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Characterisation of DMSP synthesis in marine bacteria via the methylation pathway

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Declaration

I, Kasha Cowles, declare that this thesis is a record of my own work and that I composed it. It has not been submitted in any previous application for a degree in any other university. All quotations have been distinguished by quotation marks and all sources of information are specifically acknowledged.

Abstract

Dimethylsulfoniopropionate (DMSP) is one of the Earth's most abundant organosulfur molecules, with approximately 9 billion tonnes being produced annually, and is the primary cause of the release of the climate-active gas dimethylsulfide (DMS). The molecule provides many benefits to the organisms with the ability to synthesise DMSP and many that cannot actively assimilate it from the environment. Although bacteria have been well-documented as DMSP degraders, they have only recently been identified as DMSP producers. The saltmarsh environment is a hotspot for DMSP production, with DMSP concentrations in the Stiffkey saltmarsh sediment (77.1 ± 15.0 nmol DMSP g^{-1}) much higher than the overlying pool water (0.4 ± 0.1 nmol DMSP ml^{-1}). Approximately 25 % of the bacteria isolated from the sediment could produce DMSP, which could be increased following enrichment conditions to 77 %. Whilst most isolates contained the bacterial DMSP-synthesis reporter gene, *dsyB*, several did not (*Alteromonas, Marinobacter*, and *Novosphingobium*). *Novosphingobium* was an interesting strain, as incubation with intermediates from the three DMSP synthesis pathways revealed that *Novosphingobium* only produced DMSP in the presence of intermediates from the methylation pathway. Upon further research, a bacterial methionine methyltransferase was identified and termed '*mmtN*', responsible for the methylation of methionine, producing *S*-methylmethionine (SMM). While SMM is a process found in all flowering plants, this is the first instance observed in bacteria. Upon further analysis, *mmtN* is found within a diverse range of bacteria and has been demonstrated as functional in those tested. An *mmtN*$^{-}$ disruption mutant created in *T. profundimaris* removed the ability of the strain to produce DMSP. However, DMSP synthesis was reinstated when the mutant strain was complemented with *mmtN* from *Novosphingobium*. The results presented in this thesis suggest that bacteria have more than one method for producing DMSP and details the discovery of the second DMSP synthesis gene.

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1 Introduction

1.1 The sulfur cycle

Sulfur is one of the most abundant elements on Earth, with the majority being fixed in mineral and rock forms[1]. However, approximately 1 % of the dry weight of any organism is made up of sulfur in the form of amino acids, such as cysteine and methionine[1]. Sulfur also plays critical roles within coenzymes and metalloproteins[1]. All organisms require sulfur to survive, which exists in many states; however, the most abundant form is an inorganic sulfate (SO_4^{2-})[1]. Fortunately, microorganisms can assimilate the inorganic sulfur into different organosulfur compounds, such as methionine and DMSP[2], making it available to animals that are dependent on preformed sulfur compounds[1].

Figure 1-1 shows how sulfate is assimilated to biogenic sulfur and vice versa several times throughout the sulfur cycle. Through the process of weathering, sulfur dioxide is released into the terrestrial environment, where it is oxidised in the air to produce sulfate[3], allowing the compound to be assimilated by various microorganisms and plants and turned into different organosulfur molecules[4], which animals can then consume. When the animals decompose after death, the sulfur is released back into the soil as sulfate. These are not the only known releases of sulfur into the environment; other emitters include volcanic eruptions and biomass burning[5]. The deposition from the atmosphere and run-off form lakes and rivers ultimately finds its way into the oceans[3] where it may be assimilated into cysteine, methionine and dimethylsulfoniopropionate (DMSP), and finally dimethyl sulfide (DMS)[6].

1.1.1 The assimilation of sulfur

The assimilation of sulfur into DMSP and its release as DMS begins with the production of L-methionine (Met). Met is the prerequisite for DMSO production and is formed from cysteine[7]. The organisms that uptake sulfate require energy for this reaction which is supplied as ATP[6]. The sulfate is then reduced to form a free sulfide[6]. A product from glycolysis, *O*-acetlyserine, combines with the free sulfide to produce cysteine and acetate[8]. Cysteine has multiple roles within the cell, such as the *de novo* production of methionine. This is achieved via the transfer of the cysteine thiol group to *O*-phosphohomoserine, creating homocysteine, which is methylated to produce methionine[6]. The methionine may be used in subsequent reactions through its methyl group, with *S*-adenosylmethionine (AdoMet) acting as a methyl donor.

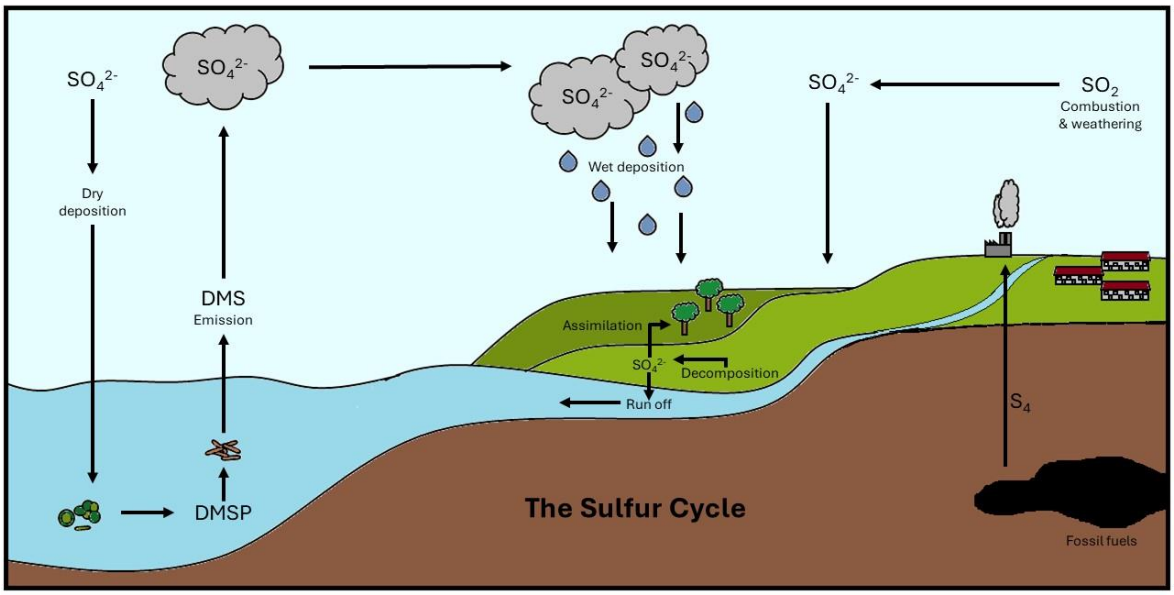

Figure 1-1 The environmental sulfur cycle. Most of the Earth's sulfur is contained within rocks and fossil fuels. However, through the combustion o fossil fuels and weathering, sulfur is released into terrestrial and marine environments. When oxidised in the air, the sulfur becomes SO_2, which reacts with the atmosphere to become SO_4^{2-}. The sulfate is returned as dry deposition and assimilated by plants and microorganisms, reducing it to organosulfur compounds. Subsequently, these compounds can be used by animals and other bacteria and re-released via decomposition. Run-off into rivers moves the SO_4^{2-} back to the ocean, where it can be assimilated into compounds such as methionine. Marine algae can produce DMSP from methionine, which is released and broken down into DMS. The DMS is emitted into the atmosphere and oxidised to form clouds. The clouds move inland, bringing biogenic sulfur to the land through wet deposition. This is the only known movement of biogenic sulfur to land from the oceans.

The biogenic sulfur compounds are well used within all stages of the sulfur cycle by marine organisms. Within the environment, DMS is created through the demethylation or cleavage of sulfur compounds, including DMSP, acrylate and 3HP. Approximately 10 % of DMS produced in the ocean is released into the atmosphere[2]; this equates to about 30 million tonnes and accounts for about 50 % of the biogenic sulfur and 10 % of the total atmospheric sulfur flux[4]. Considering the seasonal cycle, the global annual DMS flux from the oceans ranges from 13 to 37 TgS yr^{-1} [9]. When in the atmosphere, the DMS once again becomes sulfate, including in the form of dimethylsulfoxide (DMSO) and sulfuric acid; these compounds form cloud condensation nuclei (CCN). Once formed, the clouds are blown to shore, where the large volume of sulfur is returned to the terrestrial environment via deposition[1] (Figure 1-1). The return of the sulfur to the terrestrial environment is incredibly important since the land is sulfur depleted. The deposition resupplies the nutrients and creates weathering, completing the cycle and bringing more nutrients into the marine environments by run-off[10].

1.1.2 The CLAW hypothesis

As well as returning sulfur to terrestrial environments, the clouds produced resulting from DMS emissions were theorised to cause an albedo effect that creates a feedback loop which controls the local climate. This theory is termed the CLAW hypothesis after the authors who first hypothesised it[10]. Essentially, the feedback loop was suggested because DMS had been established as a major source of CCN, and the resulting clouds could be controlled by the release and oxidation of DMS[10]. During algal blooms, higher levels of DMS are produced in warmer waters, which may be because the increase in solar radiation results in better growth[3]. The increase in the atmospheric DMS would cause an increase in CCN and, subsequently, cloud formation, resulting in the sun's radiation being reflected away and thus causing the surface waters to cool. The cooling of the water would lead to a decrease in algal growth and DMS production, ultimately reducing cloud cover and allowing an increase in solar radiation and heating of the surface waters. Creating a balanced and ongoing cycle[3]. In 1991, Mount Pinatubo erupted which caused a large amount of SO_2 to enter the stratosphere, as a consequence the UV radiation absorption in the stratosphere was increased, resulting in the amount of UV radiation reaching the troposphere to decrease[11,12]. Although the CLAW hypothesis seems simple and is well referenced, the theory has not been fully validated, and even though there is some evidence that light dosage can drive DMS levels[13], it is now thought that this theory is unlikely, or at the very least, much more complicated than suggested[14].

Although the amount of sulfur released via the DMSP/DMS cycle is huge, today, it is dwarfed by the sulfur released by anthropomorphic production[5]. However, when considered locally, the sulfur produced via these cycles is very important, particularly when considering marine environments and is still considered a key step in the overall sulfur cycle[15]. The distribution and abundance of the sulfur molecules within the environment are mostly a result of the microorganisms that produce and catabolise them, creating biogeochemical cycles on a global scale. Understanding how and why microorganisms use these functions leads to a deeper understanding of the cycle as a whole.

1.2 Dimethylsulfoniopropionate

DMSP is a ubiquitous organosulfur molecule found mainly in marine but also in brackish freshwater environments. With approximately 10^9 tons produced annually[16], DMSP plays a considerable role in the Earth's sulfur cycle[1], is a signalling molecule[1] and a key nutrient source for marine organisms[17], and there have also been suggestions of DMSP providing osmoprotectant and anti-stress properties[6]. As mentioned previously, DMSP is the major

precursor for DMS, a volatile sulfur gas which is climate-active and important within the environment as, when released into the atmosphere, it can affect the Earth's albedo[18] (see above, 1.1.2) and after forming CCN and precipitating, completes the global sulfur cycle[6] (see above, 1.1.1).

1.2.1 DMSP structure

DMSP is a five-carbon sulfonium compound isolated in 1948 from the red algae *Polysiphonia fastigiata*[19]. DMSP is generated from the amino acid methionine and includes a twice-methylated sulfur molecule and propionate, which is a carboxylate (Figure 1-2). DMSP is a zwitterion which means the molecule has a negative charge at one end and a positive charge at the other; in this molecule, the methylated sulfur is positively charged and the oxygen of the carboxylate is negative[20]. The structure of DMSP can help elucidate its function within the environment; the fact that DMS is easily released from the compound via enzymatic cleavage of the sulfur-carbon bond would suggest that the methyl groups are accessible carbon sources[17]. Additionally, the structure of DMSP is highly similar to the well-studied compound glycine betaine (GBT). The only difference between these two compounds is the replacement of the sulfur molecule with nitrogen[21]. As GBT has been well studied and known to be involved in osmoregulation, it would be probable that DMSP may also have this function.

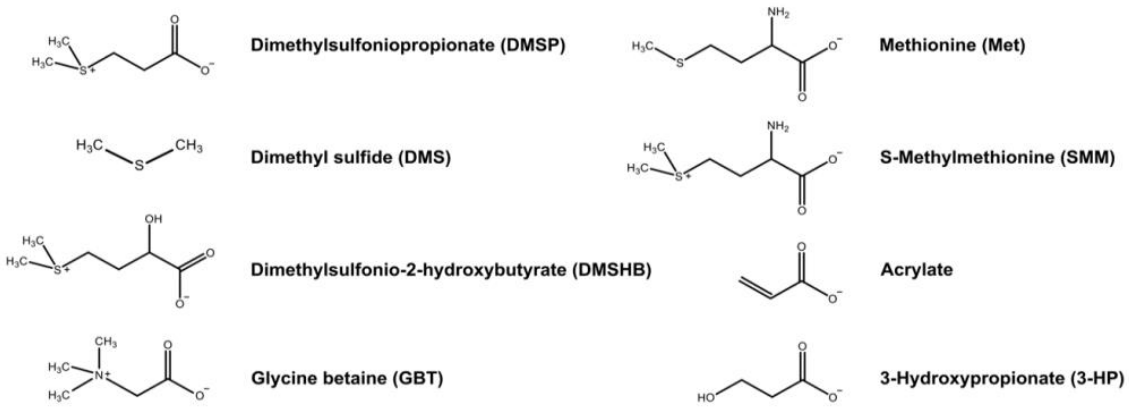

Figure 1-2 DMSP and compounds involved in DMSP cycling[22]. DMSHB and SMM are involved in DMSP production, DMS, Acrylate and 3-Hydroxypropionate are products of the breakdown of DMSP, and GBT is an osmolyte with a similarity in structure and a nitrogen-based homolog to DMSP. As DMSP has two methyl groups which may be removed during catabolism (in the form of DMS), it is classed as a C1 compound. Similarly, DMSHB and SMM also have a twice-methylated sulfur and are able to release DMS.

The ability of many organisms to synthesise and transport DMSP and its catabolites into cells suggests that they likely provide an array of advantages to the organisms (Figure 1-3). As

mentioned previously, DMSP is a crucial carbon and sulfur source for the organisms that can break it down[23], and as an osmoprotectant, it balances the cell against high salinity within the environment. The list of advantages is not conclusive, and other benefits suggested are as use as a signalling molecule[24] and as a protector against oxidative stress[20]. Understanding how these molecules are produced and why, as well as the cycles they are involved with, will give greater insight into the organisms that produce them, as well as their environment and global sulfur cycle.

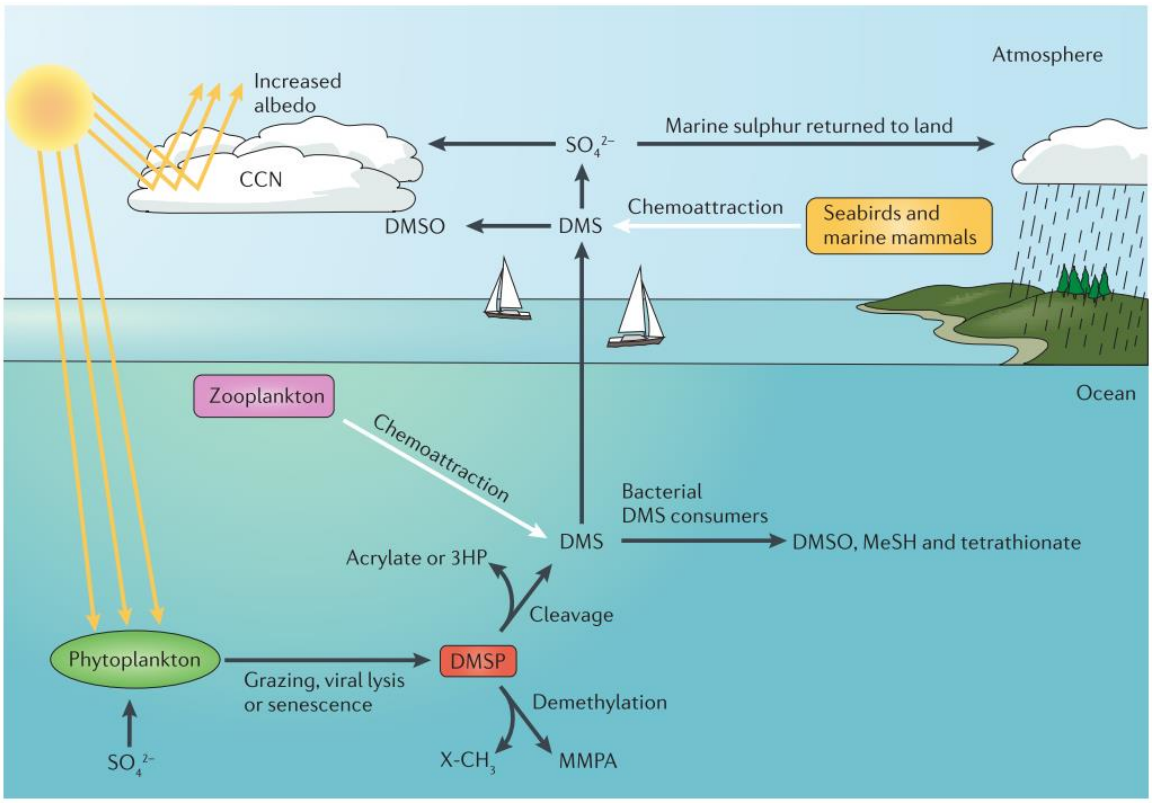

Figure 1-3 DMSP within the environment[17]. DMSP is produced by marine organisms, including algae and bacteria, and then released into the environment following cell death or lysis. Bacteria in the surrounding environment can then internalise the DMSP and cleave it to release DMS and acrylate or 3-HP. Subsequently, the oceanic DMS can be utilised as a carbon source by marine bacteria, and upon reaching the surface, 10 % is released into the atmosphere in its gaseous form. Both DMSP and DMS act as chemoattractants to many marine organisms, including zooplankton, harbour seals and petrels. When in the atmosphere, the molecules are oxidised to DMSO or SO_4^{2-}, which form CCN. When these clouds precipitate, they bring the biogenic sulfur back to the terrestrial environment.

1.2.2 How DMSP is used as a nutrient source

Upon being released into the environment, approximately 30 to 90 % of DMSP is immediately imported and catabolised by marine bacteria[24] (Figure 1-3) because DMSP and DMS are such a superb source of nutrients and, consequently, energy in a variety of microorganisms, including bacteria, bacterioplankton and phytoplankton[23]. DMSP is such an important

molecule in the environment that no known single compounds contribute as much sulfur or carbon to the food web[15]. Within DMSP-producing algae, 50 to 100 % of organic sulfur within the cells can have come from DMSP, and furthermore, it is often the preferred source of sulfur for marine bacteria[25]. As an example, SAR11 are a large clade of carbon-oxidising bacteria which represent ~25 % of the Earth's phytoplankton[26] and have been found to favour DMSP as a carbon and sulfur source; this clade lacks a complete set of assimilatory sulfate reduction genes and relies exclusively on the uptake of reduced sulfur such as DMSP and methionine[25].

Approximately 10 % of the fixed carbon within the ocean is contributed by DMSP[27], which supports about 13 % of the bacterial carbon demand within surface waters[28]. Bacteria are able to access the nutrients from DMSP in many ways, including via the very effective DMSP cleavage enzymes (*ddd* genes) and the demethylation pathway (*dmdA*), which is present in 30 % of the bacterioplankton tested in the GOS data set[27], indicating that DMSP is a hugely important source of carbon to these organisms. Furthermore, a study of the OM-RGC database[29] revealed that 20 % of bacteria in the metagenomic database contained a *ddd* gene[30].

1.2.3 Antioxidant and other properties of DMSP

DMSP is not only a valuable source of carbon and sulfur, but several other roles have also been suggested for the molecule within the environment. These predicted functions are the result of observations in the increase in DMSP production or in take in response to external stimuli[6,20,31]. One of these stimuli is oxidative stress which occurs when there is an imbalance between the production of free radical reactive oxygen species (ROS) and the ability for them to be removed from the organism[20,32]. The free radicals must be removed from the cell to prevent significant damage to cell structures, including proteins and DNA[32]. Oxidative stress can be increased by environmental conditions, such as the limitation of CO_2 and Fe, an increase in UV radiation or copper levels[33], or the presence of hydrogen peroxide, which increase the formation of hydroxyl radicals that cause damage to lipids, proteins and nucleic acids. Not forgetting that natural processes also create ROS, such as respiration and photosynthesis. DMSP and its catabolites, such as DMS and acrylate, react rapidly with the hydroxyl radicals and protect against these would be stressors[20,33].

Other environmental conditions that have been found to raise the production of DMSP include temperature decrease. In Antarctic algae, DMSP levels increase and act as a cryoprotectant

under lowered temperatures, protecting protein integrity[34,35]. As mentioned previously, DMSP and GBT share similar structures and, therefore, likely have similar functions within the organisms that utilise them. Moreover, when comparing polar and tropical species of algae, polar algae have been observed to have significantly higher levels of DMSP[36]. The higher levels of DMSP within the polar algae are suggested to act as an antifreeze, protecting the cytoplasm liquid and similarly protecting the proteins via specific molecular interactions. When investigated further, Karsten *et al.* (1996) found that DMSP protected the cells from damage during freezing and improved the activity or some enzymes to temperatures as low as 0 °C when compared to controls[36].

1.2.4 DMSP and is role as a compatible solute

Numerous studies have suggested DMSP is an osmolyte[35–37] or constitutive compatible solute[6]. Although DMSP is usually used with other solutes[38], in marine algae, it has been found to be largely dominant[39]. In environments with high salinity, several organisms produce or accumulate larger amounts of DMSP[31,40,41]. The ability of unicellular organisms to protect themselves in high salinity environments is crucial as they often lack the type of cell membrane structure which would prevent desiccation following a loss of water from the cell due to osmosis[38].

Microorganisms are not able to maintain turgor by actively transporting water molecules into the cell. Instead, they produce and/or accumulate osmoprotectants from the environment[38]. Osmoprotectants are highly soluble compounds which create a balance between the internal and external osmotic levels without disturbing the function of cellular proteins[35]. Both DMSP and GBT are efficient osmolytes. Even at nanomolar levels, DMSP enhances salinity tolerance within organisms[41]. These molecules help acclimatise cells to osmotic changes by adjusting the cell potential to match the conditions on the outside of the cell and maintain the optimum cell volume and turgour[6]. Additionally, these molecules are able to accumulate to a high concentration without affecting cellular processes due to their net neutral charge; *Sporobolus alterniflora* was reported as containing more than 29 μmol g^{-1} fresh weight[38,42] which allowed the molecule to stabilise protein structures and metabolic pathways, further protecting them from any adverse effects, such as inhibition or denaturation, that would be the result of high salinity[43]. There have been studies which suggested that DMSP synthesis in marine organisms may have arisen during the last ice age to help contend with the increase in salinity within the oceans[18], supported by the fact that DMSP is a cryoprotectant.

1.2.5 Chemoattractant properties of DMSP

There are several species of bacterioplankton and proteobacteria that use DMSP as a chemoattractant, enabling them to find, assimilate and catabolise it[24,44]. Organisms that are able to sense DMSP are at an advantage since when the organisms that produce it release it into the environment, either through death or lysis, it is in limited supply[44]. The organisms producing DMSP may intend to interact with other organisms, such as a bacterium-dinoflagellate interaction, or it could be something marine bacteria exploit[24,44]. Either way, the chemotaxis and breakdown of DMSP to DMS and its catabolites are now important within the sulfur budget[45]. It is not only microbes that are attracted to DMSP; planktivorous reef fish, sea urchins, sea birds, and harbour seals[24] are also lured to the scent, which indicates feeding activity[46]. Higher organisms use DMSP as an indirect foraging cue; algal blooms releasing DMSP alert reef fish, and subsequently, higher organisms that would feed on the fish and so on[46].

1.2.6 DMSP as an antimicrobial & anti-grazing molecule

Up to this point, DMSP has been discussed as a nutrient source, an antioxidant, an osmoprotectant, a compatible solute and a chemoattract, but it may possess additional properties, such as, as an antimicrobial or anti-grazing molecule[47]. The DMSP doesn't act as a deterrent itself, but bacteria and phytoplankton break it down to produce acrylate, which does act as a deterrent to predators, such as protozoan herbivores and copepods[47]. At high concentrations, acrylate has antimicrobial activity as it can inhibit the growth of numerous bacterial species[48]. There have been studies that indicated that the catabolism of DMSP to acrylate may not be activated by grazing but actually after ingestion of the algae[47,49]. The protozoa grazing on the algae were not detrimentally affected by the acrylate but preferentially consumed the non-DMSP-containing prey[47]. This observation suggests that the catabolism of DMSP may not exclusively be driven by the need for nutrients but may also be a defensive action[50].

1.3 Dimethyl sulfide

With around 200 million tonnes being produced per year, DMSP is the major biogenic source of DMS within the environment[17]. DMSP catabolism has important roles within the ocean, and its catabolism to DMS is an environmentally significant reaction. DMS is a volatile sulfur compound which will readily diffuse through the sea surface to the air, with around 10 % of the ocean DMS being transferred to the atmosphere as a gas[9,51,52]. As a gas, DMS can fulfil its role as a key player in the sulfur source by returning biogenic sulfur to land[1]. Although the bulk of DMS is released into the marine environments as a result of DMSP catabolism[53,54], a study

showed that DMS could also be produced via the methylation of methanethiol (MeSH), particularly in terrestrial environments[54]. Furthermore, another study found DMS could be a product of the reduction of DMSO under anoxic conditions[3]. The fact that DMS can be produced in various environments and several pathways suggest that the production may be more prolific than previously estimated.

1.3.1 How DMS is used as a nutrient source

The majority of DMS dissolved in the marine environment ins removed by microbial activity[9]. Like DMSP, DMS is a C1-sulfur compound and similarly an excellent source of carbon, sulfur and nutrients[55]. As a result, most of the DMS in the oceans is degraded by microbial activity, with some species of bacteria able to utilise DMS as a sole carbon source[3]. Additionally, several microorganisms from a broad array of environments have been observed to have DMS catabolising abilities[3]. There are two known pathways for DMS degradation, the monooxygenase pathway[55] and the methyltransferase pathway[56]. Of course, both pathways begin with their namesakes. In the monooxygenase pathway, DMS is oxidised by a DMS monooxygenase to formaldehyde and methanethiol, which is further degraded to formaldehyde, hydrogen peroxide and sulfide[3]. The methyltransferase pathway was identified when DMS degradation was observed in oxic conditions[3,56], and the pathway begins with a methyl group from DMS being transferred from a methyltransferase to an acceptor, which is further degraded to methanethiol, which is then degraded the same way as the monooxygenase pathway. Finally, DMS may be oxidised photochemically to DMSO, which can then be used as a carbon source[55].

1.3.2 Atmospheric DMS

Like DMSP, DMS has important roles within the environment. In is gaseous forms, DMS is also a chemoattractant for various organisms, including zooplankton[57], sea birds[58] and harbour seals[59]. Just like the chemotaxis of DMSP, DMS chemoattraction is thought to offer the same advantages with foraging clues[58]. Furthermore, when released into the environment as a gas, DMS can oxidise and form various products, including DMSP and sulfate aerosols, for example, SO_2[5]. As discussed before, sulfate aerosols and particles can become CCN[9], causing the formation of clouds which result in precipitation (Figure 1-3). Although DMS-related particulates are solely responsible for CCN over oceanic environments, they still represent one of the major contributors[14]. Again, these CCN cause changes in the local environment, cooling through the albedo effect, and they provide a vital step in the sulfur cycle by returning biogenic sulfur to land via atmospheric deposition[1].

1.4 DMSP production

As mentioned previously, the production of DMSP was first identified in *P. fastigiata*[19], and since this discovery, many more organisms have been identified from diverse algae[60], diatoms[60], dinoflagellates[60], haptophytes[60], plants[61,62], coral[63], and bacteria[31,64–67]. Studies in the 1990s used radiolabelled compounds of model plants and algae determined three pathways used for DMSP synthesis (Figure 1-4), with each pathway named after its primary enzymatic modification of L-methionine (Met)[42,68–70]; the methylation, transamination, and decarboxylation pathways. Although the pathways begin with the same starting product and contain similar enzymatic activities, they take part in different sequential order, and each pathway has distinct intermediates[42,68–70]. Initially, attempts were made to purify DMSP synthesis enzymes to determine the kinetic properties and characterise cofactor dependence, but these studies were unable to identify the corresponding amino acid (aa) sequences[71,72]. The characterisation of these processes using metabolic and biochemical processes was hugely valuable as they simplified the process of identifying target enzymes and became the favoured method for gene discovery.

1.4.1 The transamination pathway

1.4.1.1 Elucidation of the transamination pathway

Ulva (Enteromorpha) intestinalis was used as a model alga to determine the pathway intermediates of the transamination pathway by using [^{35}S]Met tracer and intermediate biochemistry[68]. The first step is the reversible transamination of Met to form 4-methylthio-2-oxobutyrate (MTOB) by a 2-oxoglutarate-dependent aminotransferase[71]. Interestingly, the initial aminotransferase reaction in this pathway is equivalent to the final reaction in the methionine salvage pathway found in many organisms[71,73,74], including *Escherichia coli* and *Bacillus*[74,75]. Proteins with 40 – 44 % aa identity to the aminotransferases involved in the salvage pathway exist in DMSP-producing organisms. However, no Met aminotransferase was shown to be involved in DMSP synthesis in these organisms. Therefore, it was suggested that there were likely to be specific DMSP Met aminotransferase enzymes and the observation that an unidentified enzyme in *Ulva* showed a strong preference for 2-oxoglutarate as an amino acceptor over glutamate and aspartate reinforced this idea[71]. Additionally, *Ulva* extracts have lower (μM) K_m values for Met when compared to the (mM) K_m of other organisms with Met salvage enzymes[71].

The step after the initial transamination is by reversible reduction to produce D-MTHB (4-methylthio-2-hydro-xybutyrate) via an unidentified NADPH-linked reductase[63,68,71,76]. The

penultimate step carried out by MTHB *S*-methyltransferase (MSM) is the methylation of D-MTHB to form D-DMSHB (4-dimethylsulfoxide-2-hydroxybutyrate)[63,71]; this step is the rate-limiting committed step in the transamination pathway [77] as it is specific to DMSP production and non-reversible. It is worth noting that all steps within the transamination pathway, excluding MSM, exist in other non-DMSP-producing organisms. For example, *Rhizobium leguminosarum* is able to catalyse all steps of the transamination pathway except for the MSM step[31]. Finally, D-DMSHB goes through oxidative decarboxylation by a pyridoxal-5-phosphate (PLP)-dependent oxidative DMSHB decarboxylase enzyme to produce DMSP [68,76]. The hypothesis that DMSHB and DMSHB decarboxylase activity in green algae, diatoms, haptophytes and prasinophytes predicted DMSP synthesis via the transamination pathway[68] has only recently been supported by the identification of the functional genes *DSYB* and *TpMMT*[30,78]. The presence of these genes within the available algal genomes or transcriptomes emphasizes the transamination pathway as the most common DMSP synthesis pathway.

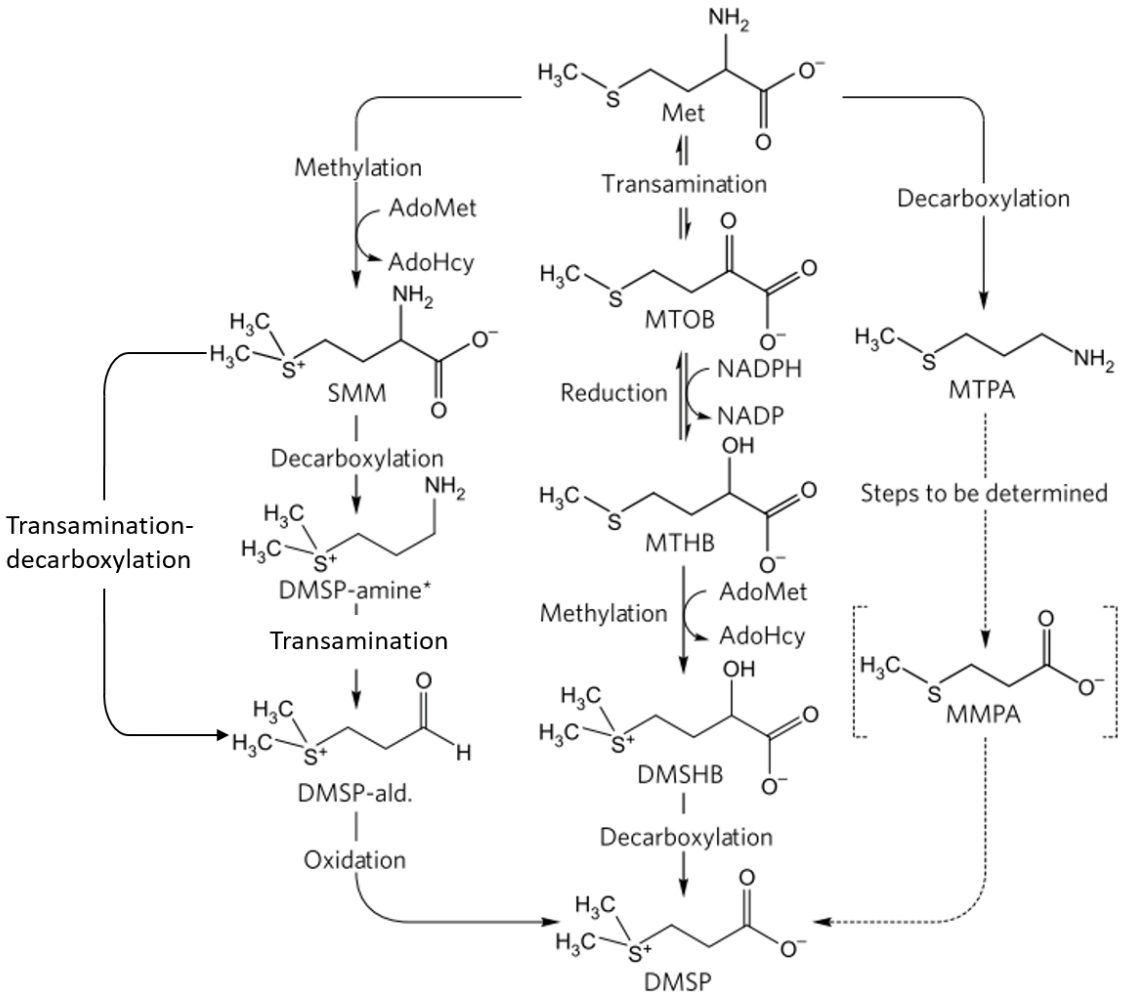

Figure 1-4 DMSP synthesis pathways[31]. The methionine pathway used by angiosperms (left), the transamination pathway used by phytoplankton, algae, and marine heterotrophic bacteria (centre), and the decarboxylation pathway used by a single dinoflagellate (right). Some organisms which use the methylation pathway can produce DMSP-ald from SMM.

1.4.1.2 Identifying candidate enzymes within the transamination pathway

As discussed earlier, many pieces of research have investigated the relationship between DMSP production and environmental factors, which have enabled the inference of the physiological roles of DMSP[6,79,80]. Moreover, studies have used these factors to increase DMSP-producing conditions along with 'omics' analysis to identify candidate genes and enzymes within the synthesis pathway[63,76,78,81,82]. Lyon *et al.* (2011) identified candidate genes by matching the abundance of proteins to that of DMSP during salinity acclimation experiments using the polar ice diatom *Fragilariopsis cylindrus*[68,71]. In addition to this study, Raina *et al.* (2013) observed that *Acropora* corals were able to produce DMSP, and both *Acropora* and its symbiont, *Symbiodinium*, contained the candidate orthologs to MTOB and MSM enzymes from *F. cylindrus*. More excitingly, DMSP production in *Acropora* was the first non-photosynthetic animal shown to do this[63]. Nevertheless, these predicted enzymes were later shown to likely not be involved in DMSP synthesis, and the supposed MSM enzymes lacked this activity[30,78].

Finally, another MSM enzyme was predicted in the *Ulva mutabilis* genome due to its homology with glycine sarcosine dimethylglycine *N*-methyltransferase (GSDMT)[81], a protein involved in glycine betaine (GB) synthesis induced by salinity[76]. Interestingly, when GSDMT was first identified[76], it was not suggested to have MSM activity. The orthologue in *Thalassiosira pseudonana* (a diatom, TpGSDMT) was shown to also have GSDMT activity[78]. Nevertheless, the proposed enzyme in *U. mutabilis* is distinct from TpGSDMT and the proteins found in *F. cylindrus* and gene transcription and proteins where upregulated by low temperatures, which correlated with an increase in the DMSP produced by sea lettuce[81]. What is very clear is that functional verification is a necessary process in determining enzymes within a pathway to avoid false interpretations.

1.4.1.3 Bacterial DMSP production via the transamination pathway

The first DMSP synthesis gene was identified from the marine heterotrophic bacteria *Labrenzia aggregata* when the strain produced DMS via its DddL DMSP lyase when grown without exogenous DMSP[31]. Surprisingly, although these organisms were well-characterised DMSP catabolisers[17,79,80], they were not known to be DMSP producers. Consequently, a diverse and abundant community of DMSP-producing bacteria were found in seawater and marine sediment samples, including alpha-, beta-, delta-, gammaproteobacterial, several firmicutes and actinobacteria[31,65–67]. When grown in the presence of intermediates from the three known DMSP synthesis pathways, the intermediates from the transamination pathway

increased DMSP synthesis by *L. aggregata*[31], and using functional genomics, a key enzyme with MSM activity was identified and termed DMSP synthesis in bacteria 'DsyB' (AOR83342). Following the deletion or disruption of *dsyB* in *L. aggregata*, DMSP production was no longer observed, and MTHB was overproduced[31]. And, as mentioned previously, DSYB enzymes from *F. cylindrus* and *Acropora* had MSM activity which was upregulated during the Lyon *et al*. (2011) salinity upshift experiments, confirming the importance of *dsyB* in the bacterial production of DMSP via the transamination pathway. The experiments were designed to show how the concentration of DMSP synthesis proteins changed with increasing saline conditions[76]. The results showed that proteins found in the Met DMSP synthesis pathway were elevated in the higher saline conditions[76].

DsyB is within the *O*-methyltransferase protein family (pfam00891) and present in more than 200 alphaproteobacteria, including Rhodobacterales, Rhizobales and Rhodospirillales, and from beta- and gammaproteobacterial, actinobacteria, and Bacteroidetes from the metagenome-assembled genomes (MAGs)[83–85]. A diverse range of DsyB proteins from this family have been shown to confer MSM activity with their natural hosts producing DMSP, making DsyB a strong reporter of DMSP production in bacteria[31]. In addition to this, Curson *et al*. (2017) better defined the family of DsyB by showing that proteins with less than 36.5 % aa identity dd not have MSM activity. For example, *Streptomyces varsoviensis* and *Bacillus mycoides* had a DsyB-like protein but less than the needed identity threshold and not an enzyme involved in DMSP synthesis[31].

Unlike other metabolite synthesis genes, *dsyB* is not linked genetically to any genes predicted to encode steps within the transamination pathway in DMSP-producing bacteria, and to date, none of these enzymes have been identified. It is possible that *dsyB* was transferred through horizontal gene transfer (HGT), conferring the ability to produce DMSP. This would be a possibility as some bacteria, for example, *Rhizobium* have all the required enzymes of the transamination pathway except MSM encoded by *dsyB*[31]. Therefore, by gaining *dsyB*, organisms like *Rhizobium* would be able to synthesise DMSP and subsequently benefit from the molecule, as mentioned previously. Although none of the other genes in this pathway have been identified, *dsyB* in *Rhodobacterales* and *Rhizobiales* strains have *isc/suf* clusters or genes closely associated with it[31,64,86,87]. The *isc/suf* genes are involved in protecting the organisms from oxidative stress and therefore compliment the properties of DMSP[20].

1.4.1.4 Eukaryotic DSYB

Within eukaryotes, proteins which were phylogenetically distinct from DsyB (<38 % aa identity to DsyB) were termed DSYB and identified in several eukaryotic genomes and transcriptomes, including *Emiliania huxleyi*, *Chrysochromulina tobin*, and *Symboidium microadriaticum* from the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP)[30,88]. Examination of the available genomic and transcriptomic data from MMETSP showed that *DSYB* was expressed in haptophytes (80 %), dinoflagellates (77 %), diatoms (18 %), Ochrophyta (18 %), and in corals, including *Acropora cervicornis*[30,84]. Some phytoplankton, including dinoflagellates, had multiple *DSYB* genes, which may correspond to their ability to produce the highest amounts of DMSP, which can be up to 8 M in some cases[60,89].

Not all organisms with *DSYB* are high DMSP producers (≥50 mM intracellular concentrations)[30,90]. In fact, examples of low, medium, and high producers are found within the organisms containing *DSYB*; *Chrysochromulina tobin* CCMP291 is a low DMSP producer with an intracellular concentration of 0.6 ± 0.1 mM DMSP, *Prymnesium parvum* CCAP946/6 is a medium producer at 54.3 ± 6.0 mM and 6.0 mM, and *Symbiodinium microadriaticum* CCMP2467 high at 282 ± 35 mM[30]. When *DSYB* was cloned from these three organisms into *Rhizobium*, the levels of DMSP produced were similar, and the cloned genes restored DMSP synthesis to *L. aggregata dsyB*$^{-}$ mutants[30]. What this shows is that the aa sequence of DSYB and DsyB probably isn't involved it the levels of DMSP produced in these organisms. Curson *et al.* (2018) observed that the transcription levels of these organisms were proportionate to the levels of DMSP being observed and are likely to be one of the mechanisms the organisms use to control the production of DMSP. The discovery of a eukaryotic version of *DsyB* is important as phytoplankton are considered to be the major producers of DMSP; *DSYB* will help identify new, and likely important, contributors within the environment.

1.4.1.5 Analysis of the DsyB/DSYB family

According to various studies, the characterisation of DsyB and DSYB has been problematic as although both genes showed *in vivo* MSM activity in *Rhizobium*, when expressed in or purified from *E. coli*, the enzymes were inactive[30,31,84]. The activity in *E. coli* containing *dsyB* or *DSYB* was reinstated if heat-denatured *P. parvum* or *L. aggregata* cell lysates were added, implying that an unknown cofactor was necessary for the SAM-dependent MSM activity mechanism to work[30,31,84]. Unfortunately, even after attempts to find the missing cofactor using mass spectroscopy, the mechanism and possible missing cofactor are still unknown[84].

To measure the affinity of DsyB and DSYB to SAM and MTHB, active DsyB was purified from *N. denitrificans* and DSYB from *P. parvum*. Both proteins were found to have similar kinetics; *N. denitrificans* DsyB had a K_m of 0.14 and 0.16 mM and a V_{max} of 365 and 368.9 nmol min^{-1} mg^{-1} protein for MTHB and SAM, respectively[30], and *P. parvum* DSYB had a K_m of 0.09 and 0.06 mM and a V_{max} of 294 and 303 $nmol^{-1}$ min^{-1} mg protein for MTHB and SAM respectively[84]. Supporting the theory that the presence of these genes is not sufficient for the prediction of DMSP productivity within an organism, even if they have similar enzymatic characteristics[30,31,84,90,91].

The crystal structure of DsyB in *N. denitrificans* DR41_21 was solved and shown to consist of four DsyB molecules arranged in an asymmetric tetramer[84] (Figure 1-5). Li *et al.* (2022) employed MSM assays on site-directed mutants to obtain structural data and suggested that DsyB used a proximity and desolvation mechanism. The way this model works is once SAM is bound, DsyB undertakes a confirmational change, from 'open' to 'closed', and promotes the binging of MTHB (Figure 1-6). When MTHB is in the active site, DsyB moves the sulfur atom from MTHB into the proximity of the SAM methyl group, allowing the methyltransferase activity to occur, releasing DMSHB and SAH (*S*-adenosylhomocysteine)[84] (Figure 1-6). Li *et al.* (2022) were also able to identify the likely SAM (Ser150, Gly173, Asp196, Asp223, Ala224 and Ser239) and MTHB (Tyr97, Gln101, Tyr129, Tyr142, Gln146 and His291) binding residues. The sequences of DsyB and DSYB appear to be highly conserved, as when aligned, the sequences and structures were aligned[84] except in the case of Asp196 and Asp223[84]. The conservation between these proteins indicates that this mechanism is universal in bacteria and algae that have DsyB or DSYB.

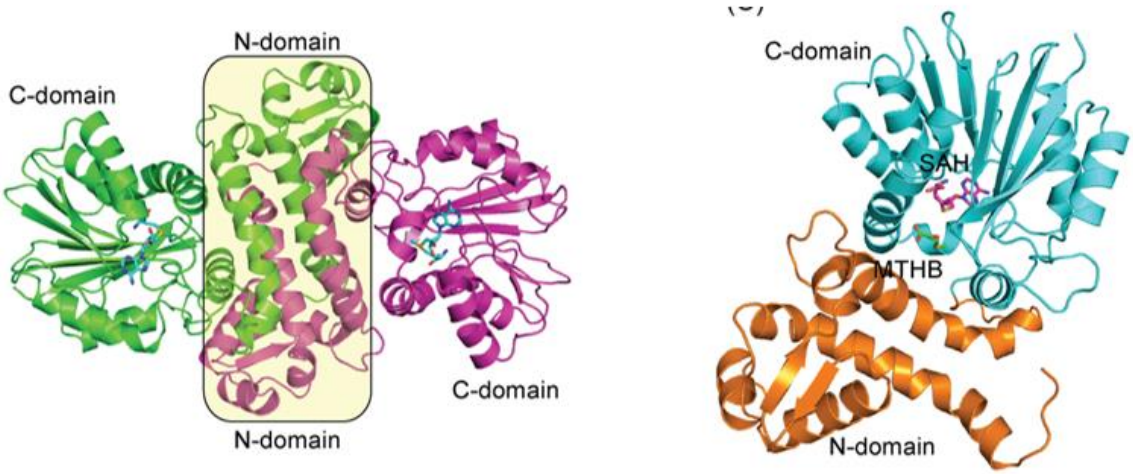

Figure 1-5 Ribbon representations of DsyB dimers, each monomer contains a C-domain and an N-domain. Left: DsyB dimer with SAM molecule in cyan sticks. Right: DsyB dimer with the SAH molecule as purple sticks and the MTHB molecule as green sticks. Adapted from Li *et al.* (2022).

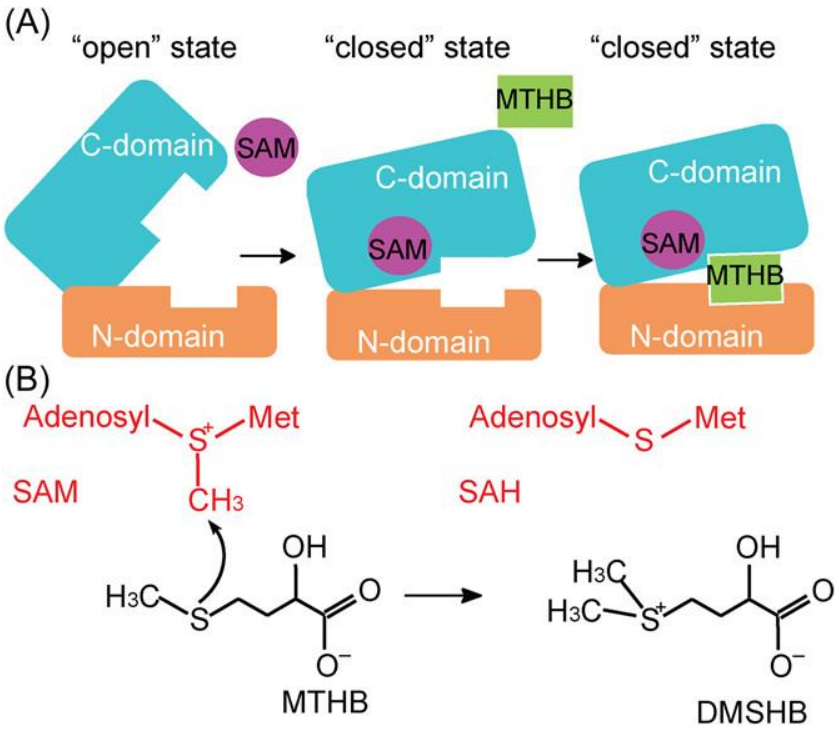

Figure 1-6 The proposed catalytic mechanism from Li *et al.* (2022). (A) Shows a schematic diagram of how DsyB undergoes a conformational change following the binding of SAM. (B) Shows how the sulfur atom of MTHB (black) attacks the methyl group of SAM (red) to produce DMSHB (black) and SAH (red).

1.4.1.6 Evolution of DsyB and DSYB

Due to the similarity between DsyB found in bacteria and DSYB found in eukaryotes, Curson *et al.* (2018) were able to analyse the evolutionary domain of origin. The analysis showed that

the diverse groups of eukaryotes with DSYB were monophyletic and summarised that DMSP likely originated in prokaryotes were it was transferred to eukaryotes multiple times, either at mitochondrial origin or by HGT[30].

1.4.1.7 The MTHB S-methyltransferase found in diatoms

When tested, most diatoms can produce low levels of intracellular DMSP (<50 mM)[60,91]; however, the DSYB enzyme is only present in 18 % of them. Using a bioinformatic approach and *Thalassiosira pseudonana* CCMP1335 (lacks DSYB) as a model, Kageyama *et al.* (2018) identified candidate MSM enzymes. The process they used is unclear, although they were able to identify two possible MSMs; the first, termed TpMT1, did not have MSM activity and was, in fact, a GSDMT enzyme involved in GB production[63,76,78]. The second MSM enzyme candidate was termed TpMT2 and was an isoform of the MSM enzyme and had in vitro MSM activity[78]. TpMT2 was subsequently named TpMMT and had a K_m or 2. 1 mM and 0.75 mM for MTHB and SAM respectively[78]. Compared to the values for DsyB, these values were considerably higher and drew questions about how efficiently these organisms produce DMSP in comparison. TpMMT has low similarity to DsyB at only 24 %, and it is a single-domain *S*-methyltransferase within the Class I SAM-dependent methyltransferase family (pfam08241).

Out of the 82 diatoms transcriptomes on MMTESP, 17 contain proteins with approximately 70 % identity to TpMMT, and 7 also contain DSYB[84]. McParland *et al.* also predicted other proteins that had a lower identity to TpMMT, 27 % to 52 %, due to the ability of the organisms to produce DMSP. However, they did not experimentally ratify MSM activity[90]. Furthermore, three of the proposed enzymes (termed 'T2') were three times bigger than TpMMT and included extra protein domains such as a dehydrogenase and aldolase. As the enzyme activity within these organisms is still unknown, it is unwise to make assumptions as to whether these proposed genes are indicators of the ability of an organism to produce DMSP. In order to be able to create molecular probes and investigate environments further, more work needs to be done to ratify and characterise these proposed enzymes.

1.4.2 The methylation pathway

The methylation pathway (Figure 1-4) was determined using radiolabelled pulse-chase experiments in *Melanthera biflora* (*Wollastonia biflora*) and *Sporobolus alterniflorus* (*Spartina alterniflora*) as model organisms[42,92–94]. Cell cultures are incubated briefly ("pulse") with radiolabelled amino acids which results in newly synthesised products incorporating the label[95]. Subsequently, the cells put into non-radioactive culture medium for various times

("chase"), and the subsequent products can be studied[95]. This allows for the process to be followed, proteins may change conformationally, be transported, or degraded. Any proteins of interest can be isolated via immunoprecipitation and resolved by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis and viewed using autoradiography[95].

The first step in both species begins in the cytosol with the SAM-dependent *S*-methylation of Met to *S*-methylmethionine (SMM) via a Met-*S*-methyltransferase (MMT)[42,69,92]. This is a process that exists in most plants and is vital in the SMM cycle in plants which regulates the levels of SAM and Met[96,97]. SMM is transported to the chloroplast, where the subsequent steps take place[98]. After the initial step, both model plants have slightly differing pathways. In *S. alterniflorus,* SMM is decarboxylated to form 3-dimethylsulfoniopropylamine (DMSP-amine) by a pyridoxal 5'-phosphate (PLP)-dependent SMM-decarboxylase, termed SMM decarboxylase (SDC)[93,99]. SDC has a K_m of 18 mM and a V_{max} of 0.28 $nmol^{-1}$ min^{-1} mg^{01} protein[93,99]. Next, DMSP-amine oxidase (DOX), with a K_m of 1.8 mM and a V_{max} of 0.37 $nmol^{-1}$ min^{-1} mg^{01} protein, oxidises DMSP amine to produce DMSP-aldehyde[93]. Finally, DMSP-aldehyde is oxidised to DMSP by a DMSP-aldehyde dehydrogenase (DDH)[42,98,100]. Unlike *S. alterniflorus*, the pathway used by *M. biflora* is still unsolved, although there are suggestions that *M. bifolora* uses a PLP-dependent transamination-decarboxylation step to produce DMSP-aldehyde directly from SMM[69,94,99]. This could be the reason no intermediates between SMM and DMSP-aldehyde were identified during labelling experiments[94]. It may be that an unusual enzyme performs both transamination and decarboxylation reactions simultaneously, explaining the missing intermediates between SMM and DMSP-aldehyde[94]. Or, equally as likely, there could be an intermediate formed that is unstable and undetectable, or the product may be produced and immediately processed to DMSP-aldehyde[94]. Like *S. alterniflorus*, the final step in the methylation pathway of *M. biflora* is the oxidation of DMSP-ald to form DMSP DDH[42,69,98,100]. DDH (*M. biflora*) was characterised further and had a K_m of 1.5 μM for DMSP-aldehyde and for its cofactors NAH and NADP, K_m of 6.9 μM and 68 μM, respectively[98]. The K_m for NAH and NADP show that NADP is the preferred cofactor[98].

Although the complete methylation pathway is not widespread among higher plants, some intermediate steps are. The ability to produce SMM from Met occurs in the cytosol of all angiosperms[98,101]. Furthermore, some higher plants can carry out the final step of the methylation pathway, the oxidation of DMSP-ald to form DMSP when supplied with DMSP-ald[98]. Studies have shown that betaine-aldehyde dehydrogenase (BADH) enzymes found in plants can catalyse the NAD-dependent oxidation of DMSP-aldehyde, creating DMSP[102]. Therefore, the conversion of SMM to DMSP-aldehyde is the step specific to DMSP producing organisms[42]. To date, there have been no DMSP-specific synthesis genes found in plants.

There was, however, one study that suggested several candidate genes for each step of the methylation pathway[103], but these are yet to be characterised.

1.4.3 The decarboxylation pathway

The decarboxylation pathway is the least understood and observed only in *Crypthecodinium cohnii*[70,72], which is yet to be determined. The pathway was predicted using incubations of ^{14}C and ^{35}S radiolabelled isotopomers of Met[70,72]. Following incubations, all of the carbon and sulfur from Met were incorporated into DMSP, except for the carboxyl carbon. When unlabelled SMM or MTOB was added to the incubation, the incorporation of Met was not inhibited. However, it was stopped by methylmercaptopropionate (MMPA), implying that MMPA was a pathway intermediate[70,72]. From these studies, it was proposed that *C. cohnii* used a pathway where Met was decarboxylated to form 3-methylthiopropylamine (MTPA), which was subsequently modified to MMPA and *S*-methylated to generate DMSP[70,72].

Since the decarboxylation pathway was suggested, a 100 kDa homodimeric PLP-dependent Met decarboxylase (MDC) enzyme was purified from *C. cohnii* extracts[72,104], supporting the proposed decarboxylation pathway. The first nine aa from the sequence of the N-terminal sequence of the MDC in *C. cohnii* were elucidated[70,72,104], but the sequence does not align with the predicted proteins found in the transcriptomes for *C. cohnii* at MMETSP. Therefore, this pathway and the MDC enzyme are thought of as uncertain.

It is worth noting that the decarboxylation pathway was dependent on the import of potential pathway intermediates into cells of *C. cohnii*. The uptake of intermediates at not be at the same level for the different intermediates or even used in the strain. It may be that *C. cohnii* uses a different pathway. When the transcriptome of *C. cohniii* from MMETSP was studied further, five copies of *DSYB* were identified[30,84]. These DSYB sequences contained the conserved MTHB and SAM-binding residues and, therefore, likely use MTHB and not MMPA as a substrate[30,84]. It may be possible that *C. cohnii* has more than one competing DMSP synthesis pathway.

1.5 Regulation of DMSP production

There are many suggested functions proposed for DMSP in the organisms that produce or assimilate it. However, very few have been fully confirmed, yet there are multiple conditions which seem to stimulate of inhibit the ability of an organism to produce DMSP, and this can be exploited to yield information on potential roles.

1.5.1 Regulation by nutrients presence

Within the oceans, there are large areas of nutrient deficiency, including nitrogen, which is known to play a role in DMSP regulation[105]. Furthermore, areas where nitrate and silicate are limited in the ocean have been found to be associated with higher DMSP concentrations[106], and also such conditions have been shown to increase the synthesis of DMSP in angiosperms[69], marine algae[107], and most recently, bacteria[31]. This is due to DMSP being sulfur-based, unlike GBT, which is nitrogen-based[99]. When environments are nitrogen-limited, DMSP is the most practical osmoprotectant to synthesise, saving any nitrogen taken up by the organisms to be used in more important pathways required for survival. Many organisms maintain the ability to produce a variety of osmoprotectants, and subsequently, when nitrogen is deficient, organisms will produce DMSP, sometimes replacing GBT completely[6]. When considering the difference between marine and terrestrial environments, the marine environment has low nitrogen and high sulfur levels, while the terrestrial environment can be considered generally the opposite, largely due to the addition of fertilisers. The availability of these nutrients may explain why marine environments promote the use of sulfur-based DMSP production more favourably than for osmoprotection, whilst terrestrial environments may favour organisms to use the nitrogen-based GBT[108].

Of course, the presence of sulfur has an important role in the regulation of DMSP. For example, *Ulva pertusa* (sea lettuce) growth and production of DMSP are inhibited under sulfur-deficient conditions[77]. Sulfur-deficient environments cause DMSP activity to be inhibited by a decrease in the activity of MTHB *S*-methyltransferase and an accompanying increase in the activity of the sulfur-assimilation gene *O*-acetyl serine sulfhydrase. The change in the activity of the genes can be explained by the limited amount of sulfur and subsequent limited methionine availability, making cells produce their own. When methionine becomes limited in this way, any produced will be used for vital pathways such as AdoMet production, and therefore, DMSP synthesis becomes less important at that time[77]. However, under conditions of sulfur deprivation, the amount of DMSP taken up from the environment is increased and as well as the conversion of DMSHB to DMSP. So, whilst the organisms may

need to use the methionine that would have been used in the DMSP synthesis pathway elsewhere, the organisms still have a need to take up DMSP or produce it from DMSHB. Again, demonstrating how important DMSP is to those that utilise it.

1.5.2 Regulation by salinity

As mentioned previously, DMSP is an osmoprotectant and of often regulated by changes in salinity[6]. This is not the case for all DMSP-producing organisms; for example, *Emiliania huxleyi* produce DMSP constitutively and are not really regulated[20]. Yet, most organisms require a higher concentration of osmoprotectant in higher levels of salinity to maintain cell volume. DMSP is produced at low salinity levels, but it is usually markedly higher with increased salinity and increased activity of MTHB *S*-methyltransferase[77]. Similarly, DMSHB and DMSP uptake is increased under high salinity and can also be observed in marine bacteria[77,109]. Salinity can easily be thought of as one of the major regulators of DMSP synthesis and uptake; however, for organisms living continuously in high-salinity environments, this is not the case. For example, *S. alterniflorus* DMSP synthesis levels do not vary at the change of salinity[21], further supporting the different roles that DMSP plays in the organisms that use it. When other osmoprotectants are added, such as GBT, to already sulfur-deficient conditions, DMSP levels decrease markedly[77].

1.5.3 Regulation by temperature

The ability of DMSP to act as a cryoprotectant in some organisms would suggest that temperature may act as a regulatory condition in these[6], and decreasing temperatures have been linked to an increase in DMSP production[36,110] and subsequent protection against damage caused by freezing. Even though the incorporation of carbon into proteins is reduced at low temperatures, this does not seem to be the case for the construction of carbohydrates, which have a role in the production of acetyl-CoA and DMSP[6].

1.5.4 Regulation by light

Light can play a role in the regulation of DMSP synthesis in photosynthetic organisms because sulfate reduction is an energy-dependent process and is coupled to cell metabolism, which of course, is stimulated (but not dependent upon) light[6]. With increased light levels, photosynthetic organisms are able to produce more methionine and therefore, more methionine is available for the production of sulfur compounds such as DMSP. Additionally, during short-day incubations, carbon fixation is reduced and reserved for vital metabolic processes, reducing the amount of DMSP synthesised[6]. Multiple phytoplankton species have

been observed and shown to have a relationship between the amount of light and then levels of DMSP produced[111]. Furthermore, the DMSP production in green algae cycles annually, with levels of DMSP decreasing with decreasing daylength and vice versa[112]. What is more, *Synechococcus* has been observed to produce 15 % more DMSP when incubated in light compared to dark conditions[113].

1.6 DMSP transport

DMSP-producing organisms are not the only ones able to benefit from the many positive roles that DMSP may confer. Many non-DMSP-producing strains of bacteria and phytoplankton assimilate DMSP from the surrounding environment[114]. Again, due to sulfur being more freely available in marine environments, microorganisms use DMSP more preferably than other compatible solutes in the oceans[108]. However, it must be said that DMSP is not necessarily taken up because of its role as an osmoprotectant because its uptake is not always regulated by salinity[21]. Within the marine environment, 10 to 50 % of the DMSP is assimilated by bacteria, phytoplankton and microzooplankton[114], and therefore, assimilation plays an important role in the regulation of the sulfur emissions from the ocean to the atmosphere.

Due to DMSP being a zwitterion, it is unable to diffuse through a membrane, and therefore the organisms must use specific transporters or make use of other transporters already available to the organism[114]. DMSP and GBT appear to share transporters, as the uptake of one inhibits the other and vice versa[115], suggesting that the transporter may select one or the other depending on cell needs. Additionally, both compounds have similar kinetics[115], which means they would likely require similar transporters. Interestingly, even terrestrial organisms are able to assimilate DMSP when subject to environments of high salinity[41].

1.6.1 Betaine choline carnitine transporters

BCCT (betaine choline carnitine transporter) transporters have been proposed to act as a transporter for DMSP[108]. These transporters are associated with *dddD* and other catabolic genes in many species but are almost ubiquitous in microbes[17]. In *Escherichia coli*, the transporters are understood to transport GBT across the membranes[99], and subsequently, it was learned that the transporter also moved DMSP[116]. Interestingly, although the BCCT moves GBT and DMSP across the membrane, it is unable to move the structurally similar compound MMPA [15]. This would indicate that the positive charge found on the sulfur for DMSP and the nitrogen for GBT is needed to use these transporters, and this is a feature which many known BCCT carriers share[116]. The BCCT transporters are high-affinity uptake systems which help maintain the cells' osmotic pressure by moving ions or molecules in and out of the cell,

causing the intracellular osmotic pressure to change[116]. Often, the genes for BCCT are found to be regulated by salinity which would be necessary for a rapid response to salinity changes in the environment[116].

BCCT transporters are secondary transporters; this means that the transporter actively moves a solute in the direction of increasing electrochemical potential whilst diffusing a second solute in the direction of decreasing electrochemical potential[116]. These transporters usually have three monomers with 12 transmembrane segments predicted and variable length N- and C-terminals[116]. The N-terminals of these transporters project into the cytoplasm and help control transport activity. Additionally, BCCT can be symporters and transport different solutes in the same direction or antiporters and transport different solutes in opposite direction[99]. By moving theses solutes, the transporter creates transmembrane motive force, and the energy from this force can be used to transport DMSP into the cell[116]. There is variation in the amino acid sequences and nomenclature of BCCT between species; for example, in *E. coli* they are named CaiT, in *Corynebacterium glutamicum* they are called BetP[108], in *Marinomonas* they are named DddT[117], and in *Halomonas* they are called HTNK1[118]. The gene *dddT* is contained within the operon *dddTBCR*, where *dddD* is transcriptionally regulated by *dddR,* which responds to environmental DMSP[117,118].

1.6.2 ATP binding cassette transporter

The ABC (ATP binding cassette) transporter is the second family of transporters observed to carry DMSP across membranes but is also one of the most commonly used primary transporter found in all three domains of life[119]. As the name suggests, ABC transporters work by moving molecules across the membrane in exchange for a molecule of ATP[119]. There is variation within the family of ABC transporters, and the one used for DMSP transport is most commonly found in prokaryotes, although even within this group there is a great deal of structural variation[119]. There are three parts to the standard ABC transporter, the transmembrane protein (TMP), the nucleotide-binding protein (NBP), and the substrate-binding protein (SBP)[99]. Two TMP span the membrane barrier, creating a translocation pore, and together with two NBP that bind the ATP molecules and hydrolyse them[119]. Together, TMP and NBP form a heterodimer.

There are bacteria that contain multiple ABC transporters which have the ability to move DMSP, although they do this with varying effectiveness[99]. For example, *Bacillus subtilis* uses OpuA, OpuC, and OpuD to move GBT and DMSP across its membrane[38], and *Burkholderia*

ambiafaria uses the gene cluster *potABCD,* which encodes a DMSP transporter[99]. Similarly to BCCT, many of the ABC transporters can be linked to the *dddD* gene in multiple species[108].

1.7 DMSP catabolism

DMSP is released into the environment from organisms through grazing[28,120], viral lysis[121], or senescence[122], at which point it can be utilised by bacteria and phytoplankton as a resource and catabolised into various molecules[99]. As mentioned in the previous section, DMSP uptake requires energy, so the benefits of this transaction must be significant. Many organisms assimilate DMSP to use it as a source of nutrients, but before they can access these nutrients, they must catabolise DMSP, and they have two pathways to do this; demethylation or cleavage[123]. While some species have the ability to transport DMSP across the membrane and catabolise it via demethylation or cleavage, they are still unable to use it as a sole source of carbon[17,124]. Some organisms may not be using the DMSP but the DMS^3 or acrylate[118] as carbon sources, or it may be that DMSP is used as an exogenous sulfur source[25] but not as a carbon source. For the majority of other organisms, however, cleavage of DMSP results in a useable source of carbon.

As depicted earlier, DMSP may be taken up by bacteria to act as a compatible solute for osmotic acclimation. However, it may also be assimilated and cleaved as an indirect route for scavenging nutrients. For example, the 'messy eater' hypothesis suggests that some zooplankton species which feed on phytoplankton are attracted to the emissions of DMS[124]. When the zooplankton feed on the phytoplankton, they do not eat all of the organism resulting in 'scraps' being left in the nearby environment[124]. The leftovers, such as DMSP, are put to use by bacteria causing more DMS to be released, attracting more grazing by the zooplankton[124]. Additionally, DMS and acylate work better at scavenging hydroxyl radicals compared to DMSP, so they are just as desirable in the cell as an antioxidant.

1.7.1 DMSP demethylation

Within the cell, DMSP can be broken down and catabolised, and this is most commonly achieved by a pathway beginning with a demethylation step, allowing useful nutrients such as carbon and sulfur to be assimilated[125]. From the DMSP taken up into cells, between 50 and 90 % is processed in this way[125]. This pathway can release MeSH (methanethiol) which enables the assimilation of biogenic sulfur, which can be used during the biosynthesis of amino acids or released and consumed by phytoplankton[99]. Although the existence of this pathway was known for many years, the first gene was only discovered in 2006 and designated *dmdA*[27].

In this pathway, DMSP is first demethylated by DmdA, which has a strict substrate specificity and can be assumed to have only this specific function[53]. This process also requires FH_4 (tetrahydrofolate), which is a methyl group acceptor, converting FH_4 to Me-FH_4[27]. Me-FH_4 has important roles within the cell as it can act as a methyl donor in the synthesis of methionine and *S*-adenosyl-methionine or can be further oxidised to produce Formyl-FH_4, which is a carbon donor in cysteine and glycine synthesis[53]. The second demethylation event in this pathway is the demethylation of MMPA (methyl mercaptopropionate)[53] followed by demethiolation to produce MeSH, CO_2 and acetaldehyde[53]. During experiments with *Ruegeria pomeroyi*, MMPA-CoA thioester was discovered during the catalysation of MMPA to MMPA-CoA by DmdB (methylmercaptopropionyl-CoA ligase)[53]. The MMPA part of the thioester is dehydrogenated by the dehydrogenase DmdC resulting in MTA-CoA (methythioacryloyl-CoA)[53]. In a mutant of *R. pomeroyi*, the ability to produce MTA-CoA was removed, and subsequently, the mutant was unable to grow with MMPA as a sole carbon source, which would suggest that the breakdown of MMPA is vital[53]. The final part of this pathway is catalysed by DmdD, which catalyses multiple steps[126]. Firstly the incorporation of a molecule of H_2O results in the synthesis of Mas-CoA (malonate semialdehyde), which is hydrolysed by another H_2O to release the CoA group and the spontaneous degradation of acetaldehyde[126]. Acetaldehyde can then be further converted to acetate by acetaldehyde dehydrogenase[53].

1.7.2 Cleavage of DMSP to DMS

As mentioned earlier, there is a second pathway for DMSP catabolism via enzymatic cleavage resulting in DMS and 3HP or acrylate[17,117,127], and all have important roles in the environment and in industry[128]. The lysis or cleavage enzymes responsible for the breakdown of DMSP to DMS in marine bacteria are termed Ddd enzymes, and homologues can be found in many diverse species[99]. To date, there are eight different *ddd* (including Alma1) genes which encode a varied group of peptides and processes which ultimately result in the catabolism of DMSP and the production of DMS[50].

1.7.2.1 DMSP lysis via dddD

The first gene involved in DMSP catabolism was *dddD* in *Marniomonas* sp. MWYL1, a marine bacterium with the ability to produce DMS when supplemented with DMSP[117]. DddD is a member of the type III acyl-coenzyme A (CoA) transferase family. The *dddD* gene is linked to genes involved in the ancillary degradation of the 3C lysis product and the regulation of these, *dddTBCR*[117]. *E. coli* contains a similar enzyme, CaiB, which moves CoA to carnitine[129]. DddD

contains a polypeptide linker in between two CaiB-like domains that are intertwined with a catalytic aspartate on the C-terminus for CoA transfer[129]. As DddD has the aspartate, it would suggest that DddD works in a similar way to CaiB and uses two steps to breakdown DMSP, firstly by transferring CoA to DMSP, creating 3HP-CoA followed by rapid hydrolysis and release 3HP[129]. The idea that 3HP-CoA is created as an intermediate has still not been confirmed [129], but it may be that the lack of detection is due to the rapidness with which it is converted to 3HP[99]. The *dddD* gene can be found in many species of bacteria and is often found close to an operon containing *dddT*, a BCCT which moves DMSP in or out of the cell[17].

1.7.2.2 DMSP lysis via dddL, dddQ, dddW and dddK

Many species of bacteria that catabolise DMSP were found to contain *dddD*, but there were many that did not, such as the alphaproteobacterium *Sulfitobacter* EE-36[50]. As *Sulfitobacter* was able to degrade DMSP but did not have a DddD homologue, it was suggested that it used a different pathway for catabolism. Curson *et al*. (2008) observed that *Sulfitobacter* lysed DMSP to form DMS and acrylate, and the gene responsible was termed *dddL*[50]. The gene *dddL* is found mainly (but not exclusively) in *Roseobacters* and encodes a small transmembrane peptide, DddL, which does not function like DddD[50].

DddL works by cleaving the carbon-sulfur bond of DMSP, producing acrylate, and interestingly, bacteria that use this process keep the majority of acrylate outside of the cell and within the growth medium[50]. The fact that the acrylate is mostly outside of the cell would suggest that DddL works on periplasmic DMSP and cleaves it outside of the cell, and this could provide benefits to the bacteria. The acrylate may act as a deterrent to other organisms as, at high concentrations, it has antimicrobial activity and deters predators[47]. Or, acrylate could be involved in signalling, and instead of being toxic to predators, it may actually send an anti-grazing signal[47,49]. Some strains containing *dddL* are unable to use acrylate as a sole carbon source, and this therefore, supports the theory that DMSP has other functions than as a nutrient source[50]. Additionally, *dddL* is not usually associated with any transporters like *dddD*, which helps explain the transmembrane properties of the enzyme[50].

DddQ, DddW and DddK are similar to DddL in that they are all small polypeptides that have C-terminus domains which form cupin pockets and bind transition metals[17]. Other than this similarity, the rest of the structure differs, and they are organised within different protein families that have evolved the cupin separately[108]. DddQ was identified in *Roseovarius nubinhibens* when knock-outs of *dddP* did not fully prohibit DMSP degradation[130]. DddW was

additionally found in *Ruegeria pomeroyi* DSS-3 and, to date, has only been found in two strains of *Roseobacter*[17,131]. Most recently, DddK was identified in *Pelagibacter* HTCC1062[132]. Interestingly, *Pelagibacter* contains both the cleavage and the demethylation pathway and is able to use these pathways simultaneously, balancing them with changing cellular sulfur demands[132].

1.7.2.3 DMSP lysis via dddP

Following the discovery of DddD and DddL, DddP was identified in *Roseovarius nubinhiens*[133]. Again, DddP was unlike the previous Ddd proteins, all of which are in different protein families, and emphasised how widespread these DMSP degrading enzymes are. Since the discover of *dddP*, homologues have been identified in multiple species[17]. DddP consists of a homodimer and is part of the M24 metallopeptidase family[133]. Unlike other metallopeptidases, DddP does not need a metal co-factor to function, and it cleaves the sulfur-carbon bond instead of an amino group[127]. Out of all the DMSP lyases, DddP is likely to be the most abundant, and it can be found in marine and terrestrial environments, which would suggest that there have been multiple HGT events in the past[133].

1.7.2.4 DMSP lysis via dddY

DddY was discovered in *Alcaligens faecalis* M3A[134], a betaproteobacterium, but it had previously been purified by de Souza and Yoch in 1995[135]. Interestingly, *A. faecalis* has the ability to grow on DMSP or acrylate as a sole carbon source[136]. DddY is classed as a DUF as its protein family is unknown, yet it is strongly anticipated to be a periplasmic protein[17] because when purified in 1995, it was thought to be periplasmic or associated with the outer membrane of the cell[135], and later confirmed by factionation[134] and would make this the only DMSP lyase to work outside of the cytoplasm[134]. A periplasmic DMSP lyase would be beneficial when compared to a cytoplasmic DMSP lyase as it would remove the need to transport the DMSP inside the cell, meaning the process would be less costly in terms of energy[135].

As with many other DMSP lyases, DddY is found in many species, such as beta-, gamma- and epsilonproteobacteria, and as the gene is so widespread, it was most likely distributed via HGT. Interestingly, *dddY* is the only DMSP lyase not found in any alphaproteobacteria[134]. The organisms that do contain *dddY* are all microaerobic and found mostly in marine and sediment environments. The *dddY* in *Shewanela* species was closely associated with genes

involved in anaerobic respiration, which could mean these organisms are catabolising DMSP to produce acrylate that can be used as an electron acceptor[134].

1.7.2.5 Switching between lysis and demethylation

Organisms are proposed to make the most of these two catabolic pathways by switching between them depending on which is most suitable[28]. There have been several suggested methods of regulation for the switch, such as nutrient supply, light and temperature[137]. *Roseobacter* uses the cleavage pathway preferentially under higher UV-A, whereas in environments of low UV-A, they use the demethylation pathway[137]. The switching in *Roseobacter* in response to UV-A might be because of the antioxidant function of DMS that protects the organism from reactive oxygen species[137]. Additionally, increased temperature resulted in increased cleavage of DMSP and decreased demethylation[137]. Another study found that DMSP demethylation was preferred when DMSP was the prevalent organic sulfur source, allowing organisms to use the biogenic sulfur to meet their needs instead of losing it as its gaseous form[123]. In comparison, if the surrounding environment contains other biogenic sulfur sources, then the DMSP cleavage pathway is preferred since DMSP is not as needed as a sulfur source[123].

1.8 Conclusion and research gap

Clearly, DMSP has important roles in many areas, from the sulfur cycle and the available nutrients within the environment to the protection it offers organisms that can use it. Until fairly recently, DMSP production was thought to be carried out exclusively by marine eukaryotes. However, as discussed previously, Andrew Curson *et al*. (2017) discovered that bacteria share this ability, revealing an understudied area of DMSP production in these organisms. Due to the importance of DMSP production within the environment, the progression of research is vital since bacterial DMSP synthesis may have a significant influence on the synthesised DMSP and may influence the current model of sulfur cycling.

This thesis aims to test the hypothesis that bacteria are important contributors to the total concentration of DMSP within an environment by:

1. Determining the diversity and abundance of DMSP-producing bacteria within an environment.
2. Utilising culture-dependent techniques to isolate DMSP-producing bacteria from Stiffkey saltmarsh sediment.

3. Identifying important bacterial DMSP-producers and determining the method of DMSP synthesis by these organisms using culture-dependent techniques.

2 Materials and methods

2.1 Chemical synthesis

DMSP was synthesised as described in Todd *et al.* (2010) using DMSP (Sigma-Aldrich) and acrylic acid (Sigma-Aldrich). Both DMSP-amin and SMM were synthesised as described by Curson *et al.* (2017). Met, MTOB, MTHB and MTPA were obtained from Sigma Aldrich.

2.2 Media preparation and growth conditions

Novosphingobium BW1 and *Thalassospira profundimaris* were grown in YTSS[138], MB medium[139] or MBM (Marine Basal Medium) 35 PSU (practical salinity units) unless otherwise stated, 10 mM mixed carbon source (1 M stock containing 200 mM of glucose, glycerol, pyruvate, succinate and sucrose), and either 0.5 or 10 mM NH_4Cl at 30 °C. The salinity and nitrogen concentration of MBM was altered by adjusting the quantity of sea salts (Sigma-Aldrich) and NH_4Cl within the media. Methylated sulfur compounds were only added to MBM in specific experiments investigating the effect of those compounds on various strains. *Escherichia coli* was grown in LB (Luria-Bertani) complete medium[140] at 37 °C. *Rhizobium leguminosarum* was grown in TY (tryptone yeast) complete media[141]. *Roseovarius indicus* was cultured in MBM media (as above). *Pseudobacteriovorax antillogorgiicola* was grown in MB medium at 30 °C. *Nocardiopsis chromatogenes* and *Streptomyces mobaraensis* were grown GYM Streptomyces Medium 10% NaCl (DSMZ medium 1159) at 30 °C. *Corallococcus coralloides* was grown in VY/2 agar or SP – medium[142]. *Stigmatella aurantiaca* was grown in VY/2 agar or CY agar[142] at 30 °C. Where necessary, the media was supplemented with antibiotics in the following concentrations (unless stated otherwise): ampicillin (100 μg/ml), gentamycin (20 μg/ml), kanamycin (20-200 μg/ml), neomycin (80 μg/ml), rifampicin (20 μg/ml), spectinomycin (200 μg/ml), streptomycin (400 μg/ml), and tetracycline (5 μg/ml). The use of antibiotics in experiments allowed for the selection of particular strains and/or vectors. The strains used throughout this study are listed in Table 2-1.

Table 2-1 Strains used throughout the study.

Strain	Description	Reference
Corallococcus coralloides	The strain was used to test the functionality of *mmt*.	DSMZ Culture Collection [143]
Escherichia coli 803	Used for routine transformations.	[144]
Escherichia coli BL21	Allows overexpression of cloned genes in pET vectors.	[145]
Novosphingobium sp. BW1	Wild-type strain, isolated from Stiffkey Saltmarsh.	[65]
Pseudobacteriovorax antillogorgiicola	The strain was purchased from DSMZ to test the functionality of *mmt*.	DSMZ Culture Collection [146]
Rhizobium leguminosarum J391	A streptomycin-resistant derivative of the wild-type strain 3841 was used for library screening and expression of genes cloned in plasmid pLMB509.	[147]
Stigmatella aurantiaca	Strain purchased from DSMZ and used to test the functionality of *mmt*.	DSMZ Culture Collection [148]
Thalassospira profundimaris WP0211 (DSM 17430)	Wild-type strain.	DSMZ Culture Collection[149]
Thalassospira profundimaris WP0211 Rif^R	Rifampicin-resistnat derivative of *T. profundimaris* WP0211.	[65]
Thalassospira profundimaris WP0211 Rif^R (*mmtN*$^-$)	*T. profundimaris* WP0211 Rif^R with a mutation in *mmtN*.	[65]

2.3 Transformations into *E. coli*

2.3.1 Making chemically competent cells

Starter cultures of 5 ml LB inoculated with *E. coli* (803/BL21) were incubated at 37 °C, 200 rpm overnight. A 1:100 dilution of the starter culture was inoculated into 100 ml LB and incubated at 37 °C, 200 rpm for 2-3 h (to OD_{600} 0.2-0.4). The culture was split into 50 ml sterile falcon tubes and spun in a centrifuge at 4 °C, 6000 rpm for 5 minutes to retrieve the cells. The falcon tubes were kept on ice, the supernatant removed, and both pellets were gently mixed with 10 ml ice-cold 0.1 M $CaCl_2$ and incubated on ice for 30 minutes. The incubation in $CaCl_2$ causes the cell wall to become positively charged, attracting the negatively charged DNA and resulting in a higher number of successful transformations. The cells were centrifuged as before, and the supernatant was removed. To resuspend the pellets, 2 ml of 0.1 M $CaCl_2$ was added to one of the falcon tubes, and then once resuspended, this was used to resuspend the second pellet. Cells were left on ice for at least 30 minutes or at 4 °C overnight.

2.3.2 Heath shock transformations

To 100 µl competent cells, 4 µl of DNA was added and incubated on ice for 30 minutes. Heat shock at 42 °C for 3 minutes and then immediately put on ice. Add 500 µl LB to the cells and incubate at 37 °C for 1 h. Aliquots of 100 µl were plated onto LB agar containing selective antibiotics. The remaining culture was spun at max speed in a tabletop microcentrifuge for 2 minutes. Most of the supernatant was poured off, and the pellet was resuspended in the remaining liquid and plated onto LB with selective antibiotics. The plates were incubated at 37 °C overnight.

2.4 Polymerase Chain Reaction (PCR)

Sequences were amplified using PCR in a Thermal Cycler in a 25 µl, 50 µl, or 100 µl reaction. The standard 25 µl reaction contained 12.5 µl MyFi™ DNA Polymerase (enzymes/buffer/dNTPs/DMSO), 1 µl template (~100 ng), 1 µl of 100 pmol of forward and reverse primers (**Table 2-2**) and 9.5 µl water. Each PCR had a negative control where the template was replaced with water and a positive control using genomic DNA. In some cases, DNA was amplified from single bacterial colonies to allow large-scale isolate screening. A sterile loop was used to pick a single colony and mix it with 200 µl of sterile water; 1 µl of this solution was used as a template.

Table 2-2 Primers used throughout the study.

Primer name	Sequence (5' to 3')
16S	
27F	AGAGTTTGATCCTGGCTCAG
1492R	GGTTACCTTGTTACGACTT
Positive cosmid insert amplification	
M13 uni (-43)	AGGGTTTTCCCAGTCACGACGTT
M13 rev (-29)	CAGGAAACAGCTATGACC
SAM-Methyltransferase - *Novosphingobium*	
Forward	cggatcccatATGTCTGACGCAGATGACTCC
Reverse	ggaatTCACTCTACCTTGGGGATACC
SAM-Methyltransferase – *Roseovarus*	
Forward	cgaattccatATGACCGATTTCAAAACGCCCG
Reverse	cccggatccTCAACGATTGGACGGATCGGTTTCC
Diaminopimelate decarboxylase - *Roseovarius*	
Forward	cggatcccatatGAGGCCTGGTGGGCGCGGGAAG
Reverse	cgaatTCAATCCAGCAGCAGCACCTCTTTTGC
Aspartate aminotransferase - *Roseovarius*	
Forward	cggatcccatATGAATGCACTCGCGGAAACCG
Reverse	cgaatTCACAGCCGGCCGGCCTCCAGGTCG
SAM-Methyltransferase - *Streptomyces*	
Forward	cggatcccatATGCCGTCCGAGCACACGATG
Reverse	cgaatTCATCGCCGGTCCTCCTCGTCGG
SAM-Methyltransferase - *Thalassospira*	
Forward	cggatcccatATGCAACATGCTTTAGAAGAGAGC
Reverse	cgaattcTTAGGCCGGTGTGCCGCGAATGAC
SAM-Methyltransferase - *Nocardiopsis*	
Forward	cggatcccatATGAGAACAGAGACCGGACCGCC
Reverse	cgaattCTACGTGGCGGGTGTGCCCCTGAC
LacZ fusion - *Thalassospira*	
Forward 1	cgcgctgCAGGGATCATCGGGATCAAAG
Forward 2	cgcgcgaaTTCGCTTGGCCCGCTTGCCC
Forward 3	cgcgctctAGATGGCCTATGACGACGGTG
Reverse	cgcgctctAGAACACCGTAAATCGGTTC

N.B. Restriction sites included in primers underlined

2.5 Visualisation and extraction of DNA

2.5.1 Agarose Gel Electrophoresis

Gels were made to 1 % (w/v) agarose using 1x TAE Buffer (50x stock made up of 242g Tris base, 57.1 ml glacial acetic acid, 100 ml 500 mM EDTA pH 8.0, water to 1 L. A 1x solution contains 40 mM Tris, 20 mM glacial acetic acid, and 1 mM 500 mM EDTA pH 8.0). The agarose was melted and cooled to 50 °C, 3 µl Ethidium Bromide (10 mg/ml) was added and then poured into gel trays. Samples and a 1 KB Plus DNA ladder (Invitrogen) for reference were loaded into wells. Gels were usually run at 90 V for 60 minutes. The separation of the DNA fragments was visualised using a UV gel imaging doc.

2.5.2 PCR purification (Roche)

The Roche High Pure PCR Product Purification Kit was used to recover DNA following PCR amplification using five times the volume of Binding Buffer to the PCR mix. The purified PCR product was eluted into a 1.5 ml microcentrifuge tube using 35 – 50 µl sterile water.

2.5.3 Gel extraction (QIAGEN)

Following gel electrophoresis, DNA was extracted using the QIAquick Gel Extraction Kit. Dissolved gel samples were precipitated with one volume of isopropanol and 10 µl 3M Sodium acetate. DNA was eluted by adding 35 µl of sterile water to the centre of the column membrane. After 5 minutes, the column was centrifuged for 1 minute at full speed using a tabletop microcentrifuge.

2.6 DNA extraction (linear and plasmid)

2.6.1 Phenol chloroform DNA extractions

A starting culture of 5 ml LB was inoculated from a single colony and incubated at either 30 °C or 37 °C with shaking overnight. From the starter culture, 1.5 ml was dispensed into a microcentrifuge tube, and the cells were pelleted by centrifugation at maximum speed for two minutes. The supernatant was discarded, the pellet was resuspended in 250 µl Buffer P1 to which 250 µl of Buffer P2 was added and mixed by inversion, and 350 µl Buffer P3 was added immediately after. The samples were left on ice for 5 minutes and centrifuged at maximum speed for 10 minutes. The supernatant was removed into a clean microcentrifuge tube and mixed with 400 µl Phol:Chloroform:Isoamyl Alcohol 25:24:1 (v/v). This solution was vortexed for 5 to 10 seconds until the mixture was homogenised and cloudy. The samples were centrifuged again at maximum speed for two minutes, after which the top aqueous layer was removed to a clean microcentrifuge tube containing 800 µl of 100 % ethanol and mixed by

inversion. The tubes were spun at maximum speed for 10 minutes, the supernatant was discarded, and 500 µl of 70 % ethanol was added to the pellet. Samples were spun a final time at maximum speed for 2 minutes, and the ethanol was removed. The pellet was air-dried for 5 to 10 minutes before resuspending in 35 µ of nuclease-free water. The DNA was quantified using a nanodrop.

2.6.2 Minipreps (QIAGEN)

The QIAprep Miniprep involves preparing and clearing bacterial lysate, binding he DNA to the QIAprep membrane and washing and eluting the plasmid DNA. The first step involves the alkaline lysis method developed by [150] and the neutralisation and adjustment of the lysate to high-salt conditions. The lysate is then purified using the QIAprep silica membrane which allows for selective absorption of plasmid DNA that is in high-salt buffer and elution in the presence of low-salt buffer. Any RNA, cellular protein, or metabolites are not held in the silica membrane and so come out in the flow through. Next, the column is washed with Buffer PB to remove endonucleases and salts with a wash of Buffer PE. Finally, the purified DNA is eluted from the QIAprep column with water.

The QIAprep Spin Miniprep Kit was used to extract plasmid or cosmid DNA from 3 ml of 5 ml starter cultures of LB incubated overnight at either 30 °C or 37 °C. The column was eluted by adding 35 µl of nuclease-free water to the membrane, leaving it for one minute and then centrifuging at maximum speed for one minute to collect the DNA. Plasmids used in this study are presented in **Table 2-3**.

Table 2-3 Plasmids used throughout the study.

Plasmid	Description	Reference
pLAFR3	Wide host-range cosmid vector used for the construction of genomic libraries.	[151]
pET21a	Allows the expression of cloned genes in *E. coli*.	Merck Millipore
pRK2013	Helper plasmid used in triparental matings.	[152]
pK19-Spec	Plasmid used in creating *mmtN* SCO knockout in *T. profundimaris*	[17]
pLMB509	Used to express *mmtN* in *T. profundimaris* mutant.	[153]
pBIO0438	pLAFR3 cosmid from BW1 library, ~21 kb genomic DNA including *mmtN*.	This study

pBIO0762	pLAFR3 cosmid from BW1 library, ~30 kb genomic DNA including *mmtN*.	This study
pBIO21N1	BW1 *mmtN* cloned into pET21A	This study
pBIO509N	BW1 *mmtN* clones into pLMB509	This study
pBIO21T2	*T. profunfimaris* WPO211 *mmtN* cloned into pET21a	This study
pBIO19TK	Disruption mutant for *T. profundimaris mmtN* in pK19-Spec	This study
pBIO21R3	*R. indicus mmtN* cloned into pET21a	This study
pBIO21N4	*N. chromatogenes mmtN* cloned into pET21a	This study
pBIO21S5	*S. morbaraensis mmtN* cloned into pET21a	This study

2.6.3 QIAGEN Plasmid Midipreps

The QIAGEN Plasmid Midiprep Kit performed high-quality, high-concentration plasmid extractions with the QIAGEN-tip 100 column on 100 ml of culture. To elute the DNA from the column, 5 ml of Buffer QF was added. The DNA was then precipitated by adding 3.5 ml room temperature isopropanol. The mixture was aliquoted into 1.5 ml microcentrifuge tubes and centrifuged at maximum speed for 30 minutes. Following centrifugation, the supernatant was removed, and the DNA pellets were washed with 500 µl 700 % ethanol and centrifuged again at maximum speed for 10 minutes. The ethanol was aspirated, and the pellet air dried for 5 to 10 minutes. Finally, the DNA was re-dissolved in a suitable volume of nuclease-free water and quantified using a nanodrop. Plasmids were stored at -20 °C.

2.6.4 Promega Genomic DNA extractions Promega

The Wizard® Genomic DNA purification kit extracted genomic DNA from bacterial isolates. Following the nuclei lysis step, the solution was incubated for 5 minutes at 80 °C and then cooled to room temperature. To the cell lysate, 3 µl of RNase Solution was added and mixed and then incubated at 37 °C for 45 minutes. To rehydrate the genomic DNA, 35 µl of nuclease-free water was added and incubated at either 65 °C for 1 hour or 4 °C overnight. The genomic DNA was stored at -20 °C.

2.7 Restriction digests using FastDigest enzymes

Thermo Scientific FastDigest restriction enzymes were used to digest DNA. A 20 µl reaction comprised of up to 16 µl of DNA (depending on the DNA concentration), 1 µl of Enzyme 1, 1 µl Enzyme 2 (if required), 2 µl FastDigest Buffer and distilled water (up to the required volume), was added to a microcentrifuge tube and incubated at 37 °C for 60 minutes. The solution was inactivated by incubation at 80 °C for 5 minutes. For vectors that required dephosphorylation,

1 µl of alkaline phosphatase, 2.5 µl of alkaline phosphatase buffer, and 1.5 µl of nuclease-free water were added and incubated at 37 °C for 60 minutes. The mixture was inactivated by incubating at 80 °C for 5 minutes. The digested DNA was visualised on a 1 % agarose gel to ensure correct-sized fragments.

2.8 Quantification of DMSP

2.8.1 GC quantification of DMS/DMSP/SMM

Gas chromatography assays were used to determine a sample's quantity of DMS, DMSP, or SMM. The measurements were taken from 2 ml glass vials which contained 300 µl liquid samples, and were sealed with PTFE/rubber crimp caps. From these vials, the DMS was measured from the headspace using a flame photometric detector (Agilent 7890A GC fitted with a 7693 autosampler). For DMS, 200 µl of culture and 100 µl of water were placed in the vial and sealed. The vials were placed in the shaking incubators at 30 °C overnight and assayed on the GC the following day. For DMSP and SMM, the samples needed to be treated to allow the release of DMS by alkaline lysis. For DMSP, 200 µl of culture was placed into the vial, then 100 µl of 10 M NaOH was added, and the vials were crimped immediately to avoid the loss of released DMS. Again, samples were placed in the dark in the 30 °C shaking incubator overnight and assayed the next day. SMM measurements were treated the same, except after sealing the vials, they were incubated at 80 °C for 10 minutes.

To quantify the amount of DMS, DMSP, or SMM present, each experiment required an eight-point calibration curve using the appropriate compound as a standard (**Figure 2-1**). These standards were prepared as previously mentioned. The limit for detection in the headspace was 0.015 nmol of DMS in water.

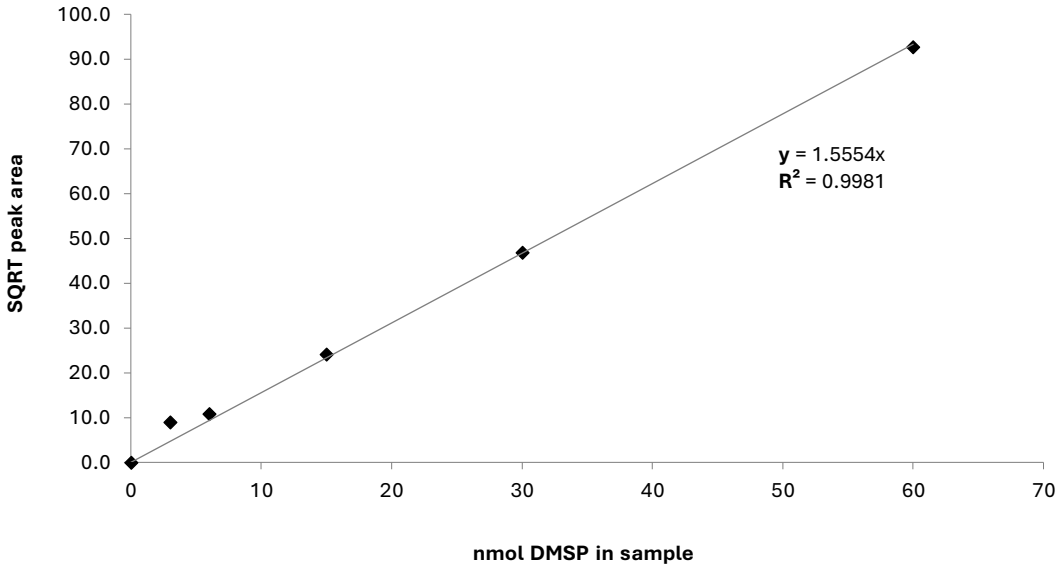

Figure 2-1 A calibration curve using known concentrations of SMM (ranging from 1.5 nmol to 30 nmol SMM) to determine the amount of DMS released. The DMS in the headspace of the vials was measured following the alkaline lysis of SMM; this was achieved by adding 100 µl of 10 M NaOH to 200 µl of SMM and water solution. The vials were incubated for 10 minutes at 80 °C and then in the dark in a shaking incubator at 30 °C overnight before being assayed using GC.

2.8.2 DMSP quantification by LC-MS

LC-MS was used to confirm that the DMS detected by GC was released by DMSP and not another compound since both SMM and DMSHB lyse to DMS following alkaline hydrolysis. The process began by extracting samples. First, cells were recovered from 3 ml of culture and resuspended in 300 µl of 80 % LC-MS grade acetonitrile (extraction solvent) and mixed by pipetting. The solution was centrifuged at maximum speed for 3 minutes, and 200 µl was put into a fresh 2 ml screw cap tube. The second round of extraction began with adding another 200 µl of 80 % acetonitrile to the pellet and the remaining 100 µl supernatant. The pellet was resuspended before centrifugation again for 3 minutes. Another 200 µl of supernatant was collected, and a third round of extraction was carried out as before to produce a total of 600 µl of supernatant to be analysed by LC-MS. LC-MS was performed using a Shimadzu Ultra High-Performance Liquid Chromatography (UHPLC) system (formed by a Nexera X2 LC-30AD Pump, a Nexera X2 SIL-30AC Autosampler, a Prominence CTO-20AC Column oven and a Prominence SPD-M20AD Diode array detector) and a Shimadzu LCMS-2020 Single Quadruple Liquid Chromatograph Mass Spectrometer. Samples were analysed in hydrophilic interaction chromatography (HILIC) mode using a Phenomenex Luna NH2 column (100 x 2 mm with a particle size of 3 µm) at pH 3.75. The mass spectrometry spray chamber conditions were a

capillary voltage of 1.25 kV with an oven temperature of 30 ºC, a desolvation temperature of 250 ºC and nebulising gas flow of 1.50 L min^{-1}. Solvent A was 5 % acetonitrile and 95 % 5 mM ammonium formate in water. Solvent B was 95 % acetonitrile + 5 % 100 mM ammonium formate in water. The flow rate was 0.6 ml min^{-1}, and the gradient (% of solvent A/B) was t = 1 min, 100 % B; t = 3.5 min, 70% B; t = 4.1 min, 58% B; t = 4.6 min, 50% B; t = 6.5 min, 100% B; t = 10 min, 100% B. The injection volume was 15 µl. All samples were analysed immediately following the extraction. The targeted mass transition corresponded to $[M+H]^+$ of DMSP (*m/z* 135) in positive mode. A calibration curve was performed for the quantification of DMSP using pure DMSP standards in the extraction solvent.

2.8.3 DMSP quantification using purge-trap and GC

Sediment was weighed out into 0.5 g and mixed in 25 ml distilled water with 0.5 % H_2SO_4 and incubated at room temperature for 1 hour, upon which 5 ml of the mixture was mixed with 1 ml 10 M NaOH and incubated at room temperature overnight. The purge and trap method was used to quantify the amount of DMS within the sample[154]. The samples were purged for 20 minutes, and then any DMS was detected and quantified using an Agilent 7890B gas chromatography (GC) machine. Calibration curves were made using the same method but with 5 ml of each gradient of DMSP concentration standards. Similarly, seawater was measured as detailed but using 5 ml of water as the sample.

2.9 Protein quantification

2.9.1 Bradford assay

Bradford assays were used to determine the protein concentration within a sample. The cells from 1 ml of culture were pelleted by centrifugation for 1 minute at maximum speed and resuspended in 500 µl Tris-HCL buffer (50 mM, pH 7.5). The cells were lysed using sonification for three rounds of 10 seconds and rested on ice between sonication. Samples were centrifuged for 10 minutes at maximum speed to remove cell debris, and 20 µl of supernatant was mixed with 980 µl of Bradford Reagent and added to a cuvette. The absorption was measured at OD_{595} using a spectrometer. Known concentrations of BSA were used as standards to create a four-point protein standard graph (Figure 2-2), allowing the calculation of ug protein within each culture.

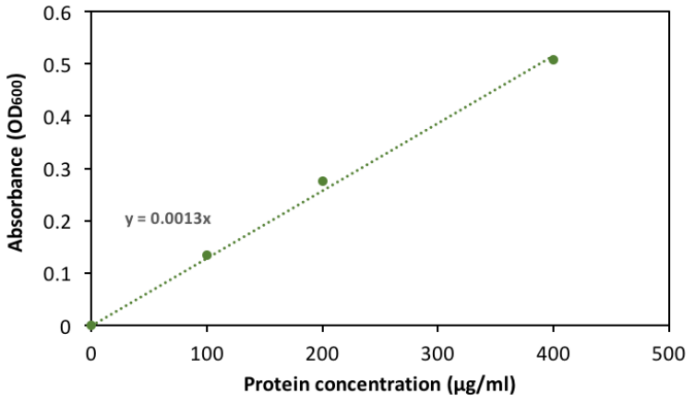

Figure 2-2 A four-point protein standard graph used to calculate the protein concentration of samples. Standards are BSA at concentrations of 0, 100, 200 and 400 µg μl^{-1} in water. Absorbance = OD_{595}. Plotted with a line of best fit.

2.10 Culture-dependent experiments

2.10.1 Sampling Stiffkey saltmarsh sediment

The site chosen for sampling sediment was on the lower marsh of Stiffkey saltmarsh (52°57'51.5"N 0°55'31.8"E). Acrylic corers, which were tapered at one end, were used to collect triplicate marine sediment samples that included the pool water as well as the oxic and anoxic layers of sediment. The concentration of DMSP was measured from 200 µl of pool water, 0.5 g of oxic sediment and 0.5 g of anoxic sediment. In addition to this, the pH, salinity, and temperature were also noted.

2.10.2 Isolating DMSP-producing bacteria

Samples from before and after the DMSP enrichment incubation were diluted and plated onto MBM minimum medium agar and incubated at 28 °C for 72 hours. Colonies with differing morphologies were picked and screened for the ability to produce DMSP. Any positive isolates were purified and identified using PCR amplification of the 16S rRNA gene using primers 27F and 1492R[155]. It is important to remember that whilst these primers are a valuable tool in aiding the identification of bacterial organisms, they were developed over 30 years ago[156,157]. There have been many studies doubting the efficiency of these primers and how appropriate they are to represent the diversity of the bacterial community, and reviewed by [158].

The PCR products were purified and sent to Eurofins Genomics (https://www.eurofinsgenomics.eu, Munich, Germany) for sequencing. The results were identified taxonomically using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). To confirm the ability of the isolates to produce DMSP, they were grown in low nitrogen conditions and screened again by GC and the quantities normalised to the cellular protein content, which were estimated using Bradford assays. The isolated strains were tested for the presence of

dsyB using degenerate primers. The bacterial isolates were stored as pure colonies by mixing 525 µl culture with 225 µl DMSP and 750 µl 50 % glycerol and frozen at -80 °C.

2.10.3 Microscopy

After growing cultures to stationary phase, 5 µl of culture was placed on a glass microscope slide and covered with a glass coverslip, taking care to avoid bubbles. Immersion oil was placed in the coverslip, and the samples were observed at 100x light magnification with the Olympus BX40 microscope with the Olympus Camedia C-7070 digital camera. Samples were considered pure when there was no noticeable variation in the morphology of the observed cells in multiple areas of the glass slide.

2.10.4 Whole genome sequencing

Strains were sent to the MicrobesNG sequencing facility (https://microbesng.uk, University of Birmingham) for Whole Genome Illumina sequencing using the Illumina MiSeq platform, which produced 2 x 250 bp paired-end reads. The reads were trimmed using Trimmomatic and in-house scripts combined with SamTools, BedTools and bwa-mem were used to assess the quality. RAST [159–161] (http://rast.nmpdr.org) was used to annotate the sequences[159] against the closest related species genomic sequence.

To screen the genomic library, the fragment of *Novosphingobium* DNA which was thought to contain the potential DMSP-producing gene was aligned to the sequenced genome. The functional genes within this fragment were analysed using BLAST to determine the likelihood of the resulting enzyme being able to process a step in the DMSP synthesis pathway in *Novosphingobium*.

2.10.5 Growth curves

Growth curves exponential growth phase to be determined for other growth experiments. Starter cultures were inoculated in 5 ml MBM and incubated at 30 °C with 200 rpm shaking (unless otherwise stated) for 16 hours or until reaching 0.6 OD_{600}. Triplicate flasks of 100 ml MBM were then inoculated with 2 ml of starter culture and incubated at 30 °C with 200 rpm shaking. Growth was measured at OD_{600} in 1 ml of culture every hour until cultures reached stationary phase. Stationary phase was determined by the OD_{600} remaining consistent for at least 3 hours. The measurements were averaged and plotted on a line graph.

2.10.6 DMSP pathway induction incubations

Triplicate cultures were inoculated in 10 ml MBM, in either standard conditions (35 PSU with 12 mM nitrogen) or MBM containing 5 or 50 PSU salt levels, or lowered nitrogen levels at 0.5 mM nitrogen. Cultures were incubated overnight at 30 °C with 200 rpm shaking, and DMSP quantity was measured using GC and normalised for protein concentration.

An induction experiment specifically for the pathway intermediates was performed on BW1. Starter cultures were adjusted to an OD_{600} of 0.5 and inoculated into 3 x 100 ml MBM, and incubated for 12 hours. The DMSP levels of the Time 0 culture were measured, and subsequently, the cultures were measured into 5 ml aliquots and mixed with 0.5 mM of each of the intermediates Met, MTOB, MTHB, DMSHB, MMPA, MTPA, SMM and DMSP-amine, individually. A control mix which contained none of the intermediates was used as a control. The cultures were incubated at 30 °C with 200 rpm shaking. Following the incubation, 200 µl of each culture was used to quantify the DMSP concentration in triplicate, and protein content was measured at 30, 60, 120 and 240 minutes.

2.10.7 Environmental conditions and DMSP production

The effect of changing environmental conditions on BW1 DMSP production began with a starter culture grown in standard media (35 PSU MBM, 12 mM nitrogen). The starter culture was inoculated into 5 ml of either 5, 35 or 50 PSU MBM with 12 mM nitrogen, 35 PSU MBM with 0.5 mM nitrogen, or standard media in triplicate. All cultures were incubated at 30 °C with 180 rpm shaking overnight. However, one of the standard media cultures was incubated at a lower temperature of 16°C. Protein content and DMSP concentration were analysed as described previously and compared. For *T. profundimaris*, triplicate cultures were inoculated into MBM of salinity levels of 5, 35, 50 and 70 PSU, with 0.5 mM nitrogen (which was set as the 'standard' MBM condition from this point) to test the effect that salinity has on the production of DMSP. Cultures were also grown in 35 PSU MBM with high nitrogen levels of 12 mM to observe the impact. Cultures were incubated overnight, and DMSP levels were quantified using 200 µl of culture.

2.10.8 Seawater incubations and DMSP production

The low levels of DMPS in the seawater were detected using purge-trap and GC, as detailed above. After growing strains in MBM at 28 °C with 180 rpm shaking overnight, they were pelleted ad washed three times and resuspended in filter-sterilised seawater. The cultures

were adjusted to an OD600 of 0.4 and diluted 1:1000 unto 20 ml filter-sterilised seawater (T0). The cultures were incubated at 25 °C with 90 rpm shaking for 21 hours (T1) and 43 hours (T2). The samples from each time point were pelleted, and the supernatants were collected. Cells were resuspended in Tris-HCl buffer (50 mM, pH 7.5), and the DMSP within the cells and the supernatant was quantified by adding 500 µl 10 M NaOH and incubating at room temperature in the dark overnight. Any DMS generated was measured using a modified purge and trap method[154] and measured with an Agilent 7890B gas chromatography with a flame photometric detector. An HP-5 (0 – 325 °C) 30 m x 320 µm capillary column (Agilent Technologies, Inc) was used to separate the sulfur gases under the oven thermal cycle, which was 50 °C to 120 °C (20 °C min^{-1}) to 180 °C (30 °C min^{-1}) to 50 °C. The GC had a detection limit for DMS of ~0.015 nmol.

2.11 Gene library construction

2.11.1 QIAGEN Genomic DNA extraction

The QIAGEN Genomic DNA extraction kit was used to extract high-quality and high-volume DNA. Following the elution of the genomic DNA with 1 x 5 ml Buffer QF. The DNA was precipitated by the addition of 0.7 x the volume of the eluted DNA of room-temperature isopropanol. The mix was inverted 10 to 20 times. The DNA was collected using a sterile 5 ml pipette tip and transferred to a microcentrifuge tube containing 1 ml of TE buffer (pH 8.0, or 10 mM Tris·Cl, pH 8.5). The DNA was dissolved in a shaker at 55 °C for approximately 2 hours.

2.11.2 Library construction

A genomic library of BW1 was constructed, which allowed the fragments to be screened in the wide-host species *R. leguminosarum* J391 following the method described in Curson et al. (2008). The genomic DNA of BW1 was extracted using the QIAGEN Genomic-tip 100/G kit, and test digestions with *Eco*RI determined the stage at which the genome was partially digested into approximately 25 – 30 kb fragments (~5 – 10 minutes) before being flash-frozen in liquid N_2 which stopped the digest, and the samples were run on a 0.5% agarose gel to assess the fragment size. After a time was confirmed, up to 10 µg of genomic DNA was partially digested, and 100 µl of the digest was transferred to a tube containing 200 µl 100% ethanol and 10 µl 3M sodium acetate (pH 4.8) and frozen in liquid N_2.

After ethanol precipitation, the DNA was quantified, and at least 2.5 µg of genomic DNA was ligated into the *Eco*RI-digested, dephosphorylated cosmid vector pLAFR3. The ligation was

ethanol precipitated, resuspended in 17 µl nuclease-free water, and 0.7 µg was packaged in preparation for transfection into *E. coli* 803 using the Stratagene Gigapack III XL Packaging mix. The mix was removed from the -80 °C freezer until partially thawed, and the genomic DNA was added and mixed by stirring with the pipette tip. Tubes were incubated for 2 hours at 22 °C before being mixed with 500 µl SM buffer and 20 µl chloroform. The supernatant containing the phage was removed and stored as glycerol. The packaged genomic DNA fragments were transfected into 803, which had been prepared by inoculation into 100 ml LB supplemented with 10 mM $MgSO_4$ and 0.2% (w/v) maltose and incubated at 37 °C for 4 – 6 hours, or 30°C overnight (not reaching above an OD_{600} of 1). The cells were pelleted by centrifuging at 500 x g for 10 minutes and resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.5. To titre the library and determine the number of clones, the packaged mix was diluted either 1:10 or 1:50 with SM buffer, and 1 µl was mixed with 200 µl host cells and incubated at 37 °C for 15 minutes, topped up with LB to 1.5 ml and plated on LB tetracycline which would select for pLAFR3 cosmids. The colonies were counted, and the number of clones in the library was calculated. In this case, the library consisted of an estimated 50,000 clones. Another transfection into *E. coli* was set up with a higher volume of packaged cosmids, 25 µl of 53 cosmid mix and *E. coli* were mixed 1:1 and incubated at 37 °C for 30 minutes, mixed with 200 µl LB and incubated for an hour, shaking gently every 15 minutes. The cells were pelleted and resuspended in 50 µl LB and plated on LB tetracycline. To test for the correct-sized fragments and ensure the fragments were different, six colonies were digested with *Eco*RI, *Bam*HI and *Hind*III.

Multiple rounds of infection into *E. coli* ensured a high number and variation of cosmids, which were pooled together and stored in glycerol at -80 °C. The clones were crossed into J391 via tr-parental mating. Approximately 1,000 transconjugants were picked to RM medium containing 0.5 mM Met, incubated overnight at 30 °C with 200 rpm shaking and screened by GC for the ability to produce SMM to J391 (because of MMT activity). The samples were prepared for GC by the addition of 100 µl 10 M NaOH and heating at 80 °C for 10 minutes, and the samples were then incubated in the dark overnight before quantifying the amount of DMS produced. Positive samples were checked by re-inoculation and repeat screening. After confirming the samples showed MMT activity, the plasmids were extracted and transformed into 803 and mobilised back into J391 by tri-parental cross before re-confirming MMT activity. Positive cosmids were digested with *Eco*RI, *Bam*HI, *Hind*III and *Pst*I to demonstrate the presence of inserted fragments in the pLAFR3 cosmid and to compare the fragments.

2.11.3 Tri-parental crossing

A tri-parental cross was used to transfer plasmids or cosmids from *E. coli* to *Rhizobium*. The process involves three strains, a host (*Rhizobium*), a donor (*E. coli* 803 containing the plasmid or cosmid of interest) and a helper (*E. coli* pRK2013[162], which is Kan^R). The helper strain contains a plasmid with the genes needed to assemble a conjugation bridge. During the incubation of the helper and donor strain, some of the helper strains will transfer the helper plasmid to the donor strains, resulting in donor strains that contain the plasmid of interest and the plasmid required to make a conjugation bridge. The donor strains with the helper plasmid are selected for by growing in the presence of antibiotics of tetracycline and kanamycin, since *E. coli* 803 is resistant to tetracycline and the helper plasmid has a kanamycin resistance cassette. The host strain, *Rhizobium* is resistant to spectinomycin and so is grown with this antibiotic in the medium. Both cultures are then washed so that they can be grown in media without any antibiotics and mixed on a filter and grown overnight. During this time, it is expected that the donor will create a conjugation bridge and transfer the donor plasmid, resulting in the host containing the donor plasmid. This is selected for by growing the culture on selective media containing kanamycin, tetracycline, and spectinomycin. Any bacteria growing should be *Rhizobium* containing the donor plasmid. This can be checked using primers specific to the donor plasmid and gel electrophoresis.

The host strain is inoculated into TY media with appropriate antibiotics and incubated at 28 °C with 180 rpm shaking overnight. The donor and helper strains are inoculated into LB with appropriate antibiotics and incubated at 37 °C with 180 rpm shaking overnight. To wash out residual antibiotics, 1 ml of the host culture was centrifuged at maximum speed to form a pellet which was subsequently resuspended in 500 μl of fresh TY media; this was repeated three times. Finally, the cells were pelleted and resuspended in 100 μl TY media. The donor and helper were treated in the same way. The 100 μl from each of the strains were added to a filter on a TY agar plate containing no antibiotics and mixed with a sterile loop. Control crosses (helper & host and donor & helper) were plated in the same way. All plates were incubated at 28 °C overnight. The filters were placed into sterile universals using ethanol-sterilised forceps, and the cells were washed off the filter using 2 ml 50 % glycerol. An appropriate dilution of the cells was plated on selective TY plates (containing kanamycin and antibiotics to select for the donor DNA). Again, plates were incubated at 28 °C and any successful crosses were confirmed using colony PCR.

2.12 Identification of MmtN enzymes and phylogenetic trees

BLAST searches to identify homologues of the MmtN protein of BW1 were performed using BLASTp at NCBI or JGI. MmtN homologues and more distantly related methyltransferases in Pfam family PF10672, which were below the predicted cut-off for MMT functionality (E values ≤ $1e^{-90}$, identity = 36 %), were aligned by ClustalW in MEGA v6 and visualised in a maximum-likelihood phylogenetic tree to observe the relatedness of the sequences.

2.13 *in vivo* and *in vitro* genetic manipulations

The plasmids (Table 2-3) were transformed into *E. coli* or transferred into *R. leguminosarum* J391 or *T. profundimaris* by conjugation in a tri-parental cross using *E. coli* pRK2013 as a helper strain. Restriction digestions and ligations were performed as in Downie et al. (1983). Table 2-2 shows the oligonucleotide primers used for molecular cloning, which were synthesised by Eurofins Genomics. The company also sequenced plasmids and PCR products.

2.14 SMM assay

The genes encoding SAM-MMT from BW1, *R. indicus, T. profundimaris, S. morbaraensis* and *N. chromatogenes* were cloned into pET21-a using specific primer amplifying fragments from genomic DNA (Table 2-2), which were digested with *Nde*I and *Eco*RI, or *Bam*HI (in the case of *R. indicus*) restriction enzymes. The plasmid clones are described in Table 2-3. The pET21a containing *mmtN* homologues were transformed into *E.* coli BL21. SMM activity was measured in the clones by growing the strains in LB complete medium in triplicate overnight at 37 °C with 180 rpm shaking. From these cultures, 1 ml was spun down in the microcentrifuge at maximum speed for 2 minutes. The cells were resuspended in 1 ml LB medium and diluted 1:1000 into 5 ml LB. The cultures were incubated for 2 hours at 37 °C with 180 rpm shaking and then induced with 0.2 mM IPTG (Sigma-Aldrich) and incubated at 30 °C with 180 rpm shaking overnight. From each culture, 1 ml was mixed with 0.5 mM L-Met (Sigma-Aldrich) and incubated for 8 hours at 30 °C with 180 rpm shaking and subsequently sampled on the GC to determine the amount of SMM produced during the incubation period. The amount of protein within the cells was calculated using the Bradford method (BioRad). Controls included media only, *E. coli* BL21, and *E. coli* BL21 with an empty pET21a vector.

2.15 Purification of MmtN and in vitro catalytic assays

2.15.1 SAM charcoal affinity testing

The ability of activated charcoal to sequester SAM was determined by mixing duplicates of 0.5 mM SAM, 0.5 mM SMM, or both with 200 μl sterile water. After quantifying the amount of DMS released from the samples, 400 μl of activated charcoal solution (38 mg ml^{-1} in 0.1 M Acetic Acid) was added and incubated for 5 minutes. The mixture was centrifuged at maximum speed for 1 minute, which removed the charcoal and the compounds within it. The supernatant was carefully removed, and the DMS measurements were taken and compared to the concentrations before charcoal.

2.15.2 Cell lysate activity of *Novosphingobium*

BW1 cells grown in YTSS were harvested by centrifugation at maximum speed for 2 minutes. The cells were resuspended in 50 mM Tris-HCl buffer and sonicated to lyse the cells. To remove debris, the samples were centrifuged at maximum speed for 2 minutes, and the lysate was removed and dialysed to remove pre-existing metabolites. The dialysis was done using dialysis tubing (SpectrumLabs) in 2 L of dialysis buffer (20 mM HEPES, 150 mM NaCl, pH 7.5) at 4 °C overnight. From the lysate, duplicates of 200 μl were mixed with 1 mM SAM, 1 mM Met, or both and incubated at room temperature for 30 minutes. The MMT activity in the samples was measured by adding 10 M NaOH, heating at 80 °C for 10 minutes, and then measuring on the GC.

2.15.3 Purifying MmtN

Cultures of *E. coli* BL21 expressing the MmtN protein were grown in LB at 37 ° C with 180 rpm shaking to an OD_{600} of 0.8 to 1.0 and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 20 °C for 16 hours. MmtN was purified with NI^{2+}-NTA resin (QIAGEN, Germany) and fractionated with gel filtration buffer (10 mM Tris-HCl pH 8.0 and 100 mM NaCl) on a Superdex-200 column (GE Healthcare, America). The purification of MmtN was carried out at 4 °C. Next was the NI^{2+}-NTA resin purification; this was achieved by removing protein impurities with wash buffer (50 mM Tris-HClpH 8.0, 250 mM NaCl and 20 mM imidazole) and eluting the purified protein from the column with elution buffer (50 mM Tris-HCl pH 8.0, 250 mM NaCl and 250 mM imidazole).

The activity of the MmtN enzyme was measured by using HPLC to detect the production of S-adenosyl homocysteine (SAH) produced by the demethylation of SAM. The optimal conditions

for MmtN activity were determined by comparing the enzyme activity under different conditions, such as temperature and pH. The highest activity was defined as 100 % activity, with the other related conditions being described as relative to this definition. The reactions were incubated for 30 minutes at temperatures between 0 °C and 60 °C and measured at 10 °C intervals, and optimal pH levels were assessed using Britton-Robinson buffer (40 mM H3BO3, 40 mM H3PO4 and 40 mM CH3COOH) with pH levels varying between 5.0 and 10.0. Finally, the K_m of the activities were determined using non-linear analysis of the initial rates and determined using 3.34 μM MmtN and 0.1 – 4 mM SAM or 0.1 – 6 mM Met.

2.16 Creating an *mmtN*⁻ mutant and phenotyping

To select *T. profundimaris* when grown with *E. coli*, it was necessary to create a *T. profundimaris* spontaneous Rif^R mutant. This was achieved by growing a concentrated number of cells on MB-Rif plates and incubating them for 48 to 72 hours. Single colonies were picked and tested for their ability to grow on agar plates containing rifampicin. Upon successfully creating a mutant, the *T. profundimaris* Rif^R was treated as the wild-type strain during the experiments comparing the performance of the *mmtN*⁻ strain with the wild-type.

To create the *mmtN*⁻ mutant, primers were designed containing sites for the restriction enzymes *Eco*RI and *Bam*HI to amplify ~500 bp fragment internal to the *T. profundimaris* WPO211 *mmtN* open reading frame and cloned into pBIO1879[130], which is a derivative of pK19mob (a suicide vector), forming pBIO19TK. Using tri-parental conjugation, pBIO19TK was transferred into *T. profundimaris* Rif^R using the helper strain *E. coli* pRK2013. Mutants with the correct recombination in the target gene were selected by growing on YTSS agar containing rifampicin (WPO211), kanamycin (pBIO1879), and spectinomycin (pBIO1879). The potential *mmtN*⁻ mutants were isolated and checked for DMSP production and further checked by digestion with *Eco*RI and *Bam*HI, and the insert was visualised using gel electrophoresis. Primers designed to the outside of the insert in the plasmid were used to ensure the correct insert, as any strains that did not give a band when visualised on a gel likely had huge inserts into the DNA. Finally, complementation of the mutant was carried out by crossing pBIO21N1 (containing *Novosphingobium mmtN*) back into *T. profundimaris mmtN*⁻ and the return of partial function was screened using methods described previously.

To investigate any phenotypes associated with the disruption of *mmtN*, the wild-type and *mmtN*⁻ strains were grown in varying conditions. The salinity and nitrogen levels were varied,

and the strains were tested for their ability to survive after freezing. Salinity was tested by growth in MBM media with 0.5 mM (now standard) with various concentrations of sea salt (Sigma-Aldrich), such as 35 PSU (approximately the level of seawater) and 50 PSU. Cell growth was measured by optical density hourly at OD_{600} until stationary phase was reached. The effect of higher nitrogen was observed by growing the strains in 35 PSU MBM with 12 mM nitrogen and monitored as before. The tolerance to freezing was measured by growth in 35 PSU MBM with 0.5 mM nitrogen, and the cultures were adjusted to the same cell density in a 1 ml aliquot. A serial dilution using 100 µl of each culture was plated on MB agar plates, and after 2 to 3 days of growth at 28 °C, the number of cells counted. The remaining 900 µl was frozen at -20 °C for 5 days. The cultures were thawed and plated and counted as before, and the percentage of cell survival following freezing was calculated. Finally, cultures of each strain were grown to stationary phase in 35 PSU MBM with 10 mM nitrogen and inoculated 1:1 into fresh media. After growth at 30 °C with 180 rpm shaking overnight, the culture was plated onto MB agar and grown at 30 °C for 2 days. Colonies were picked and tested for kanamycin and spectinomycin resistance to determine survival.

The complementation of *T. profundimaris mmtN*$^{-}$ was achieved by subcloning the *Novosphingobium mmtN* from pBIO21N1 into pLMB509 (a taurine-inducible wide-host range plasmid, Gent^{R}) using *Nde*I and *Eco*RI, creating pBIO509N. pBIO509N was mobilised into the mutant via tri-parental mating, as explained before, and selected for by growing on rifampicin and gentamycin. Colonies were tested for their ability to produce DMSP using GC.

2.17 Statistics

The measurements for DMSP production, DsyB enzyme activity, or MmtN enzyme activity were all based on the mean of at least three biological replicates per strain or condition tested. The program used for statistics throughout this thesis was Microsoft Excel[164].

3 DMSP production in marine sediment

3.1 Introduction

3.1.1 Stiffkey saltmarsh

Marine sediment environments cover two-thirds of the Earth's surface[165] and are considered hotspots for organosulfur cycling that contain DMSP concentrations with orders of magnitude greater than other areas[166–168]. The sediment within saltmarshes are essential sites of DMSP production and cycling; they are highly saline and sulfurous environments with DMSP levels significantly higher than the overlying seawater[169]. However, this had been chiefly attributed to the grass *Spartina* which produces high intracellular DMSP levels [42,61]. However, there are other diverse sources of DMSP, e.g., bacteria and algae, in these sediments that may also be significant contributors.

Stiffkey saltmarsh (North Norfolk) comprises upper and lower marsh habitat[170]. The lower marsh is flooded with high tides twice a day, and the salinity of the sediment is about the same as the seawater[171]. In contrast, the upper marsh is only covered by spring tides, in winter, precipitation can cause these areas to become hyposaline, and in summer, the area can become dry with a salinity of three to four times the concentration in seawater[171]. The lower marsh, in particular, was chosen as an area of study, due to its more stable conditions and ease of access and sample during low tide. The site was approximately an hour's drive from our laboratory at UEA, so the samples could be collected and processed quickly to reduce the impact of samples being stored in non-natural conditions. Furthermore, due to the plant life around the tidal pools, it was easy to identify the pools on subsequent visits adding extra reproducibility to the results.

3.1.2 *dsyB* gene probe

Following the discovery of *dsyB* in *L. aggregata* [31], further investigation was carried out on the presence and abundance of bacteria containing *dsyB*. Dr Beth Williams designed degenerate primers to *dsyB* by aligning known functional gene sequences to identify conserved regions and designing primer sets specifically targeting the gene[22]. Most importantly, the primers needed to amplify sequences from a diverse phylogenetic group of bacteria with similar but not identical nucleotide *dsyB* sequences[172]. The degenerate primers designed for *dsyB* were used to study the presence and abundance of this gene in marine sediment from Stiffkey saltmarsh. Indeed, it was observed that the sediment contained many bacteria with the genetic potential to synthesise DMSP and that these bacteria could hypothetically be important DMSP producers within this environment[22].

3.1.3 Culture-dependent methods of isolation

The method of isolating bacteria from a chosen environment, characterising and studying them, is a relatively easy and cost-effective way to investigate key microbes within an area and how they interact together and within the environment. Previously, Carrión *et al.* (2017) used culture-dependent and -independent methods to gain a greater understanding of a pathway where DMS is generated from the methylation of methanethiol (MeSH), known as the Mdd pathway in sediment environments[54]. The study isolated bacteria from grassland soil samples before and after a 14-day incubation period, where the samples were incubated with succinate and MeSH to enrich for bacteria that could methylate MeSH and produce DMS. Additionally, the DMS produced could potentially enrich bacteria with the ability to degrade DMS. Many diverse bacteria, including *Pseudomonas* (gammaproteobacteria), *Streptomyces* (actinobacteria), and *Bacillus* (bacilli), were isolated before the enrichment. After the enrichment, isolates were made up of *Gemmobacter*, *Phyllobacterium, Rhizobium, Sinorhizobium, Ensifer* (all alphaproteobacteria), *Pseudomonas,* and *Acinetobacter* (all gammaproteobacteria). Isolates were screened for their ability to produce DMS from MeSH. Approximately 58 % of the isolates were able to produce DMS before the enrichment, whereas roughly 96 % of isolates had the ability following the incubation[173]. This experiment clearly demonstrated how effective enrichment cultivation can be and how culture-dependent and -independent studies can complement one another. Notably, these isolates were also found in 16S rRNA gene analysis conducted on the samples pre- and post-incubation, and their relative abundances were as expected; that is, those isolated from the enrichments were more abundant in DNA isolated from the enriched samples.

It is important to remember that there are disadvantages to these techniques, with the most prominent being that only ~1 % of bacteria are viable under laboratory conditions[174,175] and results in the majority being overlooked. Nevertheless, this method is still a valuable tool; usually, the most abundant bacteria are the most abundant. Additionally, as shown in work by Carrión *et al.* (2017), combining culture-dependent and -independent methods reduces doubts about the bias of either method being done on their own drastically. It is also worth noting that there is not always the option for culture-independent studies in understudied areas. DMSP-synthesis by bacteria was only recently discovered, and whilst methods such as metagenomics and metatranscriptomics can be used to look for the presence of functional genes for that process, for example, *dsyB*, it does not help if the critical genes for the process being studied have not been identified. The discovery and characterisation of novel genes and pathways almost always require culture-dependent microbiology work on model organisms. Hence, the most utilised and most straightforward method is plate culturing[173,176], which for

this study, allowed bacteria to be isolated, purified and then screened for the ability to produce DMSP regardless of the presence or absence of known genes.

This study aimed to look for a synthesis process that is more complex than enriching for uptake or degradation pathways. It is relatively easy to enrich for processes such as the uptake and catabolism of a compound because the organisms generally gain significant benefits by degrading that compound. For example, in DMSP catabolism, some organisms can use DMSP as a sole source of carbon and/or sulfur. In this case, simple incubations of environmental samples with DMSP as the sole carbon source should be sufficient since only organisms that can use DMSP as a sole carbon source should grow and thus would be enriched. In contrast, enriching a process in which an organism does not gain a massive benefit is much more difficult. For example, although DMSP is known to be an osmoprotectant, bacteria in which essential DMSP synthesis genes have been mutated and which no longer make this compound show the same growth patterns as wild-type strains, even under high salt conditions. The best option in the case of DMSP production is to set up a mixture of conditions previously shown to enhance the production of DMSP in *Labrenzia* in Curson *et al*. (2017). These conditions include low nitrogen levels, high salinity and the presence of methionine as the precursor for DMSP. The theory here is that high salt would select for microbes requiring osmoprotectants, and low nitrogen and methionine would select for sulfur osmolytes such as DMSP production.

Furthermore, if able to isolate bacteria from the sites of interest that produce DMSP, it confirms their presence in that environment. However, as importantly, these bacteria can also be used as model organisms, characterised and genetically manipulated, to identify the processes at play in the environment and vital pathways and genes/enzymes that might be important in that bacterium and potentially in the environment. Work on such relevant models is likely more valuable than work on model bacteria that conduct the process but were isolated from a different environment. Whilst work under laboratory conditions is not the same as in the natural environment, such experiments can be used to gain knowledge of processes that are important to the environment. Additionally, the sediment environment differs greatly from those inhabited by well-known DMSP synthesisers. For example, marine sediment creates anoxic and microoxic areas that are often light-starved—suggesting that the organisms that may be producing DMSP in these areas are different to those seen elsewhere.

3.2 Chapter aims

Saltmarsh sediments are environments with high levels of DMSP production. Previous studies have reasoned this to be due to Eukaryotic organisms such as *Spartina*; however, these areas likely contain other important microbial DMSP-producers. Just as succinate and MeSH were used to enrich for DMS production via the Mdd pathway in Carrion *et al*. (2017), enrichment methods adapted from Curson *et al*. (2017) were designed to increase microbial DMSP production that should increase the success of DMSP-producing organisms. Thus, the main aim is to conduct a proof of concept experiment to enrich and isolate DMSP-producing bacteria using both culture-dependent and -independent methods. For the former, the aim is to identify and characterise the DMSP production by any bacterial isolates. Following the isolation of any DMSP-producing bacteria, they will be screened for the presence of the only known bacterial DMSP-synthesis gene, *dsyB,* by PCR. The plan also involved high-throughput sequencing in showing that the established enrichment techniques increased the microbes producing DMSP. Overall, this chapter will test the hypothesis that the DMSP found within marine sediment is partly due to bacterial synthesis.

3.3 Results

3.3.1 Stiffkey saltmarsh site characterisation and sample collection

To better understand the environment chosen for study, in-depth nutrient and salinity analysis of the Stiffkey saltmarsh characteristics was carried out with the help of Andrew Hind, UEA. Due to the Stiffkey being a tidal environment, samples were collected at low tide as the area was inaccessible at high tide. The tidal pools were between 1.5 to 2 m wide (Figure 3-1), and the temperature and conductivity of the pool were measured using a Fisherbrand accumet AP75. The salinity could be calculated from the conductivity using a three-point calibration, using Fisherbrand Traceable Conductivity Standards that are NIST Certified Reference Materials (CRM).

Figure 3-1 An example of the tidal pools at Stiffkey saltmarsh, taken during a sampling trip in September 2021.

The O_2 content was measured using a Jenway 970 and a 2-point calibration curve which was carried out in the field at ambient temperature with filtered seawater in equilibrium with air (100 % O_2 saturation) and 2 M sodium sulphite solution (0 % O_2 saturation). The surface water had an O_2 saturation of 62 %, declined to 34 % at 0.8 cm, and further to 29 % at 1.6 cm, where the water and sediment met. The dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) (Table 3-1) were obtained with a Skalar Formacs CA15 analyser and a six-point calibration that was validated against Canada Environmental Matrix Reference Material Cranberry-05, lot 0317. The TDN includes organic nitrogen species, NO_3^{1-}, NO_2^-, NH_4^+, and N_2O, but not N_2.

Table 3-1 The amount of dissolved organic carbon (DOC) and total dissolved nitrogen (TDN). Calculations were made from the mean of triplicate measurements.

	Concentration (mg L^{-1})	Standard error (mg L^{-1})
DOC	3.60	0.07
TDN	0.59	0.01

The sediment was collected using bespoke acrylic corers, driven into tidal pools to a depth of ~ 15 cm. The collected samples were transported back to the laboratory with minimal disturbance to ensure the layers were not disturbed. pH and salinity measurements were taken from the water layer using an electronic pH meter and the Fisherbrand accumet AP75 with a three-point calibration, using Fisherbrand Traceable Conductivity Standards that are NIST Certified Reference Materials (CRM) (Table 3-2). Once these measurements were taken, the water was drained off.

The oxic layer was removed and used for DMSP quantification, incubation experiments and culturing. The core was quickly split to allow samples to be taken from the anoxic level at 5, 10, and 15 cm from the surface sediment. The anoxic sediment was only used to determine the amount of DMSP present. DMSP quantification on sediment was achieved by mixing 0.1 g sediment with 100 μl sterile water in 1.5 ml GC vials. To chemically release DMS, 10 M NaOH was added to the vials, and they were crimp-sealed, vortexed for 5 to 10 seconds and incubated in the dark at room temperature overnight. The pool water samples were measured in the same way but using 200 μl pool water. The samples were tested in triplicate, and the DMS was quantified using an Agilent 7890A GC fitted with a 7693 autosampler (Table 3-2).

Table 3-2 DMSP concentration, pH, salinity, and temperature of a tidal pool at Stiffkey saltmarsh. The oxic layer is 0 – 1 cm, and the anoxic is >1 cm.

Sampling site	Location (Lat, Long)	Depth (cm)	nmol DMSP g^{-1} or ml^{-1}	Salinity (PSU)	pH	Temp (°C)
Stiffkey sediment	52.964947, 0.925655	0 – 1	77.1 ± 15.0			
		1 - 5	9.8 ± 0.8			
		5 – 10	4.6 ± 0.3			
		5 - 15	3.9 ± 0.03			
Stiffkey pool water			0.4 ± 0.1	38	7.5	17

3.3.2 DMSP production by *Sporobolus anglica* in Stiffkey

The production of DMSP within saltmarshes is often associated with the activity of *Sporobolus anglica*. *Sporobolus* sp. were collected to measure DMSP content within these plants. Furthermore, to determine how DMSP concentrations changed within the sediment in relation to the distance from these DMSP-producing plants, sediment was collected in transects moving away from the *Sporobolus* species.

From the samples of *Sporobolus* sp. collected, 0.1 g of leaf and root in quadruplicate samples were measured for DMSP content by pulverising the samples and treating them with MeOH to create a methanolic extract. The extract was quantified on GC and normalised to 1 g fresh weight (FW). The *Sporobolus* sp. roots contained 2,568.6 ± 24.5 nmol g^{-1} FW DMSP, and the leaves contained 9,579.5 0177 ± 796.9 nmol g^{-1} FW DMSP. When compared to other levels published, there are some which are very similar[21] and some which are indeed a lot higher[42], leading to the suggestion that *Sporobolus* sp. have varying abilities when it comes to the production of DMSP.

The DMSP content within *Sporobolus* sp. in Stiffkey is still considerably higher than those detected in bacteria, and thus it was hypothesised that the DMSP within the sediment closest to the plants would have the highest DMSP content and, therefore, be predominantly due to eukaryotic activity. This would also infer that bacteria and algae would have a more significant role in DMSP produced further away from the plants. To examine whether this was the case, oxic sediment was collected from a transect that began directly in contact with the *Sporobolus* plant and moved away from the plant in intervals of 10 cm. The DMSP content from these samples was quantified using GC and can be seen in Figure 3-2.

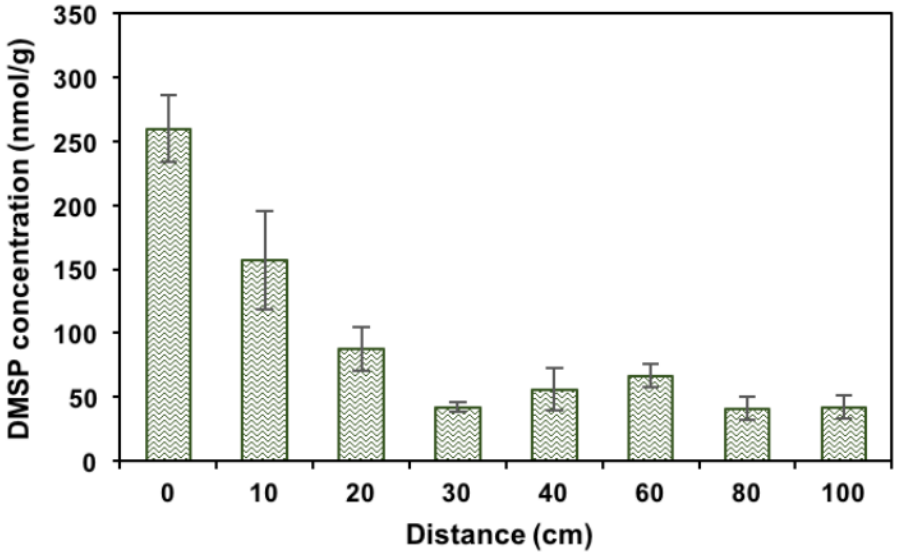

Figure 3-2 The DMSP concentration within oxic sediment in relation to the distance from *Sporobolus* sp. in Stiffkey. Sediment was collected in a transect that began directly in contact with *Sporobolus* sp. and gradually got further away. The DMSP content in the samples was quantified using gas chromatography. Error bars show standard error.

The DMSP concentration in sediment directly in contact with the *Sporobolus* plant was very high but then dropped dramatically as the distance from the plant increased to 30 cm away. However, after his point, it appeared that the DMSP concentration stabilised to an amount similar to those measured in the tidal pools, implying that *Sporobolus* does contribute to the total DSMP content. However, there are potentially other DMSP-producing organisms with major roles, which could potentially be algae and bacteria.

3.3.3 Isolating bacterial DMSP-producers and optimising production

To check whether DMSP-producing bacteria could be isolated from Stiffkey saltmarsh sediment, a serial dilution was made using 100 μg sediment in MBM to a dilution factor of 10^{-6} and 100 μl plated onto MBM agar containing a mixed carbon source (see 2.2). The plates were incubated at 28 °C for a week, and colonies with different morphologies were purified to single colonies. Once pure, the colonies were screened for the ability to produce DMSP using the GC, and for several of the strains, the presence of DMSP was confirmed with LC-MS. From the 33 strains isolated, 9 showed detectable amounts of DMS after treatment with NaOH, presumably from DMSP, and those isolates were identified by sequencing their 16S rRNA gene. Table 3-3 shows the strains identified, with alphaproteobacteria being the most abundant; however, there were also gammaproteobacterial isolated.

Table 3-3 The characterisation of the most abundant or novel bacteria isolated from Stiffkey sediment. NT = Not tested.

Isolate	Closest 16S rRNA gene identity	Intracellular DMSP concentration (pmol DMSP μg protein $^{-1}$)	Presence of *dsyB* with degenerate primers	Presence of DMSP indicated by LC-MS
S4	*Marinobacter* sp. Set72	38 ± 1.3	-	✓
S5	*Labrenzia* sp. BR-18	278.6 ± 62	+	✓
S8	*Stappia* sp. M8	153.1 ± 11.5	-	NT
E26	*Pseudooceanicola* sp. 22II1-22F33	64.4 ± 2.3	-	✓
E27	*Rhodobacter* sp. AB300d	495.4 ± 53.5	-	✓
E30	*Oceanicola* sp. Ar-45	78.5 ± 0.58	+	NT
E35	*Rhodobacteriales bacterium* JB-27	762.0 ± 403.9	+	✓
E37	*Roseobacter* sp. ARCTIC-P4	44.2 ± 3.4	NT	NT
E45	*Novosphingobium* sp. MBES04	665.8 ± 102.3	-	✓
E48	*Alteromonas genevensis* PQQ33	6.9 ± 3.5	NT	NT

The next step was to attempt to enrich for and, more specifically, isolate DMSP-producing bacteria that might contain novel DMSP synthesis genes and pathways from Stiffkey sediment. This first involved establishing conditions that increased DMSP levels in sediment samples. As detailed above, enriching a synthesis process is more complicated than for a catabolic process as it requires a clear benefit for the production of the compound. Conditions for this experiment were based on those shown to increase DMSP production in *L. aggregata*[31], including high salinity[36], high sulfur, low nitrogen[105], the addition of MTHB (the precursor for the reaction catalysed by *dsyB*)[31], and a combination of all four conditions. From the sediment collected from the tidal pools, 2 g was weighed into flasks and mixed with 30 ml MBM medium. The conditions tested were in triplicate and included a control of standard MBM. The flasks were incubated at 30 °C with 180 rpm shaking for one week. After adding NaOH, the DMSP content was analysed by GC (Figure 3-3).

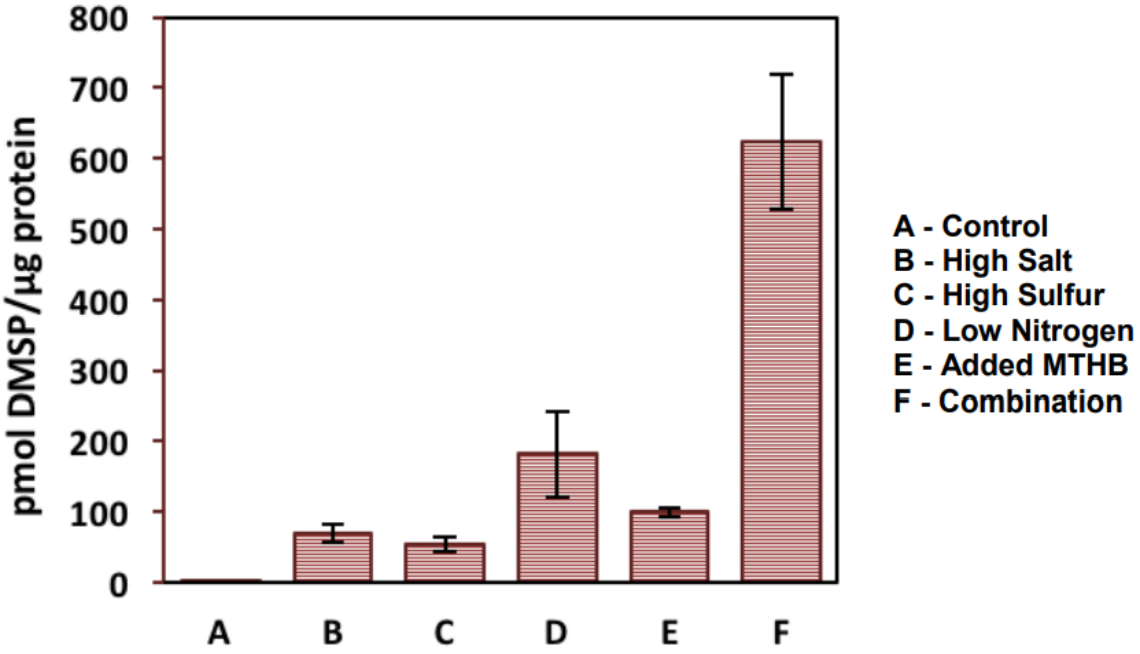

Figure 3-3 The average DMSP produced in Stiffkey saltmarsh sediment[22]. Sediment slurries were incubated in various enhancing conditions, such as increased salinity and sulfur, low nitrogen, the addition of MTHB and all of them combined. Error bars show standard error.

As Figure 3-3 shows, all the individual conditions significantly enhanced the production of DMSP in the sediment slurries, with the low nitrogen tests showing the highest level of these singlet tests. However, the highest DMSP levels were seen when all four enrichment experiments were combined, which were at least three-fold higher than any of the other conditions tested; that is, the percentage of DMSP-producing isolates rose from 27 % before the enrichment incubation to 77 %. Hence, all four were combined as the enrichment condition for the following experiments.

3.3.4 Purification of DMSP producers

As many of the isolates from the Stiffkey sediment had the same or similar phylogeny, representatives of each genus were fully classified and are summarised in Table 3-3. To obtain pure strains, single colonies were streaked onto MBM agar plates until no contaminating colonies could be seen. The colonies were inoculated into liquid media and observed using microscopy to confirm cell size and shape homogeneity. Any colonies that appeared to be contaminated were diluted to dilution factors between 10^{-4} and 10^{-6}, plated on MBM agar, and incubated at 28 °C overnight. Once the isolates were considered pure, they were tested individually for DMSP production using GC. The pure isolates were stored at -80 °C and on

agar plates at -4 °C. Following confirmation that the isolates contained DMSP, they were classified to confirm their identification (Table 3-3).

To identify the now pure isolates, the 27F/1429R primer set was used to amplify the 16S rRNA gene on the genomic DNA of the pure cultures. To confirm successful amplification, 5 μl of PCR product was visualised using gel electrophoresis and the remaining PCR product was extracted using a PCR purification kit. Eurofins Genomics sequenced the purified 16S rRNA fragments, and the phylogenetic identity was determined by submitting the sequences to a nucleotide BLAST against all sequences within the NCBI database. The top hit, usually with a >99 % identity, was used to determine the phylogeny, and an example of each can be seen in Table 3-3.

Several isolates were suspected DMSP producers, such as Labrenzia, Oceanicola, Pseudooceanicola, and Stappia, as they are all from genera containing *dsyB* and have previously been shown to produce DMSP. Similarly, *Rhodobacter* and *Rhodobacterales* are closely related to *Labrenzia,* so their ability to produce DMSP was less surprising. Interestingly, *Novosphingobium*, *Marniobacter* and *Alteromonas* were previously not known to be able to synthesise DMSP. Although *Novosphingobium* is an alphaproteobacterium, it is from the order Sphingomonodales, which had not previously been linked to DMSP production, similarly, nor had *Marinobacter* or *Alteromonas* which are gammaproteobacteria and were the first in this class to be observed as DMSP producers.

Alkaline hydrolysis can release other methylated sulfur compounds, such as SMM and DMSHB, following treatment with NaOH (most significantly in the presence of heat). Therefore it was important to confirm that the peaks of DMS were indeed due to the lysis of DMSP. To do this, the presence of DMSP was confirmed by analysis with LC-MS. The DMSP has a diagnostic retention time of 4.9 with an appropriate mass/charge ratio. Example chromatograms may be seen in **Chapter 4**.

3.3.5 Determining inducers

In order to characterise the isolated strains further, more incubation experiments were set up to investigate how variations in the growth conditions or the addition of intermediates from the transamination or other DMSP synthesis pathways influenced DMSP synthesis. The isolates were inoculated into MBM with either standard conditions (at this time, it was 20 PSU

with 12 mM N_2), 5, 35 or 50 PSU salinity, or lowered nitrogen levels. Intermediates from the transamination pathway (Met, DMSHB, MTHB or MMPA) were added separately to a final concentration of 0.5 mM. The cultures were incubated at 30 °C overnight with 180 rpm shaking. The DMSP produced was measured using GC and normalised to protein concentration (**Figure 3-4**).

There was a clear difference in the DMSP produced not only by the comparison of growth conditions but also by the various strains. *Labrenzia* and *Stappia* clearly demonstrate the expected production following induction. There is a clear increase from the standard conditions to low nitrogen and an even greater increase when incubated with intermediates from the transamination pathway, the synthesis pathway identified in *Labrenzia* by Curson *et al*. (2017) and the suspected pathway used by *Stappia*. There appeared to be no increase in the amount of DMSP produced in any of the lower or higher salinities tested. This was more surprising for the higher salinity of 50 PSU since, as discussed previously, DMSP has been suggested as an osmoprotectant. Usually, there is an increase in DMSP production between 5 and 35 PSU, but not between 35 and 50 PSU, which is thought to be because 50 PSU is too stressful for the bacteria.

Since Met is the precursor to the three DMSP synthesis pathways, it was expected that this condition would cause an increase in the synthesis of DMSP. This was indeed the case for all strains except *Rhodobacterales*. Unlike the other strains that increased by a significant amount in comparison to the standard media, for *Rhodobacterales*, the amount of DMSP produced was similar between the standard media and with the addition of Met. It may be that the Met was used in other pathways, that it may not have been taken up efficiently, or that the level used was detrimental to the growth of the organisms. Within some isolates, there appears to be a higher production of DMSP when incubated with DMSHB. This was not likely due to the release of DMS from DMSHB via chemical lysis because this requires heating to provide activation energy. It is possible that some of the bacteria can cleave DMSP to release DMS, but this has never been tested. Finally, these bacteria might contain a decarboxylase enzyme that liberates DMSP from DMSHB.

Most interestingly, *Novosphingobium* increased DMSP synthesis substantially when incubated with Met but does not any of the other intermediates from the transamination

pathway. This could be explained by the suggestion that *Novosphingobium* is able to synthesise DMSP but not via the transamination pathway. This is noteworthy since bacteria have only been observed producing DMSP via the transamination pathway, which has been believed to be most predominant in the marine environment.

3.3.6 Incubations in seawater

The experiments detailed so far have been conducted under conditions much different from those found in the natural environment. While it has been demonstrated that the isolates from Stiffkey saltmarsh sediment could produce DMSP, it was necessary to demonstrate that this was also possible *in situ* and that bacteria contributed to the total DMSP content within this environment. For these experiments, two strains were incubated in seawater, *Pelagibaca bermudensis* (which contains *dsyB*) and *Novosphingobium* (which does not contain *dsyB*, see later). Both strains were inoculated into MBM and incubated at 30 °C overnight with 180 rpm shaking. The cultures were adjusted to an OD_{600} of 0.4 and diluted 1:100 in filter-sterilised seawater (T0). The incubation was at 25 °C with 90 rpm shaking for 21 hours (T1) and 43 hours (T2). At each time point, cell pellets were collected, the supernatant removed, and the pellets resuspended in Tris-HCl buffer (50 mM, pH 7.5). Both the resuspended cells and the supernatant were tested for DMSP. In this instance, the DMS released by adding NaOH was processed via a modified purge trap method and measured using GC (Figure 3-5).

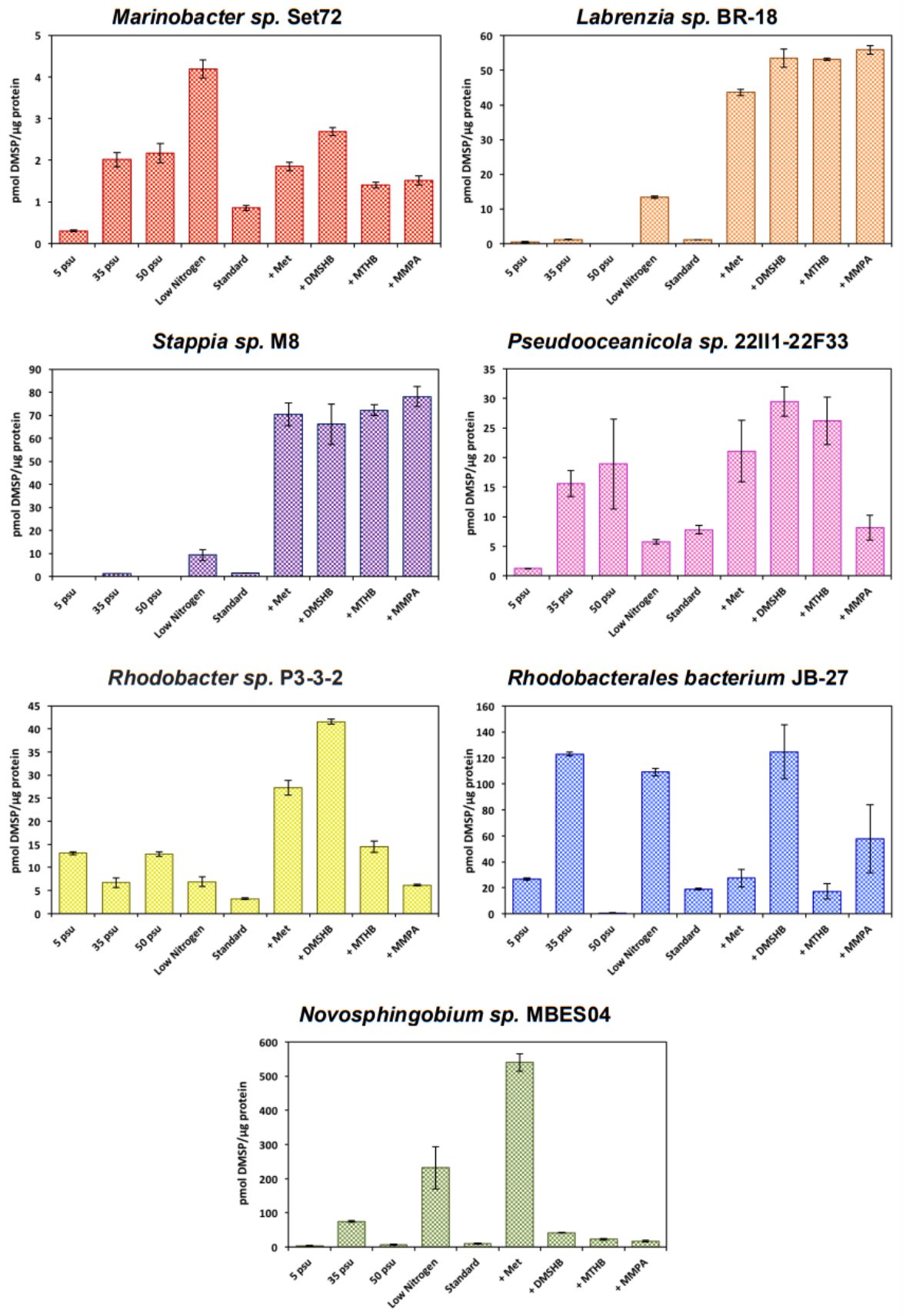

Figure 3-4 Isolates from Stiffkey saltmarsh sediment (*Marinobacte, Labrenzia, Stappia, Pseudooceanicola, Rhodobacter, Rhodobacterales,* and *Novosphingobium*) incubated in various conditions thought to increase DMSP production[22]. The cultures were grown in standard conditions or MBM with either 5, 35, or 50 PSU, low nitrogen, or the addition of Met, DMSHB, MTHB, or MMPA.

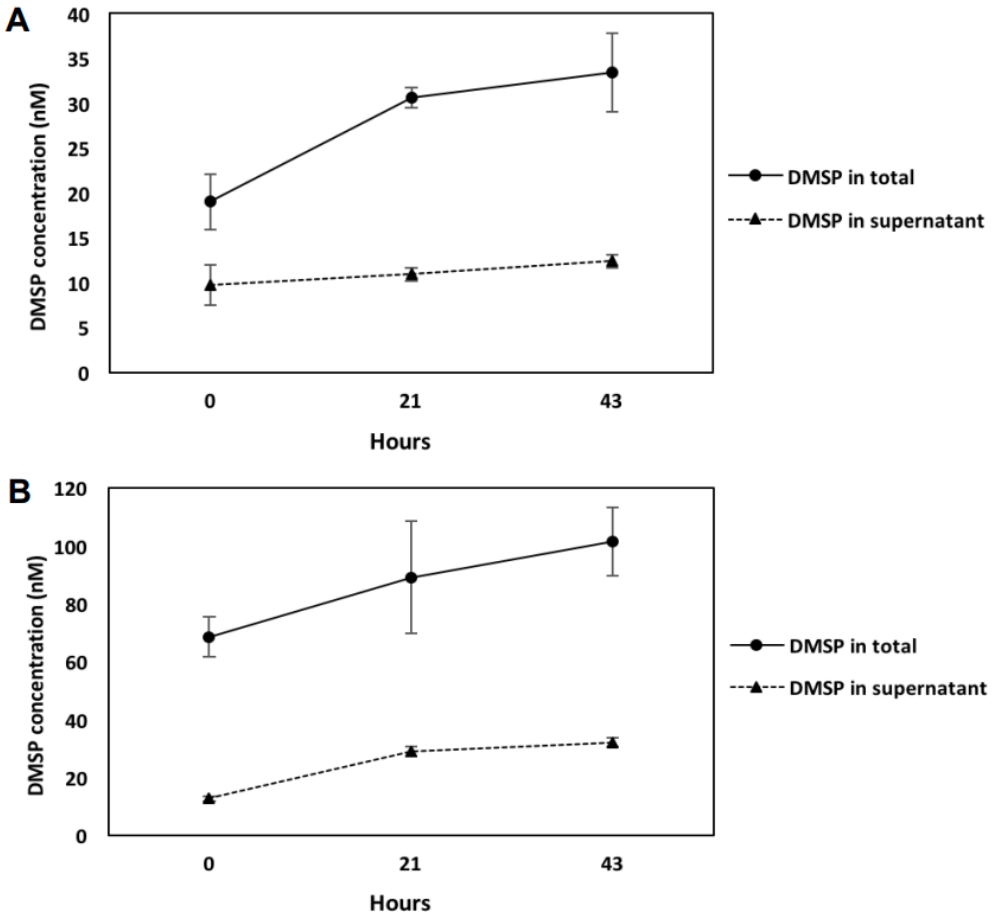

Figure 3-5 DMSP produced by *P. bermudensis* (A) and *Novosphingobium* (B) after incubation in filter-sterilised seawater[22]. DMSP in total in the pellet & supernatant. The DMSP in the samples was measured using a modified purge trap method and GC. Error bars show standard error.

From these incubations, it is clear that *P. bermudensis* and *Novosphingobium* had the ability to produce DMSP in conditions close to those found in the natural environment, and therefore it is likely that they can produce DMSP within environments such as Stiffkey saltmarsh. As **Figure 3-5** shows, DMSP within the cells is much higher than the levels found in the supernatant, implying that the cells are synthesising DMSP but not exporting it out of the cells. There is a slight increase over time in the supernatant, which may be accounted for by the release of DMSP following cell death.

3.3.7 Confirming *dsyB* presence or absence

To determine whether the strains isolated from Stiffkey saltmarsh sediment contained *dsyB* and likely used the transamination pathway for DMSP synthesis the genomic DNA was isolated from 24 strains (Table 3-3). The sequences of these isolates were aligned to identify

conserved regions of *dsyB* and construct degenerative primers (constructed by Beth Williams) with a cut off of up to five degenerate bases. These primers were used to amplify the gene fragment if it was present. To test the primers efficiency, five strains known to contain *dsyB* were used as positive controls and eukaryotes containing *DSYB* were used to test the specificity to bacteria. Negative controls also included bacterial strains unable to produce DMSP. Following PCR amplification, the PCR products were visualised using gel electrophoresis to determine if *dsyB* was present in the genome (Figure 3-6).

The band for *dsyB* can be seen in Figure 3-6 at 246 bp, and whilst a positive result can indicate the presence of *dsyB* in the genome, a negative result is less of a certainty. As expected, *Labrenzia* (S5) presented a band indicating *dsyB*, as did *Rhodobacterales* (E25, E32, E35, E41) and *Oceanicola* (E30). As mentioned previously, *Rhodobacterales* is similar phylogenetically to *Labrenzia,* and *Oceanicola* has already been demonstrated to contain *dsyB*. To confirm these strains did have *dsyB* within their genome, the bands were excised and sent for sequencing. For the other strains, there was no PCR product. As there was a larger band in several of the samples, one was excised and sequenced, which confirmed that it was junk DNA. Although related strains of *Pseudooceanicola* have been shown to contain *dsyB,* the *Pseudooceanicola* (E26) isolated from the sediment did not. Similarly, *Rhodobacter* (E27) and *Stappia* (S6, S8, E24) are from the same family as *Labrenzia*, yet they also did not produce a band in the correct location. It may be that these strains do not contain *dsyB*, or it could be that the degenerate primers were unable to amplify some of the *dsyB* sequences but not all, under the conditions used here. Interestingly, but not completely unexpected, *Novosphingobium* (E39, E42, E43, E44, E45), *Alteromonas* (E48), and *Marinobacter* (S4) did not produce a band for the presence of *dsyB*. This is likely because these strains are from the Sphingomonodales order or are gammaproteobacterial, which have not been shown to include any *dsyB*-containing organisms.

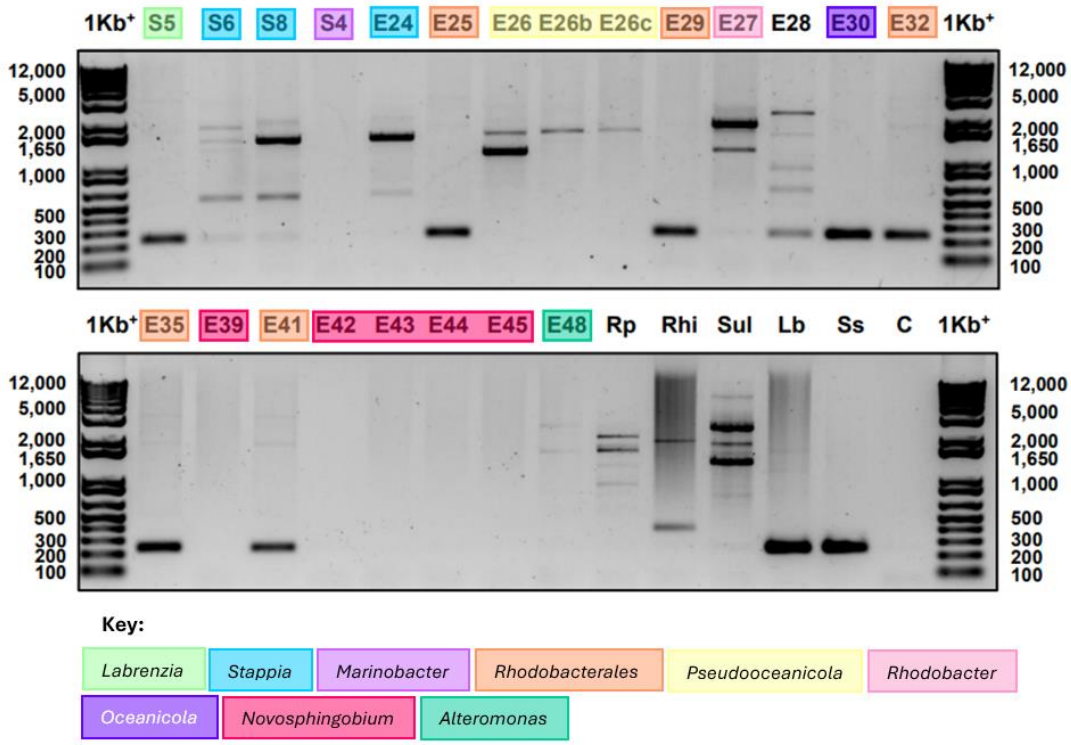

Figure 3-6 PCR amplification used to determine the presence of *dsyB*. Degenerate primers were used to amplify any *dsyB* homologues found in strains isolated from Stiffkey saltmarsh sediments[22]. Refer to Table 3-3 for isolates. Negative controls: *R. pomeroyi*, *R. leguminosarum*, and *Sulfiobacter* sp. EE-36). Positive controls: *L. aggregata* and *S. stellata*. C is the reagents with no sample.

Since the use of degenerate primers does not provide a finite answer in regards to the presence or absence of a gene, the next step was to send the strains which allegedly lacked *dsyB* for Whole Genome Sequencing (WGS). Pure cultures of *Alteromondas, Marinobacter, Novosphingobium, Rhodobacter, Rhodobacterales*, and *Stappia* were sent to Microbes NG, Birmingham, where they were sequenced using the Illumina MiSeq platform. The sequences were trimmed and checked for quality, and the reads were annotated against the closest genomic sequence available with RAST (http://rast.nmpdr.org/). This method does not provide complete genomes; however, the completeness for each of the strains was >99 %.

The WGS for the six isolates were searched for the presence of *dsyB*, and homologues were identified in *Rhodobacter, Rhodobacterals,* and *Stappia*. The three remaining strains, *Alteromonas, Marinbacter,* and *Novosphingobium,* did not contain a *dsyB* homologue. As the genomes are not complete, there is a small chance that the strains could still contain *dsyB*. Nevertheless, these strains are not closely related to any of those recognized to have *dsyB* making it more probable that these bacteria lack DsyB and likely contain a novel DMSP synthesis pathway.

3.4 Discussion

3.4.1 Summary

This chapter aimed to determine if bacterial DMSP production contributed to the total DMSP found in Stiffkey saltmarsh sediment. This was achieved using culture-dependent techniques similar to those established by Carrión *et al*. (2017) and adapting enrichment conditions using those reported to increase DMSP production in Curson *et al*. (2017).

The sampling of sediment and pool water took place at the lower marsh in Stiffkey as this area had been established as a site of high DMSP production previously due to the area comprising of conditions that indicated the likelihood of this process, such as high salinity, large fluxes of biogenic sulfur and high emissions of DMS[169,171]. With the location being a prospective DMSP hotspot, the potential to isolate and characterise DMSP-synthesising bacteria was favourable. Plate culturing dilutions of saltmarsh sediment produced many isolates, and from those tested, 27 % had the ability to produce DMSP. This percentage of DMSP-synthesising isolates increased to over double to 77 % when the sediment was incubated with enrichment conditions (high salt, high sulfur, low nitrogen and MTHB). Interestingly, *Alteromonas, Marinobacter,* and *Novosphingobium* had not been previously identified as DMSP producers and, furthermore, did not contain *dsyB*. Other DMSP producers included *Labrenzia, Rhodobacter, Rhodobacterales,* and *Stappia*, which were less surprising. *Labrenzia* has already been demonstrated to produce DMSP and is a very close relative of *Stappia*. In fact, only in 2007 were some species reclassified from *Stappia* to *Labrenzia*[177], and similarly, *Rhodobacter* and *Rhodobacterales* are also closely related to *Labrenzia*.

The more interesting strains that did not appear to contain *dsyB* following the use of degenerate primers or after searching their genomes following WGS warranted further investigation. The incubation experiments shown in Figure 3-4 showed that *Marinobacter* was likely using the transamination pathway since when incubated with intermediates from that pathway (Met, DMSHB, MTHB, MMPA), there was an increase in the production of DMSP compared to when incubated in media alone. Furthermore, the production of DMSP in the strain increased with increasing salinity and decreasing nitrogen. *Novosphingobium,* on the other hand, had a significant increase in the production of DMSP following incubation with Met compared to the standard media but showed no increase in production with the other intermediates of the transamination pathway (DMSHB, MTHB, MMPA). DMSP production in *Novosphingobium* did increase with a slightly higher salinity (35 PSU) and lower nitrogen, but this activity was not seen in the highest salinity tested (50 PSU). As explained previously, the

three DMSP synthesis pathways begin with Met, so it is possible that *Novosphingobium* is using a novel synthesis pathway that has not been observed in bacteria. To examine the production of DMSP-synthesis in *Novosphingobium* further, subsequent experiments were carried out, which are detailed in the following chapter.

3.4.2 Limitations of a culture-dependent study

This chapter has demonstrated how culture-dependent studies can be used to isolate and characterise bacteria performing certain processes within an environment. Yet, as alluded to earlier, this is not a complete picture. Mainly because only a very tiny percentage of bacteria, ~1 %, are culturable within the laboratory[175]. So, of the 10^9 bacterial cells within 1 g of sediment, it is only possible to grow 10^7. And while this is a very large number, there are still 9.9×10^7 remaining unculturable. While this may be due to a lack of necessary nutrients, incorrect media composition or unobtainable growth conditions, there are also practical elements that make a strain suitable for growth within the laboratory setting, such as growth speed. For example, when strains are isolated, there is less chance of picking the slow-growing strains when they are in competition with others that are more prolific and perhaps quicker to adapt to the new conditions. What is more, the abundance of the species, to begin with, would also affect the likelihood of a strain being cultured. However, as demonstrated here and in Carrión *et al*. (2017), certain characteristics can be enriched, increasing the likelihood of isolating strains with the desired trait.

3.4.3 Implications of studies within the laboratory

Even though the strains used in this study were isolated from an environment necessary for the production of DMSP, DMS and biogenic sulfur and were grown under conditions as close to their natural environment as possible, there is always lab bias which should be accounted for. In addition to this, experiments such as the enrichment incubation changed the natural conditions, which restricts how these observations can be related to the natural environment within the saltmarsh. Furthermore, while some bacteria are unable to survive in laboratory conditions, others thrive, with an abundance of nutrients available in more stable conditions, very unlike the changeable conditions they may usually experience. For this reason, it was necessary to include the incubation in sterilised-filtered seawater, to confirm the possibility that DMSP was being produced by these organisms *in situ* conditions.

While this work has limitations, it is an important starting block in identifying strains that may be important within DMSP synthesis, and the study can be continued and supported with

culture-independent work in the future. Culture-independent work on the sediment isolated from Stiffkey saltmarsh found that ~91 % of 16S rRNA gene sequences within the community were from bacteria, and 2.3 ± 0.6 % encompassed genera containing *dsyB*[65]. Additionally, analysis of metagenomes revealed that around 1 % of bacteria contained *dsyB* and that the *DSYB* synthesis gene was 13-fold less abundant than *dsyB*[65].

Following incubation of Stiffkey saltmarsh sediment under enrichment conditions, alphaproteobacterial and gammaproteobacterial increased, with isolates mentioned previously, including *Marinobacter* (3.2 ± 0.4 %), *Novosphingobium* (4.7 ± 0.9 %) and *Alteromonas* (20.7 ± 2.4 %)[65]. These genera represented 0.6 % of the community within the natural sediment and lacked *dsyB* within their genomes[65]. Interestingly, the *dsyB* transcripts were not increased following enrichment incubations[65]. In contrast, *mmtN*-containing (see below) bacteria increased significantly following enrichment from 1.9 ± 0.44 % to 28.7 ± 2.2[65].

3.4.4 Future work with DMSP-producing bacteria

This study focused on the bacteria contained within the oxic layer of sediment. However, DMSP was also detected within the anoxic layer, providing a currently unstudied area of potentially unknown DMSP producers. The process would be slightly more complicated than demonstrated here since any isolates would need to be grown in anoxic conditions, and that alone makes even the more straightforward experiments more challenging. Yet, the prospect is exciting since if DMSP producers were found to be occupying the anoxic layers of sediment, it would raise the question of how accurately DMSP production is estimated and what organisms are contributing the most to the total quantities. The belief is that photosynthetic organisms are the most significant DMSP contributors, which, unlike the organisms residing in the light-starved sediment, are restricted to the epipelagic zone.

4 Identification and Characterisation of MmtN

4.1 Introduction

4.1.1 The methylation pathway

When the isolates from Stiffkey saltmarsh sediment were incubated with potential inducers (**Chapter 3.3.5**), DMSP production was only increased by *Novosphingobium* when incubated with Met and not with any of the intermediates from the transamination pathway (Figure 3-4). As was the case with the *ddd* genes, it may be expected that the *Novosphingobium,* which lacked *dsyB*, could contain novel DMSP-synthesis genes. Since the decarboxylation pathway has only been observed in a dinoflagellate[104] and if the bacterium is not using a completely unknown pathway to produce DMSP, then the most likely synthesis pathway would be the methylation pathway. The possibility of bacteria utilising an additional pathway would indeed be interesting since the bacteria isolated from the saltmarsh sediment can also be found in marine sediment, which greatly increases their abundance.

There are two versions of the methylation pathway (Figure 1-4). Both begin with the methylation of Met with *S*-Adenosylmethionine (AdoMet) acting as the methyl donor to produce SMM and *S*-Adenosyl-L-homocysteine (AdoHcy)[92]. After this step, either SMM is decarboxylated to DMSP-amine[42] and oxidised to DMSP-aldehyde, or SMM goes through a transamination-decarboxylation step straight to DMSP-aldehyde[94]. The final step is the same for both, the oxidation of DMSP-aldehyde to DMSP. Both routes of synthesis via the methylation pathway have been observed in DMSP-producing organisms; for example, Gramineae (e.g. *Sporobolus*) use the two separate steps with DMSP-amine as an intermediate while Compositae (e.g. *Melanthera*) likely use a PLP-dependent transamination-decarboxylation step to produce DMSP-aldehyde. However, this part of the synthesis pathway is still unsolved [69,94,99].

The production of SMM is common within plants, and the production is well documented. The gene, *MMT*, encodes the enzyme which methylates Met forming SMM and can be found in many angiosperms[101], not just those that produce DMSP. The presence of SMM in non-DMSP producing plants such as *Zea mays* and *Sorghum bicolor* would suggest that the production of SMM is important. What is more, SMM is part of a cycle (Figure 4-1), where it is synthesised from Met by MMT activity and then used as a methyl donor itself by homocysteine (HMT) which produces two molecules of Met; one following the removal of a methyl group and another

formed when a methyl is donated to Hcy[178]. The production of SMM has been suggested to have roles in Met regulation by acting as a storage molecule for Met[6].

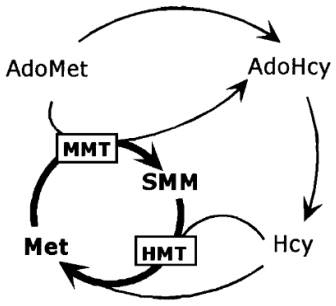

Figure 4-1 The SMM cycle. The cycle shows the reactions facilitated by MMT and HMT in bold. Adapted from Ranocha (2001).

There are species of bacteria and yeast that bypass the Met synthase pathway and use HMT to reclaim Met from SMM brought into the cells, but MMT activity and the production of SMM is a trait found only in plants[178]. The possibility of the methylation pathway being utilised by bacteria and potentially creating SMM as an intermediate is an interesting area of study.

4.1.2 Previously used methods to identify bacterial DMSP synthesis pathways

The last chapter demonstrated that there are species of bacteria with the ability to produce DMSP but lack the bacterial DMSP-synthesis gene, *dsyB*[31]. This may be because there are isoform versions of the gene, or it may be that there is a novel DMSP synthesis pathway in bacteria that uses currently unknown genes. Neither of those options are unusual, as demonstrated by the genes associated with DMSP degradation in bacteria. The majority of the *ddd* genes encode proteins that perform the same function, the lysis of DMSP to acrylate. Yet, DddK, DddL, DddQ, and DddW are small proteins with cupin pockets at the C-terminal used to bind to transition metals[130,131,179], whereas DddP is from the M24B metalloprotease family and consists of a larger polypeptide with two active sites[180]. There are also examples of different pathways being used; for example, DddD is involved in the production of DMS from DMSP and is a Class III acetyl CoA-transferase[117,129]. There are also proteins involved in DMSP metabolism which carry out the same role, such as Alma1, the first protein identified in a eukaryote as a DMSP lyase[181] with a completely different structure to the Ddd proteins but exactly the same function.

Previously, Curson *et al.* (2017) predicted the DMSP synthesis pathway in *L. aggregata* by incubating the strain with intermediates from the three known synthesis pathways (Figure 4-2). Any intermediates that were from the pathway used by *L. aggregata* would, in theory, cause an increase in the production of DMSP. As Figure 4-2 shows, more DMSP was produced when *L. aggregata* was incubated with Met, MTOB, MTHB and DMSHB, all of which are intermediates from the transamination pathway, indicating that *L. aggregata* was most likely using this pathway[31]. In contrast, intermediates from the methylation (DMSP-amine and SMM) and decarboxylation (MTPA) pathway did not significantly increase DMSP production[31].

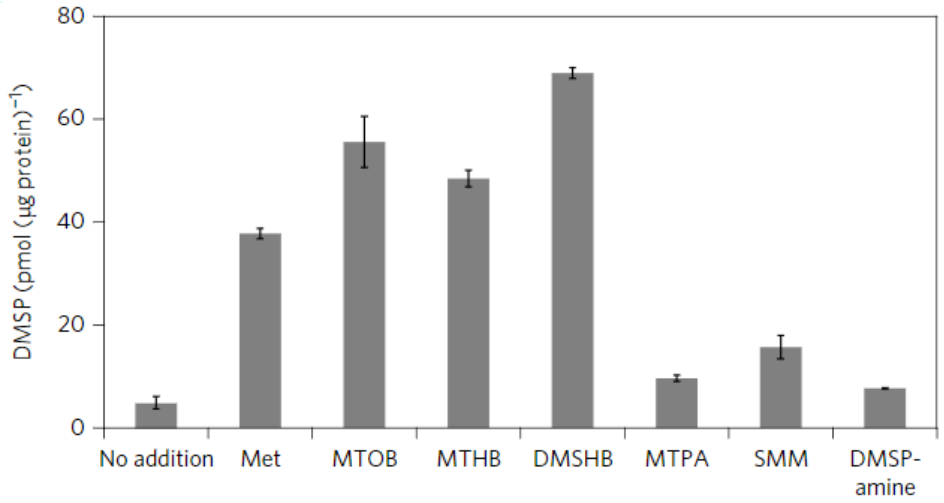

Figure 4-2 The production of DMSP by *L. aggregata* LZB033 incubated with 0.5 mM intermediate from the transamination and methylation pathway in MBM media. The DMSP released was quantified using gas chromatography. Error bars show standard deviation (n = 3). Adapted from Curson *et al.* (2017).

4.1.3 Methods of bacterial DMSP synthesis gene identification

Using the methods discussed previously, Curson *et al.* (2017) had an idea of the pathway *L. aggregata* was using and, subsequently, an idea of the type of enzymes needed for each step, allowing for a more focused approach when looking for any of the responsible genes. As previous work has identified the MTHB methyltransferase (MHM) as the rate-limiting step and specific to DMSP producers[68,71,77], this was the activity that was screened for[31]. The simplest way to achieve this was to create a genomic library consisting of 25 – 35 kb fragments of contiguous DNA cloned into a vector, creating a cosmid. The cosmids are screened for the expected activity in a heterologous host. Using bioinformatics, the genes of clones with the anticipated activity can be used to identify potential candidate genes, and these can be sub-cloned and further screened for activity. This is a method that has always been effective within the Todd lab and was the method used for finding many of the genes involved in DMSP catabolism, including *dddD*[117], *dddL*[50], *dddP*[133], *dddQ*[130], *dddY*[134], *mddA*[54], and *dsyB*[31].

The screening methods using a genomic library are much simpler than the process of screening a huge number of mutants via mutagenesis. In the identification of *dsyB*, a genomic library of *L. aggregata* was screened in the heterologous host *R. leguminosarum* J391 (J391) by identifying clones that conferred the ability to produce DMSP from MTHB[31]. The reason this was possible is that J391 has the ability to produce DMSP from DMSHB but cannot synthesise DMSHB from MTHB. Therefore, only a clone containing a gene conferring MHM activity would have the ability to produce DMSP and be detected on the GC by the release of DMS when NaOH was added. Once a clone was positively identified for the ability to produce DMSP, the ends of the cosmid were sequenced and aligned to the genome, which likely contained the gene of interest. In the case of *L. aggregata*, Curson *et al.* (2017) were able to identify a methyltransferase-like protein, which they termed *dsyB*, and subclone into J391 to test the ability to produce DSMP[31]. J391 *dsyB*$^+$ was able to produce DMSP when incubated with MTHB. To further validate the findings, an *L. aggregata dsyB*$^-$ mutant was created, which when tested, was unable to produce DMSP[31].

Utilising the methods described by Curson *et al.* (2017), it may be possible to predict the pathway used by *Novosphingobium* by incubating the strain with intermediates from the three DMSP synthesis pathways. Once a pathway is predicted, an assay identifying predicted activity could be used to screen a genomic library, as was also demonstrated in Curson *et al.* (2017) and potentially identify novel DMSP synthesis genes used by *Novosphingobium*.

4.2 Chapter aims

The previous chapter identified a strain of interest, *Novosphinobium* BW1, the first *Sphingomonadales* order alphaproteobacteria shown to produce DMSP and even more excitingly, it lacked *dsyB*, the only known bacterial DMSP synthesis gene. This chapter aimed to identify the pathway for DMSP synthesis and candidate DMSP synthesis genes using methods analogous to those successfully used by Curson *et al.* (2017).

The chapter will test the hypothesis that *Novosphingobium* BW1 produces DMSP via an alternative pathway to the transamination pathway and likely contains a novel DMSP synthesis gene.

4.3 Results

4.3.1 Characterisation of *Novosphingobium* BW1

4.3.1.1 Growth experiments with BW1

Prior to experimental design, it was essential to determine the growth characteristics of BW1. This was achieved by measuring the OD_{600} of BW1 cultures in triplicate until they reached the stationary phase (Figure 4-3). Samples were incubated at 30 °C with shaking at 180 rpm with OD_{600} reading measured hourly.

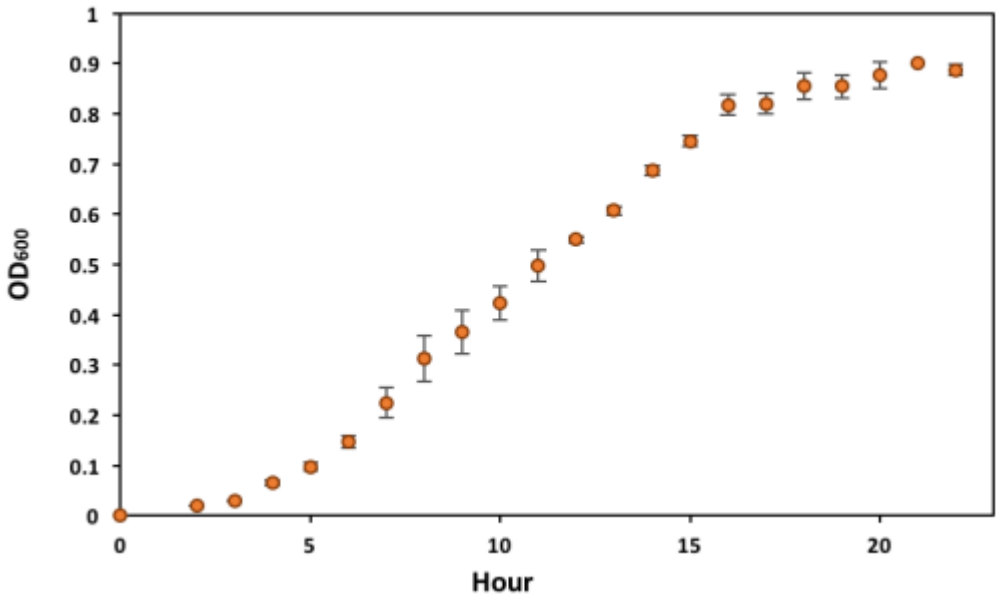

Figure 4-3 Growth curve of *Novosphingoibium* BW1[22], incubated at 30 °C, shaken at 180 rpm for 22 hours. Cultures were grown in triplicate and the cell density measured (OD_{800}) and error bars display standard error.

The effect of environmental conditions on the production of DMSP by BW1 was determined by growing cultures with various concentrations of salt and nitrogen and by altering the temperature. Figure 4-4 shows that BW1 produces higher amounts of DMSP when incubated in higher salt concentrations and at lower nitrogen levels. Lower salt concentration and temperature seem to decrease the amount of DMSP produced. All were incubated overnight at 30 °C (except for the cultures incubated at the lower temperature of 16 °C) with shaking at 180 rpm. Following incubation, protein content and the concentration of DMSP were calculated for comparison. The comparatively low amount of DMSP produced when BW1 was incubated at 16 °C would suggest that this strain does not use DMSP for protection against low temperatures. In contrast, BW1 seems to increase the amount of DMSP produced as the salinity increases, suggesting that BW1 uses DMSP as an osmoprotectant. There also seems to be an increase in DMSP synthesis under conditions when nitrogen is limited, which makes

sense since in environments where nitrogen is valuable, it would be used only in pathways important for the viability of the cell.

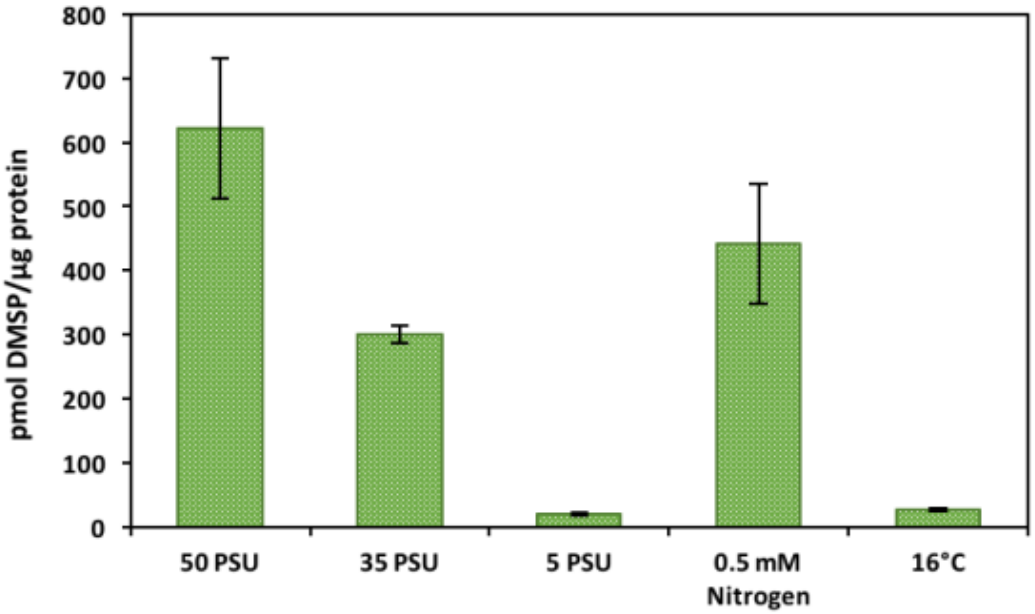

Figure 4-4 The change in DMSP production of *Novosphingobium* BW1 in differing conditions[22]. Conditions tested include high salinity (50 PSU, 10 mM NH_4Cl), normal salinity (35 PSU, 10 mM NH_4Cl), low salinity (5 PSU, 10 mM NH_4Cl), low nitrogen (35 PSU, 0.5 mM NH_4Cl), and low temperature (35 PSU, 10 mM NH_4Cl). Error bars show standard error.

4.3.2 Predicting the DMSP synthesis pathway for BW1

4.3.2.1 Identifying the likely DMSP synthesis pathway in BW1

BW1 was grown in the presence of methionine (the starting substrate in all known DMSP synthesis pathways), intermediates from the methylation pathway (SMM and DMSP-amine), the transamination pathway (MTOB, MTHB and DMSHB), the decarboxylation pathway (MTPA and MMPA), and without any intermediates (control) (Figure 4-5). The aim of this experiment was to identify intermediates that caused significant increases in DMSP production in BW1 and were, therefore, likely involved in its synthesis pathway.

Cultures of BW1 were incubated until they reached an OD_{600} of 0.5 (~12 hours). These were aliquoted and mixed individually with each intermediate to a final concentration of 0.5 mM. A control mix with nothing extra added was included. All cultures were incubated at 30 °C 180 rpm and the DMSP concentration and protein content were measured after 30, 60, 120 and 240 minutes (Figure 4-5). As expected, when grown in the presence of methionine, we saw a significant increase since Met is the universal DMSP precursor for all three pathways. None of the transamination or decarboxylation intermediates significantly increased the DMSP

produced by BW1 except for DMSHB, which was approximately 1.5-fold higher than the control. An explanation for this could be that many organisms can carry out some of the steps of the transamination pathway; often, organisms have Met aminotransferase and MTOB reductase activity and can convert Met to MTHB, or DMSHB to DMSP[31,71]. By far, the most significant increase in DMSP synthesis occurred when BW1 was incubated with SMM, a

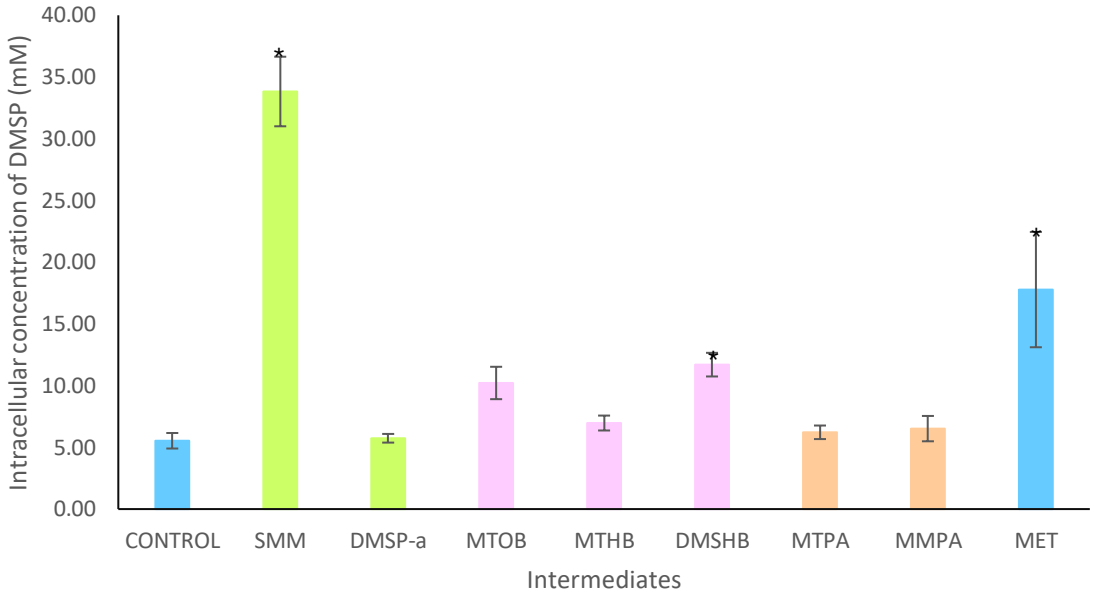

Figure 4-5 DMSP production intermediates growth experiment. BW1 was grown in the presence of intermediates from the methylation pathway (green), the transamination pathway (pink), and the decarboxylation pathway (orange). The control (blue) has no intermediates within the media. Met (blue) is the precursor to all three DMSP synthesise pathways. A two-sample t-test between the control and each intermediate was carried out, and significant differences are marked by an asterix (SMM t_{10} = -8.945, $P < 0.001$. DMSHB t_{10} = 0.305, $P < 0.001$. Met t_{10} = -2.372, $P < 0.05$).

metabolite of the methylation pathway, suggesting that BW1 used the methylation pathway, an entirely novel way for DMSP production by bacteria. However, DMSP-amine, an intermediate of the methylation pathway, did not enhance DMSP production significantly. Therefore, we can hypothesise that BW1 uses the same methylation pathway as *M. biflora* and synthesises DMSP-aldehyde from SMM by decarboxylation[6,69] (Figure 1-4), rather than the methylation pathway in *S. alterniflorus,* which uses SMM to produce DMSP-amine and then oxidised to form DMSP-aldehyde.

4.3.2.2 Demonstrating methionine methyltransferase activity in BW1

The production of SMM had never been documented before in a bacterium, making this potential discovery exciting. Therefore, the next step was to show that BW1 had methionine methyltransferase (MMT) activity, and for this to be achieved, it was necessary to differentiate between Met and SMM. Fortunately, SMM can release DMS following alkaline hydrolysis and incubation at 80 °C, whereas Met does not. DMS is detectable using GC and can quickly give

information about whether or not SMM is likely to be present in the sample. Slightly complicating this simple assay was a need for the methyl donor *S*-AdoMet (SAM) for MMT activity which also releases DMS after alkaline hydrolysis and incubation at 80 °C. Any DMS liberated from SAM would mask the peaks of SMM, making the assay ineffective. However, due to its nucleotide base region, SAM can be sequestered by the addition of activated charcoal[182]. To ensure this method would work, sterile water was mixed with SAM, SMM or both and tested for DMS before and after activated charcoal treatment using GC. As Figure 4-6 shows, when treated with activated charcoal, all of the SAM is sequestered. There is a reduction in the amount of SMM following treatment, but it is still detectable.

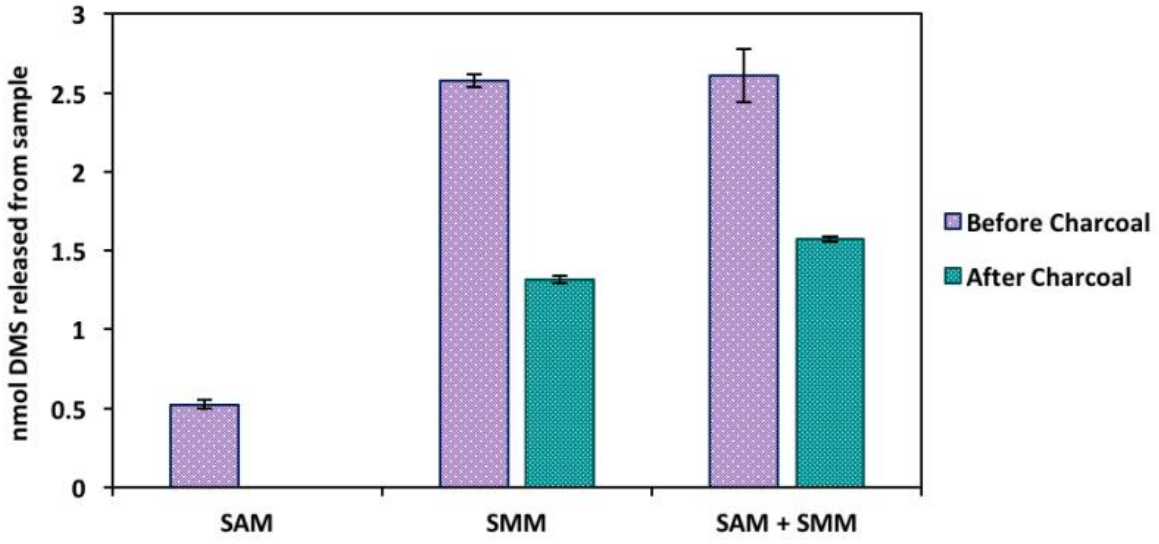

Figure 4-6 DMS measured by GC from samples containing *S*-AdoMet (SAM), SMM or both, before and after activated charcoal treatment[22]. Activated charcoal sequesters the remaining SAM.

To investigate whether BW1 had SAM-dependent MMT activity, BW1 cell lysate (see methods) was mixed with either 1 mM SAM, 1 mM Met or both. The cell extract activity was stopped following a 30-minute room temperature incubation by the addition of activated charcoal to sequester the SAM. Figure 4-7 shows that BW1 cell lysate had SAM-dependent MMT activity with no DMS detected in any of the controls. Furthermore, others have detected SMM using LC-MS. There was a possibility that the cell lysate transformed SMM into other intermediates, including DMSP, though it is unlikely since the downstream enzymes of the methylation pathway require PLP, NADPH and other metabolites, and they would have been removed during dialysis.

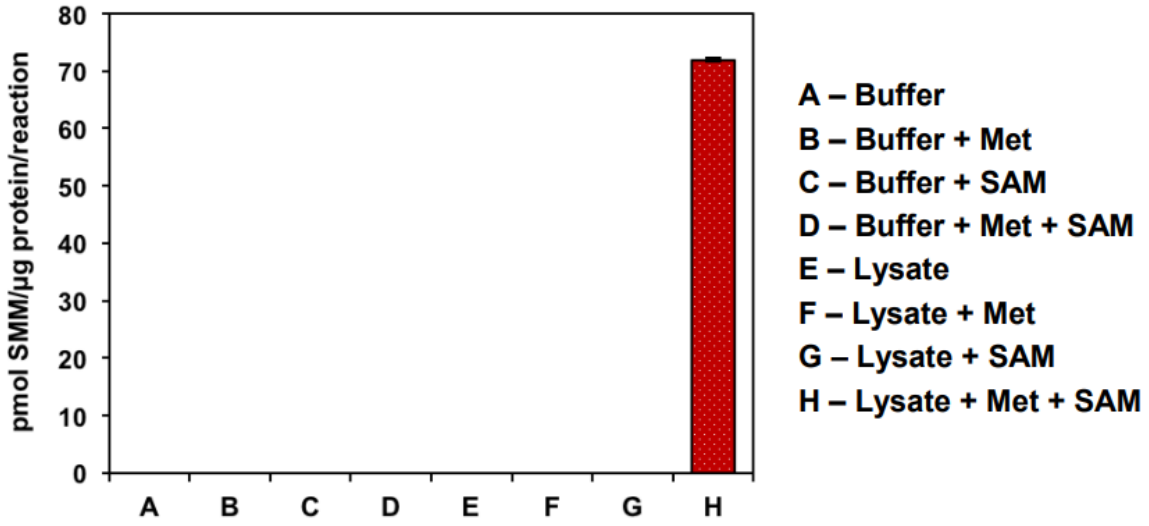

Figure 4-7 The production of SMM in the cell lysate of BW1 containing SAM-MMT[22]. This clearly shows that the cell lysate is only able to produce SMM in the presence of methionine and the methyl donor SAM. The buffer acted as a negative control, and error bars show standard error.

4.3.3 Identification of a methionine methyltransferase in BW1

4.3.3.1 Constructing and screening a gene library

As SMM was likely an intermediate in the BW1 DMSP synthesis pathway, screening for a methionine methyltransferase was an excellent starting point, especially since the transformation of Met to SMM had not previously been observed in bacteria. A BW1 genomic library (made by Dr Andrew Curson) was conjugated into *Rhizobium* J391. The construction of the library began by preparing the pLAFR3 [151] vector (Figure 4-8) using a Qiagen midiprep kit (see **2.6.3**), resuspended in 60 µl water and the DNA quantified to ensure there was at least 25 µg of DNA. The vector was digested with *Eco*RI and confirmed using gel electrophoresis. The digest was purified further with a phenol chloroform prep (see **2.6.1**) and

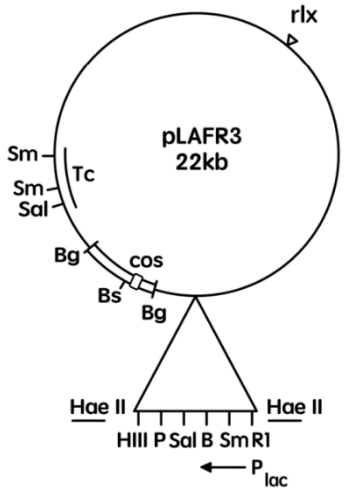

Figure 4-8 pLAFR3, a wide host range cloning vector. R1 = *Eco*R1 Adapted from Staskawicz *et al.* (1987).

dephosphorylated with a shrimp alkaline phosphatase. The dephosphorylated vector was again purified of any waste by performing another two phenol chloroform preps and resuspended in 50 μl water. The DNA was quantified to ensure that enough had been produced as 1 μg was required for the ligation reaction. To test the efficiency of the digest and dephosphorylation, 1 μl of DNA was incubated with *Eco*RI and transformed into *E.* coli 803. If after incubation overnight there were no transformants the pLAFR3 was suitable for ligation, if otherwise the digestion and dephosphorylation was repeated with more PLAFR3 and tested again. The final digestion was run on 10 μg of DNA using the same conditions as selected during the test. The reaction was stopped by adding 200 μl 100 % ethanol and 10 μl 3M Na acetate pH4.8 and frozen in liquid N_2. The sample was thawed and cleaned with a phenol chloroform prep and resuspended in 30 μl of water. The DNA was quantified to ensure there was at least 2.5 μg, which was the minimum amount needed for the ligation reaction.

The ligation reaction was set up with 2.5 μg of the digested BW1 genomic DNA and 1 μg digested and dephosphorylated pLAFR3 vector. The ligation was incubated overnight in water in the fridge to allow the ligation to slowly reduce in temperature. The DNA was quantified to ensure at least 0.7 μg of DNA was resent for packaging and that it was in a volume of 1 – 4 μl. The ligated cosmid was then ready for infection of *E. coli* 803 which was done using the Stratagene Gigapack III XL Packing mix. The library was titred to determine the number of clones in the library with dilutions of packing mix 1:10 and 1:50 used. DNA preps were carried out on six colonies which were then digested with *Eco*RI, *Bam*HI and *Hind*III to check the size of the inserts and ensure that the inserts were different. The library was amplified by repeated infection of the 803 cells. After the amplification was complete, 1 – 2 ml of LB was added to the surface of plates, the cells were scraped off using a sterile loop and the liquid was removed with a pipette. The liquid from each amplification was combined into one culture and from this 10 25 % glycerol stocks were made and stored at -80 °C.

The genomic DNA from BW1 was prepared using a Qiagen genomic kit and the DNA was quantified to check there was more than 20 μg of DNA. As with the vector, the DNA was run on an agarose gel to check that there was the correct amount of DNA present. Test digestions were set up on 10 μg of genomic DNA with the digestion times of 1, 2, 5, 10, 15, 20, 25, and 30 minutes. The digests were run on an agarose get with λ *Hind*III as a marker. The time point which had fragments of 25 – 30 kb fragments was chosen for ligation, and this was typically 5 – 10 minutes.

The genomic library contained fragments from 25 – 40 kb of the BW1 genome cloned into the *Eco*RI site of pLAFR3 (Figure 4-8). Approximately 1000 single J391 transconjugant colonies were picked and inoculated individually into minimal media containing tetracycline (selected for pLAFR3) and 0.5 mM Met and grown overnight. Each resulting culture was transferred to GC vials, heated with NaOH and screened for MMT activity, i.e. for the ability to produce DMS derived from SMM.

After screening the 1,000 BW1 genomic library clones for MMT activity, two cosmids were found to be positive for the production of SMM; cosmid 438 and cosmid 726 (Figure 4-9). Both produced a DMS peak that implied around 20.7 nmol ml^{-1} of SMM was produced in the culture, unlike the negative controls, which did not show any DMS peaks. To confirm the ability of the clones to confer SMM production from Met, the positive clones were reinoculated as before and once again examined for MMT activity by GC. Both cosmids were confirmed as positive and termed pBIO0438 and pBIO0726. To fulfil Koch's postulates, the plasmids were extracted from J391 transconjugants and transformed into *E. coli* 803 and mobilised back into J391 to confirm their MMT activity. Indeed, the subsequent strains all retained this MMT activity providing us with confidence that the gene of interest was contained within these genomic library clones.

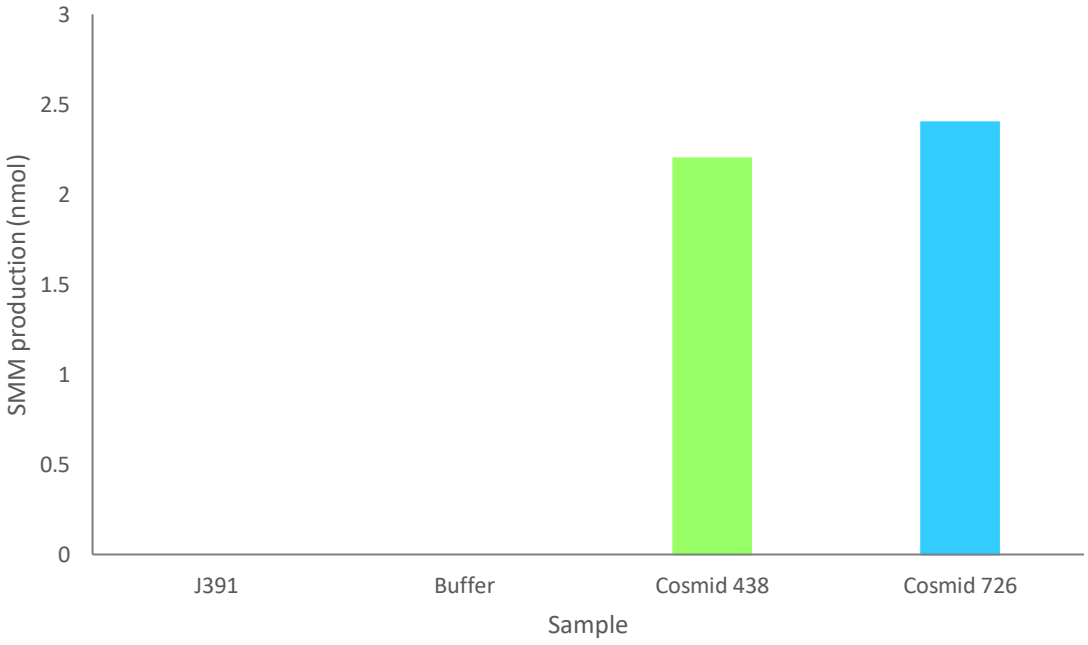

Figure 4-9 The amount of SMM produced by positive clones and controls. J391 and Buffer were used as negative controls. Both cosmids pBIO0438 and pBIO0726 produced SMM and confirmed their activity.

To analyse the clone *Eco*R1 fragments in pLAFR3 that conferred MMT activity and establish if pBIO0438 and pBIO0726 contained overlapping DNA, several restriction digests were set up using four enzymes (*Eco*RI, *Bam*HI, *Hind*III and *Pst*I) following extraction of the cosmids using the phenol-chloroform extraction method. The restriction digests showed multiple identical *Eco*RI fragments were cloned; one at ~12 kb, two on either side of 5 kb and another at 2 kb, indicating an overlapping region between the two fragments (Figure 4-10). There are differences between the fragments, such as extra bands in pBIO0726, which may be because the clones have extra fragments at each end around the section that contains the gene(s) with MMT activity.

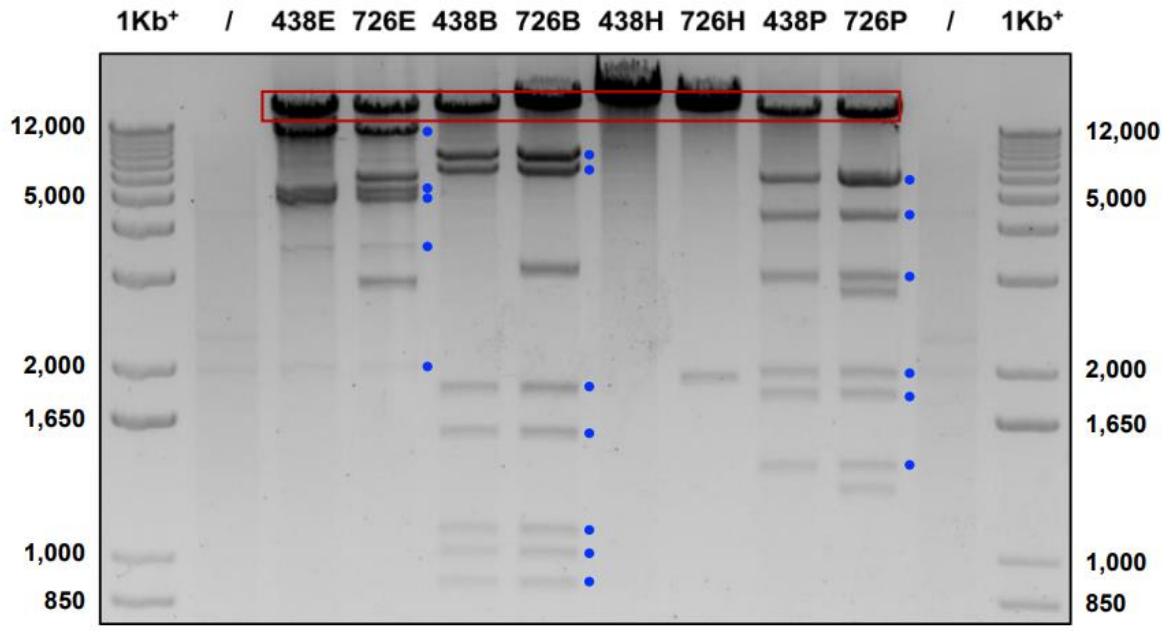

Figure 4-10 Gel electrophoresis image of the restriction digests of pBIO0438 and pBIO0726[22]. Digest enzymes: *Eco*RI (E), *Bam*HI (B), *Hind*III (H) and *Pst*I (P). The red box shows a band of ~22 kb and is probably linearised pLAFR3. Fragments contained in pBIO0438 and pBIO0726 are shown with a blue dot.

4.3.3.2 Identifying candidate genes with MMT activity

To establish the exact nucleotide sequence cloned into pBIO0438 and pBIO0726, the termini of the cloned fragments were sequenced by Eurofins Genomics using primers designed to the polylinker in pLAFR3. The sequences revealed ~500 bp at the beginning and end of the two cloned fragments that were searched for and aligned to the BW1 genome sequence (Figure 4-11). As expected from the restriction digests, the cloned fragments from BW1 were overlapping with pBIO0726 being slightly larger than pBIO0438 at 30.9 kb and 21.8 kb, respectively. The Whole Genome Sequence (WGS) of BW1 was annotated using RAST[159–161].

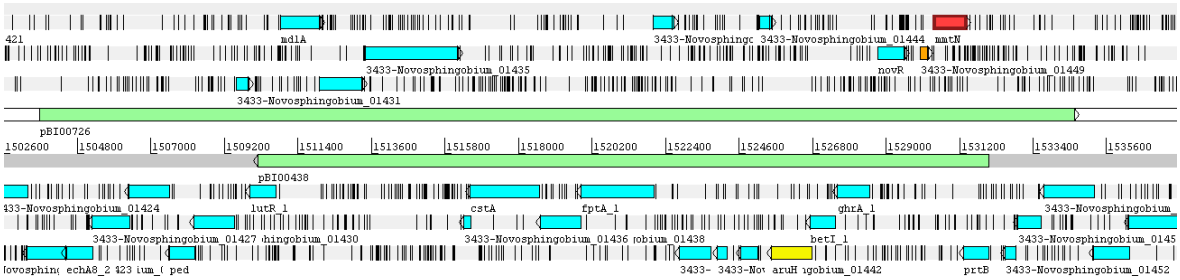

Figure 4-11 The two fragments pBIO0438 and pBIO0726 from BW1 created by partially digesting the BW1 genome. The fragments (shown as green bars) were aligned to the genome sequence from MicrobesNG to determine the fragment sizes and the genes encoded within each fragment. The red box is annotated as a Met *S*-MMT, the yellow is as an aspartate aminotransferase and the orange is a hypothetical protein. All the remaining light blue bars show coding sequences.

The potential functions of the genes within the overlapping regions could then be identified using BLASTp (Table 4-1).

One of the genes contained within the sequenced fragments was a SAM-dependent MMT (SAM-MMT) that belonged to the Met *S*-MMT family, which are known to require *S*-AdoMet as a methyl donor. This gene was termed *mmtN* (methionine methyltransferase *Novosphingobium*). Furthermore, the gene product of *mmtN* showed similarity to the plant MMT enzyme of *Arabidopsis thaliana* (AtMMT; E value 2E-18, identity 28 %), although the similarity is only to the N-terminal domain (Figure 4-12). What is more, while MmtN is 307 aa, AtMMT is 1,071 aa. The C-terminal domain of AtMMT that is not within MmtN contains a PLP binding site or an aminotransferase which is thought to be involved with regulating MMT in plants[183]. Within plants, SMM is a vital metabolite of methionine[184] (Figure 4-1), and the role of MMT in producing SMM from Met has been understood for a long time[178,184]. As Table 4-1 shows, there is a PLP-dependent aminotransferase close to *mmtN* and may potentially be involved in the regulation of *mmtN* in bacteria, or perhaps it is involved in DMSP synthesis for the amino removal step.

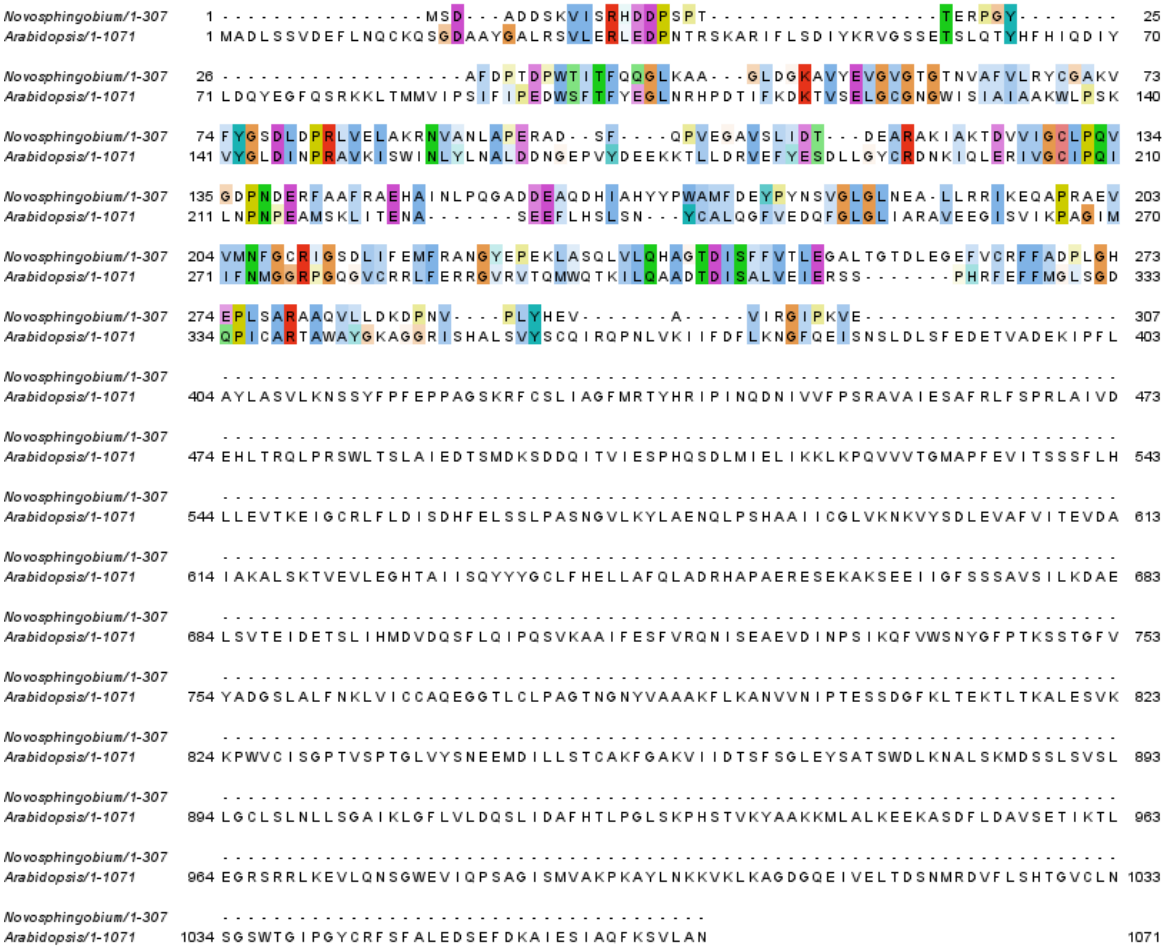

Figure 4-12 An alignment of the MMT amino acid sequence of BW1 (MmtN) and *A. thaliana* (AtMMT), responsible for the methylation of methionine to SMM via the methyl donor SAM. Whilst MmtN shares similarity to the N-terminal domain of AtMMT but does not contain the C-terminal domain which encodes a PLP binding site and an aminotransferase.

Table 4-1 Genes found within the overlapping fragments pBIO0438 and pBIO0726 of the BW1 genome. The genes are from the forward (+) and reverse (-) strands.

bp number	Annotation	BLASTp	E value	Identity (%)
1509579..1509908 (+)	L-fucose mutarotase, type 2	L-rhamnose mutarose	8E-63	90
1509970..1510656 (-)	Transcriptional regulator, *GntR* family	Transcriptional regulator, FadR family	8E-143	96
1510882..1512051 (+)	Muconate cycloisomerase	Mandelate racemase/ muconate lactonizing enzyme	0.0	93
1512135..1513322 (+)	Major facilitator superfamily MFS_1	MFS transporter	0.0	94
1513424..1516180 (+)	N-acetylglucosamine-regulated TonB-dependent outer membrane receptor	TonB-dependent receptor	0.0	95
1516370..1516543 (-)	Hypothetical small protein yjiX	DUF466 domain-containing protein	6E-27	81
1516540..1518603 (-)	Carbon starvation protein A	Carbon starvation protein A	0.0	97
1518662..1519849 (-)	Putative iron-regulated membrane protein	PepSY domain-containing protein	0.0	94
1519864..1522038 (-)	Ferrichrome-iron receptor	TonB-dependent receptor	0.0	98
1522809..1523741 (-)	Hypothetical protein	Hypothetical protein	-	-
1523952..1524233 (-)	Purple acid phosphatase	Metallophosphoesterase family protein	2E-43	84
1524642..1525136 (-)	Transcriptional regulator, *MarR* family	Transcriptional regulator, MarR family	4E-103	90
1525222..1525530 (+)	Hypothetical protein	DUF3861 Superfamily	7E-59	94
1525557..1526756 (-)	Aspartate aminotransferase	Pyridoxal phosphate-dependent aminotransferase	0.0	98
1526738..1527448 (-)	Transcriptional regulator, *TetR* family	Transcriptional regulator, TetR family	2E-148	93
1527538..1528494 (-)	D-3-phosphoglycerate dehydrogenase	Hydroxyacid dehydrogenase	0.0	96
1528718..1529536 (+)	Ribulose-5-phosphate 4-epimerase and related epimerases and aldolases	Aldolase class 2/ adducin family protein	0.0	94
1530026..1530241 (+)	Hypothetical protein	Hypothetical protein	-	-
1530445..1531368 (+)	**Methionine S-methyltransferase**	**SAM-dependent methyltransferase**	**0.0**	**95**

4.4 Discussion

4.4.1 Summary

The aim of this chapter was to identify the pathway for DMSP synthesis and candidate synthesis genes in BW1 using the methods by Curson *et al*. (2017). BW1 was of interest because although the bacterium could produce DMSP, it lacked the only known bacterial DMSP gene, *dsyB*. The first step was to understand how the production of DMSP by BW1 changed to different environmental stressors as increases in DMSP production would likely mean any genes or proteins involved in the DMSP synthesis pathway would be upregulated. The conditions used were based upon those used to identify *dsyB*[31] and included increased salinity, decrease nitrogen and lowered temperatures. The amount of DMSP produced by BW1 was increased under the conditions of higher salinity and low nitrogen as is seen in many DMSP producing organisms. However, DMSP was not increased when BW1 was grown at a lower temperature. This could be because 16 °C is not necessarily cold for an organism isolated from sediment with pool water around 17 °C on a relatively warm day.

To determine the pathway used by BW1, pure cultures were incubated with intermediates from each of the suggested DMSP synthesis pathways. As shown in Figure 4-5, the amount of DMSP produced by BW1 increased significantly when incubated with SMM, an intermediate from the methylation pathway[22]. This was unexpected as previously the methylation pathway had only been reported in angiosperms[42,69,92–94]. As SMM enhanced DSMP production but DMSP-amine did not, it could further be suggested that BW1 was possibly using the methylation pathway that decarboxylated DMSP-aldehyde to form SMM[6,42,69,178]. As mentioned earlier, SMM production in bacteria had never been documented and so made an exciting prospect to investigate further. As mentioned earlier, SMM is produced by all flowering plants. These plants also contain a separate mechanism that allow the conversion of SMM back to methionine. Whilst the function of SMM and its interconversion with methionine are unknown, there has been studies that suggest SMM as having a role in sulfur transport in the phloem[183]. One study used aphid stylet collection method and radiolabelling to analyse the movement of SMM up the phloem to the wheat ears[183]. Aphid stylet collection involves restricting aphids to a 2 cm segment of material and allowing them to feed overnight, the stylets are then severed, and the exudate allowed to air dry and collected[185]. Other studies have also verified that SMM is found ubiquitously in leaves, roots, and other organs[101,186,187]. Yet, there had been no documentation of bacteria being found to produce or utilise SMM at this point in the study.

As finding SMM producing bacterium was an exciting prospect, the study focused on the first step of the methylation pathway. As this step was the methylation of methionine, the gene of interest would be likely to encode a methionine methyltransferase (MMT). As SMM releases DMS in the presence of NaOH and heat, it was simple enough to assay the presence of the molecule using gas chromatography. However, SAM also released DMS after incubation with NaOH and heat, which could have been problematic. Fortunately, the ability to discern SMM from SAM was simple to separate as SAM is sequestered by the addition of activated charcoal. This allowed cell lysate to be assayed for the ability to produce SMM from methionine and the methyl-donor SAM. This experiment was a relatively simple way to determine whether it was at all possible for BW1 to synthesise SMM before committing to more work intense methods such as creating a gene library without having the necessary assay in place to confidently test transconjugants.

A genomic library allowed smaller fragments of the BW1 genome to be screened for MMT activity. Fragments of BW1 genome of approximately 25 – 40 kb were cloned into pLAFR3[151,188], which is a broad-host-range cloning vector that was then conjugated into *R. leguminosarum* J391 (J391). After this, the J391 transconjugants were screened for their ability to produce SMM following incubation with methionine. From the 1,000 transconjugants screened, two were positive for the release of DMS, pBIO0438 and pBIO0726. The next step was to determine what genes each fragment of BW1 contained by sequencing the termini of the fragments and aligning the BW1 genome. The overlap of the two fragment sequences revealed genes potentially involved within the synthesis of SMM. The most likely candidate was annotated as a Methionine S-methyltransferase which when searched for using BLASTp[189] returned a result of a SAM-dependent methyltransferase.

4.4.2 Future work on the importance of SMM production in bacteria

As mentioned previously, the role in SMM has been documented as a way for plants to transport sulfur up the phloem and this has been documented in *Arabidopsis* and *Triticum aestivum* (wheat)[183]. Another study found that knocking out the ability to produce SMM in *Arabidopsis* and *Zea mays* did not affect the growth and development of the plants but did increase the level of Ado-Met by up to 160 %, although the free Met pool did not differ[96]. There were also suggestions that SMM was produced by plants for a hypothetical plant-specific reaction[186], although this seems less likely with the results brought forward in this chapter.

Whilst plants might use SMM to transport sulfur up the phloem and to different areas of the plant, it might be used slightly differently in bacteria. Furthermore, whilst all flowering plants produce SMM, not all plants have the ability to produce DMSP. This would be of interest to study in bacteria, as so far, the aim of this study has focused on SMM and the relationship to the DMSP synthesis pathway. A more in-depth study of the presence of SMM production in bacteria might give a better understanding about the evolutionary pathway of the gene and perhaps hint towards the benefits of having this process. Furthermore, it could be that the addition of SMM in bacteria could provide an extra benefit to bacteria in addition to the DMSP synthesis pathway.

5 Ratification of *mmtN*

5.1 Introduction

As mentioned in **Chapter 4.1.3**, the methodology of identifying DMSP synthesis genes has been developed in the Todd lab over the years and used to identify many different genes in the production and catabolism of DMSP[31,117,190]. BW1 was shown to produce DMSP without the presence of *dsyB* and when incubated with intermediates from the three known DMSP synthesis pathway more DMSP was produced in the presence of SMM, an intermediate from the methylation pathway.

5.1.1 SMM

SMM is a metabolite produced by all flowering plants. Similarly to DMSP, there have been many studies about the reasons for this process to be beneficial, but there is still no consensus. Many studies suggest that SMM is important in the long-distance transportation of reduced sulfur[191–193]. Lee *et. al.* (2008) suggested that methionine is produced in non-seed tissue and converted to SMM which can be transported through the phloem to the reproductive organs and seeds where it is later converted back to methionine by homocysteine S-methyltransferase[183,194].

A study by Tan *et. al.* (2010) used transgenic pea (*Pisum sativum*) plant, *MMP1*) that expressed the yeast SMM transporter *S-Methylmethionine Permease1* targeted to the phloem and seeds. *MMP1* had higher concentrations of SMM in the phloem, likely due to an increase in phloem loading[193]. Expression studies, alongside xylem sap analyses supported the increase of sulfur uptake and assimilation in the roots of *MMP1*[193]. Furthermore, there was the assimilation of nitrogen in the roots[193]. Ultimately, the *MMP1* plants had an increase in seed sulfur and nitrogen which affected plant growth and seed number, suggesting that SMM has important roles in plant sulfur and nitrogen metabolism[193]. And yet, despite the well documented presence of SMM in plants, this metabolite had never been documented in bacteria.

5.1.2 Studying gene expression, the proteins they encode, and homologues in other organisms

There are many ways to gather information regarding a gene or protein of interest, and by doing so it helps to build a better picture of the purpose for related processes. By studying the regulation of the genes involved in DMSP catabolism and acrylate degradation, Todd *et. al.* (2010) found that the relationship between the catabolic pathways was different to what had

been proposed. Previously, there had been suggestions that DMSP was cleaved by DMSP-lyase and formed DMS, acrylate and a single proton[15]. Further studies suggested that the acrylate was not seen in these bacteria due to it not accumulating to high enough levels for detection and that DMSP was converted to acrylate, then 3HP (3-OH-propionate) and then subsequently other downstream products[135,136,195]. Todd *et. al.* (2010) cloned an Nsil fragment which contained *dddC, dddA, acuN, acuK, dddT,* and *dddD* (pBIO1672), then they created individual in-frame delections in *dddC, dddA, dddT, dddD, acuN,* and *acuK* by removing approximately 90% of each gene[118]. Interestingly, they found that *acuN* and *acuK*, involved acrylate degradation, are not involved in the catabolism of DMSP in *Halamonas* HTNK1[118]. Fundamentally, 3HP is a catabolite derived from the degradation of DMSP and acrylate independently, and thus it is not that acrylate is a part of degradation pathway for DMSP[118]. By comparing the effects on each mutant, it was possible to separate two independent processes which happen to contain the same catabolite.

Purifying a protein allows further experiments, for example it is possible determine what substrate an enzyme works on, whether an additional co-factor is required for activity, and what kind of specificity the molecule has. When purified DSYB and MTHB were incubated independently, they had no S-adenosyl methionine-dependent MMT activity[30]. However, when DSYB and MTHB were incubated with the heat-denatured cell lysates of *Prymnesium parvum*, activity was observed, suggesting that a co-factor present in the cell lysate was required for activity. The structure of a protein can also help to determine the mechanisms used to perform the reaction. The structure and activity of DddK was examined in the presence of different metal ions[196]. Schnicker *et. al.* (2017) showed that DddK contained a double-stranded β-helical motif and used different metal ions as cofactors for its catalytic activity.

How processes are distributed throughout an environment can also provide information on environmental benefits, evolutionary trends, and show up other organisms that may share the same and/or similar traits. Many functional genes for DMSP production and degradation have been identified to date, and these were used to study the genetic potential of microorganisms in various marine environments[197]. Song *et. al.* (2020) found that sediment samples contained higher concentrations than surface water and that the genetic potential to synthesise DMSP was higher than for phytoplankton in all samples. As mentioned in **Chapter 1.4**, it has long been thought that phytoplankton were the biggest producers of DMSP, but with new ways to study organisms we're not able to culture or sample areas we previously weren't able to we are able to make more reliable assumptions about these processes.

5.2 Chapter aims

Following on from the work in the last chapter and determining that BW1 produces DMSP likely using the methylation pathway with its own version of MMT. The next step was to provide evidence to support the claim that BW1 did indeed produce SMM similar to that of flowering organisms. Again, following methods used to identify *dsyB* in *L. aggregata* to elucidate other bacterial strains with the gene in the marine environment, test their functionality and to begin to look at what effect disrupting the gene may have on the species that produce DMSP via this method.

The chapter will test the hypothesis that *Novosphingobium* BW1 contains a novel DMSP synthesis gene.

5.3 Results

5.3.1 Confirming BW1 MmtN as an MMT enzyme

Having established that pBIO0438 and pBIO0726 contain *mmtN,* we wanted to show that this gene encoded a Met S-methyltransferase generating SMM. This was achieved by designing primers to either end of *mmtN*, amplifying the gene and cloning it into the *E. coli* expression vector pET21a (to yield pBIO21N1), which allowed its over-expression in *E. coli*. Bl21 containing an empty pET21a plasmid was used as a negative control for comparison. The cultures were inoculated into LB and induced with 0.2 mM IPTG, and incubated at 30 °C. Following the addition of Met, cultures were incubated for a further 8 hours at 30 °C. The amount of SMM produced was determined by measuring DMS after SMM lysis, and the amount of protein was quantified. As shown in Figure 5-1, when MmtN was expressed in BL21, the strain was able to produce SMM but only when incubated with Met and SAM, confirming that *mmtN* does have MMT activity and was likely responsible for the production of SMM in BW1 and potentially other bacteria containing the gene. To be confident that *mmtN* was involved in DMSP production, the next step was to mutate the gene in a host organism and observe how MMT activity and subsequent DMSP production were affected.

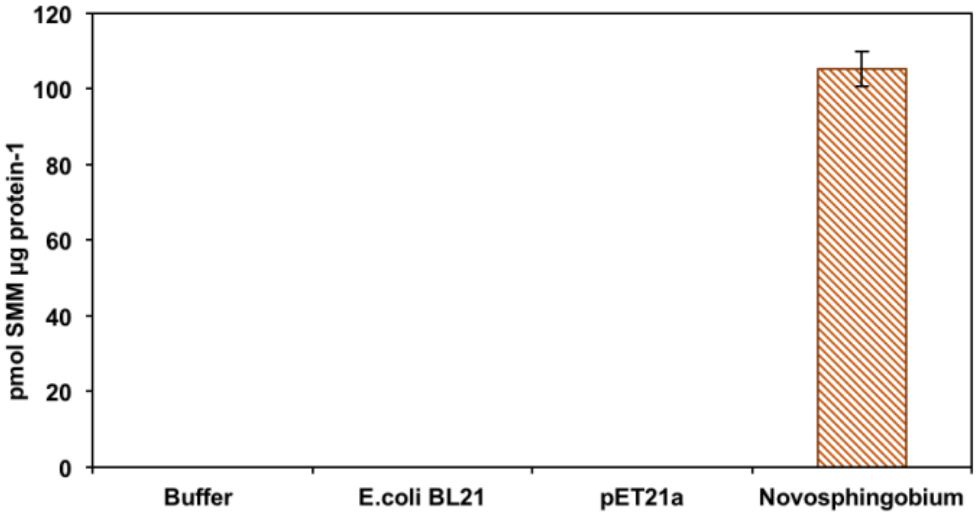

Figure 5-1 The production of SMM in BL21 containing pET21a with *mmtN*[22] confirming that the cloned *mmtN* confers the ability to produce SMM. The buffer, BL21, and BL21 with an empty pET21a plasmid were used as negative controls. Error bars show standard error.

5.3.1.1 Other bacterial strains with mmtN

Using the sequence of *mmtN* it was possible to identify other bacterial strains with the potential ability to produce SMM with a homologue of *mmtN*. The sequence from BW1 was used to search the NCBI database[189]. Which revealed strains potentially able to produce DMSP via the methylation pathway and able to synthesis SMM (**Figure 5-2**).

To investigate the DMSP-producing capabilities of other strains containing MmtN and whether or not their enzyme homologues were functional, four bacterial species were ordered from DSMZ Culture Collection (Table 5-1). Two alphaproteobacteria, *Roseovarius indicus* and *Thalassospira profundimaris*, and two actinobacteria, *Streptomyces mobaraensisi* and *Nocardiospis chromatogenes* were cultured, checked to be pure, identified by 16S and then assayed for DMSP production. Interestingly, *R. indicus* also contains *dsyB,* suggesting that this bacterium has more than one method for synthesising DMSP. During the growth of these strains, the alphaproteobacteria were relatively easy and grown in 35 PSU MBM with 0.5 mM without complication. The actinobacteria, however, were not so straightforward and after

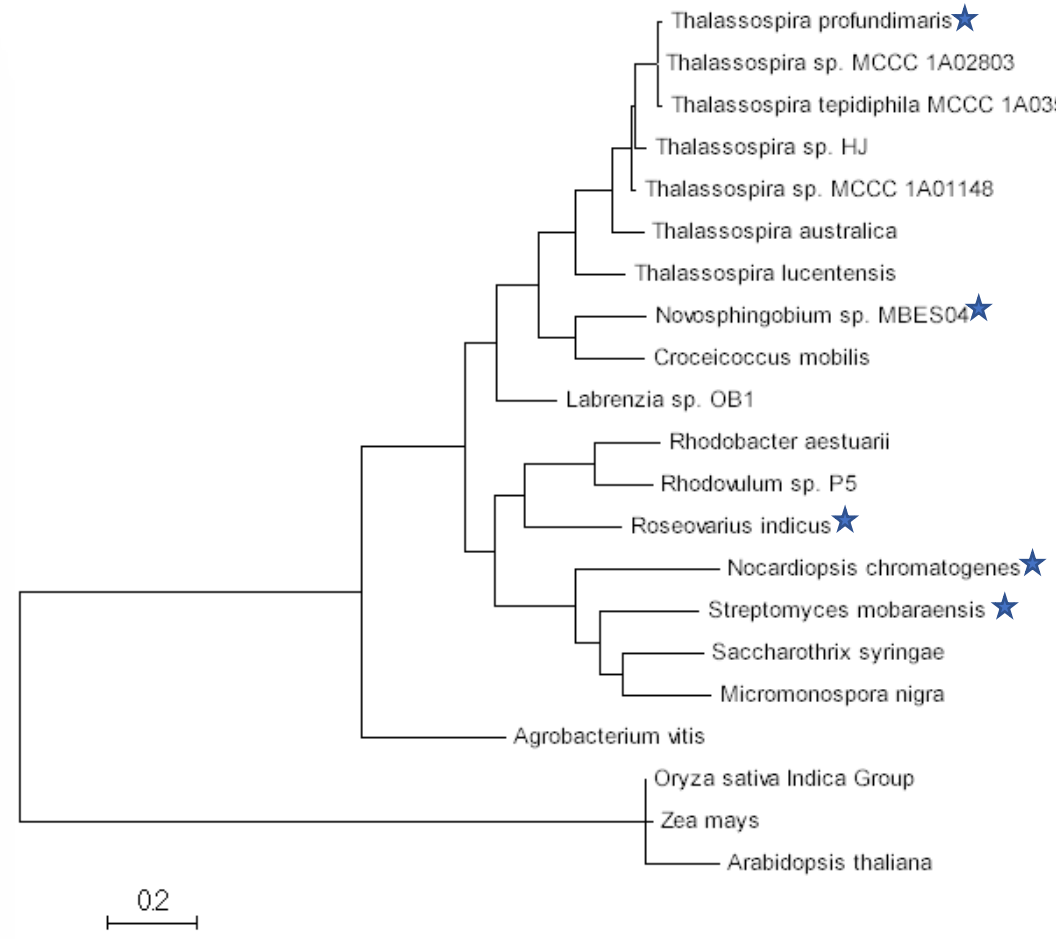

Figure 5-2 Phylogenetic tree showing organisms containing MmtN retrieved from the NCBI database. The scale bar indicates the number of substitutions per site and the stars indicate strains ordered for further study and which were subsequently shown to produce DMSP.

trying various growth mediums, including actinobacteria-specific recipes, all attempts proved futile. Fortunately, the actinobacteria were happy to grow on plates, so the DMSP estimations for these strains were obtained using whole cells taken from plates, and this should be noted when comparing results. For *R. indicus, T. profundimaris* and *N. chromatogenes*, the production of DMSP was confirmed by LC-MS.

As with BW1, the next step was to clone the *mmtN* homologues from these organisms and confirm that they conferred MMT activity to *E. coli*. The process was much the same as before, and the genes were cloned into pET21a and expressed in BL21. Compared to the negative controls, all *E. coli* strains expressing MmtN produced SMM (Figure 5-3). From this data, it was clear that *mmtN* encoded a functional enzyme in *T. profundimaris, S. mobaraensis* and *N. chromatogenes*. Less clear is the functionality in *R. indicus,* which showed activity but was much lower and requires further investigation. Interestingly, BL21 expressing *mmtN* from *S. morbaraensis* produced the highest amount of SMM, despite *S. morbaraensis* producing quite low levels of DMSP itself (Table 5-1). The reason for this is unknown, but it is highly possible that DMSP is produced for a different role in *S. morbaraensis* compared, for example, to *T. profundimaris*. Indeed, this supports the hypothesis that, unlike *T. profundimaris mmtN*, *S. morbaraensis mmtN* is predicted to co-transcribed with a non-ribosomal peptide synthase gene whose product might utilise DMSP as an intermediate in the production of a more complex molecule, see later.

Table 5-1 Bacterial strains used to test DMSP and SMM production in the presence of *mmtN*. Identity percentage based on similarity to *mmtN* from BW1. The E value and Identity % show the similarity to *mmtN* from BW1.

Organism	E Value	Identity (%)	Intracellular DMSP concentration (pmol ug^{-1} protein)	The presence of DMSP confirmed with LC-MS	Presence of *dsyB* in the genome
Thalassospira profundimaris	3e-147	69	54.3 ± 3.6	✓	×
Roseovarius indicus	7e-132	64	6.02 ± 1.2	✓	✓
Streptomyces mobaraensis	7e-91	54	3.9 ± 0.7	Untested	×
Nocardiopsis chromatogenes	2e-91	51	1.5 ± 0.05	✓	×

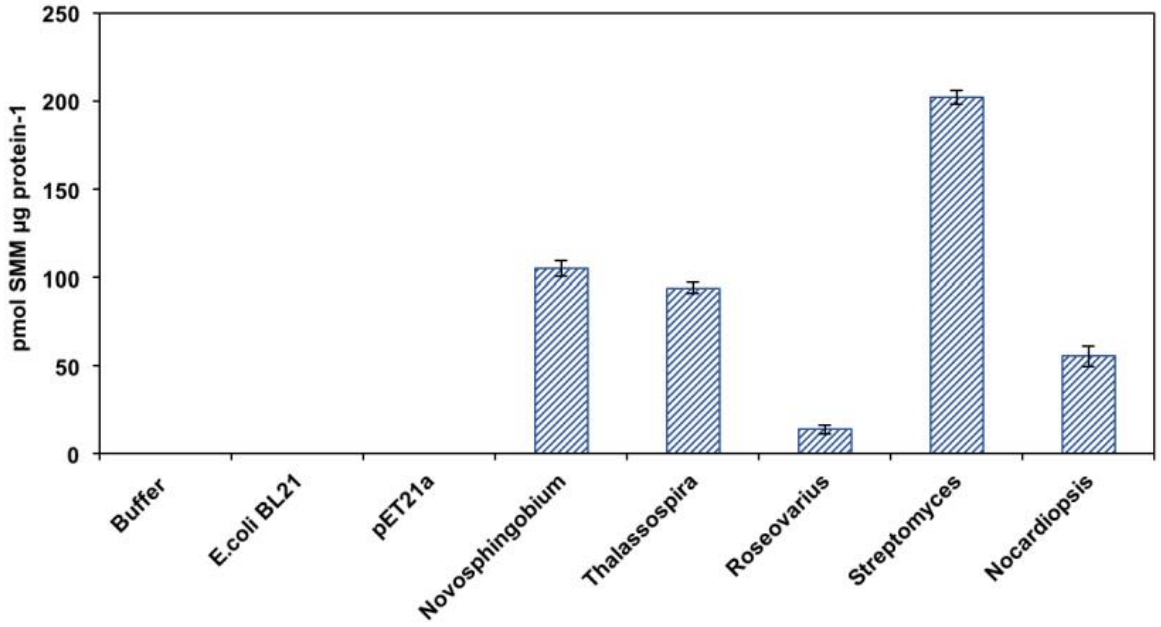

Figure 5-3 The production of SMM in BL21 containing pET21a with *mmtN* from BW1, *T. profundimaris*, *R. indicus*, *S. mobaraensis,* and *N. chromatogenes*[22]. The buffer, BL21, and BL21 with an empty pET21a plasmid were used as negative controls. *Novosphingobium, Thalassospira, Roseovarius, Streptomyces,* and *Nocardiopsis* produced SMM. Error bars show standard error.

5.3.1.2 Purifying the MmtN protein

From the detailed experiments, it may be assumed that *mmtN* confers *in vivo* MMT activity. However, it was necessary to demonstrate that MmtN had *in vitro* MMT activity and characterise its enzyme characteristics. For the protein purification work, *mmtN* was subcloned into pET22b (Novagene, America), allowing the protein to be fused to a C-terminal His-tag. This would allow MmtN to be purified using a metal affinity column. The following work and characterisation were done with Chun-Yang Li (Shandong University, Jinan, China).

The MmtN protein from BW1 was overexpressed in BL21 grown in LB at 37 °C, induced with 0.5 mM IPTG for 16 hours at 20°C, purified with Ni^{2+}-NTA resin and subsequently fractionated using gel filtration buffer on a Superdex-200 column at 4 °C. An example of purified MmtN and DsyB can be seen in Figure 5-4, with sizes of 33.55 kD and 36.94 kD, respectively. The proteins were judged to be more than 95 % pure. Pure MmtN enzyme was incubated with SAM and Met, and the production of SAH (*S*-adenosyl homocysteine) was measured, which could be detected by HPLC. SAH is produced following the demethylation of SAM when acting as a methyl donor for Met to generate SMM. This confirmed that MmtN has *in vitro* activity.

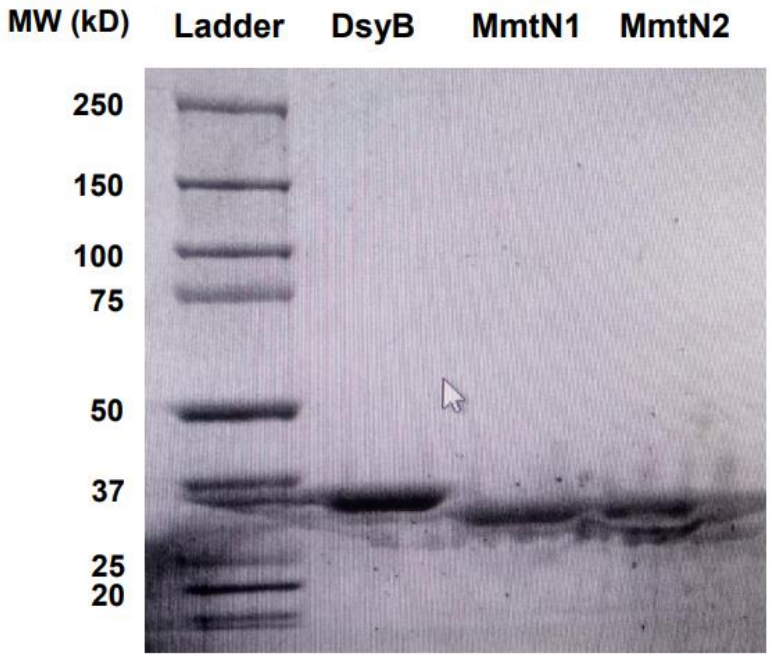

Figure 5-4 Purified proteins of DsyB and MmtN visualised on an SDS PAGE protein gel[22]. Run against a prestained precision protein ladder.

Different temperatures and pH conditions were tested to determine the optimal conditions for MmtN activity. After measuring the highest amount of activity, this was defined as 100 % activity and allowed all the other conditions to be described relative to this. For temperature, this was testing reaction mixes between 0 °C and 60 °C at 10 °C intervals for 30 minutes (Figure 5-5**a**). pH levels were between pH 5.0 and pH 10.0 using Britton-Robinson at discrete intervals (Figure 5-5**b**). Finally, the kinetic parameters (K_m) were determined using non-linear analysis that was based on the initial rates using 3.34 μM MmtN and 0.1 to 0.4 mM SAM (Figure 5-5**c**) or 0.1 to 6 mM Met (Figure 5-5**d**).

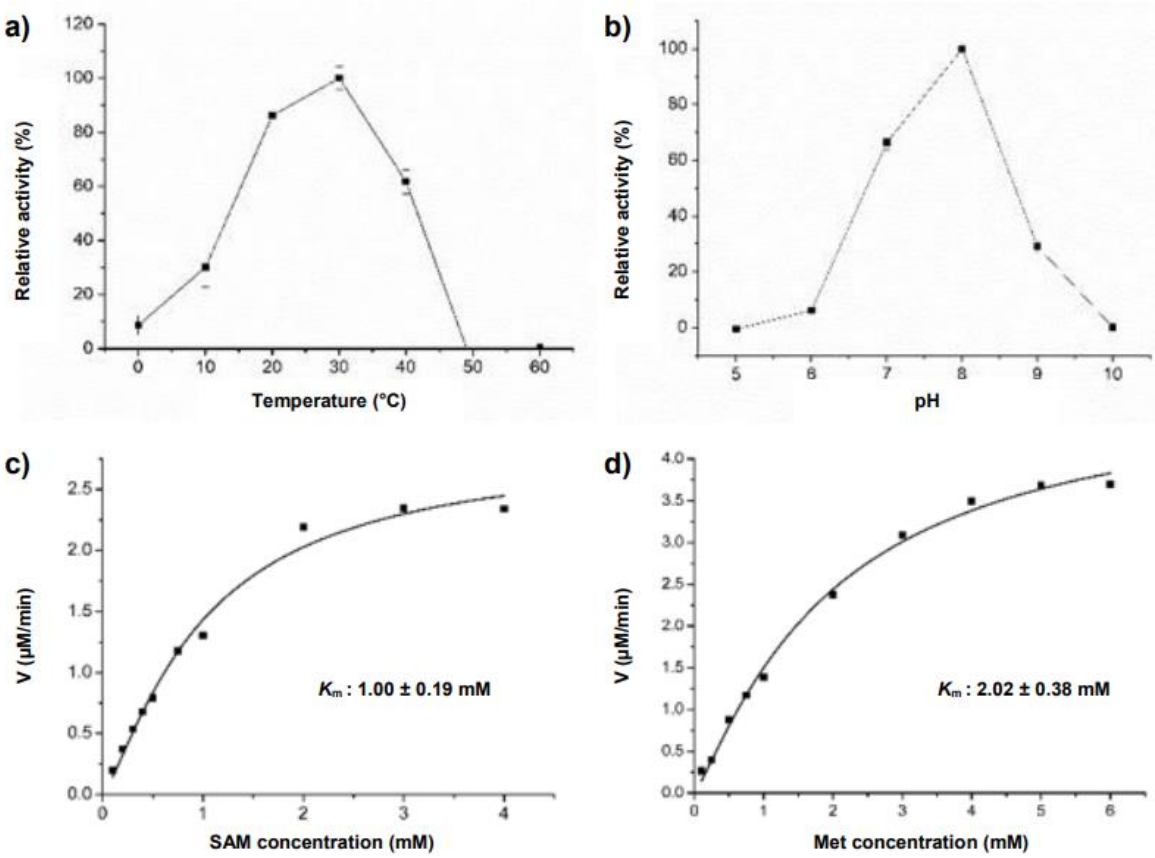

Figure 5-5 MmtN characterisation[22]. (a) The effect of temperature on MmtN activity, 100 % at 30 °C. (b) The effect of pH on MmtN activity, 100 % at pH 8.0. (c) SAM demethylation by MmtN is shown by a non-linear fit curve. K_m 1.00± 0.19 mM. (d) Met methylation by MmtN shown by a non-linear fit curve. K_m 2.02 ± 0.38 mM.

Now the optimal activity conditions had been determined; all subsequent experiments were performed using them with an amount of Met and SAM always more than was required. The purified MmtN (3.34 μM), Met (2.5 mM) and SAM (2.5 mM) were mixed in reaction buffer at pH 8.0 and incubated at 30 °C for 30 minutes. At this point, the reaction was stopped, and the concentration of SAH was detected by HPLC on a Sunfire C18 column (Waters, Ireland). The methylation activity of MmtN on other substrates was also tested (MTHB, MMPA and L-Gly), as was MmtN on its own as a control (Figure 5-6). Clearly, the only reaction condition where SAH was produced was when MmtN, SAM and Met were incubated together. Confirming the assumption that MmtN methylates Met using SAM as a methyl donor while freeing SAH.

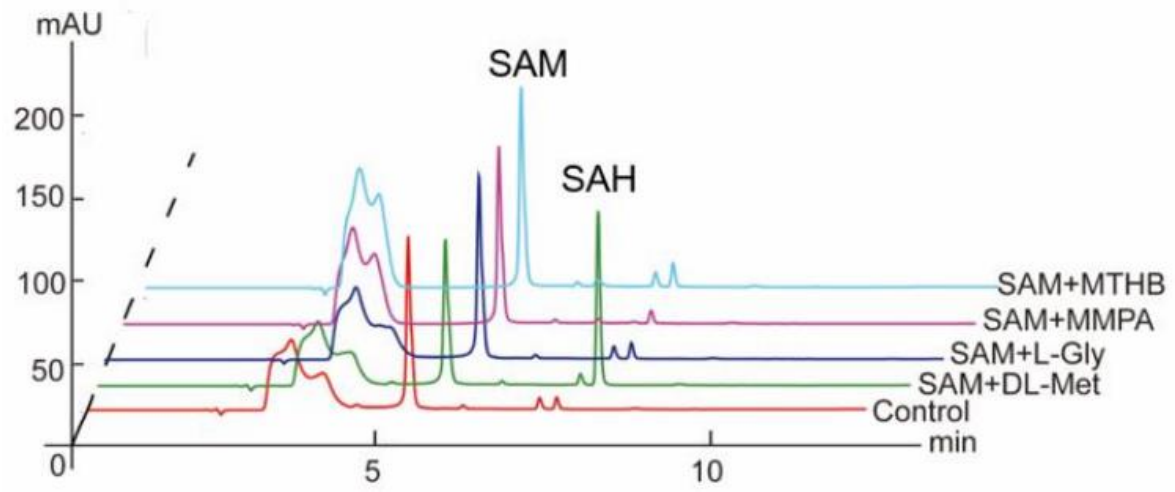

Figure 5-6 The methylation activity of MmtN on MTHB, MMPA, L-Gly and Met using the intensity of absorbance on HPLC (wavelength detected, 260 nm)[22]. Coloured curves show different reaction systems with different substrates.

5.3.2 Creating a disruption mutant of *mmtN*

5.3.2.1 Choosing a candidate strain

To demonstrate that *mmtN* was solely responsible for the production of SMM and was central to DMSP in BW1, the gene needed to be mutated in the BW1 genome. This would test the hypothesis that mutating *mmtN* would either reduce or completely remove DMSP synthesis in the strain, depending on whether it was a sole enzyme with MMT activity in BW1. This gene mutagenesis was achieved by disrupting the *mmtN* in the genome of the wild-type strain using a suicide plasmid. Three strains were considered for the generation of an *mmtN*$^{-}$, BW1, *R. indicus* and *T. profundimaris*. The actinobacteria were difficult to grow, so they were not considered. Furthermore, since *R. indicus* contained *dsyB* as well as *mmtN,* it was removed from the running, leaving BW1 and *T. profundimaris*.

To determine which of the two strains would be easiest to work with, they were tested for antibiotic resistance since the methods for mutation being used would rely on selection by antibiotics and strains with multi-drug resistance would be unsuitable. Cultures were grown to stationary phase in rich media and then plated onto agar containing various antibiotics, which can be seen in Table 5-2. Growth was checked following an incubation of 48 hours (Table 5-2).

Table 5-2 The growth of BW1 and *T. profundimaris* on agar-containing antibiotics.

Antibiotic	Concentration (μg ml^{-1})	Growth of BW1	Growth of *T. profundimaris*
Gentamycin	20	✓	×
Kanamycin	200	✓	×
Neomycin	20 or 40	✓	×
Rifampicin	20	✓	×
Spectinomycin	200	✓	×
Streptomycin	200	✓	×
Tetracycline	5	✓	✓

Clearly, BW1 was not an appropriate choice for mutagenic gene work because it was resistant to all of the antibiotics tested. Therefore, *T. profundimaris* became the strain of choice for mutation since it was only resistant to tetracycline, and this antibiotic was not required for any of the vectors used in this study. As with BW1, it was appropriate to start by gaining a better understanding of how *T. profundimaris* grew and how its production of DMSP was affected by different salinity and nitrogen conditions (Figure 5-7). The levels of DMSP produced by *T. profundimaris* increased with salinity, with the greatest amount produced at 70 PSU, showing that *T. profundimaris* has a high tolerance for salt, making it well adapted to the saltmarsh sediments from which it was first isolated. Interestingly, increasing the levels of nitrogen caused a complete lack of DMSP production, which may be due to *T. profundimaris* being able to synthesise GBT (**Error! Reference source not found.**) and would use preferably in environments where the nitrogen concentration was high.

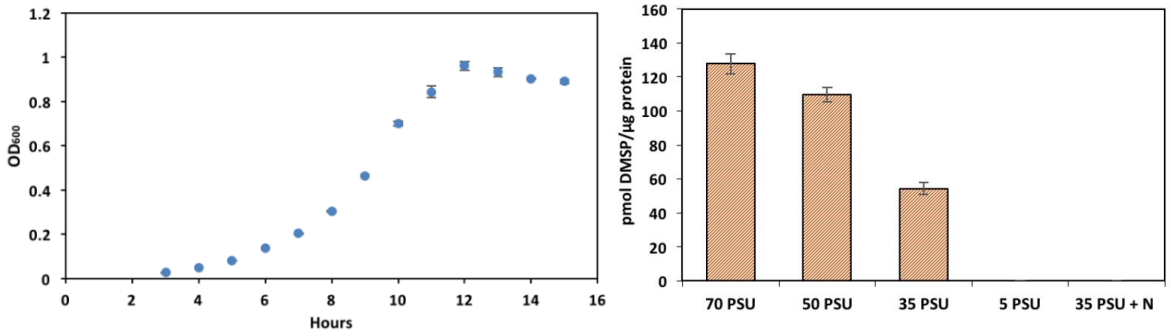

Figure 5-7 The growth of *T. profundimaris* to stationary phase, error bars show standard error (left). The production of DMSP under various salinity and nitrogen concentration conditions. The DMSP concentration decreases with the decreasing salinity. Error bars show standard error (right). Adapted from Williams (2019).

5.3.2.2 Disrupting mmtN in T. profundimaris

The single homologous recombination method using pBIO1879 (pK19spec)[131] was used to generate a mutant in *T. profundimaris,* and the mutagenesis was achieved with guidance from Andrew Curson. Firstly, a spontaneous *T. profundimaris* Rif^R mutant was isolated, which allowed for the selection of *T. profundimaris* over *E. coli* in conjugation experiments. This was achieved by growing a high density of *T. profundimaris* cells on plates containing rifampicin and allowing time for spontaneous mutants to arise. In the case of these experiments, *T. profundimaris* Rif^R is treated as a wild-type in comparison to the *mmtN*$^-$ strain.

Single crossover (SCO) gene disruption is a method by which a fragment of the gene (e.g., *mmtN*) is cloned into pBIO1879[131], a derivative of pK19mob[198] containing a $Spec^R$ cassette. pBIO1879 is a suicide vector which only replicates in *E. coli*, so when mobilised into a host strain, the origin of replication does not work, and so the plasmid is not maintained unless it is integrated into the genome via homologous recombination. Recombination is most likely to happen between the cloned fragment and the complement sequence of the host's genome. The integration of the plasmid into the gene disrupts and renders it non-functional, and mutants are selectable by spectinomycin, kanamycin and rifampicin. To begin the mutation, primers were designed for either side of a central region of the *mmtN* gene in *T. profundimaris* and were used to amplify a ~ 500 bp fragment which was digested and cloned into pBIO1879. Subsequently, this was transformed into 803 competent cells and, using tri-parental mating, mobilised into *T. profundimaris* Rif^{R152}. *T. profundimaris mmtN*$^-$ were selected by plating on YTSS media with rifampicin, spectinomycin and kanamycin, which would select for pBIO19TK integration. The potential mutants were checked by PCR using primers outside of the cloned fragment insert, and any that did not give a PCR product were examined by GC for their DMSP production phenotype. No PCR product was expected because when the plasmid (~ 5.7 kb) is inserted into the genome, the potential product would be too large for PCR. The ability of the resulting *T. profundimaris mmtN*$^-$ mutant was assayed for its ability to produce DMSP by GC and LC-MS. Both methods showed that the *T. profundimaris mmtN*$^-$ mutant no longer accumulated DMSP compared to the wild-type strain (Figure 5-8). This demonstrated that a deletion of *mmtN* removed DMSP production and therefore confirmed that *mmtN* is required for DMSP synthesis in *T. profundimaris*.

The next step was to show that the absence of DMSP production in the *T. profundimaris mmtN*$^-$ mutant was indeed due to the mutation of *mmtN*. This was done by genetic complementation, i.e. by introducing cloned wild-type *mmtN* gene on a plasmid into the *T. profundimaris mmtN*$^-$

mutant. The *mmtN* gene from BW1, which was cloned into pET21a, was subcloned into pLMB509[30,147,153] and mobilised into the *T. profundimaris mmtN*$^-$ mutant and tested on the GC for DMSP production. As expected, cloned *mmtN* complemented the loss of DMSP production phenotype of the *T. profundimaris mmtN*$^-$ mutant strain (Figure 5-9). However, the genetically complemented strain did not return DMSP production back to the wild-type levels. This could be due to the taurine-inducible promoter in pLMB509, which is not a natural promoter for *mmtN* and may have resulted in lower expression in comparison to the wild-type. Irrespective of the difference between DMSP levels in the mutant and the complemented strain, it is consistent with *mmtN* being essential for DMSP production in *T.* profundimaris and encoding its key Met *S*-methyltransferase enzyme.

Interestingly, LC-MS data showed that although the *T. profundimaris* wild-type strain only produced DMSP, the *mmtN*$^-$ produced very large amounts of GBT (**Figure 5-8**), further supporting the theory that GBT and DMSP are switchable in their roles as osmoprotectants. The ability for organisms to switch between the two would also suggest a lack of obvious phenotypes in the *mmtN*$^-$ strain since it may be able to compensate for the absence of DMSP by utilising GBT.

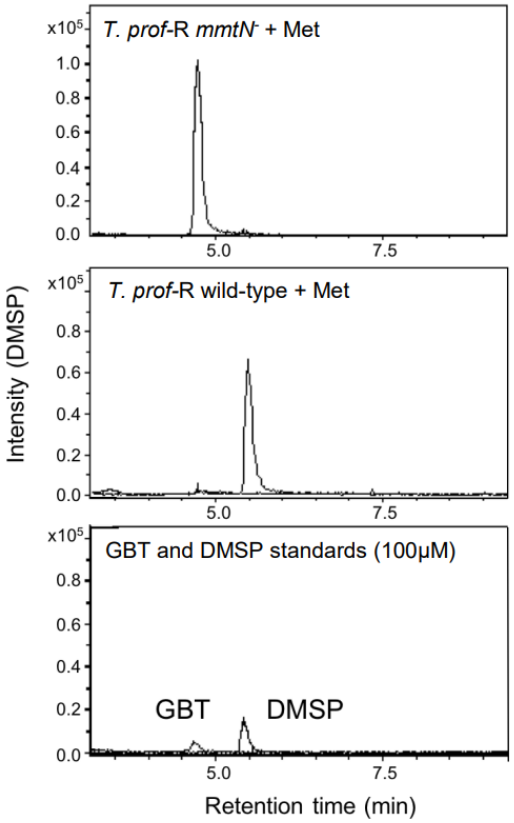

Figure 5-8 *T. profundimaris* wild-type and *mmtN*⁻ mutant showing peaks of DMSP (dashed line, *m/z* = 135) and GBT (continuous line, *m/z* = 118)[65]. The mutant is unable to produce SMM and subsequently DMSP, the bacterium seems to compensate for this by producing GBT.

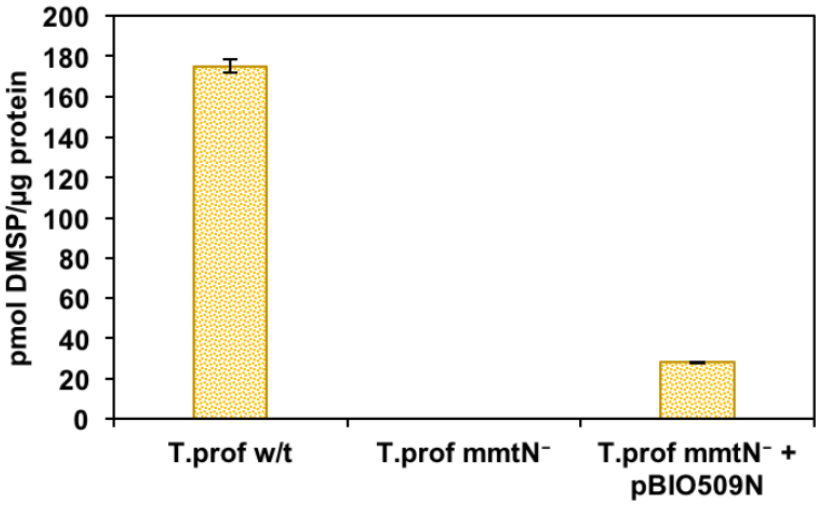

Figure 5-9 The amount of DMSP produced by wild-type *T. profundimaris*, *T. profundimaris mmtN*⁻, and the *T. profundimaris mmtN*⁻ complemented with *mmtN* from BW1. The mutant looses the ability to produce DMSP, this is reinstated when complemented with *mmtN*, although not to the wild-type levels.

5.3.2.3 *Phenotypic characterisation of the T. profundimaris mmtN⁻*

The *T. profundimaris mmtN⁻* mutant allowed any phenotypes caused by a lack of DMSP production to be observed, such as impacted growth or survival, suggesting that DMSP was necessary for growth or cell function. Due to the effect of salinity on the production of DMSP already observed (Figure 5-7**-right**), this was the first condition used. Cultures of MBM with a salinity of 35 PSU or 50 PSU with 0.5 mM, or 35 PSU with 12 mM nitrogen as a control, nitrogen were inoculated with *T. profundimaris*^{R} and *T. profundimaris mmtN⁻* in triplicate. All cultures were incubated at 30 °C at 200 rpm for 14 hours. The growth was measured hourly until the cultures reached stationary phase, which was judged by cultures having a similar OD_{600} for 3 hours (Figure 5-10).

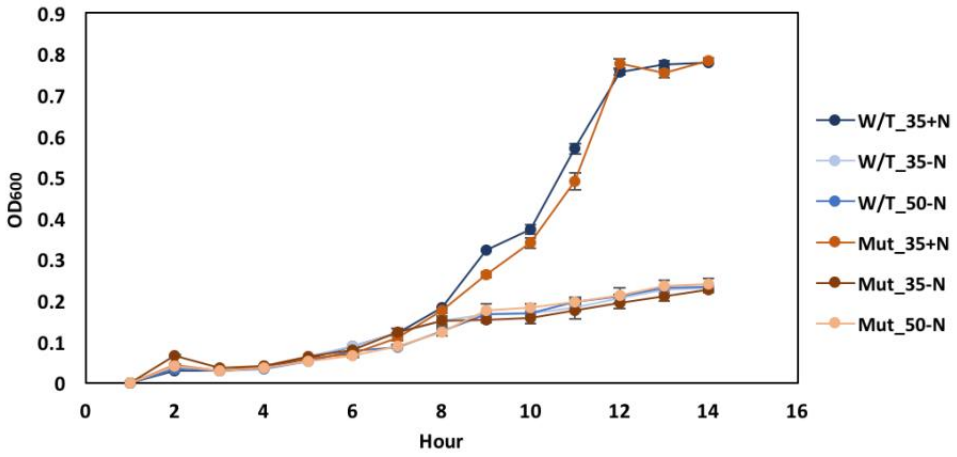

Figure 5-10 The growth of *T. profundimaris* wild-type (W/T) and *mmtN⁻* mutant (Mut) grown in 35 PSU or 50 PSU with 0.5 mM nitrogen (-N) and 35 PSU with 12 mM nitrogen (+N)[22]. Error bars show standard error.

Unfortunately, but somewhat expected, there was no significant difference between the growth of *T. profundimaris* wild-type and *T. profundimaris mmtN⁻* in any of the salinities tested. When grown in 50 PSU media, the growth is impeded, yet the growth continually increases, regardless of the presence of *mmtN*, again suggesting the ability of GBT (or others in the case of -N) to take place as an osmoprotectant. Perhaps the DMSP concentration increases in high salinity due to other processes, or it may not be required for growth.

Next, a competition experiment was conducted between *T. profundimaris* wild-type and *mmtN⁻* where the cells were grown under various conditions. The wild-type and mutant were inoculated from fresh plates into 35 PSU MBM with 12 mM nitrogen and incubated overnight at 30°C; these conditions were used as 'before stress'. The cultures were mixed at a ratio of 1:1, and a serial dilution was plated onto MBM agar and incubated at 28 °C until single colonies were visible. Colonies were picked and streaked in the same place on plates with

and without kanamycin, allowing for the mutant (Kan^R) and the wild-type (Kan^S) to be differentiated between. This method was also carried out under high-stress conditions such as low nitrogen (35 PSU and 0.5 mM nitrogen) and high salinity (50 PSU and 0.5 mM nitrogen). The percentages were calculated and compared between before and after stresses (Table 5-3). As with the previous experiment, there appeared to be no obvious phenotype, although there is almost always a higher percentage of wild-type colonies. This seems to be the same for higher salinity (50 PSU) but decreases to 1:1 when in 35 PSU.

Table 5-3 Ratio growth of *T. profundimaris*^{R} wild-type and *T. profundimaris mmtN*$^-$ mutant[22]. Strains were grown in competition with and without stress.

Growth condition	Replicate	Wild-type growth (%)	*mmtN*$^-$ mutant growth (%)
Before stressed growth		63	37
50 PSU -N	1	64	36
	2	64	36
	3	61	39
	Average	**63**	**37**
35 PSU -N	1	61	39
	2	50	50
	3	50	50
	Average	**54**	**46**

Finally, the effect of temperature was tested and compared. *T. profundimaris*^{R} and *mmtN*$^-$ were grown in 35 PSU MBM with 0.5 mM nitrogen until stationary phase. The OD_{600} was adjusted to 0.3, the cells were centrifuged at maximum speed, and the pellets were washed with MBM. A serial dilution was plated on YTSS and incubated until colonies were visible. Colonies were counted to calculate cfu ml^{-1}. Following this, cultures were frozen for a week, defrosted, and a serial dilution plated on YTSS and incubated until colonies formed, and then the cfu ml^{-1} was calculated. The cfu ml^{-1} was compared before and after freezing (Figure 5-11).

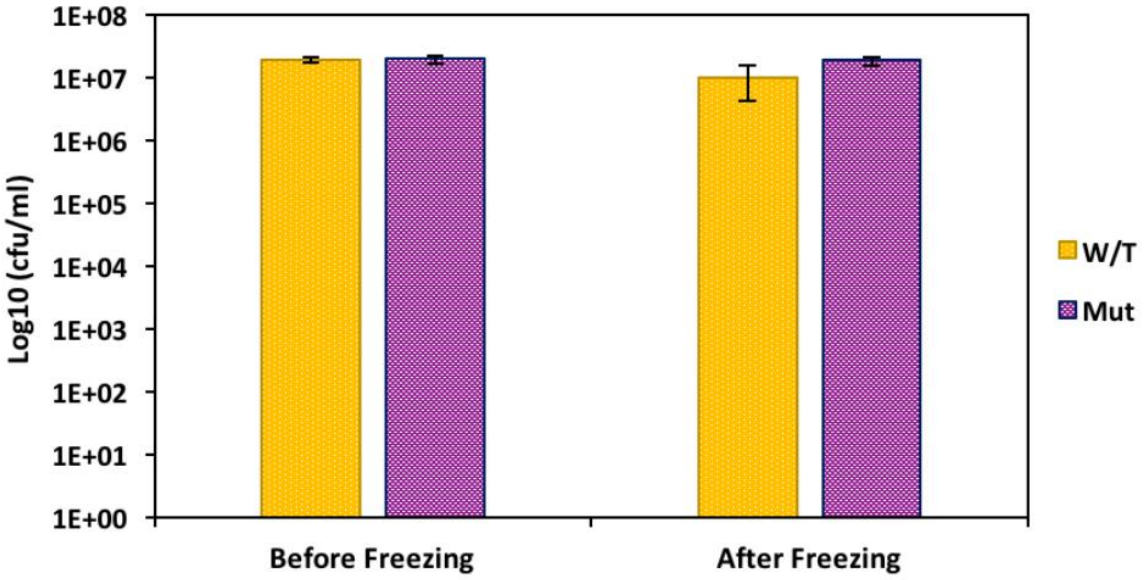

Figure 5-11 The effect of temperature on *T. profundimaris* wild-type and *mmtN*⁻ mutant[22]. The cfu ml^{-1} was calculated before and after freezing (-20 °C) for 1 week and plotted on a logarithmic scale. Error bars show standard error.

Alas, none of the characterisations performed could identify any observable phenotypes. However, it may be that the conditions which would present a phenotype have not been tested, such as growth under oxidative stress.

5.3.3 The presence of *mmtN* in other isolates from Stiffkey

Only BW1 contained *mmtN* from the three strains sent for WGS that did not contain *dsyB*. And it appears that neither *Alteromonas* nor *Marinobacter* isolated from Stiffkey has *mmtN*. However, there is an *Alteromonodaceae* bacterium on the JGI database that has *mmtN*, suggesting that some bacteria within the order may have it. Additionally, it seemed that other *dsyB*-containing bacteria also contained *mmtN*, such as *Rhodobacterales* (E value 3E-145, identity 69 %). Although this is not unusual since *R. indicus* also contains both (Table 5-1), and there are many species which contain multiple *ddd* genes[131,199].

5.3.4 Other methylation pathway candidates

The MmtN aa sequence was used to search for other $MmtN^+$ bacteria by searching the NCBI database using BLASTp. The results showed that there were 22 bacteria that had > 50 % identity, including Alphaproteobacteria, 4 Actinobacteria and a single Gammaproteobacterium (Figure 5-2). Although there was a variety of genera covered, all of the strains appeared to be of marine origin. Furthermore, there were species already known

to be DMSP producers in the list, such as *Labrenzia* and *Sagittula*. Moreover, the bacteria containing *mmtN* had greater variation than those containing *dsyB*.

The methylation pathway requires a decarboxylase, transaminase and a reductase in addition to the *S*-methyltransferase. In *R. indicus*, *mmtN* appears to be upstream of several genes that could make up part of a DMSP-synthesis operon in the form of aspartate aminotransferase and a diaminopimelate decarboxylase (**Figure 5-12**). The whole genome sequences of *mmtN*-containing bacteria were mined for decarboxylase and transaminase, and some contained both within close proximity to *mmtN*. However, there were also examples that did not contain this group of genes. For example, BW1 has an aspartate aminotransferase but no full diaminopimelate decarboxylase, and many *Thalassospira* and *Labrenzia* had the aspartate aminotransferase within reasonable distance to *mmtN*. Although the last three mentioned did have smaller hypothetical proteins, which showed low identity to the missing genes. What is more, many contained a pyridoxal phosphate-dependent aminotransferase which may be performing a similar role. When the putative DMSP-amine aminotransferase from *T. profundimaris* was mutated (using methods used to create the *mmtN*$^-$ mutant), the DMSP production in the bacterium reduced by 73 % compared to the wild-type strain, which would suggest at least one of the genes downstream of *mmtN* in *T. profundimaris* is linked to the production of DMSP in this organism[65].

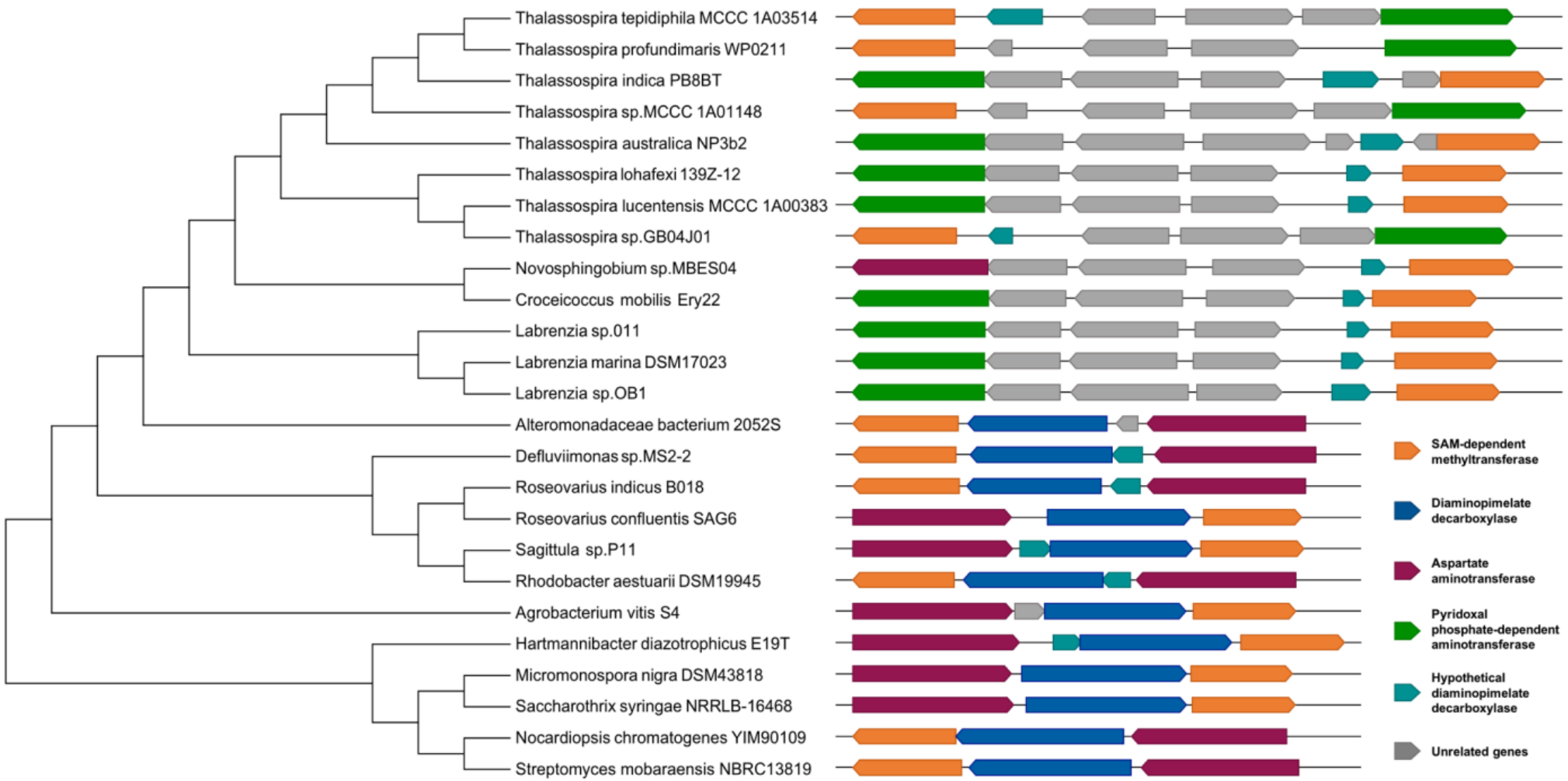

Figure 5-12 A tree showing the synteny between genes surrounding *mmtN* and how these *mmtN*-containing organisms relate to each other[22]. Genes of interested are highlighted.

5.4 Discussion

5.4.1 Summary

Due to the work presented in this chapter, there is now a second DMSP synthesis gene, *mmtN*, which encodes a SAM-dependent MMT responsible for the first step in the methylation pathway, and the possibility of another pathway used by bacteria. This discovery is made even more important by the fact that the product made by MmtN is SMM, a compound which, until these experiments, was considered to only be produced by plants.

Furthermore, *mmtN* is not limited to BW1 or even alphaproteobacterial but can be found in actinobacterial species as well as a gammaproteobacterium. When the *mmtN* gene was disrupted in *T. profundimaris*, the strain was unable to produce DMSP, confirming the role of *mmtN* in DMSP production. Lastly, experiments to determine any phenotypes due to loss of the DMSP synthesis pathway were carried out, and unfortunately, from the conditions tested, there were no phenotypes observed.

5.4.2 The environmental abundance of *mmtN*

Without the identification of microbial DMSP synthesis genes, the prediction of potential DMSP-producing organisms using complex metagenome, metatranscriptome and metaproteome data would be impossible. Before these genes were available, key DMSP producers were predicted only by the taxa that were thought to produce DMSP, and the levels were inferred[60,91]. Studies investigating the presence of *mmtN* in marine environments found that up to 0.2 % of bacteria contained *mmtN*[30,65,66,84,200,201]. Although *dsyB* genes were usually found to be more abundant than those of *mmtN* (37.5-fold), there were exceptions. *mmtN* was expressed higher than *dsyB* in the South Atlantic and South Pacific Oceans. A recent study by Teng *et al.* (2021) found that *mmtN* was more abundant in the polar oceans (0.09 %) compared to the non-polar oceans (0.004 %), suggesting that bacteria may have a more significant role in DMSP production in high-altitude polar regions compared to low altitude locations[200]. Furthermore, *mmtN* appears to be evenly distributed between the sea surface microlayer (SSM) and the subsurface seawater (SSW), which is unlike other DMSP-producing organisms, which are mostly found in the SSM[202–205]. However, *mmtN* is still 4-fold more abundant in the SSM than the sea SSW. As DMSP was previously thought to be important within the SSM, this makes the SSW an interesting and understudied environment.

A vertical profile of the East China Sea showed DMSP synthesis genes throughout the water profile[67], indicating that DMSP production is important throughout the water column and sediment. Another profile from the Eastern China marginal seas also found DMSP synthesis genes throughout the water column, although in this case, only *dsyB* was detected and not *mmtN* [197]. Finally, the most significant was the vertical profile of the Challenger Deep within the Mariana Trench (the deepest site on Earth)[66]. Again, DMSP synthesis genes were found all the way through the column, but interestingly for *dsyB*, abundance levels decreased with depth until 2 km deep (from 0.9 % of bacteria to 0.4 %), where the abundance steadily increased until a total depth of > 6 km was reached with 4 % of bacteria containing the gene[66]. Although *mmtN* did not increase with water depth, it did reach a maximum level of 1.2 % of bacteria at 8 km deep[66]. The DMSP synthesis genes consistently found within all of these water profiles suggest that bacterial DMSP is important within the marine environment.

5.4.3 Future work with *T. profundimaris mmtN*$^-$

All bacteria that can produce DMSP increase production under high saline conditions, and in the case of LZB033, *dsyB* transcription is seen to increase[31], with salinity causing the greatest fold change. Therefore, it would be expected that knocking out a gene within the synthesis pathway (*dsyB* or *mmtN*) would impact survival or at least impact growth. Unfortunately, this is not the case for either of the known genes[31]. It might be that DMSP production is helpful to the organisms that use it but not vital to survival, or it may be that the true condition in which DMSP is necessary has not been tested. In the case of *T. profundimaris mmtN*$^-$, only a few conditions have been tested, so there may be other conditions which would confer a visible phenotype. Most likely is the theory that DMSP and GBT synthesis are interchangeable, and it would certainly be beneficial for organisms to utilise more than one osmoprotectant since their natural environments change and fluctuate, meaning that they would need to adapt if they were to survive. This is supported by the increased production of GBT by *T. profundimaris mmtN*$^-$ in comparison to the wild-type strain. Furthermore, Ana Bermejo Martinez created mutants of the genes for GBT synthesis for LZB033 and found no growth phenotypes when put under the same conditions tested in this chapter. To really understand how DMSP and GBT are used would be to knock out both genes and screen for double knock-out mutations.

It is worth remembering that genetic studies for DMSP-producing bacteria are limited to two alphaproteobacteria which produce DMSP, so the results here are unlikely to reflect all. Mutants of *mmtN* and *dsyB* in other bacteria that lack GBT and use DMSP as a sole osmolyte would be very interesting. Furthermore, with all the suggested functions of DMSP, it would be expected that there are other bacteria which regulate DMSP synthesis in a different way to *T. profundimaris* or LBZ033. For organisms where the production of DMSP is essential, such as

it is with some phytoplankton, it would be expected that preventing DMSP synthesis would produce a more damaging phenotype.

At the other end of the spectrum, organisms that contain more than one DMSP-synthesis pathway, such as *R. indicus*. *R. indicus* contains *dsyB* of the transamination pathway and *mmtN* of the methylation pathway, which would seem to cause redundancy in at least one of the pathways. There might be intermediates within the pathways which give additional benefits to the organisms producing them. Although DMSP is the final step for the pathways here, there are many processes which may provide advantages under different circumstances. It could be that DMSP is so important within *R. indicus* that the presence of a backup pathway increases survival chances.

Not forgetting SMM, which in plants is not specific to DMSP production. There may be a difference between losing SMM and losing DMSP. It would be interesting to see if there are bacteria which are able to produce SMM but not DMSP and look at how they differ from the DMSP-producing strains.

5.4.4 Further characterisation of *mmtN* and DMSP production in bacteria

The phenotyping of an *mmtN*$^{-}$ mutant is not the only method for understanding the role of the gene within its host organism. The purified protein could be used to determine its structure via X-ray crystallography and understand the reaction mechanism. Additionally, the structure may elude to any other environmental factors affecting DMSP productivity. Additionally, there has not been any characterisation of the transcriptional or translation regulation of *mmtN* within any organism, and this is crucial to understand how DMSP is produced. This could be achieved by creating lac fusions or with RT-qPCR, such as was done by Curson *et al.* (2017) and (2018). Furthermore, gaining a better understanding of how DMSP production is regulated. It may be that the transcription of genes involved in DMSP production is all controlled by a single regulator in response to an environmental cue.

As discussed earlier, after evolutionary analysis of *DSYB* and *dsyB,* the gene contained in the alphaproteobacterial was thought to be in the sister clade to the eukaryotic gene. However, subsequent analysis showed that the gene first appeared in prokaryotes and has been transferred to eukaryotes, likely on multiple occasions. These transferences may have taken place through endosymbiosis at mitochondrial origin or perhaps more recently via HGT. With MmtN being homologous to the plant MMT, it would be reasonable to think that one

descended from the other, and it would be most interesting to carry out an evolutionary analysis of *mmtN* and *MMT*. Preliminary work by Lewis Spurgeon suggests that there was a gene duplication event early in this protein families evolution, resulting in the development of ~ 1000 aa MMT (found in all flowering plants and some bacteria) and a shorter MmtN without the C-terminal PLP-binding domain in bacteria. However, both proteins retained the same function, and possibly the C-terminal domain of MMT has an additional function. Additionally, whilst *mmtN* can be used as a reporter of DMSP since all tested bacteria containing it had the ability to produce DMSP, *MMT* cannot be used as a reporter since not all strains containing it produced DMSP. This would be expected since there are only a few plants that can make DMSP, yet all have MMT.

Although this work has confirmed *mmtN* as an MMT which produces SMM, there is no certainty about the subsequent steps in the pathway. This could be achieved using methods documented by Gage *et al*. (1997) to establish the DMSP pathways; by incubating *T. profundimaris* with labelled Met and tracing the intermediates with either HPLC, LC-MS, or NMR. Another method would be to mutate the suspected genes in *T. profundimaris* and observe how DMSP production is affected and if any phenotypes are observed.

Furthermore, it may be that some organisms are using DMSP synthesis to produce more complex molecules, such as those explored in work published by Trottmann *et al*. (2020), which found that in the pathogenic bacteria *Burkholderia pseudomallei*, DMSP was a key intermediate in the production of the cyclopropanol unit. As mentioned previously, *S. mobaraensis* contained an *mmtN* homologue and showed a peak of DMSP when screened by GC. An investigation into why and how *Streptomyces* produce DMSP would be particularly interesting as they are well known for synthesising secondary metabolites and may shed light on other pathways where DMSP acts as an intermediate.

6 Discussion

As one of the most abundant organosulfur compounds on Earth, DMSP plays an important role[16]. The bacteria, algae, corals, and plants that have the ability to synthesise or take up the molecule use it for nutrients[17] or to protect themselves from stressful environments. The anti-stress compound has suggested roles, including osmoprotection[6], antigrazing[47], antioxidant[6,20,31]. Yet, DMSP is most well known as a precursor for the climate-active gas DMS[28]. Whilst the catabolism of DMSP has been studied extensively, the study of DMSP synthesis using molecular biology is relatively newer, and the first synthesis gene was only recently discovered[31]. The DMSP, *dsyB*, identified in the bacteria *Labrenzia aggregata* was an exciting discovery as up until this point, the production of DMSP by bacteria had not been documented, and since the DMSP-producing eukaryotes are limited to where they are able to grow, bacteria have much fewer restrictions. Furthermore, the bacterial contribution to the production of DMSP had not been considered, so there was a need to address this lack of knowledge. The work in this thesis aimed to gain a better understanding of the role bacteria have in the production of DMSP by:

1. Determining the abundance of DMSP-producing bacteria within Stiffkey saltmash sediment through bacterial isolation.
2. Using culture-dependent techniques to observe how DMSP production might be important within the saltmarsh environment.
3. Identifying DMSP producers and determining their method of DMSP synthesis.

As DMSP is such an important compound within the environment, the study of its production and distribution are valuable areas of research. Through this study, the experiments were designed to study the production of the wider bacterial community and the diversity of species with the ability to produce the molecule, but also how a single species produces the molecule through the use of genetic manipulations. This study provides insight into the production of DMSP within saltmarsh sediment through the development of techniques and methods which may be used to further study DMSP-producing bacteria in other environments.

6.1 Key findings in this thesis

6.1.1 Bacteria contribute to DMSP production in saltmarsh sediment

The production of DMSP within Stiffkey saltmarsh was previously attributed to *Sporobolus* species[42,169], and indeed the experiment in **Chapter 3** of DMSP concentrations from sediment transects did show a high level of DMSP directly under the *Sporobolus* plants. However, there

was a consistent level of DSMP further away from the plants, suggesting that the plants were not the only organism producing DMSP. Most likely, DMSP is being produced by bacteria or algae as well. Around the edges of the tidal pools at Stiffkey saltmarsh, there were algal mats which may have caused the higher concentration nearer the *Sporobolus*, but also the possibility that some of the plant's roots may have been included in the samples.

Furthermore, when initially isolating bacteria from the sediment samples collected from Stiffkey without enrichment, 25 % had the ability to produce DMSP, and with enrichment increased to 77 %. From the bacteria isolated, some were already known to produce or suspected to produce DMSP, such as *Labrenzia*, *Stappia, Rhodobacter*, and *Rhodobacterales*. Conversely, there were some surprising strains also, for instance, *Novosphingobium, Marinobacter* and *Alteromonas,* which have not been previously shown to be DMSP producers. Upon further investigation, these strains were determined not to have *dsyB* and were therefore predicted to have *dsyB* isoforms or perhaps novel genes and DMSP synthesis pathways.

6.1.2 *Novosphingobium* contains *mmtN*, a novel DMSP-synthesis gene

Chapter 4 and 5 described the discovery of *mmtN* as a SAM-dependent methyltransferase enzyme BW1. MmtN selectively methylates Met via S-methylation and does not catalyze intermediates from other DMSP synthesis pathways or glycine, a substrate for betaine synthesis, indicating that MmtN is a specific S-methyltransferase for the DMSP methylation pathway[206]. Compared to the N-terminal domain of the plant MMT enzyme that catalyzes SAM-dependent Met methylation to SMM, MmtN was less than 30 % identical but was a third of its size as it lacks the C-terminal aminotransferase domain of MMT that is known to have a regulatory function in plants[183]. While *mmtN* encoding functional MMT were found in some diverse alpha- and gammaproteobacteria and actinobacteria that produced DMSP, they were far less common than those with *dsyB*. They were primarily located in operons with genes that likely encode the downstream enzymes of the bacterial Met methylation pathway[65,207]. This data confirmed that *mmtN* was an effective reporter gene for DMSP synthesis. When *mmtN* was knocked out in *T. profundimaris*, this alphaproteobacterium no longer produced DMSP[65], confirming the importance of this gene for DMSP synthesis. MmtN enzymes were less efficient than plant MMT equivalents, with higher K_m values for Met and SAM[65,206,207], and the ecological significance of this is unknown. However, these millimolar values are in the range of those reported for DsyB, DSYB, and TpMMT and are not uncommon for enzymes transforming Met[30,78,84,208,209]. Furthermore, the *mmtN* mutant did not have a growth impairment compared

to the wild-type *T. profundimaris* strain under conditions such as salinity and temperature stress, as was the case with the *L. agreggata dsyB*- strain, leaving unanswered questions as to the role that DMSP plays in these bacteria[31].

The crystal structures of MmtN from *R. indicus* were determined to understand its mechanism, which forms a trimer around a central phosphate ion and utilizes the proximity and desolvation mechanism to S-methylate Met and generate SMM[84,206]. SAM stabilizes in the active site of MmtN through hydrogen bonds to specific residues, and Met is stabilized by hydrogen bonds to other residues, allowing the sulfur atom to attack the methyl group of SAM, forming SMM and SAH[206]. The proposed mechanism is believed to be universal to DMSP-producing bacteria with MmtN since all bacterial MmtN enzymes analyzed contained the SAM-binding residues Asp69, Ser101, Leu102, Cys121, and Arg132 for Met binding, and a "GxGxG" signature sequence, which was highly conserved in the SAM-dependent methyltransferase family. Some bacterial MmtN proteins substituted residues Glu127 and Glu250, which are involved in the binding of Met, with aspartate[206]. Using the MmtN structure and key conserved residues, the diversity of organisms with functional MmtN enzymes was increased, and organisms with this enzyme were grouped into three distinct groups, likely with different roles for MmtN.

6.1.3 Group I MmtN

Group I mmtN genes are present in alpha-, gamma-proteobacteria, and actinobacteria, which produce DMSP. These genes encode MMT proteins with more than 50 % amino acid identity, making them reliable indicators of bacterial DMSP synthesis[65,206,207]. Studies have shown that group I mmtN is located near genes encoding enzymes involved in the methylation pathway for DMSP synthesis[65,207]. In *S. mobaraensis*, genes linked to *mmtN* encode functional enzymes involved in the downstream steps of the methylation pathway, including SMM decarboxylase (termed SMMDC), DMSP-amine aminotransferase (termed DMSPAAT), and DMSP-aldehyde dehydrogenase (termed DMSPADH)[207]. These genes are conserved in actinobacteria with *mmtN*. Some alphaproteobacterial *Agrobacterium* and a gammaproteobacterial *Alteromonadaceae* bacterium with *mmtN* also contain *S. mobaraensis SMMDC* and *DMSPAAT* genes[207]. However, most alphaproteobacteria with *mmtN* likely harbour genes encoding isoforms of these enzymes[65]. Knocking out the candidate *DMSPAAT* gene in *T. profundimaris* resulted in a 73 % reduction of DMSP levels compared to the wild-type strain, supporting its role in DMSP production[65].

Bacteria with Group I MmtN enzymes can be divided into two subgroups based on whether their *mmtN* genes are linked to a non-ribosomal peptide synthesis (NRPS) gene or not[206]. In most bacteria with a Group I MmtN, *mmtN* is not linked to an NRPS gene, while in all actinobacteria and some alphaproteobacteria, *mmtN* is located in an operon that also contains an NRPS gene. It has been suggested that bacteria with an *mmtN* associated with an NRPS gene might synthesize DMSP as an intermediate in producing more complex secondary metabolites[206,210]. However, further experimental work is needed to verify this hypothesis, and, likely, the intracellular levels and roles of DMSP in these two subgroups would be different, with higher levels predicted in those lacking the NRPS gene.

6.1.4 Group II MmtN

Group II MmtN enzymes were discovered in archaeal *Candidatus* Woesearchaeota, Candidate Phyla Radiation (CPR) bacteria, and the animalcule *Adineta steineri*, which only share about 30 % amino acid identity with Group I MmtN proteins[206]. These enzymes contained the "GxGxGx" signature sequence and most conserved SAM and Met binding residues. Synthesized representative genes from this group have shown that they encode functional MmtN enzymes in *in vitro* assays, suggesting that these host archaea and CPR bacteria generate SMM from Met[206]. However, since these organisms have not yet been cultured, it is unknown whether they also produce DMSP. The absence of known *SMMDC*, *DMSPAAT*, and *DMSPADH* genes, and the fact that their Group II mmtN is not located near genes predicted to have these roles, suggest that it is unlikely that they synthesize DMSP by the methylation pathway. Therefore, a Group II mmtN in an organism cannot be used as an indicator for DMSP production.

The Group II mmtN genes were found to be closely associated with putative homocysteine S-methyltransferase (HMT) and, in some cases, a Met-tRNA ligase-encoding gene involved in Met metabolism. In plants, MMT and HMT are critical enzymes of the SMM cycle that regulate the levels of Met and SAM in plants[97,101]. MMT generates SMM and SAH from Met in an irreversible reaction using SAM as a methyl donor, whereas SMM can be converted back to Met in another irreversible methylation reaction catalyzed by HMT, where homocysteine acts as a methyl acceptor[97,101]. It has been suggested that organisms with Group II MmtN proteins might have a similar regulatory SMM cycle as in plants[206]. For archaea and CPR bacteria with Group II *mmtN* linked to a Met-tRNA ligase gene, it was proposed that they use SMM as a non-standard amino acid to initiate protein translation, possibly as an adaptation to their symbiotic lifestyle[206].

6.1.5 Group III MMT

Notably, certain deltaproteobacteria and Oligoflexia *Pseudobacteriovorax* species have MmtN-like proteins more similar to the plant MMT enzyme than Group I or II MmtN[65,206]. These proteins, called Group III MMT enzymes, are comparable in length to plant MMT enzymes and contain the same C-terminal aminotransferase domain. However, only one of the four bacterial strains examined produced DMSP, indicating that this longer Group III *MMT* gene is not a reliable indicator for bacterial DMSP synthesis[65]. Furthermore, the bacterial *MMT* gene is not associated with downstream DMSP synthesis, *NRPS*, or other Met cycling genes, making it difficult to determine the potential function of SMM production in these microorganisms[206]. The significant difference (24 % aa identity) between MmtN and MMT enzymes, which perform the same reaction, is noteworthy[207]. Additionally, bacterial downstream enzymes SMMDC, DMSPAAT, and DMSPADH are more closely related to bacterial enzymes with other functions than to plant proteins[207], suggesting that the DMSP methylation pathway may have originated independently in bacteria due to the promiscuity of many PLP-dependent enzymes and aldehyde dehydrogenases.

6.2 Other DMSP synthesis genes

6.2.1 TpMMT

Most diatoms produce DMSP at low intracellular levels[60,91], but only a few have a DSYB enzyme. Researchers used a bioinformatic approach to identify candidate MSM enzymes in the diatom *Thalassiosira pseudonana*, which lacks DSYB[78]. They identified two candidate S-methyltransferases, one of which, called TpMT2, was found to be an isoform MSM enzyme with in vitro MSM activity[78]. Although other TpMMT-like proteins are found in diatoms, they cannot be used as indicators of DMSP production. TpMMT has deficient aa identity to DSYB/DsyB and MmtN (24 % and 29 % respectively), the Met S-methyltransferase involved in DMSP synthesis via the methylation pathway in some bacteria. Several other candidate genes were proposed for the aminotransferase, reductase, and decarboxylase enzymes from the transamination pathway in *T. pseudonana*, but transcription of these candidate genes was not elevated in *T. pseudonana* grown under raised salinity conditions that increased DMSP accumulation[78].

6.3 Recommendations for future research

The identification of the second DMSP-synthesis gene, *mmtN*, shows that bacteria are able to produce DMSP via more than one pathway. While it is possible to speculate what this pathway

might be, further studies are necessary to determine what these intermediate steps are. A way of achieving this could be to use radiolabelled compounds and track the production of intermediates in DMSP-synthesis mutants with LC-MS, HPLC, or NMR. Since Met is used in other metabolic pathways, it may be more pertinent to use labelled SMM. Additionally, the production of SMM is a process found within all flowering plants and has now been shown to also be utilised by some bacteria. It would be interesting to determine whether this is only a step within the DMSP synthesis pathway or if SMM has any other role within the cell.

Furthermore, the *T. profundimaris mmtN*$^-$ mutant showed no apparent phenotypes under the conditions tested, yet there are many other suggested benefits for DMSP synthesis. Growing the mutant under oxidative stress by the addition of H_2O_2 or by treatment with UV may result in a phenotype presenting. *T. profundimaris* can easily able to grow at salinities of 70 PSU, and perhaps even higher concentrations could be tested. It may be that the mutant is able to compensate for the mutation through the production of GBT, which was observed by LC-MS. By creating a double mutant that lacks both the ability to produce DMSP and GBT, it may be possible to narrow down what these compounds are used for within these organisms.

7 References

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8 Appendix

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LETTERS

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Bacteria are important dimethylsulfoniopropionate producers in coastal sediments

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Dimethylsulfoniopropionate (DMSP) and its catabolite dimethyl sulfide (DMS) are key marine nutrients[1,2] that have roles in global sulfur cycling[2], atmospheric chemistry[3], signalling[4,5] and, potentially, climate regulation[6,7]. The production of DMSP was previously thought to be an oxic and photic process that is mainly confined to the surface oceans. However, here we show that DMSP concentrations and/or rates of DMSP and DMS synthesis are higher in surface sediment from, for example, saltmarsh ponds, estuaries and the deep ocean than in the overlying seawater. A quarter of bacterial strains isolated from saltmarsh sediment produced DMSP (up to 73 mM), and we identified several previously unknown producers of DMSP. Most DMSP-producing isolates contained *dsyB*[8], but some alphaproteobacteria, gammaproteobacteria and actinobacteria used a methionine methylation pathway independent of DsyB that was previously only associated with higher plants. These bacteria contained a methionine methyltransferase gene (*mmtN*)—a marker for bacterial synthesis of DMSP through this pathway. DMSP-producing bacteria and their *dsyB* and/or *mmtN* transcripts were present in all of the tested seawater samples and *Tara* Oceans bacterioplankton datasets, but were much more abundant in marine surface sediment. Approximately 1×10^8 bacteria g^{-1} of surface marine sediment are predicted to produce DMSP, and their contribution to this process should be included in future models of global DMSP production. We propose that coastal and marine sediments, which cover a large part of the Earth's surface, are environments with high levels of DMSP and DMS productivity, and that bacteria are important producers of DMSP and DMS within these environments.

Approximately eight billion tonnes of dimethylsulfoniopropionate (DMSP) is produced by phytoplankton in the Earth's surface oceans annually[9]. However, surface sediment from saltmarsh ponds, an estuary and the deep ocean (with high pressures and no light) contained levels of DMSP (5–128 nmol g^{-1}) that were up to three orders of magnitude higher than the overlying seawater (0.01–0.70 nmol ml^{-1}; Fig. 1a,b, Supplementary Tables 1a and 2), a phenomenon that has been previously observed[10,11]. The concentration of DMSP decreased with sediment depth, being much lower in anoxic sediment, but even in anoxic sediments the concentration of DMSP was approximately an order of magnitude higher than in the overlying seawater (Supplementary Table 1a). This study focused on DMSP synthesis in coastal surface sediments, where DMSP concentrations were highest. The DMSP-producing cordgrass *Spartina* is proposed to be the major source of DMSP and DMS in many saltmarshes[12,13]. Indeed, high levels of DMSP were found in *Spartina anglica* roots and leaves around the sampled ponds, and the highest levels of sediment DMSP were detected adjacent to this cordgrass (Supplementary Fig. 1a,b). However, *S. anglica* rhizosphere and phyllosphere samples contained bacteria with the genetic potential to synthesize DMSP (Supplementary Table 3), and we cannot dismiss the possible contribution of bacteria to DMSP levels found in *S. anglica* and/or the surrounding environment. Furthermore, surface sediment DMSP concentrations stabilize approximately 20 cm away from the *Spartina* (Supplementary Fig. 1b). Yarmouth Estuary, samples from which also had high levels of DMSP (Supplementary Table 1a), lacked *Spartina* and was populated with *Aster tripolium*. *A. tripolium* is a halophyte not previously known to accumulate DMSP, but which was found to contain DMSP at much lower levels than *S. anglica* (Supplementary Fig. 1a). As with DMSP standing-stock concentrations, the rates of DMSP and DMS synthesis were much higher in surface sediment than the overlying water samples (Table 1, Supplementary Fig. 2). These data suggest that a sizeable amount of DMSP in the sediment may result from microbial biosynthesis, rather than solely from sinking particles or DMSP-producing plants. We propose that surface coastal and marine sediments in general, which cover more than 70% of the Earth's surface[14], are highly active environments for microbial biosynthesis and catabolism of DMSP, generating the climate-active gas DMS.

Microbial community analysis was performed on surface sediment samples obtained from Stiffkey saltmarsh to identify potential DMSP producers. The community was dominated by bacteria (~91% of 16S ribosomal RNA gene sequences; Supplementary Fig. 4), of which around $2.3 \pm 0.6\%$ belonged to genera that include species containing *dsyB*—a reporter gene for bacterial DMSP synthesis[8] (Supplementary Figs. 5 and 6, Supplementary Tables 4 and 5). Furthermore, metagenomic analysis predicted that approximately 1% of bacteria contain *dsyB*, spanning a range of functional methylthiohydroxybutyrate (MTHB) methyltransferases[8] (Supplementary Fig. 7). This abundance was higher than

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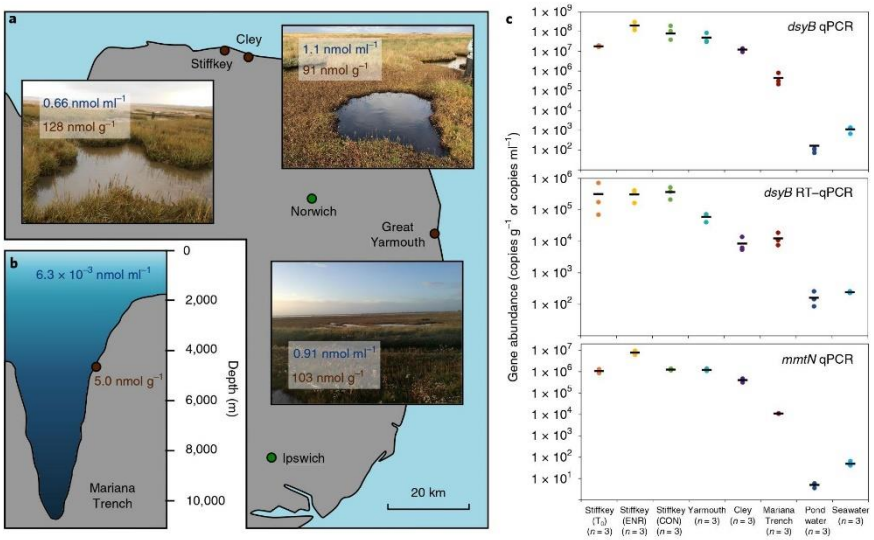

Fig. 1 | DMSP synthesis in tested marine sediments. a,**b**, The mean standing stock concentration of DMSP in surface sediment (brown) and the overlying water (blue) from (**a**) two saltmarshes (Stiffkey and Cley) and an estuary (Yarmouth; $n = 3$ biologically independent samples), and (**b**) the surface seawater (blue) and 4,500 m deep surface sediment (brown) from the Challenger Deep of the Mariana trench ($n = 3$ biologically independent samples). **c**, Analysis of DNA (quantitative PCR (qPCR)) and mRNA (qPCR with reverse transcription (RT-qPCR)) isolated from natural sediment samples obtained from Stiffkey saltmarsh at time (T_0) and after incubation (control (CON) and enriched (ENR) for DMSP production); Yarmouth Estuary sediment, Cley saltmarsh sediment and surface sediment samples from the Mariana Trench at a depth of 4,500 m; and pond water samples from Stiffkey saltmarsh and coastal seawater samples from Great Yarmouth. qPCR was performed using degenerate primers designed to target the DMSP synthesis genes *dsyB* and *mmtN*. The black line represents the mean of $n = 3$ independent samples.

Table 1 | Rates of DMSP synthesis and DMS production after 24 h using ^{3}H-methionine, determined from saltmarsh and estuary samples from north Norfolk

Sampling site	Rate of DMSP synthesis (fmol DMSP g^{-1} min^{-1} or ml^{-1} min^{-1})	pmol DMSP m^{-2} h^{-1}	pmol DMS g^{-1} captured after 24 h
Stiffkey			
Sediment	263	158	1.89
Water	0.57		
Yarmouth			
Sediment	135	81.5	0.04
Water	2.27		
Cley			
Sediment	145	85.8	1.89
Water	1.13		

most DMSP lyases (enzymes that cleave DMSP, releasing DMS) apart from the genes encoding DddD, DddL and DddP (present in 1.1%, 4.8% and 6.6% of bacteria, respectively; Supplementary Table 7), which are likely important DMS-producing enzymes in these sediments. Eukaryotic plastid 16S rRNA genes, predominantly from diatoms, represented around 9% of the community sequences (Supplementary Fig. 4). *Asterionellopsis*, a member of the Fragilariophyceae family with low or undetectable levels of intracellular DMSP[15], was the most abundant diatom (~6% of 16S rRNA gene community data (Supplementary Fig. 5)). A 3 µm diameter chain-forming *Asterionellopsis glacialis* (strain PR1) isolated from Stiffkey sediment, with 99% 16S rRNA gene identity to the dominant *Asterionellopsis* in the amplicon data, produced low levels of intracellular DMSP (0.21 mM; Supplementary Fig. 8). No DMSP synthesis genes have been identified in the Fragilariophyceae family of diatoms, but in Stiffkey metagenomes the eukaryotic DMSP synthesis gene *DSYB*[16] was approximately 13-fold less abundant than *dsyB* (Supplementary Table 7). The plastid 16S rRNA gene sequences of other DMSP-producing eukaryotes were detected at very low levels, including *Phaeodactylum* (0.4%) and *Thalassiosira* (0.3%). Given the abundance of DMSP-producing diatoms and bacteria in Stiffkey sediments, both are probably important DMSP producers in such photic marine environments.

Incubation experiments were conducted to enrich for and isolate DMSP-producing bacteria from sediment samples obtained from Stiffkey. DMSP production in enriched sediment slurries was enhanced by incubation in enrichment medium with increased salinity, addition of MTHB and reduced nitrogen levels (conditions that enhance the synthesis of DMSP in *Labrenzia*[14]). Over 14 d, DMSP levels were consistently highest (day 4 onwards) in microbial particulates from enriched samples (Supplementary Fig. 9). The proportion of DMSP-producing bacterial isolates (Supplementary Table 8) in the enriched sediment increased to 71% from 25% in natural sediment. This supports these incubation experiments as an effective method to enrich for DMSP-producing bacteria.

Enriched and control microbial community profiles greatly differed from those in natural sediment, probably due to the addition

of medium, mixed carbon source and/or other differences from the natural conditions (such as temperature and dissolved oxygen) during the incubation (Supplementary Figs. 4, 5, 7 and 10). Importantly, the enriched microbial community had several clear genus-level differences compared with the natural and control samples. The abundance of the known DMSP producers *Oceanicola* (2.1 ± 0.01%) and *Ruegeria* (4.5 ± 0.1%) was significantly increased in the enriched samples (Supplementary Figs. 5 and 10), alongside genera of DMSP-producing alphaproteobacterial and gammaproteobacterial isolates from this study, such as *Marinobacter* (3.2 ± 0.4%), *Novosphingobium* (4.7 ± 0.9%) and *Alteromonas* (20.7 ± 2.4%). Bacteria of these three genera—which made up around 0.6% of the natural sediment community and lacked *dsyB* in their available genomes—likely contributed to the enhanced levels of DMSP that we observed in enriched samples (Supplementary Fig. 9). The abundance of *dsyB*-containing bacterial genera (11.7% and 10.5%; Supplementary Table 5), the *dsyB* gene (determined by qPCR and metagenomics; Fig. 1c, Supplementary Table 7) and *dsyB* transcripts (Fig. 1c) showed no significant differences between control and enriched samples. However, the diversity of DsyB varied somewhat between the enriched and the control samples (Supplementary Fig. 6). It is possible that bacteria with the more abundant DsyB variants in the enriched samples may contribute to the higher DMSP levels observed, for example, by producing higher intracellular DMSP concentrations. Alternatively, there may be additional unknown genes and/or pathways associated with the synthesis of DMSP that contributed to the enhanced levels of DMSP observed.

Novosphingobium sp. BW1 was used to investigate *dsyB*-independent DMSP production pathways. Of the known DMSP synthesis pathway intermediates[17] (Fig. 2a), DMSP production by BW1 was significantly enhanced by adding methionine (Met)—the universal DMSP precursor—and *S*-methyl-methionine (SMM), a common plant metabolite[18,19] and intermediate of the methylation pathway in DMSP-producing plants, including *Spartina*[20] (Fig. 2b). Met (0.90 ± 0.01 mM) and another intermediate in this pathway, DMSP-amine (0.13 ± 0.02 mM), were detected in BW1 cell extracts by high-performance liquid chromatography (HPLC; Supplementary Fig. 11) and SMM was detected by liquid chromatography–mass spectrometry (LC–MS), further supporting the methylation pathway as the probable pathway for the synthesis of DMSP in BW1. Addition of DMSP-amine did not enhance DMSP production, possibly due to the ability of BW1 to import DMSP-amine, or because DMSP-amine may not induce the expression of DMSP synthesis genes. BW1 cell extracts had *S*-adenosyl-Met (SAM)-dependent Met-methyltransferase (MMT) activity, converting Met to SMM (3.6 μmol min^{-1} μg $protein^{-1}$). Although some bacteria catabolize SMM[19,21], none have previously been shown to possess MMT activity. Addition of 4-methylthio-2-oxobutyrate (MTOB) also enhanced DMSP production in BW1 (2.5-fold), but to a lesser extent than Met or SMM (7- and 13-fold, respectively), perhaps indicating that BW1 has an active Met salvage pathway that generates Met from MTOB[22].

By screening a BW1 genomic library, we identified a gene termed *mmtN* that confers MMT activity (EC2.1.1.12; Supplementary Table 8, Supplementary Fig. 12). Purified MmtN showed SAM-dependent MMT activity but did not methylate related compounds, including methylmercaptopropionate (MMPA), glycine and MTHB (Supplementary Figs. 13a and 14). MmtN homologues (≥54% amino acid identity) exist in many marine alphaproteobacteria, one gammaproteobacterium and some actinobacteria, representatives of which produced DMSP, and whose *mmtN*-like genes were cloned and functionally ratified (Fig. 3, Supplementary Fig. 12, Supplementary Table 8). A recent biochemical study showed that MmtN from *Streptomyces mobaraensis* and *Rhodovulum* sp. P5 had MMT activity[23], with kinetic parameter (K_M) values comparable to those reported here for *Novosphingobium* MmtN (Supplementary Fig. 14).

Thus, *mmtN*—like *dsyB*[8] and *DSYB*[16]—is another robust reporter gene for the potential of an organism to synthesize DMSP. Bacteria containing *mmtN* were less abundant than those with *dsyB* in tested seawater and sediment samples (Fig. 1c, Supplementary Tables 5 and 7). However, the abundance of *mmtN*-containing bacteria was higher in the enriched incubation samples compared with the control samples, suggesting that MmtN-dependent DMSP production may be a considerable contributor to the increased levels of DMSP seen under the enrichment conditions (Fig. 1c, Supplementary Tables 5 and 7).

The *mmtN* gene is required for the synthesis of DMSP in *Thalassospira profundimaris*, given that an *mmtN*$^-$ mutant did not produce DMSP, and function was restored by complementation with cloned *mmtN* (Fig. 2c, Supplementary Table 8, Supplementary Fig. 13b). Further work is required to elucidate the complete MmtN-dependent DMSP synthesis pathway, which probably involves a suite of genes (two distinct types) that encode a putative aminotransferase, dehydrogenase and decarboxylase adjacent to *mmtN* in many marine bacterial genomes (Supplementary Fig. 12). Liao and Seebeck[23] found that *S. mobaraensis* candidate gene products from one such suite of genes (SMM decarboxylase, DMSP-amine aminotransferase and DMSP-aldehyde dehydrogenase; Fig. 2a) had the expected enzyme activities. We also show that a mutation in the putative DMSP-amine aminotransferase (TH2_03140), part of the second suite of genes (Supplementary Fig. 12) in *T. profundimaris*, caused a 73% reduction in the level of DMSP compared to wild-type *T. profundimaris*. This suggests that at least one of these linked genes encodes a downstream enzyme in the DMSP biosynthesis pathway in *T. profundimaris*. The *mmtN*$^-$ mutant showed no significant growth reduction or competitive disadvantage compared with the wild-type strain in response to increased salinity and/or reduced nitrogen conditions, which were known to enhance DMSP production in this bacterium (Supplementary Fig. 15). Similar results were found with a *Labrenzia dsyB*$^-$ mutant[8], which, like *T. profundimaris*, also produces the nitrogenous osmolyte glycine betaine (GBT). Indeed, the *T. profundimaris mmtN*$^-$ mutant displayed enhanced levels of GBT production compared with the wild type, suggesting that GBT and/or other osmolytes produced by these bacteria compensate for the loss of DMSP (Fig. 2c, Supplementary Fig. 13b).

MmtN proteins form a distinct group (Fig. 3), but share ≤30% identity with the N-terminal methyltransferase domain of distantly related and larger (33 kDa versus 115 kDa) plant MMT enzymes (PLN02672; Fig. 3). These plant enzymes contain an extra C-terminal aminotransferase domain (pfam00155) that is thought to have a regulatory role[24]. The differences in length, amino acids and domains between the bacterial MmtN and plant MMT enzymes are probably responsible for the approximately tenfold higher K_M values observed for the bacterial MmtN[24]. Genes encoding plant-like MMT enzymes exist in some bacterial genomes, most of which are deltaproteobacteria (Fig. 3), and four such bacteria were tested for DMSP production. Only *Pseudobacteriovorax antillogorgiicola* DSM103413 produced DMSP, albeit at low levels (Supplementary Table 8). Thus, in contrast to *mmtN*, the presence of the plant-like MMT in an organism is not a good indicator of DMSP production. Within the group containing functional MmtN proteins, we did not find monophyly among the major bacterial groups, suggesting that *mmtN* may have transferred between bacteria by horizontal gene transfer. The high level of sequence divergence between bacterial *mmtN* and plant-like MMT genes suggests that these pathways are ancient, arising independently in bacteria and plants, or possibly through ancient horizontal gene transfer.

DMSP-producing bacteria (containing DsyB and/or MmtN) are predicted by qPCR to constitute 0.1–3.6% of bacteria in the tested marine sediment samples obtained from saltmarsh ponds, an estuary and the deep ocean (Supplementary Table 9). Indeed, the percentage of DMSP producers predicted by metagenomic analysis is

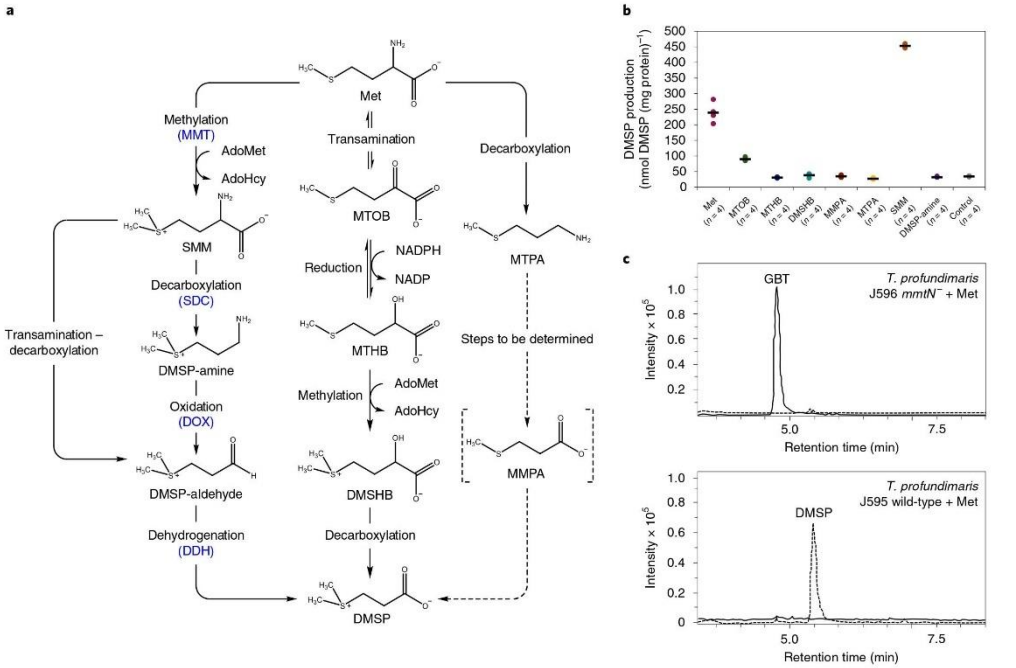

Fig. 2 | DMSP-biosynthesis pathways and bacterial production of DMSP. a, Predicted pathways for DMSP biosynthesis in higher plants and bacteria containing *mmtN* (*Spartina*, *Wollastonia*; left); macroalgae (*Ulva*, *Enteromorpha*), diatoms (*Thalassiosira*, *Melosira*), prymnesiophytes (*Emiliania*), prasinophytes (*Tetraselmis*) and algae that contain *DSYB* and bacteria that contain *dsyB (middle);* and the dinoflagellate *Crypthecodinium* (right). The dotted line represents a suggested—but as yet unconfirmed—pathway. Enzymes involved in the *Spartina* pathway are shown in blue. SDC, SMM decarboxylase; DOX, DMSP-amine oxidase; DDH, DMSP-aldehyde dehydrogenase; DMSHB, 4-dimethylsulfonio-2-hydroxybutyrate; MTPA, 3-methylthiopropylamine. In *Wollastonia* SMM is converted to DMSP-aldehyde via an unconfirmed process, not through DMSP-amine. **b**, Production of DMSP in *Novosphingobium* sp. BW1 with or without (control) pathway intermediates (0.5 mM) in marine basal medium (MBM) minimal medium (10 mM succinate as carbon source, 10 mM NH_4Cl as nitrogen source). The black line represents the mean value of $n = 4$ independent samples. Two-tailed Student's *t*-test ($P < 0.05$): Met ($P = 0.001$), SMM ($P = 0.000001$) and MTOB ($P = 0.0002$) were all significantly different compared with the control. **c**, LC–MS chromatograms for DMSP (dashed line; $m/z = 135$) and GBT (continuous line; $m/z = 118$) in *T. profundimaris* J595 wild type (contains *mmtN*), compared with the J596 *mmtN*$^-$ mutant. These experiments were repeated twice with similar results. Panel **a** adapted from ref. [8], Springer Nature Ltd.

approximately 1.1% (Supplementary Table 7), which, when applied to the estimated 1.99×10^{10} bacterial cells g^{-1} of sediment from Stiffkey surface sediment (Supplementary Table 10), suggests an abundance of around 1×10^8 DMSP-producing bacteria g^{-1} of sediment, with intracellular levels of DMSP ranging from 0.66 mM to 73 mM (Fig. 1c, Supplementary Tables 7 and 9). DMSP-producing bacteria were much less abundant in the Ocean Microbial Reference Gene Catalogue Metagenomic Database (OM-RGC[25]; predominantly surface seawater samples) and in tested coastal seawater samples compared with the surface sediment, but they still represent 0.3–0.6% of a reported 1×10^6 bacteria ml^{-1} in seawater [26] (Fig. 1c, Supplementary Tables 7 and 9). These predictions are likely underestimations, as some of the isolated bacteria lacking *dsyB* and *mmtN* were shown to produce DMSP (for example, *Marinobacter*, which represents approximately 0.5% of the natural sediment community), probably through unidentified DMSP synthesis genes and pathways. The *dsyB* gene was transcribed in all of the tested samples, but transcription was more than three orders of magnitude higher, per unit mass, in surface sediment than in pond water and surface seawater (Fig. 1c). Furthermore, *dsyB*[16] and *mmtN* transcripts are omnipresent or mostly present, respectively, at varied levels in *Tara* Oceans bacterioplankton metatranscriptome databases (Supplementary Tables 11 and 12). In seawater incubation experiments, *Novosphingobium* sp. BW1 (*mmtN*$^+$), *Pelagibaca bermudensis* (*dsyB*$^+$) and *Labrenzia* LZB033 (*dsyB*$^+$) produced DMSP and contributed to the dissolved pool, demonstrating activity even under conditions closer to the natural environment (Supplementary Fig. 16, Supplementary Table 13). These data are consistent with a large global biomass of DMSP-producing bacteria actively synthesizing DMSP in marine sediment and seawater environments. Our study shows that bacteria probably contribute to levels of DMSP in seawater environments, but further work is required to evaluate their importance. Additionally, the contribution of bacteria—and, in some cases, that of benthic algae—to total DMSP levels is likely to be far higher in marine surface sediments, which, per unit mass, are more productive than the overlying seawater. Moreover, although the content of DMSP in the anoxic saltmarsh sediment is far lower than the oxic surface layer (Supplementary Table 1a), it is still approximately five to tenfold higher than that of the overlying seawater, and is an environment

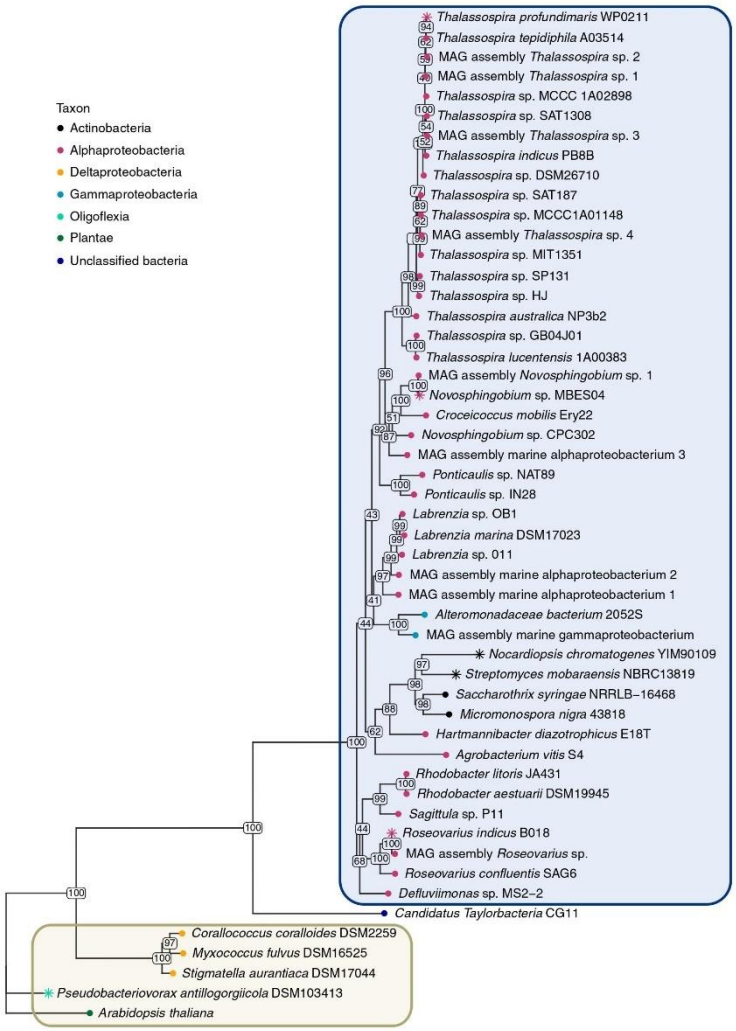

Fig. 3 | Maximum-likelihood phylogenetic tree of MmtN proteins. Species are colour-coded according to taxonomic class, as shown in the key, with proteins shown to be functional marked with an asterisk. Bootstrap support for nodes is indicated. Bacterial MmtN proteins (blue) and the larger MMT proteins (cream) are outlined. The phylogenetic tree is based on 47 protein sequences.

in which bacterial DMSP production is unstudied. This study challenges the notion that DMSP production is mainly an oxic and photic process and suggests that global models for the production of DMSP and DMS should consider marine surface sediments and bacteria as important contributors.

Methods

General scientific practices. *Chemical syntheses.* DMSP was synthesized from DMS (Sigma-Aldrich) and acrylic acid (Sigma-Aldrich) as described by Todd et al.[27]. DMSHB, DMSP-amine and SMM were synthesized as described previously[3]. Met, MTOB, MTHB and MTPA are commercially available and were obtained from Sigma-Aldrich.

Quantification of DMS/DMSP/SMM by GC. All of the gas chromatography (GC) assays involved measurement of headspace DMS, either directly produced or produced through alkaline lysis of DMSP or SMM using a flame photometric detector (Agilent 7890A GC fitted with a 7693 autosampler) and a HP-INNOWax 30 m × 0.320 mm capillary column (Agilent Technologies, J&W Scientific). Unless otherwise stated, all GC measurements for DMSP and SMM were performed using 2 ml glass vials containing 0.3 ml liquid samples and sealed with PTFE/rubber crimp caps. To quantify DMSP, 0.2 ml of overnight culture was added to a 2 ml vial,

0.1 ml 10 M NaOH was added, vials were crimped immediately, incubated at 22 °C overnight in the dark and monitored by GC. To quantify the production of SMM, 0.2 ml of culture and 0.1 ml of 10 M NaOH were sealed in glass vials and heated at 80 °C for 10 min before incubating overnight and sampling. An eight-point calibration curve was produced by alkaline lysis of DMSP and SMM standards in water. The detection limit (per 300 µl sample in 2 ml GC vial) was 0.015 nmol for DMSP and 1.5 nmol for SMM.

Detection of DMSP, GBT and SMM by LC–MS. LC–MS was used to confirm that bacteria were producing DMSP and at similar levels to those determined by GC, which ruled out the possibility that DMS detected by GC was due to compounds other than DMSP. The method used to detect DMSP and GBT was described previously by Curson et al.[3]. The detection of SMM was performed using the same method. All of the samples (15 µl) were analysed immediately after being extracted. The targeted mass transition corresponded to $[M+H]^+$ of DMSP (m/z = 135), GBT (m/z = 118) and SMM (m/z = 165) in positive mode. To confirm the presence of the compounds in the biological samples, standards were also run (10–50 µM).

Quantification of Met, DMSP and SMM by HPLC. HPLC methods were developed to determine the levels of Met, SMM and DMSP-amine as fluorescent adducts after precolumn derivatization with *ortho*-phthaldialdehyde (OPDA)[28], using mercaptoethanol rather than mercaptopropionic acid as the thiol reagent. Samples (50 µl) were mixed with 50 µl derivatization reagent (5 mg OPDA in 5 ml methanol, buffered with 35 ml 1 M potassium borate buffer, pH 10.4, mixed with 84 µl mercaptoethanol) and reacted for 3 min before injecting a 10 µl sample onto a 4.6 × 250 mm Synergi Hydro-RP (Phenomenex) column and eluted according to Caddick et al.[28]. Fluorescent adducts were detected using a Jasco FP-920 fluorescence detector set as follows: excitation, 332 nm; emission, 445 nm; bandpass filter, 18 nm; and gain, 10. Met, SMM and DMSP-amine standards yielded correlation coefficients of >0.999, >0.999 and >0.995, respectively, for five-point calibration in the range 1–20 µM. A five-point calibration for Met in the range 0.2–2 µM in seawater medium yielded a correlation coefficient >0.984. The limit of detection of Met, at 3× noise, of the chromatogram, was estimated to be approximately 0.02 µM in samples. Seawater was filtered using a 0.45 µm syringe filter. For the detection of Met dissolved in the sediment, 0.3 g sediment was diluted with 3 ml ESAW artificial seawater medium and then centrifuged at 500*g* for 20 min. The supernatant was removed and filtered using a 0.45 µm syringe filter before analysing for Met.

Quantification of DMSP by purge-and-trap analysis. Total DMSP samples of seawater and sediment were fixed with 50% (v/v) H_2SO_4 and stored at room temperature for 2 d. For seawater samples, 250 µl 50% H_2SO_4 was added directly to 25 ml of seawater and then sealed. For sediment samples, 0.5 g of sediment was initially mixed with 25 ml distilled water and then added to 250 µl 50% H_2SO_4 and sealed. For analysis, 1 ml of 10 M NaOH was injected into 5 ml of the preserved seawater sample and then sealed and incubated in the dark at 22 °C for 16 h. To measure the DMSP concentration in sediment, the samples were centrifuged at 5,000*g* and 5 ml of the supernatant of the preserved mix was used. The liberated DMS was measured using the purge-and-trap method[35]. In brief, sulfur gases were sparged from the sample with nitrogen and trapped in a loop of tubing immersed in liquid nitrogen. The trapped gases were desorbed with hot water (above 90 °C) and analysed by GC.

The DMSP content in seawater was determined by mixing 25 ml seawater with H_2SO_4 (to 0.5%). This mix was incubated at room temperature for 2 d and then 5 ml was mixed with 1 ml 10 M NaOH and incubated at 22 °C for 16 h in the dark. A modified purge-and-trap method was then used as described by Zhang et al.[30] to collect the DMS released by the sample. The samples were purged for 20 min and then compounds were detected by GC.

Site characterization. *Environmental parameters of Stiffkey saltmarsh.* The oxygen saturation was measured at the water surface, half depth (80 mm) and above the water/sediment interface (160 mm) using a Jenway 970 and a two-point calibration with filtered seawater in equilibrium with air (100% oxygen saturation) and a 2 M sodium sulfite solution (0% oxygen saturation). To measure dissolved organic carbon (DOC) and total dissolved nitrogen (TDN), we performed triplicate measurements using a Skalar Formacs CA15 analyser, with a six-point calibration. TDN represents the sum of all dissolved nitrogen-containing species (excluding dinitrogen (N_2)), including organic nitrogen species and nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and nitrous oxide (N_2O).

Nutrient analysis was performed using a Seal AA3 AutoAnalyzer at CEFAS, Lowestoft. The levels of phosphate[31], ammonium[32], and nitrate and nitrite[33] were measured as described previously.

Sampling sediment. The majority of the enrichment and isolation research described in this study was performed on surface sediment samples obtained from Stiffkey saltmarsh, UK (latitude 52.9643° N, longitude 0.9255° E; Supplementary Table 1a,b). Triplicate samples of marine sediment were collected using sterile acrylic corers at a distance of at least 40 cm from the banks of the ponds. Cores were immediately transported to the laboratory and processed on arrival. DMSP content was measured in samples (200 µl) taken from the overlying water (~15–20 cm deep), from the surface sediment layer (0.1 g in 100 µl sterile water, top 1 cm), and from three anoxic depths (5 cm, 10 cm and 15 cm). Surface sediment and water (~20–30 cm deep) from Cley saltmarsh, UK (latitude 52.9586° N, longitude 1.0473° E) and Yarmouth Estuary, UK (latitude 52.6133° N, longitude 1.7162° E) were also sampled for comparison of rates of DMSP production as well as expression of key genes involved in the synthesis of DMSP. Finally, the R.V *Dong Fang Hong 2* cruise (September 2016) sampled surface seawater and deep-sea surface sediment (4,500 m depth) from the Mariana Trench (latitude 10.4091° N, longitude 142.3569° E) using a box corer.

DMSP/DMS rate experiments using ^{3}H-methionine. Experiments to establish rates of DMS and DMSP production in surface sediment from Stiffkey, Yarmouth and Cley were undertaken as follows. For seawater samples, 10 ml seawater was added to a 30 ml universal bottle. For sediment samples, 1 g sediment and 10 ml autoclaved seawater were added to a 30 ml universal bottle. Autoclaved sediment and seawater were used as negative controls. L-[methyl-^{3}H]-methionine (85 Ci mmol^{-1}; Perkin Elmer) was added to a final concentration of 6 nM (185 kBq) and samples were incubated at 22 °C for the times specified. For DMSP measurements, at each timepoint (T = 30 min, 60 min, 90 min, 180 min and 240 min), 1 ml of seawater or sediment and seawater slurry were removed to a new 30 ml universal bottle containing 13 µl of 20% (v/v) H_2SO_4 (to prevent further bacterial activity, stabilize the DMSP and convert ^{3}H volatiles to non-volatiles) and mixed. A 1.5 ml centrifuge tube containing a DMS trap was placed in the top of the universal bottle. The DMS trap consisted of half of a 25 mm GF/F glass microfibre filter folded and soaked in 100 µl 3% (v/v) hydrogen peroxide to collect headspace DMS, as described by Slezak et al.[34]. To release the ^{3}H-DMS from any ^{3}H-DMSP present, 1 ml of 10 M NaOH was added to the seawater or slurry in the universal bottle containing the trap. The universal bottles were then sealed, shaken gently and incubated at 22 °C for 24 h to trap ^{3}H-DMS. The filters were then removed and placed in a 20 ml polyethylene vial containing 4 ml scintillant (Ecoscint A) and the vials mixed. The samples were left in the dark for 1 h before scintillation counting using a Hidex 300 SL scintillation counter. DMS measurements were performed in the same way as described for DMSP except that the DMS trap was placed directly into the universal bottle containing the seawater or sediment slurry without added NaOH. This enabled the capture of DMS that was produced and released into the headspace—through microbial cleavage of any ^{3}H-DMSP—in the trap. Filters were removed after 24 h and ^{3}H was measured, as described for the DMSP samples. Recorded values for counts min^{-1} were used to calculate the rate of DMSP and DMS production expressed as fmol g^{-1} min^{-1} or fmol ml^{-1} min^{-1} for sediment or seawater samples, respectively, and DMS production in sediment over a 24 h period was expressed as nmol g^{-1}.

Rates of DMSP or DMS production were calculated on the basis of the amount of labelled product produced (that is, ^{3}H-DMS). For the rates of DMSP production in sediment, experiments were performed with the labelled ^{3}H-Met substrate in tracer amounts (<0.6%) relative to the dissolved ambient Met concentration, estimated here to be 3.94 ± 0.89 µM for Stiffkey, 2.71 ± 0.20 µM for Cley and 1.04 ± 0.88 µM for Yarmouth[35]. The rate derived from the labelled product was then multiplied according to the factor of the dissolved ambient Met concentration relative to the concentration of added labelled ^{3}H-Met (6 nM). For the rates of DMSP production measured in seawater obtained at Stiffkey, the dissolved ambient Met concentration was 0.34 ± 0.06 µM and calculations were performed as described for DMSP in sediment to correct by the factor of dissolved ambient Met relative to labelled ^{3}H-Met added. This value for the concentration of dissolved ambient Met in seawater was used for all of the sites. The values were converted to pmol DMSP m^{-2} h^{-1} and pmol DMSP cm^{-3} h^{-1} for sediment and seawater, respectively, by normalizing wet to dry sediment using a factor of 0.5 g cm^{-3}, which was determined from weight and drying measurements[36] on sediments comparable to those of Stiffkey. Finally, it was assumed that this type of active oxic sediment makes up the top 1 cm of sediment, and we converted rates cm^{-3} to rates m^{-2}.

For measurements of DMS produced from dissolved ambient Met over 24 h in sediment, we calculated values from the labelled ^{3}H-DMS that was produced. As with experiments for rates of DMSP production in sediment described above, labelled ^{3}H-Met was used as substrate in tracer amounts (<0.6%). The amount of labelled ^{3}H-DMS produced was corrected by the factor of the dissolved ambient Met concentration in sediment at each location (see above) relative to the added labelled ^{3}H-DMSP concentration (6 nM).

These estimations of rates of DMSP and DMS production were performed under laboratory conditions that do not consider the ambient Met already within cells, thus, we advise caution in their extrapolation beyond this level.

Analysis of the rate of DMSP cleavage. Experiments to approximate the rate of DMSP catabolism, generating DMS, in surface sediment and overlying pond or seawater from Stiffkey, Yarmouth and Cley were performed as follows. For pond or seawater samples, 10 ml samples were added to 140 ml serum vials in triplicate. For sediment samples, 1 g sediment and 10 ml autoclaved seawater was added to a 140 ml serum vial in triplicate. DMSP was added to a final concentration of 0.1 mM alongside controls with no DMSP, and vials were immediately crimp-sealed. Headspace concentrations of DMS were measured at T = 0 min, 30 min, 60 min,

90 min and 120 min by GC (as described above) using manual injections. These measurements were used to calculate the rate of DMS production, expressed as nmol g^{-1} min^{-1} or nmol ml^{-1} min^{-1} for sediment or seawater samples, respectively.

Analysis of DMSP in S. anglica, the surrounding surface sediment and *A. tripolium*. Plant and sediment samples were obtained during low tide from ponds in Stiffkey and Cley saltmarsh and from Yarmouth Estuary. *A. tripolium* and *S. anglica* plants were carefully uprooted and placed into sterile plastic bags. Surface sediment from Stiffkey was sampled as described above; samples were collected every 10cm along a 100 cm transect, moving away from *Spartina*. Plant material was washed to remove sediment and then separated into different tissue types (roots and leaves for *S. anglica* and stems and leaves for *A. tripolium*) using ethanol sterilized scissors and tweezers. The phyllosphere and rhizosphere of *S. anglica* were sampled by washing 10 g leaves and 5 g roots in 10 ml sterile water with vortexing for 5 min, and repeating five times. The five washates from these samples were centrifuged for 10 min at 15,000*g* and DNA was extracted from the pellets. This DNA was used as a template for qPCR analysis to test for the presence and abundance of *dsyB* and *mmtN*, and the values from each of the five washates were pooled to give the total gene abundance in the phyllosphere and rhizosphere, normalized to the weight of plant tissue washed (Supplementary Table 3).

Between 1 g and 5 g (fresh weight) of tissue was ground to fine powder particles with liquid nitrogen using a pre-cooled sterile ceramic pestle and mortar. To measure DMSP content, approximately 0.1 g (fresh weight) of the ground material was added to 2 ml glass GC vials, 300 µl 10 M NaOH was immediately added, and the vials were sealed with 11 mm crimp caps with rubber/PTFE septa and mixed. For the transect samples, 10–20 g of sediment was mixed thoroughly to ensure a homogenous sample. Replicates of approximately 0.1 g (wet weight) of this mix were placed into GC vials and mixed with 300 µl 10 M NaOH before crimp-sealing, as described above. Samples were left overnight in the dark at 22 °C before analysis by GC (see the 'Quantification of DMS/DMSP/SMM by GC' section).

Isolation of A. glacialis. To isolate the epipelic diatoms present on the surface of saltmarsh pond sediment, samples were taken by scraping the top 0.5–1 cm surface layer of the sediment. These samples were then subsampled and inoculated into 250 ml flasks containing F/2 medium (made with 0.2 µm filtered sterile pond water from Stiffkey, 32 practical salinity units (PSU)); as described previously by Guillard and Ryther[37]). Several monoclonal isolates of pennate diatoms, including *Asterionellopsis*, were established using the single-colony isolation technique described by Andersen et al.[38]. Isolates were allowed to grow for 2–3 weeks at a constant temperature of 22 °C under a 12 h:12 h light:dark photoperiod with a constant photon flux of 120 µE m^{-2} s^{-1} (QSL-100 Quantum Scalar Irradiance Meter, Biospherical Instruments) provided by Philips MASTER TL-D 58W/840 white tubes. Isolates from enriched cultures were then further purified, and unnecessary contaminating picoplankton were removed by dilution. Once purified, strains were transferred to 42-well plates and allowed to grow for approximately 2–3 weeks. Cultures were then treated with multiple rounds of antibiotic treatments (400 µg ml^{-1} streptomycin, 50 µg ml^{-1} chloramphenicol, 20 µg ml^{-1} gentamicin and 100 µg ml^{-1} ampicillin) to remove as many bacteria as possible. Clonal cultures were then transferred and up-scaled to culture flasks (Nunc EasYFlask with Filter Caps, 75 cm^2 cell culture area, Thermo Fisher Scientific) containing 20–40 ml F/2 medium (0.2 µm-filtered sterile 50:50 pond water and ESAW artificial seawater, 35 PSU). The isolate used in this study was *A. glacialis* strain PR1. To culture *A. glacialis* PR1 for the quantification of DMSP, 30 ml of stock culture (3×10^5 cells ml^{-1}) was inoculated into 200 ml F/2 medium (made with ESAW artificial seawater medium, 35 PSU) in triplicate. Growth of the culture was monitored every day by cell counting using a Zeiss Primovert inverted optical microscope and a Sedgewick-Rafter Counting Cell. Cells were collected after 24 d (in stationary phase; Supplementary Fig. 8) and assayed for DMSP as described previously[16]. The cell volume of *A. glacialis* PR1 used for intracellular DMSP calculations was 654 $µm^3$ and this was based on previous calculations as described by Naz et al.[39].

DNA extraction and PCR amplification of rRNA genes from A. glacialis PR1. PR1 cells were harvested by centrifuging 100 ml of culture containing 3.34×10^5 cells ml^{-1} for 10 min at 5,000*g*, and genomic DNA was extracted as described by Yin et al.[40], with the following modifications. Cell disruption was achieved through bead beating at 6 m s^{-1} for 60 s with a BeadBlaster 24 bead beater (Benchmark) using silica beads (Lysing Matrix E, MP Biomedicals) in 60 µl of 10% (w/v) sodium dodecyl sulfate. After cell disruption, 6 µl of proteinase K (10 mg ml^{-1}) was added to the sample and incubated for 20 min at 65 °C; the sample was then centrifuged at 15,000*g* for 10 min. Nucleic acid extracts were precipitated in an equal volume of cold isopropanol and washed with 800 µl cold 75% ethanol, and the pellets were dissolved in 100 µl nuclease-free water and stored at −80 °C. The 16S and 18S rRNA genes were PCR amplified using the primers 8F and 1492R, and Euk_A and Euk_B, respectively. PCR was performed and the products were cloned into pGEM-T easy (Promega), sequenced and analysed (see the 'In vivo and in vitro genetic manipulations' section).

Culture-independent work. *Enrichment to enhance DMSP production in sediment from Stiffkey*. Microcosm experiments were set up to increase the production of DMSP and the abundance of organisms that produce DMSP in sediment from Stiffkey saltmarsh. Microcosms consisted of 2 g of surface sediment slurries in 30 ml MBM media of varying compositions, including a control with MBM (35 PSU, 10 mM NH_4Cl), high salinity (50 PSU), low nitrogen (0.5 mM NH_4Cl), additional MTHB (0.1 mM) or a combination of all three conditions (50 PSU, 0.5 mM NH_4Cl, 0.1 mM MTHB). Samples were incubated at 28 °C for 7 d before the content of DMSP was quantified (Supplementary Fig. 9). This experiment was scaled up for molecular microbial ecology work using 3 g sediment and 45 ml MBM with either the combined conditions (enriched media; 50 PSU, 0.5 mM NH_4Cl, 0.1 mM MTHB) or the control MBM (35 PSU, 10 mM NH_4Cl). Sediment slurries were incubated at 28 °C for 14 d. Samples were taken at regular time points, centrifuged, and the DMSP content was determined in the particulate and cell-free medium (Supplementary Fig. 9). All of these experiments were performed in triplicate.

Extraction and purification of DNA and RNA. DNA and RNA were extracted from all of the marine sediment samples at T_0 and from the sediment that was incubated for 14 d (enriched and control samples, see above) following the protocol described by Carrión et al.[41]. Samples were stored at −80 °C and RNA was purified separately (see below).

Degenerate primer design. To design degenerate primers that targeted the *dsyB* gene, 24 DsyB sequences available from GenBank were aligned using the ARB[42] project program to identify conserved amino acid positions. Two non-DsyB sequences with a cut-off value below the value used previously[8,16] were also included in the alignment to guide specific amplification of *dsyB* by the degenerate primers (Supplementary Fig. 17a). Various sets of primers with a degeneracy of ≤5 bp spanning different regions of *dsyB* were designed manually. Several different primer combinations were tested against genomic DNA from positive and negative control strains (Supplementary Table 15). The primer pair dsyB_deg1F and dsyB_deg2R (Supplementary Table 17) yielded a 246 bp fragment of genomic DNA from all of the positive control strains tested, with no non-specific bands; furthermore, no amplification bands at that size were obtained from any of the negative control strains (Supplementary Fig. 17b). Optimization of PCR conditions with these primers included annealing temperatures ranging from 60 °C to 65 °C, extension times from 15 s to 60 s and from 30 to 40 cycles. The most specific amplification was obtained with an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, an annealing step of 61 °C for 15 s and an elongation step of 72 °C for 15 s, and a final extension step of 72 °C for 5 min.

Degenerate primers for *mmtN* were designed following the same principles as described above, using the *j*-CODEHOP PCR primer design program[43] with 20 MmtN sequences from GenBank and one MMT sequence (primers were designed to amplify bacterial *mmtN*; Supplementary Fig. 17c). The primers mmtN_degF and mmtN_degR were selected for further analysis (Supplementary Table 17) because they yielded a product of the expected size (301 bp) from four positive control strains, showed the least number of non-specific bands and did not amplify the negative control (Supplementary Fig. 17d). PCR conditions for these primers were optimized using annealing temperatures between 50–60 °C, extension times of 20–45 s and 30–35 cycles. The final PCR program consisted of an initial denaturation step of 95 °C for 3 min, 35 cycles of 95 °C for 20 s, annealing at 54 °C for 30 s and elongation at 72 °C for 30 s, and a final extension step of 72 °C for 7 min.

qPCR and RT–qPCR. To study the abundance of *dsyB* and *mmtN* transcripts, RNA from environmental samples was purified using the RNase-free kit (Qiagen) and the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Absence of DNA in RNA samples was confirmed by PCR using primers 27F and 1492R[44] (Supplementary Table 17). Purified RNA samples were quantified using a Qubit RNA HS assay kit (Thermo Fisher Scientific). Reverse transcription of RNA was performed using approximately 100 µg purified RNA. Between 1 and 9 µl RNA was mixed with 1 µl 10 µM specific reverse primer (Supplementary Table 17) as described by Farhan Ul Haque et al[45]. The mixture was incubated for 5 min at 70 °C and cooled briefly on ice. Then, 1 µl deoxyribonucleotide triphosphates (dNTPs; 10 mM), 4 µl M-MLV 5× reaction buffer (Promega), 0.4 µl RNase Inhibitor (40 U $µl^{-1}$; Roche), 0.8 µl M-MLV reverse transcriptase (200 U $µl^{-1}$; Promega) and 3.8 µl nuclease-free water were added to the mixture. Finally, samples were incubated at 42 °C for 1 h and the resultant cDNA was stored at −20 °C until use.

qPCR and RT–qPCR assays were performed using a C1000 Thermal cycler equipped with a CFX96 Real-time PCR detection system (BioRad). qPCR reactions (20 µl) contained 2 µl of cDNA/DNA (2–10 ng for the 16S rRNA gene and 10–50 ng for *dsyB* or *mmtN*), 0.8 µl of each primer (10 µM) and 10 µl of SensiFAST SYBR Hi-ROX Kit (Bioline). The primers used for qPCR and RT–qPCR are described in Supplementary Table 17. The qPCR and RT–qPCR reactions consisted of an initial denaturation step of 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s and 55 °C (16S rRNA gene), 60 °C (*dsyB*) or 54 °C (*mmtN*) for 20 s, and 72 °C for 30 s. The specificity of qPCR and RT–qPCR reactions was determined from melting curves from 60 °C to 95 °C, followed by gel electrophoresis and construction of a clone library from DNA and/or cDNA that was isolated from environmental samples. Ratified sequences were between 77% and 100% identity at the derived

clones (Supplementary Table 16) were transferred into a spontaneous rifampicin-resistant derivative (strain J595) of *T. profundimaris* DSM17430 by tri-parental conjugation using the helper strain *E. coli* pRK2013. The *T. profundimaris* gene insertional mutants J596 (*mmtN*$^{-}$ mutant) and J597 (aminotransferase mutant) were isolated on YTSS agar containing rifampicin (J595), kanamycin (pBIO1879) and spectinomycin (pBIO1879). All of the mutants were ratified by PCR and checked for their ability to synthesize DMSP.

To confirm that the *mmtN* mutation in *mmtN*$^{-}$ mutant strain J596 (Supplementary Table 14) was responsible for the loss of DMSP production phenotype, cloned *Novosphingobium mmtN* (pBIO2282) was mobilized into J596 through tri-parental mating.

Phenotyping of T. profundimaris mutant. In cases where MBM was used as the minimal medium for the following experiments, this medium lacked any methylated sulfur DMSP pathway intermediates. To identify potential phenotypes for the mutations in *mmtN*, the J595 (wild type) and J596 (*mmtN*$^{-}$) strains were grown with varying levels of salt and nitrogen, or under different environmental conditions, as described previously[8]. The strains were tested against 35 PSU and 50 PSU for salt tolerance and 10 mM, 0.5 mM or 0.1 mM NH_4Cl for different levels of nitrogen, and growth was measured by OD_{600}. Tolerance to freezing was also tested, as described previously[8]. Competition experiments were performed in which cultures of the wild-type and mutant strains were grown to stationary phase in 35 PSU MBM (10 mM NH_4Cl), OD_{600} adjusted, mixed in equal parts (500 µl of both), and subjected to high salinity (50 PSU) and reduced nitrogen (0.5 mM). Before and after perturbation, aliquots of the mix were serially diluted and plated on MB agar. Single colonies were tested for kanamycin and spectinomycin resistance (mutant selection) to distinguish the wild-type strain from the mutant strain. All of the above experiments used three biological replicates for each condition.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The 16S rRNA gene amplicon sequencing, metagenomic data and whole-genome sequences generated in this study are publicly available from the NCBI Sequence Read Archive (BioProject: PRJNA522699).

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