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# Molecular Analysis of DMSOP Cycling in Bacteria

A thesis submitted for the degree of *Master of Science by Research* in Biomolecular Science

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# Abstract

The novel organosulfur compound dimethylsulfoxonium propionate (DMSOP) was recently discovered in diverse marine organisms. It is synthesised by marine bacteria and microalgae from the highly abundant molecule dimethylsulfoniopropionate (DMSP) via an unknown oxidative mechanism. DMSP and its catabolite dimethylsulfide (DMS) have key roles in the global sulfur cycle, signalling, atmospheric chemistry and potentially climate regulation. DMS can either be emitted to the atmosphere or oxidised to dimethylsulfoxide (DMSO) and other products. The discovery of DMSOP has extended the known sulfur cycle as it provides a novel pathway for the production of DMSO through cleavage of the DMSOP compound by marine bacterial DMSP lyases. In addition, this new pathway effectively limits the amount of DMSP available for DMS production via DMSP lysis. Currently there is no information on the role and/or regulation of DMSOP which further emphasises the need to bridge this gap in knowledge about the sulfur cycle.

To study the cycling of this novel compound, this project aims to investigate the molecular mechanism of DMSOP synthesis from DMSP. Furthermore, it will study how DMSOP is catabolised to generate DMSO i.e. do any of the known DMSP lyases also act on DMSOP and if so how efficient are they in doing so?

# CHAPTER 1

# INTRODUCTION

## 1.1 The Sulfur Cycle

Sulfur is the 10th most abundant element on Earth and is an example of one whose transformation and fate are critically dependent upon microbial activities (1). Sulfur's nature (particularly its wide range of stable redox states) mean that it plays important roles in central biochemistry as a structural element, redox centre and a carbon carrier, effectively making it essential for life. Although sulfur is the sixth most abundant element in microbial biomass (1), at any one given time, only very small fractions (about 1% of the dry weight of the organism) are bound in biomass (2). The biogeochemical cycle (henceforth termed as the 'sulfur cycle') that describes the transformation of sulfur is comprised of a multitude of redox reactions. A simplified schematic of the sulfur cycle is shown in Figure 1. On the reductive side, sulfate is

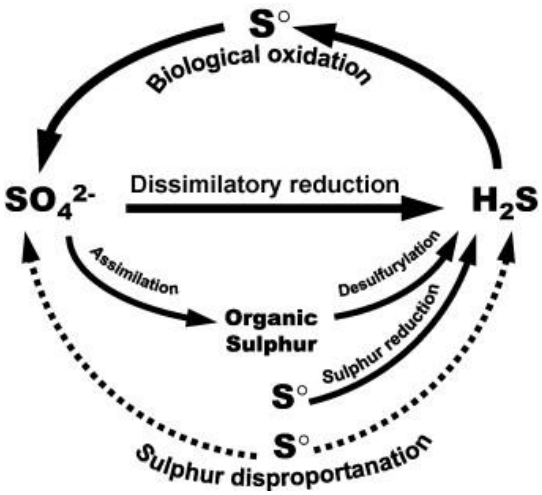


*Figure 1* – **Schematic diagram of the sulfur cycle (1).** Pathways of sulfur transformation include: biological oxidation, dissimilatory reduction, assimilation and desulfurylations, sulfur reduction and sulfur disproportionation.

converted into sulfide as it functions as an electron acceptor in the metabolic pathways used by a wide range of microorganisms (3). On the oxidative side, the reduced sulfur compounds (e.g. sulfide) serve as electron donors for phototrophic or chemolithothrophic bacteria which convert these compounds into elemental sulfur or sulfate (3). Another reaction that occurs within the sulfur cycle (seen in Figure 1) is sulfur disproportionation. This is an energy generating process, carried out by some species of sulfate reducing bacteria and other highly specialised bacteria, in which elemental sulfur or thiosulfate functions as both an electron donor and acceptor; in turn resulting in the simultaneous formation of sulfate and sulfide (3). Within the sulfur cycle, microbial transformations of both inorganic and organic sulfur

compounds have profound effects upon the biosphere and global geochemistry (1). This is due, but not limited, to the formation of compounds such as dimethyl sulfide (DMS), dimethyl sulfoxide (DMSO) and methanethiol.

The ocean is a massive reservoir for sulfur on Earth as it is dissolved as sulfate and sedimentary minerals (2). Sulfur is initially released from the terrestrial environment through oxidative weathering of rocks (4), following this, some of the now environmental sulfates are then assimilated by a variety of microorganisms and plants (5) which are then consumed by animals. These animals will then utilise the biogenic sulfur and return it to the soil as sulfate during death and decomposition (see Figure 2 ).

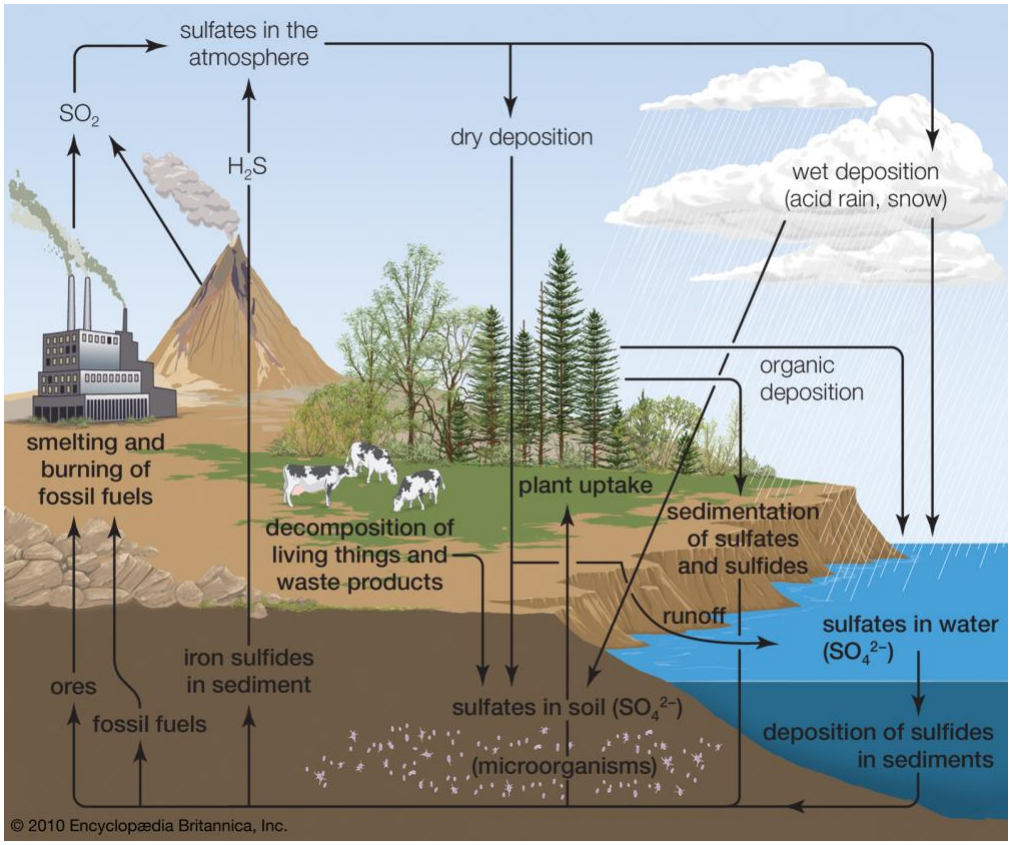


*Figure 2* – **Emissions and cycling of sulfur through terrestrial, atmospheric and marine environments (7).** Sulfur from the terrestrial environment ends up in the oceans as run off and from there can be returned to land through deposits in sediments. Microorganisms and plants can take up sedimented sulfur which is then transferred to animals when consumed. Animals will return the sulfur to the earth through death and decomposition. Sulfur in the ground can be transferred to the atmosphere through erupting and degassing volcanoes as well as burning of fossil fuels. Atmospheric sulfur is then returned to the land and oceans through wet deposition.

This sulfur then ends up in the oceans as 'run off' from the terrestrial environment. Although biological processes and oxidative weathering of continental sulfur remain important to the

global sulfur cycle (4), they have been strongly overtaken by anthropogenic emissions; mainly due to the burning of fossil fuels (2). Atmospheric sulfates produced from anthropogenic emissions, as well as from erupting and non-erupting degassing volcanoes (6), also end up in the oceans as a wet deposition (through acid rain and snow) and through subsequent dry deposition and run off (see Figure 2). The 'acid rain' component of the wet deposition is a significant problem in the environment as it can kill sensitive aquatic organisms but also can degraded and damage buildings and monuments made from marble or stone (7). Within the ocean, sulfate is assimilated into cysteine, methionine and dimethylsulfoniopropionate (DMSP) via a methionine enzymatic biotransformation (8) (see Figure 3) and is released back into the atmosphere as DMS (9).

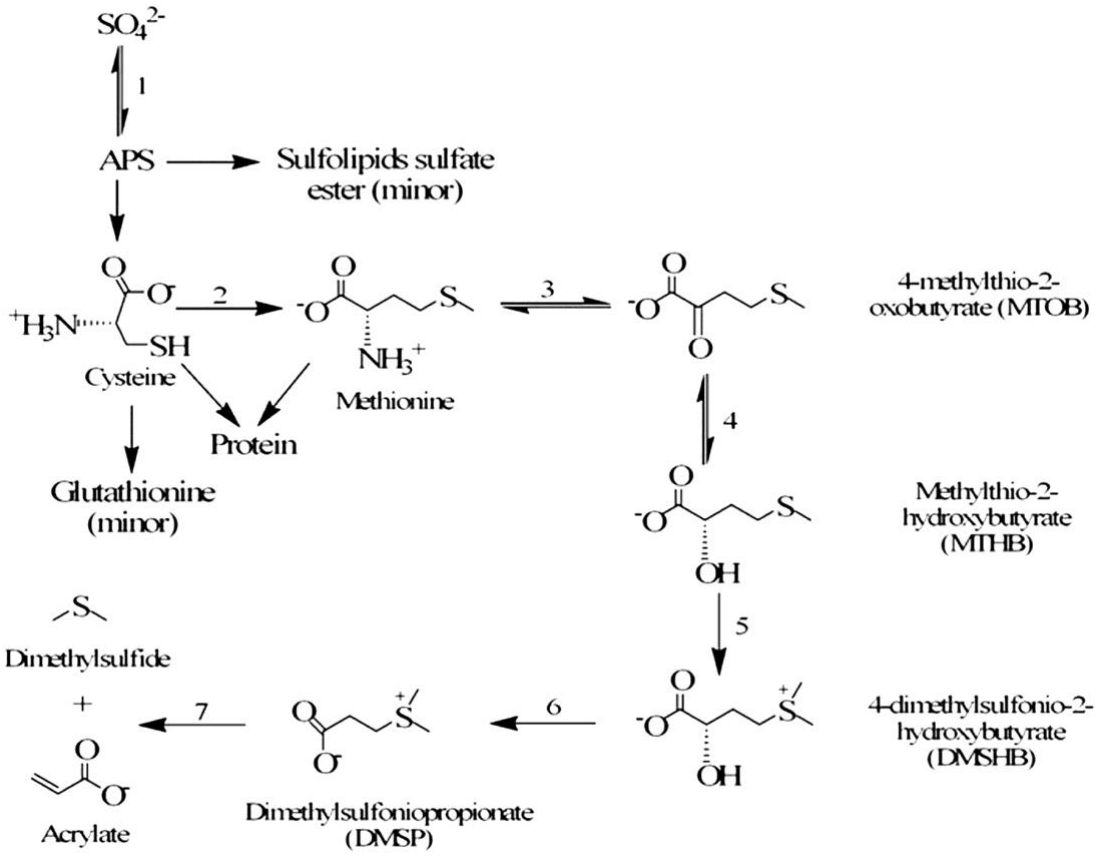


*Figure 3* –**The biosynthetic pathway of DMSP/DMS by marine algae through assimilatory sulfate reduction, via methionine enzymatic biotransformation.** The reaction processes involved in seawater sulfate assimilation by marine algae species are as follows: [1] carrier-bound sulfate reduction, [2] transsulfuration to methionine biosynthesis, [3] transamination, [4] reduction, [5] methylation, [6] oxidative decarboxylation, and [7] cleavage/degradation. (8)

## 1.2 Dimethylsulfoniopropionate (DMSP)

Dimethylsulfoniopropionate (DMSP) is a vastly abundant organosulfur molecule first isolated in 1948 from the red algae *Polysiphonia fastigiata* (10) and is ubiquitous in marine surface waters (11). Approximately $10^9$ tonnes of DMSP is produced annually by marine organisms such as micro and macro-algae, some species of halophytic plants (12), corals (13), as well as phytoplankton of classes Dinophyceae (dinoflagellates) and Prymnesiophycaea (coccolithophores) (14). More recently, it was discovered that marine heterotrophic bacteria produce DMSP as well (15). The precise function of DMSP within the organisms that produce it is largely unknown however several anti-stress roles have been suggested as follows. Cosquer *et al* found that a culture of *Escherichia coli* incubated with nanomolar concentrations of DMSP had increased salt tolerance (16). DMSP shares structural similarity and properties to its nitrogen analogue glycine betaine (GBT) and as such under nitrogen limiting conditions, it can act as an intracellular osmolyte – replacing GBT (9). At low temperatures, several species of algae have been shown to have an increased rate of DMSP production, suggesting that it can act as a cryoprotectant to stabilize proteins and protect from cold influenced denaturation (17). Within algae, DMSP (and its catabolites), has also been suggested to act as an antioxidant. Furthermore, a Mariana Trench study by Zheng *et* al showed DMSP has a physiological function for protecting bacterial producers against hydrostatic pressure (18). Levels of DMSP (and its breakdown products – DMS, acrylate, dimethylsulfoxide (DMSO) and methane sulfonic acid) were shown to increase in abundance and readily scavenge hydroxyl radicals as well as other reactive oxygen species in response to oxidative stressors (19).

DMSP is also of environmental importance as it is the major precursor molecule for the volatile, climate active gas DMS; produced through DMSP lyase enzymes. DMS is the major source of sulfur emitted from the seas to the atmosphere where it is oxidised to sulfate, sulfur dioxide, methanesulfonic acid as well as other products that can act as cloud condensation nuclei (CCN) (see Figure 4) (20). The research of Chin *et al* has found that DMS oxidation accounts for over 80% of $SO_4^{2-}$ in the tropical upper troposphere and even though the total flux of DMS is less than half of that of the anthropogenic sulfur emissions, the longer residence time of DMS oxidation products in the atmosphere as well as the large global

production of DMS results in a vast contribution to atmospheric sulfur levels and thus a large effect on the global climate (21). The sulfur released into the atmosphere as DMS is returned to the land through precipitation, thus completing the sulfur cycle.

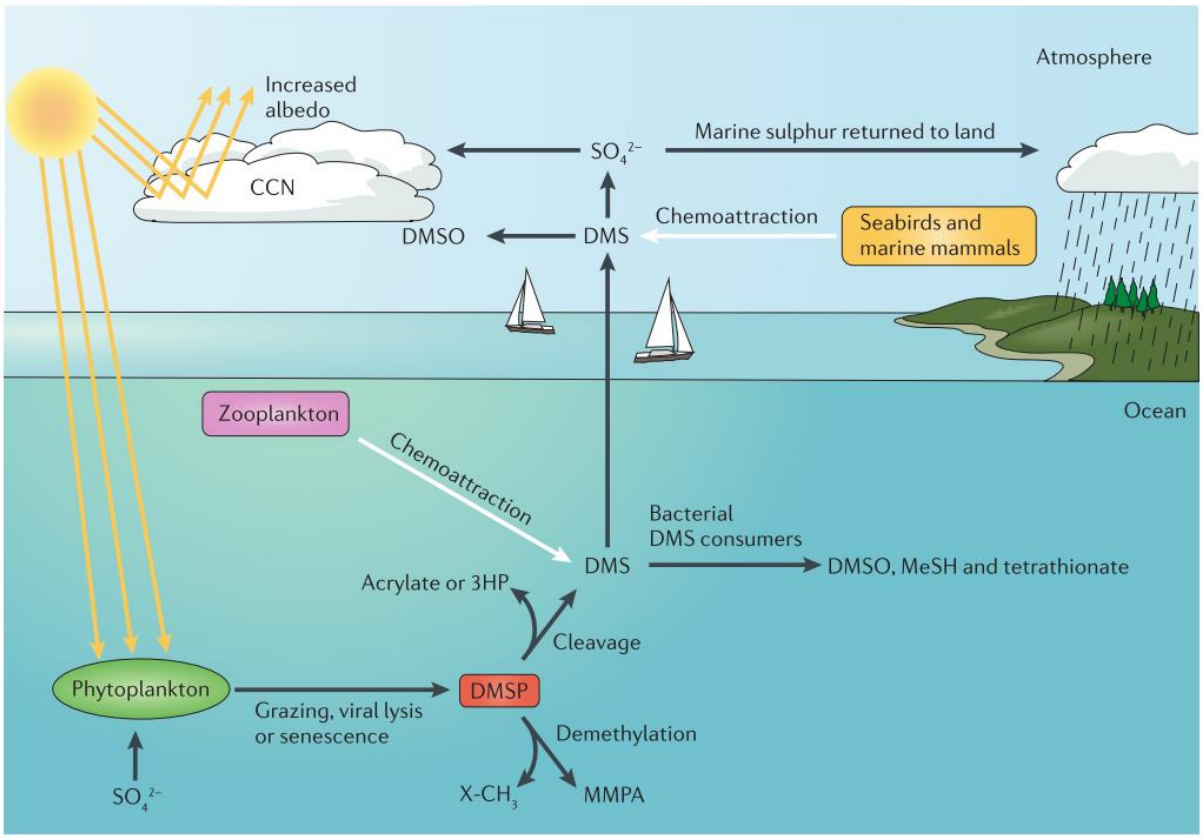


*Figure 4* – **DMSP within the environment** (18). DMSP is released into the marine environment from phytoplankton due to cellular lysis and is then catabolized by heterotrophic bacteria. This can be done in two ways, a demethylation pathway yielding methylmercaptopropionate (MMPA), or a lysis pathway yielding dimethylsulfide (DMS). DMS acts as a chemoattractant for many organisms but is also the precursor to sulfate molecules that can act as cloud condensation nuclei (CCN). Sulfur released into the atmosphere through DMS is returned to the land through precipitation.

## 1.3 The 'CLAW' Hypothesis

The relationship between DMS and global climate is theorised in the 'CLAW' hypothesis (named for its authors) (22) which states that increased levels of solar radiation and thus higher planetary temperatures encourage the growth of DMSP-producing marine phytoplankton and therefore the total levels of DMSP increase. As a result, there is an increased amount of atmospheric DMS, due to DMSP lysis, which increases the abundance of CCN that act to reflect solar radiation, decreasing global temperatures, growth of marine phytoplankton and DMSP levels, generating a negative feedback loop.

In 2011, Quinn *et al* (23) published a paper questioning the reality of 'CLAW' which states that over the last 20 years of research, sources of CCN to the remote marine boundary layer (MBL) are more complex than was first recognised in 'CLAW'. They state that bubble bursting at the surface of the ocean is the major sources of aerosol mass in the MBL and that it introduces both organic and inorganic components of sea water to the atmosphere. These inorganic components are derived from sea salt and the organic mainly from phytoplankton. Thus the concentrations of CNN in the remote MBL are the result of both emissions of sea salt and organics within sea spray as well as DMS-derived particulates in the upper troposphere (23).

## 1.4 Synthesis of DMSP

First purified from the marine red algae *Polysiphonia fastigiata* and *Prevotella nigrescens* in 1948 (24), DMSP was thought to be synthesised exclusively from marine eukaryotes including: algae and single celled marine phytoplankton (9), corals (25) and many angiosperms (10). Interestingly, the angiosperms *Wollastonia biflora* and *Spartina sp.* (26) (both DMSP producers) are terrestrial which makes them somewhat of an exception to other marine eukaryotes. More recently, the work of Curson *et al* (15) in 2017 found that the alphaproteobacterium *Labrenzia aggregata* LZB033, isolated from the East China Sea, was also able to produce DMSP, further deepening our understand of how widespread the ability to synthesise DMSP really is.

Currently, there are three known pathways for the synthesis of DMSP; the methylation pathway - used by angiosperms and as recently shown, also by some heterotrophic bacteria, the transamination pathway - used by heterotrophic bacteria, corals and algae, and the decarboxylation pathway – only studied in a single dinoflagellate (see Figure 5) (27). All of these pathways begin with methionine before diverging into three separate routes – depending on the organism.

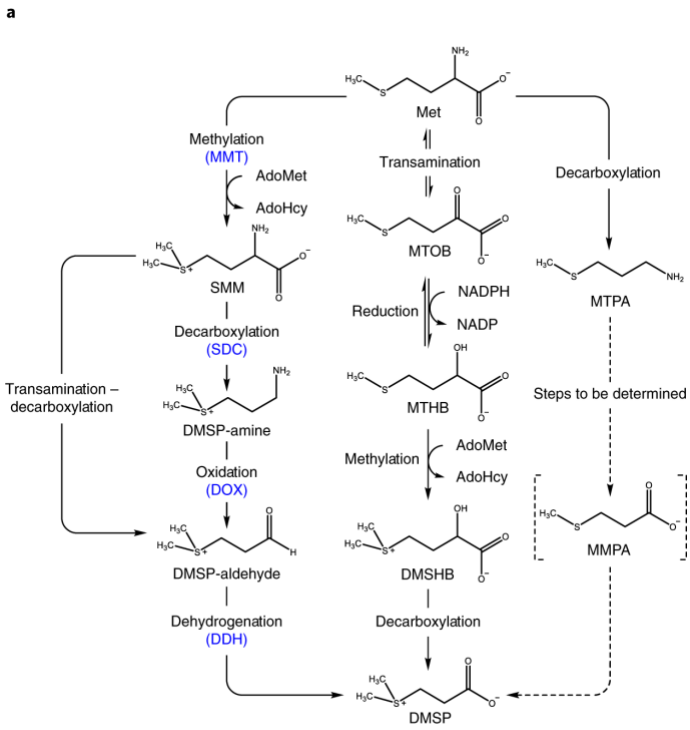


*Figure 5* – **DMSP synthetic pathways; methylation (left), transamination (middle), decarboxylation (right).** The methylation pathway is used by organisms that contain the *mmtN* synthesis gene, the transamination pathway is used by organism that contain the *dsyB* synthesis gene. The single dinoflagellate *Crypthecodinium* is known to use the decarboxylation pathway – the dotted line represents a suggested (but as yet unconfirmed) pathway.

### 1.4.1 Methylation pathway

The ability to synthesis DMSP is not widespread among higher plants only *Wollastonia biflora* (Compositae)*, Spartina sp.* and *Saccharum* (Gramineae) (9)) have evolved the ability to do so. Many of the proposed functions of DMSP within single celled organisms (cryoprotectant, osmolyte etc) would not appear to be as beneficial to higher plant producers. DMSP production from higher plants is thought of as a singular 'Methylation' pathway with many of the produced intermediates being that same between the Compositae and the Gamineae however, the central steps differ enough to be considered significant (28).

The initial step (see Figure 5) involves the methylation of *L*-methionine to *S*-methylmethionine (SMM) via the methyl donor AdoMet (29). This reaction is catalysed by the enzyme *S*-adenosylmethionine:methionine *S*-methyltransferase (MMT) (30) and takes place in the cytosol after which the SMM is transported into the chloroplast for the rest of the

pathway (31). Following the production of SMM, the methylation pathway diverges between the Compositase and Gamineae. In Compositae, the SMM is converted into DMSP-aldehyde via a pyridoxal 5'-phosphate (PLP) dependent transamination-decarboxylation reaction where the amino group is transferred to 2-oxoglutarate with the $CO_2$ being released through decarboxylation (28). Due to this appearing to occur as a two-step process, it is assumed that an unstable intermediate is formed before the DMSP-aldehyde but has yet to be isolated (28).

Within Gramineae, the removal of the amino and carboxyl groups from SMM to convert it to DMSP-aldehyde is done using a different method. An additional intermediate (DMSP-amine) has been identified during this process in Gramineae (32). Initially, SMM undergoes a PLP-catalysed decarboxylation reaction forming DMSP-amine (33) which is then converted to DMSP-aldehyde via the removal of its amino group. This reaction is transamination as before but an oxidative deamination that is not dependent on PLP, potentially due to an oxygen-dependent amine oxidase enzyme (28). Following the formation of DMSP-aldehyde, the pathways converge again and DMSP is produced through dehydrogenation.

Recent work by Williams *et al* (27) in 2019 found that the salt marsh bacterial isolate *Novosphingobium* BW1 produced DMSP via the methylation pathway – previously thought only to be used by plants. *Novosphingobium* BW1 contains the *mmtN* (Met S-methyltransferase) DMSP synthesis gene which converts methionine to SMM. This discovery has furthered our understanding of how widespread the methylation pathway may be as it no longer is confined to higher plants.

### 1.4.2 Transamination pathway

The transamination pathway (see Figure 5) is most widely used by marine algae, both red and green, as well as diatoms. As the name suggests, the first step is the reversible transamination of methionine to form 4-methylthio-2-oxobutyrate (MTOB) (34) (as opposed to methylation to form SMM) in which an amine group is transferred from methionine to a keto acid (2-oxoglutarate) (35). Once the MTOB is formed, it is reduced to 2-hydroxy-4-methylthio butanoic acid (MTHB) via an NAD(P)H-linked reductase (35). Following this, MTHB is methylated on the sulfur molecule by the methyl group donor, S-Adenosyl-L-methionine

(SAM), to produce DMSHB (35). The production of DMSHB from MTHB is thought to be the committing step in the transamination pathway as, unlike the previous steps, is irreversible (35). The DMSHB intermediate is also only found in the context of DMSP synthesis whereas the previous steps are also found in species that are unable to produce DMSP. The work of Ito *et al* showed that levels of DMSHB synthesis in algae are directly linked to the regulation of DMSP production in the organism (36) which indicates that the DMSHB molecule is a key intermediate for DMSP production via transamination. The last step in the pathway sees the conversion of DMSHB to DMSP via an oxidative decarboxylation, releasing $CO_2$ (28).

The transamination pathway is thought to be the most widespread of the three described DMSP synthetic pathways. It has been reported to be utilised not only in the algae and diatoms mentioned previously, but also in the corals *Acropora millepora* and *Acropora tenuis* (25) and more recently in some marine bacteria (15). The work of Curson *et al* in 2018 on marine bacterium *Labrenzia aggregata* resulted in the identification of the first DMSP synthesis gene, *dsyB*, which is a methyltransferase that converts 2-hydroxy-4-(methylthio) butatonic acid (MTHB) into 4-(dimethylsulfonio)-2-hydroxy-butanoate (DMSHB) (15). A year later in 2018, Curson *et al* elucidated the functional *dsyB* homologue, *DSYB,* in many phytoplankton and corals (37). DSYB, localised in the chloroplasts and mitochondria (sites of DMSP synthesis) in haptophyte *Prymnesium parvum*, is a methylthiohydroxybutryate methyltransferase enzyme (37). Additionally, the work of Kageyama *et al* found another DMSP synthesis gene encoding TpMMT in diatom *Thalassiosira pseudonana* which catalyses the production of DMASHB from MTHB (38).

### 1.4.3 Decarboxylation pathway

Of the decarboxylation pathway, little is known. The pathway was first observed in the dinoflagellate species *Crypthecodinium cohnii* (39). Dinoflagellates are some of the highest DMSP producers in the marine environment with some species containing intracellular concentrations of up to 0.5M DMSP (40). These organisms form large algal blooms which results in significant amounts of DMSP being released into the marine environment. However, only one intermediate and the enzyme responsible have been identified. It hypothesised that

within *C. cohnii,* methionine is converted into 3-methylthiopropylamine (MTPA) by an L-methionine decarboxylase (39) (see Figure 5). The rest of this pathway is yet to be determined however it is predicted that only one other intermediate is missing, either 3-methylmercaptopropionate (MMPA) (28) or 3-methylthiopropionate (MTP) (39).

## 1.5 DMSP Catabolism

Environmental importance notwithstanding, DMSP is also important due to its potential to act as a source of fixed carbon, sulfur and energy for marine microorganisms (12). As a result, DMSP is an invaluable compound in the marine microbial food web and is unsurprisingly catabolised for its raw components by a vast number of bacterial communities. Upon its release into the marine environment (usually through cellular lysis caused by grazing zooplankton, senescence and viral infection (11)) DMSP supports between 1-13% of the microbial carbon demand in surface waters as well as being a major source of sulfur for these organisms as well (41). As the uptake of DMSP is not simple and can require energy, the benefits of it must be significant for organisms to utilise it as a source of nutrients. Catabolism of DMSP occurs through two main pathways; demethylation and lysis which provide carbon and energy to the cell (42).

### 1.5.1 DMSP Demethylation

The most prevalent route of DMSP catabolism is through demethylation. The demethylation pathway begins as the name suggests with the removal of a methyl group from DMSP by a tetrahydrofolate (THF)-dependent enzyme, DmdA to form 5-methyl-THF and methylmercaptopropionate (MMPA) (see Figure 6) (43). The gene encoding DmdA was first discovered by Howard *et al* in 2006 (44) and has a very strict substrate specificity which suggests its sole purpose is in DMSP catabolism (11). The presence of THF is also essential for this enzyme as it acts as the methyl acceptor for the reaction, the then Me-THF can become a methyl group donor in subsequent reactions such as methionine synthesis (11).

The resulting MMPA molecule is demethiolated as the catabolic pathway proceeds. For a long time, catabolism of MMPA was unknown and thought to follow several different routes however in 2011, Reisch *et al* found that demethiolation resulting in MeSH and $CO_2$ release is the one used in at least members of the Roseobacter and SAR11 clades (45). Roseobacter

model organism *Ruegeria pomeroyi* DSS-3 was found to make the MMPA-CoA thioester intermediate, the reaction of which is catalysed by methylmercaptopropionyl-CoA ligase (DmdB) and requires a molecule of ATP (45). The MMPA then undergoes a dehydrogenation reaction, catalysed by the dehydrogenase DmdC, and forms a double bond to make MTA-CoA. This stage in catabolism is essential to the breakdown of MMPA as mutant *R. pomeroyi* who no longer have the ability to undergo the dehydrogenation step were unable to grow on MMPA as sole carbon source (45). The conclusive steps of the demethylation pathway are catalysed by the crotonase family member DmdD (46). Initially, DmdA catalyses the hydration of MTA-CoA followed by the immediate release of MeSH to form the malonate semialdehyde-CoA (Mas-CoA). This intermediate then undergoes a hydrolysis reaction to release the CoA moiety (46). The subsequent acetylaldehyde molecule is able to be converted to acetate via an acetaldehyde dehydrogenase (11).

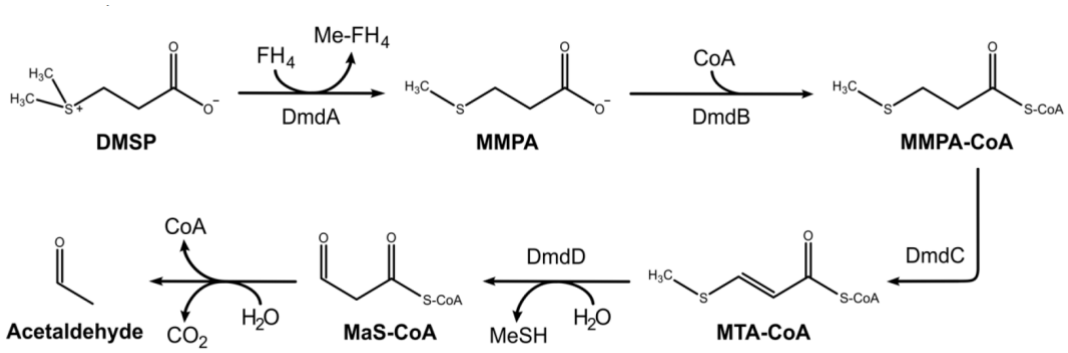


*Figure 6* – **The demethylation and demethiolation catabolic pathway for DMSP degradation.** Conversion of DMSP to MMPA is catabolised by the tetrahydrofolate (THF) dependent enzyme DmdA. Subsequent addition of coenzyme to MMPA to form MMPA-CoA is catalysed by DmdB. The MMPA-CoA undergoes oxidation to MTA-CoA via DmdC, followed by transformation by DmdD through addition of water which forms a brief intermediate. Release of MeSH transforms the intermediate into MaS-CoA which is finally converted to acetaldehyde via hydrolysis reaction releasing CoA and carbon dioxide.

### 1.5.2 DMSP Lysis

The other pathway available for DMSP catabolism is the lysis or cleavage pathway. The primary enzymes that act on DMSP in the lysis pathway are the Ddd enzymes, or DMSP lyases in bacteria or the only identified algal lyase Alma1 (47). In general, the model accepted for cleavage of DMSP by the DMSP lyases is that the process will yield acrylate and DMS, however, some bacteria will generate 3-hydroxypropionate (3-HP) instead of acrylate (see

Figure 7) (48). The lyase enzymes that catalyse these processes are now known. One type, the DddD proteins, were the first of the DMSP lyases to be discovered in 2007 by Todd *et* al in the marine bacterium *Marinomonas* sp MWYL1 (49). The DddD CoA transferase enzymes will cleave DMSP to generate 3-HP directly after which it is oxidised via a DddA enzyme to form Mal-SA that is subsequently converted to Acetyl-CoA by a DddC enzyme.

Of the other DMSP lysing enzymes, 6 bacterial enzymes are known to cleave DMS from the carbon-sulfur bond in DMSP to form the acrylate metabolite. These enzymes are known as DddL, DddP, DddQ, DddW, DddY and DddK (50). These enzymes are all cupin containing with the exception of DddP which is a metallopeptidase (51). Several years after the identification of these bacterial DMSP lyases, the first algal DMSP lyase, termed Alma1, was identified in *Emiliania huxleyi* (47). More recently, a new DMSP lyase enzyme, DddX (belonging to the acyl – CoA synthetase superfamily) which is distinct from the other known DMSP lyases, was discovered (51). DddX catalyses the conversion of DMSP to DMS via a two-step process; ligation of DMSP with CoA to form the intermediate DMSP-CoA that is then subsequently cleaved to produce DMS and acryloyl-CoA (51). It was found that acrylate production occurred mainly outside of the cell in a lot of DMSP catabolisers leading to the theory that DMSP cleavage doesn't serve solely nutritional purposes but can act as a defensive response (50,52). As the acrylate molecule itself is toxic at high concentration to microorganisms, it can act as a deterrent molecule to various predators such as protozoan herbivores (52). Once acrylate has been formed, its conversion to 3-HP is catalysed by AcuNK after which the pathway of 3-HP degradation is the same as when it is formed directly from DMSP via DddD.

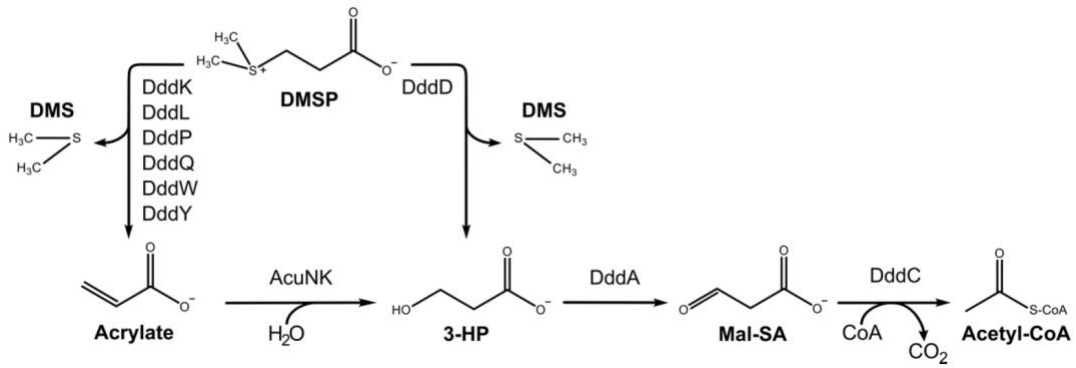


*Figure 7* – **The lysis/cleavage pathway for DMSP degradation.** Lysis of DMSP yields DMS via one of two ways controlled by various ddd genes. Enzymes controlling the lysis of DMSP to acrylate are DddK/L/P/Q/W/Y. Acrylate is then converted to 3-HP via AcuNK. Alternatively, DddD, catalyses the lysis of DMSP to 3-HP directly. Once the 3-HP molecule has been formed, DddA catalyses its oxidation to Mal-SA where DddC catalyses the addition of a coenzyme A moiety to form acetyl-CoA.

## 1.6 Dimethyl Sulfoxide (DMSO)

Much of what we know about the marine organosulfur cycle is that it is fuelled by small sulfur-containing zwitterionic osmolytes, primarily produced by planktonic algae (53), with DMSP being a central molecule in this process. Volatile DMS, produced from DMSP catabolism, is the main source of organosulfur in the atmosphere (see Figure 8) – as previously described. Concentrations of DMSP and DMS within the surface waters can be detected to substantial amounts (1.3–8.8 nM and 1 to 40 nM respectively) (54) however even greater concentrations (at least 1-2 orders of magnitude more) of dimethyl sulfoxide (DMSO) have been measured throughout the water column (55).

DMSO is non-volatile and soluble in all proportions of water, as such it does not have the same potential to influence the global climate as can DMS. Despite this, biotransformations between DMSO and DMS allow for some climactic influence to be exerted by DMSO (55). The role of DMSO within the marine organosulfur cycle remains poorly understood however it is generally accepted to be a dominant sink for DMS within the surface ocean (54). DMS is converted into DMSO primarily via photochemical oxidation which was first known to be initiated by hydroxyl radicals (55) but it is now known that halogen species (such as bromide radicals) are also implicated in this process (56). Considering that bromine is a major

constituent of seawater (approximately 65 $mgL^{-1}$) (55) the finding that it is involved in atmospheric photochemistry of DMS has made this an important field of research since its discovery in 1996.

Conversely, biological generation of DMSO is a slightly better but still quite poorly understood field despite the significance of microbial consumption of DMS being well known. Several species of aerobic bacteria have been reported to consume DMS but will produce $CO_2$ as a bioproduct instead of DMSO. The first reported microbial biosynthesis of DMSO from DMS was demonstrated by anoxygenic phototrophic purple sulfur bacteria (53) and phototrophic green sulfur bacteria where the process provides electrons for carbon fixation (58). Since then, later work has shown that during the growth of chemoheterotrophic bacteria DMS oxidation to DMSO also occurs (57). Work by Vila-Costa *et al* (59) associated a wider range of bacteria including *Alphaproteobacteria* and *Flavobacteria* (*Bacteriodetes*) as having the ability to oxidise DMS to DMSO. DMS dehydrogenase (see Figure 8), discovered in *Rhodovulum sulfidophilum,* is the main enzyme implicated in the production of DMSO from DMS and to date is the only such enzyme to be characterised (58). DdhABC DMS dehydrogenase is expressed during phototrophic growth of *R. sulfidophilum* in the presence of DMS and is a soluble, trimeric, periplasmic protein consisting of a catalytic molybdenum subunit (DdhA) which contains an iron/sulfur cluster, a subunit containing 4 iron/sulfur clusters (DdhB) as well as a subunit containing a single heme *b* group (DdhC) (58). Despite the operon encoding DdhABC being known, no molecular studies have been published on the regulation of DMS dehydrogenase to date.

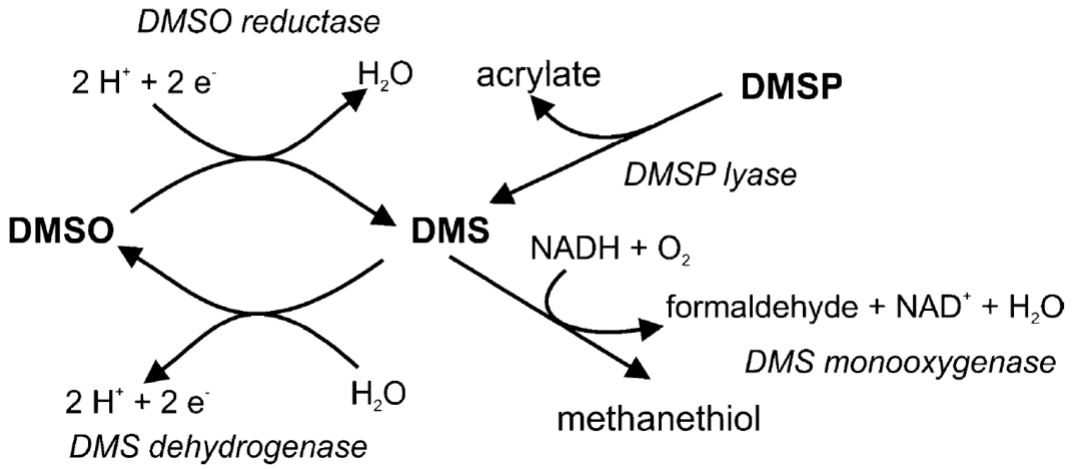


*Figure 8* – **Enzymatic interactions of DMS with DMSP, DMSO and methanethiol (54).** DMSP is converted to DMS through enzymatic lysis via DMSP lyases, additionally yielding acrylate. DMS can be converted to methanethiol via DMS monooxygenase but also DMSO via DMS dehydrogenase. DMSO can also be converted back to DMS via DMSO reductase.

Building upon the understanding of DMSO bio generation within the oceans, a 2016 paper by Lidbury *et al* elucidated a trimethylamine monooxygenase (Tmm) within the model marine heterotrophic bacterium *Ruegeria pomeroyi* (which is usually involved in the oxidation of trimethylamine (TMA) to trimethylamine *N*-oxide (TMAO)) that can convert DMS into DMSO at comparable rates of TMA to TMAO (60). Due to how widespread the gene encoding Tmm is within bacterial cells inhabiting the surface waters of the ocean (including the large marine *Roseobacter* and SAR11 clades), it is hypothesised that a significant proportion of the observed DMSO production in the surface oceans occurs via Tmm oxidation of DMS via Tmm-containing heterotrophic bacteria (60).

In addition to being a dominant sink for DMS, DMSO is also a source of it within the ocean. The reduction of DMSO to DMS is catalysed by a class of metalloenzymes found in a variety of bacterial phyla but are widespread throughout all the domains of life (61) known as the DMSO reductases (58). The DMSO reductases fall under a class of mononuclear molybdenum (Mo) containing enzymes i.e. they contain a single Mo metal centre at their active site (see Figure 9 for a schematic diagram) (58). The best studied examples of DMSO reductases are from the γ-Proteobacterium *Escherichia coli* and the α-Proteobacteria *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* (58). There are two main types of DMSO reductases within their enzyme family, the Dor- (found in *Rhodobacter* species) and the Dms- (found in

*Escherichia* species) types (58). Both of these enzyme types are found in the bacterial periplasm with the difference between the two being that the Dms- types are membrane-bound through additional subunits DmsB and DmsC whereas the Dor- types are soluble and have only transient interactions with the membrane-bound DorC- type cytochromes that act as electron donors for these systems (58). Expression of these enzymes is induced by the absence of oxygen where DMSO reductase acts as the terminal electron acceptor during anaerobic respiration (58).

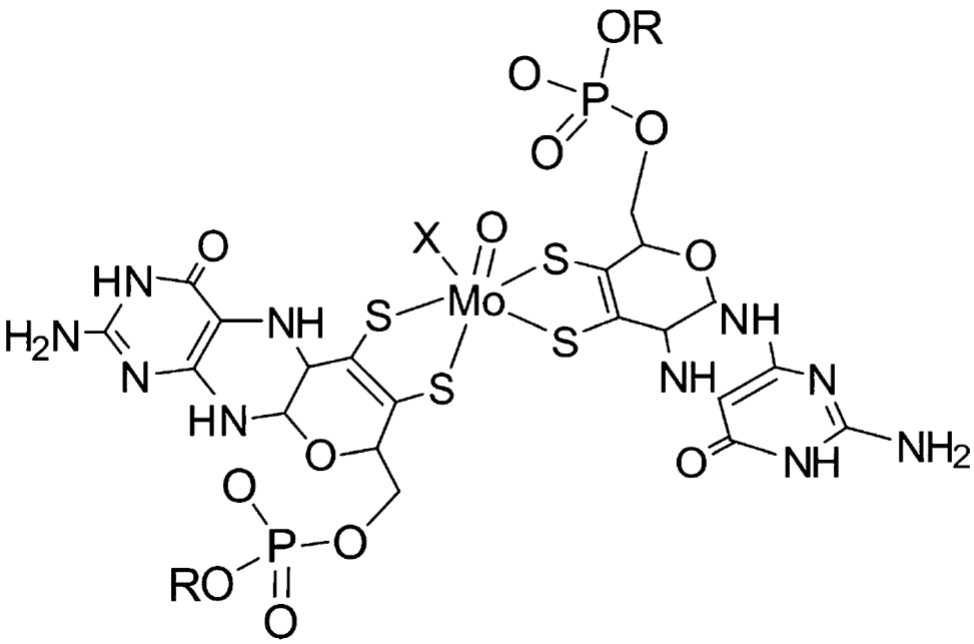


*Figure 9* – **Schematic representation of the bis-MGD cofactor present in enzymes of the DMSO reductase enzyme family.** Only the cofactors, the central molybdenum atom and the direct ligands to the molybdenum are shown, X = amino acid ligand to the molybdenum centre.

Of interest to note is the unusual DMSO respiration system found in the organism *Shewanella oneidensis* which is neither a Dor- or Dms- type but does have some similarities with the *E. coli* Dms-type. This DMSO reductase system, unlike the others in *E. coli* and *R. sphaeroides,* is not found in the periplasmic space but instead is located on the outer membrane and thus has coined the term 'extracellular respiration' (58). Due to its location, it has also been hypothesised that its extracellular position allows for facilitation of particulate substrate-bound DMSO (58).

## 1.7 Dimethylsulfoxonium propionate (DMSOP)

In 2018, Thume *et al* (53) published in Nature the discovery of a novel organosulfur compound that extends the known marine sulfur cycle; dimethylsulfoxonium propionate (DMSOP). Using methods previously established in the labs of Georg Pohnert (62) for the direct detection of zwitterionic metabolites such as DMSP (which prior to the establishment of these methods, were only able to be detect via indirect methods). Thume *et al* noticed a discrepancy between their analytical data and previous determinations of DMSP and DMSO within algal samples. Undertaking an investigation into these discrepancies, Thume *et al* used various analytical methods including, but not limited to, electrospray ionization high-resolution mass spectroscopy (ESI-MS) and ultra-high-pressure liquid chromatography (UHPLC), they tentatively assigned the sulfoxonium species DMSOP as the metabolite responsible for the discrepancies in their previous determinations of DMSP and DMSO concentrations in algae. In order to confirm this a $^{13}$C-labelled DMSOP was synthesised via a $RuCl_3$/sodium hypochlorite-mediated oxidation of DMSP to use a reference compound which was confirmed through Nuclear Magnetic Resonance Spectroscopy (NMR) and Tandem Mass Spectroscopy (MS/MS) (53). When added to cultures of algal extract, this standard co-eluted with the unknown sulfoxonium species when subjected to liquid chromatography – MS, confirming the identity of DMSOP.

The DMSOP zwitterionic metabolite is widely distributed in phytoplankton (see Table 1) and the total flux of DMSOP is estimated by Thume *et al* to be in the tetragram range. The discovery of this novel sulfoxonium species provides a previously unknown pathway for the production of biogenic DMSO within the sulfur cycle (see Figure 10) which, given the interactions between DMSO and DMS, could have potential impacts on climate regulation. In addition, the authors note only one other natural product containing the dimethylsulfoxonium moiety (2-hydroxyehtyl dimethylsulfoxonium chloride) and as such, the discovery of this novel, highly polar, zwitterionic compound presents the opportunity to investigate a nearly completely unexplored structural family (53).

*Table 1* – **Known algal producers of DMSOP**

| Class | Species |
|---|---|
| Haptophyte | *Isochrysis galbana* |
| | *Prymnesium parvum* (axenic) |
| | *Prymnesium parvum* |
| Diatom | *Coscinodiscus waileslii* |
| | *Entomoneis paludosa* |
| | *Eucampia zodiacus* |
| | *Skeletonema* |
| Coccolithophore | *Emiliania huxleyi* |
| Dinoflagellate | *Protocentrum minimum* |
| | *Symbiodinium microadriaticum* |

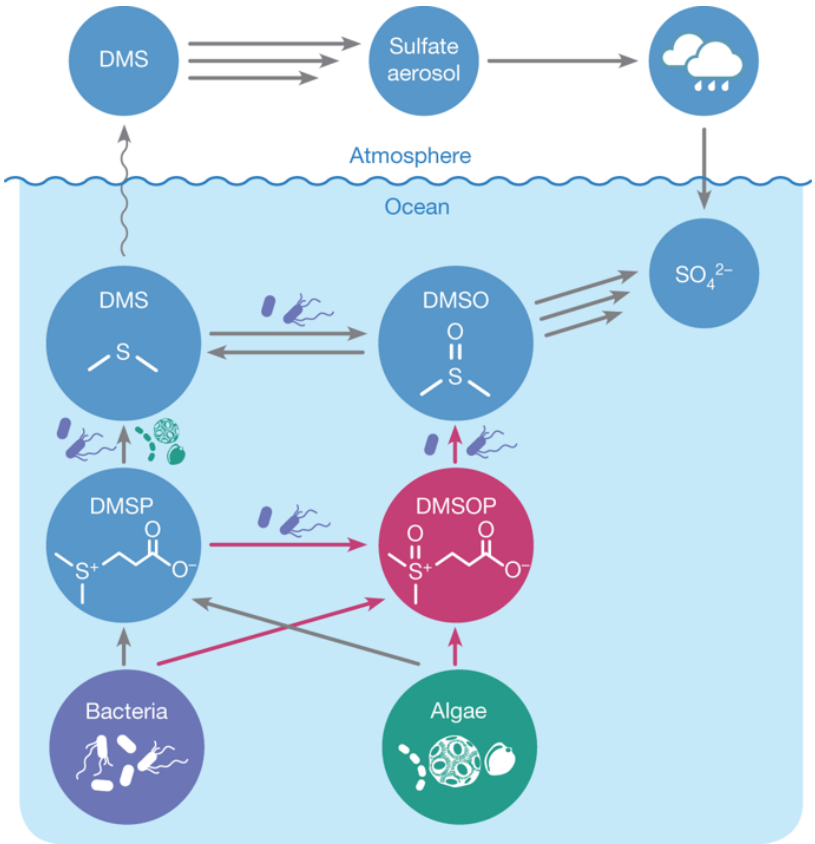


*Figure 10* – **Extended marine organosulfur cycle (49).** Bacteria and algae are shown to produce DMSOP but it can also be produced from DMSP. The DMSOP extends the sulfur cycle by providing a previously unknown pathway for the production of DMSO. The known pathway for DMSO production is conversion of DMSP to DMS and then DMSO. The new pathway is conversion of DMSP to DMSOP to DMSO.

Currently, very little is known about this biogenic compound. The main known producers of it are the bloom-forming dinoflagellate *Prorocentrum minimum*, the diatom *Skeletonema costatum* and the haptophytes *Prymnesium parvum*, *Isochrysis galbana* and *Emiliania huxleyi* - all of which produce DMSOP at micromolar to millimolar cellular concentrations, corresponding to 0.13–1.2% of DMSP in the algae (53). Additionally, the presence of DMSOP was also able to be detected in the medium of *P.parvum* axenic cultures indicating it is also expressed extracellularly. Thume *et al* sampled sea water at many different locations including: the northwest Pacific Ocean, northwest Atlantic Ocean, Arctic Ocean and Mediterranean Sea (see Figure 11). They were able to detect the presence of DMSOP at all of these sites with an average concentration of about 0.14 ± 0.18 nM, this is a good indicator of a probably universal distribution of the sulfoxonium species within oceanic surface waters (53). Of marine, DMSP-producing bacteria, only one has been tested and shown to produce DMSOP: *Pelagibaca bermudensis* (0.32 ± 0.049 pmol $\mu g^{-1}$ protein, approximately 0.1% of DMSP) (53), which is a known DMSP producer. As DMSOP has both eukaryotic and bacterial origins, it stands to reason that other marine bacterial with the ability to synthesis DMSP would also have the ability to synthesis DMSOP.

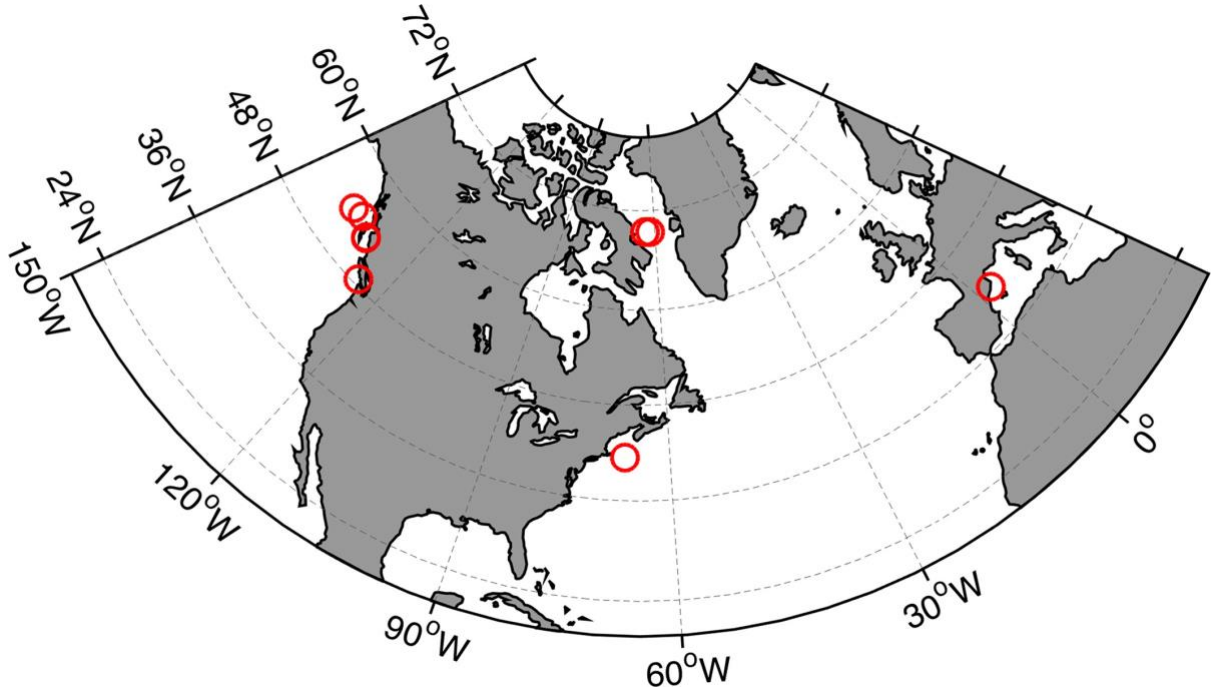


*Figure 11* – **Sampling site (indicated in red) where Thume *et al* were able to detect the presence of DMSOP (49).**

### 1.7.1 Synthesis

Using $^{13}$C-labelled DMSP and DMSOP (see Figure 12), the biosynthesis of DMSOP was able to be studied in *P. bermudensis*. Following addition of $^{13}$C-labelled DMSP to batch cultures of *P. bermudensis*, the incorporation rates of the DMSP (revealed through high-resolution MS) were 3.7 ± 0.6% after 18 hours incubation (53). This experiment also revealed that the bioprocess for the production of DMSOP is the direct oxidation of DMSP to DMSOP via a currently unknown DMSOP synthase enzyme instead of an initial demethylation, subsequent oxidation to the sulfoxide and then remethylation (53). This direct oxidation is also consistent with previously described functions of DMSP as an antioxidant due to its high intracellular concentrations or potential upregulation during oxidative stress in marine algae i.e. as oxidative stress increases, intracellular DMSP concentrations increase as a way to scavenge oxygen radicals before they cause cellular damage (53). Additionally, batch cultures of *I. galbana* exhibiting oxidative stress as a decrease in photosynthetic efficiency showed a 300% increase of cellular DMSOP during the late exponential/stationary phase (see Figure 13) (53) further emphasising an antioxidant based role for DMSOP.

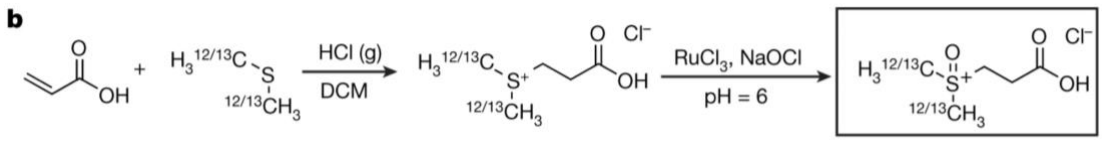


*Figure 12* – **Synthesis of $^{13}$C-labelled DMSOP by $RuCl_3$/sodium hypochlorite-mediated oxidation of DMSP (49).**

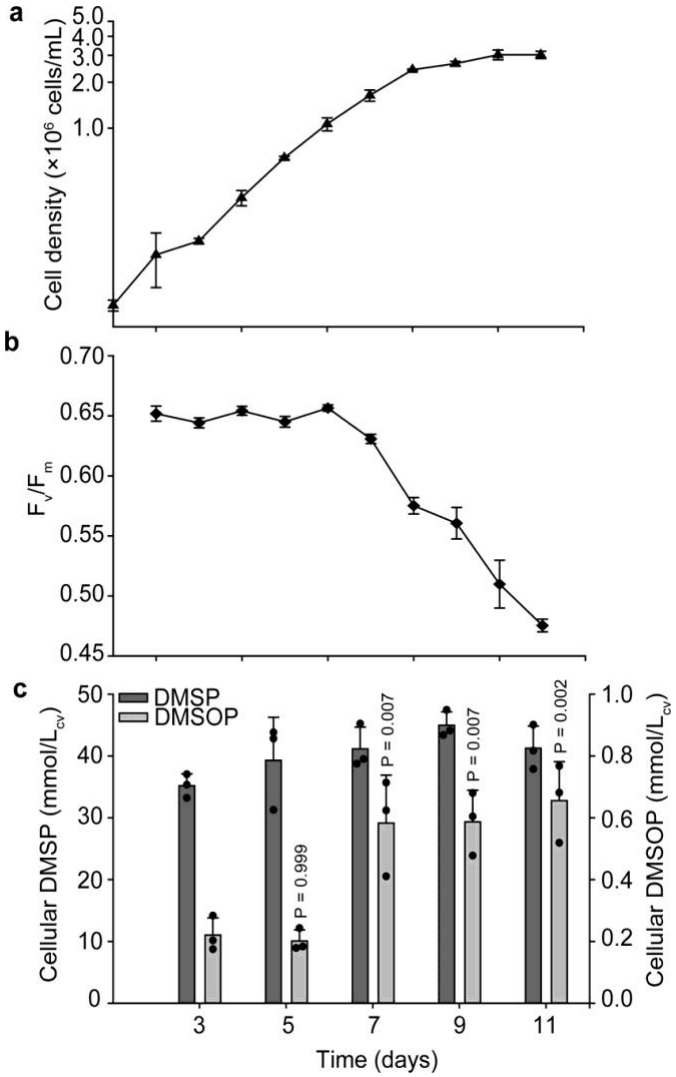


*Figure 13* – **Growth (a) and photosynthetic efficiency (b) of *I. galbana* cultures. (c) Cellular DMSP and DMSOP concentrations.** The data are mean ± s.d. of *n* = 3 independent cultures. Statistical analysis revealed a significant difference in cellular DMSOP concentrations compared to day 3 was detected from day 7 onwards (49).

### 1.7.2 Catabolism

Despite the stability of DMSOP over several weeks in 0.2 µM filter-sterilised sea water at room temperature, Thume *et al* have shown that microbial transformations of DMSOP contribute to its degradation within the ocean (53). Using the $^{13}$C-labelled DMSOP as before, the authors investigated the catabolism of DMSOP to DMSO by common marine bacterial DMSP degraders. To date, only four species (*Sulfitobacter* sp*., Ruegeria pomeroyi, A. faecalis and Halomonas* sp.) have been shown to break down DMSOP using similar pathways to DMSP degradation (lysis or demethylation) (see Figure 14) (53).

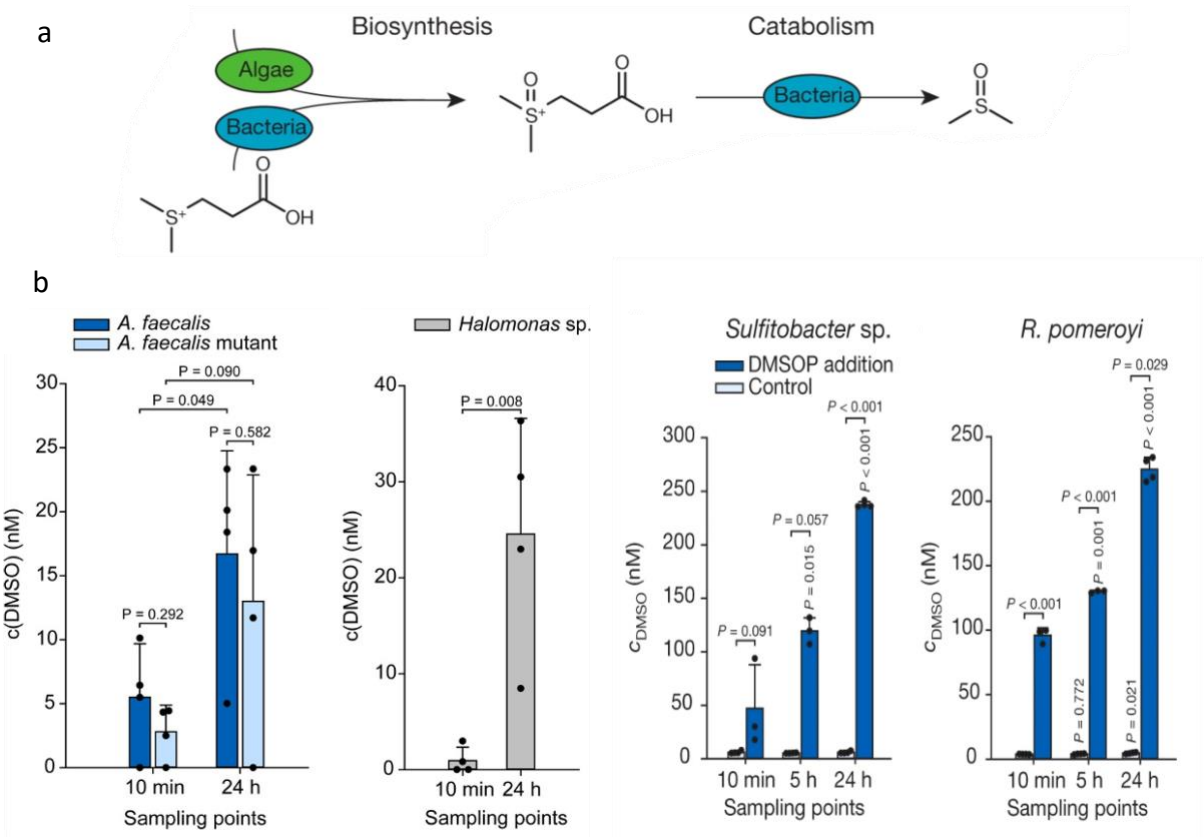


*Figure 14* – **(a) Schematic of DMSOP synthesis and catabolism. (b) DMSO release (concentration (*c*) given as mean) for bacteria *A. faecalis, Halomanas sp., Sulfitobacter sp.* and *R. pomeroyi* over time (49).**

The discovery of this novel compound has a left a resounding research gap within the known marine sulfur cycle and indeed our knowledge of the mechanism of DMSOP production which has opened the doors to further experimentation and investigation.

## 1.8 Objectives

The main objectives of my master's thesis are to study the novel compound DMSOP published in Nature (2018) by Thume *et al* in terms of its production and its cycling by microorganisms. The objectives can be broken down as follows:

### 1.8.1 Identification of the DMSOP synthase enzymes

The primary aim of this project was to identify the molecular mechanism behind the production of DMSOP from DMSP by the model marine Roseobacter *Pelagibaca bermudensis*.

- Initially this was attempted through screening of a genomic library of *Pelagibaca bermudensis* (which is known to produce DMSOP) within the heterologous host *Rhizobium leguminosarum* J391 and assaying for DMS production from DMSOP via Gas Chromatography (GC). Due to sensitivity issues with the GC assay, additional methods of screening for DMSOP production were used as follows. Nuclear Magnetic Resonance (NMR) spectroscopy allows for the accurate detection of DMSOP production within a cell lysate. Detection of DMSOP was also achieved through a methyl viologen assay as it was discovered that the DMSO reductase enzyme would also reduce DMSOP and thus change the colour of the methyl viologen which was characterised through UV/Vis spectroscopy.

### 1.8.2 Identification and characterisation of enzymes with DMSOP lyase activity

The one paper on DMSOP shows that the known DMSP lyase in *Alcaligenes faecalis* (DddY) does not function on DMSOP. However, preliminary work done by Curson and Walsham in Todd's lab shows this not to be the case and that many of the known DMSP lyase enzymes can also function on DMSOP. The aim of this section of work was to characterise the ability of DMSP lyases to function on DMSOP as substrate and to look at the ability of organisms with these enzymes to use DMSOP as a source of sulfur and carbon.

- Bacterial strains containing the various DMSP lyases were grown and incubated with DMSOP or DMSP. Headspace DMS produced from DMSOP or DMSP lysis, was measured by GC to determine the relative activity of the various DMSP lyases on DMSP and DMSOP. This is in collaboration with protein biochemists in Qingdao, China, who are analysing the activity of these in comparison to DMSP.

- The ability of the strains to grow on DMSOP and DMSP as a sole carbon source is also being determined. For this experiment, carbon sources of DMSP, DMSOP and succinate (control) are being used to investigate the how well the strains are able to grow on DMSOP.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Media Preparation and growth conditions

All of the bacterial strains used in this study have been detailed in Table 2, with their growth media detailed in Table 3. Where necessary, antibiotics were added to the media at the following concentrations: streptomycin (400 µg $ml^{-1}$), kanamycin (200 µg $ml^{-1}$) and tetracycline (200 µg $ml^{-1}$).

*Table 2* – **A list of strains used in this study along with what they were used for and their growth medias.**

| Strain | Description | Media | Strain reference |
|---|---|---|---|
| *Rhizobium leguminosarum* J391 | Streptomycin-resistant derivative of wild type strain 3841 used for library screening | RM, TY | Young *et al* (63) |
| *Escherichia coli* 803 | Strain used in tri-parental mating to transfer cosmids to *Rhizobium* | LB | Wood *et al* (64) |
| *Escherichia coli* 803 (pRK2013) | Helper strain used in tri-parental mating | LB | Wood *et al* (64), Figurski *et al* (65) |
| *Alcaligenes faecalis* M3A | Wild type strain, *dddY*$^{+}$, used in growth studies on DMSOP | LB, M9 | De Souza and Yoch (66) |
| *Alcaligenes faecalis dddY*$^{-}$ | *dddY*$^{-}$ mutant strain used in growth studies on DMSOP | LB, M9 | Curson *et al* (67) |
| *Ruegeria pomeroyi* DSS-3 | Wild type strain, *dddW*$^{+}$, *dddQ*$^{+}$, *dddP* , used in growth studies on DMSOP | YTSS, MBM | González *et al* (68) |
| *Oceanimonas doudorofii* | Wild type strain, *dddP*$^{+}$, used in growth studies on DMSOP | LB, M9 | Baumann *et al* (69) |
| *Halomonas caisteri* HTNK1 | Wild type strain, *dddD*$^{+}$, used in growth studies on DMSOP | YTSS, MBM | Todd *et al* (70) |
| *Labrenzia aggregata* LZB033 | Wild type strain, *dddL*$^{+}$, used in growth studies on DMSOP | YTSS, MBM | Curson *et al* (15) |

| | | | |
|---|---|---|---|
| *Shewanella oneidensis* MR-1 | Strain from which DMSO reductase was purified and used in enzyme assays with DMSOP | LB, MBM | Myers *et al* (71) |

*Table 3* – **A list of medias used in this study to culture the bacterial strains used.**

| Media | Type | Reference |
|---|---|---|
| TY (Tryptone Yeast) | Rich | Beringer (72) |
| LB (Luria-Bertani) | Rich | Sambrook *et al* (73) |
| M9 | Minimal | Miller (74) |
| YTSS (Yeast Tryptone Sea Salts) | Rich | González *et al* (75) |
| MBM (Marine Basal Medium) | Minimal | Zheng *et al* (18) |

## 2.2 Preparation and Mobilization of *P. bermudensis* genomic library

A genomic library of *P.berumdensis* was prepared by Dr Andrew Curson, UEA, as described in Curson *et al* (50) using the pLAFR3 plasmid. Tri-parental crossing was utilised to transfer cosmids from *E. coli* to *Rhizobium*. This involved the three strains: *Rhizobium* (the host strain), *E. coli* 803 (the donor strain containing the genomic library to be conjugated into the host strain) and a kanamycin resistant *E. coli* 803 pRK2013 (the helper strain). A 5ml culture of *Rhizobium* was grown in TY media supplemented with streptomycin (400 µg $ml^{-1}$) at 28°C with shaking overnight. The helper plasmid *E. coli* 803 (pRK2013) and donor strain were both grown in 5ml LB cultures each supplemented with antibiotics kanamycin (200 µg $ml^{-1}$) and tetracycline respectively (200 µg $ml^{-1}$) at 37°C with shaking overnight. A 1ml aliquot of the host was centrifuged at maximum speed for 1 min. The supernatant was removed, and the pellet resuspended into 500 µl fresh TY media. This process was repeated three times to wash out residual antibiotics, before being centrifuged again and resuspended in 100 µl TY media. The donor and helper strains were also treated in the same way and resuspended in 100 µl TY media. A sterile filter was placed on a TY agar plate containing no antibiotics using sterile

forceps. Aliquots of 100 µl of each strain were added to the filter and mixed using a sterile loop. Control crosses were also set up with just the helper and host, and just the donor and helper. Plates were incubated at 28°C overnight. Following incubation, sterile forceps were used to remove the filters and place them into sterile universal tubes. The cells were washed off the filter using 2 ml of 50% glycerol before being plated. The transconjugates were selected for on TY media plates containing streptomycin (400 µg $ml^{-1}$), kanamycin (200 µg $ml^{-1}$) and tetracycline (200 µg $ml^{-1}$).

## 2.3 Principles of Gas Chromatography (GC)

Gas chromatography is one of the most widely utilised analytical techniques to separate and analyse volatile compounds (76). Both organic and inorganic matter in states; gas, liquid and solid (usually dissolved in a volatile solvent) are able to be analysed on the GC with molecular weights ranging from 2 to over 1000 Da (76). A simplified schematic of a typical gas chromatograph is shown in Figure 16. In essence, an inert carrier gas (mobile phase), such as helium, flows continuously from a large gas cylinder through the inlet, the column and the detector with the flow rate continually carefully controlled to ensure reproducible retention times and minimal detector drift and noise (76). A sample is injected into the inlet where it is vaporised and carried into the column. The column itself is typically within the ranges of 15-30 m long and is coated on the inside with a thin film of a high boiling liquid called the stationary phase (76). The sample is separated between the mobile and stationary phases as the different constituents pass through the column at varying rates based on their chemical and physical properties and subsequent interactions with the stationary phase. Following the column, the sample constituents (contained within the carrier gas) pass through the detector at varying speeds and they exit is recorded electronically. The electronic signal is then sent to a data system where a chromatograph is generated (76).

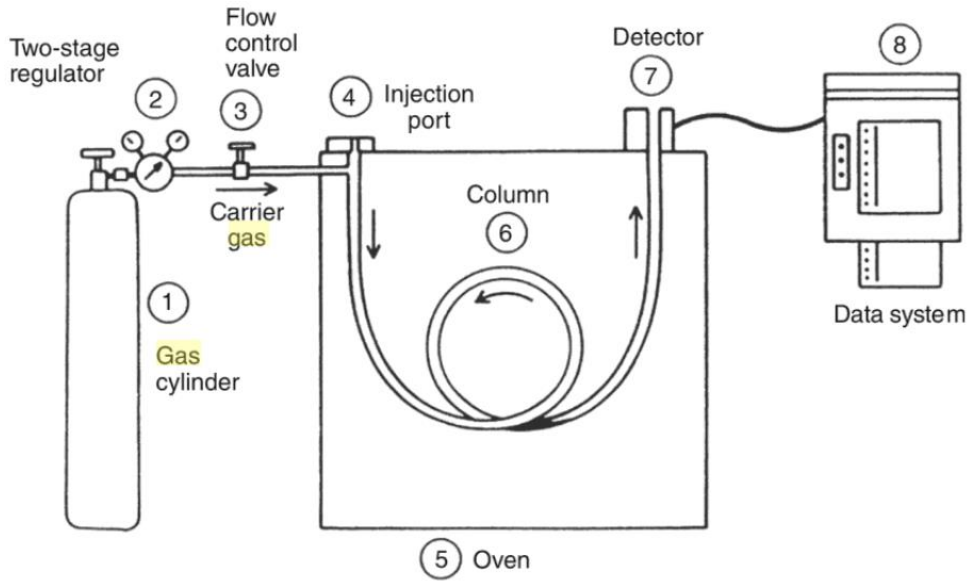


*Figure 15* – **Schematic of a typical gas chromatograph.** [1] Gas is provided via a gas cylinder where it is passed through a two-stage regulator [2] and a flow control valve [3]. The gas then passes through the injection port [4] with the sample and carries the sample through the column [6] in the oven [5]. At the end of the column is the detector [7] which sends a digital signal to the data system [8] where the sample can be analysed.

## 2.4 Gas Chromatography (GC) screening for DMSOP synthase activity

So far ~187 colonies of transconjugated *Rhizobium* have been screened. Colonies were picked on selective TY plates (as described above) and inoculated into 1 ml TY media containing succinate (1mM), $NH_4Cl$ (1mM), DMSP (1mM) and tetracycline (200 µg $ml^{-1}$). These cultures were then incubated at 28°C overnight with shaking. These cultures would then undergo the GC screening procedure.

To quantify DMSOP-dependent DMS production, 200 µl aliquots of 10M NaOH were added to the 1ml of cultures and these were left overnight in the dark to allow complete lysis of DMSP and DMSOP. The following day samples were incubated for 2 hours at 80°C in open air to allow for removal of dimethyl sulfide (DMS) gas produced from DMSP cleavage. This step allows for any background DMS produced via DMSP to be removed from the samples to ensure that any DMS produced in subsequent steps would be from DMSOP. Addition of the NaOH also cleaves the DMSOP in the samples to DMSO.

Following the incubation step, 200 µl of the reaction mixture is transferred to 2 ml glass GC vials and 100 µl of $SnCl_2$ (880 mM) under acidic conditions was added before the vial was immediately crimped with PTFE/rubber crimp caps and incubated at 55°C for 90 minutes to convert remaining DMSO (produced from DMSOP cleavage) to DMS. The vials were incubated one final time at 22°C overnight in the dark before being read by GC. DMS concentrations were estimated by comparison to a standard curve generated from varying concentrations of DMSO samples treated in the same way.

## 2.5 Principles of Nuclear Magnetic Resonance Spectroscopy (NMR)

The first technique explored as a replacement for screening via GC was Nuclear Magnetic Resonance Spectroscopy (NMR). This analytical technique was first developed in 1946 by scientists at Stanford and M.I.T. (USA), NMR was established over the following 50 years as the premier technology available to chemists to determine the detailed chemical structures of the complex molecules they were synthesising (77). Since then, the principles of the technology have been used in the online process analyser market and extensively in medical radiology fields with the more widely recognised Magnetic Resonance Imager (MRI) machine (77). The principles that sanction the NMR phenomenon can be described through quantum mechanics, see below. However, in Layman's terms, information can be yielded about a molecules' chemical structure based on the alignment of the magnetic nuclear spins of its atoms within an applied, external magnetic field.

### 2.5.1 Quantum Mechanics Underpinning the NMR phenomenon

Within quantum mechanics, subatomic particles (protons, electrons and neutrons) are imagined as spinning on their axis (77). Within atoms such as $^{12}C$, these spins are paired in opposite directions such that the nucleus has no overall spin. However, in atoms such as $^{13}C$ and $^{1}H$, the nucleus does possess a spin. Figure 17 shows the nuclear shell model of $^{12}C$ and $^{13}C$ which can be used to show why $^{13}C$ is NMR active while $^{12}C$ is not.

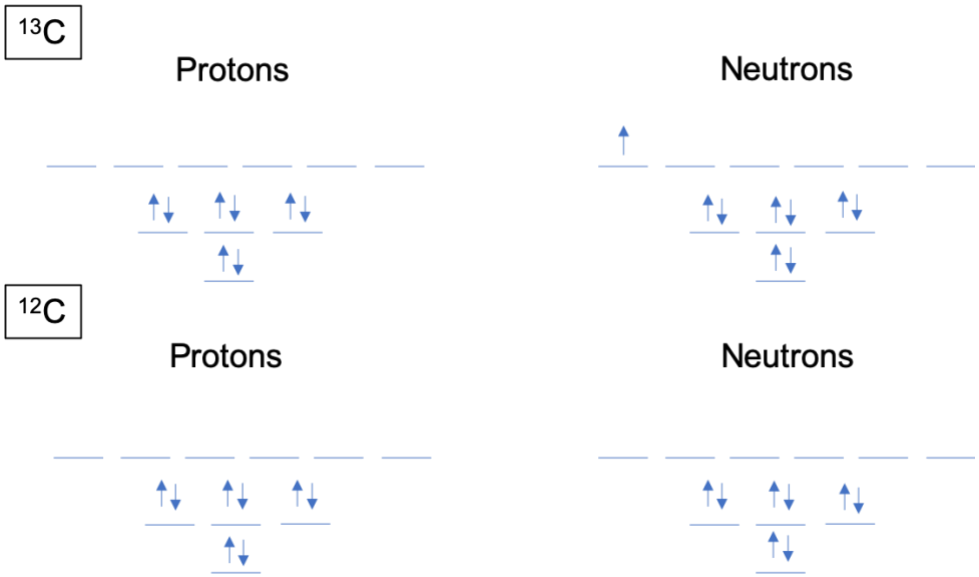


*Figure 16* – **Nuclear shell model of $^{12}C$ and $^{13}C$ showing the arrangement of electrons.**

The nuclear spin model has the following rules:

- Atomic neutrons pair with neutrons while the protons pair with protons
- The number of available states is determined by Pascal's triangle
- Spins fill the orbitals in accordance with Hund's rule where:
    a) Every orbital within a subshell is singly occupied before it can be doubly occupied
    b) All nucleons within singly occupied orbitals have the same spin orientation

Following these rules, we can see in Figure 17 that $^{13}C$ contains an additional neutron compared with $^{12}C$ and as such it singly occupies a subshell. Thus, $^{13}C$ contains an overall atomic spin of ½ and can be detected by the NMR instrument.

The rules for determining the net spin of the nucleus of an atom are as follows:

- If the number of protons and neutrons are both even, then the nucleus has no spin

- If the number of protons plus neutrons is odd, then the nucleus has a half integer spin (i.e.$\frac{1}{2}, \frac{3}{2}, \frac{5}{2}$)
- If the number of protons and neutrons are both odd, then the nucleus has an integer spin (i.e. 1, 2, 3) (77)

The overall spin of an atom, termed 'I', is of importance as quantum mechanics tells us that a nucleus of spin I will have 2I + 1 possible orientations. Using this rule, we can see that a nucleus with spin ½ will have two possible orientations which, in the absence of a magnetic field, are of equal energy. When an external magnetic field is applied, the energy levels of each orientation are split and each level is given a magnetic quantum number 'm' (77). Figure 18 shows the splitting of the two energies. These nuclear magnetic moments of a nucleus can align with the externally applied magnetic field (the strength of which is noted by '$\mathbf{B_0}$') in only 2I + 1 ways; with or against the applied field $\mathbf{B_0}$. The energetically preferred orientation is aligned with $\mathbf{B_0}$ whereas the higher energy orientation is aligned against $\mathbf{B_0}$. In terms of rotational axes, the spinning nucleus can never be aligned exactly parallel or anti-parallel with the direction of $\mathbf{B_0}$, instead, the nucleus must precess about this field at an angle (77). The angular velocity is given by the expression:

$$w_o = g\boldsymbol{B}_0$$

Where, $w_o$ refers to the Larmor frequency (precession rate). The constant 'g' is the magnetogyric ratio and it relates the magnetic moment 'm' and the spin number 'I' for any specific nucleus in the following expression:

$$g = 2pm/hI$$

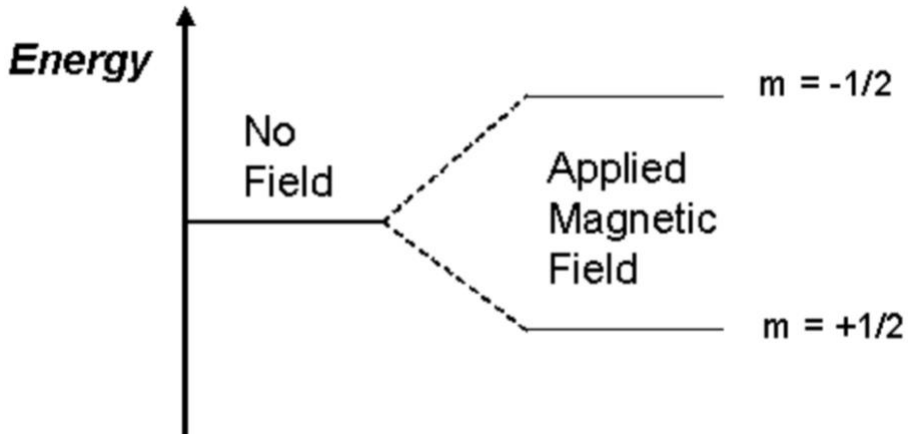


*Figure 17* – **Energy levels for a nucleus with spin quantum number ½.** The energetically preferred orientation (m = +1/2) has its magnetic moment aligned parallel with the applied field and is often the notation $\alpha$. The higher energy, anti-parallel orientation (spin = -1/2) is referred to as $\beta$ (34).

Each nucleus will have a characteristic value of 'g' (the constant of proportionality between the nuclear angular momentum and the magnetic moment) which for a proton is $2.674 \times 10^4$ $gauss^{-1}sec^{-1}$ (77). The precession process of the nucleus will generate an electric field of frequency $w_o$ and if this sample is then irradiate with radio waves (MHz), the precessing proton can then absorb the energy be promoted to the less favourable, higher energy state (77). We call this absorption resonance as the frequency of the applied radiation and the precession coincide and 'resonate'. The type of NMR used in this work was Fourier Transform NMR or FT-NMR. In FT-NMR, all the frequencies in the spectrum were irradiated simultaneously with a single radio frequency (RF) pulse generated by a single oscillator (77). Following the RF pulse, nuclei will return to a thermal equilibrium and a time domain emission signal (called a free induction decay (FID)) is recorded by the instrument. A frequency domain spectrum is then calculated by Fourier Transform of the FID with the heights of each peak representing the number of nuclei resonating and each specific frequency (77). Where peak signals are on the x axis of a spectrum is known as their 'chemical shift' (recorded in part per million, ppm) or their resonance frequency relative to standard in the magnetic field (usually tetramethylsilane (TMS) as it has a chemical shift of 0 (77). Chemical shift can be affected by multiple factors including electron deshielding but will not be further described here.

## 2.6 Nuclear Magnetic Resonance Spectroscopy (NMR) detection of DMSOP

Detection of DMSOP production was attempted using 500 MHz $^{1}$H-NMR in cell lysates. 5 ml cultures of *L. aggregata* were grown in rich media overnight at 28°C with shaking. 2.5 ml aliquots of the cultures were lysed through sonication for 5 repeats (30 seconds each), being kept on ice in between. Following sonication, samples were centrifuged at max speed for 10 minutes before the supernatant was removed and transferred to a new tube.

A 1 ml aliquot of the cell lysate supernatant was added to 1 ml of a $H_2O$: $D_2O$ mix (90:10 v/v respectively) and mixed by inversion 3-4 times. Of this sample, 600 µl was loaded into an NMR tube before being run. The detection signal used to identify DMSOP in the spectrum is the singlet signal at approximately 3.89 ppm (see Figure 15) and the peak intensity was recorded. Concentrations of DMSOP in the sample were calculated by comparing the peak intensity to a calibration curve of known peak intensities for different concentrations of DMSOP

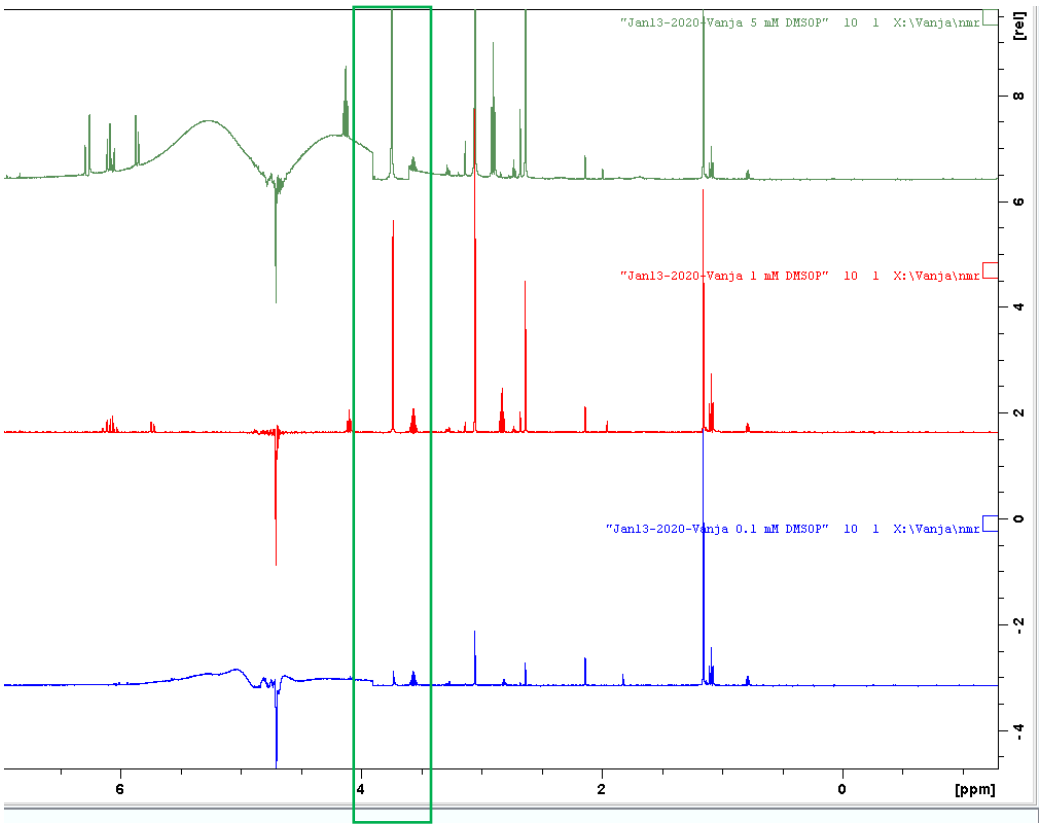


*Figure 18* – **$^{1}$H- NMR spectra showing the detection peak for DMSOP at 3.89 ppm (indicated by green box).** Three different concentrations of DMSOP are shown: 5 mM (top – green), 1 mM (middle – red) and 0.1 mM (bottom – blue).

## 2.7 DMSO reductase assay

DMSO reductase assays were conducted to determine the relative activity of DMSOP as sole substrate for DMSO. This assay were being developed as a way to screen for the presence of DMSOP in cell culture.

DMSO reductase (purified from *Shewanella oneidensis* MR-1 by K. Usman, UEA) activity was assayed by monitoring the oxidation of reduced methyl viologen (MV) by DMSOP through UV/Vis spectrophotometry whereby the colour change from blue/purple (reduced MV) to colourless (oxidised MV) is measured.

Enzyme assays were performed under sparged, anaerobic conditions (to maintain the reduced state of methyl viologen) in 2 ml glass cuvettes containing 1960 µl 20 mM HEPES buffer (pH 7.8), 20 µl of 100 mM methyl viologen and 20 µl of 0.0541 ng/µl DMSO reductase. Anaerobic sodium dithionite (125 µM) solution is made by dissolving 33 mg of sodium dithionite in 4 ml of 20 mM HEPES buffer (which had been sparged for 15 minutes prior to addition). The spectrophotometer (HITACHI U-3310) was set to 600 nm wavelength with intervals of 1 – 600 seconds. The spectrophotometer was blanked with a cuvette containing only HEPES, DMSO reductase and non-reduced methyl viologen. Following this, small amounts of the sodium dithionite solution is added to the separate reaction cuvette with a Hamilton syringe until the background absorbance is around 1.5 nm. Once the background absorbance is stable, known concentrations of DMSOP are injected via a Hamilton syringe into the cuvette, inverted 2-5 times to mix and the absorbance measured. The background absorbance and change in absorbance per minute was measured and the data subsequently analysed. Varying concentrations of DMSOP were assayed to construct a Michaelis-Menten plot to measure the $K_m$ and $V_{max}$ of the enzyme with DMSOP as substrate.

## 2.8 Anaerobic growth curves and analysis of DMSOP/DMSO consumption by *S. oneidensis* (in collaboration with K. Usman and J. Wang, UEA)

### 2.8.1 Growth Curve

A 10 ml culture of LB was inoculated with a single colony of *S. oneidensis* taken from an LB agar plate. The culture was allowed to grow overnight at 30°C with shaking after which 1 ml of the culture was used to inoculate 100 ml LB media and allowed to grow for a further 24 hours. Following the 24-hour growth period, the entire 100 ml culture was spun down at 4000 rpm at 4°C for 15 minutes and the supernatant was discarded. A 25ml aliquot of fresh was used to resuspend the pellet and the culture was spun a second time under the same conditions and the supernatant was discarded. The resulting pellet was resuspended in 5 ml of fresh minimal media and diluted to a final $OD_{600}$ of 8. A 500 µl aliquot of the diluted culture was inoculated into 250 µl of minimal media containing:

- 20 mM lactate and 10 mM DMSO
- 20 mM lactate and 10 mM DMSOP
- 20 mM lactate and 10 mM fumarate
- Media without an electron donor or acceptor

Optical density measurements were taken every 6 hours for 72 hours and the results analysed.

### 2.8.2 Consumption of DMSOP/DMSO

Following each 6-hour sampling interval, 100 µl of cell culture (20 mM lactate + 10 mM DMSOP for DMSOP consumption analysis and 20 mM lactate + 10 mM DMSO for DMSO consumption analysis) was drawn out of a Hungate tube and transferred to a 2 ml GC vial and heated to 80°C (with the lids off to remove DMS from the system) for 90 minutes. This step inactivates the cells and prevents further growth. Following this, 100 µl of 10 M NaOH was added to the DMSOP condition only – this allows any residual DMSP in the sample to convert to DMS and DMSOP to convert to DMSO. Following addition, the sample was heated again as before with the lids off to allow removal of DMSP-derived DMS. Once the heating step was complete for the DMSOP condition, both DMSOP and DMSO condition samples received 100

µl $SnCl_2$ (880 mM) under acidic conditions and were immediately crimp sealing with PTFE/rubber crimp caps. The sealed samples were incubated a final time at 55°C for 90 minutes to let DMSO in the sample convert to DMS. The samples were left to headspace at room temperature in the dark overnight before being analysed by GC.

## 2.9 Growth Curves – DMSP Lyases

Growth curves of various bacterial strains containing the different DMSP lyases were constructed to monitor how well they could grow on DMSOP as sole carbon source compared with DMSP and succinate (positive control). Strain information can be found in Table 4. The strains were first grown in rich media at 30°C with shaking for 2 days before the cultures were adjusted to an $OD_{600}$ of 1 and washed three times in minimal media. A 20 µl aliquot of the washed cells was used to inoculate 1 ml of minimal media (within a 24 well plate) containing 1 mM of either DMSP, DMSOP or succinate as well as a no carbon source negative control. Each condition was done in triplicate. The plate was then run on a plate reader at 30°C for 2 days. $OD_{600}$ readings were taken every 30 minutes with 5 seconds of medium intensity orbital shaking taking place before each read.

*Table 4* – **Rich and minimal media used to grow strains containing the various DMSP lyases.**

| Strain | Rich Media | Minimal Media | DMSP lyase(s) |
|---|---|---|---|
| *Alcaligenes faecalis* WT | LB | M9 | *dddY* |
| *Alcaligenes faecalis dddY*$^-$ | LB | M9 | *dddY*$^-$ |
| *Ruegeria pomeroyi* DSS-3 J470 | YTSS | MBM | *dddW, dddQ, dddP* |
| *Oceanimonas doudorofii* | LB | M9 | *dddP* |
| *Halomonas caisteri* HTNK1 | YTSS | MBM | *dddD* |
| *Labrenzia aggregata* | YTSS | MBM | *dddL* |

## 2.10 DMSP and DMSOP catabolism by DMSP lyases

Headspace DMS produced from lysed DMSOP and DMSP was measured by gas chromatography. 5 ml starter cultures of strains containing different DMSP lyases were grown in rich media, 50 µl of each strain was used to inoculate a fresh 5 ml rich culture containing 1 mM DMSOP or DMSP. These were incubated at 30°C with shaking for 2 days. For DMSOP samples, following incubation, GC samples were prepared by adding 200 µl NaOH to 0.5 ml samples of each culture in Eppendorfs and incubating at 80° for 2 hours with the lids off to remove any DMS produced from lysed DMSP. 200 µl samples of the incubated cultures were transferred to 2 ml GC vials before adding 100 µl of $SnCl_2$ under acidic conditions and immediately crimp sealing with PTFE/rubber crimp caps. The vials were incubated at 55°C for 90 minutes and then again one final time at 22°C overnight in the dark before being monitored by GC. For DMSP samples, following incubation, GC samples were prepared by adding 200 µl NaOH to 200 µl sample of each culture in 2 ml GC vials and immediately crimp sealing with PTFE/rubber crimp caps. Samples were incubated at 80° for 2 hours and then again one final time at 22°C overnight in the dark to headspace before being monitored by GC. For both DMSP and DMSOP samples, protein content will be estimated by Bradford assays.

# CHAPTER 3

# RESULTS - METHOD DEVELOPMENT FOR DMSOP DETECTION

## 3.1 Introduction

To be able to screen for DMSOP synthase activity, it is necessary to be able to reliably detect DMSOP (and related compounds DMSP and glycine betaine) within cell extracts for varying organisms. Several methods were investigated including an already established gas chromatography (GC) method (which has proven successful in detecting concentrations of DMSP as low as 0.015 nmol) as well as methods not previously used in the Todd lab, e.g. Nuclear Magnetic Resonance Spectroscopy (NMR) and DMSO reductase assays.

## 3.2 Screening of *P. bermudensis* library using GC to detect DMSOP synthase activity

A total of 187 colonies of *Rhizobium* containing the *P. bermudensis* library (each clone predicted to contain ~30kb insets) have been screened to date. Of these, several positive colonies were identified as having a large amount DMS in the headspace (produced from reduced DMSO), see Table 5. However, when the clones were rechecked or isolated and reconjugated back into *Rhizobium* none of the phenotypes were reproduced (one example is shown in Table 4). The variability in detection with the GC method is likely the cause of false positives in the screening procedure, as a result, there was a need to develop a more reliable screen.

*Table 5* – **Headspace DMS measured from colony 125 (peak area) against *Rhizobium* and media only controls.**

| Sample | DMS Peak Area (Replicate 1) | DMS Peak Area (experiment repeat) | |
|---|---|---|---|
| | | Replicate 2 | Replicate 3 |
| Media only control | 8505.54 | 8596.01 | 9413.06 |
| *Rhizobium* control | 6115.70 | 15,416.10 | 16,408.50 |
| Colony 125 (*P. bermudensis* library) | 128,586.00 | 1195.39 | 540.98 |

## 3.3 Outcomes for GC detection of DMSOP

So far efforts to detect DMSOP via a gas chromatography-directed method have proven difficult. Direct detection of DMSOP is not possible via GC and as such an indirect detection needs to be employed whereby DMSOP is chemically converted to DMSO which is subsequently chemically converted to DMS that can be detected via GC. This multi-step process introduced a large margin of error at each reactionary stage. Additionally, the sample preparation is very time consuming involving long incubation periods. Due to the nature of the chemical conversion, we also cannot be certain that the DMS we are detecting is purely produced via DMSOP degradation as the *P. bermudensis* organism is a known producer of DMSP. The chemical conversion of the DMSOP to DMSO via addition of NaOH will also cause the cleavage of DMSP to DMS. As a result, there could remain a lingering amount of DMSP-derived DMS contamination in the sample, potentially giving false positives. Limitations of this method led to the exploration of alternative assays for DMSOP detection.

## 3.4 Detection of DMSOP via $^{1}$H-NMR

The sensitivity/reproducibility issues with using GC methods primarily led me to investigate the use of NMR to detect the actual DMSOP molecule. Pure samples of DMSOP (5 mM) were run on $^{1}$H-NMR to determine the chemical shifts and splitting pattern of the peaks in conjunction with what was expected from the literature. Figure 19 shows the $^{1}$H-NMR spectra of 5 mM DMSOP. In addition to DMSOP, related compounds DMSP and glycine betaine were also run (see Figure 20 and 21 respectively) as they are of importance to the work being done in the Todd lab.

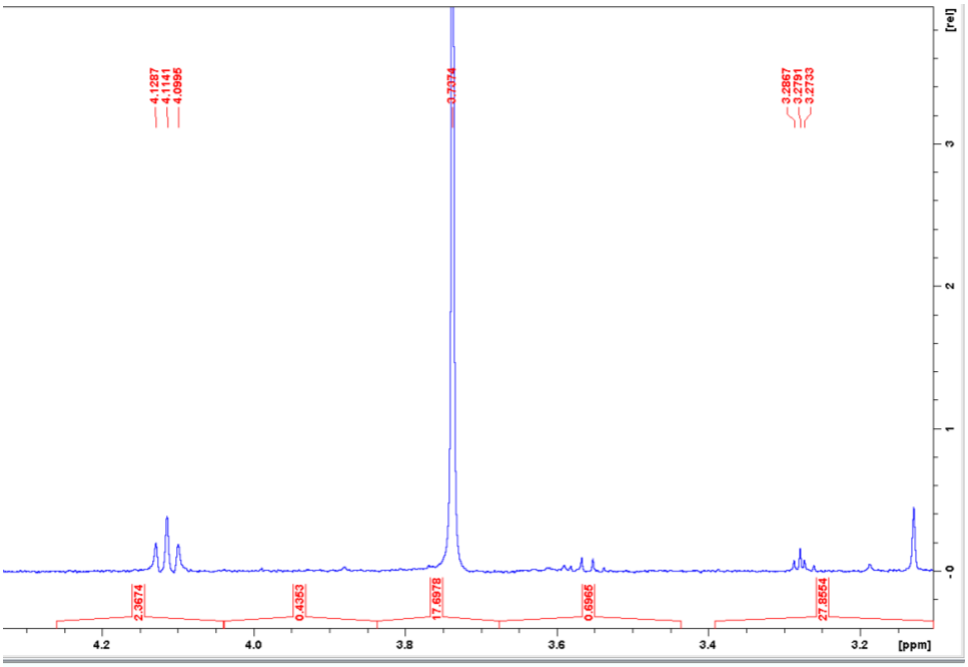


*Figure 20* – **$^{1}$H-NMR spectrum of 5 mM sample of DMSOP.** The singlet signal at 3.7 ppm was used as the identifying peak and corresponds to the hydrogen atom environments present on the methyl groups adjacent to the sulfur molecule.

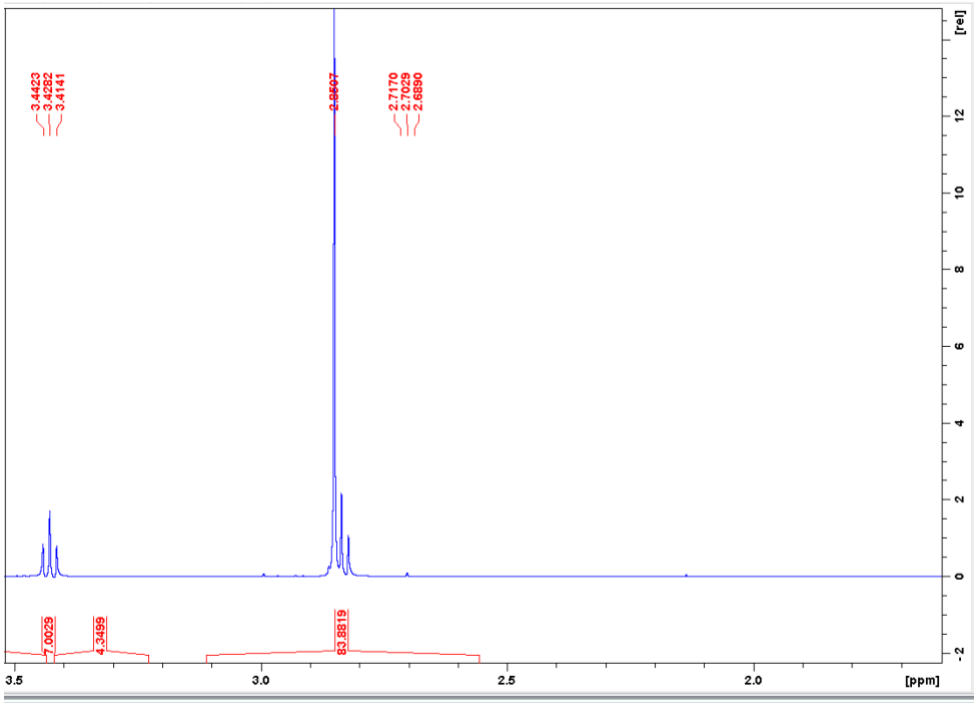


*Figure 21* – **$^{1}$H-NMR spectrum of 5 mM sample of DMSP.** The singlet peak at 2.85 ppm was used as the identifying peak and corresponds to the hydrogen environments on the methyl groups adjacent to the sulfur molecule.

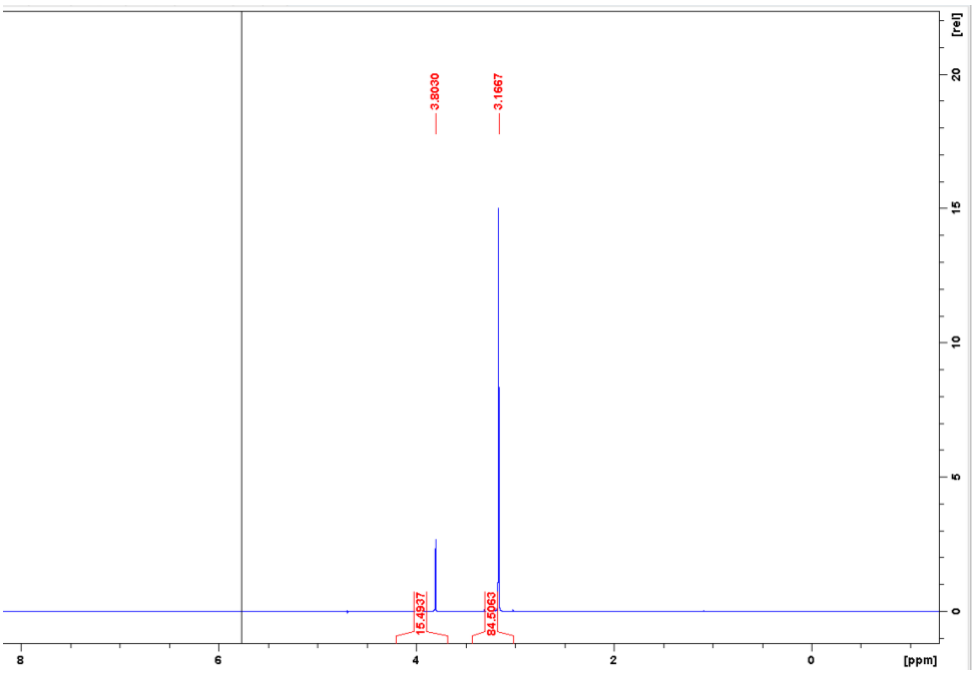


*Figure 22* – **$^{1}$H-NMR spectrum of 5 mM sample of glycine betaine.** The larger singlet peak at 3.16 ppm was used as the identifying peak and corresponds to the hydrogen environment on the (CH) adjacent to the nitrogen molecule.

Once the chemical shifts of the identifying peaks had been established, calibration curves of these compounds were constructed using known concentrations of each compound against the peak areas of the identifying peaks. Figures 22, 23 and 24 show the calibration curves of

DMSOP, DMSP and glycine betaine respectively. The sensitivity for detection was determined to be around 0.1 mM for each compound.

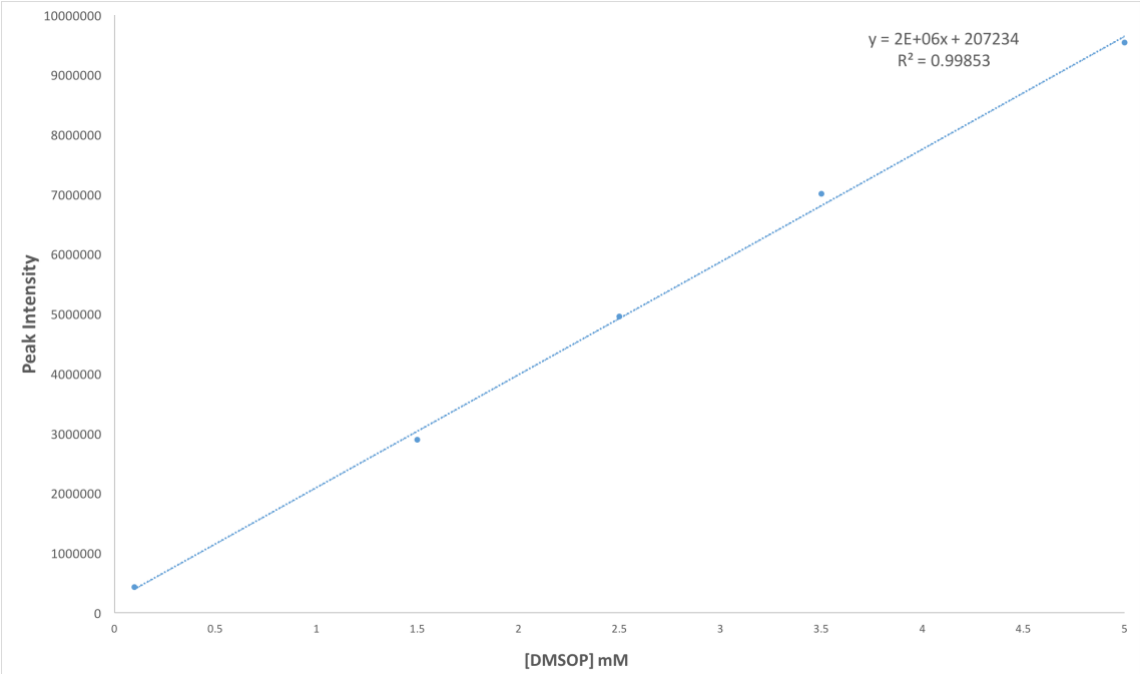


*Figure 23* – **Calibration curve of DMSOP for $^{1}$H-NMR.** Concentration range is from 0.1 mM to 5 mM DMSOP. Each concentration point is an average of 3 replicates.

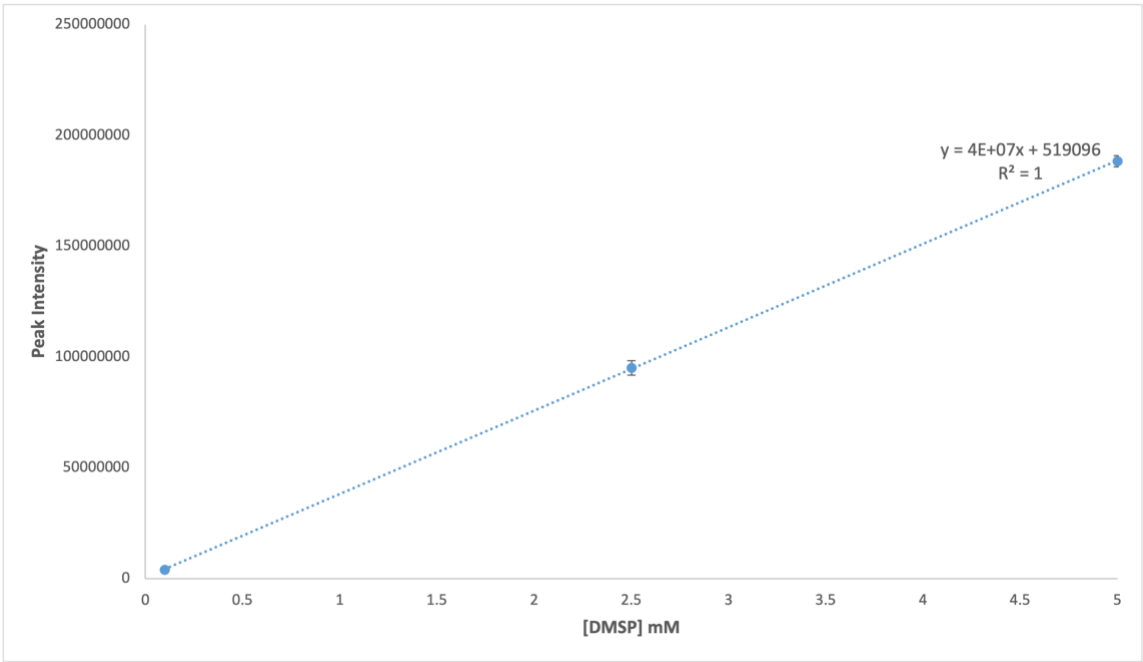


*Figure 24* – **Calibration curve of DMSP for $^{1}$H-NMR.** Calibration range is from 0.1 mM to 5mM DMSOP. Each concentration point is an average of 3 replicates.

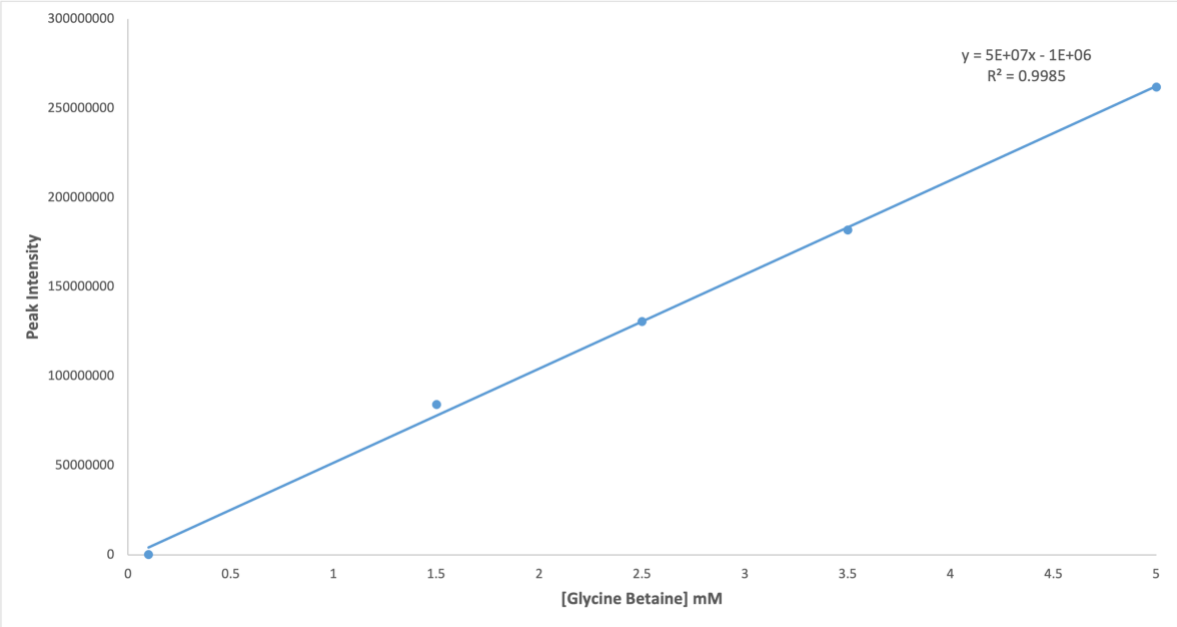


*Figure 25* – **Calibration curve of glycine betaine for $^{1}$H-NMR.** Calibration range is from 0.1 mM to 5 mM glycine betaine. Each concentration point is an average of 3 replicates.

The constructed curves would subsequently provide a reference for the concentrations of each compound able to be identified within cell extracts. The first cell extract to be tested using NMR to detect for DMSOP, DMSP and glycine betaine was the known DMSP producer *L. aggregata* (Figure 25).

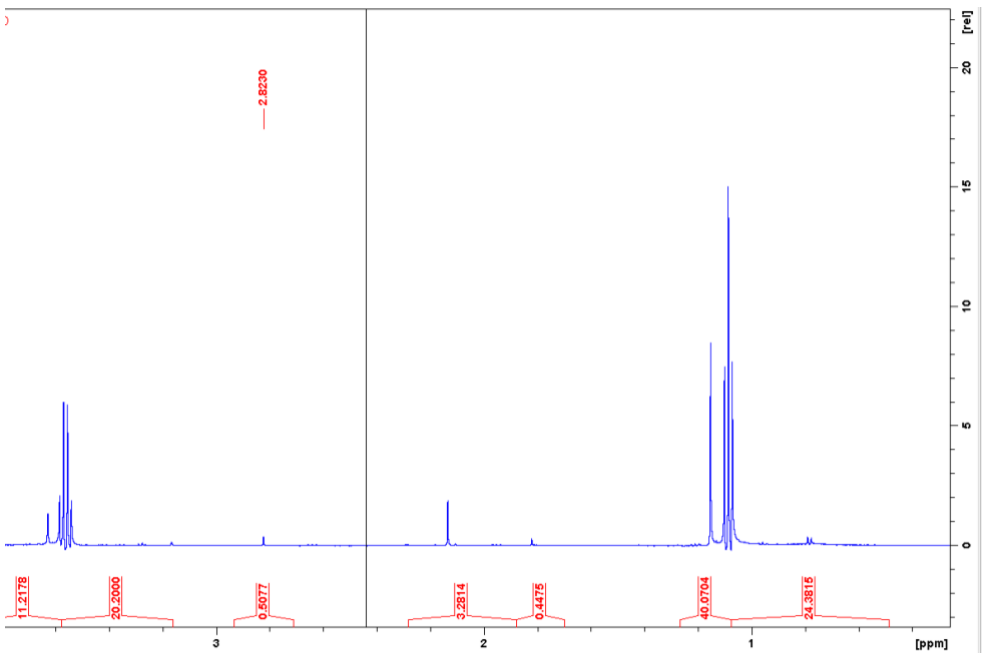


*Figure 26* – **$^{1}$H-NMR spectra for *L. aggregata* cell lysate sample.** DMSP detection peak is shown at 2.8 ppm.

The spectra in Figure 24 shows a singlet peaks at 2.8 ppm indicating the presence of DMSP. The peak intensity was used in conjunction with the DMSP calibration curve to estimate the concentration of DMSP within the samples to approximately 0.1 mM in *L. aggregata*. Due to this value being on the lower end of the constructed calibration curve, a more concentrated cell lysate sample was planned to be used in following runs to more accurately determine the detection of DMSP in the sample. Larger batch cultures of *L. aggregata* (50 – 100 ml) were planned to be grown, spun down and resuspended in 5 ml of sterile distilled water prior to sonication. The reasoning being that the larger volume of cells should increase the concentration of DMSP and potentially any DMSOP that may be present in the sample and thus putting the concentrations of these compounds firmly within the scope of detection. More testing and validation experiments needed to be done on this method to approve its use for detection of DMSOP, DMSP and glycine betaine within cell extracts. Specifically, it would be useful to know whether lysate samples from the known bacterial DMSOP producer *P. bermudensis* would generate a DMSOP detection signal using NMR for detection however due to the COVID-19 pandemic and subsequent lockdown, I became unable to finish this work. Despite this, the preliminary results do show a potential to use NMR as a way to detect for other compounds of interest within the Todd lab in addition to DMSOP, DMSP and glycine betaine. In terms of using NMR as a screening method for DMSOP synthase activity in *P. bermudensis* genomic library cosmids, it is not well suited. NMR is not a high throughput technique and would be better used in conjunction with the GC screening assay as a way to conclusively determine the presence of DMSOP in potentially interesting colonies found from GC.

## 3.5 DMSO reductase assay

In addition to using NMR to solve sensitivity issues with the GC assay for DMSOP detection, I also investigated whether DMSO reductases would work on DMSOP. Initially my plan was to use DMSO reductase to measure the DMSO formed by chemical lysis of DMSOP. However, I established that the DMSO reductase purified from *Shewanella oneidensis* MR-1 could also use DMSOP as a substrate and the kinetics were determined. From the graph (see Figure 26), the $K_m$ and $V_{max}$ were determined as 1.01 ± 0.84 mM and 100.3 ± 26 mM $s^{-1}$ respectively. As a

comparison the $K_m$ and $V_{max}$ for DMSO as a substrate (determined by Kabiru Usman, UEA) are 0.19 ± 0.056 mM and 146 ± 13 mM $s^{-1}$ respectively. This shows that while DMSOP is not the preferred substrate for DMSO reductase it still has a reasonable affinity to the enzyme. More concentrations should be added to the graph in Figure 16 to gain a more accurate fit of the line and thus more trusted values for the enzyme kinetics. However, this is a significant finding as it identifies a novel step in the organosulfur cycle that has not yet been reported. Due to the COVID-19 pandemic, I ran out of time to sufficiently optimise this assay for use in screening for DMSOP in cell cultures, but it does provide a good platform from which to build upon in the future. Additionally, in future experiments it would be interesting to examine whether other bacterial species with a DMSO reductase can effectively substitute DMSOP for DMSO as an effective terminal electron acceptor in anaerobic respiration to determine how widespread this ability is within the marine sulfur cycle.

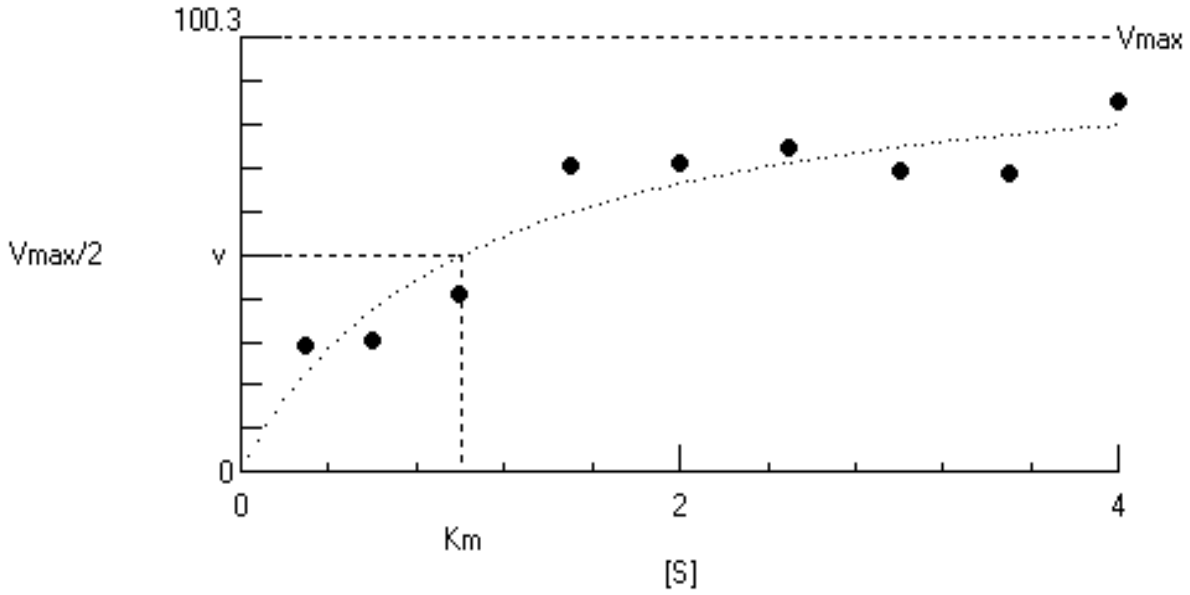


*Figure 27* - **Michaelis-Menten plot for DMSO reductase using DMSOP as substrate.** Substrate (DMSOP) concentration is given in mM and Vmax is mM $s^{-1}$.

## 3.6 Anaerobic growth of *S. oneidensis* (comparison of DMSO/DMSOP as terminal electron acceptor – in collaboration with K. Usman and J. Wang, UEA)

The finding that DMSO reductase from *S. oneidensis* can act on DMSOP has extended what we currently know of the sulfur cycle. In addition to using this mechanism to create a suitable

screening method for DMSOP in *P. bermudensis*, it also became important to understand to what extend *S. oneidensis* can use DMSOP over DMSO as a terminal electron acceptor during growth. Figure 27 shows the relative growth of *S. oneidensis* under different electron accepting conditions and Figure 28 shows the depletion of DMSO/DMSOP over the 72 hours of growth. The results show that *S. oneidensis* is able to utilise both DMSO and DMSOP effectively as electron acceptors during growth although better growth is achieved in the DMSO condition. From the GC results, we can see that total DMSOP was depleted in the first 24 hours of growth while DMSO was not depleted until 36 hours post inoculation.

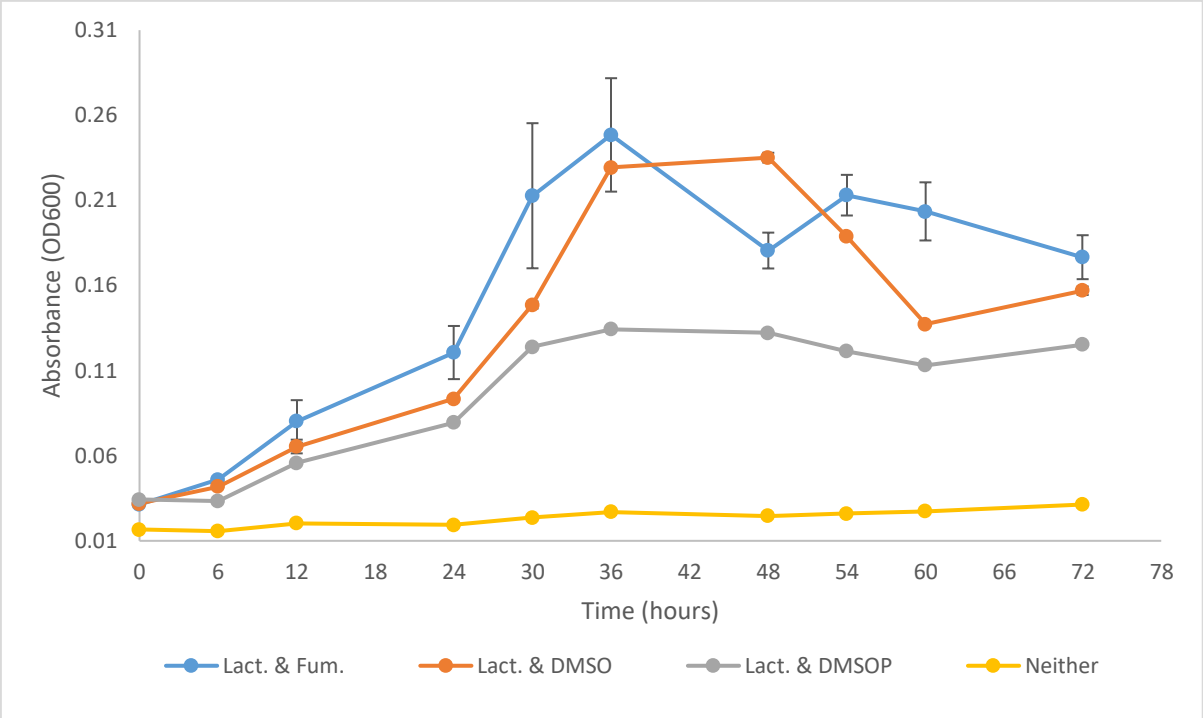


*Figure 28* – **Growth curves of *S. oneidensis* on lactose with different electron accepting conditions.** Each data point is averaged over 3 replicates. Most amount of growth is seen in the Lact.& Fum condition followed by Lact & DMSO and then Lact & DMSOP.

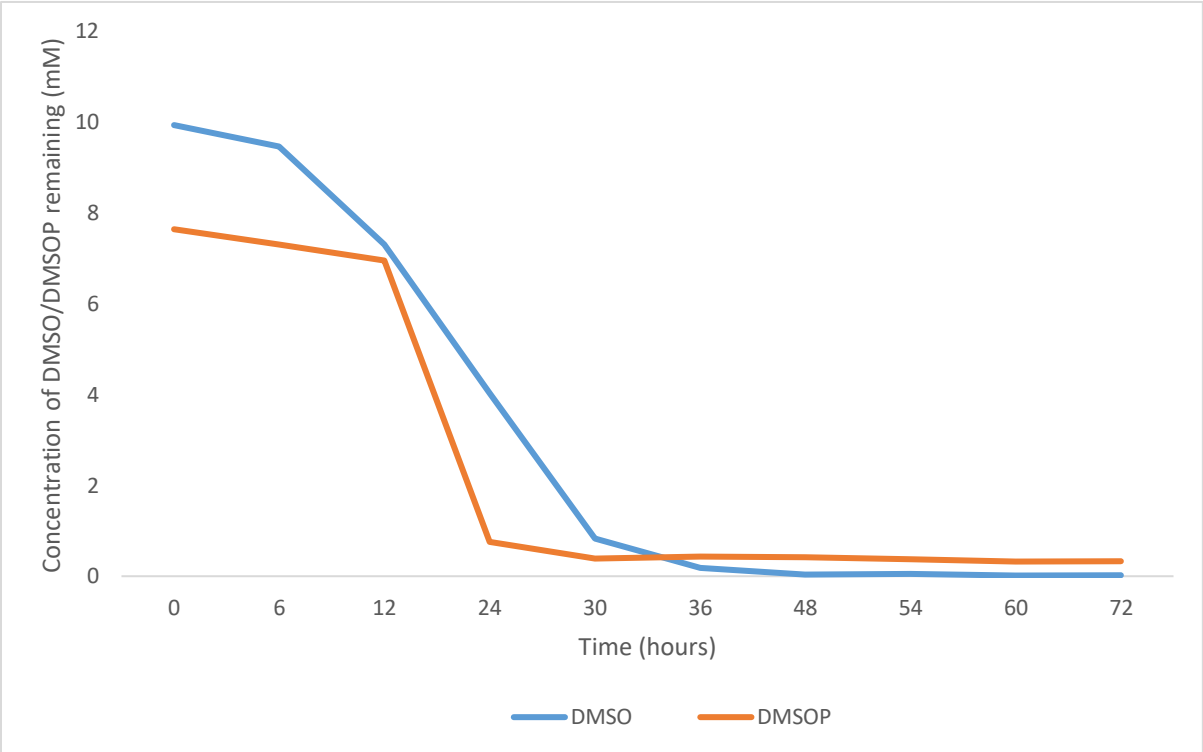


*Figure 29* – **Depletion of DMSO and DMSOP in conditions Lactose + DMSO and Lactose + DMSOP respectively over time.** Results are averaged over three replicates. Decrease in concentration of DMSO/DMSOP remaining is seen over time.

## 3.7 Conclusions

To summarise, three different methods for detection of DMSOP were explored in this section of work: GC, NMR and DMSO reductase assay. The GC assay initially seemed promising as it provided a way to screen hundreds of samples at a time with the aid of the automated sampler. Several positive colonies came out of this screening method with high peak areas for DMS (assumed to be derived from DMSOP). However, upon returning to the original colony and repeating the assay multiple times to test for DMSOP-derived DMS via GC, the same high values for the DMS peak area were not able to be achieved. This led to the realisation that these initially promising results were most likely false positives because of sensitivity issues with the screen. In addition, there wasn't confidence in the assay's ability to remove all present DMSP-derived DMS from the samples which could also skew the results, again leading to false positives. The multiple chemical conversions required in the GC assay also introduced large margins of error at each reactionary stage. Limitations in the GC assay because of the indirect quantification of DMSOP and sensitivity issues made it an unreliable method for screening DMSOP leading to NMR being explored as screening assay for more direct quantification of the DMSOP molecule.

Despite its ability to directly quantify DMSOP (as well as DMSP and glycine betaine) within cell extracts, the NMR method has a significant drawback in terms of how many samples can be screened. As hundreds of samples need to be tested, the lack of high throughput machinery with the NMR assay makes it more suitable as a confirmation technique to be used in conjunction with another assay such as the GC assay potentially. Any 'positive' results from the GC can quickly be confirmed with NMR to determine if the reading is a true reflection of DMSOP concentration. In addition, more validation needs to be done with using NMR to detect compounds of interest within cell extracts. As seen from this work, large amounts of cell culture need to be used to get accurately detectable intracellular concentrations of the compound of interest. The spectra of cell culture (Figure 25) also shows multiple unknown peaks because of the crude extract, possible ways to improve this and optimise the process is to go through purification steps. All in all, there is much scope to improve the NMR assay

for detection of compounds in cell culture however more testing and validation experiments will need to be done to achieve an optimised system.

Perhaps the most interesting of all three screening techniques investigated is the DMSO reductase assay. The finding that DMSO reductase can utilise DMSOP as sole substrate is novel and extends what is currently known about the global sulfur cycle. Many questions come up from this finding and it opens the door to further exciting work including:

- Finding how abundant DMSO reductase is across marine organisms and organisms that are known to produce DMSP/DMSOP?
- Whether the organisms that contain it are actively contributing to global DMSP levels by acting on DMSOP within the marine environment and whether this means our current estimations of DMSP levels are wrong?
- How the DMSO reductase/DMSOP system is regulated?

In terms of moving forward with the DMSO reductase assay as a screening technique for DMSOP synthases, much work is needed. To date, no cell extract samples have been tested on the DMSO reductase assay. As with the NMR assay, it is likely that large volumes of culture will need to be prepared to get a high enough concentration of DMSOP to be detected by the assay. In addition, due to the crude nature of the cell extract sample, it is possible that there might be assay interfering compounds within the lysate which may need to be removed and the sample purified in some way before the assay can be done. In addition, the cuvette method that the assay has been performed with to date to generate the enzyme kinetics with DMSOP as substrate is not a high throughput method. The assay would benefit from being optimised to perform in a 96 well plate where multiple samples from different cosmids of the *P. bermudensis* will be able to be assayed in one go. However with this, validation experiments will need to be conducted and standard curves will need to be constructed to see whether the assay will perform within a 5% error margin of the original cuvette protocol. Despite this, the DMSO reductase assay is perhaps the most promising of the three screening techniques investigated and the work conducted with it so far provides a lot of scope for further experimentation to be done. In addition to the screening possibilities that the finding

that DMSO reductase can act on DMSOP gives us, we also have scope to do further experimentation on what the impacts of this finding are in the marine environment. From the growth curve experiments, we can see that while *S. oneidensis* grows better with DMSO, it's preferred substrate, as a terminal electron acceptor, it can still use DMSOP to grow to similar levels. There is much we still don't understand about how DMSOP is generated and catabolised in the marine environment and this work gives a good standing for further experiments to be conducted to fill this gap in our knowledge of the marine sulfur cycle.

# CHAPTER 4

# RESULTS - CATABOLISM OF DMSOP VIA DMSP LYASES

## 4.1 Introduction

To study all aspects of DMSOP cycling, it's necessary to understand not only how the molecule is synthesised but also how it is broken down. This section of work focuses on the catabolism of DMSOP by various DMSP degraders and to what extent they can use DMSOP as a source of carbon and sulfur for growth. Preliminary work done in Todd's lab by Curson and Walsham invalidated the claims made in the Thume DMSOP paper that the known DMSP lyase, DddY, in strain *Alcaligenes faecalis* does not function to break down DMSOP as it does DMSP. Following on from that, I planned to test the other known DMSP lyases for their ability to catabolise DMSOP. This was done via the construction of growth curves to discern the growth phenotypes of strains containing the known DMSP lyases (as described in table 4) while they were growing in minimal media containing DMSOP, DMSP and succinate. Additionally, GC methodology was employed to measure any DMSOP -derived DMS present following incubation of each of the DMSP lyase-containing strains with DMSOP.

The DMSP lyases can be placed into two groups for ease of explanation: Group 1 (those that produce DMS and acrylate) and Group 2 (those that produce DMS and 3-HP). It is important to test both to determine which potential pathway the degradation of DMSOP might proceed by. Table 6 shows information regarding the strains, their Ddd machinery and which group they belong to.

*Table 6* – Strain information regarding the DMSP lyase enzymes they possess.

| Strain | DMSP lyase(s) | Group |
|---|---|---|
| *Alcaligenes faecalis* WT | *dddY* | 1 |
| *Alcaligenes faecalis dddY*$^-$ | *dddY*$^-$ | |
| *Ruegeria pomeroyi* DSS-3 J470 | *dddW, dddQ, dddP* | |
| *Oceanimonas doudorofii* | *dddP* | |
| *Labrenzia aggregata* | *dddL* | |
| *Halomonas caisteri* HTNK1 | *dddD* | 2 |

## 4.2 DMSOP lyases - Growth Curves

Bacterial cultures containing the various DMSP lyases were tested for their ability to grow on DMSOP (1 mM) as sole carbon source in comparison to DMSP (1 mM), succinate (1mM) and no added carbon. While several strains showed the ability to grow on DMSOP, like DMSP, the use of the plate reader to construct such growth curves did not allow for them to grow as effectively as they do in larger culture volumes. In all cases the $OD_{600}$ levels were much lower than the levels seen in previous larger culture studies - which usually have final $OD_{600}$ values in excess of 1 during stationary phase. Growth curves for *H. caisteri* and *R. pomeroyi* can be seen in Figures 29 and 30 respectively. The strains grew as expected with the most growth being seen with succinate as sole carbon source followed by DMSP and finally DMSOP.

All strains tested thus far (*R. pomeroyi*, *Halomonas caisteri* HTNK1) all grew to a final $OD_{600}$ of around 0.33 however previous experiment conducted in the Todd lab would suggest that this is a low value as both strains would usually grow much better. This could be the result of using a 6 well plate to grow the strain instead of a much better aerated flask culture. Due to the COVID-19 pandemic, strains containing the other DMSP lyases (Table 4) were not able to be tested with this method to determine their relative affinities for DMSOP as a substrate for growth.

Further optimisation needs to be done with this method. The low optical density values for the stationary phases of the two strains tested in this growth study suggest that something is hindering the growth of these organisms. In future a comparative study should be conducted using shake flask cultures where samples will be taken manually for the optical density to be recorded at regular intervals during growth. This will allow us to determine if the values we get from the plate study are comparative and therefore true. Nevertheless, the plate data has indicated that bacteria with DMSP lyases can use DMSOP as a carbon source albeit not as effectively as DMSP.

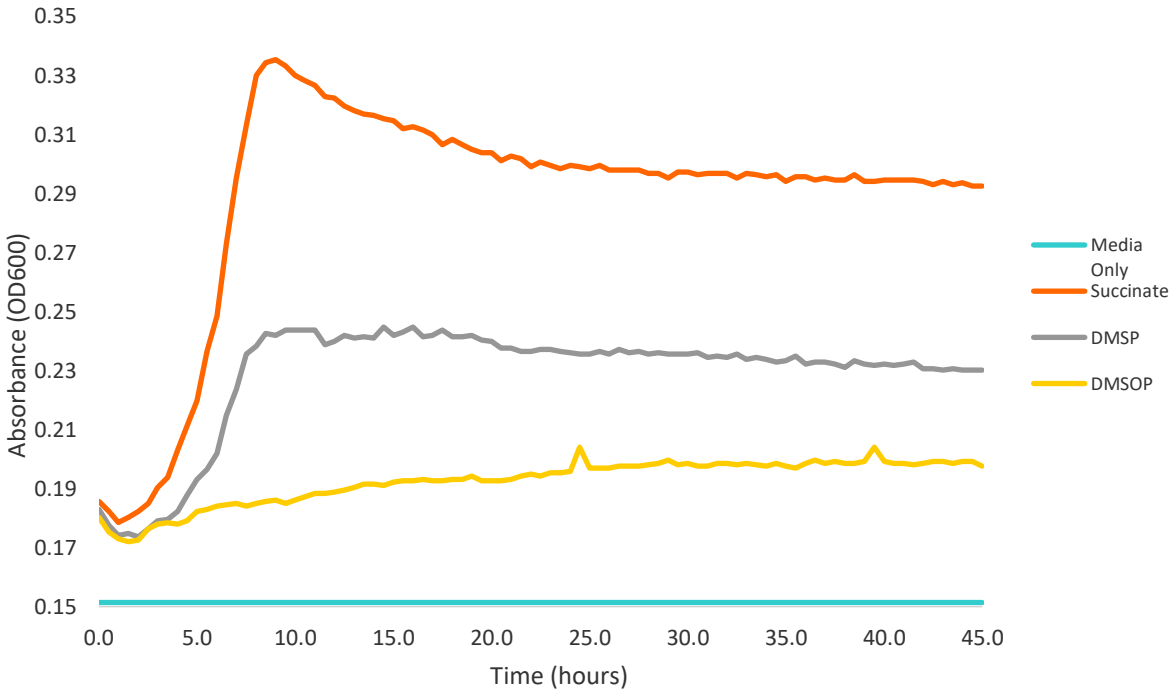


*Figure 30* - **Growth of *Halomonas caisteri* HTNK1 over 48 hours using sole carbon sources: succinate, DMSP and DMSOP.** Time is shown in hours and optical density was measured at 600 nm. *H. caisteri* is shown to be able to grow on DMSOP as sole carbon source however at a much lower amount than when using DMSP or succinate as sole carbon source.

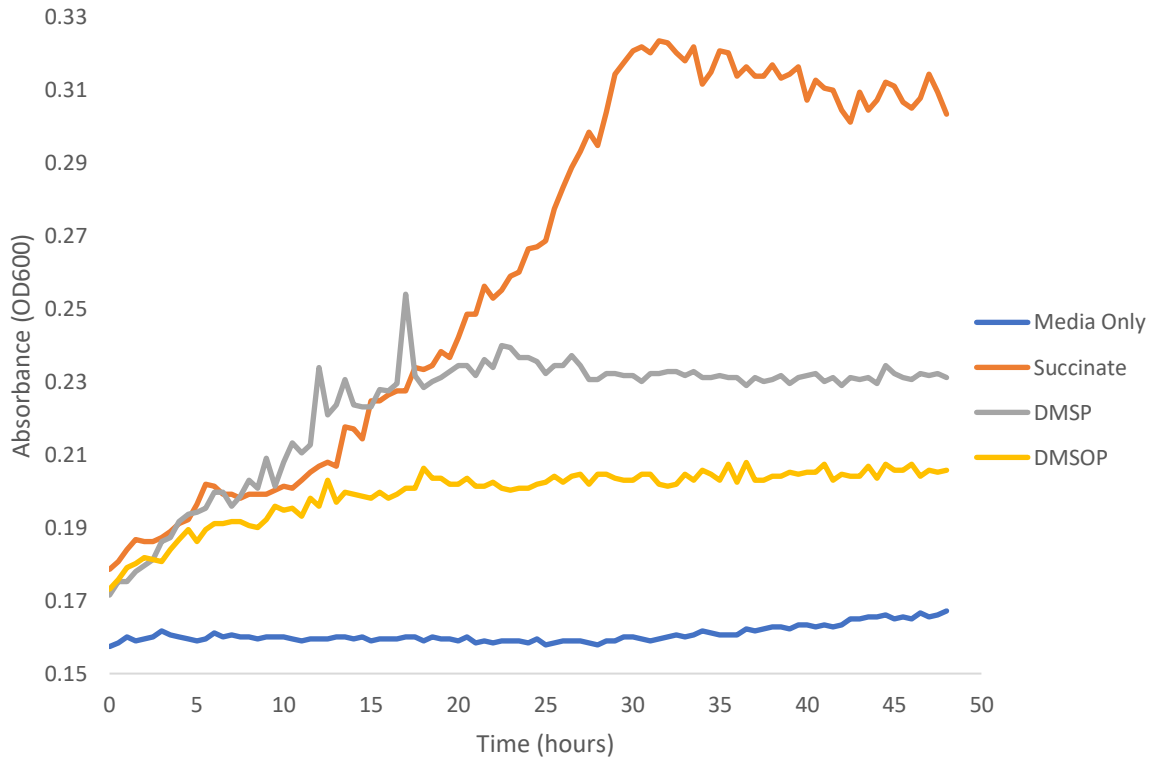


*Figure 31* - **Growth of *Ruegeria pomeroyi* J470 over 48 hours using sole carbon sources: succinate, DMSP and DMSOP.** Time is shown in hours and optical density was measured at 600 nm. *R. pomeroyi* is shown to be able to grow on DMSOP as sole carbon source however at a much lower amount than when using DMSP or succinate as sole carbon source.

## 4.3 DMSOP catabolism by DMSP lyases (planned work)

Gaseous headspace DMS was to be measured by GC following chemical lysis of any DMSOP present in the cell cultures of strains containing the various DMSP lyases. This data would be compared to headspace DMS produced from lysis of DMSP to determine the relative efficiency of the various DMSP lyase enzyme with DMSOP. Due to the COVID-19 pandemic and subsequent lockdown, I was unable to complete this work.

## 4.4 Acknowledgment of the work done by Yu-Zhong Zhang *et al*

Collaborative work on DMSP lyases acting upon DMSOP (to produce DMSO and acrylate) with scientists in Qingdao, China has yielded information on the enzyme kinetics of various cupin superfamily DMSP lyases with DMSOP as substrate (see Table 7). The $K_m$ values obtained from this work were in the same millimolar level as DMSP (shown in Table 7 from Lei *et* al (78)) indicating that the DMSP lyases have a high affinity for DMSOP in addition to their preferred substrate DMSP. It is likely that due to the similar $K_m$ values to DMSP, the tested lyase enzymes likely follow a similar catalytic mechanism to break down DMSOP and DMSP. It is possible that this high affinity for DMSOP means that there are no specific DMSOP lyases in nature and that marine organisms utilise their DMSP lyases to metabolize DMSOP in addition to DMSP with the only limiting factor to DMSOP catabolism being its abundance in the environment. The kinetic information produced from this study will help to inform further work that can be done to aid our understanding of the global sulfur cycle including: the mechanisms behind how the DMSP lyases act upon DMSOP and the relative effect DMSP lyase-containing organisms have upon the production of DMSO from DMSOP in the environment.

*Table 7* – **Kinetic parameters for cupin superfamily DMSP lyases with DMSOP (averaged over three replicates) as well as DMSP lyases with DMSP as substrate for comparison.**

| Strain | DMSP lyase(s) | Yu-Zhong Zhang *et al* $K_m$ and $k_{cat}$ DMSOP | Lei Lei *et al* $K_m$ and $k_{cat}$ DMSP |
|---|---|---|---|
| ***Alcaligenes faecalis*** | DddY | **41.0** ± 6.3 mM<br>**26.5** ± 1.8 $s^{-1}$ | **2.56** mM<br>**0.9 x $10^3$** $s^{-1}$ |
| ***Pelagibacter ubique*** **HTCC1062** | DddK | **24.1** ± 3.4 mM<br>**14.8** ± 0.9 $s^{-1}$ | **5.1** mM<br>**3.1** $s^{-1}$ |
| ***Ruegeria pomeroyi*** **DSS-3** | DddW | **11.2** ± 1.6 mM<br>**26.7** ± 3.9 $s^{-1}$ | **4.5** mM<br>**17.33** $s^{-1}$ |
| ***Ruegeria lacuscaerulensis*** **ITI_1157** | DddQ | **9.6** ± 1.6 mM<br>**40.0** ± 6.5 $s^{-1}$ | **39.1** mM<br>**1.05 x$10^{-1}$** $s^{-1}$ |

# CHAPTER 5

# DISCUSSION AND CONCLUSIONS OF RESULTS

The primary focus of this report is the study of the novel organosulfur compound DMSOP, which was discovered and characterised by Thume *et al* (2018), in terms of its production and cycling by marine microorganisms. The molecular method behind the production of DMSOP via DMSP is unknown and as such it is important to elucidate it to understand why this molecule is made by the organisms that have the ability to produce it. Thume *et al* showed that marine Roseobacter *Pelagibaca bermudensis,* contained this ability and produced DMSOP. To understand why DMSOP was being produced I began a screening experiment of a *P. bermudensis* genomic library within the heterologous host *Rhizobium leguminosarum* to test for the expression of DMSOP and consequently which genes were being expressed. The screening was done via a chemical catabolism of DMSOP to gaseous DMS which would then be quantified via GC. Of the 187 colonies screened, a few showed promising results however upon repeating these colonies, the results were not reproducible. For a single colony (for example colony 125 showed in table 5) the DMS peak area could range from as high as 128,586 to as low as 540.98. This was well outside of the acceptance range of variance and thus highlighted inconsistencies within the methodology. Errors could have been introduced to the quantification of DMSOP due to the chemical catabolism that it undergoes during the experimental procedure. As DMS is also produced from any present DMSP within the sample, it could also affect the total DMS detected by the GC. Although care was taken to release any DMS produced during the process by allowing it to be released to the environment following the addition of NaOH – some DMS could still have remained. Additionally, we are unable to control for any DMSO already dissolved in the growth media which again, could have accounted for the discrepancies we saw when trying to quantify DMSOP. If this experiment was to be repeated, a potential way to control for DMSP derived DMS would be to sparge the samples before the addition of $SnCl_2$/HCl. However, DMSP derived DMS as well as dissolved DMSO contamination could not account for the large disparity that was seen between the repeats of samples. Therefore, the sensitivity of the GC-based assay comes into question.

Moving on from GC-based methodology I explored a few different options to address the problems I was having for DMSOP screening. Of the avenues explored, the two methods that stood out were NMR spectroscopy and enzymatic assay screening using DMSOP as a substrate. As DMSP has in the past been shown to be quantifiable by NMR methods it seemed

reasonable to assume that the similar compound DMSOP could also be quantifiable this way. Indeed, when Thume *et al* were first investigating the sulfoxonium species DMSOP by mass spectrometry analysis, they synthesised DMSOP by $RuCl_3$/sodium hypochlorite-mediated oxidation of DMSP to use as a reference compound and subsequently confirmed its structure by NMR. A standard curve of known concentrations of synthesised DMSOP was constructed to test the limits of NMR sensitivity. It was determined that the lower limit of detection for DMSOP was 0.1 mM which would be suitable to detect DMSOP concentrations within highly concentrated cell extracts. Using NMR methods for screening were advantageous in that the DMSOP compound itself was able to be detected rather than the derivatives of its catabolism, as with GC methodology, which introduced a level confidence in the results and subsequently would lead into further genetic analysis to find the unknown DMSOP synthase. Unfortunately, NMR also has significant drawbacks most notably in that it is not a high throughput method. This makes it unsuitable for the screening of multiple colonies of the *P. bermudensis* genomic library and thus it was disregarded as a method for screening but rather it will be used to confirm DMSOP's presence in colonies where there is suspected DMSOP synthase activity, perhaps detected via GC or other methods.

The other method investigated for screening for DMSOP synthase activity was an enzymatic assay where a colour change was quantified via UV-vis spectrometry. It was theorised that the DMSO reductase enzyme – which catalyses the reduction of the structurally similar molecule DMSO to DMS during anaerobic respiration would also be able to catalyse DMSOP. The redox potential of methyl viologen is the basis of this assay. When in its reduced form, methyl viologen is colourless however upon oxidation it turns violet/blue. In the presence of DMSO reductase, DMSOP is reduced to yield DMSO and the electrons are in turn used to oxidise the methyl viologen – yielding the colour change. The intensity of the violet/blue colour determines how much of the DMSOP has been reduced. These readings at different concentrations of DMSOP were used to be able to construct the Michaelis – Menten plot to determine the relative affinity of the enzyme towards DMSOP as a substrate. As previously stated, DMSOP has a $K_m$ value (1.01 ± 0.84 mM) relatively close the $K_m$ value of DMSO (0.19± 0.056 mM) – the preferred substrate. This indicates that DMSO reductase has quite a high affinity for DMSOP as a substrate and likely follows the same catalytic mechanism. The affinity of DMSO reductases for DMSOP is an important finding as it extends what is currently known

about the global sulfur cycle – providing a previously unknown pathway to produce DMSO. Despite the important finding, more work is needed on this assay in terms of using it for screening the *P. bermudensis* genomic library to determine DMSOP synthase activity. To date, no cell extracts have been tested using this method, it is unclear whether purification steps will need to be undertaken before accurate readings can be seen therefore additional testing is required. It is likely that alterations of the DMSO reductase assay for DMSOP synthases screening will need to be done to optimise the assay for a 96 well plate which will make the technique high throughput and allow for multiple cosmids to be screened at once. Positive colonies could be further confirmed through NMR before genetic analysis is done to identify the genes involved.

Additionally, because of the screening work and the finding that DMSO reductase (purified from *S. oneidensis* MR-1) can use DMSOP as substrate, further work was done in collaboration with K. Usman and J. Wang at UEA to try to understand the relationship between *S. oneidensis* (and perhaps other DMSO reductase- producing marine organisms) and DMSOP in the marine environment. Initial studies from the growth curves have established that not only can *S. oneidensis* use DMSOP as a terminal electron acceptor during anaerobic respiration but it also grows to similar levels compared to the DMSO condition. Interestingly, the GC work done shows that the DMSOP was depleted faster than DMSO in the cultures. Further work needs to be done to fully understand the mechanism of DMSOP catabolism in the marine environment and more DMSO reductase- containing organisms will need to be tested to get a better understanding of how widespread the ability to use DMSOP as a substrate by DMSO reductases is and what the relative effect is in oceans. Nevertheless, this work is a good starting point and furthers what we currently know about the sulfur cycle.

In terms of future work, the enzyme kinetics for DMSO reductase activity with DMSOP have been somewhat crudely determined and more concentrations of DMSOP will need to be tested to get a better fit of the curve for the Michaelis – Menten plot and thus more trusted values for the $K_m$ and $V_{max}$. In addition, the finding that DMSO reductases work on DMSOP opens the door to further investigation in the field. Bioinformatics techniques could be utilised to determine other organisms encoding DMSO reductases and how widespread they are in the marine environment. Organisms found to contain DMSO reductases could be grown

in the presence of DMSOP, and the growth phenotype could be determined relative to DMSO. This work could give us a better understanding of how DMSO is produced in the marine environment from DMSOP and how widespread the ability to metabolise DMSOP is. In addition, it would also give us an insight into whether different types of DMSO reductases (i.e Dor- or Dms- types) have better affinities for DMSOP as a substrate.

For the secondary focus of my master's thesis, I was investigating the ability of DMSP lyases to act on DMSOP as well as the ability of organisms containing DMSP lyases to grow on DMSOP as a sole carbon source. For the growth experiments, organisms containing the various DMSP lyases (see table 2) were grown on DMSP, DMSOP, succinate (positive control) and a no carbon source negative control. All organisms were grown in 1 ml cultures containing the various carbon sources and $OD_{600}$ absorbance measurements were taken via a plate reader. For all the strains tested, they all showed similar trends in which they grew the best on succinate followed by DMSP and then DMSOP. These trends show that organisms containing the DMSP lyases tested can indeed utilise DMSOP as a sole carbon source for growth albeit to a lower extent than DMSP. Unfortunately, although the expected trends were seen, all the cultures grew to unacceptable levels for the final $OD_{600}$ readings in the stationary phases of bacterial growth. The typical optical density measurements in the stationary phase of these kinds of growth experiments conducted in the Todd lab are usually in excess of 1. The low OD values in the growth curves could potentially be due to the small volume of 1 ml being used for the cultures that does not allow them to grow as they would normally in larger cultures. As such, further work can be done on this by growing the strains in larger volumes and manually taking optical density measurements to construct growth curves.

In terms of future work, there is much that can be done. The other DMSP lyase-containing organisms have not been grown to date on DMSOP, DMSP and succinate and thus their phenotypes have not been determined. It is possible that there may be some variation between the affinity of each of the different lyases for DMSOP. In addition, the planned work testing the catabolism of DMSOP by the organisms containing the various DMSP lyases was not completed and could provide further insights to the relative activity of each enzyme on DMSOP as a substrate. The mechanism by which the DMSP lyases catalyse the catabolism of

DMSOP to DMSO and acrylate is currently unknown however through the collaborative work by scientist in Qingdao, China, we know that the $K_m$ values for the DMSP lyases with DMSOP is similar to that with DMSP as a substrate. As a result, the catalytic mechanism is likely to be similar, if not the same. However, this will need to be proven beyond speculation, which in turn leaves room to explore the possibilities for further research that the finding entails.

In this thesis, the aims were:

1. Identification of the DMSOP synthase enzyme(s)
2. Identification and characterisation of DMSP lyases with DMSOP lyase ability

Of these aims, neither were met to any conclusive level. With regards to DMSOP synthases, screening methods were developed by GC, NMR and DMSO reductase assays. Due to the global COVID-19 pandemic, I was unable to complete this work, however it does provide a good foothold for future work to be done to identify the DMSOP synthase genes as the methods are in place. The secondary aim was also not completed to a conclusive level. Growth curves were being constructed to determine to what extent organisms containing the various DMSP lyases could grow on DMSOP as sole carbon source (and thus have the ability to catabolise it for its raw components). Of these growth curves, only 2 of the 6 planned strains were able to be grown in the time frame I had and while the trends are what was expected, the low final OD values bring into question the validity of the results. In terms of catabolism of DMSOP to be measured by headspace DMS, this work was planned and would have utilised a similar method of detection as with the initial GC screening method for DMSOP synthase however again, I was unable to see this to completion due to the national lockdown. All in all, the work in this thesis does provide a good foundation for future experimentation, in particular to note is the extension of the known sulfur cycle with the discovery that DMSO reductase can act on DMSOP as a substrate. This discovery opens up the field to a wider range of experimentation including how widespread this ability is amongst other DMSO reductase - containing organisms and thus how impactful it is upon what is currently known as the sulfur cycle.

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