterotrophic community (Morris *et al.*, 2002, Sjöstedt *et al.*, 2014) and peptides mapping to Tmm from SAR11 have been detected in this region (Sowell *et al.*, 2008). Therefore, this clade may be largely responsible for the organic carbon-stimulated increase in DMSO production ($\sim$70–90%) from DMS oxidation in the Sargasso Sea (Vila-Costa *et al.*, 2006).

As Tmm can oxidize DMS to DMSO, understanding the regulation of this enzyme is vital in order to determine the factors that control the loss of DMS loss from marine surface waters. Our data reveal that Tmm expression is controlled at both the transcriptional (through the repressor, TmoR) and post-transcriptional level because the TmoR knockout mutant did not have a methylamine-independent DMS oxidation phenotype (Fig. 5D). Post-transcriptional regulation in bacteria can be mediated by the occurrence of *cis*-acting antisense RNA molecules (asRNA) which can modulate translation of a given target gene through a variety of mechanisms (Georg and Hess, 2011; Sesto *et al.*, 2013). In the cyanobacterial strains, *Synechocystis* sp. PCC6803 and *Prochlorococcus* sp. MED4, asRNA can help promote translation through interactions with the primary gene transcript (Stazic *et al.*, 2011; Sakurai *et al.*, 2012). Interestingly, the diazotrophic cyanobacterium, *Trichodesmium erythraeum* IMS101, has a *tmm* homolog (53.6% identity to that of *R. pomeroyi*) and an associated asRNA molecule (Pfreundt *et al.*, 2014) and we also detected a putative promoter (P_{astmm}) located on the antisense strand of *tmm* within the coding region (Fig. 7). We therefore propose a model for Tmm regulation in *R. pomeroyi* (Fig. 7), which includes the presence of an asRNA molecule that helps to stabilize the primary transcript of *tmm*, thus protecting it from degradation by RNases. Based on our experimental data, we hypothesize that transcription of the asRNA molecule (as*tmm*) is induced by DMA through an as yet unknown regulator. In support of this model, RT-PCR assays targeting the antisense strand of *tmm* revealed the presence of a potential asRNA molecule that is upregulated in the presence of TMA (Supporting Information Fig. S1). Although other posttranscriptional and posttranslational regulation mechanisms of Tmm activity may also occur in this bacterium, such as protein modification and bacterial riboswitches (Bastet *et al.*, 2011), further research regarding the possible involvement of asRNA on Tmm activity is certainly warranted to better understand the regulation of this ecologically important enzyme.

DMS oxidation to DMSO can enhance chemoorganoheterotrophic growth in some marine bacteria (Boden *et al.*, 2011; Green *et al.*, 2011), but the molecular mechanism for the increase in growth yield in these isolates is unknown. The phototrophic bacterium *Rhodovulum sulfidophilum* can utilize DMS to DMSO (performed by DMS dehydrogenase) as an electron donating reaction for photoautotrophic growth (McDevitt *et al.*, 2002), however oxidation of DMS by Tmm does not result in any net gain of electrons or reducing equivalents. Indeed, Tmm actually consumes NADPH for the conversion of TMA to TMAO and DMS to DMSO (Chen *et al.*, 2011). This begs the question 'why do these bacteria possess an enzyme that shares a similar high affinity for two differing substrates?' The FAD-binding site of Tmm and closely related flavin-containing monooxygenases (FMOs) has been likened to a 'cocked gun' often present in a reduced state within the cell, awaiting a suitable substrate (Krueger and Williams, 2005). Bacterial and mammalian FMOs produce both superoxide anion radicals and hydrogen peroxide through spontaneous NADPH oxidation in the absence of these substrates (Williams *et al.*, 1985; Krueger and Williams, 2005; Alfieri *et al.*, 2008). Indeed, in FMO purified from rabbit liver, up to 41% of the total NADPH oxidized resulted in hydrogen peroxide production (Tynes *et al.*, 1986). Co-oxidation of DMS by Tmm may therefore have a role in the prevention of free radical formation and subsequent oxidative stress when TMA stocks are depleted. In addition, DMSO can accumulate to higher concentration within the cell compared with DMS (Simó *et al.*, 2000) and does indeed act as a highly effective antioxidant in a number of algal species during times of physiological stress (Lee and De Mora, 1999; Sunda *et al.*, 2002; Riseman and DiTullio, 2004; Bucciarelli *et al.*, 2013).

In conclusion, we show that marine heterotrophic bacteria of the MRC can rapidly oxidize DMS to DMSO using the enzyme Tmm. The widespread occurrence of this enzyme in key microbial taxa that are ubiquitous in the marine environment represents a significant molecular mechanism for the oxidation of this volatile compound to DMSO in the marine surface waters. We speculate that this methylamine-dependent co-oxidation pathway of DMS may be a significant route for DMSO production in the oceans.
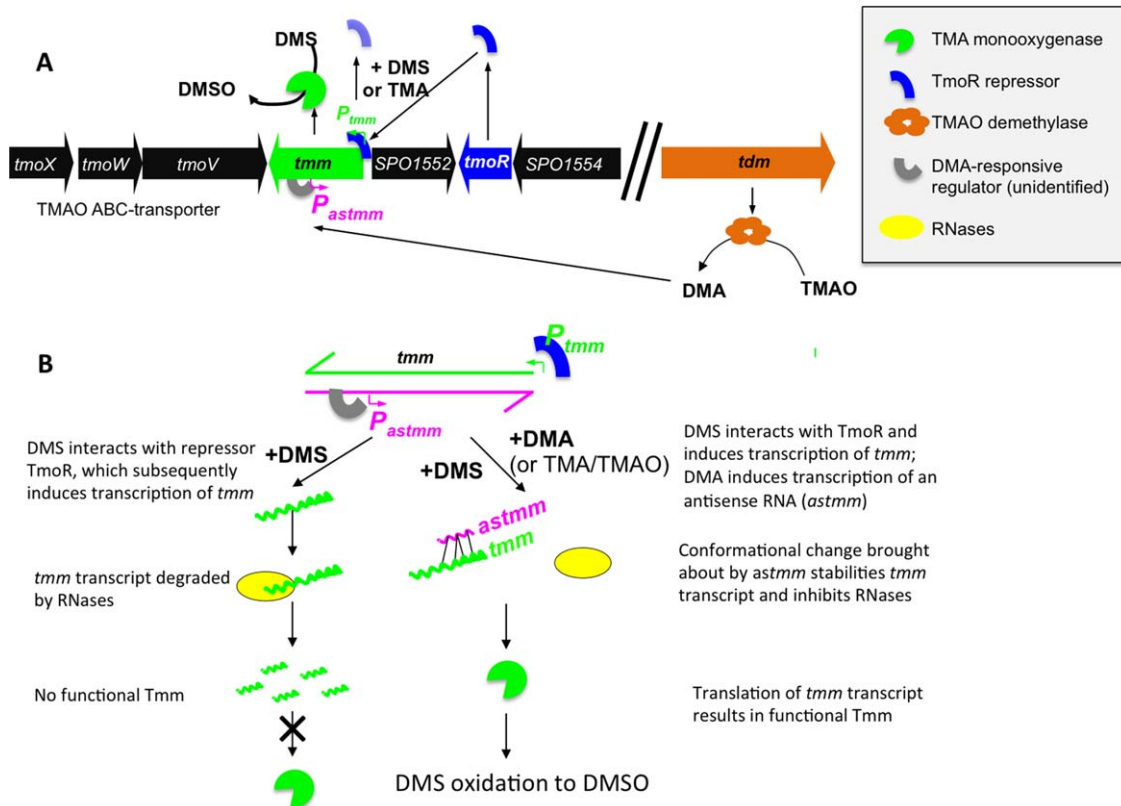
Fig. 7. Proposed model of transcriptional and post-transcriptional regulation of trimethylamine monooxygenase (Tmm) in *R. pomeroyi*.
A. The transcriptional regulator TmoR acts as a repressor of *tmm*. In the presence of TMA and DMS, TmoR is released from the promoter (P*tmm*), allowing transcription of *tmm*. A promoter on the antisense strand of *tmm* (P*astmm*) is predicted. Transcription of the antisense regulatory RNA (asRNA) is directly regulated by DMA.
B. The role of the putative asRNA is likely to stabilize *tmm* transcript by preventing its degradation by RNases. In the absence of methylated amines, DMS leads to the transcription of *tmm*, but not asRNA, resulting in the degradation of *tmm* prior to translation. However, in the presence of methylated amines and DMS, both *tmm* and *astmm* are transcribed which subsequently form a duplex that protects *tmm* transcript from nuclease degradation. TmoXWV encodes an ABC-type transporter for TMAO. SPO1552 is annotated as an unknown periplasmic protein. SPO1554 encodes a putative ammonium transporter.

Experimental procedures

Cultivation of Ruegeria pomeroyi *DSS-3 and other* MRC *isolates*

A complete list of *R. pomeroyi* DSS-3 strains used in this study can be found in Tables 1 and Supporting Information S1. All bacterial strains were maintained on 2216 marine broth (Difco) agar plates (1.5% w/v). For growth experiments, *R. pomeroyi* was grown at 30°C in 125-ml serum vials in triplicate using the defined marine ammonium mineral salts (MAMS) medium using a 5% (v/v) inoculum. Vitamins were added as described previously (Chen, 2012). Glucose (10 mM) or DMSP (5 mM) was used as the main carbon substrate and either ammonium or TMA as a sole nitrogen source (0.5 mM). Starter cultures were grown in glucose and ammonium in the presence or absence (+/−) of TMA (0.5 mM). Other MRC isolates were grown in triplicate at either 30°C or 18°C in 125-ml serum vials with rubber stoppers in MAMS using a 5% inoculum (v/v). Ammonium (NH_4^+), TMA, TMAO, DMA or MMA (0.5 mM) was used as the sole N source. Succinate (5–10 mM) was used as the sole carbon source +/− DMS (0.5 mM). Two MRC isolates (*Phaeobacter* sp. TMAL401,

Roseobacter sp. TMAL402) isolated from seawater collected from the L4 sampling station, off the coast of Plymouth (Devon, UK) in June 2012, were also used in this study. The seawater samples were enriched using a defined medium as described previously (Chen, 2012) with TMA (0.5 mM) as the sole carbon source. These isolates were obtained by plating out liquid enrichments in increasing dilutions onto MAMS agar plates with TMA (3 mM) as the sole carbon source.

Quantification of methylated amines, DMS, DMSP and DMSO

Quantification of methylated amines and ammonium was achieved by cation-exchange ion chromatography with a Metrosep C4/250-mm separation column and a conductivity detector (Metrohm) as described previously (Lidbury *et al.*, 2014). The eluent used for separation was prepared as follows (10× stock solution): double distilled water (ddH$_2$O) up to 1 L, nitric acid (1.4 M), 500 ml acetone, 350 ml 2, 4-pyridinedicarboxylic acid monohydrate. Eluent stock solution was stored at 4°C and diluted with ddH$_2$O prior to use.

R. pomeroyi cultures incubated with DMS were grown in 125-ml serum vials and sealed with rubber stoppers. Quantification of DMS in headspace gas was measured by injecting 100 μl of a headspace gas sample into a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Corporation, Columbia, USA) fitted with a 30 m × 0.32 mm ID SHIM-1 × 3.0 μm capillary dimethylpolysiloxane column (Shimadzu Corporation, Columbia, USA.). Helium was used as the carrier gas (column flow rate, 2 ml min^{-1}) and the column temperature was 180°C. A flame photometric detector was used to detect DMS. DMS concentrations were calculated by regression analysis based on a five-point calibration with standard DMS solutions in MAMS.

DMSP concentrations in the cultures were measured as DMS following alkaline hydrolysis as reported previously (Miller and Belas, 2004). An aliquot of the culture sample was added to a 25-ml serum vial with the addition of the same volume of either 5 M NaOH or distilled water. Solutions of pure DMSP at 0.1–1 mM dissolved in distilled water were prepared along with each experiment. After overnight incubation, DMS resulting from alkaline hydrolysis of DMSP was measured as described above. DMSO was determined according to a minor modification of the method of Jonkers (Jonkers et al., 1996): DMSO was measured as DMS after reduction with acidified stannous chloride (20 g SnCl$_2$ in 100 ml 37% HCl, v/v) for 90 min at 55°C.

Activity assay of DMS oxidation to DMSO by Tmm, DMSO demethylation by Tdm and TMA oxidation to TMAO by Tmm

E. coli BLR (DE3) expressing either Tmm from *R. pomeroyi* DSS-3 or Tdm from *R. pomeroyi* was used for activity assays (Chen et al., 2011; Lidbury et al., 2014). *E. coli* cells were grown at 37°C to an OD$_{540}$ of 0.6, and isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.2 mM. Cells were broken by passing three times through a French pressure cell (American Instrument) at 110MPa. Cell debris was removed by centrifugation at (21 000 x g) for 15 min. Overexpressed Tmm was purified using a His-tag protein purification kit (Novagen) as described in the manufacturer's protocol (Merck KGaA, Darmstadt, Germany). Tmm activity was measured by following the decrease in absorbance at 340 nm of NADPH (Sigma-Aldrich). Enzyme assays were performed in triplicate at 22°C. A 2-ml mixture contained 0.26 mg purified enzyme, 10 mM PIPES (pH 7.6), and 0.25 mM NADPH. The reaction was initiated by adding the substrate, and the decrease in NADPH and DMS was measured via UV-visible spectrophotometer and gas chromatography respectively. Enzyme assays were carried out with varying concentrations of DMS (0.05 mM, 0.1 mM, 0.15 mM, 0.2 mM, 0.25 mM, 0.3 mM, 0.35 mM, 0.4 mM). DMSO concentrations were then determined as described above. Tdm activity assays were performed as previously described (Zhu et al., 2014).

Construction of the mutant, ΔtmoR::Gm

To construct a *tmoR* mutant, a region towards the 5′ end (with PstI and XbaI sites engineered in) and a region towards the 3′

end (with a HindIII and XbaI engineered in) of the target gene (Spo1553) was amplified. The two regions, along with a gentamicin gene cassette inserted at an XbaI site between the two regions, were cloned into the cloning vector, pGEM-T (Promega). Primers used for PCR amplification are listed in Supporting Information Table S2. The entire construct was ligated into the suicide vector pK18mobsacB, harbouring a kanamycin resistance cassette, at sites PstI and HindIII. The resulting plasmid was transformed into *E. coli* S17-1 via electroporation and mobilized into *R. pomeroyi* via conjugation, using ½ YTSS as the medium (DSMZ). Transconjugants were selected for on the sea salts minimal medium with gentamicin (10 μg ml^{-1}) and MMA (3 mM) as a sole nitrogen source. Double crossover mutants were selected by their sensitivity to kanamycin (80 μg ml^{-1}) and homologous recombination was confirmed by PCR and subsequent DNA sequencing.

Construction of the P$_{tmm}$:lacZ fusion report probe and quantification of β-galactosidase activity

The 250 bp 5′ untranslated region of *tmm* (SPO1551) of *R. pomeroyi* was cloned into the reporter probe plasmid, pBIO1878 (Todd et al., 2012) using the restriction sites KpnI and PstI. The plasmid was mobilized into *R. pomeroyi* wild type and the Δ*tmoR::Gm* mutant by conjugation with *E. coli* S17-1. A mixed cell suspension was plated onto minimal medium plates containing MMA as the sole nitrogen source plus spectinomycin (175 μg ml^{-1}) to select against *E. coli* S17-1. Activity assay for β-galactosidase was performed as described in the Supporting Information (Lidbury et al., 2014).

Complementation of the mutant, Δtmm::Gm, with tmm of R. pomeroyi or Roseovarius sp. 217

The Δ*tmm::Gm* mutant was constructed previously (Lidbury et al., 2014). To complement this mutant, the promoter of *tmm* from *R. pomeroyi* DSS-3 was amplified with an XbaI and an NdeI site engineered into the 5′ and 3′ end, respectively, and sub-cloned into pGEM-T. The promoter sequence was then released via enzymatic digestion and inserted into the pET28a plasmid, containing the *tmm* from *R. pomeroyi* or *Roseovarius* sp. 217 (Chen et al., 2011). The combined construct was released from pET28a and inserted into the broad-host range plasmid, pBBR1MCS-km at sites XbaI/EcoRI. The resulting plasmids were transformed via electroporation into *E. coli* S17-1 and then mobilized into the *R. pomeroyi* Δ*tmm::Gm* mutant via conjugation. Transconjugants were selected for using kanamycin (80 μg ml^{-1}) as described previously (Lidbury et al., 2014). The Δ*tdm::Gm* mutant and the complemented *tdm* mutant were constructed previously (Lidbury et al., 2014).

Extraction and amplification of nucleic acids

All DNA extractions were performed using the FastDNA^{TM} SPIN Kit for Soil (MP Biomedicals, LLC, CA, USA) according to the manufacturer's instructions. For cultivated bacterial isolates, 1–5 ml of liquid culture was centrifuged (8000x g for 5 min) to generate a cell pellet. For RNA work, all glassware, water and solutions were treated with diethylpyrocarbonate

(DEPC) (or prepared with DEPC-treated water where appropriate) by shaking overnight at 37°C in a 0.1% (v/v) solution prior to autoclaving. Total RNA was isolated from *R. pomeroyi* using the hot acid-phenol method of Gilbert and colleagues. (Gilbert *et al.*, 2000). The quality of the RNA was analysed by running 1–5 μl on a 1% (w/v) TBE-agarose gel. DNA was removed by one treatment using RNase-free DNase (Promega) and the DNA-free RNA was purified using an RNeasy spin column (Qiagen, Crawley, UK) following the manufacturer's instructions (RNA-clean up protocol). Removal of DNA was confirmed by the absence of a PCR product of the 16S rRNA gene, using the primer set 341F/518R (Supporting Information Table S2) in reactions using 1 μl or 4 μl (150–200 ng of RNA equivalent) of RNA template undergoing 35 cycles of PCR. In addition, minus (−) reverse transcriptase (RT) controls were performed in parallel to cDNA library preparation to ensure no DNA contamination was affecting the results from RT-PCR. PCR amplification of *tmm* cDNA was performed using the primers tmm_RTF: 5′-CCGGCTACAAG-CATTTCTTC-3′/tmm_RTR: 5′-GATGTCTTCGCCCTTGTGTT-3′. 150 ng of cDNA was used as a template. Conditions for PCR amplification were as follows: 3 min denaturation step at 95°C, followed by 30 cycles of, 95°C for 1 min, an annealing step (45–60°C dependent on primers) of 30 s, an elongation step at 72°C for an appropriate length of time (30 s for every 500 bp), followed by a final elongation step at 72°C for 5 min.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Quantification of β-galactosidase activity

Table S1. A list of *R. pomeroyi* DSS-3 strains and plasmids used in this study

Table S2. Primers used in this study. Bases underlined represent the restriction sites engineered in for enzymatic digestion.

Fig. S1. RT-PCR assays targeting the antisense strand of *tmm* of *R. pomeroyi* cells (duplicate cultures, 1 and 2) grown on either ammonium (NH_4^+) or trimethylamine (TMA) as the sole nitrogen source respectively. The presence of an antisense RNA was observed in TMA-grown cultures. Controls were performed for RT-PCR where no reverse transcriptase was added (-) in the reverse transcription reactions. Positive and negative PCR controls were also set up where genomic DNA of *R. pomeroyi* or ddH$_2$O was used as the template, respectively.