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# Bacterial Metabolism of C<sub>1</sub> Sulfur Compounds

Rich Boden and Lee P. Hutt

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## Abstract

The metabolism of  $C_1$  organosulfur compounds by the *Bacteria* is important in the biogeochemical cycling of sulfur and carbon and in climate regulation in terms of mediating release of, e.g., dimethylsulfide from the oceans. Herein we review the canon of work on the metabolism of dimethylsulfide, dimethylsulfoxide, dimethylsulfone, methanesulfonate, dimethyldisulfide, and methanethiol, in terms of dissimilation to formaldehyde or carbon dioxide when used as carbon and energy sources by methylotrophs or autotrophs, oxidation to sulfite prior to assimilation as sulfur sources, and use as respiratory terminal electron acceptors. We discuss the enzymology of the metabolism of these compounds and propose a revision to the Enzyme Commission classification to some of them where multiple enzymes are clearly grouped under one name at present. We also provide methodologies for enzyme assays, for the safe handling and quantification of these compounds, and for the synthesis of carbon-14, carbon-11, sulfur-34, and sulfur-34 compounds for use in physiological and ecological studies.

## 1 Introduction and Overview

The diversity of one-carbon ( $C_1$ ) compounds containing sulfur is given in Table 1, including both organic and inorganic examples, and the abbreviations for them used in this chapter. The bacterial metabolism of  $C_1$  organosulfur compounds is largely limited in understanding to methanethiol (MT), dimethylsulfide (DMS), dimethylsulfoxide (DMSO), dimethylsulfone (DMSO<sub>2</sub>), methanesulfonate (MSA), and dimethyldisulfide (DMDS), but as Table 1 shows, there is considerable scope for broadening our understanding with many unstudied compounds with regard to microbiology.

The roles, sources, sinks, and chemistry of the  $C_1$  organosulfur compounds in the environment (atmospheric chemistry, etc.) are touched on in this chapter where important, but for good reviews on core aspects of the subject, the reader should consult Kelly (1996), Keine (1993, 1996), Wood (1996), and Kelly et al. (1993) for short reviews and perspectives that, while over 20 years old, are very useful – for a longer and more up-to-date review on the subject, Schäfer et al. (2010) give much

**Table 1** Diversity and properties of one-carbon (C<sub>1</sub>) sulfur compounds, defined by their absence of carbon-carbon bonds

Compound and abbreviation	Melting point (°C)	Boiling point (°C)	Formula
<i>Organic</i>			
Methanethiol (MT)	-123	+6	CH <sub>3</sub> SH
Methanethial (CH <sub>2</sub> S) <sup>a</sup>	<i>N.D.</i>	<i>N.D.</i>	CH <sub>2</sub> S
1,2,3-trithiane (Thioform)	215	230	(CH <sub>2</sub> ) <sub>3</sub> S <sub>3</sub>
Dimethylsulfide (DMS)	-98	+35	(CH <sub>3</sub> ) <sub>2</sub> S
Dimethylsulfoxide (DMSO)	+19	+189	(CH <sub>3</sub> ) <sub>2</sub> SO
Dimethylsulfone (DMSO <sub>2</sub> )	+109	+248	(CH <sub>3</sub> ) <sub>2</sub> SO <sub>2</sub>
Dimethylsulfide (DMDS)	-85	+110	(CH <sub>3</sub> ) <sub>2</sub> S <sub>2</sub>
Dimethyltrisulfide (DMTS)	-68	+170	(CH <sub>3</sub> ) <sub>2</sub> S <sub>3</sub>
Dimethyltetrasulfide (DMQS)	<i>N.D.</i>	+243	(CH <sub>3</sub> ) <sub>2</sub> S <sub>4</sub>
Dimethylsulfite (DSMO <sub>3</sub> )	<i>N.D.</i>	+126	(CH <sub>3</sub> O) <sub>2</sub> SO
Dimethylsulfate (DMSO <sub>4</sub> )	-32	+188	(CH <sub>3</sub> O) <sub>2</sub> SO <sub>2</sub>
Lenthionine	+60	<i>N.D.</i>	(CH <sub>2</sub> ) <sub>2</sub> S <sub>5</sub>
1,4,2-dithiazole-5-thione	+78	+280	CHNSCSS
1,3,4-oxathiazol-2-one	+36	+226	CHNSCOO
Methanesulfonate (MSA) <sup>b</sup>	-	-	CH <sub>3</sub> SO <sub>3</sub> <sup>-</sup>
Methanesulfinate (MSiA) <sup>b</sup>	-	-	CH <sub>3</sub> SO <sub>2</sub> <sup>-</sup>
Methylmethanesulfonate (MMSA)	+20	+202	CH <sub>3</sub> SO <sub>3</sub> CH <sub>3</sub>
Thiourea	+182		C(NH <sub>2</sub> ) <sub>2</sub> S
<i>Inorganic</i>			
Carbon disulfide (CS <sub>2</sub> )	-112	+46	CS <sub>2</sub>
Carbon monosulfide (CS)	<i>N.D.</i>	<i>N.D.</i>	CS
Carbonyl sulfide (COS)	-139	-50	COS
Thiocarbonate (TC) <sup>b</sup>	-	-	CO <sub>2</sub> S <sup>2-</sup>

<sup>a</sup>Spontaneously oligomerizes into 1,3,5-trithiane

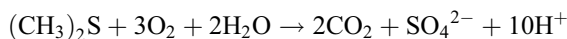
<sup>b</sup>Used in the form of the sodium or potassium salts, which are currently both commercially available for both MSA and MSiA

more depth. Obviously, the reader is directed to Charlson et al. (1987) for the overarching significance of these compounds in the environment, particularly DMS.

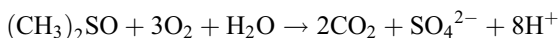
In this chapter, we cover the physiological pathways of C<sub>1</sub> organosulfur compound metabolism, as well as give detail on each enzyme involved and the structure, function, ecology, and evolution thereof. Since the assay methods for these enzymes have not been curated in any other text, we have included them for completeness. We also give consideration to the practicalities and safety of working with C<sub>1</sub> organosulfur compounds, as well as methods for their determination in the microbiology laboratory and methods for the synthesis of stable (carbon-13, sulfur-34) and radiolabeled (carbon-11, carbon-14, sulfur-35) compounds for use in physiological and ecological studies – this is particularly important since some of the key questions and gaps in our understanding can (at present) only be resolved using such methodologies and these labeled compounds are not commercially available, or, where they are, it is for >£20,000 (US\$26,500, €23,000) as a custom synthesis, which

prohibits work for many laboratories. Since these methods are not especially complex, we have included them with hopefully enough detail for the non-chemist to reproduce them without too much difficulty!

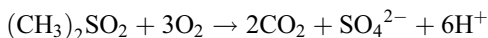
The oxidation of C<sub>1</sub> organosulfur compounds to sulfate or partially to other C<sub>1</sub> organosulfur species is well known in methylotrophic and heterotrophic *Bacteria*, respectively. For illustration of the bioenergetics of these oxidations, those of dimethylsulfide (DMS), dimethylsulfoxide (DMSO), dimethylsulfone (DMSO<sub>2</sub>), methanethiol (MT), and dimethyldisulfide (DMDS) to sulfate and carbon dioxide are given, along with the oxidation of DMS to DMSO found in heterotrophs (cf. Boden et al. 2011a), with their Gibbs energy changes ( $\Delta G^\circ$ ) determined from parameters obtained from Zhdanov (1985), Ross (1985), and Galus (1985), on the basis of 298.15 K, 1 bar, and all solutes at 1 M concentration. For comparison, consider the energy requirement of ATP and NAD(P)H biosynthesis, viz.,  $\text{ADP} + \text{HPO}_4^{2-} \rightarrow \text{ATP} + \text{H}_2\text{O}$ ,  $\Delta G^\circ = +46.1$  kJ/mol ATP produced, and  $\text{NAD}^+ + \text{H}^+ + e^- \rightarrow \text{NADH}$ ,  $\Delta G^\circ = +83.4$  kJ/mol NADH produced (that of NADPH is very similar; thus we have not shown it) – Kelly (1978) made use of these values to ascertain “best case scenario” maxima for ATP and NAD(P)H generation from electron donors, e.g., for the first reaction given, the oxidation of DMS to sulfate and carbon dioxide, thus, could at most yield 23 mol ATP or 12 mol NAD(P)H, which can then be related to the energetic requirements of the various carbon assimilation pathways and viability as an electron donor can be examined – as such, Gibbs energy changes can be a very useful tool when comparing electron donors in a biologically meaningful way.



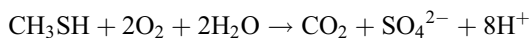
$$\Delta G^\circ = -1,068 \text{ kJ/mol DMS oxidized}$$



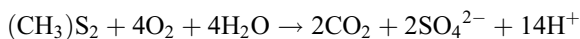
$$\Delta G^\circ = -1,216 \text{ kJ/mol DMSO oxidized}$$



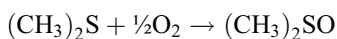
$$\Delta G^\circ = -1,262 \text{ kJ/mol DMSO}_2 \text{ oxidized}$$



$$\Delta G^\circ = -656 \text{ kJ/mol MT oxidized}$$



$$\Delta G^\circ = -1,347 \text{ kJ/mol DMDS oxidized}$$



$$\Delta G^\circ = -89 \text{ kJ/mol DMS oxidized}$$

## 2 Handling, Quantifying, and Synthesizing C<sub>1</sub> Organosulfur Compounds

### 2.1 Quality, Storage, and Disposal

While DMSO<sub>2</sub> is a stable, easy to store and handle compound, DMSO and DMS pose some complexities. Both are commonly contaminated even when “99.9999%” analytical grade – the moment they are opened, they start to oxidize, unless kept under argon. We detail procurement options in the following sections, but DMS and DMSO should be stored at room temperature in non-actinic glass. Neat DMS is best stored in the fume hood itself as even closed bottles sealed in paint cans of vermiculite still smell. Neat DMSO should be stored in a desiccator over silica gel in vacuo, and the bottle of DMSO should contain 4 Å molecular sieve – it is often shipped with it in place, but adding more from time to time is best. DMSO<sub>2</sub> does not require any special storage and solutions are very stable.

For disposal of DMS and glassware contaminated with it, oxidation to DMSO<sub>2</sub> with hypochlorite is used, and Menger and Elrington (1990) report an excellent microemulsion (“the Menger microemulsion”) that accomplishes this oxidation very rapidly, with a reduction in smell, which we highly recommend. DMSO and DMSO<sub>2</sub> do not require any special disposal.

#### 2.1.1 Preparation and Use of Dimethylsulfide Solutions in Culture Work

For all culture work and storage of stock solutions of DMS, gray rubber or butyl rubber septa (vaccine stoppers) should ideally be used. Most work is conducted in septa-sealed containers, and the approximate equivalencies are that we use 30 mL serum bottles where one would normally use Universal bottles and 250 mL or 2,000 mL “QuickFit” Erlenmeyer flasks with a ground-glass socket where a normal Erlenmeyer would normally be used. The latter are sealed using Suba-Seal vaccine stoppers – Sigma-Aldrich sells a range of sizes and colors, and we have found the white ones more resistant to DMS than the traditional red. Suba-Seals are prepared by standing upside-down on a sheet of foil and spraying with three to four coats of Teflon Dry Lubricant Spray (from an electronics supplier) and drying thoroughly before stacking into glass measuring cylinders (they fit inside of one another neatly) and sealing with foil before autoclaving. Alternatively, they can be wrapped individually, with the “inside” end of the packet sealed with a twist and the “outside” end folded closed – this enables the “outside” end to be identified and opened very easily (Dr Ann P. Wood, personal communication). The same coating is applied to serum bottle septa which are autoclaved in beakers. The coating should be replenished after every use. Spent vaccine stoppers are soaked in 1:10 solutions of domestic hypochlorite bleach for 24 h to remove any DMS and are then washed, dried, coated, and re-autoclaved. For culture work done in serum bottles and QuickFit flasks, gas samples for analysis can easily be removed from the headspace with an appropriate syringe – it is important to use only the 19 gauge (1.1 mm outside diameter) needles (orange colored) and “Luer-Lok” syringes, firstly to avoid “coring” the septum and

secondly to avoid pungent solutions leaking out. For gas samples, a tap or “switch” should be between the needle and syringe. Where no gas sampling is needed, we use ground-glass QuickFit stoppers, clipped or wired into place, which are very useful if just bulking up cells.

For agar plates, small pieces (1" by 1") of filter paper (or the discarded “pads” used in between nitrocellulose filters during storage) should be autoclaved in glass Petri dishes. Basal medium is made and poured with no carbon source (a pH indicator such as 1 mL saturated aqueous bromocresol purple per liter is useful in solid media – yellow coloration of the medium indicates full oxidation to sulfate; deeper purple is tetrathionate formation). Once plates have set and been inoculated, a piece of sterile paper is added to the inside of the lid and then in the fume hood, and 5  $\mu$ L DMS is added to the paper. Plates are then stacked in a gas jar, along with an open “DMS bomb” comprising a Bijou tube stuffed with tissue to which 100  $\mu$ L DMS has been added. The jar is then sealed and growth will be observed usually within a week. Owing to long incubation times, some workers add glass Petri dishes containing silica gel to minimize condensation; however, we have found silica gel is often laden with fungal spores and so we do not use it.

For liquid cultures where precision is needed or for analytical work, our protocol is to buy DMS in small bottles from Acros Organics (10–20 mL) and, once opened, dispose of them after a few weeks as DMSO, CS<sub>2</sub>, etc. will start to form. We assume neat DMS is sterile and add 73  $\mu$ L to 120 mL glass serum bottles containing 100.00 mL glass-distilled, sterile water (from a volumetric glass pipette). We then gently fill the headspace with argon from a 20 mL syringe to push out the majority of air and seal with crimp seal with a gray rubber septum (see note above re: septa preparation). After shaking for 24 h, this yields a 10.00 mM stock, which is stable at room temperature in the dark for 2–3 weeks. For most culture work, we add this to 2–4 mM final concentration – other work has been successful at *c.* 50  $\mu$ M concentrations with many repeated additions (Schäfer 2007), but we find this inconvenient as most organisms in our hands tolerate at least 2 mM. For the chemostat, 15 mM DMS in the medium feed is easily prepared by sterilizing a glass Universal and lid in a glass beaker and in the fume hood, adding 22.0 mL DMS into the tube and adding the lid with sterile tongs. It is then taken to the reactor where 20 L of medium have been prepared in a glass carboy with a hard-rubber stopper or, better still, with a QuickFit “udder” flanged lid to minimize rubber components, which can leak DMS over time. The lid of the Universal is unscrewed as aseptically as possible, the lid of the carboy quickly opened, and the Universal “depth charged” by dropping it in. By the time it hits the bottom and the lid comes off, the lid of the carboy will have been tightly sealed again – this takes practice but it is not difficult to master. After equilibrating overnight with stirring, this will yield a 15 mM solution – there may be an “oil slick” of DMS visible on the surface – this will dissolve and come back out of solution in response to temperature and pressure like the FitzRoy storm glass (Bolton and Ray 1992), but since concentration of substrate in the medium feed should be monitored anyway, it will not pose any issues. While high concentrations in the medium feed can be made up to about 100 mM before saturation is reached, the smell can be unbearable if the chemostat malfunctions, so we do not recommend

this! All O-rings and tubing on the chemostat should be Viton<sup>®</sup> and, at the pumps, Viton<sup>®</sup> Flex. The effluent gas should be passed through a Drechsel bottle of a 1:1 solution of domestic bleach containing a few drops of Antifoam 289 or Antifoam 204 (Sigma-Aldrich) – or better still, the Menger microemulsion – and the treated gas then passed out of a window or into the fume hood. A charcoal filter such as the Carbon Cap 150 (Whatman), which also contains a HEPA filter, is also a prudent addition after the scrubbing bottle, but when used with DMS in the off-gas, reconditioning them should be done in an oven in the fume hood or with a vacuum oven, as the smell will be very strong.

### 2.1.2 Preparation and Use of Dimethylsulfoxide Solutions in Culture Work

Anhydrous analytical-grade DMSO is usually shipped in a bottle with a septum containing molecular sieve (4 Å) to keep it dry, which helps to keep it pure – if the DMSO smells of DMS or like oysters, sweetcorn, etc., it should be disposed of, unless the user can be bothered to purify it by refluxing for 4 h over calcium oxide under reduced pressure, drying over calcium hydride, and fractionally distilling at reduced pressure under argon – we just buy another bottle! DMSO can be autoclaved but this tends to break down some of it, so we add it after autoclaving, usually neat as it is sterile as sold – a 50 mL culture requires 71 µL DMSO to give a 20 mM solution. For growth on agar plates, it can be added after autoclaving, but the plates become quite hygroscopic and look and feel “damp,” causing one’s loop to slide around – they should thus be stored in a sealed box. Rather than drying the air and risking cracking the plates, we just flush the box with dry nitrogen or argon to omit water vapor as much as possible.

### 2.1.3 Preparation and Use of Dimethylsulfone Solutions in Culture Work

DMSO<sub>2</sub> solutions are autoclavable and very stable, and no special handling is needed – cultures can be grown in flasks with foam bungs, and DMSO<sub>2</sub> can be added to solid media in the normal way before autoclaving. We usually use 20 mM in liquid cultures but 10 mM is more than enough in agar plates. For the chemostat, we have used 0.1 M DMSO<sub>2</sub> for freshwater/soil organisms (Boden et al. 2011b), but in NaCl-containing media, the 0.1 M H<sub>2</sub>SO<sub>4</sub> produced during growth, and thus the 0.2 mol NaOH added per liter by the reactor to neutralize it, results in a heavy precipitation of large crystals of NaCl and Na<sub>2</sub>SO<sub>4</sub> into the medium; thus for marine work, lower concentrations are clearly necessary.

DMDS is very similar to DMS which is covered in Sect. 2.2.1 in terms of disposal, handling, and storage. MT being a gas and 10 times more toxic than hydrogen cyanide (*LD*<sub>50</sub> rat, 2 h, 3.3 ppm vs. 36 ppm for HCN) presents a much bigger handling issue. Thankfully the odor threshold of MT is 1 ppb versus 5,000 ppb for HCN, and it is very disagreeable; thus the user is far more likely to leave the area in the event of a leak before a toxic concentration can be reached.



### **2.1.4 Preparation and Use of Methanethiol Solutions in Culture Work**

MT dimerizes in the presence of oxygen to form DMDS over time, and so stock solutions must be carefully prepared. In brief, sterile glass-distilled water is deaerated by bubbling with argon for 30 min and is then bubbled with gaseous MT in a serum bottle sealed with a butyl rubber crimp seal using two needles. The off-gas is passed through a Drechsel bottle of domestic bleach, which converts most of the waste gas to MSA, preventing risk – this should be done in a ducted fume hood, and it should be ensured that no one is working on the roof as even the diluted gas is toxic and very pungent. In some jurisdictions, it is important to notify the local authorities and gas companies before carrying this out, since the highly diluted smell of MT carried downwind from a fume hood can lead to fear of a natural gas leak some distance from the source. Once done, the solution is stored under a slight overpressure of argon, at 4 °C, and will be around 0.3 M but should be quantified before use, of course. For the chemostat it is in theory possible to use the gas directly, diluted in air, but the off-gas would be so pungent and potentially deadly in the event of the organism dying that we do not condone this! For cultures, MT is usually added to 1–5 mM, and for the chemostat, concentrations of 10–20 mM are usual, but considering the smell if the organism dies, keeping the concentration in the chemostat low is prudent – the same tubing, carboys, and so on as DMS should be used.

### **2.1.5 Preparation and Use of Methanesulfonate Solutions in Culture Work**

At one time, sodium methanesulfonate was hard to obtain, and thus methanesulfonic acid was titrated against NaOH and the sodium salt produced, but now it is readily obtained, as are other alkanesulfonates. It is water soluble, stable to autoclaving, and handled much like DMSO<sub>2</sub>. In cultures, concentrations of 10–20 mM would usually be used, and in the chemostat, 0.1 M is possible, but higher concentrations are not used for the same reason given for DMSO<sub>2</sub>.

## **2.2 Determination of C<sub>1</sub> Organosulfur Compounds in Cultures**

In the well-equipped and well-funded laboratory with access to gas chromatography (GC) and high-performance liquid chromatography (HPLC), many of these compounds are easy to determine, and the process can even be automated; however, not all laboratories have access to such equipment, and so we have given colorimetric and other methods here also.

### **2.2.1 Dimethylsulfide**

DMS is not difficult to quantify although GC is often thought to be the only way to do it; there are others which we summarize herein, since there are many laboratories worldwide that do not have such instrumentation or wish to use it during fieldwork.

### Gas Chromatography of Culture Headspace

For laboratories with access to a GC with a suitable detector, this is a very good option and can be automated. A 1 m column of 4 mm caliber packed with Porapak™ Q (80/100 MT, Phase Separations Ltd) held at 200 °C with oxygen-free nitrogen at 30 mL/min as the carrier gas can be used with a flame ionization detector (FID), with both it and the injector held at 250 °C. One of us has used this (Boden et al. 2010, 2011a, b) method injecting 100 µL volumes of headspace gas and using a calibration curve to determine the DMS concentration in the headspace. Relative to *n*-hexane, DMS has a retention time of 0.53, where *n*-hexane is typically eluted around 4 min in our hands.

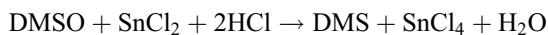
### Iodometric Determination of Culture Extracts

0.2–0.5 mL volume of culture is extracted using 5 mL *iso*-octane (2,2,4-trimethylpentane) in tightly sealed tubes (10 mL serum bottles work very well) by shaking for 5–10 min at room temperature. After allowing the phases to separate, 2 mL of the *iso*-octane fraction is removed and reacted with 2 mL 0.2% (*w/v*) elementary iodine in *iso*-octane, and the absorbance at 300 nm determined using an optical quartz cuvette. This method works well in the 0–6 mM range (Smith 1988; Kanagawa and Kelly 1986) – we have used it in our laboratory and find it a very suitable alternative to gas chromatography for those who do not have access to the equipment or who require more rapid determination as a large number of samples can be analyzed in quite a short period of time.

### 2.2.2 Dimethylsulfoxide

Quantitation of DMSO is reliant on reduction to DMS, which can be quantified using any of the methods in Sect. 2.1.2 – thus in the following sections, we only cover the reduction steps.

#### Reduction to DMS Using Stannous Chloride



0.1 mL culture is placed in a 10 mL serum bottle, and 0.9 mL 0.1 M stannous chloride in concentrated (*viz.*, 37% (*v/v*) and not 32%*v* (*v/v*)) HCl is added and a vaccine stopper rapidly added and crimped into place. Bottles are incubated at 90 °C for 2 h and are then allowed to fully equilibrate to room temperature before determination of DMS. DMSO is determined based on calibration curves made using 0.8–8.0 mM DMSO. There is no interference from DMSO<sub>2</sub>, but if samples contain DMS, it should be determined in one aliquot and another used for this assay and the former subtracted from the latter to give DMSO (Anness 1981).

#### Reduction to DMS Enzymatically

Effectively the same reaction can be done as above but using the enzyme dimethylsulfoxide reductase (EC 1.8.5.3). A recombinant enzyme kit was available from Glycomar Ltd. (UK), but at the time of writing, this does not seem to be on sale.

The procedure has been outlined previously in full by Hatton et al. (1994), using the enzyme from *Rhodobacter capsulatus*.

### 2.2.3 Dimethylsulfone

#### Reduction to MT Using Zinc Amalgam and Vanadium

Meites and Meites (1948) described a strong reducing agent comprising zinc amalgam and vanadyl sulfate that could reduce oxygen to water, and we have found this suitable for DMSO<sub>2</sub> determination in cultures by reduction to methanethiol, which can be determined as outlined elsewhere in this chapter. To 100 mL 0.55 M mercuric chloride solution, 100 g fine zinc powder is added and mixed vigorously for 2 h at room temperature. The zinc amalgam formed is recovered by filtration at the pump and is washed with about a liter of water before suspending in 100 mL 0.1 M vanadyl sulfate solution with rapid stirring. Just before use, concentrated H<sub>2</sub>SO<sub>4</sub> is added with rapid stirring until bubbles of hydrogen are evident on the surface of the amalgam. About 5 mL of this reagent is added to 30 mL serum bottles with 1 mL of the culture supernate. It is sealed with a butyl rubber bung and crimped and incubated at 70 °C for 2 h. After equilibration to room temperature, headspace methanethiol was determined and DMSO<sub>2</sub> calculated from calibration curves made using 1–50 mM DMSO<sub>2</sub> standards. If DMSO and DMS are present, they will also be reduced and so should be determined separately and subtracted.

#### Gas Chromatography of Culture Supernates

Methods for DMSO<sub>2</sub> determination at concentrations found in the laboratory have been reported, and these could be (semi)automated to reduce workload burden versus the other methods outlined above, all of which require GC-FID determination of DMS or MT, whereas the methods in this section are “one stop.”

A GC with mass spectrometer (GC-MS) can be used with a DB-WAX 30 m capillary column of 0.25 mm caliber and 500 nm film thickness (J&W Scientific). The column is held at 40 °C for 1 min and then rises to 230 °C at 10 °C/min. Helium is used as the carrier gas at 1 mL/min with the injector and transport line at 250 °C and 230 °C, respectively. The MS is used in electron impact mode at 70 eV with the ion source at 230 °C and quadrupole analyzer at 150 °C. 1 µL samples are injected (samples (diluted if need be) are prepared by removing biomass by centrifugation and reacting 2 mL supernate with 0.2 mL 17.5% (v/v) HCl and 20 mL 2,2-dimethoxypropane containing 1.19 µg/mL [D<sub>U</sub>]-DMSO as the internal standard; after 30 s, it is vortexed and incubated for 10 min; 27 g Na<sub>2</sub>CO<sub>3</sub> is added and after 10 min centrifuged at 13,000 g for 10 min), and DMSO<sub>2</sub> is detected and quantified by selected ion monitoring at 79 m/z. Solutions of 5–500 µM DMSO<sub>2</sub> can be determined in this way (Takeuchi et al. 2009).

An alternative method uses a 3 m deactivated glass column of 2 mm caliber packed with 3% SP1500 stationary phase on Carboxen B (80/120 MT, Supelco). Helium is used as the carrier gas at 18 mL/min and an electrolytic conductivity detector is used. The column is held at 165 °C, the injector at 220 °C, and detector at 175 °C. 1 µL samples are injected, and solutions containing concentrations of

0.1–23 mM can be determined in this way (a peak for DMSO appears before DMSO<sub>2</sub>), so they can be determined in mixtures in this way (Lang and Brown 1991).

Further GC-FID methods have been reported (Park and Lee 2015; Šatinský et al. 2014) as well as GC-FPD (flame photometric detector, Ogata et al. 1979) methods, all of which could be applied to DMSO<sub>2</sub> determination in cultures.

#### 2.2.4 Methanethiol

MT can be determined from headspaces with the GC method given for DMS, with a relative to *n*-hexane retention of about 0.3.

#### Spectrophotometric Determination

Culture (5.00–20.00 mL) is added to a 25 mL volumetric flask with water to a total volume of 22.0 mL. A reagent solution is prepared by mixing 3 volumes of 0.5% *N,N*-dimethyl-*p*-phenylenediamine hydrochloride in concentrated hydrochloric acid with 1 volume of an iron-nitrate reagent. This is prepared by dissolving 33.8 g ferric chloride hexahydrate in 250 mL glass-distilled water and then adding 250 mL nitric acid, prepared by dissolving 36 mL boiled concentrated nitric acid in water and diluting to volume. 2.0 mL of this reagent solution are added to each flask, which is then mixed well and allowed to incubate for 30 min at 20 °C, before measuring absorbance at 500 nm against a reagent blank (adapted by the authors from the method of Lodge (1988) for measuring thiols in air).

#### 2.2.5 Dimethyldisulfide

DMDS can be determined using GC using the methods given for DMS but has a relative to *n*-hexane retention of about 2.2, which is very long and gives a somewhat splayed peak that takes time to elute – spectrophotometric assay can be more convenient.

#### Spectrophotometric Determination

To 1 mL culture, 2 mL *n*-hexane is added in a 10 mL serum bottle and a vaccine stopper added. After shaking for 10 min at room temperature, the phases are allowed to separate, and the upper phase is removed into an optical quartz 1 cm pathlength cuvette and the absorbance at 260 nm determined versus an *n*-hexane blank.

#### 2.2.6 Methanesulfonate

Many complex methods have been developed for the detection of MSA in seawater at low ppm levels (e.g., Kolatis et al. 1989), which can no doubt be adapted for use in culture work. Otherwise ion chromatography in a carbonate-bicarbonate buffer or NaOH-acetonitrile, with a suppressed conductivity detector, is the best option (Jagota et al. 1995), as simple colorimetric assays are not possible.

### 2.3 Isotopic Labeling for Ecological and Physiological Studies

Radiolabeled or stable-isotope-labeled compounds are useful as tracers in physiological or respirometric work or for stable-isotope-labeled metabolomics or stable-isotope probing (SIP) in molecular ecology. High chemical purity and defined isotopic purity are obviously important, as is ease of preparation. It is important to note that all procedures must be undertaken in a ducted fume hood and that it is prudent to undertake “dry runs” using non-labeled reagents until confident. For radiolabeling, work must be done in accordance with local regulations, and at many institutions now there is a paucity of fume hoods licensed for radioisotope work, and this should be checked, obviously. Disposal using oxidation methods outlined in Sect. 2.1.2 should be observed, and then any radioisotope waste (which would contain [ $^{14}\text{C}$ ]- or [ $^{35}\text{S}$ ]-dimethylsulfone and/or [ $^{35}\text{S}$ ]-sulfate) should be disposed of as liquid waste and should not contain any volatiles at that stage. Decontamination of glassware should be undertaken for 7 days with the radioisotope methods before transferring into 5% (v/v) Decon 90 or equivalent for a further 24 h, and then one should proceed following local rules for disposal of the isotope in solution as dimethylsulfone.

In this section we give the basic semimicromethod for carbon-13 and micro-method for carbon-14, as well as adaptations for sulfur-34 and sulfur-35. Carbon-11 can also be used, which decays via  $\beta^+$  decay and electron capture, with energies of 0.96 MeV or 1.98 MeV, respectively, and a half-life of 20.3 min. [ $^{11}\text{C}$ ]-carbon dioxide can be obtained through proton bombardment of nitrogen-14 at very high specific activities (*c.* 100 GCi/mol) – this necessitates working in very small volumes in a full-sealed reaction system. This is then reduced with lithium aluminum hydride to yield [ $^{11}\text{C}$ ]-methanol, which is reacted with hydroiodic acid to produce the [ $^{11}\text{C}$ ]-methyl iodide required for the synthesis of [ $^{11}\text{C}$ ]-DMS, as detailed by Marazano et al. (1977). Bombardment is done in a cyclotron, which necessitates collaboration for most workers – Moses (1964) and Aronoff (1956) cover useful methodologies for nanoscale radiochemistry that may be of use for carbon-11 work.

Glassware should be washed very thoroughly in advance followed by 24 h in 10% (v/v) nitric acid, washing with glass-distilled water (not “MilliQ,” etc. which sometimes contain low levels of  $\text{C}_1$  compounds that can disrupt reactions) and drying in an oven. Joints should be *lightly* greased with high-vacuum grease.

In any syntheses that use natural abundance sodium sulfide nonahydrate, crystals should be cleaned of sulfur oxyacids before use. Filter paper is mounted in a Büchner funnel in a flask, set up for filtration at the pump. With the vacuum already turned on, the crystals are shaken directly onto the filter paper from a new, unopened jar of  $\geq 99.5\%$  purity  $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ . 100 mL ice-cold glass-distilled water is rapidly poured over the crystals and is immediately removed with the vacuum. Crystals are blotted completely dry with filter paper and are quickly weighed into the reaction vessel. If it is intended to wait before using them, flush the flask with argon, seal with a glass stopper, and store at  $-20\text{ }^\circ\text{C}$ .

### 2.3.1 Synthesis of [<sup>13</sup>C<sub>U</sub>]-Dimethylsulfide

[<sup>13</sup>C<sub>U</sub>]-DMS ( $M = 64.13$  Da) has a density of 0.873 g/mL, melts at  $-98$  °C, and boils at 38 °C. It can be prepared in a single working day from sodium sulfide and [<sup>13</sup>C]-methyl iodide, which is readily available. The cost of synthesis of about 1 g of [<sup>13</sup>C<sub>U</sub>]-DMS in terms of reagents (not including those used in the work-up) is (May 2018) £346 (US\$468, €395), which is significantly more economical than paying a chemical supply company to undertake it as a custom synthesis. The following methodology was developed by one of us (RB) and is adapted from McAllan et al. (1951) and Beerli and Borschberg (1991). Since publication of the original summary method (Neufeld et al. 2008), we have added more efficient purification methods and thus report this here.

6.4 g Na<sub>2</sub>S·9H<sub>2</sub>O (26.4 mmol) is dissolved in 6.4 mL glass-distilled water in a two-neck 25 mL round-bottomed flask in the fume hood, which is stirred vigorously with a glass stirring “flea” and a magnetic stirrer, and is closed with a glass stopper at the top neck. The side neck has a thermometer held in a ground-glass adaptor and which reaches the liquid in the flask. Once fully dissolved, the flask is surrounded with an ice water bath and the contents are stirred until 0 °C. The stopper is replaced with a 10 mL pressure-equalizing addition funnel, to which 2.18 mL (5.00 g, 35 mmol) [<sup>13</sup>C]-methyl iodide is added. The latter is of  $\geq 99\%$  isotopic and chemical purity and is usually supplied with a length of copper wire as a stabilizer – care must be taken not to transfer this to the addition funnel. Over 30 min, the [<sup>13</sup>C]-methyl iodide is added at intervals, taking care to maintain the temperature at 0 °C – a little ammonium chloride can be added to the ice bath if needed, but care should be taken not to cool the flask contents too far below the specified temperature. The addition funnel is removed and replaced with a glass stopper, and the flask contents are stirred very rapidly for 4 h, taking care to keep the reaction temperature as specified. After this, the ice bath is replaced with fresh wet ice to cool the flask contents for 15 min, and the flask is then opened, and 1 mL 2.5 M NaOH is added, with stirring, followed by 1 mL 0.1 M Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, which lyses any excess methyl iodide into methanol and elementary iodine, the latter being reduced to iodide, such that it will not contaminate the product. The flask is moved to a water bath and is topped with a ported Hickman still-head in turn topped with a dry-ice trap that has the lower tubing olive sealed off with Viton<sup>®</sup> tubing and a clamp, so that it can be used as a cold-finger. The trap is charged with dry-ice, and the water bath is heated to maintain the flask contents, with stirring, at 30–35 °C. Over 1–2 h, the Hickman head will fill with essentially pure [<sup>13</sup>C<sub>U</sub>]-DMS, from which it can be removed with a Pasteur pipette into a serum bottle. The theoretical yield is 1.12 g (17.5 mmol, 1.28 mL), and while 88% yields of 0.98 g (15.4 mmol, 1.12 mL) have been reported (Beerli and Borschberg 1991), removal of all of the product from the Hickman head directly is difficult – washing out the head with sterile glass-distilled water will obtain a solution of [<sup>13</sup>C<sub>U</sub>]-DMS which can easily be quantified and stored in a serum bottle under argon and used when more dilute samples are needed, so as not to waste it – we have used this solution in stable-isotope probing ecology studies (Neufeld et al. 2008), for example. Alternatively, a very small volume of water can be used, and liquid product can be separated from it in a separatory funnel, but there are few solvents fully immiscible with the product. If desired, the neat product can be

purified further by shaking with twice with 5 volumes of glass-distilled water and then twice with 5 volumes of 10% (w/v) NaOH, followed again with 5 volumes of water. The product can then be dried with anhydrous calcium chloride and is stored in non-actinic glass under argon at  $-20\text{ }^{\circ}\text{C}$ .

### 2.3.2 Synthesis of [ $^{14}\text{C}_U$ ]-Dimethylsulfide

Carbon-14 has a half-life of 5,730 years and decays by  $\beta$ - emission at 0.156 MeV, which is a “soft beta” emission that is easy to handle. Given the long half-life, both starting materials and the product can be stored indefinitely without appreciable decay, but radiolysis should be taken into account if stored for decades before use – it may be prudent to repurify.

[ $^{14}\text{C}$ ]-methyl iodide at specific activities of 50–60 mCi/mmol in 5 mCi (*c.* 8  $\mu\text{L}$ , users should refer to batch-specific analyses) aliquots in break-seal flasks from American Radiolabeled Chemicals, Inc. These can be opened using a Schlenk line, or a heavy glass stirring “flea” can be put into the neck and the neck sealed with a greased Suba-Seal held in place with a cable tie. The flask is frozen in liquid nitrogen to condense the product and is then shaken to break the glass seal. 2.19 mL (35 mmol, 4.97 g) natural abundance (“[ $^{12}\text{C}$ ]-” hereafter) methyl iodide is injected into the flask, which is allowed to warm to room temperature to equilibrate the label into the liquid, giving a final specific activity of about 0.14 mCi/mmol – if higher specific activities are needed, the synthesis can be scaled down to a micro synthesis, which we describe herein – if using the semimicro synthesis, the method for [ $^{13}\text{C}_U$ ]-DMS can be adapted accordingly. For micro synthesis, 10 mCi [ $^{14}\text{C}$ ]-methyl iodide is dissolved as described above in 7 mmol (0.44 mL) [ $^{12}\text{C}$ ]-methyl iodide to give a specific activity of 1.42 mCi/mmol, thus 2.84 mCi/mmol in the final product of the reaction – given for most physiological work or for microautoradiography, dilution to about 1  $\mu\text{Ci}$  in 5 mM DMS is sufficient; this specific activity is sufficient. If higher specific activities are needed, the isotope should be diluted less and the reaction scaled down further.

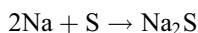
All synthetic work should be done in accordance with local rules. If working with  $>5$  mCi of carbon-14, it is usual to use 10 mm Perspex shielding and detection badges. To a 5 mL conical reaction vial, add 1.28 g  $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$  (5.28 mmol) and 1.28 mL glass-distilled water, and stir with a magnetic, PTFE-coated “spin vane,” with the lid of the vial in place until the entire solid material has dissolved. Meanwhile, withdraw the [ $^{14}\text{C}$ ]-labeled methyl iodide (about 0.45 mL) from the flask using a 0.5 mL glass syringe and a long needle. Transfer the reaction vial into a crystallizing dish on top of the stirrer and fill with an ice water slush. Replace the lid of the vial with one ported for a micro Liebig condenser in the reflux orientation and run cold water through it from the bottom up. Place a thermometer port onto the top and lower a thermometer down into the sulfide solution, taking care to stay above the stirring vane. Prepare a drying column to connect to the top of the condenser with anhydrous calcium chloride, but do not connect it. Maintaining the temperature of the liquid in the vial at  $0\text{ }^{\circ}\text{C}$ , slowly inject the [ $^{14}\text{C}$ ]-methyl iodide using a long needle so that it can be added down the condenser directly into the liquid – this should be added slowly with vigorous stirring, over 30 min, and the syringe and needle then rinsed with the liquid in the reaction vial. Put the drying column onto the

top of the condenser and maintain the system at 0 °C for 4 h, ensuring the condenser stays cold. At the end of the reaction, transfer the reaction vial into a crystallizing dish of water on a stirring hot plate. Add 0.2 mL 2.5 M NaOH and then 0.2 mL 0.1 M Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. Attach a ported microscale Hickman still-head, topped with dry-ice-loaded microscale cold-finger as described in the carbon-13 method, above, to the top of the reaction vial. Load a thermometer down the still-head into the region just above the liquid and heat the water bath until the thermometer reads 35–38 °C – by keeping the temperature at this level for 1–2 h, the product is distilled into the Hickman head. A theoretical yield would be 0.22 g (3.5 mmol, 0.26 mL), with specific activity of 2.84 mCi/mmol – but for the more typical 88% yield, 0.20 g (3.1 mmol, 0.05 mL) at the same specific activity is obtained. The neat product is removed from the Hickman head via the port and is added to a 3 mL reaction vial containing 1.5 mL 10% (w/v) NaOH, which is capped and shaken and then cooled in an ice water to reduce pressure and to allow the layers to separate. The entire mixture is drawn into a filter Pasteur pipette of suitable volume (made by pushing a tiny ball of glass wool down into the tip of a Pasteur pipette using a length of stiff wire), and the heavy (aqueous) phase [<sup>14</sup>C<sub>U</sub>]-DMS is released into a second, clean vial, and the aqueous phase is disposed of as low-level waste. This process is repeated three times and then repeated using water in place of NaOH three more times. The product is then dried over a small amount of anhydrous calcium chloride and is then filtered through a glass Pasteur pipette stuffed with a loose plug of glass wool into the beginning of the constriction and is collected in a 1 mL reaction vial, which is flushed lightly with argon and sealed. Alternatively, it can be cooled to –50 °C and dispensed into glass ampoule blanks which are flushed quickly with argon and rapidly sealed using two blowtorches positioned so that their flames cross. [<sup>14</sup>C<sub>U</sub>]-DMS should be stored at –20 °C under 10 mm thick Perspex – it is worth noting that even in well-sealed reaction vials, the product may escape, so serum bottles or ampoules are preferential. The specific activity can be confirmed by liquid scintillation counting in Ultima Gold™ XR liquid scintillation cocktail (Perkin-Elmer) or, equivalent, in a glass scintillation vial, using 1 µL in 10 mL cocktail, and counting for replicates of 1 h after leaving for 24 h in the dark beforehand. Counts should be normalized against quench curves made using nitromethane as the quench and a suitable carbon-14 standard such as [<sup>14</sup>C<sub>1</sub>]-acetate or [<sup>14</sup>C<sub>U</sub>]-glucose. The cost of this synthesis, *pace* the work-up, and isotopic determination is (May 2018) £2,750 (US\$3,709, €3,118).

### 2.3.3 Synthesis of [<sup>34</sup>S]-Dimethylsulfide

Sulfur-34 is a stable isotope suited to some isotope-fractionation experiments with various groups of the *Bacteria*. It can also be used as a tracer but sensitivity is not as high as with a radioisotope.

Sodium [<sup>34</sup>S]-sulfide is not currently (May 2018) commercially available, but [<sup>34</sup>S<sub>U</sub>]-cyclooctasulfur (i.e., S<sub>8</sub> elementary sulfur) can be obtained from Sigma-Aldrich at 90 at.% as a custom-packaged product. This can be reduced using solvated electrons generated by elementary sodium in anhydrous ammonia:





This procedure is beyond the scope of this chapter and requires a skilled radiochemist, but to prepare the necessary 26.4 mmol sodium sulfide, 0.90 g [ $^{34}\text{S}_\text{U}$ ]-cyclooctasulfur (26.4 mmol S) is reacted with at least 1.21 g (52.8 mmol) elementary sodium – though allowing for loss during work-up etc., it is probably prudent to double this. The carbon-13 synthesis (above) can then be followed using the purified sodium [ $^{34}\text{S}$ ]-sulfide product and [ $^{12}\text{C}$ ]-methyl iodide – this will yield a similar amount in moles of product, but of  $M = 64.00$  Da, at 90 at.%, so the overall  $M$  is 63.81 Da.

### 2.3.4 Synthesis of [ $^{35}\text{S}$ ]-Dimethylsulfide

Sulfur-35 has a half-life of 87.51 days (about 3 months) and decays by  $\beta^-$  emission at 0.167 MeV, which is a “soft beta” emission that is easy to handle. Given the short half-life, both starting materials and the product decay – a large specific activity should be ordered and work conducted immediately and the product used with rapidity. The decay product is chlorine-35, which means decay results in lysis of the product, thus repurification every 3 months is needed, and the decay should be taken into account when calculating how much to use in experiments. After 9 months (three half-lives), the [ $^{35}\text{S}$ ]-DMS should be disposed of as low-level waste.

Sodium [ $^{35}\text{S}$ ]-sulfide of 50–100 mCi/mmol can be obtained from American Radiolabeled Chemicals, Inc., as a solid in 5 mCi batches (*c.* 8–16 mg, users should refer to batch-specific analyses). Using the washing procedures described above, a solution of normal isotopic  $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$  is prepared (6.4 g in 6.4 mL water), and a few mL are added to the vial containing the isotope, which is then sealed and incubated on a roller for 30 min, before tipping into a clean 50 mL round-bottomed flask. The remaining stock solution is then used to wash the entire labeled compound out of the original container – as evidenced with a sensitive Geiger-Müller counter. This can be used with [ $^{12}\text{C}$ ]-methyl iodide per the carbon-13 protocol, above, with quantification done according to that for carbon-14, to produce a theoretical yield of 1.14 g (17.5 mmol), but 88% yields of 1.00 g (15.4 mmol) are more likely in practice – this being of about 0.29 mCi/mmol. If higher specific activities are needed, raise the concentration of the label and/or use the microscale method outlined for carbon-14, above. The cost (May 2018) of preparation of about 1 g of 0.29 mCi/mmol product – excluding work-up and determination costs – is about £1,750 (US\$2,363, €1,984). This is an economical labeling method, albeit a short-lived label; thus a full work plan should be ready for use as soon as it is synthesized.

### 2.3.5 Cognate Preparation of [ $^{13}\text{C}_\text{U}$ ]-, [ $^{14}\text{C}_\text{U}$ ]-, [ $^{34}\text{S}$ ]-, and [ $^{35}\text{S}$ ]-Dimethylsulfoxide

Herein we give procedures based on Beerli and Borschberg (1991) with amounts in mmol rather than g or mL as the various labels would all have to be given, and the user will need to scale anyway to the amount obtained from the previous procedures. Workers thus need to convert to mass, etc. remembering to use the correct  $M$  for the product and not that of the standard unlabeled compound!

After preparation of the relevant DMS and purifying, it should be introduced (23.2 mmol) into a round-bottomed flask containing a glass-coated “flea” (scale-down to a microprocedure accordingly if need be) with three necks, one sealed with

a glass stopper, one with a thermometer capable of working at  $-100\text{ }^{\circ}\text{C}$ , and a third left open. The flask is cooled in dry-ice-acetone slush atop a magnetic stirrer until the contents are  $-78\text{ }^{\circ}\text{C}$ . 13 mmol 96% *m*-chlorobenzoic acid in 35 mL diethyl ether is added via a pressure-equalizing addition funnel slowly (added to the open neck) over 90 min with stirring. The flask contents are brought to room temperature, and 0.018 mmol Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> and 14.0 mmol K<sub>2</sub>CO<sub>3</sub> are added with stirring, which is continued for 16 h. Contents of the flask are filtered at the pump and the filtrate washed with 3 vol dichloromethane. The filtrate is distilled in a Kugelrohr short-path distillation apparatus at 0.13 atm with the cage at  $170\text{ }^{\circ}\text{C}$ , which yields about 10 mmol labeled DMSO, which should be dried over 4 Å molecular sieve and stored under argon at  $-20\text{ }^{\circ}\text{C}$ .

### 2.3.6 Cognate Preparation of [<sup>13</sup>C<sub>U</sub>]-, [<sup>14</sup>C<sub>U</sub>]-, [<sup>34</sup>S]-, and [<sup>35</sup>S]-Dimethylsulfone

Per the previous section, we use mmol herein rather than mass, etc. These procedures are based on McAllan et al. (1951). Suitable methods for recrystallization of very small amounts of (radiolabeled) solid material are found in Aronoff (1956).

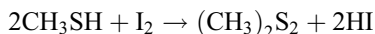
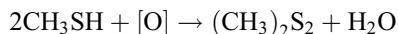
23.2 mmol of the purified, labeled DMS is dissolved in 47 mL glacial acetic acid in a three-neck flask cooled in ice and equipped with a glass-coated “flea,” on a magnetic stirrer. 24 mL “100 volume” hydrogen peroxide is added from a pressure-equalizing addition funnel with rapid stirring, and with the other flask necks closed, slowly over an hour. The flask is removed from the ice bath and a Dimroth or Friedrichs condenser attached to the middle neck (the others are closed), topped with a drying column of calcium chloride. The condenser is cooled with water and the flask incubated at room temperature with stirring for 48 h, before refluxing for 4 h. The flask is transferred to a rotary evaporator and heated under reduced pressure to drive off the acetic acid, leaving behind the solid DMSO<sub>2</sub>, which is recrystallized to constant melting point from diethyl ether, then dried in vacuo, and stored at  $-20\text{ }^{\circ}\text{C}$ .

### 2.3.7 Synthesis of Labeled Methanethiol

The most common means of synthesis of MT is from methanol and hydrogen sulfide using alumina or reduced molybdenum sulfide catalysts. Since [<sup>13</sup>C]-methanol and [<sup>14</sup>C]-methanol are readily available and hydrogen [<sup>35</sup>S]-sulfide and hydrogen [<sup>34</sup>S]-sulfide can both be synthesized from labeled elementary sulfur (summarized in Sect. 2.3.3), the synthesis of all labeled versions of MT alone or with dual labeling is possible; however, the synthetic methods are complex, require fastidious levels of dryness and anoxia, and thus are beyond the means of most biologists – collaboration with a skilled radiochemist is probably needed, who should consult Paskach et al. (2002) and Roberts (2000) for practical details. Kaufmann (2015) details means to synthesize methanethiol from DMS, which could be used for cognate preparation from DMS labeled as described herein.

### 2.3.8 Cognate Preparation of [ $^{13}\text{C}_\text{U}$ ]-, [ $^{14}\text{C}_\text{U}$ ]-, [ $^{34}\text{S}$ ]-, and [ $^{35}\text{S}$ ]-Dimethyldisulfide

Oxidation of MT with air or with elementary iodine will yield DMDS:



Isotopically labeled DMDS can thus be synthesized from any labeled MT, using molecular oxygen in the presence of  $\text{Fe}^{3+}$  or  $\text{Cu}^{2+}$ , or catalyzed by the use of ultraviolet light (Koval' 1994). Hydrogen peroxide, ferric chloride (Field 1977), elementary iodine (Bock and Rittmeyer 1992), and potassium ferricyanide (Koval' 1993) are also widely used in these reactions. Koval' (1994) reviews the methodologies, which should be adapted for use with labeled MT at an appropriate scale.

### 2.3.9 Cognate Preparation of [ $^{13}\text{C}_\text{U}$ ]-, [ $^{14}\text{C}_\text{U}$ ]-, [ $^{34}\text{S}$ ]-, and [ $^{35}\text{S}$ ]-Methanesulfonate

Labeled sodium methanesulfonate can be prepared by neutralizing labeled methanesulfonic acid, which is made by oxidizing appropriately labeled MT using *m*-chloroperoxybenzoic acid, which is described for microscale synthesis by Feil et al. (1988).

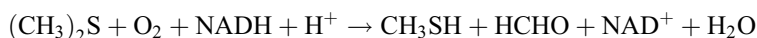
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## 3 Pathways of $\text{C}_1$ Organosulfur Compound Metabolism

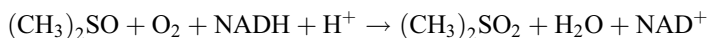
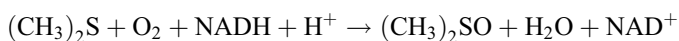
In this section we cover the core metabolic pathways that have been identified for  $\text{C}_1$  organosulfur compounds. These compounds can be assimilated as carbon or sulfur sources –for the former, they are usually dissimilated to formaldehyde or formate (methylotrophs) or carbon dioxide (autotrophs) first; for the latter they are oxidized to dimethylsulfone ( $\text{DMSO}_2$ ) and then to methanesulfonate (MSA) before assimilating at the level of sulfite – this is usually in heterotrophs under sulfur starvation. DMSO can additionally be used as a respiratory terminal electron acceptor in some *Bacteria*. When utilized as a carbon source, the carbon assimilation is via the serine cycle (*Alphaproteobacteria*) or the Quayle pathway (ribulose monophosphate pathway, *Gammaproteobacteria* and *Betaproteobacteria*). In contrast, the autotrophs dissimilate the carbon to carbon dioxide and could assimilate it via any one of the six known bacterial carbon dioxide fixation pathways – though thus far, all known examples use the Calvin-Benson-Bassham (CBB) cycle. There is increasing evidence from sulfur-35 work that the sulfur moiety of DMS can be assimilated into biomass, though given the abundance of sulfate in most environments, it is hard to see a circumstance in which this would be an evolutionary advantage, *pace* in the example of organisms using every possible assimilation source at the same time to beat the competition – “thiovidity,” if one were to need a term to describe using every source of sulfur in an environment at the same time (from Gr. neut. n. *θειον* (*theion*), sulfur, brimstone, transliterated into Latin as *thium*; L. fem. n. *aviditas*, greed; thiovidity, sulfur-greed), rather than switching from sulfate or thiosulfate to

using, e.g., DMS because there was no thio/sulfate left, as is in the case in many organisms in which expression of these enzymes has been demonstrated (Kertesz 1996; van der Ploeg et al. 1996). In this section, we summarize the various catabolic pathways that have been described, with enzymes covered in the next section.

It is worth noting that several DMS monooxygenases, several DSMO monooxygenases, several DMSO reductases, etc. have been purified and characterized. There are clearly distinct classes – the dissimilatory enzymes used to convert these compounds into, e.g., formaldehyde for assimilation as a carbon source and the assimilatory enzymes used when these compounds act as sulfur sources, in which they are ultimately oxidized to sulfite, and the respiratory enzymes as well. We have introduced the convention herein of referring to them as such for clarity, since these enzymes are functionally distinct, e.g., the DMS monooxygenase DmoAB sensu Boden et al. (2011b) and De Bont et al. (1981) catalyzes



and is involved in the use of DMS as a carbon and energy source in *Hyphomicrobium* spp. and *Thiobacillus* spp., whereas the DMS monooxygenase DsoABCDEF of Horinouchi et al. (1997, 1999) catalyzes both the oxidation of DMS and DMSO:



and is involved in the use of DMS and DMSO as sulfur sources in *Acinetobacter* sp. 20B. We discuss the nature of many of these enzymes and whether they are bona fide monooxygenases.

The chemical series of DMS, DMSO, and DSMO<sub>2</sub> in terms of level of oxidation means that their catabolic and sulfur-assimilatory pathways overlap. DMSO can also be used as a terminal electron acceptor, resulting in the formation of DMS – DSMO<sub>2</sub> probably can as well but this has not yet been observed – though we anticipate that it occurs. The S-oxidation of DMS and DMSO to form DMSO and DMSO<sub>2</sub>, respectively, without any oxidation of the carbon occurs in chemolithoheterotrophs such as *Sagittula stellata* (Boden et al. 2011a), whereas full mineralization into inorganic sulfur oxyanions and carbon dioxide occurs in both chemolithoautotrophs such as *Thiobacillus thioparus* (Smith 1988) and in methylotrophs such as *Hyphomicrobium* spp. (Suylen et al. 1996) – but there is a *hinterland* in which “internal” chemolithoheterotrophy occurs (Boden et al. 2010 used this term, but we have since favored “endochemolithoheterotrophy” (Boden and Hutt 2018a, b), in the sense that the electron donor (thiosulfate) is formed endogenously during DMS catabolism, in contrast to “exochemolithoheterotrophy” which would be, e.g., growth on glucose with exogenously supplied thiosulfate). Endochemolithoheterotrophy has been observed in *Methylophaga* spp. (De Zwart 1997; De Zwart et al. 1996; Boden et al. 2010) and in *Xanthobacter* spp. (Padden 1997; Padden et al. 1997, 1998) – the latter are wont to growing endochemolithoheterotrophically and autotrophically

at the same time on some substrates, which represents the maximal use of trophic modes at the same time – we have elsewhere (Boden and Hutt 2018b) proposed the term “voracotrophy” for this circumstance.

While DMS, DMDS, and MT are toxic, with organisms tolerating only concentrations <5 mM or so in usual batch cultures, DMSO, DMSO<sub>2</sub>, and MSA are non-toxic and thus better tolerated – organisms will tolerate DMSO to relatively high concentrations (0.2 M) as it is not very toxic, though interestingly in *Hyphomicrobium denitrificans* WU-K217, it grew best at 75–150 mM, growing poorly at both low and high concentrations – possibly owing to osmotic effects (Murakami-Nitta et al. 2002). *H. denitrificans* WU-K217 was isolated on 20 mM DMSO as the sole carbon and energy source, but the precise source is not clearly reported. This indicates that for DMSO-isolated organisms, testing growth in this range of concentrations is important as it could lead to higher specific growth yields and thus easier work, particularly with protein or mRNA.

### 3.1 De Bont Pathway

De Bont et al. (1981) isolated *Hyphomicrobium* sp. S from enrichment cultures on 13 mM DMSO as the sole carbon source, inoculated with soil, incubated under air at 30 °C. The organism assimilated carbon using the serine cycle and performed a stoichiometric conversion of DMS or DMSO sulfur to sulfate. The pathway of dissimilation was proposed to be



with two formaldehyde molecules being liberated in the second and third steps. Given that the first step occurred in the absence of air but the second did not, an NADH-dependent DMSO reductase and NADH-dependent DMS monooxygenase were implicated for these steps, followed by a methanethiol oxidase (evidenced by hydrogen peroxide production at this step and catalase activity). Formaldehyde could be assimilated via the serine cycle or dissimilated via formaldehyde dehydrogenase and formate dehydrogenase (both yielding NADH) to carbon dioxide.

### 3.2 Visscher-Taylor Pathway

Visscher and Taylor (1993a) studied two strains that they designated as members of the genus *Thiobacillus* and that were isolated in previous studies. Visscher isolated (Visscher et al. 1991) *Thiobacillus* sp. T5 from enrichment cultures on 1.3 mM DMS as the sole carbon and energy source, incubated under air at 25 °C, inoculated with pieces of a microbial mat collected from the West Frisian island of Texel (Tessel) in the North Sea off the Netherlands. Visscher and Taylor (1993b) isolated *Thiobacillus* sp. ASN-1 from enrichment cultures on 0.5 mM DMS as the sole carbon and energy

source, with nitrate as the terminal electron acceptor, inoculated with sediment from a cordgrass (*Spartina* L.) marsh on Sapelo Island, GA, USA.

The two putative *Thiobacillus* isolates (NB: since strain ASN-1 could be grown on multicarbon compounds, it is not a true *Thiobacillus*) were studied and were found to have two different pathways for DMS catabolism. *Thiobacillus* sp. T5 catabolized DMS via the De Bont pathway (see Sect. 3.1), but *Thiobacillus* sp. ASN-1 had an oxygen-independent first step, in which a putative DMS methyltransferase (correctly, a demethylase) transferred a methyl group from DMS onto a carrier, which was then oxidized to formate. The methanethiol remaining was oxidized via a methanethiol oxidase to sulfide, which was then oxidized to sulfate, presumably via the same mechanisms as in the De Bont pathway.

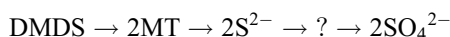
### 3.3 Smith-Kelly Pathway

Smith (1988) examined two strains of *Thiobacillus thioparus* (identities verified by Boden et al. 2012), viz., *Thiobacillus thioparus* E6 and *Thiobacillus thioparus* Tk-m – the latter was also examined by Kanagawa and Kelly (1986). *T. thioparus* E6 (= DSM 5369) was isolated by Smith from an enrichment culture on 0.5 mM dimethyldisulfide (DMDS) under air, inoculated with pond water from Coventry, UK. *T. thioparus* Tk-m (= DSM 5368) was isolated by Kanagawa and Mikami (1989) from a mixed culture (Tk-1), originally obtained from enrichment culture on 19 mM *O,O*-dimethylphosphorodithioate (Kanagawa et al. 1982; Kanagawa and Kelly 1986).

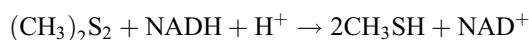
Just as De Bont et al. (1981) had noted that the dissimilation of DMSO and DMS was stepwise, such that the latter was an intermediate of the dissimilation of the former, Smith (1988) observed that dimethyldisulfide (DMDS) and MT share a similar relation, such that the De Bont pathway proceeds:



and the Smith-Kelly pathway proceeds:



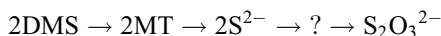
with an NADH-dependent DMDS reductase catalyzing the first reaction:



and the subsequent oxidation of MT proceeds per the De Bont pathway, with the carbon oxidized to carbon dioxide for autotrophic assimilation. Smith and Kelly (1988) proposed that thiosulfate and/or tetrathionate may be intermediates in the oxidation of sulfide to sulfate.

### 3.4 De Zwart-Kuenen Pathway

De Zwart (1997) and De Zwart et al. (1996) isolated *Methylophaga sulfidovorans* LMD 95.210<sup>T</sup> from enrichment cultures inoculated with marine mat samples collected in 1993 from an intertidal estuarine region off Roelshoek, Zeeland, Netherlands. Cultures were supplemented with 1 mM DMS as the sole carbon and energy source. DMS metabolism in *M. sulfidovorans* was similar to the Visscher-Taylor and De Bont pathways, but rather than sulfate being the end product, thiosulfate was formed by unknown means:

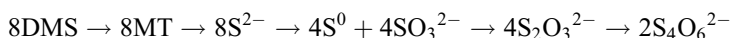


Exochemolithoheterotrophic growth at the expense of exogenous sulfide was observed, in common with other *Methylophaga* spp. (cf. Sect. 3.5).

### 3.5 Boden-Kelly Pathway

Boden et al. (2010) studied the type strain of *Methylophaga thiooxydans* (DSM 22068<sup>T</sup>), which was previously isolated (as *Methylophaga* sp. DMS010) from enrichment cultures inoculated with four pooled, non-axenic cultures of *Emiliania huxleyi* (Lohm.) Hay and Mohler, isolated variously from the Sargasso Sea (obtained in 1987 and 1960), English Channel (1957), and South Pacific (1991). These cultures were grown on 0.05 mM DMS as the sole carbon and energy source (Schäfer 2007).

Similar to the Visscher-Taylor pathway, *M. thiooxydans* has an oxygen-independent oxidation of DMS to MT via a DMS demethylase and then proceeds per the De Bont pathway as far as hydrogen sulfide, which is disproportionated by a sulfite reductase (EC 1.8.1.2) and sulfide-cytochrome *c* reductase (EC 1.8.2.3) to yield sulfite and elementary sulfur, respectively, which then combine chemically via the Suzuki and Silver (1966) reaction to thiosulfate (Boden et al. 2010). This is then oxidized to tetrathionate via a cytochrome *c*-linked thiosulfate dehydrogenase (EC 1.8.2.2) – electrons from this terminal step are used to generate proton-motive force ( $\Delta p$ ) and thus ATP, which was demonstrated in whole cells and was inhibited by the uncoupling agents 2,4-dinitrophenol, carbonyl cyanide *m*-chlorophenylhydrazine (CCCP), and carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazine (FCCP). The DMS oxidation pathway in *M. thiooxydans* is effectively that of *M. sulfidovorans* “plus one” as it has an additional step, and of course the route of thiosulfate formation is resolved:



This study (Boden et al. 2010) was the first report of the formation of a polythionate from an organosulfur precursor and the first report of the coupling of DMS and organosulfur compound cycling in the environment to the cycling of

polythionates and reduced sulfur species, which are then metabolized to sulfate by ubiquitous marine chemolithoautotrophs such as *Thiomicrospira*, *Thiomicrothrix*, *Hydrogenovibrio*, *Guyparkeria*, and *Halothiobacillus* spp. This additional (vs. *Methylophaga sulfidovorans*) terminal step oxidizing thiosulfate to tetrathionate via the ubiquitous cytochrome *c*-linked thiosulfate dehydrogenase (Denkmann et al. 2012) gives the organism an ecological “leg up” in two ways:

### 1. Endochemolithoheterotrophic growth on DMS rather than heterotrophic growth on DMS

i.e., chemolithoheterotrophic growth at the expense of endogenously produced thiosulfate, rather than growth on DMS without energetic gain in this additional step – this leads to higher specific molar growth yields ( $Y_{MAX}$ ) and is thus a clear evolutionary advantage. See also Boden and Hutt (2018a) for a detailed discussion of this.

### 2. Production of a toxic end product

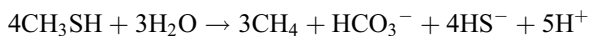
Tetrathionate is toxic to many *Gammaproteobacteria*, for example, and production of it will no doubt inhibit or kill the competition (Palumbo and Alford 1970; Boden et al. 2010).

Evidence from inhibitor studies suggested that the DMS demethylase (EC 2.1.1.x) was corrinoid linked, since it was inhibited strongly by *n*-iodopropionate (cf. Brot and Weissbach 1965) and *n*-butyl iodide in the dark. It was also inhibited by ethyl vinyl sulfide (EVS), mersal, and arsenite – the former is a suicide inhibitor of *S*-adenosylmethionine thioester *S*-methyltransferase, in which it acts as an alternative cofactor, forming the methyl ethyl vinyl sulfonium anion, which binds to amino acids in the active site (Warner and Hoffman 1996). It is known that corrinoid cofactors are bound by vicinal thiol groups in the active site of enzymes (Hogenkamp 1968); thus inhibition by arsenite – which binds strongly to thiol groups – would support a corrinoid demethylase.

## 3.6 Padden-Wood Pathway

*Xanthobacter tagetidis* DSM 11105<sup>T</sup> was isolated from compost surrounding the rootball of a *Tagetes* sp., through enrichment culture on thiophene-2-carboxylate (T2C) as the sole carbon and energy source, and, in common with other *Xanthobacter* spp., grows chemolithoautotrophically on thiosulfate and methylotrophically on methanol, MT, DMS, etc. but only aerobically. When resting cell suspensions were incubated with 5 mM MT under air or under nitrogen, the former produced hydrogen sulfide, in line with the presence of an MT oxidase, but the latter produced carbon dioxide and methane over about 3 h, but no sulfide was detected – all intermediates and end products were determined using GC-MS, so are likely to be valid data – which Padden (1997) determined as



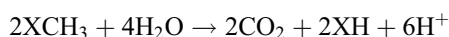
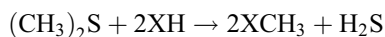


which was similar to pathways used in methanogenic *Archaea* and thus somewhat surprising in one of the *Bacteria* (Zinder and Brock 1978). This hydrolysis of MT in a member of the *Bacteria* has not been observed elsewhere and in some ways does not make biological sense, since in vivo, *Xanthobacter* spp. cannot oxidize methane; thus the methane produced would be wasted, and only the bicarbonate (25% of the MT carbon oxidized) would be assimilated. Padden (1997) felt that this was not a biological step and instead represented something caused by a paucity of oxygen in these incubations and may represent a malfunctioning enzyme; however, methane is produced by some *Alphaproteobacteria* during the acquisition of phosphate from methylphosphonate via a complex pathway of proteins encoded by the *phn* operon, which we summarize (Yao et al. 2016; Grossart et al. 2011; Karl et al. 2008):

$\text{CH}_3\text{PO}_3\text{H}_2 + \text{ATP} + \text{H}_2\text{O} \rightarrow \text{CH}_4 + 5\text{-phospho-}\alpha\text{-D-ribose-1,2-cyclicphosphate} + \text{pyrophosphate} + \text{adenine}$

As such, Padden could have potentially observed an as yet unknown *sulfur* acquisition pathway, in which MT was being oxidized purely for the sulfide moiety.

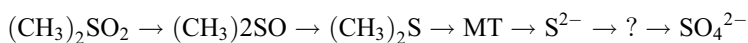
DMS was metabolized in samples incubated without oxygen as far as sulfide; thus it was proposed that MT oxidase does not participate and that a “double” DMS demethylase per that of Visscher and Taylor (1993a, b) but acting on both carbons was used, with all carbon assimilated at the level of carbon dioxide (Padden 1997; Padden et al. 1997, 1998):



Note that the Padden-Wood pathway does not proceed from DMS to MT as is the case in the de Bont pathway, Boden-Kelly pathway, Smith-Kelly pathway, and Visscher-Taylor pathway – but may represent an alternative use of the DMS demethylase for CBB cycle uptake rather than uptake at the level of formaldehyde by the serine cycle or Quayle pathway.

### 3.7 Borodina-Wood pathway

Borodina (2002) and Borodina et al. (2000, 2002) note that the pathway of  $\text{DMSO}_2$  catabolism in *Hyphomicrobium*, *Arthrobacter*, and *Pseudarthrobacter* species examined was the De Bont pathway of DMS catabolism plus a DMSO reductase and a  $\text{DSMO}_2$  reductase:

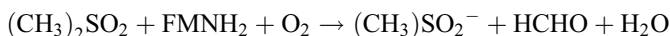


The organisms containing the Borodina-Wood pathway were all soil isolates from garden soil obtained from Radford-Semele, Warwickshire, UK, from enrichment cultures on 20 mM DMSO<sub>2</sub>.

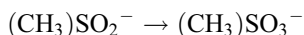
### 3.8 Kino-Wicht pathway

In *Hyphomicrobium* sp. WU-OM3, DMSO<sub>2</sub> was oxidized via unknown steps to methanesulfonate (MSA) – which we presume are the assimilatory DMSO<sub>2</sub> monooxygenase of Wicht (2016), which was then in turn oxidized to sulfate (Kino et al. 2004). DMS was not produced as an intermediate in this pathway.

For sulfur assimilation, the assimilatory DMSO<sub>2</sub> monooxygenase (SnfG, EC 1.14.14.35) is used by *Pseudomonas putida* DS1 under sulfur limitation (Wicht 2016), which is an FMN-dependent monooxygenase similar to DMS monooxygenase (Boden et al. 2011b). SnfG catalyzes the oxidation of DMSO<sub>2</sub> to methanesulfinate (MSiA):



The MSiA ((CH<sub>3</sub>)SO<sub>2</sub><sup>-</sup>) is then chemically oxidized to methanesulfonate (MSA):



The MSA is then further oxidized to formaldehyde and sulfite, the latter of which is assimilated.

In *Hyphomicrobium* spp., which have the ability to assimilate formaldehyde as a carbon and energy source, it is likely that the sulfur assimilation pathway can also lead to carbon assimilation and thus growth – the terminal oxidation of sulfite to sulfate was probably a means of stock control and dealing with too much sulfite, which can be toxic, or the spontaneous oxidation of sulfite to sulfate in the presence of oxygen. The Kino-Wicht pathway is an unusual hybrid situation, which may be entirely a laboratory artifact of a *Hyphomicrobium* sp. growing under sulfate-limited growth, forcing it to use DMSO<sub>2</sub> as a sulfur source and co-assimilating the carbon; alternatively, it could represent an additional assimilation pathway from carbon that is used when both carbon and sulfur are needed: when sulfate is present, carbon is assimilated via the Borodina-Wood pathway, but if sulfate is absent, the Kino-Wicht pathway allows both sulfur and carbon to be assimilated together.

### 3.9 Koch-Dahl Pathway

Very recently, Koch and Dahl (2018) have studied DMS dissimilation in *Hyphomicrobium denitrificans* X<sup>T</sup>. Like other *Hyphomicrobium* spp. (De Bont et al. 1981; Borodina 2002; Borodina et al. 2000, 2002; Boden et al. 2011b), the

organism oxidized DMS to sulfate – as is the fate in the majority of DMS oxidizing *Alphaproteobacteria* that are in culture. The key finding of this study was in confirming the pathway of the downstream oxidation of sulfide produced by MT oxidase. The authors found that a heterodisulfide reductase (Hdr, EC 1.8.98.1) was expressed during methylotrophic growth on DMS and demonstrated that it is involved in the oxidation of sulfide, which proceeded identically to the De Bont pathway until hydrosulfide ( $\text{HS}^-$ ) was produced. This then interacted with hydrosulfite to yield thiosulfate, either fully chemically as observed by Boden et al. (2010) or by direct action of enzymes from the Kelly-Friedrich thiosulfate-oxidizing multienzyme system, viz., SoxXA and SoxB (the precise nature of this coupling was not entirely clear in the paper). Thiosulfate could be oxidized by a cytochrome-linked thiosulfate dehydrogenase (TsdA, Denkmann et al. 2012) to tetrathionate; alternatively, the thiosulfate could be oxidized further. The sulfonate-sulfur of thiosulfate could be oxidized to sulfate by SoxXA and SoxB, while the sulfane-sulfur was bound to SoxYZ, from which it could be transferred via the sulfur carrier protein TusA (Dahl et al. 2011) to the Hdr complex, which oxidized the sulfane-sulfur originating from thiosulfate, now in the form of the “outer” sulfur of a bound disulfane ( $-\text{SS}^-$ ) group on TusA, to hydrosulfite ( $\text{HSO}_3^-$ ), which was recycled to react with more hydrosulfide to yield thiosulfate. The authors also noted that the organism could be grown in the presence of exogenous thiosulfate, as was also the case with other *Hyphomicrobium* spp. and *Methylophaga* spp. – in this work, thiosulfate was oxidized to tetrathionate stoichiometrically, which was not further oxidized – there was no sufficient data to demonstrate the coupling of this to the generation of  $\Delta p$  and thus robustly demonstrate exochemolithoheterotrophy (cf. Boden and Hutt (2018a) for the requirements needed to robustly demonstrate this trait) or to demonstrate that the oxidation of thiosulfate from DMS by TsdA was endochemolithoheterotrophy, but it is likely to be the case. The fate of sulfur from DMS was shown to be sulfate in this study, but since tetrathionate is an alternative fate in theory, it could be that the organism produces the latter as an “overflow” reaction when the pool of thiosulfate in the cell becomes too large or does so only when the energy demands require it. The work of Koch and Dahl (2018) probably provides the end of the De Bont pathway and Borodina-Wood pathways, though it would need demonstrating during growth on DMSO and  $\text{DMSO}_2$ , of course, and demonstrates a further difference between the metabolism of DMS in the *Alphaproteobacteria* from the *Beta*- and *Gammaproteobacteria*.

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## 4 Enzymology of $\text{C}_1$ Organosulfur Compound Metabolism

In this section we have given revised enzyme nomenclature where the evidence is clear that multiple separate enzymes (functionally and evolutionarily) grouped under one umbrella name and/or Enzyme Commission (EC) number at present. Figure 1 accompanies this section and gives a summary of the canon of  $\text{C}_1$  organosulfur research over the last 40 or so years. We give summaries of enzyme assay methodologies where they have been published and are demonstrably not subject to false positives, etc.



ways. In the work of Boden et al. (2011b), the enzyme was purified from cells grown in a DMSO<sub>2</sub>-limited chemostat ( $D = 0.03 \text{ h}^{-1}$ , with 40 mM DMSO<sub>2</sub>) using a mix of gel filtration and affinity chromatography. The purified enzyme was found to be 72 kDa in size, comprising two subunits, DmoA (53 kDa) and DmoB (19 kDa) – the former is an FMNH<sub>2</sub>-dependent DMS monooxygenase and the latter is an NADH-dependent flavin oxidoreductase – an overall structure in common with those of the bacterial luciferase family – the DmoA subunit was shown to be related to the pristinamycin IIA synthase (SnaA, *Streptomyces* sp. DSM 40338), nitrilotriacetate monooxygenase (NtaA, EC 1.14.14.10, *Aminobacter aminovorans*), EDTA monooxygenase (EmoA, EC 1.14.14.10, EC, *Mesorhizobium* sp. BNC1), alkanesulfonate monooxygenase (SsuD, EC 1.14.14.5, *Pseudomonas putida*), and dibenzothio-phenesulfone monooxygenase (DszA, EC 1.14.14.21, *Rhodococcus* sp. IGTS8). The enzyme is stimulated by Fe(II) and Mg(II) ions, and if the metals of the enzyme were depleted with EDTA, Fe(II) and Mg(II) ions restored activity. It was inhibited strongly by umbelliferone, Hg(II), Cd(II), and Pb(II) ions and was wholly dependent on NADH and FMN rather than NADPH or other flavins.

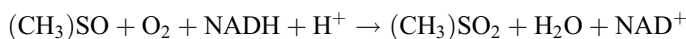
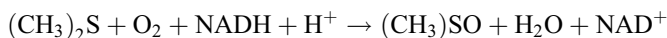
DMS monooxygenase is assayed at the optimum growth temperature of the organism by filling a 1 mL optical quartz cuvette with 0.85 mL 20 mM PIPES-HCl pH 7.4, supplemented with 1 mM NADH, 3  $\mu\text{M}$  FMN, 5  $\mu\text{M}$  dithiothreitol, and 5  $\mu\text{M}$  ferrous ammonium sulfate. 50  $\mu\text{L}$  cell-free extract is added, and the endogenous rate of NADH oxidation is monitored at 340 nm ( $\epsilon = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ ). 100  $\mu\text{L}$  10 mM DMS solution is then added and the rate of DMS-dependent NADH oxidation is determined.

## 4.2 Assimilatory Dimethylsulfide S-Monooxygenase (EC 1.8.1.x)

This enzyme (originally named “DMS monooxygenase,” but this is ambiguous – this enzyme specifically oxidizes the sulfur moiety; thus we have recommended the above name) has been characterized by recombinant expression from *Acinetobacter* sp. 20B (Horinouchi et al. 1997, 1999) and catalyzes the oxidation of DMS to DMSO and also that of DMSO to DMSO<sub>2</sub>, in the assimilation of sulfur from these species – the DMSO<sub>2</sub> monooxygenase of Wicht (2016) discussed later in this chapter is the next step in the assimilation of sulfur:



The enzyme catalyzes the reactions



It is a multicomponent monooxygenase (DsoABCEDF), which has very high sequence homology to the NAD(P)H-linked phenol 2-monooxygenases (EC 1.14.13.7, MopKLMNOP or DmpKLMNOP), which are also termed phenol

hydrolases, which are soluble diiron monooxygenases (SDIMO), a family that includes the soluble methane monooxygenase (sMMO, MmoXYZBCD, EC 1.14.13.25) involved in obligate methanotrophs such as *Methylococcus capsulatus* Bath during copper starvation, or in facultative methanotrophs such as *Methylocella* spp. as their only primary methane oxidizing enzyme (Smith and Murrell 2011). As such, this enzyme has no functional, structural, or evolutionary relationship with the DMS monooxygenase EC 1.14.13.131, and thus we recommend it is moved to a separate EC number (EC 1.8.1.x), with the name “assimilatory dimethylsulfide S-monooxygenase” to better describe physiological function and the action of the enzyme, removing confusion.

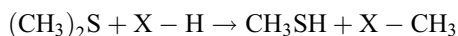
The enzyme is assayed in a discontinuous manner in 10 mL serum bottles containing 3 mL PIPES-HCl pH 7.4 supplemented with 1 mM DMS and 1 mM NAD(P)H, to which 0.1 mL cell-free extract is added. The concentrations of DMS and DMSO are both monitored, with the former disappearing and the latter appearing. This should be done over 5 min or so, as in longer incubations, the DMSO will oxidize to DMSO<sub>2</sub>. For rapidity, the assay could be conducted in the barrel of a water-jacketed glass syringe, with contents ejected into tubes held in ice-salt at intervals to stop the reaction for assay of products and substrate.

It is worth noting that the enzyme can be qualitatively assayed using the oxidation of indole to indigo (Horinouchi et al. 1997), which is not catalyzed by the dissimilatory DMS monooxygenase (Boden, unpublished data). This could be useful in screening organisms or looking for induction during chemostat experiments, in the same way that the oxidation of naphthalene to 1-naphthol is used for sMMO.

It is also worth noting that the oxidation of DMSO to DMSO<sub>2</sub> has been observed in many *Eukarya* (e.g., Williams et al. 1965) and could proceed via an evolutionarily related enzyme, though this has not been studied to date.

### 4.3 Dissimilatory Dimethylsulfide Demethylase (EC 2.1.1.x)

Thus far, this enzyme has not been purified or identified in proteomic or transcriptomic studies (Boden et al. 2011b) and can only be detected as the oxygen-independent oxidation of DMS to MT:



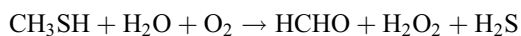
where *X* is some manner of corrinoid cofactor (Boden et al. 2010).

The enzyme is assayed in 10 mL serum bottles with butyl rubber stoppers coated with PTFE. 1 mL cell-free extract is introduced into vials and gassed with argon for 15 min to exclude air. 0.1 mL 10 mM DMS in 0.2 M PIPES-HCl pH 7.4 is added, to give a final concentration of 1 mM DMS. Bottles are incubated with shaking at the optimum growth temperature of the organism, and DMS and MT are both determined at intervals (5 min) using methods given elsewhere in this chapter. A control using boiled cell-free extract or formaldehyde-fixed cell-free extract is important to include, as is one with water in place of biomass.

It is worth noting that an as-yet-hypothetical DMS hydrolase (discussed in Boden et al. 2010) could also give a similar result, but methanol would be detectable as well as MT, and the dissimilation of DMS by living cells could be inhibited by cyclopropanol, which inhibits methanol dehydrogenase (EC 1.1.2.7).

#### 4.4 Methanethiol Oxidase (EC 1.8.3.4)

This enzyme has been purified from organisms including *Thiobacillus thioparus* Tk-m (Gould and Kanagawa 1992), *Hyphomicrobium* sp. EG (Suylen 1988; Suylen et al. 1987), and *Rhodococcus rhodochrous* (Kim et al. 2000).



The enzyme is inhibited by diphenyleioidonium in *M. thiooxydans* (Boden et al. 2010), a common oxidase inhibitor, since it reacts with the hydrogen peroxide produced by the enzyme to form a radical that attacks enzyme cofactors (O'Donnell et al. 1993). It is around 40–50 kDa, varying between organisms, and was inhibited by ammonium ions and by cyanide in *Hyphomicrobium* sp. EG (Suylen 1988) and by solutes in *R. rhodochrous* (Kim et al. 2000). In *Hyphomicrobium methylovorum* VS (Eyice et al. 2018), the monomer, MtoX, was reported at 46.2 kDa, found in homotetramers of 185 kDa. The same study reported the *mtoX* gene in *M. thiooxydans*, *Ruegeria pomeroyi* DSM 15171<sup>T</sup>, and various *T. thioparus* strains, including *T. thioparus* Tk-m from which it was purified previously (Gould and Kanagawa 1992). The MtoX homotetramer contains a tryptophan tryptophanylquinone (TTQ) prosthetic group and four Ca and two Cu atoms per MtoX monomer.

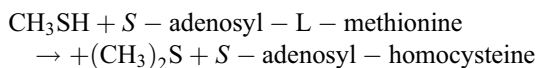
The enzyme can be assayed using discontinuous methods only and by quantification of MT decreasing and (crucially) sulfide, formaldehyde, or hydrogen peroxide increasing. We have found that the Cline (1969) method for sulfide is very effective. Formaldehyde determination by the Hantzsch reaction (Nash 1953) is very prone to interferences in biological systems (Cinti and Thal 1977) – we have observed that even MilliQ deionized water will give a false-positive result, presumably from glycine traces – and in the laboratory in which C<sub>1</sub> compounds and methylotrophs are handled, this is more of an issue. We have made use of 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald<sup>®</sup>), which, in alkaline solution, gives a rapid reaction with formaldehyde to yield a deep purple adduct that is readily determined at 550 nm against calibration curves (Quesenberry and Lee 1996). For determination of hydrogen peroxide, the titanium sulfate (Eisenberg 1943) and 4-aminoantipyridine with phenol (Zhou et al. 2006) methods are both effective colorimetric methods that are easy and rapid.

To assay the enzyme, 10 mL serum bottles containing small glass stirring “fleas” are filled with 5 mL of a 1 mM solution of MT in water, which is thoroughly degassed beforehand (see later in this chapter re: handling of MT) to prevent dimerization. 0.5 mL 0.1 M PIPES-HCl pH 7.4 is added, followed by

0.05–0.10 mL cell-free extract. After rapid stirring on a magnetic stirrer (in a water bath at the optimal growth temperature), samples are withdrawn using a syringe. Keeping the needle in situ and using a tap or valve is convenient. Those samples are rapidly added to serum bottles held in an ice-salt slush at  $-5^{\circ}\text{C}$  to halt biological activity (lids replaced after adding the solution). This is continued at a range of time points over 5–10 min, and then each bottle thawed if necessary and from it aliquots carefully measured for analytical determinations. It is prudent to add zinc acetate to aliquots for sulfide determination as it “fixes” the sulfide in a form that will still react for assay but is no longer labile.

#### 4.5 Methanethiol S-methyltransferase (EC 2.1.1.334)

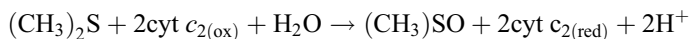
This enzyme has recently been identified in *Pseudomonas* spp. that can assimilate C<sub>1</sub> sulfur compounds – it catalyzes the reaction



producing DMS from MT. It comprises a single subunit, MddA, which is membrane-associated. Homologues of the *mddA* gene were found in many taxa from the *Proteobacteria* to the *Actinobacteria*, and it was found to be extremely abundant in soil metagenome studies (Carrión et al. 2015), which proposed that this is the dominant route of DMS production in soils.

#### 4.6 Dimethylsulfide-Cytochrome c Reductase (EC 1.8.2.4)

Also known as “dimethylsulfide dehydrogenase,” this very well-characterized enzyme was purified from *Rhodovulum sulfidophilum* SH1 and catalyzes



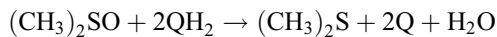
It is a molybdoprotein related to the respiratory DMSO reductases (EC 1.8.5.3), dissimilatory DMSO reductases (EC 1.8.1.x), and trimethylamine *N*-oxide reductases (TorA, EC 1.7.2.3) and comprises the subunits DdhABC (McDevitt et al. 2002a, b). This *Rhodovulum sulfidophilum* strain is reported to grow autotrophically, using the DMS as the electron donor (Hanlon et al. 1994), and is found in the periplasm.



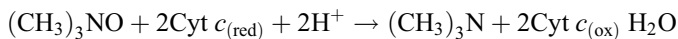
## 4.7 Respiratory Dimethylsulfoxide Reductase (EC 1.8.5.3)

Two DMSO reductases exist, both currently united as EC 1.8.5.3, even though this level of hierarchy relates to quinol donors – which is the case in the respiratory form – and not NAD(P)H per the dissimilatory form.

The respiratory DMSO reductase (EC 1.8.5.3) comprises the DmsABC subunits, wherein DmsA is functionally and structurally very similar to the dissimilatory DMSO reductase (cf. next section); DmsB is an iron-sulfur protein, and DmsC is a transmembrane unit that anchors the enzyme and accepts electrons from the quinol pool, which are then passed through DmsB to DmsA and on to DMSO (Bilous et al. 1988; Bilous and Weiner 1985):



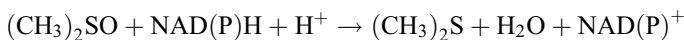
DMSO is thus used as a terminal electron acceptor by members of the *Enterobacteriaceae*, for example, with *Escherichia coli* and so on producing DMS as their respiratory end product. This is an analogous process to the use of trimethylamine *N*-oxide (TMAO) as their terminal electron acceptor and releasing trimethylamine, using trimethylamine *N*-oxide reductase (TorECA, EC 1.7.2.3):



Both TMAO reductase and DMSO reductase are molybdoproteins, with molybdopterin cofactors bound in TorA and DmsA, respectively (Zhang et al. 2008).

## 4.8 Dissimilatory Dimethylsulfoxide Reductase (EC 1.8.1.x)

We propose the separation of this enzyme from EC 1.8.5.3 since it is coupled to NAD(P)H and not to quinols. While the dissimilatory enzyme is comprised of only one subunit versus the respiratory one, it is very similar to the DmsA subunit of the latter. As the DmsC membrane anchor and DmsB iron-sulfur protein are absent in the dissimilatory form, it is soluble and found in the periplasm rather than being membrane bound per the respiratory form. In *Hyphomicrobium* spp. it catalyzes the dissimilation of DMSO to DMS at the expense of NAD(P)H:



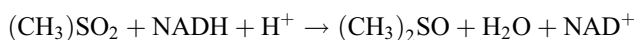
Borodina et al. (2000, 2002) identified DMSO reductase in extracts of DMSO<sub>2</sub>-grown *H. sulfonivorans* S1<sup>T</sup>, *Arthrobacter methylotrophus* DSM 14008<sup>T</sup>, and *Pseudarthrobacter sulfonivorans* DSM 14002<sup>T</sup>, in terms of both enzyme activity and through Western blots using antibodies raised to the respiratory form, which of course shares DmsA.

The enzyme is assayed in a Thunberg cell (Ahlgren 1925) containing 1 mL water, 0.15 mL 1.0 M PIPES-HCl pH 7.6, and 50 μL cell-free extract in the main chamber, which is then deoxygenated by bubbling with argon for 15 min. The side arm is filled

with 0.3 mL 50 mM DMSO and 1.5 mL 2 mM methyl viologen (MV, reduced with dithionite) in 50 mM PIPES-HCl pH 7.6 added to the main chamber. The whole apparatus is then evacuated for 10 min and is then sealed. Endogenous MV oxidation is monitored at 600 nm ( $\epsilon = 1.13 \text{ mM}^{-1} \text{ cm}^{-1}$ ), and then the contents of the side arm are added to the main chamber, and the enzyme activity is monitored for 5–10 min. Activity is expressed in nmol methyl viologen oxidized  $\text{min}^{-1} (\text{mg protein})^{-1}$ .

#### 4.9 Dissimilatory Dimethylsulfone Reductase (EC 1.8.1.17)

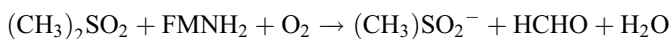
This enzyme has not been purified thus far but has been demonstrated in terms of enzyme activity in *Hyphomicrobium*, *Arthrobacter*, and *Pseudarthrobacter* spp. (Borodina 2002; Borodina et al. 2000, 2002). Much like the DMSO reductase, it catalyzes the NADH-dependent dissimilation of DMSO<sub>2</sub> to DMSO:



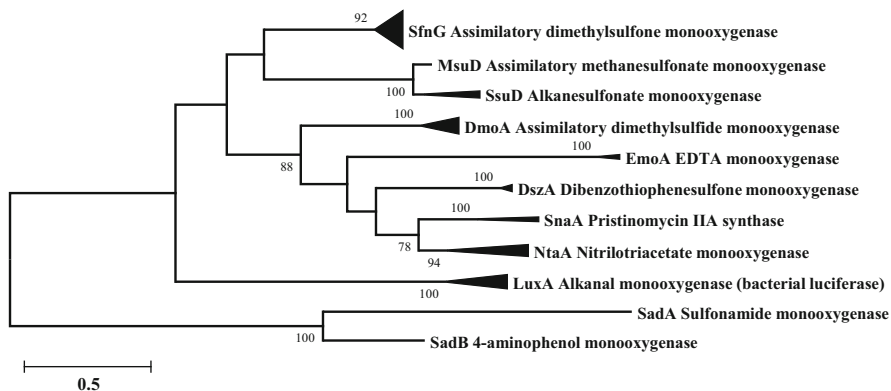
Very little is known about this enzyme, but it can be easily assayed using the same methodology as that we have given for dissimilatory DMSO reductase but using 0.3 mL 50 mM DMSO<sub>2</sub> in lieu of DMSO in the side arm of the Thunberg cell.

#### 4.10 Assimilatory Dimethylsulfone Monooxygenase (EC 1.14.14.35), Assimilatory Methanesulfonate Monooxygenase (EC 1.14.14.34), and Dissimilatory Methanesulfonate Monooxygenase (EC 1.14.13.111)

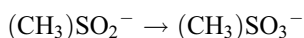
Wicht (2016) demonstrated this enzyme in *Pseudomonas putida* DS1 based on previous work by Endoh et al. (2003a, b, 2005) that uses methylated sulfur species as sulfur sources. The enzyme comprises the catalytic subunit (SnfG, 40.3 kDa), which is an FMN-dependent monooxygenase similar to the large subunit of dissimilatory DMS monooxygenase (DmoAB, EC 14.13.13.1, Boden et al., 2011b), and which is coupled in vitro to an NADH-dependent FMN reductase (SnfF, 20.3 kDa), as an SnfGF heterodimer of 60.6 kDa which is distinct both in size and sequence to the c. 72 kDa DmoAB (Fig. 2). This is part of the sulfur assimilation pathway from DMS, DMSO, DMSO<sub>2</sub>, and methanesulfonate that is used during sulfur starvation in, e.g., *Pseudomonas* spp., with DMS and DMSO being oxidized to DMSO<sub>2</sub> (stepwise) by assimilatory DMS S-monooxygenase (EC 1.8.1.x) covered elsewhere in this chapter. SnfGF then catalyzes the oxidation of DMSO<sub>2</sub> to methanesulfinate (MSiA):



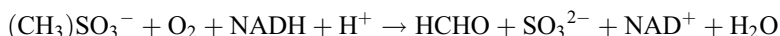
The MSiA ( $(\text{CH}_3)_2\text{SO}_2^-$ ) is then chemically oxidized to methanesulfonate (MSA,  $(\text{CH}_3)_2\text{SO}_3^-$ ):



**Fig. 2** Maximum likelihood tree constructed from amino acyl sequences from large subunits of FMN-dependent two-component monooxygenases, showing the distinction between the different monooxygenase groups – those involved in  $C_1$  organosulfur metabolism and otherwise. Amino acyl sequences were obtained from the GenBank and Integrated Microbial Genomes (IMG) databases and were aligned with MUSCLE (Edgar 2004) and model-tested in MEGA X (Kumar et al. 2018) to find the most suitable model on the basis of the lowest Bayesian information coefficient (BIC). Tree was built using the Le and Gascuel (2008) model with gamma distribution to model evolutionary rate differences (five categories, gamma parameter = 4.8871). 5,000 bootstrap replications were undertaken, and numbers at nodes give the percentage of replicates in which these taxal clusters were found. Tree shown has the highest log likelihood ( $-14237.94$ ) of all replications. Positions at which  $<95\%$  of sequences had a residue were discarded – final analysis used 310 residues. Sulfonamide degradation enzymes SadA and SadB were used as the outgroup



The MSA is then further oxidized to formaldehyde and sulfite by MsuDE, the assimilatory methanesulfonate monooxygenase (EC 1.14.14.34), and the sulfite is assimilated into biomass as sulfur amino acids:

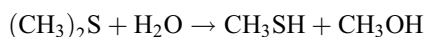


MsuDE is another two-component FMN-dependent monooxygenase and should not be confused with the dissimilatory methanesulfonate monooxygenase (EC 1.14.13.111) found in methylotrophic *Bacteria* such as *Marinosulfonomonas methylotropa*, *Afipia felis*, and *Methylosulfonomonas methylavora* (Baxter et al. 2002). The latter comprises the subunits MsmABCD and is a hybrid enzyme in many senses, with hydroxylase subunits MsmA and MsmB relating to Rieske-centered dioxygenases and electron transfer subunits MsmC and MsmD relating to monooxygenases (De Marco et al. 1999). Recently, *msmA* sequences were obtained from metagenomic and genomic libraries of marine and estuarine environments, including *Filomicrobium* and *Candidatus Puniceispirillum marinum*, further demonstrating the diversity of MSA utilizing methylotrophs (Henriques and De Marco 2015). MsmABCD oxidizes methanesulfonate to sulfite and formaldehyde by the same mechanism given above for MsuDE (Thompson et al. 1995; Kelly et al. 1994).

Assimilatory DMSO<sub>2</sub> monooxygenase and assimilatory MSA monooxygenase can be assayed per the method we give for dissimilatory DMS monooxygenase but using DMSO<sub>2</sub> or MSA, respectively, in lieu of DMS. For dissimilatory MSA monooxygenase, the conventional assay has been to use 5,5'-dithiobis (2-nitrobenzoic acid), Ellman's reagent, to detect the sulfite produced; however, since this cross-reacts with cysteine, cystine, MT, sulfide, etc., we recommend that pararosaniline (Magenta™ O, Basic Red 9) and formaldehyde are used to determine sulfite, modifying the usual enzyme assay method of Higgins et al. (1996) accordingly – we use the method of Yoshiko et al. (1968) for sulfite – though of course ion chromatography or indeed formaldehyde assay could be used (cf. MT oxidase assay methods). It is worth noting that a rapid-screen version has also been published which may be useful in protein purification or in screening clones (Jamshad et al. 2007) or to check if the enzyme is being expressed in chemostat work when switching substrates.

#### 4.11 Putative Dissimilatory Dimethylsulfide Hydrolase

In Boden et al. (2010), it was noted that aside from the dissimilatory DMS demethylase already discussed, a second oxygen-independent route to MT was possible in theory – though it may not be thermodynamically feasible, of course – and would yield methanol rather than formaldehyde:



Were this enzyme (a DMS hydrolase) present, the addition of a methanol dehydrogenase inhibitor such as cyclopropanol would lower the specific growth yield as assimilation would only be possible from the MT-carbon and not the methanol-carbon – as such, its existence in *Methylophaga thiooxydans* was ruled out, but it could be another route of dissimilation. No evidence exists for this “enzyme,” but it remains a possibility.

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## 5 Ecological Theory and Strategies

Ecological studies pertaining to the C<sub>1</sub> organosulfur compounds thus far relate to isolation work and study of functional diversity using SIP, metagenomics, etc. An understanding of the ecological strategies and underpinning evolution is needed since some interesting questions now arise. Canonically, most organisms seem to “prefer” sulfite, sulfate, or thiosulfate as sulfur sources (Le Faou et al. 1990), whereas others “prefer” reduced sulfur such as methionine or dimethylsulfoniopropionate (DMSP) as their sulfur source (Tripp et al. 2008), and, indeed, seemingly cannot use sulfate, etc. Additionally, the assimilation of sulfur from, e.g., alkanesulfonates, taurine, MSA, DMS, etc. has been considered a “sulfur starvation” (Kertesz 1996; van der Ploeg et al. 1996) response, and (thio)sulfate and sulfite

(hereafter “inorganics”) are the “preferred” sulfur sources. In *most* common environments, inorganics will dominate – in the oceans, the standing concentration of sulfate is *c.* 30 mM (Canfield and Farquhar 2009), whereas DMS is *c.* 2 nM (Kloster et al. 2006) and DMSP is *c.* 5 nM (Asher et al. 2017). If we make the assumption of totally equal mixing in a homogenous water column, an organism that can *only* use DMSP as a sulfur source would have to travel enormous distances versus one that uses inorganics in order to be able to assimilate sulfur. In this sense, the latter seems an unlikely strategy, since while it is using a resource with less competition, it is a *scarce* resource *and* is in an environment in which the more-competed-for resource is 600,000 times more abundant.

For the methylotroph growing on DMS and using sulfate as its sulfur source (e.g., *H. sulfonivorans*) versus the heterotroph growing on acetate but using DMS as its sulfur source (e.g., *P. putida*), some ecological and evolutionary considerations can be made. In an oxic, surface soil, the standing concentrations of DMS are up to *c.* 45 nmol/kg in soils (Lomans et al. 1997), sulfate *c.* 50  $\mu$ mol/kg (Reussi Calvo et al. 2009), and acetate *c.* 1 mmol/kg (Sigren et al. 1997). Obviously this is a heterogeneous environment, but even so, the methylotroph is dealing with much lower concentrations of its C source versus the heterotroph – though it will be adapted for oligotrophic life (Hirsch 1986) – but the S source for the methylotroph is much more abundant than that of the heterotroph. As such, one would expect the heterotroph, if the canon of sulfate being the preferred source is correct (Le Faou et al. 1990), to use sulfate and to only consume DMS if the sulfate becomes limiting or if the cell enters a microenvironment low in sulfate. Of course, in nature, the methylotroph will be assimilating acetate and other multicarbon compounds, since it is of a facultative nature.

Should we then take the methylotroph from the last paragraph (which uses the De Bont pathway of DMS dissimilation) and the same environment also and add a second methylotroph that uses the Kino-Wicht pathway, oxidizing DMS ultimately to sulfite to assimilate the sulfur and assimilating the “waste” formaldehyde produced along the way – so acting much the same as the heterotroph from the last paragraph but using DMS for sulfur *and* carbon. In this circumstance, the second organism is using a low-abundance compound as a carbon and sulfur source and thus may have very limited growth – alternatively, it could be assimilating acetate per the heterotroph and only using the DMS for sulfur, but not wasting the formaldehyde as the heterotroph would – we could consider it as growing heterotrophically but with “methylotrophic mopping up” to avoid wasting  $C_1$  moieties formed during sulfur assimilation, perhaps, rather than necessarily growing methylotrophically.

It is hard to rationalize these various strategies in terms of traditional macroecological lifestyle models that have been applied to microbial systems, such as that of *r* and *K* selection (MacArthur and Wilson 1967; Pianka 1970) or, indeed, those that have not been applied to microbial systems, such as plant-focused Grime’s triangle, or universal adaptive strategy theory (Grime 1979). The Silvertown-Franco demographic triangle (Silvertown et al. 1992) may provide a possible means in the future to compare effectively the properties of these organisms on the basis of their

kinetic and biochemical parameters under ecophysiologicaly relevant conditions, such as cellular affinities for substrates (cf. Boden and Hutt 2018b).

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## 6 Research Needs

The canon of work on C<sub>1</sub> sulfur compound assimilation has grown enormously since the days of De Bont et al. (1981) and their seminal study, with the majority of the enzymes now known and purified or recombinantly expressed. There are, however, still holes in the story:

- (a) **The interplay of C and S assimilation.** We need to understand the evolutionary and physiological (regulatory) rationale for the evolution of C<sub>1</sub> organosulfur compound dissimilation for energy and for C assimilation versus oxidation in the “opposite direction” for S assimilation and any thermodynamic or kinetic influences or limitations that make this the case.
- (b) **Enzymology.** The elusive dissimilatory DMS demethylase has still not been purified or identified after 25 years since Visscher and Taylor (1993a) first noted it, and a second “double demethylase” as observed by Padden (1997) could also exist, or a single demethylase could be acting twice. This enzyme needs identification, purification, and characterization. It is possible that the methanethiol S-methyltransferase (EC 2.1.1.334) could act in reverse to catalyze a similar reaction, but it lacks the properties of the enzyme observed by Visscher and Taylor (1993a) and Boden et al. (2010).
- (c) **Sulfur oxidation and energy metabolism.** We already know that in some organisms such as *Methylophaga* spp., *Thiobacillus* spp., and *Hyphomicrobium* spp., the downstream oxidation of sulfide provides reducing equivalents ([H]) and thus proton-motive force ( $\Delta p$ ), which is consumed by ATP synthesis or, in *Thiobacillus* spp., in NAD(P)H generation by reverse electron transport. We need to understand the mechanisms and diversity of these oxidations and the full range of end products – sulfate, thiosulfate, and tetrathionate have been identified thus far – that organisms growing on these compounds produce and why.
- (d) **Ecology and strategism.** Through ecophysiological work, modeling and chemostat competition studies, etc., we need to understand the whys and wherefores of the different S and C uptake pathways, their benefits and ecological relevance, and their evolution.

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