Bacterial SBP56 identified as a Cu-dependent methanethiol oxidase widely distributed in the biosphere

Özge Eyice[a,b1], Nataliia Myronova[a,1], Arjan Pol[c], Ornella Carrión[d], Jonathan Todd[d], Tom J. Smith[e], Stephen J. Gurman[f], Adam Cuthbertson[a], Sophie Mazard[a,2], Monique A.S.H. Mennink-Kersten[c], Timothy D.H. Bugg[g], K. Kristoffer Andersson[h], Andrew W.B. Johnston[d], Huub J.M. Op den Camp[c,3] , Hendrik Schäfer[a,3]

Author contributions: HS, HOdC, NM, AP, KKA, TJS, SJG, JT, TDHB, AWBJ designed research; OE, NM, OC, JT, TJS, SJG, MMK, AP, HS, KKA, AC, SM performed research; HS, JT, TJS, KKA, AWBJ, AP, HOdC wrote the paper with editorial help of co-authors.

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

[b] School of Biological and Chemical Sciences, Queen Mary University of London, London, E1 4NS, United Kingdom

[c] Department of Microbiology, Institute for Water and Wetland Research, Faculty of Science, Radboud University, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands

[d] School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, United Kingdom

[e] Department of Biosciences and Chemistry, Sheffield Hallam University, Howard Street, Sheffield, S1 1WB, United Kingdom

[f] Department of Physics and Astronomy, University of Leicester, Leicester, LE1 7RH, United Kingdom

[g] Department of Chemistry, University of Warwick, Coventry, CV4 7AL, United Kingdom

[h] Department of Bioscience, University of Oslo, PO Box 1066 Blindern, Blindernveien 31, 0371 Oslo, Norway

[1] These authors contributed equally to the work

[2] present address: Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW, Australia 2109

[3] Corresponding authors:

Hendrik Schäfer, Email: h.schaefer@warwick.ac.uk; Phone: +44 2476 575052
Huub J.M. Op den Camp, Email: h.opdencamp@science.ru.nl; Phone: +31243652657

Short title: Identification of the bacterial methanethiol oxidase

Keywords: Methanethiol, methanethiol oxidase, Cu enzyme, dimethylsulfide, dimethylsulfoniopropionate, sulfur cycle, selenium-binding protein family

ABSTRACT

Oxidation of methanethiol (MT) is a significant step in the sulfur cycle. MT is an intermediate of metabolism of globally significant organosulfur compounds including dimethylsulfoniopropionate (DMSP) and dimethylsulfide (DMS), which have key roles in marine carbon and sulfur cycling. In aerobic bacteria, MT is degraded by a methanethiol oxidase (MTO). The enzymatic and genetic basis of MT oxidation have remained poorly characterized. Here we identify for the first time the MTO enzyme and its encoding gene (*mtoX*) in the DMS-degrading bacterium *Hyphomicrobium* sp. VS. We show that MTO is a homotetrameric metalloenzyme that requires Cu for enzyme activity. MTO is predicted to be a soluble periplasmic enzyme and a member of a distinct clade of the Selenium-binding protein (SBP56) family for which no function has been reported. Genes orthologous to *mtoX* exist in many bacteria able to degrade DMS, other one-carbon compounds or DMSP, notably in the marine model organism *Ruegeria pomeroyi* DSS-3, a member of the Rhodobacteraceae family which is abundant in marine environments. Marker exchange mutagenesis of *mtoX* disrupted the ability of *R. pomeroyi* to metabolise MT confirming its function in this DMSP-degrading bacterium. In *R. pomeroyi*, transcription of *mtoX* was enhanced by DMSP, methylmercaptopropionate and MT. Rates of MT degradation increased after pre-incubation of the wildtype strain with MT. The detection of *mtoX* orthologues in diverse bacteria, environmental samples and its abundance in a range of metagenomic datasets point to this enzyme being widely distributed in the environment and playing a key role in global sulfur cycling.

INTRODUCTION

Methanethiol (CH_3SH; methylmercaptan, MT) is a foul-smelling gas with a low odor threshold. As a malodorous compound that can be detected by the human nose at very low concentration (odor threshold 1-2 ppb,(Devos et al 1990)) it plays a significant role in causing off-flavors in foods and beverages and it is one of the main volatile sulfur compounds causing halitosis in humans (Awano et al 2004, Tangerman and Winkel 2007). The production and degradation of MT are major steps in the biogeochemical cycle of sulfur (Figure 1). Sources of MT include the methylation of sulfide in anoxic habitats, demethiolation of sulfhydryl groups and degradation of sulfur-containing amino acids (Bentley and Chasteen 2004, Lomans et al 2001, Lomans et al 2002). MT is produced in the marine environment as an intermediate of dimethylsulfoniopropionate (DMSP) degradation by the demethylation pathway. In this pathway, initial demethylation of DMSP to methylmercaptopropionic acid (MMPA) is carried out by the DMSP-dependent demethylase (DmdA) (Howard et al 2006). Subsequent degradation of MMPA occurs via MMPA-CoA to methylthioacryloyl-CoA and then to acetaldehyde and MT by the enzymes DmdB, DmdC and DmdD, respectively (Reisch et al 2011b). MT is also produced as an intermediate of dimethylsulfide (DMS) degradation (Bentley and Chasteen 2004, Lomans et al 1999a, Lomans et al 2002, Schäfer et al 2010).

Only few measurements of MT in the environment have been reported. Analysis of volatile sulfur compounds in freshwater ditches demonstrated that MT was the dominant volatile organic sulfur compound reaching concentrations of 3-76 nM in sediments and 1-8 nM in surface freshwater (Lomans et al 1997). Measurements of MT concentrations in the surface ocean water are scarce. Studies reporting MT

measurements in seawater suggest a typical range of approximately 0.02 to 2nM (Kettle et al 2001, Ulshöfer et al 1996, Xu et al 2001).

Microbial uptake and degradation of MT are important sinks for MT. Despite low MT concentrations in seawater, radiotracer experiments showed that trace levels of MT (0.5 nM) were rapidly taken up and incorporated into biomass by marine bacterioplankton (Kiene et al 1999). Besides this assimilation, MT degradation through its utilization as a carbon and energy source in methanogenic archaea, sulfate-reducing bacteria, and aerobic bacteria (Lomans et al 1999b, Lomans et al 2001, Lomans et al 2002, Schäfer et al 2010) and its methylation to DMS by the recently described methyltransferase MddA (MddA: methanethiol-dependent DMS) (Carrión et al 2015) contribute to biological MT removal.

The molecular basis of MT degradation remains poorly understood. In aerobic sulfur-oxidizing and methylotrophic bacteria including strains of *Thiobacillus* (Gould and Kanagawa 1992, Lee et al 2002), *Rhodococcus* (Kim et al 2000) and *Hyphomicrobium* (Suylen et al 1987) MT is degraded by a MT oxidase (MTO) to formaldehyde, hydrogen sulfide and hydrogen peroxide; however, inconsistent data have emerged from these studies. Estimated molecular weights of MTOs characterized previously have ranged from approximately 29-61 kDa. The MTO from *Hyphomicrobium* sp. EG was reported to be a monomer of 40-50 kDa that was insensitive to metal-chelating agents (Suylen et al 1987). In *Thiobacillus thioparus* (Gould and Kanagawa 1992) MTO also appeared to be a monomer with a molecular weight of approximately 40 kDa; however a later study of MTO in *T. thioparus* reported a different molecular weight for MTO of 61 kDa (Lee et al 2002). MTO from *Rhodococcus rhodochrous* was reported to have a molecular weight of

64.5 kDa (Kim et al 2000). The genetic basis of MT degradation has not been identified, constituting a gap in fundamental knowledge of a key step in the global sulfur cycle.

Here we report new insights into the biochemistry, genetics and environmental distribution of methanethiol oxidases in bacteria. We purified and characterized MTO from *Hyphomicrobium* sp. VS a DMS-degrading methylotrophic bacterium that was isolated from activated sewage sludge and which has MTO activity during growth on DMS as a sole carbon and energy source (Pol et al 1994). We identified the gene encoding MTO, *mtoX*, in *Hyphomicrobium* sp. VS and detected orthologous *mtoX* genes in a wide range of bacteria including methylotrophic, sulfur-oxidizing and DMSP-degrading bacteria. We then genetically analyzed its function and transcriptional regulation in a model isolate of the Rhodobacteraceae family, *Ruegeria pomeroyi* DSS-3, which produces MT during degradation of DMSP by the demethylation pathway (Reisch et al 2011a). The development of *mtoX*-specific PCR primers allowed testing environmental samples for the presence of *mtoX*-containing populations. This analysis suggested that the genetic potential of MT degradation is present in a wider spectrum of phylogenetic lineages than previously realized based on bacterial cultures. This was also reflected by the presence of *mtoX* genes from uncultivated organisms in diverse habitats based on screening of metagenomic datasets, which suggests that MTO is widely distributed in the biosphere.

MATERIALS AND METHODS

Growth of *Hyphomicrobium* sp. VS. *Hyphomicrobium* sp. VS was grown in continuous culture in a Fermac 300 series fermenter (Electrolabs, UK) as described previously (Boden et al 2011) using PV mineral medium using either DMS (12 mM) as sole substrate or in combination with methanol (both substrates 12 mM). The culture was

held at 30°C, aerated with sterile air at 1.5 L/min, and stirred at 200 rpm. pH was adjusted to 7.4 ± 0.1 by automatic titration with 1 M NaOH. *Hyphomicrobium* sp. VS was initially grown for 24 h in a 1 L volume in sterilized medium supplemented to 25 mM with methanol before beginning the addition of medium containing DMS. Overflow was collected in a vessel held on ice. Cells were harvested daily, washed with 25 mM 1,4-Piperazinediethanesulfonic acid (PIPES, pH 7.2) and resuspended in the same buffer. Concentrated cells were frozen in liquid nitrogen and stored at -80°C.

Protein purification and characterization. Thawed cells (approximately 1.5 g dry weight) were washed with 25 mM PIPES (pH 7.2), centrifuged at 12,000 *g* for 20 min at 4 ˚C and resuspended in 50 mM N-[Tris(hydroxymethyl)methyl]glycine (TRICINE, pH 8.2) supplemented with DNAse I (1µg ml^{-1}) and 1 mM benzamidine. A crude cell extract (approximately 600mg protein) was prepared by breaking the suspended cells using a Constant Cell Disrupter (Constant Systems, Daventry, UK) three times at 25 MPa and 4°C. Unbroken cells and debris were removed by centrifugation in a Beckmann JA20 at 12,000 *g* for 25 min at 4°C followed by removal of membrane fractions by centrifugation of the supernatant at 144,000 *g* for 90 min (BECKMAN rotor SW28). The final supernatant (approximately 300mg protein) was concentrated using an Amicon stirred cell with PM10 ultrafiltration membrane (Millipore). Aliquots of concentrated supernatant (~10mg/ml; 0.5ml) were applied to an anion-exchange MonoQ 10 column (GE Lifesciences, Little Chalfont, UK) equilibrated with precooled (4 ˚C) 10 mM TRICINE (pH 8.2) supplied with 1 mM benzamidine. An increasing (0-1 M) NaCl gradient was used to elute fractions, which were assayed for MTO activity (see below). Fractions with MTO activity were concentrated using an Amicon stirred cell with a PM10 ultrafiltration membrane. Concentrated MonoQ 10 fractions containing mainly MTO were subjected to gel-filtration using a Superdex 75 column (GE Lifesciences) equilibrated in precooled

(4˚C) 10 mM TRICINE (pH 8.2) supplied with 1 mM benzamidine. Fractions containing active MTO showed a single dominant polypeptide on SDS-PAGE and were collected and concentrated as described above before storage at –80˚C. Further detail about protein purification is given in Supplementary Tables S1 and S2. Analytical gel filtration was done using a Superdex 75 column equilibrated with 10 mM TRICINE, pH 8.2, 1 mM benzamidine, 0.15 M NaCl, at a flow rate of 1 ml/min.

MTO activity assays. Routine analysis of enzyme activity was done by measuring MT degradation using gas chromatography (GC) for which MT was analyzed in headspace samples (100 µl) using an Agilent gas chromatograph (Agilent Technologies, Cheshire, UK) fitted with a 30 m x 0.32 mm column (DB-1). Helium was used as the carrier gas at a temperature of 200°C. The gas chromatograph had a flame ionisation detector (FID). Alternatively, MT was measured in headspace samples using a GC-2010plus (Shimadzu, Milton Keynes, UK) equipped with a Shim-1 column (30m, 0.5mm i.d.), at a temperature of 180°C, with Helium as carrier gas and a Flame Photometric Detector. MTO activity was assayed in 10mM TRICINE, pH 8.2 at 30°C, typically using 0.1-0.5mg of protein per assay. Alternatively, MTO activity was measured as substrate induced O_2 consumption in a Clark type oxygen electrode with and without addition of catalase (0.1mg) and in the presence and absence of $ZnSO_4$ (1mM). The formation of formaldehyde by MTO was quantified using the Purpald reagent (Sigma-Aldrich, Gillingham, UK) as described previously (Boden et al 2011). Standard formaldehyde solutions were prepared from methanol-free formaldehyde in the range of 0-1mM.

Protein electrophoresis. SDS-PAGE electrophoresis was carried out using standard protocols using precast gels supplied by Bio-Rad (Hemel Hempstead, UK) run in 1x Tris(hydroxymethyl)aminomethane (Tris) Glycine buffer.

Metal analysis. Quantification of various elements contained in purified MTO was performed using inductively coupled plasma mass spectrometry (ICP-MS) at the ICI Measurement Science Group, Wilton, Middlesbrough, UK.

Electron paramagnetic resonance (EPR) spectroscopy. The EPR spectral properties of MTO were examined under various reducing (5mM ascorbate, 1mM dithionite) and oxidizing conditions (1.8mM sodium hexachloroiridate (V)), and under enzyme assay condition in the presence of substrate (all at 25°C). All analyses were carried out with a preparation of MTO of 9.2mg/ml in 10mM Tricine, pH 8.2 (with 1 mM benzamidine) on a Bruker EleXsyS 560 SuperX spectrometer fitted with a Bruker ER41116DM dual mode cavity and an Oxford ESR 900 Helium Flow Cryostat. EPR spectra of oxidized MTO were recorded at temperatures of 7K and 13K after addition of sodium hexachloroiridate (V) (1.8mM final concentration) using a microwave frequency of 9.66 GHz, microwave power of 0.63 mW, a modulation amplitude of 7 Gauss and a time constant of 81ms. Further EPR spectra (four scans) were also recorded in the presence of enzyme substrates ethanethiol (1mM) and oxygen (0.2mM similar to assay conditions) using instrument settings as detailed above, except for a microwave power of 0.2mW, a modulation amplitude of 7.6 Gauss at a temperature of 15K.

X-ray Spectroscopy Analysis of Methanethiol Oxidase. X-ray absorption spectra were obtained in fluorescent mode on station B18 of the Diamond Light Source (Didcot, UK). This uses the technique of quick EXAFS (QuEXAFS), where the monochromator rotates at a constant rate during data acquisition. The fluorescence was detected using a nine element germanium solid state detector. Data were obtained at the Cu K edge for a variety of samples and standards. All data were obtained with the samples at 77K in a cryostat. To minimize radiation damage the beam was rastered across the sample, which was moved between each scan. Each scan took about 20 minutes to acquire.

Copper metal (foil), CuO and CuS were used as reference samples. The copper-containing enzyme tyrosinase (Sigma Aldrich, Gillingham, UK) was used as additional reference. Five samples of purified MTO were analysed: as-isolated enzyme; enzyme treated with the oxidising agent sodium hexachloroiridate (2 mM); enzyme treated with the substrate methanethiol; enzyme treated with the reducing agent sodium dithionite (1 mM). Detailed information about processing of data is provided in the supplementary information.

Identification of the gene encoding MTO in *Hyphomicrobium* sp. VS. N-terminal sequence data for MTO were obtained from gel slices of Coomassie stained SDS-PAGE gels by ALTA Bioscience, University of Birmingham (UK). Internal peptide sequences were determined by the Biological Mass Spectrometry facility in the School of Life Sciences, University of Warwick, as described previously (Schäfer et al 2005). We sequenced genomic DNA of *Hyphomicrobium* sp. VS using Illumina technology. After quality trimming 26,777,191 reads with an average length of 60 bp were obtained. Reads were assembled using a combination of the CLCBio (Aarhus, Denmark) and Edena assemblers (Hernandez et al 2014). No gap-closing was performed. This resulted in a draft genome consisting of 347 contigs (average length 9125 bp) with a total size of 3,722,323 bases (See Supplementary Table S3). Peptide sequences were matched against proteins predicted by the annotation pipeline. The draft genome assembly for *Hyphomicrobium* sp. VS is available on the MaGe Microscope platform at http://www.genoscope.cns.fr/agc/microscope/mage/index.php (Vallenet et al 2013). The sequence of the contig containing *mtoX, SCO1/senC and mauG* has been deposited with the National Center for Biotechnology Information under accession number KY242492.

Phylogenetic analysis. Nucleic acid sequences were imported into Arb (Ludwig et al 2004) and translated before aligning using clustalx as implemented in Arb. A phylogenetic tree was derived using amino acid sequence data based on the Arb Neighbour Joining method, using alignment columns corresponding to positions 85-300 of the MtoX polypeptide of *Hyphomicrobium* sp. VS and the PAM (Point Accepted Mutation) distance correction as implemented in Arb. Bootstrapping (100 iterations) was carried out in MEGA 5 (Tamura et al 2011).

Genetic analysis of *mtoX* in *Ruegeria pomeroyi* DSS-3. Locus SPOA0269 was identified by blast search as a homolog of *mtoX* in *R. pomeroyi*. Two PCR primer pairs were designed to amplify the flanking regions of SPOA0269 (5'-GCGAATTCTCGAAGCCATCGCTGG-3' with 5'-CGGGATCCCATCGCCAGGGCACCGG-3' and 5'-CGGGATCCTGGGCCTGGGCCGCGCGC-3' with 5'-CCCAAGCTTCGGGGTCCGCCGGGTCAGG-3'). The resulting PCR products were digested with *BamH*I ligated together to form a clone with a truncated version of SPOA0269 (2/3 deletion in frame of the gene). The resulting fragment was digested with *Eco*RI and *Hind*III and then cloned into pK18sac. Then a spectinomycin resistance ($Spec^R$) cassette was cloned into a unique *BamH*I site within the truncated version of the gene. This construct was transferred by tri-parental conjugational mating with *E. coli* containing the mobilising plasmid pRK2013 as the helper strain (Figurski and Helinski 1979) into rifampicin resistant *R. pomeroyi* J470 (Todd et al 2011) (20 μg ml^{-1}). Colonies were selected based on resistance to spectinomycin (200 μg ml^{-1}) and sucrose (5%), but sensitivity to kanamycin (20 μg ml^{-1}). Such colonies were checked by PCR and by Southern blotting to show that they were mutated in SPOA0269.

Enzymatic assays of MTO activity in *R. pomeroyi* DSS-3. For the measurement of MT consumption by *R. pomeroyi* whole cells, *R. pomeroyi* DSS-3 wild type and *mtoX*$^{-}$ strains were grown overnight at 28 °C in MBM (Baumann and Baumann 1981) or MBM supplemented with 200 μg·ml^{-1} spectinomycin, respectively, using succinate (10 mM) as a carbon source and NH_4Cl (10mM). Cultures were spun down and pellets were washed three times with fresh MBM. After that, cell suspensions were adjusted to an OD600=1.4 and inoculated (1/10 dilution) into 120 ml serum vials containing 20 ml MBM plus 0.5 mM MT. Vials were incubated at 28 °C and MT concentration in the headspace was measured at time 0 and after 6 h by GC as described in (Carrión et al 2015). Chemical degradation of MT in the medium control was subtracted from the MT removed in *R. pomeroyi* cultures to calculate rates of biological degradation of MT. Samples were pelleted, resuspended in Tris-HCl buffer 50 mM, pH 7.3 and sonicated (5 x 10s) with an ultrasonic processor VC50 sonicator (Jencons). The protein content of the samples was estimated by the Bradford method (BioRad). Rates of biological MT disappearance are expressed as nmol·min^{-1}·mg protein^{-1} and represent the average of three biological replicates.

For MTO *in vitro* assays *R. pomeroyi* DSS-3 wild type and *mtoX*$^{-}$ were grown as above in the presence and absence of 0.5 mM MT for 6 h and pelleted. Cell pellets were washed three times with Tris-HCl buffer 50 mM, pH 7.3. Pellets were resuspended in 20 ml of Tris-HCl buffer and sonicated (as above). 5 ml of cell lysates were placed in 20 ml serum vials to which 0.25 mM MT was added. MT concentration in the headspace was measured at time 0 and after 2 h of incubation at 28 °C by (GC) as described previously (Carrión et al 2015). Cell protein content and rates of biological degradation of MT were determined as described above.

There was no difference in the growth of the *mtoX* mutant strain compared to *R. pomeroyi* DSS-3 wild type in the presence of MT (0.5mM).

Transcriptional analysis of *mtoX* in *Ruegeria pomeroyi* DSS3 and *Rhizobium leguminosarum*. The region of the *R. pomeroyi* DSS3 genome that likely spanned the promoter of the SPOA0268-0272 operon was amplified from genomic DNA using primers GCGAATTCATCGAACCGCAATAGACCAC and GCCTGCAGGATCTTGGGCATATAGGGCG and cloned into the *lacZ* –reporter plasmid pBIO1878 (Todd et al 2012) to form an *mtoX-lacZ* fusion plasmids. The *mtoX-lacZ* fusion plasmid was digested with *Nsi*I and *Pst*I and religated to delete a ~800bp 3' fragment and form a SPOA0268-*lacZ* fusion plasmid. These plasmids were transferred by tri-parental conjugational mating (as above) into *R. pomeroyi* J470 and transconjugants were selected on rifampicin (20 μg ml^{-1}) plus spectinomycin (200 μg ml^{-1}). Transconjugants were grown overnight in marine basal medium (MBM) with succinate (10 mM) as carbon source (González et al 1997). The media either contained or lacked 5 mM DMSP, 1mM MMPA, 0.1mM MeSH or 0.1mM DMS. The cells were assayed for β-galactosidase activity essentially as described previously (Rossen et al 1985).

Identification of MTO homologs in bacterial genomes. MTO homologs were identified in microbial genomes based on BLASTP searches against assembled genomes at Integrated Microbial Genomes (IMG)(Markowitz et al 2009). MtoX amino acid sequences of *Hyphomicrobium* sp. VS, *Ruegeria pomeroyi* DSS-3 (locus SPOA0269) and *Methylophaga thiooxydans* (MDMS009_768) were used as queries. All hits used in further analysis had an e-value of $1e^{-151}$ or lower and a minimum pairwise identity at the level of the entire polypeptide of 52% or higher. Based on preliminary analyses showing support for a signal peptide in MTO, the start codons of two orthologous genes

that appeared truncated were corrected to start at alternative start codons further upstream (locus GPB2148_3671 in marine gammaproteobacterium HTCC2148 was extended by 26 amino acids, while MDMS009_211 in *Methylophaga thiooxydans* was extended by 46 amino acids) as they appeared to have incomplete N-termini. Orthologs from *Phaeobacter* sp. LSS9 (714 amino acids) and *Comamonadaceae* bacterium EBPR_Bin_89 (335 amino acids) were excluded as the length of the polypeptides significantly deviated from the remaining range observed (410 to 491 amino acids). Sequences were aligned using CLUSTALW.

Detection of *mtoX* homologs in metagenomic datasets. Metagenomic datasets were obtained from the CAMERA (Sun et al 2011) project website and searched for *mtoX* homologs using tblastn and the amino acid sequences of MtoX of *Hyphomicrobium* sp. VS, *Methylophaga thiooxydans* (locus tags MDMS009_211 and MDMS009_768) and *Ruegeria pomeroyi* DSS3 (SPOA0269) as queries with a cut-off in e-value of $1e^{-20}$. In case of libraries that represented short read data (ie less than 125bp) a cut-off value of $1e^{-05}$ was used. Similarly, the metagenomic datasets were searched for homologs of the DMSP demethylase *dmdA* (*R. pomeroyi* locus SPO1913) and the bacterial housekeeping gene *recA* from *E. coli* at a cut-off of $1e^{-20}$ in order to estimate the fractional abundance of *mtoX*-containing cells in the bacterial community and compare it to that of the DMSP demethylase gene *dmdA*.

Testing of MT oxidation in bacterial isolates. The potential to degrade MT by a range of pure cultures was assessed by monitoring changes in the MT concentration in the headspace after addition of 100µM MT (Supplementary Table S4). Mineral salts medium was used to monitor MT oxidation without any other carbon source added. For *Methylococcus capsulatus* Bath and *Methylocystis* sp. ATCC 49242 were tested for MT oxidation in NMS medium (Whittenbury et al 1970) that contained methane in addition

to MT (20% v/v and 40% v/v methane added to the headspace for *M. capsulatus* and *Methylocystis*, respectively). *Pseudovibrio gallaeciensis* and *P. ascidiaceicola* were grown in marine broth (Difco) to which MT was added. Sterile controls were incubated for each medium used in order to account for chemical MT degradation.

PCR amplification and cloning of MTO from enrichment cultures and environmental samples. PCR primers were designed based on an alignment of bacterial *mtoX* homologs (Supplementary Table S5). Primers were custom synthesised by Invitrogen Life Technologies (Paisley, UK) and initially tested using *Hyphomicrobium* sp. VS and *Methylophaga thiooxydans* DNA as template showing that a combination of primers 44F1/2 and 370R1/2/3 successfully amplified *mtoX* fragments from these two reference isolates. Further optimization of PCR conditions was carried out with DNA from additional bacterial isolates containing *mtoX* homologs and those showing potential for MT degradation (Supplementary Table S4). Unless noted otherwise, the PCR conditions used were 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1.5 min, followed by 72°C for 5 min.

The presence and diversity of *mtoX* genes in enrichments and environmental samples was assessed using the newly designed primers on DNA extracted from *Brassica* rhizosphere soils enriched with dimethylsulfide or methanol (Eyice and Schäfer 2015), DNA samples of $^{13}C_2$-DMS stable isotope probing experiment carried out with soil samples (Eyice et al 2015), *Brassica oleracea* rhizosphere soil, and surface sediment from the river Dene (Wellesbourne, Warwickshire, UK). DNA was extracted from 2ml of enrichment samples or 0.5g of soil/sediment samples using the FastDNA Spin kit for Soil (MP Biomedicals) according to the manufacturer's instructions. In addition, *mtoX* diversity was assessed in surface sediments of a coastal saltmarsh (Stiffkey, Norfolk, UK). Five replicate sediment samples were obtained from the surface 5mm oxic

sediment layer of a small saline pool along transects starting at a patch of *Spartina anglica* plants at the periphery of the pool, extending 50cm towards its center. The pH of the pool was 8.0, the water temperature was 16°C. Samples were transported back to the laboratory on ice, before being centrifuged at 14,000 RPM to remove the water and retain the sediment pellet. Samples were stored at -20°C prior to DNA extraction. Extraction of DNA from the sediment samples was performed using a Qbiogene FastDNA® SPIN Kit for Soil, according to the manufacturer's instructions. Eluted DNA was stored at -20°C. PCR on Stiffkey sediment samples was done using primers MtoX41Fmodv2_inos and MTOX346Rmod (compare Supplementary Table S5) using a cycling regime consisting of a 95°C hot start followed by 40 cycles of denaturation for 45 seconds, annealing for 45 seconds and elongation for 60 seconds at 95°C, 52°C and 72°C, respectively. A final extension step of 72°C for 6 minutes followed. All PCR products were cloned in pCRTOPO 2.1 (Invitrogen Life Technologies, Paisley, UK). DNA sequencing of randomly chosen clones was carried out at the University of Warwick Genomics Centre using BigDye Terminator v3.1 cycle sequencing kit and ABI Prism 7900HT or ABI3100 sequence detection system (Applied Biosystems, UK). Sequences of *mtoX* genes obtained from environmental samples have been deposited at the NCBI under accession numbers KY056824-KY057025.

RESULTS

Purification and characterization of methanethiol oxidase from *Hyphomicrobium* sp. VS. We purified the native MTO enzyme from soluble extracts of *Hyphomicrobium* sp. VS grown on DMS or a combination of methanol and DMS using anion exchange (MonoQ) chromatography followed by size-exclusion chromatography using a

Superdex-75 column and another MonoQ column. Fractions exhibiting MTO activity and those adjacent on the final column run were analyzed on SDS-PAGE (Supplementary Figure S1A). From this it could be concluded that fraction 18 which exhibited MTO activity was dominated by a single polypeptide with an estimated molecular weight of 46 kDa. All other analyses were performed with this fraction. ESI-mass spectroscopy of this fraction revealed a polypeptide with a molecular mass of 46,186 Da (Supplementary Figure S2). Analysis of MTO by native gel electrophoresis suggested a molecular weight of approximately 180-200 kDa (Supplementary Figure S1B). Reanalysis of the excised band by SDS-PAGE resulted in a single band of 46 kDa (result not shown). Analytical gel filtration suggested an apparent size of 200 kDa (Supplementary Figure S1C) also indicating that MTO of *Hyphomicrobium* sp. VS is a homotetrameric enzyme.

The purified enzyme degraded MT and ethanethiol, but not methanol, methylamine or dimethylsulfide. When MT was the substrate, we found evidence for the production of formaldehyde, hydrogen sulfide and hydrogen peroxide, although we did not quantify the latter. The O_2 dependency of MT conversion was shown by measuring activity with an oxygen sensor (Clark type). The ratio O_2/MT consumed was around 0.75±0.05 (with 1.2 to 26µM MT converted). This is lower than the 1.0 expected from the proposed stoichiometry ($CH_3SH + O_2 + H_2O \rightarrow HCOH + H_2S + H_2O_2$) and most likely caused by a very small contamination with highly active catalase reforming additional oxygen from hydrogen peroxide. This has been observed before (Suylen et al 1987). The remaining slow oxygen consumption after MT was depleted (rate dropping from 1.8 µM O_2/min to 0.2 µM O_2/min after 5 µM MT was depleted) was attributed to sulfide oxidation. Apart from being a (competitive) substrate for MTO, the sulfide produced in the MT oxidation

was shown to be an inhibitor. This effect was also demonstrated before for the MT oxidase purified from *Hyphomicrobium* strain EG (Suylen et al 1987). Adding Zn ions to the assay buffer to trap the produced sulfide resulted in 20 % faster initial MT conversion rates (when tested at 5 µM MT, oxygen respiration increased from 5.5 to 6.7 µM O_2/min) and completely abolished the sulfide oxidation. Upon acidification of the final reaction mixture at least 75 % of the added MT sulfur was recovered as hydrogen sulfide. After including Zn^{2+} in the assay mixture, O_2 consumption rates were constant (zero order kinetics) over almost the whole MT concentration range tested (1 - 20 µM MT). From this it can be concluded that the K_m value is below 1 µM MT which is below the detection limit of the respiration measurements. Using gas chromatographic analysis of MT (detection limit 0.05 µM MT) MT consumption rates at much lower concentrations could be tested. This resulted in a very low affinity constant (K_m) for MT of 0.2-0.3 µM. The K_m for MT of the MTO was at least 10 x lower than previously reported values for *Hyphomicrobium* sp. VS (5-10 µM) and *T. thioparus* (31 µM) (Gould and Kanagawa 1992, Pol et al 1994). This may be explained by the trapping of sulfide in our assays. The V_{max} was about 16 µmol mg^{-1} protein min^{-1} (Supplementary Figure S3). Formaldehyde was formed stoichiometrically, we observed formation of 4.1nmol (±0.5) from 4nmol of MT and 36.4nmol (±2.6) from 40nmol MT.

MTO of *Hyphomicrobium* sp. VS is a metalloenzyme and Cu is involved in the redox process of MT oxidation. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) showed that the purified enzyme preparation contained 3.5 mol Ca and 1.4 mol of Cu per mol of MTO tetramer (Supplementary Table S6). To further assess the potential role of Cu and Ca for MTO activity we carried out chelation experiments using ethylenediamine tetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA).

Incubation of the enzyme with EDTA but not EGTA reduced the activity of MTO by 44% suggesting that Cu but not calcium plays a role in the catalytic activity of MTO (Table 1).

A role of Cu in enzyme function was also supported by Electron Paramagnetic Resonance (EPR) Spectroscopy and extended X-ray absorption fine structure (EXAFS) for which a detailed description of the results is provided in the Supplementary Data. In brief, the EPR signals of resting and oxidized MTO samples did not have well-resolved signals that would be expected from Cu(II) mono-nuclear Cu site(s) (Supplementary Figure S4). Instead, there were signals that were probably due to two magnetically interacting Cu(II) centres, similar to similar to Cu_A in cytochrome *c* oxidase or nitrous-oxide reductase, which are both binuclear copper centres, as well as Cu model complex possibly also without bridging sulfur (Antholine et al 1992, Monzani et al 1998, Solomon et al 1996, Kaim et al 2013). The changes in features in the EPR spectra with addition of substrate also indicated changes in the coordination of Cu when substrate binds (Figure S5), which could indicate direct interaction of the substrate with the Cu centre. Although at this point the exact nature of the Cu environment and status cannot be fully resolved, the data suggest that it is likely a binuclear site, as the data do not support a single atom Cu(II) centre. Analysis of MTO by means of extended X-ray absorption fine structure (EXAFS) were consistent with the EPR data in that the oxidation state of the copper was between 1 and 2. The data indicated that the copper in the resting enzyme (in the absence of substrate) was coordinated by four nitrogen atoms with a Cu-N bond distance of 1.99 Å. EXAFS data from samples treated with substrate (methanethiol) or the reducing agent sodium dithionite showed that the copper was somewhat more reduced than the as-isolated, which was in line with an increased Cu-N bond length shown by the EXAFS data. The substrate-treated sample

had fewer Cu-N ligands (2-3) than in the as-isolated enzyme. These observations are consistent with changes in oxidation state and coordination of the copper centres upon interaction with the substrate (Supplementary Figures S6 and S7) and support a role of Cu in the function of the enzyme.

The identification of the gene encoding MTO reveals that MTO is a homologue of the Selenium-binding protein family (pfam SBP56), has a conserved genomic context and that MTO is a periplasmic enzyme. The gene encoding MTO was identified based on N-terminal and *de novo* peptide sequencing against a draft genome sequence of *Hyphomicrobium* sp. VS. N-terminal sequencing of the purified MTO resulted in the identification of 15 amino acids, DETXNSPFTTALITG, with position X potentially a cysteine residue, indicating a processed N-terminus. In addition to the N-terminal sequence, internal peptide sequences were obtained (see Supplementary Figure S8). Using peptide data in BLAST searches against the draft genome of *Hyphomicrobium* sp. VS available on MicroScope (Vallenet et al 2013) we identified the gene encoding MTO designated hereafter as *mtoX* (locus tag HypVSv1_1800007). A contig of 18.4 kb was assembled and confirmed by PCR and sequencing that contained a genomic region including the *mtoX* and additional genes downstream that are likely to be involved in its maturation (Genbank accession number KY242492). The *mtoX* gene is 1308 bp in size, encoding a polypeptide of 435 amino acids. Signal-P analysis (Bendtsen et al 2004) indicated that MTO contained a signal peptide with a predicted cleavage site at position 24 resulting in an N-terminus identical to the one determined experimentally of the purified MTO polypeptide. No transmembrane helices were identified by the software TMHMM (Krogh et al 2001) in the sequence representing the processed polypeptide, suggesting MTO to be a soluble periplasmic enzyme. The

calculated molecular weight of the processed periplasmic MTO was 45,905 Da (46,192 Da assuming 4 Ca and 2 Cu in addition), in good agreement with the observed MW on SDS PAGE and the molecular weight estimated by ESI-MS (46,186). A conserved domain search with the predicted MTO amino acid sequence confirmed its homology to members of pfam05694, the SBP56 super family. A BLASTP search with the MTO protein sequence revealed hits with high homology in all three domains of life; including against *Bacteria* (50-79% identity), *Archaea* (26-29% identity) and *Eukarya* (human SELENBP1 26% identity). The highest identities (77-79 %) were with proteins annotated as selenium-binding proteins from other *Hyphomicrobium* species. Despite this similarity to known Se binding proteins, no Se was found as judged by ICP elemental analysis. However, there are many cases of metalloproteins in which members of the same polypeptide family contain different specific metal co-factors , e.g. in proteins of the FUR regulator superfamily (Fillat 2014).

Genes downstream of *mtoX* in *Hyphomicrobium* sp. VS are predicted to encode homologs of the copper chaperone SCO1/SenC (Interpro: IPR003782) and of MauG, a protein with sequence similarity to diheme cytochrome *c* peroxidases which are required for the synthesis of tryptophan tryptophylquinone (TTQ) prosthetic groups (Wang et al 2003). The MTO, SCO1/SenC and MauG-encoding genes formed an operon-like structure (Figure 2). Based on the Cu content of MTO, the SCO1/SenC domain protein may be involved in MTO maturation. In *Paracoccus denitrificans* , the *mauG* gene encodes an enzyme responsible for posttranslational modification of the methylamine dehydrogenase pre-protein to produce a protein-derived TTQ cofactor (Wang et al 2003).

Phylogeny and distribution of *mtoX* in bacterial genomes. Homologs of *mtoX* were identified by BLASTX searches and a phylogenetic analysis was carried out based on alignment of predicted amino acid sequences. This showed that MTO from *Hyphomicrobium* sp. VS belongs to a clade annotated as selenium-binding proteins (Supplementary Figure S9). In addition, the cluster with *Hyphomicrobium* sp. VS-MTO-like SBP included many organisms known to degrade one-carbon compounds (including DMS and MT), DMSP (e.g. the model bacterium *Ruegeria pomeroyi* DSS-3), or sulfur-oxidizing bacteria. In many of these organisms *mtoX* was also co-located with the *SCO1/senC* and *mauG* genes, or with genes encoding these two protein domains fused in a single gene as for instance in *Methylophaga thiooxydans* (Figure 2, Supplementary Table S7). In this marine gammaproteobacterium that degrades DMS via MT (Boden et al 2010), expression of polypeptides identified as selenium-binding protein was demonstrated during growth on DMS by peptide sequencing (Schäfer 2007).

The capacity to degrade MT was tested in selected isolates. All tested bacterial strains containing the *mtoX* gene could degrade MT supporting a role for MTO in MT oxidation in these bacteria including *Ruegeria pomeroyi* DSS-3 (see below), *Hyphomicrobium denitrificans* (DSM1869), *Methylococcus capsulatus* (Bath), *Methylocystis* sp. ATCC 49242, *Methylophaga thiooxydans* DMS010, *Thiobacillus thioparus* TK-m, *Thiobacillus thioparus* E6, *Phaeobacter galleciensis* (DSM 17395) and *Pseudovibrio ascidiaceicola* (DSM 16392). Complete degradation of MT was observed within 2 days and was compared to sterile controls in which MT was not degraded over the same time period. In comparison, several strains that lacked the *mtoX* gene could not degrade MT, e.g. *Methylophaga marina* and *Methylobacterium extorquens* AM1 (Supplementary Table S4).

Genetic analysis of *mtoX* in *Ruegeria pomeroyi* DSS-3. *R. pomeroyi* DSS-3 produces MT as an intermediate whilst catabolizing DMSP via the demethylation pathway (Reisch et al 2011b). The *R. pomeroyi mtoX* gene (SPOA0269), located on a megaplasmid, encodes a protein with 57% and 71% identity and similarity, respectively, to the MTO of *Hyphomicrobium* VS. To study the role of *mtoX* in MT degradation, SPOA0269 was replaced with a spectinomycin cassette in the *R. pomeroyi* genome.

MT removal assays conducted at the whole cell level (0.5 mM MT) showed that wild type *R. pomeroyi* had a rate of MT removal (23 ± 1 nmol MT min^{-1} mg $protein^{-1}$) ~ 14-fold higher than those observed for *mtoX*$^-$ mutant cultures (1.7 ± 1.4 nmol MT min^{-1} mg $protein^{-1}$) supporting a role for MTO in MT oxidation (Table 2). The enzyme responsible for the low level MT removal activity remaining in the *mtoX*$^-$ mutant was not identified.

Assays of MTO activity in cell lysates of wild type and *mtoX*$^-$ mutants with or without prior incubation with MT (0.5 mM) further support the role of the *mtoX* gene in MT oxidation and showed its activity to be inducible. Cell lysates of wild type *R. pomeroyi* that had not been pre-incubated with MT consumed MT (0.25mM) at a rate of 39 ± 11 nmol MT min^{-1} mg $protein^{-1}$. In wild type cultures pre-incubated with MT, the degradation rate increased 4-fold to 139 ± 26 nmol MT min^{-1} mg $protein^{-1}$. Cell lysates of *mtoX*$^-$ mutants did not remove MT under the same conditions, irrespective of being pre-incubated in presence or absence of MT (Table 2). Thus the *R. pomeroyi* gene SPOA0269 likely encodes a functional MTO enzyme whose level of MT oxidation was up-regulated by exposure to MT.

The transcription of *Ruegeria pomeroyi* DSS3 *mtoX* is enhanced by MT. *R. pomeroyi* DSS-3 had a similar conserved *mtoX* gene neighbourhood in which there is likely co-transcription with a gene encoding a SCO1/SenC domain protein (SPOA0270) and a

mauG-like gene (SPOA0271) (Figure 2). Directly upstream of *mtoX* in *R. pomeroyi* is an IclR family transcriptional regulator (SPOA0268), and this gene arrangement is conserved in marine Roseobacter clade bacteria (Supplementary Table S7). We noted in microarrays carried out in (Todd et al 2012) that the transcription of the predicted operon (SPOA0268-0272) containing *mtoX* was significantly enhanced (2-5 fold) by growth of *R. pomeroyi* in the presence of DMSP. To confirm these observations transcriptional *lac* fusions were made to the SPOA0268 and *mtoX* genes and assayed in *R. pomeroyi* in the presence of potential inducer molecules. Consistent with the microarray results, transcription of both SPOA0268 and *mtoX* was enhanced by DMSP, MMPA and most significantly by MT (~14 fold for *mtoX*), but not DMS (Figure 3). These results are consistent with the cell lysate assays and MT being the inducer molecule since both DMSP and MMPA are catabolized to MT by DMSP demethylation.

Diversity of *mtoX* in environmental samples. The diversity of *mtoX* in environmental samples was assessed by PCR using newly designed primers 44F1/2 and 370R1/2/3 (Supplementary Table S5) which had been optimized by testing against a range of bacterial isolates. PCR with these primers resulted in amplicons of the expected size (approx. 987 bp) (Supplementary Figure S10). Performing the PCR with DNA extracted from samples that were shown or would be expected to contain bacteria capable of methanethiol degradation (based on their known degradation of DMS and DMSP for instance) also yielded bands of the correct size. These samples included DNA extracted from DMS enrichment cultures from *Brassica* rhizosphere soil, bulk agricultural soil (Eyice and Schäfer 2016), rhizosphere sediment of *Spartina anglica* (a DMSP-producing plant) obtained from Stiffkey salt marsh (Norfolk, UK) and surface sediments of Stiffkey saltmarsh. Saltmarshes are known to be environments with high turnover of DMSP, DMS and MT (e.g. Kiene 1988a, 1988b). Stiffkey saltmarsh samples used here had high

DMS oxidation rates and enrichment of organisms containing *mtoX* genes was readily observed (Kröber and Schäfer; Pratscher et al., unpublished data).

Annealing temperatures used in these PCRs varied between 53°C and 60°C. The *mtoX* amplicons obtained with the *Brassica oleracea* rhizosphere DMS enrichment were cloned and clones chosen at random were sequenced. The *mtoX* gene sequences obtained belonged to two clades closely related to *Thiobacillus thioparus* (Figure 4). Amplification efficiency of *mtoX* from DNA extracted from Stiffkey saltmarsh sediment samples was more variable and the primers were refined further (MtoX41Fmodv2_inos and MTOX346Rmod, Supplementary Table S5) to introduce degeneracies that improved their performance with these samples (result not shown). Surface sediment *mtoX* gene diversity was investigated in a tidal pool in Stiffkey saltmarsh using five independent samples from two transects across the pool. The analysis of randomly chosen clones from *mtoX* gene libraries prepared for these five surface sediment samples showed a high diversity of *mtoX* genes in the saltmarsh environment (Figure 4), whilst there appeared to be little variation in *mtoX* diversity between samples from the saltmarsh according to terminal restriction fragment length polymorphism analysis (result not shown). This showed Stiffkey saltmarsh *mtoX* sequences to belong to several distinct clades that lacked cultivated representatives. The *mtoX* sequences from Stiffkey saltmarsh clustered more closely with *mtoX* of gammaproteobacteria rather than those of alpha- or betaproteobacteria. The most closely related *mtoX* from cultivated strains were those of marine gamma proteobacterium HTCC2148, *Sedimenticola selenatireducens* and *Dechloromarinus chlorophilus*. MTO encoding genes detected in DNA extracted from the DMS enrichments with *Brassica* rhizosphere soil and ^{13}C-DNA of DMS-SIP experiments of soil and lake sediment samples (Eyice et al 2015) were

related to betaproteobacterial taxa such as *Thiobacillus thioparus* and *Methyloversatilis* spp. (Figure 4).

Detection of *mtoX* in metagenomic datasets. Homologs of *mtoX* of *Hyphomicrobium* sp. VS, *Methylophaga thiooxydans* and *Ruegeria pomeroyi* were also detected in metagenomic datasets (Table 3). The relative abundance of *mtoX* containing bacteria was estimated based on the frequency of detection of *mtoX* in comparison to *recA*, a universal housekeeping gene present in all bacteria and compared to that of *dmdA*, the DMSP demethylase. The relative abundance of *mtoX* varied across the different datasets (0-46%) and, in most cases, was lower than that of *dmdA*.. Based on this analysis it is difficult to delineate a general abundance pattern of *mtoX* containing bacteria in different environments, however it demonstrates that *mtoX* can be an abundant gene in some microbial communities. Selected *mtoX* sequences of sufficient length from the global ocean survey (GOS)(Rusch et al 2007) and other metagenomic datasets were included in the phylogenetic analysis (Figure 4). GOS *mtoX* formed distinct clades, some of which were closely related to saltmarsh sediment *mtoX* types, or to the marine gammaproteobacterium HTCC2080, suggesting that most of the *mtoX* detected in metagenomics studies are originating from previously uncultured bacteria.

DISCUSSION

New insights into biochemical, genetic and environmental aspects of bacterial methanethiol oxidation presented here address a major knowledge gap in the biogeochemical sulfur cycle and the fundamental understanding of MT degradation by bacteria. Data presented here indicate that MTO is a periplasmic enzyme that is present in a wide range of bacteria, not limited to those known to produce MT as a metabolic intermediate during DMS and DMSP degradation, such as *Hyphomicrobium* VS,

Thiobacillus spp. and *Ruegeria pomeroyi* DSS3. The *mtoX* gene was also found in diverse cultivated bacteria that had not previously been recognized for their potential to degrade methanethiol. Homologous genes are also present in *Archaea* and *Eukarya* (including humans). In addition, the overall diversity of *mtoX* in environmental samples suggests that the potential for MT oxidation is also present in diverse uncultivated microorganisms and that MTO is a widely distributed enzyme in different terrestrial and marine environments, many of which have demonstrated potential for degradation of methylated sulfur compounds. MTO requires copper for its catalytic activity, and in *R. pomeroyi* the gene encoding MTO is induced by MT. The enzyme from *Hyphomicrobium* sp. VS has a very high affinity for MT, with a K_m (0.2-0.3 μM) at least 10-fold lower than those previously reported, which may explain the low MT concentrations found in the environment.

Distinct molecular weights for MTOs from *Hyphomicrobium*, *Thiobacillus* and *Rhodococcus* strains have been reported previously. Based on high sequence homology of *mtoX* genes found in several *Hyphomicrobium* and *Thiobacillus* strains and the fact that previously purified MTOs from *Hyphomicrobium* sp. EG (Suylen et al 1987) and *Thiobacillus thioparus* (Gould and Kanagawa 1992) had similar molecular weights to the MTO of *Hyphomicrobium* sp. VS suggests that the previously purified MTOs are similar enzymes. Although previous studies reported MTO as a monomeric enzyme in *Hyphomicrobium* sp. EG and *T. thioparus* Tk-m (Gould and Kanagawa 1992, Suylen et al 1987), rather than a homotetramer as in this study, these differences may be due to sensitivity of the MTO's oligomeric state to pH. At pH 8.2, we found tetrameric MTO, but when we carried out analytical gel filtration at pH 7.2, as used by Suylen et al (Suylen et al 1987) MTO was detected in monomeric and tetrameric state (result not shown).

Other observed differences between these MTOs may be due to different analytical approaches that were employed. For instance, a role of metals in MTO activity was previously ruled out based on chelation experiments, but these can fail to deplete the metals from the enzyme depending on variations in incubation conditions. The presence in and role of Cu for the functioning of the enzyme from *Hyphomicrobium* sp. VS is supported by ICP-MS analysis, changes in EPR spectra recorded with MTO in resting, reduced and oxidized state, and by chelation experiments showing a reduced activity of the enzyme. The presence of genes encoding putative Cu chaperones (SCO1/SenC) in close proximity to *mtoX* homologs in many bacterial genomes provides further circumstantial evidence for a role of copper in MT oxidation and provides a focus for future genetic and biochemical studies.

Besides the presence of a *mauG* homolog, involved in maturation of a protein-derived TTQ cofactor in methylamine dehydrogenase, we found supporting evidence that the MT oxidase also contains a TTQ co-factor. The PDB database contains the structure of the heterologously expressed SBP56 protein of *Sulfolobus tokodaii* (PDB entry: 2ECE). Analysis of the structure of this non-matured protein (no copper, no TTQ) made it possible to identify the putative ligands involved in copper binding (histidines) and TTQ synthesis (tryptophans) in the *Sulfolobus* homologue (Supplementary Figure S11). Alignments of the tryptophan and histidine residues identified showed strict conservation over the three domains of life. EPR and EXAFS analyses suggest that Cu in MTO of *Hyphomicrobium* sp. VS is coordinated by four nitrogen atoms, which would fit with the strictly conserved histidine residues which in *Hyphomicrobium* sp. VS MTO are His89, His90, His140, His412 (Supplementary Figures S8 and S12). The structural information and the presence of the *SCO1/senC* and *mauG*-like genes support the presence of a TTQ cofactor and 2 copper atoms per monomer; further, if we assume 4

Ca and 2 Cu per monomer the calculated mass exactly fits the ESI-MS analysis: 46,193 Da vs. 46,186 Da. The arrangement of the genes *mtoX*, *SCO1/senC* and *mauG* encoding MTO, a copper chaperone, and homologue of the enzyme known to be involved in maturation of a protein-derived TTQ cofactor in methylamine dehydrogenase was highly conserved in a wide range of bacteria (Figure 2 and Supplementary Table S7).

The role of MTO in metabolism of MT and DMSP as well as its transcriptional regulation were demonstrated in *Ruegeria pomeroyi* showing that this enzyme plays an important role in metabolism of DMSP. Transcriptional fusions of the IclR type regulator upstream also demonstrated that MT as well as DMSP and MMPA (which are degraded to MT) induced MTO transcription. Interestingly, despite the presence of a functional MTO, it has long been known that *R. pomeroyi* DSS-3 liberates MT when grown in the presence of DMSP, this being one of the products of the DMSP demethylation pathway (Reisch et al 2011b). Thus, under these circumstances the MTO does not have sufficient activity to oxidise all the DMSP-dependent MT that is formed. However, we noted (unpublished) that the *mtoX*$^{-}$ mutant *R. pomeroyi* DSS-3 released more MT (~1.5-fold) when grown in the presence of DMSP than did the wild type.

The identification of the gene encoding MTO in bacteria has allowed assessing the distribution of the enzyme in the environment and identified its evolutionary relationship to the selenium-binding protein family (SBP56), a protein family that has as yet an unresolved function. Metal analysis by ICP-MS did not show the presence of Selenium in MTO. SBP56 is a highly conserved intracellular protein (Bansal et al 1989). Previous reports stated that it is involved in the transport of selenium compounds,

regulation of oxidation/reduction and late stages of intra-Golgi protein transport, but its exact role has remained unclear (Ishida et al 2002, Jamba et al 1997, Porat et al 2000). Homologs of SBP56 were found in human, mouse, fish, horse, birds, abalone and plants such as *Arabidopsis thaliana* and maize in addition to bacteria and archaea (Flemetakis et al 2002, Jamba et al 1997, Self et al 2004, Song et al 2006). The human SBP56 homologue has been shown to be a methanethiol oxidase (Pol et al, Nature Genetics, in revision). To what extent the other SBP56 have similar function to MTO needs to be addressed, but a possible relationship of SBP56 with C1 metabolism was previously pointed out based on the presence of the SBP56 encoding gene in the vicinity of genes encoding selenocysteine-containing formate dehydrogenases in the genome of *Methanococcus vannielli* and *M. maripaludis* (Self et al 2004).

Homologs of *mtoX* are present in a wide range of bacteria, and metagenomes from marine pelagic, coastal, hydrothermal and terrestrial environments, including DMS stable isotope probing experiments of soil and lake sediment samples. Based on processes that contribute to MT production in marine and terrestrial environments, a wide distribution of this enzyme is not surprising. The diversity of *mtoX*-containing organisms present in the environment is currently not well represented by isolated organisms, which suggests that the ability to degrade MT is more widely distributed than currently realized. This lack of environmentally relevant model bacteria limits our ability to appreciate which organisms are important as sinks for MT in different environments, how the expression of MTO in these organisms is regulated and which other degradative capabilities they may have. Using a stable isotope probing approach with $^{13}C_2$-DMS, we recently identified *Methylophilaeceae* and *Thiobacillus* spp. as DMS-degrading bacteria in soil and lake sediment (Eyice et al 2015). The finding of *mtoX*

genes in representatives of *Thiobacillus* and *Methylophilaceae* is consistent with the role that MT has as a metabolic intermediate in previously characterized DMS-degrading bacteria such as *Thiobacillus* spp. and adds further weight to the suggestion that certain *Methylophilaceae* have the metabolic potential to degrade DMS. The detection of *mtoX* in a saltmarsh environment is in agreement with such environments being hotspots of organic sulfur cycling (Dacey et al 1987, Steudler and Peterson 1984) based on production of DMSP and DMS by benthic microalgae, macrophytes and macroinvertebrates (Otte et al 2004, Van Alstyne and Puglisi 2007), and MT production through anaerobic processes in the sediment (Lomans et al 2002).

Overall, this study adds to our fundamental understanding of a key step in the sulfur cycle. The identification of the gene encoding this enzyme reveals its homology to a protein super family of which homologs are present in organisms ranging from bacteria to humans, but for which only sketchy functional information has been reported previously. The outcomes of this study will therefore facilitate future investigations of the role of MTO homologs in a wide range of organisms by providing testable hypotheses regarding its physiological relevance in these organisms. At the same time, the identification of the gene encoding MTO as well as its metal dependence will provide key foci for investigation of the diversity and distribution of MTO and potential constraints on its activity such as metal availability on MT degradation rates in the environment as well as aspects of the catalytic mechanism of MTO.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

We are grateful to Sue Slade for mass spectrometry analyses, Rich Boden for providing *Thiobacillus* strains and DNA, Colin Murrell and Julie Scanlan for providing *Methylococcus capsulatus* (Bath), Lisa Stein for supplying *Methylocystis* sp. ATCC 49242 and technical support from Diego Gianolio at DIAMOND Synchrotron. We gratefully acknowledge the help of Joachim Reimann identifying the potential copper ligands and TTQ forming amino acids and producing Supplementary Figures S10 and S11. We are also grateful for funding that supported this study which came from the Biotechnology and Biological Sciences Research Council (grant reference BB/H003851/1) to HS and TDHB, a Natural Environment Research Council (NERC) Advanced Fellowship (NE/E013333/1) and project grant (NE/H008918/1) to HS, beam time support from DIAMOND Synchrotron (SP8769) to HS, NM, TJS, and SJG; NERC project grants (NE/J01138X/1 and NE/M004449/1) to JDT; a grant supporting KKA's work from the Research Council of Norway (grant nr. 214239); a PhD stipend from the University of Warwick to ÖE; and an ERC Advanced Grant 669371 (VOLCANO) to HOdC.

Supplementary Information is available at the ISME Journal's website

References

Antholine WE, Kastrau DHW, Steffens GCM, Buse G, Zumft WG, Kroneck PMH (1992). A comparative EPR investigation of the multicopper proteins nitrous-oxide reductase and cytochrome-*c*-oxidase. *Eur J Biochem* **209:** 875-881.

Awano S, Koshimune S, Kurihara E, Gohara K, Sakai A, Soh I *et al* (2004). The assessment of methyl mercaptan, an important clinical marker for the diagnosis of oral malodor. *J Dent* **32:** 555-559.

Bansal MP, Oborn CJ, Danielson KG, Medina D (1989). Evidence for two selenium-binding proteins distinct from glutathione peroxidase in mouse liver. *Carcinogenesis* **10:** 541-546.

Baumann P, Baumann L (eds) (1981) The marine Gram negative eubacteria: genera *Photobacterium, Beneckea, Alteromonas, Pseudomonas and Alcaligenes.* Springer, 1302-1331pp.

Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004). Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* **340:** 783-795.

Bentley R, Chasteen TG (2004). Environmental VOSCs - formation and degradation of dimethyl sulfide, methanethiol and related materials. *Chemosphere* **55:** 291-317.

Boden R, Kelly DP, Murrell JC, Schäfer H (2010). Oxidation of dimethylsulfide to tetrathionate by *Methylophaga thiooxidans* sp. nov.: a new link in the sulfur cycle. *Environ Microbiol* **12:** 2688-2699.

Boden R, Borodina E, Wood AP, Kelly DP, Murrell JC, Schäfer H (2011). Purification and characterization of dimethylsulfide monooxygenase from *Hyphomicrobium sulfonivorans*. *J Bacteriol* **193:** 1250-1258.

Carrión O, Curson ARJ, Kumaresan D, Fu Y, Lang AS, Mercade E *et al* (2015). A novel pathway producing dimethylsulphide in bacteria is widespread in soil environments. *Nat Commun* **6:** 8.

Dacey JWH, King GM, Wakeham SG (1987). Factors controlling emission of dimethylsulfide from salt marshes. *Nature* **330:** 643-645.

Devos M, Patte F, Rouault J, Laffort P, Gemert LJ (1990). Standardized human olfactory thresholds. *Standardized human olfactory thresholds*. Oxford: IRL Press.

Erb TJ, Evans BS, Cho K, Warlick BP, Sriram J, Wood BM *et al* (2012). A RubisCO-like protein links SAM metabolism with isoprenoid biosynthesis. *Nat Chem Biol* **8:** 926-932.

Eyice Ö, Namura M, Chen Y, Mead A, Samavedam S, Schäfer H (2015). SIP metagenomics identifies uncultivated *Methylophilaceae* as dimethylsulfide degrading bacteria in soil and lake sediment. *ISME J* **9:** 2336-2348.

Eyice Ö, Schäfer H (2016). Culture-dependent and culture-independent methods reveal diverse methylotrophic communities in terrestrial environments. *Arch Microbiol* 198: 17-26

Figurski DH, Helinski DR (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. *Proc Natl Acad Sci USA* **76:** 1648-1652.

Fillat MF (2014). The FUR (ferric uptake regulator) superfamily: Diversity and versatility of key transcriptional regulators. *Arch Biochem and Biophys* **546**: 41-52.

Flemetakis E, Agalou A, Kavroulakis N, Dimou M, Martsikovskaya A, Slater A *et al* (2002). *Lotus japonicus* gene *Ljsbp* is highly conserved among plants and animals and encodes a homologue to the mammalian selenium-binding proteins. *Mol Plant Microbe Interact* **15:** 313-322.

González JM, Mayer F, Moran MA, Hodson RE, Whitman WB (1997). *Sagittula stellata* gen. nov., sp. nov., a lignin-transforming bacterium from a coastal environment. *Int J Syst Bacteriol* **47:** 773-780.

Gould WD, Kanagawa T (1992). Purification and properties of methyl mercaptan oxidase from *Thiobacillus thioparus* TK-m. *J Gen Microbiol* **138:** 217-221.

Hernandez D, Tewhey R, Veyrieras J-B, Farinelli L, Østerås M, François P *et al* (2014). *De novo* finished 2.8 Mbp *Staphylococcus aureus* genome assembly from 100 bp short and long range paired-end reads. *Bioinformatics* **30:** 40-49.

Howard EC, Henriksen JR, Buchan A, Reisch CR, Buergmann H, Welsh R *et al* (2006). Bacterial taxa that limit sulfur flux from the ocean. *Science* **314:** 649-652.

Ishida T, Ishii Y, Yamada H, Oguri K (2002). The induction of hepatic selenium-binding protein by aryl hydrocarbon (Ah)-receptor ligands in rats. *J Health Sci* **48:** 62-68.

Jamba L, Nehru B, Bansal MP (1997). Redox modulation of selenium binding proteins by cadmium exposures in mice. *Mol Cell Biochem* **177:** 169-175.

Kaim W, Schwederski B, Klein A (2013). *Bioinorganic Chemistry - Inorganic Elements in the Chemistry of Life: An Introduction and Guide*, 2nd Edition edn, vol. 2nd Edition.

Kettle AJ, Rhee TS, von Hobe M, Poulton A, Aiken J, Andreae MO (2001). Assessing the flux of different volatile sulfur gases from the ocean to the atmosphere. *J Geophys Res Atmos* **106:** 12193-12209.

Kiene RP (1988a). Dimethyl sulfide metabolism in salt marsh sediments. *FEMS Microbiol Ecol* **53**: 71-78.

Kiene RP, Capone DG (1988b). Microbial transformations of methylated sulfur compounds in anoxic salt marsh sediments. *Microb Ecol* **15**: 275-291.

Kiene RP, Linn LJ, Gonzalez J, Moran MA, Bruton JA (1999). Dimethylsulfoniopropionate and methanethiol are important precursors of methionine and protein-sulfur in marine bacterioplankton. *Appl Environ Microbiol* **65:** 4549-4558.

Kim S-J, Shin H-J, Kim Y-C, Lee D-S, Yang J-W (2000). Isolation and purification of methyl mercaptan oxidase from *Rhodococcus rhodochrous* for mercaptan detection. *Biotechnol Bioprocess Eng* **5:** 465-468.

Krogh A, Larsson B, von Heijne G, Sonnhammer ELL (2001). Predicting transmembrane protein topology with a hidden markov model: application to complete genomes. *J Mol Biol* **305:** 567-580.

Lee H-H, Kim S-J, Shin H-J, Park J-Y, Yang J-W (2002). Purification and characterisation of methyl mercaptan oxidase from *Thiobacillus thioparus* for mercaptan detection. *Biotechnol Bioprocess Eng* **7:** 375-379.

Lomans BP, Smolders AJP, Intven LM, Pol A, Op Den Camp HJM, Van Der Drift C (1997). Formation of dimethyl sulfide and methanethiol in anoxic freshwater sediments. *Appl Environ Microbiol* **63:** 4741-4747.

Lomans BP, Maas R, Luderer R, Op den Camp HJ, Pol A, van der Drift C *et al* (1999a). Isolation and characterization of *Methanomethylovorans hollandica* gen. nov., sp. nov., isolated from freshwater sediment, a methylotrophic methanogen able to grow on dimethyl sulfide and methanethiol. *Appl Environ Microbiol* **65:** 3641-3650.

Lomans BP, Op den Camp HJ, Pol A, van der Drift C, Vogels GD (1999b). Role of methanogens and other bacteria in degradation of dimethyl sulfide and methanethiol in anoxic freshwater sediments. *Appl Environ Microbiol* **65:** 2116-2121.

Lomans BP, Luderer R, Steenbakkers P, Pol A, van der Drift C, Vogels GD *et al* (2001). Microbial populations involved in cycling of dimethyl sulfide and methanethiol in freshwater sediments. *Appl Environ Microbiol* **67:** 1044-1051.

Lomans BP, van der Drift C, Pol A, Op den Camp HJM (2002). Microbial cycling of volatile organosulfur compounds. *Cell Mol Life Sci* **59:** 575-588.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar *et al* (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32:** 1363-1371.

Markowitz VM, Mavromatis K, Ivanova NN, Chen IM, Chu K, Kyrpides NC (2009). IMG ER: a system for microbial genome annotation expert review and curation. *Bioinformatics* **25:** 2271-2278.

Monzani E, Quinti L, Perotti A, Casella L, Gullotti M, Randaccio L *et al* (1998). Tyrosinase models. Synthesis, structure, catechol oxidase activity, and phenol monooxygenase activity of a dinuclear copper complex derived from a triamino pentabenzimidazole ligand. *Inorg Chem* **37:** 553-562.

Otte ML, Wilson G, Morris JT, Moran BM (2004). Dimethylsulphoniopropionate (DMSP) and related compounds in higher plants. *J Exp Bot* **55:** 1919-1925.

Pol A, Renkema GH, Tangerman A, Winkel EG, Engelke UF, de Brouwer APM *et al* Mutations in SELENBP1 cause extra-halitosis due to methanethiol oxidase deficiency. *Nature Genetics, in review*.

Pol A, Op den Camp HJM, Mees SGM, Kersten MASH, van der Drift C (1994). Isolation of a dimethylsulfide-utilizing *Hyphomicrobium* species and its application in biofiltration of polluted air. *Biodegr* **5:** 105-112.

Porat A, Sagiv Y, Elazar Z (2000). A 56-kDa selenium-binding protein participates in intra-Golgi protein transport. *J Biol Chem* **275:** 14457-14465.

Reisch CR, Moran MA, Whitman WB (2011a). Bacterial catabolism of dimethylsulfoniopropionate (DMSP). *Front Microbiol* **2:** 172.

Reisch CR, Stoudemayer MJ, Varaljay VA, Amster IJ, Moran MA, Whitman WB (2011b). Novel pathway for assimilation of dimethylsulphoniopropionate widespread in marine bacteria. *Nature* **473:** 208-211.

Rossen L, Shearman CA, Johnston AWB, Downie JA (1985). The *nodD* gene of *Rhizobium leguminosarum* is autoregulatory and in the presence of plant exudate induces the *nodA,B,C* genes. *EMBO J* **4:** 3369-3373.

Rusch DB, Halpern AL, Sutton G, Heidelberg KB, Williamson S, Yooseph S *et al* (2007). The Sorcerer II Global Ocean Sampling expedition: Northwest Atlantic through Eastern Tropical Pacific. *PloS Biol* **5:** 398-431.

Schäfer H, McDonald IR, Nightingale PD, Murrell JC (2005). Evidence for the presence of a CmuA methyltransferase pathway in novel marine methyl halide-oxidising bacteria. *Environ Microbiol* **7:** 839-852.

Schäfer H (2007). Isolation of *Methylophaga* spp. from marine dimethylsulfide-degrading enrichment cultures and identification of polypeptides induced during growth on dimethylsulfide. *Appl Environ Microbiol* **73:** 2580-2591.

Schäfer H, Myronova N, Boden R (2010). Microbial degradation of dimethylsulphide and related C1-sulphur compounds: organisms and pathways controlling fluxes of sulphur in the biosphere. *J Exp Bot* **61:** 315-334.

Self WT, Pierce R, Stadtman TC (2004). Cloning and heterologous expression of a *Methanococcus vannielii* gene encoding a selenium-binding protein. *IUBMB life* **56:** 501-507.

Solomon EI, Sundaram UM, Machonkin TE (1996). Multicopper oxidases and oxygenases. *Chem Rev* **96:** 2563-2606.

Song L, Zou H, Chang Y, Xu W, Wu L (2006). The cDNA cloning and mRNA expression of a potential selenium-binding protein gene in the scallop *Chlamys farreri*. *Dev Comp Immunol* **30:** 265-273.

Steudler PA, Peterson BJ (1984). Contribution of gaseous sulfur from salt marshes to the global sulfur cycle. *Nature* **311:** 455-457.

Sun SL, Chen J, Li WZ, Altintas I, Lin A, Peltier S *et al* (2011). Community cyberinfrastructure for Advanced Microbial Ecology Research and Analysis: the CAMERA resource. *Nucleic Acids Res* **39:** D546-D551.

Suylen GMH, Large PJ, Vandijken JP, Kuenen JG (1987). Methyl mercaptan oxidase, a key enzyme in the metabolism of methylated sulfur-compounds by *Hyphomicrobium* EG. *J Gen Microbiol* **133:** 2989-2997.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28:** 2731-2739.

Tangerman A, Winkel EG (2007). Intra- and extra-oral halitosis: finding of a new form of extra-oral blood-borne halitosis caused by dimethyl sulphide. *J Clin Periodontol* **34:** 748-755.

Todd JD, Curson ARJ, Kirkwood M, Sullivan MJ, Green RT, Johnston AWB (2011). DddQ, a novel, cupin-containing, dimethylsulfoniopropionate lyase in marine roseobacters and in uncultured marine bacteria. *Environ Microbiol* **13:** 427-438.

Todd JD, Kirkwood M, Newton-Payne S, Johnston AWB (2012). DddW, a third DMSP lyase in a model Roseobacter marine bacterium, *Ruegeria pomeroyi* DSS-3. *ISME J* **6:** 223-226.

Ulshöfer VS, Flock OR, Uher G, Andreae MO (1996). Photochemical production and air-sea exchange of carbonyl sulfide in the eastern Mediterranean Sea. *Mar Chem* **53:** 25-39.

Vallenet D, Belda E, Calteau A, Cruveiller S, Engelen S, Lajus A *et al* (2013). MicroScope—an integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. *Nucleic Acids Res* **41:** D636-D647.

Van Alstyne KL, Puglisi MP (2007). DMSP in marine macroalgae and macroinvertebrates: Distribution, function, and ecological impacts. *Aquat Sci* **69:** 394-402.

Wang YT, Graichen ME, Liu AM, Pearson AR, Wilmot CM, Davidson VL (2003). MauG, a novel diheme protein required for tryptophan tryptophylquinone biogenesis. *Biochemistry* **42:** 7318-7325.

Whittenbury R, Phillips KC, Wilkinson JF (1970). Enrichment, isolation and some properties of methane-utilizing bacteria. *J Gen Microbiol* **61:** 205-218.

Xu X, Bingemer HG, Georgii H-W, Schmidt U, Bartell T (2001). Measurements of carbonyl sulfide (COS) in surface seawater and marine air, and estimates of the air-sea flux from observations during two Atlantic cruises. *J Geophys Res Atmos* **106:** 3491-3502.

Figure Legends

Figure 1. Simplified schematic showing the role of methanethiol (MT) as an intermediate in the metabolism of sulfur compounds. Abbreviations: $DMSO_2$, dimethylsulfone; DMSO, dimethylsulfoxide; DMSP, dimethylsulfoniopropionate; MMPA, methyl mercapto-propionic acid; DOM, dissolved organic matter; MTA, 5'-methylthioadenosine; DMDS, dimethyldisulfide; HS^-, sulfide ion; SO_3^{2-}, sulfite ion; S^0, elemental sulfur; $S_2O_3^{2-}$, thiosulfate; $S_4O_6^{2-}$, tetrathionate; SO_4^{2-}, sulfate. A single arrow does not imply a single biotransformation step.

Figure 2. Genomic context of *mtoX* genes in selected bacteria showing the clustering of *mtoX* with genes encoding proteins containing SCO1/SenC and/or MauG domains, see inset for definition of colouring and patterns to particular gene annotation. As discussed in the text, in some instances, genes are encoding fusion proteins of SCO1 and *mauG* domains. Further information about the presence of SCO1 and MauG domain encoding genes in the vicinity of *mtoX* genes is given in Supplementary Table S7.

Figure 3. Transcriptional regulation of *Ruegeria pomeroyi* DSS3 SPOA0268 and the methanethiol oxidase gene encoded by SPOA0269, assessed by beta galactosidase transcriptional fusion assay using various potential inducers. DMS, dimethylsulfide; DMSP, dimethylsulfoniopropionate; Mt, methanethiol; MMPA, methylmercaptopropionic acid. Values are reported in Miller units.

Figure 4. Phylogenetic analysis of translated methanethiol oxidase genes obtained from public databases, selected bacterial isolates by PCR, clone libraries of enrichment cultures and DNA extracted from surface sediments of Stiffkey salt marsh. The tree was based on an alignment of full length and partial MtoX sequences in Arb and was derived using the Neighbour Joining algorithm and PAM correction implemented in Arb from a region comprising amino acid positions 85 to 300 of the *Hyphomicrobium* VS MtoX polypeptide. Bootstrap values (100 iterations) were derived in Mega 5, only those supporting terminal nodes with a confidence of 75% or higher are shown. Taxa shown in bold tested positive for MT oxidation.

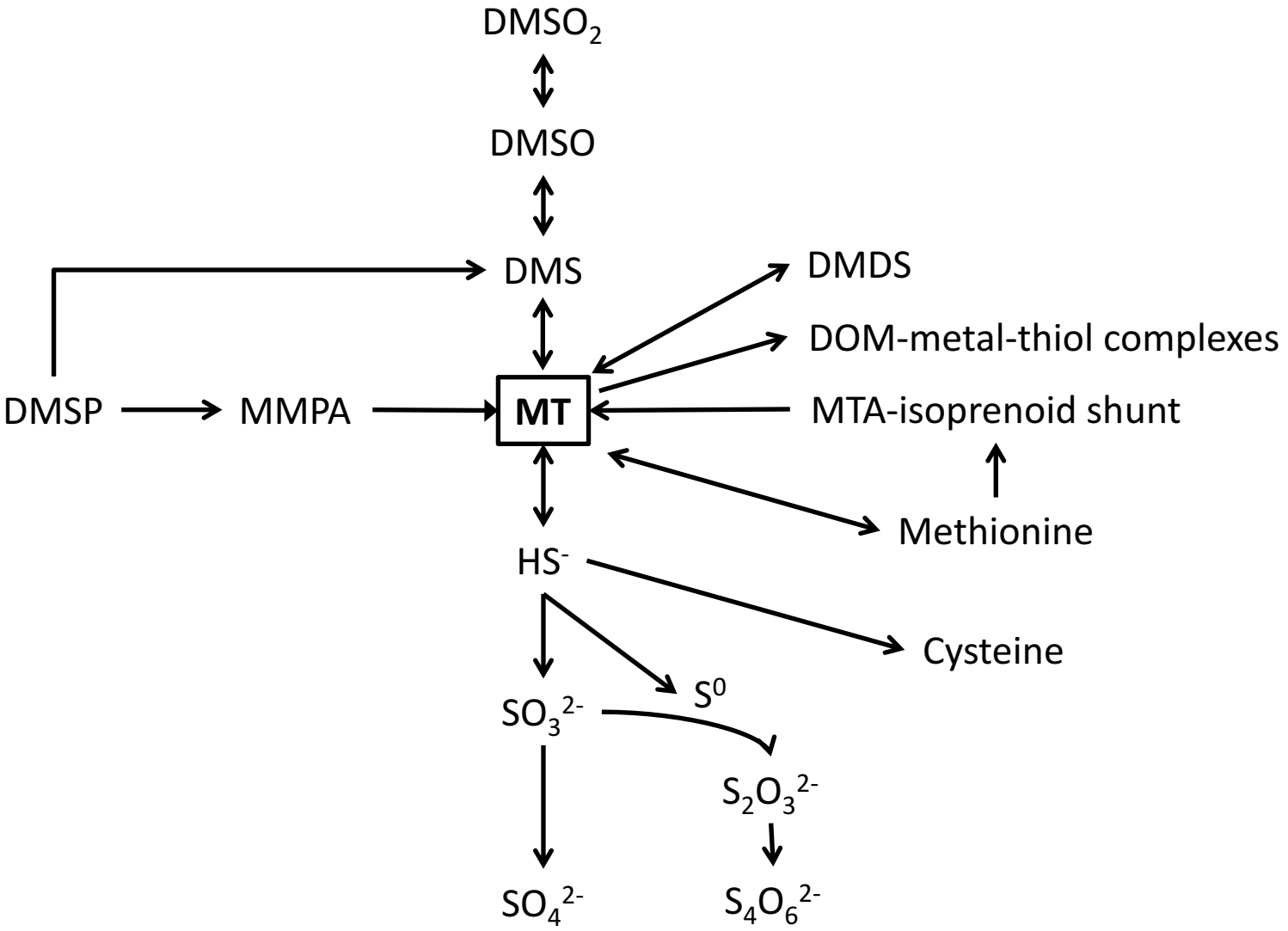

Figure 1. Simplified schematic showing the role of methanethiol (MT) as an intermediate in the metabolism of sulfur compounds. Abbreviations: $DMSO_2$, dimethylsulfone; DMSO, dimethylsulfoxide; DMSP, dimethylsulfoniopropionate; MMPA, methyl mercapto-propionic acid; DOM, dissolved organic matter; MTA, 5'-methylthioadenosine; DMDS, dimethyldisulfide; HS^-, sulfide ion; SO_3^{2-}, sulfite ion; S^0, elemental sulfur; $S_2O_3^{2-}$, thiosulfate; $S_4O_6^{2-}$, tetrathionate; SO_4^{2-}, sulfate. A single arrow does not imply a single biotransformation step.

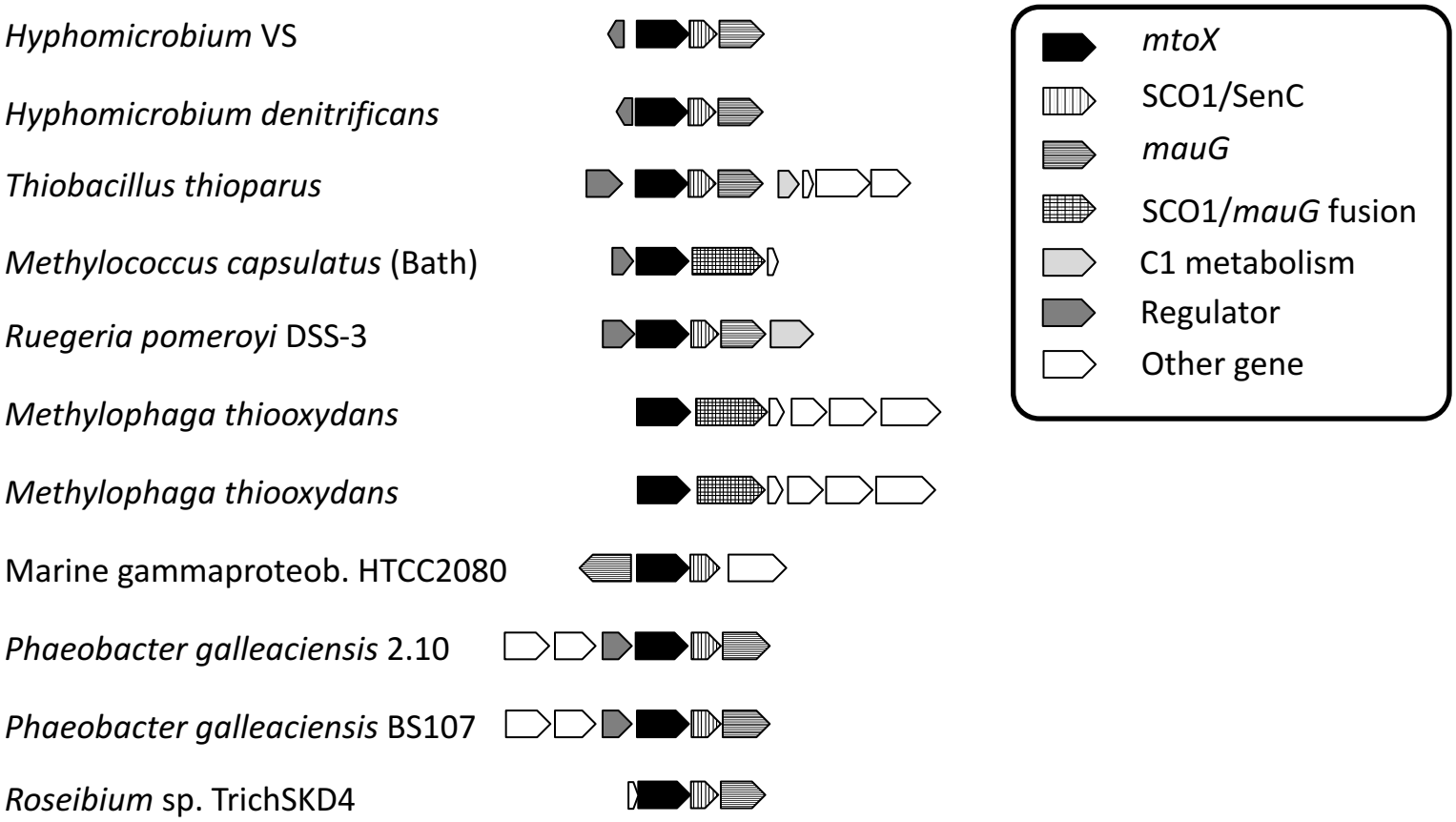

Figure 2. Genomic context of *mtoX* genes in selected bacteria showing the clustering of *mtoX* with genes encoding proteins containing SCO1/SenC and/or MauG domains, transcriptional regulators or genes otherwise implicated in C1 metabolism; 'other gene' denotes genes that have function other than those indicated above. See inset for definition of patterns to particular gene annotation. As discussed in the text, some genes encode fusion proteins of SCO1 and *mauG* domains. Further information on the presence of SCO1 and MauG domain-encoding genes in the vicinity of *mtoX* genes and other genes in the genetic neighbourhood of *mtoX* is given in Supplementary Table S5.

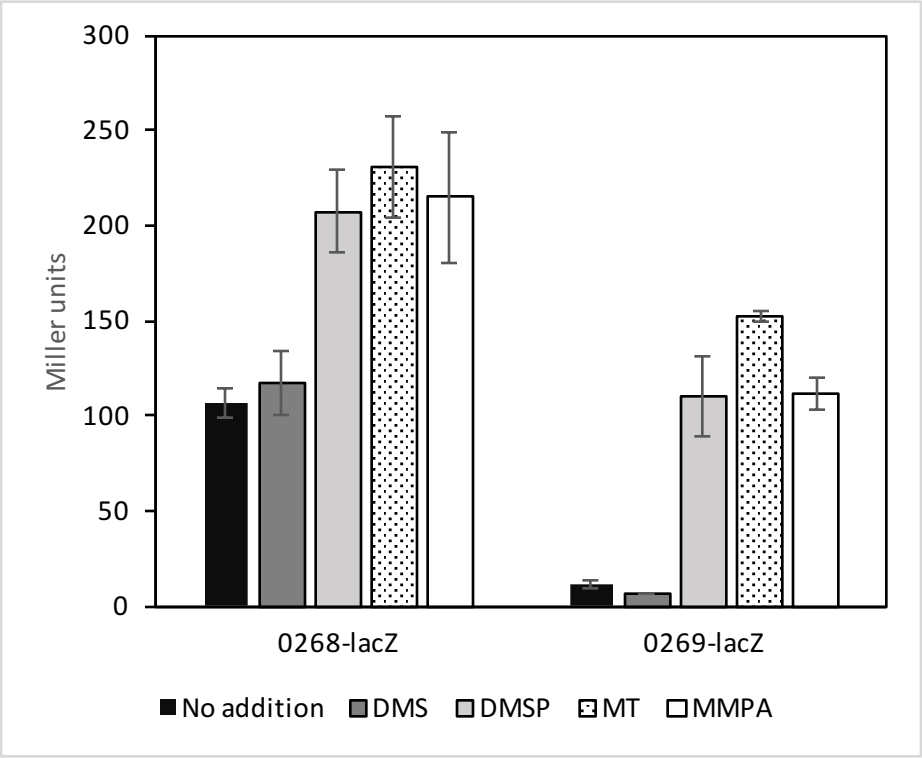

Figure 3. Transcriptional regulation of *Ruegeria pomeroyi* DSS3 SPOA0268 and the methanethiol oxidase gene (SPOA0269), assessed by beta galactosidase transcriptional fusion assay using various potential inducers. DMS, dimethylsulfide; DMSP, dimethylsulfoniopropionate; MT, methanethiol; MMPA, methylmercaptopropionic acid. Values are reported in Miller units; standard errors, from three replicates, are shown.


28 Saltmarsh sediment
Alteromonadaceae bacterium 2141T.STBD.0c.01a
Alteromonadaceae bacterium 2141T.STBD.0c.01a
Alteromonadaceae bacterium 2719K.STB50.0a.01
98 Sedimenticola sp. CUZ
Sedimenticola selenatireducens AK4OH1
Sedimenticola thiotaurini SIP-G1
99 2 Saltmarsh sediment
Thiothrix disciformis DSM 14473
Leucothrix mucor DSM 2157
99 Ferrimonas sediminum DSM 23317
99 Ferrimonas kyonanensis DSM 18153
Ferrimonas futtsuensis DSM 18154
98 Methylococcaceae sp. B42 Ga0078419
Methylohalobius crimeensis 10Ki
70 Methylophaga thiooxydans (locus MDMS009_768)
98 Methylophaga thiooxydans (locus MDMS009_211)
Methylophaga sulfidovorans DSM 11578
Pseudoalteromonas denitrificans DSM 6059
3 Saltmarsh sediment
28 Saltmarsh sediment
9 Saltmarsh sediment
96 Sedimenticola sp. CUZ
Sedimenticola selenatireducens AK4OH1, DSM 17993
Dechloromarinus chlorophilus NSS
99 6 Saltmarsh sediment
Beggiatoa alba B18LD
99 7 Thioalkalivibrio spp.
Thiorhodococcus drewsii
15 Saltmarsh sediment
99 Saltmarsh sediment clone Cc010
Saltmarsh sediment clone Hh015
Marine metagenome (GOS)
14 Saltmarsh sediment
99 4 Marine metagenome (GOS)
95 2 Marine metagenome (GOS)
95 Marine gamma proteobacterium HTCC2080
Marine metagenome (GOS)
99 Saltmarsh sediment clone Aa014
Marine gamma proteobacterium HTCC2148
Saltmarsh sediment clone Aa002
Saltmarsh sediment clone Bb017
Saltmarsh sediment clone Hh003
Gammaproteobacteria sp. genome_bin_26
Methylobacter whittenburyi ACM 3310
Methylobacterium marinus A45
83 Methylobacter sp. BBA5.1
96 Methylobacter luteus IMV-B-3098T
Methylococcaceae-55
Methylomonas sp. LW13
93 Methylomonas sp. 11b
99 Methylomonas sp. MK1
93 Methylomonas methanica MC09
Methylomicrobium album
91 Methylosarcina lacus-69
Methylosarcina lacus LW14
93 Methylosarcina-21
95 Methylomicrobium agile ATCC 35068
Methylosarcina fibrata AML-C10
97 Methylomicrobium buryatense 5G
95 Methylomicrobium alcaliphilum
Methylomicrobium kenyense AMO1
77 Methylomarinum vadi IT-4
99 Composite genome from Trout Bog Hypolimnion pan-assembly
99 Composite genome from Trout Bog Epilimnion pan-assembly
Composite genome from Trout Bog Epilimnion pan-assembly
Crenothrix polyspora
Methyloglobulus morosus KoM1
Methylocaldum sp. 175
Methylocaldum szegediense O-12
Methylococcaceae sp. 73a
98 Methylococcus capsulatus ATCC 19069
Methylococcus capsulatus str. Bath
99 Phaeobacter gallaeciensis 2.10
Phaeobacter gallaeciensis BS107
Phaeobacter inhibens T5, DSM 16374
Ruegeria pomeroyi DSS-3
Oceanicola sp. MCTG156(1a)
Pseudovibrio ascidiaceicola DSM 16392
84 Pseudovibrio sp. Tun.PHSC04-5.I4
93 Pseudovibrio sp. FO-BEG1
97 Pseudovibrio sp. JE062
82 Leisingera nanhaiensis DSM 24252
Roseibium sp. TRICHSKD4
Magnetospira sp. QH-2
Saltmarsh sediment clone Cc015
Marine metagenome (GOS)
138 Thiobacillus thioparus T-km, T. thioparus T5, T. sp. E6, T. thioparus Novellus, Rhizosphere soil DMS enrichment, Soil DMS-SIP soil and lake sediment DMS-SIP
94 7 Rhizosphere soil DMS enrichment
92 2 Soil DMS SIP metagenome
91 75 OE7-1; ctg_13822000007; from Soil DMS SIP metagenome
Rhizosphere soil DMS enrichment clone R1DMS_26
99 Rhizosphere soil DMS enrichment clone R1DMS_22
83 Thiobacillus denitrificans DSM 12475
Methyloversatilis sp. FAM1
99 Rhodocyclaceae bacterium RZ94
Methyloversatilis sp. RZ18-153
99 NC10 bacterium 'DUTCH SEDIMENT'
99 Hyphomicrobium denitrificans
Hyphomicrobium zavarzinii ATCC 27496
Bradyrhizobium japonicum USDA 4
Hyphomicrobium sp. VS
99 OE7_6 ctg 6774000013 of Soil DMS SIP metagenome
99 OE7_7 ctg2759000008 of Soil DMS SIP metagenome
MN7_1; ctg7937000008 of Lake sediment DMS SIP metagenome
unclassified betaproteobacterium ENR4
Verrucomicrobium sp. LP2A
Comamonas badia DSM 17552
Burkholderia sp. JOSHI_001
90 Betaproteobacteria sp. genome_bin_9 Ga0077526
Methylocystis sp. strain SB2
Methylocystis rosea SV97T.
Methylocystis sp. SC2
98 Methylocystis parvus OBBP
Methylocystis sp. Rockwell ATCC 49242
Methylocapsa acidiphila B2
99 Rugamonas rubra ATCC 43154
94 Janthinobacterium sp. HH01
Janthinobacterium sp. GC3
Freshwater sediment metagenome (Lake Washington)
97 6 Methylobacterium spp.
88 Microvirga sp. WSM3557
98 Skermanella stibiiresistens SB22
Skermanella aerolata KACC 11604
Haloferula sp. BvORR071
99 Candidatus Methylomirabilis oxyfera sp. Australia
Candidatus Methylomirabilis oxyfera sp. Australia
Saltmarsh sediment clone E000
Singulisphaera acidiphila MOB10
0.10

Figure 4

Table 1. Effect of chelators on the activity of *Hyphomicrobium* VS methanethiol oxidase

Sample	Specific activity [μmol MT min^{-1} mg^{-1} protein]
MTO - no chelator	12.9 ± 1.5
MTO - EDTA treated	5.7 ± 1.1
MTO - EGTA treated	12.5 ± 0.9

Table 2. MT consumption by whole cells and lysates of *R. pomeroyi* DSS-3 wild type and mtoX$^-$ strains (n=3). MT consumption is expressed as nmol MT removed·min^{-1}·mg protein^{-1}

Sample	MT consumption (nmol MT min^{-1}·mg protein^{-1})
Whole cell assays with 0.5mM MT	
Wild type	23.4 ± 1.2
mto X$^-$	1.7 ± 1.4
Cell lysate assays with 0.25 mM MT from cells preincubated in the presence (0.5 mM, +MT) or absence of MT (-MT)	
Wild type -MT	39.2 ± 14.4
Wild type +MT	139.2 ± 25.5
mto X$^-$ -MT	no MT degradation
mto X$^-$ +MT	no MT degradation

Table 3. Analysis of metagenomic datasets for the presence of *mtoX, dmdA* and *recA* homologues

			number of hits			estimate			
Metagenome name (CAMERA project name)	**Biome**	**nr of sequences**	***mtoX***	***dmdA***	***recA***	**% of cells with *mtoX***	**% of cells with *dmdA***	**See footnote**	**CAMERA/imicrobe/NCBI dataset accession**
Antarctica Aquatic Microbial Metagenome	Antarctic Lake	64626265	230	533	504	45.6	106		PRJNA33179
Botany Bay Metagenomes	coastal marine pelagic	15538531	95	551	511	18.6	108		CAM_PROJ_BotanyBay
Western Channel Observatory Microbial Metagenomic Study	coastal marine pelagic	7354754	46	622	623	7.4	100		CAM_PROJ_WesternChannelOMM
Metagenomic Analysis of the North Atlantic Spring Bloom	marine pelagic	6784781	8	268	510	1.6	53		CAM_PROJ_BATS
Microbial Community Genomics at the HOT/ALOHA	marine pelagic	5687251	10	524	534	1.9	98		CAM_PROJ_HOT
North Pacific metagenomes from Monterey Bay to Open Ocean (CalCOFI Line 67)	marine pelagic	5618147	7	4	117	6.0	3		CAM_P_0000828
Monterey Bay transect CN207 sampling sites	coastal marine pelagic	5248980	19	230	514	3.7	45		CAM_P_0000719
Guaymas Basin deep-sea Metagenome	marine deep water	4970673	56	69	340	16.5	20		CAM_P_0000545
Marine Metagenome from Coastal Waters project at Plymouth Marine Laboratory	coastal marine pelagic	1444540	3	79	172	1.7	46		CAM_PROJ_PML
Marine Bacterioplankton Metagenomes	marine pelagic	1314590	1	80	239	0.4	33		CAM_PROJ_Bacterioplankton
Sargasso Sea Bacterioplankton Community	marine pelagic	606285	11	21	91	12.1	23	a	CAM_PROJ_SargassoSea
Sapelo Island Bacterioplankton Metagenome	coastal marine pelagic	354908	9	14	30	30.0	47	b	CAM_PROJ_SapeloIsland
Washington Lake Metagenomes	lacustrine	252427	4	12	75	5.3	16		PRJNA30541
Two HOT Fosmid end depth profiles (HOT179 and HOT186)	marine pelagic	194593	2	20	54	3.7	37		CAM_P_0000828
Waseca County Farm Soil Metagenome	soil	139340	1	4	16	6.3	25	c	CAM_PROJ_FarmSoil
Hydrothermal Vent Metagenome	marine hydrothermal vent	49636	1	0	28	3.6	0		CAM_PROJ_HydrothermalVent

a) The distribution of hits against sampling sites ('control' or 'DMSP') in the Sargasso Sea Bacterioplankton study was as follows: *mtoX* 7 control, 4 DMSP; *dmdA* 4 control, 17 DMSP; *recA* 42 in control, 49 in DMSP

b) Due to the very short reads in Sapelo Island Bacterioplankton Metagenome an e-value cutoff of 1e-05 was used. Hits at that level had a high pairwise similarity, for *dmdA* , there were shorter 100% identity hits with higher e-values than the cut-off used, which were therefore rejected by this approach suggesting this as a stringent cut-off value

c) The *dmdA* hits in the Waseca County Farm Soil study had low maximum pairwise identities between 24 and 29% at the amino acid level.