

The interplay of dimethylsulfoniopropionate and glycine betaine production in marine cyanobacteria

By

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I, Keanu Stuart Walsham, 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.

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Abstract

Dimethylsulfoniopropionate (DMSP) is a key chemical in the global sulfur cycle and the main source of dimethylsulfide (DMS), a global cooling gas. DMSP is hypothesized to have several stress protection roles and is accumulated by a broad range of organisms. Glycine betaine (GB), a nitrogenous chemical found in every domain of life, has a similar role. Production of DMSP in cyanobacteria, an abundant phylum of oxygenic photosynthetic prokaryotes found in nearly environment on Earth, has only been investigated in a few species and the enzymes involved in its production have not been identified. In contrast, GB synthesis in cyanobacteria has been more thoroughly investigated and the proteins involved in this process have been elucidated. In this study, we identified a novel MTHB SAM-dependent methyltransferase, termed DsyC, which is found in many cyanobacteria including *Prochlorococcus* spp., *Synechococcus* spp. and *Trichodesmium erythraeum* IMS101. Interestingly, this protein also methylates intermediates of the GB methylation pathway and HB methylation pathway, demonstrating its role as a multifunctional DMSP-linked methyltransferase. Axenic *Synechococcus* spp and *Prochlorococcus* spp. were shown to produce DMSP but there was no relationship between DMSP and GB production under nitrogen limitation. This suggests that marine cyanobacterial DMSP production could be a major contributor to the global pool. Additionally, DMSP production was seen in species not containing DsyC, suggesting that other uncharacterised methyltransferases may catalyse this reaction. Investigation of an intertidal microbial mat demonstrated the presence of DsyC containing species, including cyanobacteria such as *Coleofasciculus chthonoplastes* and *Lyngbya aestuarii*. Together with algae containing another novel MTHB SAM-dependent methyltransferase, DSYU, this environment had between 400 – 700 μM DMSP g^{-1} sediment. Given the abundance of global saltmarshes, many dominated by cyanobacteria and algae, this suggests that these ecosystems could also be major contributors to DMSP production. Together, these results demonstrate the importance of DsyC in DMSP synthesis, the role cyanobacteria play in global production of DMSP and in modulating the Earth's climate.

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Author's Declaration

The research described in this thesis was conducted at the University of East Anglia between October 2019 and September 2023 under the supervision of Dr David Lea-Smith and Prof Jonathan Todd from the University of East Anglia. No part of this thesis has previously been submitted for a degree at this or any other academic institution. All data described here are original and were obtained by the author unless except in the cases outlined below and attributed in the text.

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Abbreviations

pH- Potential of Hydrogen	ATP- Adenosine Triphosphate
DMSP- Dimethylsulfoniopropionate	ABC- ATP-Binding Cassette
DMS- Dimethylsulfide	BCCT- Betaine Choline Carnitine Transporter
Pg- Petagram	TMP- Transmembrane Protein
MeSH- Methanethiol	NBP- Nucleotide Binding Protein
TCA- Tri-citric acid	ADP- Adenosine Diphosphate
MMPA- 3-methiolpropionate	GB- Glycine Betaine
MTPA- Methylthiopropionate	SAM- S-Adenosyl Methionine
MTOB- 4-methylthio-2-oxobutyrate	SAH- S-Adenosyl-L-Homocysteine
MTHB- 4-methylthio-2-hydroxybutyrate	GSMT- Glycine Sarcosine Methyltransferase
DMSHB- 4-dimethylsulfonio-2-hydroxy- butyrate	SDMT- Sarcosine Dimethylglycine Methyltransferase
SMM- S-methylmethionine	DMT- Dimethylglycine methyltransferase
DMSP-amine- Dimethylsulfoniopropionate-amine	NAD- Nicotinamide adenine dinucleotide
DMSP-aldehyde- Dimethylsulfoniopropionate-aldehyde	NADH- Nicotinamide adenine dinucleotide
MMPA-CoA- 3- methylmercaptopropionyl-CoA	NADP- Nicotinamide adenine dinucleotide phosphate
MTA-CoA- 3-methylthioacryloyl-CoA	Mya- Million years ago
HSCoA- Coenzyme A	
CO2- Carbon dioxide	

NOE- Neoproterozoic Oxygenation Event

GG- Glucosylglycerol

GGA- Glycosylglycerate

PSU- Percent Salinity Units

UV- UltraViolet

fg- femtogram

RuBP- Ribulose 1,5-bisphosphate

ppt- parts per thousand

LB- Luria broth

°C- Degree Celsius

IPTG- Isopropyl B-D-1
Thiogalactopyranoside

μE- microeinsteins

ESW- Enriched Sea Water

DMSO- Dimethylsulfoxide

μL- microliters

min- minutes

s- seconds

DTT- Dithiothreitol

EDTA- Ethylenediaminetetraacetic acid

TEMED- Tetramethylethylenediamine

SDS-PAGE- sodium dodecyl sulfate–
polyacrylamide gel electrophoresis

GC- Gas Chromatography

¹H NMR- Proton Nuclear Magnetic Resonance

HPLC- High Performance Liquid Chromatography

BLAST- Basic Local Alignment Search Tool

SSN- Sequence Similarity Network

PCR- Polymerase Chain Reaction

D₂O- Deuterium oxide

BSA- Bovine Serum Albumin

CTAB- Cetyltrimethylammonium bromide

L-Met- L-Methionine

Tg- Teragram

ROS- Reactive Oxygen Species

NCBI- National Centre for
Biotechnology Information

bp- Basepairs

KDa- Kilo daltons

Kb- Kilo basepairs

h- hour

mM- Millimolar

M- Molar

μM- Micromolar

L- Litre

mL- Millilitre

LLII/III/IV- Low Light Clade II/III/IV

HLIII/IV- High Light Clade III/IV

µg- Microgram

pmol- Picomolar

nmol- Nanomolar

HB- Homoserine Betaine

GMB- Glutamate Betaine

FPD- Flame Photometric Detector

ppm- Parts per million

TMS- Tropical Marine Salts

Mha- Million hectares

km- Kilometre

cm- Centimetre

g chl a- Grams chlorophyll a

Chapter 1 - Introduction

1.1 Role of Osmolytes

Water is regarded as being one of, if not the most, important molecule to life. However, water is not a consistent environment and an organism's ability to cope with changes to its external or internal water environment is crucial to its survival (1). Life adapts to these changing conditions by employing the use of osmolytes.

Osmolytes are small, organic molecules which are found almost universally in cells and play a critical role in maintaining cellular osmotic balance. Sometimes called osmoprotectants, these solutes carry no net physiological pH charge by having both a positive and negative charge and are non-toxic at high concentrations (2). Osmoprotectants raise the osmotic pressure in the cytoplasm and at the same time, stabilise proteins and membranes under unfavourable temperature or salinity conditions (2).

Synthesis of organic osmolytes convergently evolved among bacteria, plants, and animals (3). Osmolytes come in many forms, such as polyhydric alcohols, free amino acids and their derivatives, and combinations of methylamines (3). Osmolytes are compatible solutes since they do not interfere with cellular macromolecules and have broad interchangeability with one another (1). In addition to their roles in protecting against hyperosmotic or hypoosmotic stress, osmoprotectants have other unique protective properties such as antioxidant activity, providing redox balance, and detoxifying sulfides (1). Furthermore, osmolytes stabilise macromolecules and counteract perturbants in non-interchangeable ways by aiding protein folding and helping ligand binding (1). Their other protective roles include combatting hydrostatic pressure in deep sea organisms, stabilising membranes at freezing temperatures and protecting proteins from denaturation due to high temperatures (1).

The concentration of osmoprotectants typically increases during periods of stress (2), since they counteract negative environmental effects. For example, under osmotic stress the cell is influenced by impermeable solute concentrations that affect the equilibrium of the solution within the cell, which means water can move into and out of

the cell through the lipid bilayer or aquaporin channels to achieve equilibrium. The induced water flow across the membrane alters cellular volume which results in cellular responses to maintain homeostasis of the cells water content. When the solute concentration inside the cell is higher than outside this results in water molecules flowing inwards which causes the cell to swell and increases turgor pressure (**Fig. 1.1C**). On the other hand, when the solute concentration is higher outside of the cell then water will migrate outwards which shrinks and shocks the cell (4) (**Fig. 1.1B**).

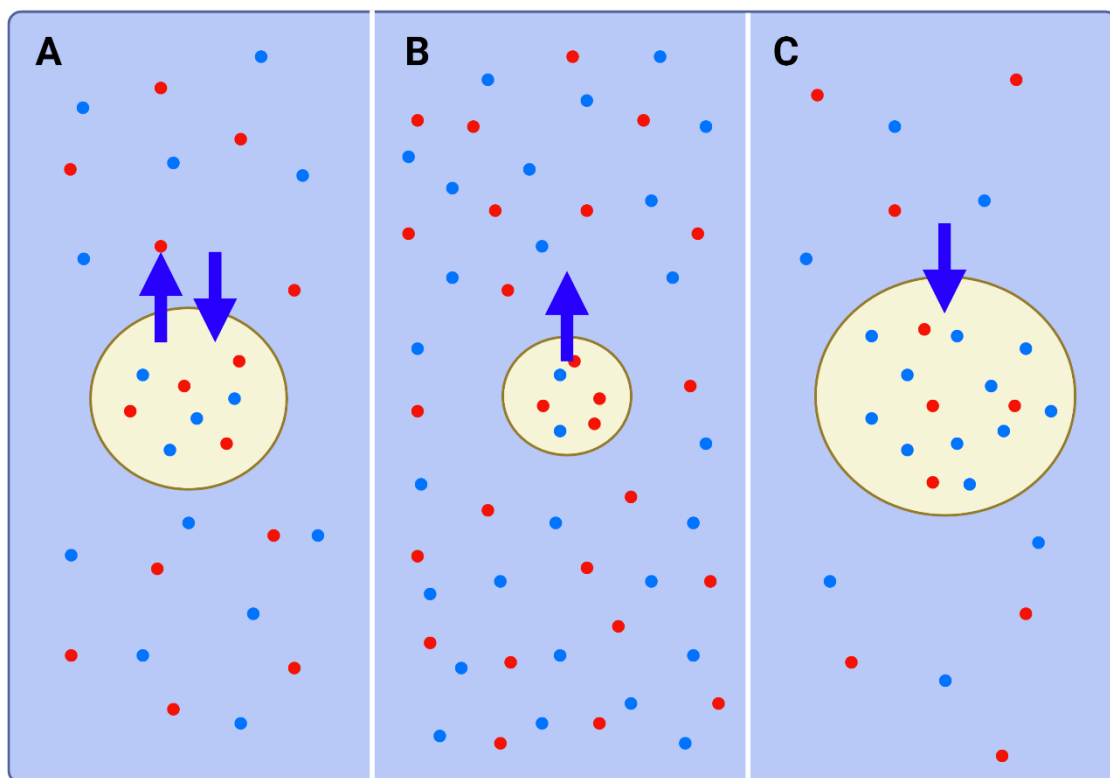


Fig. 1.1 Schematic displaying osmotic shock on cells experiencing solute imbalance.

A) Cell in equilibrium when the internal solute concentration matches the external solute concentration. **B)** Water leaves the cell when the external solute concentration is greater than the internal solute concentration, thus shrinking the cell. **C)** Water enters the cell when the external solute concentration is less than the internal solute concentration, thus expanding the cell.

To acquire osmolytes, the organism must either transport the osmolyte into their cytoplasm via transmembrane importers or synthesise the solutes via internal metabolic processes (5). Typically, lower salt tolerant species tend to use

disaccharides as organic osmolytes. Conversely, halophilic, and halotolerant species generally use sugar polyols and nitrogen containing solutes (6).

1.2 Dimethylsulfoniopropionate

Dimethylsulfoniopropionate (DMSP) is one of Earth's most abundant organosulfur molecules. It functions as a signalling molecule, a key nutrient for marine microorganisms and is a major precursor to the environmentally important gas dimethylsulfide (DMS) (7). Furthermore, DMSP is a zwitterionic compound, having a positive and negative charge. This unique chemical composition allows DMSP to function as a compatible solute, thus helping organisms maintain their internal water balance under a variety of environmental conditions (7).

DMSP is predominantly produced by marine organisms, and partially by some terrestrial organisms (8). These include phytoplankton (9), macroalgae (10), bacteria (11), plants (12) and some animals (13). The internal concentration of DMSP and rates of production vary wildly between organisms, and for a long period it was believed that eukaryotic algae were the main producers. However, in recent years studies have demonstrated that heterotrophic marine bacteria are significant contributors to global DMSP output, with the combined production from eukaryotes and prokaryotes totalling around two Pg of DMSP per year (14).

Since DMSP is a zwitterion, it can only be released into the environment through damaged membranes or via active transport utilising transmembrane proteins (15). Therefore, DMSP can only enter the environment through exudation, grazing or cell lysis (15). The alternative is for DMSP to be degraded into DMS by DMSP lyases, which convert DMSP into DMS and acrylate through cleavage of the N-terminus methyl group (16) or via demethylation to methanethiol (MeSH) by the DmdA enzyme (16).

DMSP released into the environment is readily taken up by chemoheterotrophic bacteria (8). Some of these bacteria degrade DMSP through to DMS and acrylate, using acrylate as a carbon source funnelled into the TCA cycle (17). However, DMSP turnover exceeds DMS production (8) as the majority of DMSP is demethylated into 3-

methiolpropionate (MMPA), which is further demethylated into 3-mercaptopropionate, releasing MeSH (8).

DMSP has numerous potential physiological roles in organisms which accumulate it under different environment conditions (18). The roles may vary between species, possibly due to where DMSP localises within the cell (19), (20). Some suggested roles include: osmoprotection (21), sulfur and carbon storage (21), herbivore deterrence (22), antioxidation (23), hydrostatic pressure protection (24) or as an intermediate in toxin production (25).

1.2.1 Biosynthesis of DMSP

In the marine environment, around two Pg of DMSP is synthesised annually (8), constituting approximately 10% of all fixed carbon (26). Despite some producers being found in terrestrial environments, DMSP is likely more abundant in the marine environment due to the higher concentration of sulfates (21). Major contributors include microalgae such as coccolithophores, dinoflagellates and diatoms (27,28), the macroalgae polysiphonia (29), corals like *Acropora* sp. (13), coastal angiosperms such as *Spartina* (30) and *Wollastonia* (12), heterotrophic bacteria such as *Labrenzia aggregata* (11) and non-marine higher plants such as sugarcane (31). Particulate levels of DMSP in marine surface water can range widely from nanomolar to micromolar concentrations, with higher concentrations observed during phytoplankton blooms (32).

There are three distinct and unique pathways in which DMSP can be synthesised *de novo* from methionine (32). The decarboxylation pathway (**Fig. 1.2A**) was discovered in the dinoflagellate, *Cryptothecodinium cohnii* (33), the transamination pathway (**Fig. 1.2B**) is found in some marine bacteria and algae (11,28) and finally the methylation pathway (**Fig. 1.2C**) is favoured by angiosperms and bacteria (18,34).

The methylation pathway was the first DMSP synthesis pathway to be elucidated (12) and has four steps. The initial step involves methylation of methionine to S-methylmethionine (SMM) via MmtN (34) or MMT (35), which are characterised Met S-methyltransferase enzymes from bacteria and plants, respectively. SMM is then

decarboxylated to DMSP-amine and this is then oxidised to DMSP-aldehyde before final dehydrogenation to DMSP (34).

The transamination pathway was identified in *Ulva intestinalis* (36) and contains the first DMSP synthesis enzyme to be characterised, a methylthiohydroxybutyrate (MTHB) methyltransferase termed DSYB. The first step of this pathway is the transamination of methionine to 4-methylthio-2-oxobutyrates (MTOB), which is then reduced to MTHB via a NADPH-linked reductase. MTHB is then methylated to 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB) via methyltransferases such as DsyB or DSYB, and finally DMSHB is decarboxylated to DMSP by a DMSHB carboxylase (11,28).

The first step involves decarboxylation of methionine to methylthiopropylamine (MTPA) via methionine decarboxylase. MTPA is then deaminated and oxidised to methylmercaptopropionic acid (MMPA) before demethylation to DMSP (33). However, the other reactions in this pathway have not been identified, nor have the enzymes catalysing the remaining steps.

All pathways involve a crucial step of methylation by S-methyltransferases which is the committing and often rate limiting step of the pathway (11). These S-methyltransferases are often used as robust reporters of DMSP synthesis (35). Other steps in the pathway are not unique to DMSP production. For example, mobilisation of the *dsyB* gene into the heterologous host *Rhizobium leguminosarum* was enough to constitute DMSP biosynthesis activity in a non-DMSP producer (11).

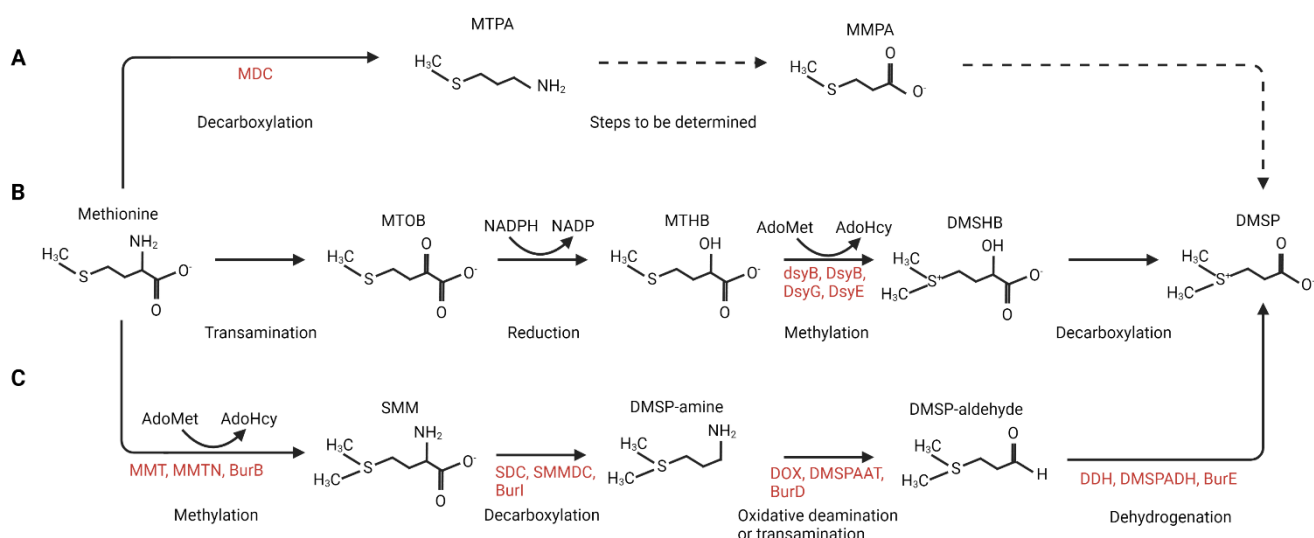


Fig. 1.2 The three pathways of DMSP synthesis from methionine. **A)** In the decarboxylation pathway, methionine is decarboxylated into MTPA which is transformed into MMPA and finally DMSP via an unknown mechanism. **B)** in the transamination pathway, methionine is transaminated into MTOB which is reduced to MTHB, that is methylated to DMSHB, which is decarboxylated to DMSP. **C)** In the methylation pathway, methionine is methylated to SMM, which is decarboxylated to DMSP-amine, that is deaminated or transaminated to DMSP-aldehyde, which is dehydrogenated to DMSP.

1.2.2 Catabolism of DMSP

The biogeochemical significance of DMSP was first suggested due to its role as a major source of DMS production (17). DMS is abundant in all marine environments and is the crucial sulfuric gas which completes the steady flow of sulfur between marine and terrestrial environments, via its ascension into the atmosphere and deposition via rainfall (8). DMS functions as the primary natural source of sulfur in the atmosphere and is readily oxidised to sulfate, sulfur dioxide, methanesulphonic acid and other products which act as cloud condensation nuclei (16). DMSP can also be converted to methanethiol (MeSH), a more reactive volatile sulfur species, which contributes little to the atmospheric flux of sulfur (37). The majority of DMSP is metabolised to MeSH, with between 2-21% of DMSP being lysed to DMS (14).

Overall, there are three different fates for DMSP sulfur. First, the production of volatile species and their ascension to the atmosphere. Second, the assimilation by microorganisms. Last, oxidation followed by release or reassimilation (37). Of these, a DMSP incubation experiment showed the largest proportion of DMSP sulfur was incorporated into protein and dissolved non-volatile compounds, with the remainder (~15%) being internalised but not further metabolised, owing to the physiological benefits of obtaining DMSP (37).

Some DMSP producers cleave their own DMSP to DMS, but the majority of DMSP is catabolised after it has entered the water column after viral lysis, zooplankton grazing or senescence of algal cells (38). DMSP then becomes available for assimilation and degradation by free living bacterioplankton or attached bacteria (38). DMSP is catabolised via two pathways, the DMSP lyase pathway and the DMSP demethylation

pathway. Species catabolise DMSP to yield either acrylate (lyase pathway) or MMPA (demethylation pathway), which enter various metabolic pathways and provide a source of energy to the organism (17).

The DMSP demethylation pathway (**Fig. 1.3A**) involves the sequential enzymatic demethylation of DMSP to produce MeSH, formaldehyde and then ultimately formate (16). Alphaproteobacteria, and those in the Roseobacter clade and SAR11, contain enzymes which are specific and efficient at catalysing the demethylation pathway (7). The key enzyme, DmdA, belongs to the glycine cleavage T-protein superfamily, catalyses the committing and first demethylation step converting DMSP to 3-methylthiopropionate (MMPA) (39). MMPA is then converted to MMPA-CoA by a MMPA-CoA ligase, termed DmdB, and then to methylthioacrylyl-CoA (MTA-CoA) via MMPA-CoA dehydrogenase, labelled DmdC. The final step is the hydration of MTA-CoA by MTA-CoA hydratase, otherwise termed DmdD or an acrylate utilisation hydratase, called AcuH (7).

DmdA is a narrowly distributed protein almost solely found in alphaproteobacteria, whereas DmdB and DmdC is found in up to 60% of surface ocean bacteria, as well as in terrestrial and other environments (37). Conversely, DmdD is absent from many marine bacteria which exhibit demethylation pathway activity, but AcuH is found in a wide range of microorganisms (7). It is worth noting that DmdD is much more efficient at catalysing hydration of MTA-CoA than AcuH, which has an additional role in metabolising acrylate derived from the lyase pathway (7).

The DMSP lyase pathway (**Fig 1.3B**) involves the enzymatic cleavage of DMSP by lyases, which yields DMS and acrylate (17). There is a high abundance of biodiversity in bacterial DMSP lyases, called 'Ddd' enzymes, which belong to three known protein superfamilies: the cupin superfamily, the M24B metallopeptidase family and the class III CoA transferase family (14). However, there is only one known eukaryotic DMSP lyase, Alma1, which has little similarity to the bacterial DMSP lyases and is a member of the aspartate racemase superfamily (14). Multiple studies have demonstrated the significant contribution individual marine phytoplankton and phytoplankton blooms, make to DMSP lyase activity after the lyases were initially discovered in *E. huxleyi* (8).

Many bacterial DMSP lyases have been discovered and all catalyse the same reaction to produce DMS and acrylate, except for DddD which produces 3-hydroxybutyrate (17,40). The similarities in function are surprising since the enzymes differ drastically in size and sequence. Of the lyase enzymes, the most abundant are DddP and DddD, with DddW, DddQ and DddL being less common. In contrast, DddY, DddK and Alma1 are not abundant in marine microorganisms (7). The formation of DMS and acrylate from DMSP is due to a β -elimination reaction. However, the lyases developed different catalytic mechanisms catalysing this reaction (7). For example, DddP implements an ion shift, where a moveable Fe binds to the carboxyl group of DMSP and anchors it into the active site. Here, two conserved residues, tryptophan and tyrosine bind to the sulfur in DMSP. This allows for the abstraction of a proton by aspartate from the alpha carbon of the DMSP carboxyl group, thus cleaving the carbon-sulfur bond and forming a double bond between the alpha and beta carbons of DMSP to produce acrylate (41). Another example is DddQ which binds DMSP to its metal cofactor, causing an oxygen on the conserved tyrosine residue to interact with the alpha carbon of DMSP. The resultant conformational change enables the abstraction of the proton from the DMSP carboxyl group by the oxygen atom of tyrosine (42).

The enzymes involved in the demethylation and cleavage pathways are hypothesised to be adapted versions of enzymes that were already contained within bacterial genomes which evolved in response to the availability of DMSP as a substrate (37). Enzymes involved within the demethylation pathway likely evolved from enzymes involved in fatty acid β -oxidation (43).

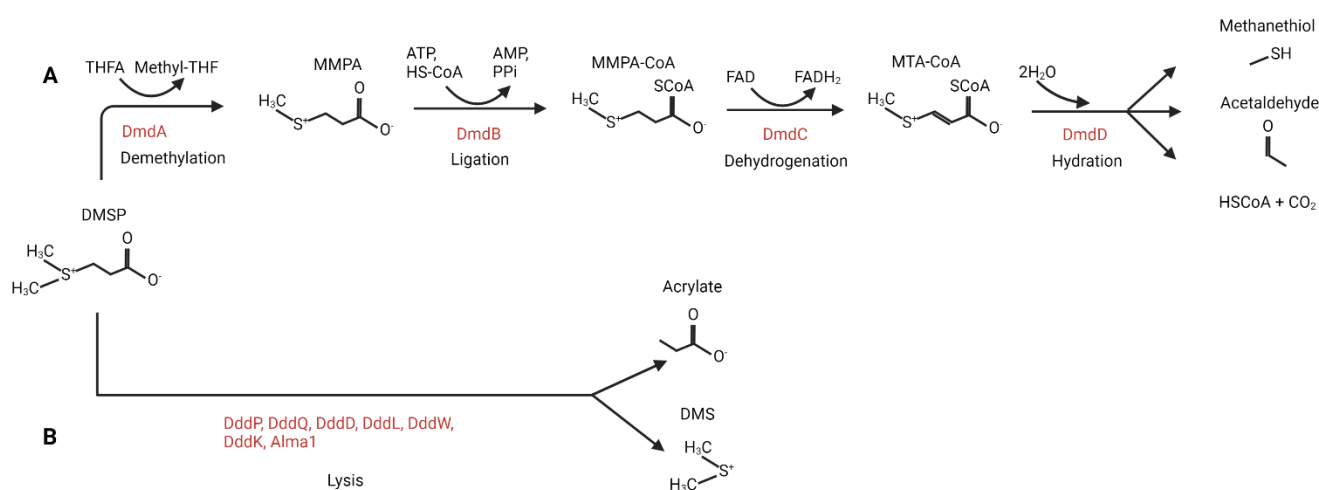


Fig. 1.3 The two pathways of DMSP catabolism. A) In the demethylation pathway, DMSP is demethylated to MMPA which is then ligated to Coenzyme-A to form MMPA-CoA, which in turn is dehydrogenated to MTA-CoA. Finally, MTA-CoA is hydrated through to MeSH, acetaldehyde, HSCoA and CO₂. **B)** In the lyase pathway, DMSP is lysed to acrylic acid and DMS in a one-step cleavage reaction.

1.2.3 Transport of DMSP

Many organisms have efficient ways of assimilating DMSP from the environment via active transport for storage or downstream metabolism (44). Furthermore, it is energetically favourable for a microorganism to take up a compatible solute, such as DMSP, over synthesis *de novo* (45).

DMSP uptake has been demonstrated in phytoplankton using double labelled ¹³C₂²H₆-DMSP (46). Similar uptake has been observed in *Roseobacter* and SAR11 clade bacteria (47), cyanobacteria including *Synechococcus* and *Prochlorococcus* (48,49), bacilli like *Bacillus subtilis* (50), enterobacteria such as *E. coli* (51), *Rhizobia* (*Ensifer meliloti*) (52) and pseudomonads like *Pseudomonas dooudoroffii* (53). Most microorganisms assimilate DMSP in addition to other sulfur sources, but in the case of SAR11 bacteria they depend on DMSP uptake for sulfur acquisition (54).

DMSP is transported via two different families of transporters, the betaine-choline-carotene-transporter (BCCT) family and the ATP binding cassette (ABC) family. The enzymology and mechanisms of action for these two systems are fundamentally different (54).

BCCTs are ubiquitous in many organisms and were first identified as glycine betaine transporters, hence their name. However, it was recently discovered that BCCTs could also transport DMSP due to the structural similarity between the two osmolytes (54). BCCT protein sequences are diverse and the proteins have various names, for example BetP in *Corynebacterium glutamicum* (54), OpuD in *B. subtilis* and BetU in *E. coli* (55). Structures of BCCT's show they are asymmetric homotrimers with a terminal domain which protrudes into the cytoplasm, where the primary binding motifs are composed of aromatic residues that are involved in cation- π -interactions (56). In terms of genomic location, the genes encoding subunits in the BCCT system in the

marine bacterium *Marinomonas* MWYL1, termed DddT, is encoded within the *dddTBCR* operon and is located next to a *dddD* DMSP lyase (54). Homologs have been found in *Sagittula stellata* E37 and *Halomonas* HTNK1. In *Halomonas* HTNK1 the *dddT* gene is clustered next to genes encoding for DMSP degradation proteins (40).

DMSP importing ABC transporters are found in many organisms. Similar to BCCTs, they do not have a unified nomenclature, with *E. coli* ABC transporter components called ProV, ProW and ProX whereas in *B. subtilis* the components are named OpuAA, OpuAB and OpuAC (54). These three components form two subunits and are found in all ABC importers and include a transmembrane protein (TMP), a nucleotide binding protein (NBP) and a substrate binding protein (SBP) (57). Two components form a heterotrimeric structure of two TMPs and two NBPs, and the SBP captures and delivers transported molecules to this structure (58,59). Afterwards, ATP binds to the NBP and hydrolyses to ADP and inorganic phosphate which releases the energy required for a conformational change to channel the substrate through the membrane (58). Like BCCT, genes encoding for ABC subunits have been found near genes encoding for DMSP lyases. For example, in *Burkholderia ambifaria* AMMD, *Rhizobium* sp. NGR234, *Hoeflea phototrophica* DFL-43 and Rhodobacterales bacterium KLH11, the *dddD* gene is found next to ABC subunit genes, which suggests the involvement of the ABC transporter in DMSP uptake (60).

1.3 Glycine Betaine

Glycine betaine (GB), much like DMSP, is a compatible osmolyte which is acquired by organisms to protect against abiotic stress (61). For example, in plants GB is accumulated in the chloroplast, where it safeguards photosystem II effectiveness under stressful conditions like high salinity or oxidative stress by reducing membrane permeability, lipid peroxidation and hydrogen peroxide concentrations (61). Structurally, GB is a zwitterionic, quaternary ammonium containing osmolyte with five carbons. It has a positively charged nitrogen atom which is bound to three methyl groups. This methylated structure means GB has the typical properties of many other organic osmolytes (62).

GB is found in nearly every domain of life. Since the initial identification of its utilisation in *Halorhodospira halochloris* (63) biosynthesis of GB has been discovered in many other organisms. Most halophilic and halotolerant bacteria can synthesise and utilize betaine as an effective osmolyte. This includes cyanobacteria and sulfur bacteria living in hyperosmotic environments (64) and some heterotrophic bacteria such as *E. coli*, *B. subtilis* and *Arthrobacter globiformis* (65,66). Most heterotrophic bacteria cannot synthesise GB but have effective GB importers to internalise environmental betaine to counter osmotic stress(67).

Some archaea can import and accumulate GB, predominantly methanogenic species (68). However, there are only a few archaea, which live in hyperosmotic areas, that can synthesise GB. The pathway for this production, along with the enzymes involved was elucidated in the halophilic archaeon, *Halorubrum* sp. 2020YC2, and differs from the biosynthetic pathways in bacteria (69).

GB synthesis pathways have also been found in some Fungi, notably *Aspergillus fumigatus*, where GB was not involved in stress protection but was used as a metabolic intermediate in the catabolism of choline and as a nutrient source (70).

1.3.1 Biosynthesis of GB

There are two pathways for GB biosynthesis, the choline oxidation pathway and the glycine methylation pathway. Unlike DMSP, GB can be synthesised from two different precursor molecules, the quaternary ammonium cation choline or the amino acid glycine (62). Animals, plants and microorganisms all utilise the choline oxidation pathway, while some microorganisms synthesise GB via the glycine methylation pathway (62).

The first GB synthesis pathway identified was the glycine methylation pathway (**Fig. 1.4A**), named after the first step of the pathway involving methylation of glycine. Every step in this pathway is a methylation step, utilising the methyl donor S-adenosyl methionine (SAM). Glycine is methylated through to sarcosine, which is methylated to dimethylglycine and finally methylated to GB (71). This pathway has been characterised in the archaebacterium *Methanohalophilus portucalensis*, the cyanobacteria *Aphanothece halophytica* and *Synechococcus* sp. WH8102, the sulfur

bacterium *Ectothiorhodospira halochloris* and the halophiles *H. halochloris* and *Actinopolyspora halophila* (72–74). Three enzymes are involved in this pathway, a glycine-sarcosine methyltransferase (GSMT), a sarcosine dimethylglycine methyltransferase (SDMT) and a dimethylglycine methyltransferase (DMT), all of which have been isolated and characterised from all of the aforementioned microorganisms (73,74).

The two-step choline oxidation pathway (**Fig. 1.4B**) uses a diverse range of enzymes. In *E. coli*, a choline dehydrogenase (CHD) utilises oxygen as an electron acceptor to catalyse both oxidation steps (75). In *B. subtilis*, this first step is catalysed by a NAD dependent type III alcohol dehydrogenase (GbsB) (65). Lastly, in *A. fumigatus* a choline oxidase (ChoAp) catalyses this first step and uses oxygen as the electron acceptor (70).

The enzyme involved in the second part of the pathway, a NAD(P)⁺-dependent betaine aldehyde dehydrogenase (BADH), is widely conserved in bacteria and fungi. BADH oxidises betaine aldehyde to GB and uses NADP⁺ as the electron acceptor (65,70,75). There is evidence in *A. globiformis* of a single enzyme which can catalyse both steps of this pathway using molecular oxygen as the oxygen acceptor, resulting in production of hydrogen peroxide **Fig. 1.4C** (66).

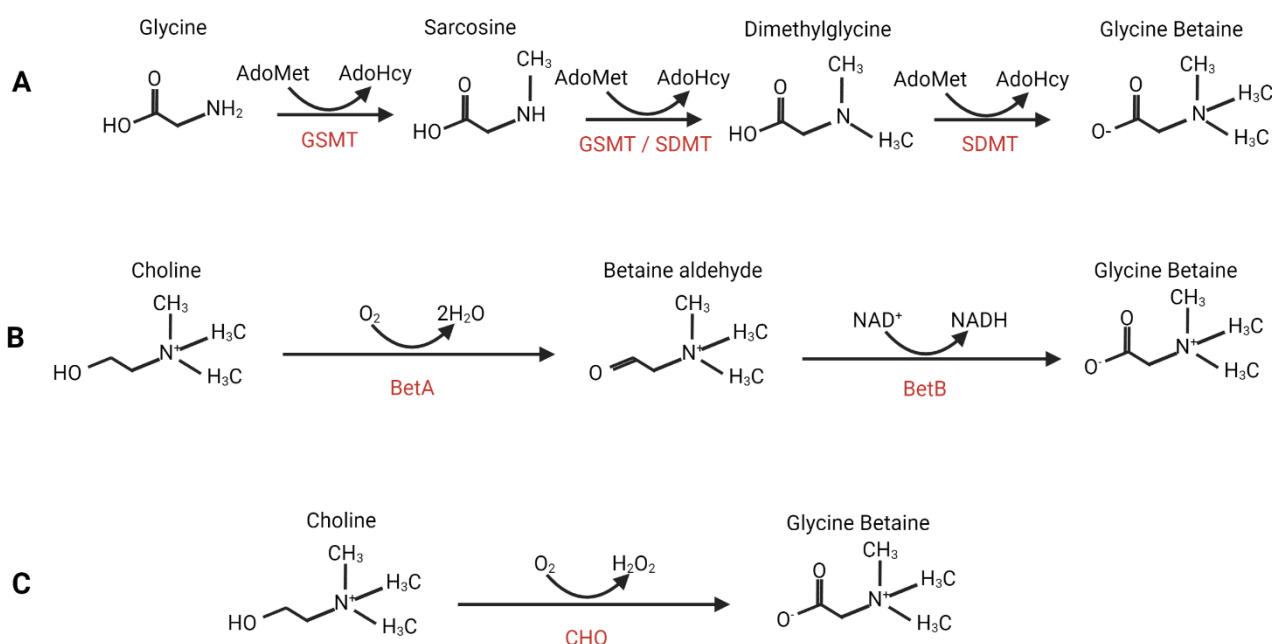


Fig. 1.4 The three pathways of glycine betaine synthesis. **A)** The glycine methylation pathway involves the stepwise methylation of glycine to sarcosine and then dimethylglycine before final methylation to glycine betaine. **B)** The choline oxidation pathway, whereby choline is oxidised to betaine aldehyde, which is reduced to glycine betaine. **C)** The one-step choline oxidation pathway, whereby choline is oxidised to glycine betaine in a one-step reaction in *Arthrobacter globiformis*.

1.3.2 Catabolism of GB

Many microbes can utilise GB as a sole carbon, nitrogen and energy source (76–78). Free GB is internalised by microbes after release by plant roots (79), microbes (80) or decaying plant and animal matter (81,82). The catabolism of GB allows organisms to obtain glycine, choline and methionine. One key product from GB catabolism is methane, which is a potent greenhouse gas. GB catabolism is estimated to account for upwards of 90% of coastal methane emissions (83). Therefore, understanding what contributes to the breakdown of GB is of key importance. There are multiple pathways by which GB can be broken down and its products utilised, which are split into anaerobic and aerobic reactions.

The GB demethylation pathway is the best understood. This involves the sequential, aerobic breakdown of GB to dimethylglycine, through to sarcosine and finally glycine via successive methyl transfer steps (84). This pathway has been studied in *Sinorhizobium* (77), *Corynebacterium* (85) and *Arthrobacter* (86) species. Various enzymes have been identified within this pathway. In *Rhizobium meliloti*, the conversion of GB to dimethylglycine is catalysed by betaine homocysteine methyltransferase (77). Dimethylglycine is demethylated to sarcosine via a dimethylglycine oxidase in *A. globiformis* (86). In *A. globiformis* and *Corynebacterium* sp. U-96, sarcosine is demethylated to glycine by a heterotetrameric sarcosine oxidase (85,86). The first complete pathway of GB demethylation was characterised in *Pseudomonas aeruginosa*. A GB demethylase, dimethylglycine demethylase and sarcosine demethylase, the last encoded by *soxBDAG*, was elucidated alongside the first bacterial transcriptional regulator of GB catabolism (84).

The other GB catabolism pathway is an anaerobic Stickland-type reaction which yields trimethylamine and acetate and was identified in *Clostridium sporogenes* (87).

Afterwards, trimethylamine is degraded to methane (87). Some marine bacteria in anoxic coastal sediments catabolise GB using this pathway although the abundance of the genes of this pathway in microbial datasets was relatively low (88). Recently, a widespread and previously uncultivated bacterial family *Candidatus* 'Betainaceae' was discovered and is prevalent in many hypoxic environments, including saltmarshes (89). This new family of bacteria may have a key role in methanogenesis from hypoxic coastal sediments (89).

1.3.3 Transport of GB

Most sequenced bacteria contain genes putatively encoding the transport machinery necessary to import GB, which are more prevalent than those encoding enzymes required to synthesize GB. Like DMSP, synthesis of GB is energetically expensive (88), providing an evolutionary incentive for organisms to import it. Like other osmolytes, GB must be actively transported into the cell, often against the concentration gradient (90). There are two families of GB transporters, the ATP-Binding Cassette (ABC) transporters and Betaine-Choline-Carnitine transporters (BCCT), which are often the same transporters for DMSP(44), and their function and mechanism of action was discussed earlier in Section 1.2.3. The majority of bacteria possess a variety of both ABC and BCCT systems to import GB although some species such as *Corynebacterium glutamicum* only contain the BCCT system (91). Following transport, GB is either used for its osmoprotective and stress protection properties or it is catabolised as an energy source (77).

GB transport has been thoroughly investigated in *P. aeruginosa*, where extracellular GB is detected through an Ara-C family transcriptional activator which leads to upregulation of genes encoding GB transport and catabolism proteins (92). Transport of GB is not only associated with osmoprotection but also with stress protection. For example, in *E. coli*, *B. subtilis*, *Listeria monocytogenes* and *Virgibacillus pantothenicus*, there is an increase in expression of genes encoding BCCT proteins under raised and lowered temperatures (44).

1.4 Cyanobacteria

Cyanobacteria, sometimes referred to as blue-green algae, are oxygenic photosynthetic bacteria which are found globally in most ecosystems (93). There are around 150 genera of cyanobacteria containing over 2000 species (94). They form a monophyletic group within bacteria, and they do not branch deeply within the bacterial domain, thus they may have been one of the last major groups to diverge within bacteria (95). Cyanobacteria originated around 2.6 billion years ago (96) and were critical to the evolution of life on Earth by oxygenating the planet's atmosphere in the Great Oxygenation Event (97). Cyanobacteria can grow and reproduce in some extreme environments such as Antarctic lakes, hot springs, alkaline soda lakes, brine pools, deserts and polar regions (98). When excess organic matter such as nitrogen and phosphorous enrich water, cyanobacteria will form blooms often leading to eutrophication (98). Typically, cyanobacterial classification has relied on morphological details such as, size, shape, arrangement, cell division, nitrogen fixation and gas vacuoles (99). However, advances in sequencing technology meant cyanobacteria are now classified based on conserved genes like the nitrogenase encoding *nifD*, RNase P encoding *rnpB* and a photosystem II protein encoding *psbA* (99).

Plant and algal chloroplasts are believed to have been derived from cyanobacteria and many key processes are conserved throughout the photosynthetic lineage (100,101). Due to the large numbers of cyanobacteria globally, and in the oceans, they are responsible for around 25% of net global carbon productivity and 50% of net marine carbon productivity (102). In addition to being prolific carbon fixers, many cyanobacteria also fix atmospheric nitrogen. After carbon, nitrogen is the second most important element for living organisms, contributing up to 10% of dry mass (103). Cyanobacteria can take up and utilise many nitrogen-based molecules, such as ammonium (104), nitrate or nitrite, urea and amino acids (103). However, these molecules can be limited in the environment, especially in the open oceans, where the ability of cyanobacteria to fix nitrogen is crucial.

Cyanobacteria can form dense blooms that are defined as a marked discolouration of the water (105). Common bloom forming genera include *Aphanizomenon*, *Trichodesmium*, *Nodularia*, *Microcystis*, *Cylindrospermopsis*, *Planktothrix* and *Dolichospermum* (105). When cyanobacteria bloom, they cause a drastic deterioration of the water quality. This increases water turbidity, smother submerged vegetation

(106), induce hypoxia and anoxia (107) and may result in release of cyanotoxins which are harmful to aquatic life and humans (108).

1.4.1 Marine cyanobacteria

Marine cyanobacteria evolved around 2000 Mya and coincided with the development of polysaccharide sheaths in some species (109). This contributed to a second and higher increase in oxygen levels known as the Neoproterozoic Oxygenation Event (NOE), which occurred 800 to 500 Mya (110). Lineages to evolve at the end of the pre-Cambrian include *Trichodesmium* and *Nodularia*, *Crocospaera* and relatives, and unicellular nitrogen fixers (109). These genera evolved from different sources, for example, *Trichodesmium* evolved out of benthic mats. *Nodularia*, single celled nitrogen fixers, *Synechococcus* and *Prochlorococcus* evolved from freshwater species (109). The species that evolved from freshwater ancestors adapted to marine environments by acquiring the genes to encode for proteins that synthesise compatible solutes such as glucosylglycerol (GG), glucosylglycerate (GGA) and GB (109).

In marine environments cyanobacteria are abundant, most notably in oligotrophic subtropical and tropical oceans (97), where picophytoplankton such as *Synechococcus* and *Prochlorococcus* dominate. These are probably the most abundant microorganisms in oceans globally with total cell numbers of around $7.0 \pm 0.3 \times 10^{26}$ and $2.9 \pm 0.1 \times 10^{27}$, respectively (102). However, despite this abundance, only a few cyanobacterial lineages evolved to colonise the open oceans such as *Crocospaera* and relatives, *Candidatus Atelocyanobacterium thalassa* (UCYN-A), *Trichodesmium*, *Synechococcus* and *Prochlorococcus* (109).

Some cyanobacteria are diazotrophs and are the main source of fixed nitrogen in the oceans (103). As part of this interaction, they supply fixed nitrogen to other photoautotrophic microbes which is crucial because marine primary production is limited by nitrogen in ~75 % of surface waters (111). Therefore, diazotrophic cyanobacteria exert control over primary productivity and the export of organic carbon to the deep ocean (112), including in tropical and subtropical oceans (113). In these environments, known open ocean nitrogen fixing cyanobacteria are placed into three groups: i) filamentous non-heterocystous forming (*Trichodesmium*), ii) filamentous heterocyst forming symbionts with unicellular eukaryotic algae (*Richelia* and

Calothrix), iii) unicellular cyanobacteria (*Crocospaera* and *Cyanothece*) (112). *Trichodesmium* are the dominant N₂ fixing organism, forming aggregates called 'puffs' which collate into vast blooms (103). *Trichodesmium* dominate the water column by controlling their buoyancy through generating gas vesicles, an attribute lacking in most other N₂ fixing cyanobacteria (112). However, in some cases, unicellular diazotrophic cyanobacteria can outnumber *Trichodesmium* (112).

There are three groups of unicellular cyanobacteria, UCYN-A, UCYN-B and UCYN-C, which was discovered via phylogenetic analysis of the *nifH* nitrogenase encoding gene (112). UCYN-A has a broader geographical range than *Trichodesmium* and contributes to global N₂ fixation in higher latitudes (114). Altogether, these unicellular diazotrophs are ubiquitously distributed in the oceans and contribute significantly to marine N₂ fixation (111). In conclusion, larger diazotrophs (>10 µm) dominate the tropical belt whilst unicellular cyanobacterial diazotrophs are globally distributed (111).

1.4.2 Saltmarsh cyanobacteria

At a similar time to when marine bacteria begun colonising the open oceans, the Earth's environment were changing and factors such as the developing ozone layer meant cyanobacteria could colonise shallow water ecosystems (99). An ancient macrocyanobacterial clade evolved to establish thick, laminated mats and stromatolites (109). To adapt to this new environment, benthic cyanobacteria evolved traits such as the ability to create an extracellular sheath to protect from desiccation (115) and ultraviolet radiation (116). In saltmarshes, cyanobacteria are typically most abundant during the summer, but their presence in marshes from Southern California have been recorded throughout the year (117).

Cyanobacterial dominated laminated mats are found in saltmarshes which harbour a wide range of life, from heterotrophic bacteria through to higher plants and animals. Microbial mats are typically composed of cyanobacteria, green algae, diatoms, purple sulfur bacteria and green sulfur bacteria, as well as older decayed mat components (118). The microbial mat stabilises and precipitates the salt marsh sediment and microbial mat processes contribute significantly to the global sulfur cycle (118).

Within the mat, unicellular and filamentous cyanobacteria occupy both aerobic and anaerobic environments (119). The cyanobacterial types within these two groups include coccoids (*Chroococcus*, *Anacystis*), colonial (*Merismopedia*), heterocystous (*Anabaena*, *Nostoc*, *Calothrix*) and non-heterocystous (*Oscillatoria*, *Lyngbya*, *Microcoleus*, *Phormidium* and *Schizothrix*) species (117). Here, cyanobacteria play a key role in the microbial food web via their substantial contribution to net primary productivity, including nitrogen fixation, nutrient cycling and ecosystem stabilising (120). For example in a young salt marsh in Southern California, cyanobacteria contributed anywhere from 17 – 100 % of net primary productivity of this salt marsh, thereby supporting the local macrofauna (121).

Salt marsh cyanobacterial dominated biofilms must be able to tolerate repeated and rapid fluctuations in temperature, light and salinity (122). These stresses are more extreme the closer to the shore they are situated, due to the lower occurrences of tidal cover. For instance, some areas may not receive tidal cover for 5 days around neap tides and up to 21 days when coupled with low amplitude Spring tides (122). Therefore, salinity ranges in upper saltmarsh areas can range from 16 to 210 PSU, whereas lower saltmarsh areas range from 18 to 70 PSU and mudflats are 22 to 39 PSU (123). Cyanobacteria adapt to this environment by migrating away from the surface (122), producing UV blocking molecules and osmolytes (124).

1.4.3 Transport and synthesis of DMSP in cyanobacteria

Like many other marine organisms, cyanobacteria can import and utilise DMSP. Only a few cyanobacterial species have been reported to produce DMSP (125,126) and therefore it is suspected that most species import it. Genes encoding for DMSP and GB transporter proteins are found in some low light adapted clade IV *Prochlorococcus* (127) and in a range of *Synechococcus*. These genera import DMSP likely produced by phytoplankton (49). Together, *Prochlorococcus* and *Synechococcus* contributed to around 10-34% of total DMSP sulfur assimilation in the Mediterranean, Gulf of Mexico and Sargossa sea (49). Of the cyanobacteria that import DMSP, only one strain (*Synechococcus* KORDI-100) has been found to encode a putative but uncharacterised DMSP lyase (128). In the Mediterranean, DMSP uptake by *Synechococcus* is higher under solar radiation than in dark conditions, in direct

contrast to free-living heterotrophic bacteria in which DMSP import is inhibited under sunlight (129). Cyanobacterial import of DMSP during daylight may be related to the potential role of DMSP as an antioxidant.

Few studies have investigated DMSP production and regulation in cyanobacteria. *Trichodesmium erythraeum* IMS101 (IMS101) cultured under iron-depleted conditions exhibited a 45-fold increase in DMSP concentration from 0.05 mmol L⁻¹ up to 2.27 mmol L⁻¹ (130). This is lower than production from a broad range of phytoplankton (5 ± 7 mmol to 22 ± 32 mmol) (131). However, production could be maximised in iron limited oligotrophic oceans (130). During blooms, *Trichodesmium* trichome density varied between 6 x 10³ L⁻¹ to 9.72 x 10⁶ L⁻¹, corresponding to 0.15 – 280 nmol L⁻¹ DMSP under iron-replete conditions or 2.2 – 4100 nmol L⁻¹ DMSP under iron-limited conditions (130).

Synechococcus, *Prochlorococcus* and *Crocospaera* species have been shown to produce low intracellular levels of DMSP (132). DMSP production was quantified in *Synechococcus* and *Prochlorococcus* species at $2.8 \times 10^{-4} \pm 1.8 \times 10^{-4}$ and $5.1 \times 10^{-3} \pm 7.8 \times 10^{-3}$ fg/cell, respectively (125). These levels were lower than observed in picoeukaryotes such as *Imantonia* sp. and *Pyconococcus* sp. at 46.5 ± 73.7 fg/cell (125). *Synechococcus* and *Prochlorococcus* were estimated to produce a maximum of 2.5% of total DMSP in the subtropical Atlantic Ocean, although not all species may produce DMSP (125).

Like open-ocean cyanobacteria, marine benthic cyanobacteria have also been screened for their ability to produce DMSP. In one study, benthic filamentous cyanobacteria, including *Lyngbya*, *Oscillatoria*, *Nostoc*, *Calothrix*, *Anabaena* and *Nodularia* species gathered from shallow coastal waters and microbial mats in North Germany were assayed for their DMSP content after growth in nutrient-replete media. DMSP concentrations ranged from 0.007 mmol DMSP (g chl a)⁻¹ to 0.583 mmol DMSP (g chl a)⁻¹ (126), which was a lot less than comparable studies conducted in *Emiliania huxleyi* (15.4, 17.9 and 158 mmol DMSP (g chl a)⁻¹) (126). However, these cyanobacteria were not tested under nutrient limitation and were only isolated from one location in North Germany. Therefore, our knowledge of DMSP production in marine benthic cyanobacteria is limited. In addition, no DMSP transporters have been characterised in filamentous cyanobacteria.

1.4.4 Transport and synthesis of GB in cyanobacteria

GB is a major osmolyte in marine and most halophilic groups of cyanobacteria (133). For example, GB is accumulated in *Halotheca* sp. PCC7418, *Synechocystis* DUN52 and *Dactylococcopsis salina* (134,135). In the case of *Halotheca* sp. PCC7418, internal GB concentrations can range between 0.3 to 1 M depending on salinity (136). GB production is upregulated following an increase in salinity and used to protect ribulose-1,5-bisphosphate (RuBP) carboxylase activity in the presence of KCl or K₂SO₄ (136,137). Enzyme protection by GB has been observed in other cyanobacterial species, including *Spirulina subsalsa* and *Synechocystis* DUN52 (134).

Cyanobacteria synthesise GB via the glycine methylation pathway and/or the choline oxidation pathway (Section 1.3.1). For example, both pathways are present in *A. halophytica* (138) while *Synechocystis* sp. PCC 7418 (PCC 7418) utilises the methylation pathway (73). PCC7418 GSMT and DMT methyltransferases were upregulated around 1.6 – 2.5 fold by salinity shock treatment from 0.5 M to 2.5 M NaCl, in turn leading to a higher intracellular concentration of GB (73). Other cyanobacteria, including *Synechococcus* sp. WH8102, synthesised GB via the methylation pathway, with a similar increase in intracellular GB concentration observed following an increase in salinity (74). Furthermore, *Prochlorococcus* in the low light adapted (LLIV) clade contain the same gene arrangement as the GSDMT locus in *Synechococcus* sp. WH8102, suggesting they synthesize GB via the same pathway (74).

In addition to their ability to synthesise GB, many cyanobacteria import GB from the environment, most notably under conditions of fluctuating salinity (134). PCC7418 contains the betaine transporter, BetT, a BCCT transporter, which when expressed in the freshwater cyanobacterium, *Synechococcus elongatus* PCC7942, conferred on this species the ability to grow in seawater (139). Uptake of exogenous GB for its osmoprotective properties and not catabolism was demonstrated in one cyanobacterium, *A. halophytica* (140).

1.4.5 Other osmolytes in marine cyanobacteria

Cyanobacteria utilise osmoprotectants other than DMSP and GB, for example glucosylglycerol (GG), which is found in salt tolerant and marine cyanobacteria (141).

In *Synechocystis* sp. PCC6803, GG biosynthesis is a two-step enzymatic reaction involving a phosphate synthase and GG phosphate phosphatase, which is regulated by the salinity concentration (142,143). Furthermore, *Synechocystis* sp. PCC6803 was also shown to transport GG via GgtA, which was upregulated under salt stress (142).

Glucosylglycerate (GGA) is another osmoprotectant utilised by cyanobacteria. Genes encoding putative GGA biosynthetic enzymes were found in *Synechococcus* sp. PCC 7002 and *Prochlorococcus marinus* SS120 but the intracellular concentration of GGA was low, thus leading to speculation that GGA is not a major osmolyte but plays a role under low nitrogen conditions in replacing glutamate (144). Furthermore, GGA accumulation is regulated by salt and nitrogen in *Synechococcus* sp. PCC7002 and when subjected to salinities of 500 mM NaCl, GGA accumulation is increased (144).

Homoserine betaine (HB) was identified as an osmoprotectant in *Trichodesmium* spp., which lacked other osmolytes including GB, GG, GGA, sucrose and trehalose, even when grown up to 43 ppt salinity (145). The gene, *tery_2447*, was upregulated following salt stress and shown to encode a protein capable of methylating L-homoserine to homoserine betaine, using SAM as a methyl donor (145).

1.5 Overall aims of this project

Previous work on characterising DMSP production in cyanobacteria has led the community to believe they are low producers. However, these studies have several flaws which will be addressed throughout this thesis. For example, none of these studies looked at axenic cultures of cyanobacteria, so it is possible that DMSP production derived from associated microbes. Additionally, past work has not investigated which gene(s), or their protein products, are responsible for synthesizing DMSP in cyanobacteria and which pathway is used. Furthermore, many cyanobacteria have not been screened for their DMSP production under relevant stressful limitations such as low nitrogen, higher salinity, cold temperatures, high pressure and elevated reactive oxygen species. Moreover, limited environmental studies on cyanobacterial DMSP production have only been conducted in a few extreme environments, such as intertidal zones, where it might be expected to be highly elevated. Also, given the chemical similarity between GB and DMSP, it is unknown whether the same enzyme(s)

could be synthesising both compounds, mirroring the transporters which show specificity for both osmolytes.

Specifically, the following research aims will be addressed in this project:

1. What are the gene(s) and protein(s) involved in DMSP production in cyanobacteria, in particular in *Trichodesmium erythraeum* and which pathway does *T. erythraeum* use for DMSP production.
2. What are the functional characteristics of novel MTHB methyltransferases in cyanobacteria.
3. How widespread are these genes in the environment, and what other, if any, cyanobacteria utilize the encoded proteins for DMSP production.
4. What is the contribution of cyanobacteria to DMSP production in extreme environments, specifically an intertidal zone in saltmarsh sediment.

Chapter 2 - Methods and materials

2.1 Chemical synthesis

DMSP was synthesised from DMS (Sigma-Aldrich), and acrylic acid (Sigma-Aldrich) as outlined in Todd *et al*, 2010 (40). Unless stated all other chemicals were commercially available and acquired from Sigma-Aldrich.

2.2 Media preparation and growth conditions

2.2.1 *E. coli* growth conditions

E. coli was grown at 37 °C with orbital shaking in Luria-Bertani (LB) (146) complete medium. For general cloning, multiple strains were used and for overexpression *E. coli* BL21 DE3 was used. A full list of strains used in this study and their purpose is denoted in **Table 2.1**. Where indicated, antibiotics were added to the media at the following concentrations: ampicillin (100 µg ml⁻¹), gentamicin (20 µg ml⁻¹), kanamycin (40 µg ml⁻¹), tetracycline (5 µg ml⁻¹), spectinomycin (200 µg ml⁻¹).

2.2.2 *Labrenzia aggregata* growth conditions

L. aggregata was grown at 28 °C with orbital shaking in Yeast-Tryptone Sea Salts (YTSS) (147) complete medium or in marine basal medium (MBM) at 35 practical salinity units (PSU), unless otherwise stated. The salinity was adjusted from 5 PSU up to 50 PSU by altering the concentration of sea salts. In MBM, 10 mM succinate was used as the carbon source and either 10 mM or 0.5 mM NH₄Cl was used as the nitrogen source. If indicated, 0.5 mM methionine, 0.5 mM MTHB and either 0.1 mM isopropyl β-D-1 thiogalactopyranoside (IPTG) or 10 mM taurine was used. Where indicated antibiotics were added to the media at the following concentrations: gentamicin (80 µg ml⁻¹), rifampicin (20 µg ml⁻¹), tetracycline (10 µg ml⁻¹), spectinomycin (200 µg ml⁻¹).

2.2.3 *Synechococcus* growth conditions

Synechococcus sp. were grown at 22 °C with orbital shaking in SN medium (148). Unless otherwise stated, *Synechococcus* was grown under 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of white light, in 35 PSU. For high light experiments, the light intensity was increased to 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Under various starvation and regulation experiments, NaNO_3 , ferric ammonium citrate or K_2HPO_4 was removed from the growth media. Under high salinity, the salinity was increased to 40 PSU by removing the freshwater component of SN media and replacing it with more seawater. Likewise, at the lower salinity of 30 PSU, the ratio of freshwater to seawater in SN media was doubled.

2.2.4 *Prochlorococcus* growth conditions

Prochlorococcus sp. were grown in acid-washed glassware in Pro99 medium (149) prepared with 0.2 μm filtered, autoclaved seawater collected from Vineyard Sound, MA or the Sargasso Sea. *Prochlorococcus* sp. were incubated under either a 14:10 light:dark cycle at 24 °C or a constant light flux at 21 °C. Light levels were 30 – 40 $\mu\text{mol Q m}^{-2} \text{ s}^{-1}$ for high-light adapted strains or 10 – 20 $\mu\text{mol Q m}^{-2} \text{ s}^{-1}$ for low-light adapted strains. Under nitrogen starvation experiments NH_4Cl was removed from the growth media.

2.2.5 Filamentous cyanobacteria growth conditions

Lyngbya aestaurii UTEX 2515 was grown at 22 °C with orbital shaking in enriched seawater medium (ESW) (150) at 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of white light. *T. erythraeum* IMS101 was grown in YBC-II (151) medium at 28 °C with orbital shaking with 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of white light.

2.2.6 Growth of filamentous cyanobacteria on solid agarose medium

For growth of *L. aestaurii* UTEX 2515 on solid media, aliquots of 300 ml ESW media were prepared with 1.5 % agarose added to the mixture before autoclaving. Afterwards, 150 ml of ESW – agarose was decanted into a phytatray II (Merck) and left to cool. Using EtOH sterilised forceps, a 0.3 g mass of *L. aestaurii* UTEX 2515 was segregated from its parent culture and streaked across the surface of the ESW – agarose before being planted on the surface of the agarose. Cultures were grown at

22 °C, 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of white light on a 12:12 hour cycle for 1 week before further experimentation.

2.2.7 Long term storage of prokaryotes

A universal of 10 ml of relevant rich growth medium with antibiotics was inoculated with a single colony of the strain to be stored and grown at the strain's optimal parameters. Into a sterile 2 ml screw cap microcentrifuge tube, 750 μL culture was added along with 500 μL 50% glycerol and 225 μL DMSO. The cultures were then submerged into liquid nitrogen and stored promptly at -80 °C.

Table 2.1 – List of strains used in this study.

Strain	Description	Reference
<i>E. coli</i> 803	Used for transformations.	(152)
<i>E. coli</i> BL21DE3	Used in protein overexpression.	(153)
<i>E. coli</i> DH5 α	Used for transformations.	New England Biolabs
<i>E. coli</i> XL10 Gold	Used for transformations.	Agilent Technologies
<i>E. coli</i> pRK2013	Conjugation into <i>L. aggregata</i>	(154)
<i>E. coli</i> S17. Δ pir	Conjugation into <i>Synechococcus</i> sp.	(155)
<i>L. aggregata</i> LZB033	Wild-type strain of DMSP/GB producer.	(11)
<i>Gynuella sunshinyii</i>	DMSP and GB producer.	(156)
<i>L. aggregata</i> LZB033 Δ dsyB	Deletion mutant of <i>dsyB</i> .	(11)
<i>L. aggregata</i> LZB033 Δ GSDMT	Deletion mutant of <i>gsdmt</i> .	(157)
<i>L. aggregata</i> LZB033 Δ dsyB Δ GSDMT	Deletion mutant of <i>dsyB</i> and <i>gsdmt</i>	(157)
<i>Synechococcus</i> sp. WH8103	Single celled non-axenic cyanobacterium culture.	Roscoff Culture Collection (RCC) 2366

<i>Synechococcus</i> WH8020	sp.	Single celled non-axenic cyanobacterium culture.	RCC 2437
<i>Synechococcus</i> WH8016	sp.	Single celled non-axenic cyanobacterium culture.	RCC 2535
<i>Synechococcus</i> WH8102	sp.	Single celled non-axenic cyanobacterium culture.	RCC 539
<i>Synechococcus</i> WH7803	sp.	Single celled non-axenic cyanobacterium culture.	RCC 752
<i>Prochlorococcus</i> MIT9313	sp.	Single celled axenic cyanobacterium culture.	Gifted by Steven Biller, Wellesley College
<i>Prochlorococcus</i> MIT9312	sp.	Single celled axenic cyanobacterium culture.	Gifted by Steven Biller, Wellesley College
<i>Prochlorococcus</i> MIT9303	sp.	Single celled axenic cyanobacterium culture.	Gifted by Steven Biller, Wellesley College
<i>Lyngbya</i> UTEX2515	<i>aestaurii</i>	Filamentous, nitrogen fixing, benthic and non-axenic cyanobacterium.	UTEX 2515
<i>Trichodesmium</i> <i>erythraeum</i> IMS101		Filamentous, nitrogen fixing and non-axenic cyanobacterium.	Gifted by Tracey Lawson, University of Essex
<i>Rubidibacter lacunae</i>		Single celled non-axenic cyanobacterium culture.	UTEX LB 2944

2.3 Protein overexpression and purification

2.3.1 Protein overexpression

A universal of 10 ml LB was inoculated with a single colony of *E. coli* BL21DE3 transformed with a pMALC2X-1039 vector and incubated overnight at 37 °C. A 1 in 100 volume of starter culture was inoculated into LB with antibiotics with a 1 in 200 addition of 20% glucose. This was grown at 37 °C for 2 hours or until an OD₆₀₀ of 0.6. The culture was left at room temperature for 10 min before 0.5 mM IPTG was added. Then, the culture was incubated overnight at 16 °C with shaking. Afterwards, the

culture was centrifuged at 4000 RPM, 4 °C for 20 min. This was then resuspended in 1 in 20 volume of lysis buffer (see protein overexpression buffers) at 4 °C before being frozen overnight at -20 °C. The next day, the sample was thawed on ice and the cells were lysed via cell disruption, before being centrifuged at 9000 RPM for 30 min to separate the sample into soluble and insoluble fractions. The supernatant was then transferred to a fresh falcon tube for downstream processing.

2.3.2 Protein extraction buffers

Buffer 1 (no DTT) was made by adding 200 mM NaCl to 20 ml 1M Tris-HCl (pH 7.4). Lysis buffer (DTT) was made by the addition of 1 mM DTT, 1 mM EDTA (pH 8) and 200 mM NaCl to 20 ml 1M Tris-HCl (pH 7.4). Washing buffer was composed of 1 mM EDTA and 200 mM NaCl added to 20 mL 1M Tris-HCl (pH 7.4). Elution buffer was made by the addition of 200 mM NaCl to 20 ml 1M Tris-HCl (pH 7.4) with 10 mM maltose monohydrate.

2.3.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Whole cell lysate, insoluble, soluble, wash steps and purified fractions of protein overexpression and purification were analysed via SDS-PAGE. Each gel was composed of a stacking gel and a resolving gel. SDS-PAGE running buffer was diluted from a 10X stock made up of 3% TRIS, 14.4% glycine and 1% SDS in MilliQ ultrapure water. Aliquots of 10 µL protein samples were added to 40 µL loading buffer, if the sample was too thick to run then it was further diluted by the addition of more loading buffer. The sample was then boiled for 10 min at 100 °C before loading. Gels were loaded with a Precision Plus Protein Dual Colour™ standard (BioRad). Afterwards, gels were resolved by running at 200 V for 30 min and then 120 V for 90 min, before staining with SimplyBlue™ (Invitrogen) coomassie blue stain overnight at room temperature with shaking. Following staining, gels were washed with dH2O for 1 h before imaging (Chemidoc XR).

2.3.4 Maltose Binding Protein purification

In a 2 ml microcentrifuge tube, 1 ml amylose resin (NEB) was added. The resin was equilibrated with 1.5 ml washing buffer (see protein extraction buffers) and then centrifuged at 1000 RPM for 1 min. This was repeated 4 times. The resuspended amylose resin was then mixed with 25 ml of soluble fraction protein lysate and mixed for 1 h at 4 °C. Then, the mixture was centrifuged at 1000 RPM for 1 min and the supernatant was discarded. A volume of 10 ml washing buffer was added to the protein lysate and this was centrifuged at 1000 RPM for 1 min. This was repeated 3 times. Then the pellet was resuspended in 10 ml washing buffer and transferred to a PD10 gravity flow column. A volume of 10 ml buffer 1 was added to the column and allowed to clear by gravity flow. Afterwards, 1 ml of elution buffer was added to the column and allowed to drip by gravity flow. This was repeated once. The eluate was collected and decanted into microcentrifuge tubes and stored at -80 °C for further downstream processing.

2.3.5 Protein *in vitro* assays

Impure soluble and purified methyltransferase protein were assayed for their activity. To a 400 µL reaction volume of 10 mg ml⁻¹ (unless otherwise stated) of SAM dependent methyltransferase, 0.1 mM MTHB and 0.1 mM SAM was added. The reaction was incubated overnight at room temperature. For kinetics experiments, a stepwise gradient of MTHB was added and incubation times were adjusted. After the reaction, 800 µL of 30 mg ml⁻¹ activated charcoal in 0.1 M acetic acid was added to the reaction. This was centrifuged at 14,000 RPM for 10 min. The supernatant was retained, and 200 µL was added to 100 µL 10 M NaOH before heating at 80 °C for 10 min. This was then incubated overnight at room temperature, in the dark, before analysis via gas chromatography (Section: 2.7.1).

2.4 In silico analyses and sequence analysis

2.4.1 Identification of target methyltransferases in cyanobacteria

BLAST searches were conducted to identify homologues of known DMSP synthesis SAM-dependent methyltransferases in cyanobacteria. Target proteins were identified

through BLASTp search of the National Center for Biotechnology Information (NCBI) database (158).

2.4.2 Multiple Sequence Alignment and gene neighbourhood analysis of target SAM-dependent methyltransferases in multiple cyanobacteria

Homologous sequences recovered from BLASTp search were aligned using ClustalW (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). Gene neighbourhood analyses were constructed using the suite of tools from the Enzyme Function Initiative (159). First, a sequence similarity network (SSN) was constructed using the EFI-Enzyme Similarity Tool. Next, the SSN was inputted into the EFI-Gene Neighbourhood Tool to interrogate the sequence map, which produced the gene neighbourhood diagrams.

2.4.3 Phylogenetic tree of DsyC and other DMSP synthases

Protein sequence alignments were conducted using ClustalW (<https://www.genome.jp/tools-bin/clustalw>) (160). Phylogenetic trees were constructed using MEGA (161).

2.4.4 Sequence optimization and gene synthesis

Genes to be synthesized were codon optimised for expression in *E. coli* using the Codon optimization tool at Interactive DNA Technologies (IDT) (<https://eu.idtdna.com/pages/tools/codon-optimization-tool>). Internal restriction enzyme sites such as, EcoRI, NdeI, XbaI, BamHI, BsaI, HindIII, PstI and BpiI were removed. Genes were synthesized into pMK-RQ or into pMALC2X-1039 by IDT.

2.4.5 Analyses of community composition

Qiime2 was used to analyse the 16S rRNA gene amplicon data. The dada2 (162) module removed low quality sequences and cluster sequences at 100 % identity. The classify-sklearn (163) module assigned a taxonomy for each sequence and Phyloflash (164) determined the prokaryotic community from metagenomes based on 16S rRNA

gene reads, with Silva v138.1 (165) as a reference database. The microeukaryotic community was analyzed based on the 18S rRNA metagenomic gene reads retrieved by BBmap, each read was assigned a taxonomy based on IDTAXA. Both identification and taxonomic assignment of 18S reads were based on the PR2 database (166). Non-Protistan sequences, defined as those assigned to the Metazoe, Embryophyta and Fungi were filtered out before downstream processes.

2.4.6 MAG recovery

To improve the quality of recovered MAGs, triplicate metagenomic samples were coassembled by MEGAHIT v1.0.2 (167) with default parameters. Co-assembly results of each sample were imported to MetaWRAP (168) to recover bacterial and archaeal MAGs. Both `bin_refinement` and `reassembly_bins` modules from MetaWRAP were performed to refine recovered MAGs and improve completion and N50 of newly recovered MAGs, respectively. All MAGs were dereplicated using dRep v2.3.2 (169) with default settings. Genome completeness and contamination were estimated by CheckM v1.0.12 (170) and only MAGs with a completeness > 50 % and contamination < 10 % were retained for downstream analysis. Taxonomic assignment of each genome was determined by the `classify` module of GTDB-Tk v1.7.0 (171). Gene annotation of each MAG was performed with Prokka v1.12 (172). The relative abundances of MAGs in metagenomes and metatranscriptomes were estimated using CoverM v0.6.1 (<https://github.com/wwood/CoverM>), using default parameters.

2.4.7 Identification of DMSP/DMS cycling related genes

Functionally ratified protein sequences (not shown), DSYB, DSYU, TpMMT, Alma1, MmtN, DsyB, DddD, DddK, DddP, DddQ, DddW, DddL, DddY, DmdA, DMSOR, Tmm, DsoB, DdhA, MddA, MddH, DmoA were used as reference sequences for Hmsearch and BLASTp search. Hidden Markov models (HMM) were created and their homologues from metagenomes were obtained using `hmmsearch` (<http://hmmer.org>). Prokaryotic gene homologues were searched in metagenomes and eukaryotic gene homologues were searched in metatranscriptomes. Then, these homologues were further curated by BLASTp. Only sequences of a minimum 40% amino acid identity and 70% query coverage to the corresponding ratified proteins were retained. Next,

these remaining sequences were used to construct a maximum-likelihood phylogenetic tree with both functional and non-functional ratified sequences by MAFFT (173) (sequence alignment) and FastTree (174) (tree construction). Proteins clustered to non-functional sequences were discarded. To determine the taxonomy of the remaining DMSP/DMS cycling genes, their protein sequences were aligned against the NCBI NR database using BLASTp. The taxon of best hit from each query was recorded. Gene relative abundance was calculated based on the equation below, whereby R equals the number of mapped reads to this gene, L is the gene length and M is the total reads number in the metagenome.

$$Relative\ Abundance = \frac{\frac{R}{L} * 1000000}{M}$$

2.5 *In vitro* genetic manipulation

2.5.1 Polymerase Chain Reaction (PCR)

Genes and flanking regions of genes were amplified by PCR in a thermocycler (BioRad) using cycling conditions outlined in **Table 2.2**. Typically, 50 µL reactions were set up consisting of: 1 µL template DNA, 1 µL forward primer, 1 µL reverse primer, 0.5 µL Phusion DNA polymerase, 1 µL 10 mM dNTPs, 1.5 µL DMSO, 10 µL 5X Phusion HF buffer and 34 µL nuclease free water. Oligonucleotide primers used in this study are listed in **Supplementary Table 1**. Primers were synthesised by IDT. Colony PCRs were conducted by picking a single colony off a solid agar plate with a sterile toothpick and mixing this with 100 µL dH₂O. 1 µL of this was used as template DNA.

Table 2.2 – Thermocycler protocol for PCR with Phusion polymerase.

Temperature	Time	Number of cycles
98 °C	30 s	X 1
98 °C	10 s	X 35
60 °C	30 s	
72 °C	30 s	

72 °C	10 min	X 1
4 °C	∞	X 1

2.5.2 Agarose gel electrophoresis

All PCR and cloning products were visualised and analysed on agarose gels. Gels consisted of a 1% w/v agarose in 1 x TAE buffer (40 mM TRIS base, 20 mM acetic acid and 1 mM EDTA in dH₂O). For gels, buffer was added to the agarose, melted, and cooled to 50 °C before 1.5 µL of SybrTM Safe (Invitrogen) was added per 100 ml. Gels were set in appropriately sized gel trays for approximately 30 min at room temperature. Gels were run for 1 h at 100 V, with increases to time for better band separation. Gels were visualised under a UV lamp in a Chemidoc XR imaging lab (BioRad).

2.5.3 PCR purification

Once the correct PCR product had been determined via agarose gel electrophoresis, the remaining PCR product in the PCR tube was purified using a QIAquick PCR purification kit (Qiagen), according to the manufacturer's instructions. The final product was eluted into 30 µL sterile D₂O in a sterile microcentrifuge tube for downstream processing.

2.5.4 Agarose gel extraction

Bands of interest were excised from a gel using an ethanol sterilised scalpel. These were then weighed and dissolved in the appropriate volume of Buffer QG (Qiagen). Processing of the DNA was carried out according to the manufacturer's instructions. DNA was eluted from the QiaQuickTM membrane into 30 µL dH₂O.

2.5.5 Cloning into pMAL-C2X via Golden Gate Assembly

A domesticated pMAL-C2X was used for Golden Gate Assembly (GGA). Internal BsaI sites were removed, and two recognition sites were added, *AGGT* and *TCCA*. The gene of interest was amplified through PCR. The following reagents were used in GGA

cloning procedures: 1 μ L pMAL-C2X-1039 (100 ng/ μ L), 1 μ L insert DNA (100 ng/ μ L), 1.5 μ L 10x T4 DNA buffer, 1.5 μ L 10x BSA, 1 μ L *Bsa*I, 1 μ L T4 DNA ligase and dH₂O to a final volume of 15 μ L. The reaction was carried out in a thermocycler (BioRad) using cycling conditions listed in **Table 2.3**.

Table 2.3 - Thermocycler protocol for Golden Gate Assembly reaction.

Temperature	Time	Number of cycles
37 °C	3 min	X 25
16 °C	4 min	
50 °C	5 min	X 1
80 °C	5 min	X 1

2.5.6 Cloning into pk18MobSacB via Gibson Assembly

In silico design of GA components was carried out using NEBuilder Assembly tool (<https://nebuilder.neb.com/#/>). pk18MobSacB was digested with the restriction enzymes *Eco*R1 and *Bam*H1 for 2 h at 37 °C in a reaction mixture of: 2 μ L pk18MobSacB (100 ng/ μ L), 1 μ L of each restriction enzyme, 2 μ L restriction enzyme buffer and dH₂O to a final volume of 20 μ L. For the Gibson assembly reaction the mixture consisted of: 0.5 pmol of each fragment, 10 μ L of 2X GA Master Mix (NEB) and dH₂O up to 20 μ L. The reaction was transferred to a thermocycler and incubated at 50 °C for 1 h, before being transformed into NEB 5-alpha Competent *E. coli* cells, following the manufacturer's instructions.

2.5.7 Restriction digestion and ligation assemblies

In silico design of all digestion/ligation assemblies was carried out using benchling (<https://www.benchling.com/>). All digestions used NEB restriction enzymes. An aliquot of 1 – 16 μ L DNA was used in the reaction along with 2 μ L buffer, 1 μ L of each enzyme and dH₂O to a final volume of 20 μ L. The reaction was then mixed by inversion and centrifuged for 10 s at 13,000 RPM. The reaction was incubated at 37 °C for 1 h and then heat killed at 80 °C for 10 min. The reaction was then visualised by agarose gel

electrophoresis (2.5.2 agarose gel electrophoresis) and where necessary plasmid DNA was dephosphorylated using shrimp alkaline phosphatase (NEB), according to the manufacturer's instructions.

Ligation reactions were carried out using T4 DNA ligase (NEB) according to the manufacturer's instructions. Reactions were incubated overnight in ice, left at room temperature. The mixture typically consisted of; 14 μL digested insert, 2 μL digested vector in a 7:1 insert/vector ratio but this was altered to 9:1 or 11:1 insert/vector to improve reaction efficiency if initial ligations were unsuccessful.

2.5.8 Metagenomic DNA extraction from saltmarsh sediments

Saltmarsh sediment samples were collected as outlined in Section 2.8.1. Then, 0.25 g of surface sediment containing visible photosynthetic organisms was added to a sterile PowerBead tube and was gently vortexed. The sediment was lysed in a Ribolyser (FastPrep system, MP biomedical) for three rounds of 1 min at 6 ms^{-1} vigorous shaking. DNA was then extracted using a DNA PowerSoil Kit (Qiagen) according to the manufacturer's instructions, eluted in 100 μL solution C6 and stored at $-20\text{ }^{\circ}\text{C}$. Subsequent metagenomic sequencing was conducted via Novogene Sequencing shotgun metagenomics (WOBI) using a 20 μL aliquot of DNA at a 100 ng/ μL concentration.

2.5.9 Metatranscriptomic RNA extraction from saltmarsh sediments

Saltmarsh sediment samples were collected as outlined in Section 2.8.1. Then, 2 g of surface sediment containing visible photosynthetic organisms was added to a 15 ml PowerBead tube. The sediment was lysed in a Ribolyser (FastPrep system, MP biomedical) in three rounds of 1 min at 6 ms^{-1} vigorous shaking. RNA was isolated using a RNeasy PowerSoil Total RNA kit (Qiagen) according to the manufacturer's instructions. The final pellet was resuspended in 100 μL solution SR7. Following extraction, genomic DNA was removed by treating the sample with two applications of TURBO DNA-free DNase (Ambion) according to the manufacturer's instructions. The samples were then decanted into 20 μL aliquots and immediately flash frozen in liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$. RNA quality was checked via nanodrop (ThermoScientific), agarose gel electrophoresis and 16S amplification. Subsequent

metatranscriptomic sequencing was carried out via Novogene sequencing shotgun metatranscriptomics (WOBI) using a 20 µL aliquot of RNA at 100 ng/µL concentration.

2.5.10 Plasmid miniprep extraction

A 10 ml LB culture with appropriate antibiotics was inoculated with a single *E. coli* (transformed with relevant plasmid) colony and incubated at 37 °C overnight. 3 ml of Cells were then pelleted via centrifugation at 13,000 RPM for 2 min. The supernatant was removed, and the pellet was resuspended in 200 µL of resuspension solution from GenElute™ Plasmid Miniprep kit (Sigma-Aldrich). The plasmid was prepared following the manufacturer's instructions and eluted in 30 µL dH₂O. DNA was stored at -20 °C for downstream applications.

2.5.11 Plasmid midiprep extraction

For plasmids to be used in cloning and long-term storage, a 10 ml LB culture with appropriate antibiotics was inoculated with a single *E. coli* (transformed with relevant plasmid) colony and incubated at 37 °C overnight. Afterwards, 100 ml LB with the same antibiotics was inoculated with 1 ml of the starter culture and incubated at 37 °C overnight. The culture was harvested by centrifugation at 6,000 RPM at 4 °C for 10 min and the plasmid extracted using the Qiagen Plasmid Midiprep kit according to the manufacturer's instructions. Following the final precipitation steps of the protocol, 120 µL dH₂O was added to the pellet and rehydrated at room temperature for 10 min. DNA was stored at -20°C for long term storage and downstream applications.

Table 2.4 - List of plasmids used in this study.

Plasmid	Description	Reference
pk18MobsacB	Suicide plasmid for mutagenesis.	(175)
pLMB509	Broad host range plasmid for expression in <i>L. aggregata</i> .	(176)
pRK2013	Conjugation plasmid used in helper strain.	(177)

pMAL-c2x	Overexpression plasmid with maltose binding protein.	New England Biolabs
pET16b	Overexpression plasmid.	Merck Millipore
pMK-RQ	Synthesis plasmid from IDT.	Integrated DNA Technologies
pET21a	Overexpression plasmid.	Merck Millipore
pLMB509-TDsyC	pLMB509 with <i>T. erythraeum dsyC</i> .	This study.
pLMB509-WDsyC	pLMB509 with <i>Synechococcus</i> sp. WH8103 <i>dsyC</i> .	This study.
pLMB509-Ω-TDsyC	pLMB509 with <i>T. erythraeum dsyC</i> and spectinomycin cassette.	This study.
pLMB509-Ω-WDsyC	pLMB509 with <i>Synechococcus</i> sp. WH8103 <i>dsyC</i> and spectinomycin cassette.	This study.
pMAL-c2x-TDsyC	pMAL-c2x with <i>T. erythraeum dsyC</i> .	This study.
pMAL-c2x-WDsyC	pMAL-c2x with <i>Synechococcus</i> sp. WH8103 <i>dsyC</i> .	This study.
pMAL-c2x-L4-1	pMAL-c2x with <i>dsyC</i> .	This study.
pMAL-c2x-L4-2	pMAL-c2x with <i>dsyC</i> .	This study.
pMAL-c2x-L4-3	pMAL-c2x with <i>dsyC</i> .	This study.
pMAL-c2x-L4-4	pMAL-c2x with <i>dsyC</i> .	This study.
pMAL-c2x-L4-5	pMAL-c2x with <i>dsyC</i> .	This study.
pMAL-c2x-L4-6	pMAL-c2x with <i>dsyC</i> .	This study.
pMAL-c2x-K1	pMAL-c2x with <i>dsyB</i> -like.	This study.
pMAL-c2x-K2	pMAL-c2x with <i>dsyB</i> -like.	This study.

pMAL-c2x-K3	pMAL-c2x with <i>dsyB</i> -like.	This study.
pMAL-c2x-K4	pMAL-c2x with <i>dsyB</i> -like.	This study.
pMAL-c2x-K5	pMAL-c2x with <i>dsyB</i> -like.	This study.
pk18MobsacB-WH8103	pk18MobsacB with flanking regions to <i>dsyC</i> in <i>Synechococcus</i> sp. WH8103.	This study.
pk18MobsacB-WH8020	pk18MobsacB with flanking regions to <i>dsyC</i> in <i>Synechococcus</i> sp. WH8020.	This study.
pLAFR	Cosmid for cloning of large fragment sizes, used in gDNA library preparation.	(178)
pLAFR-Lyngbya	pLAFR containing gDNA from <i>L. aestaurii</i> UTEX2515.	This study.

2.6 In vivo genetic manipulation

2.6.1 Chemically competent *E. coli* preparation

A starter culture was prepared by inoculating 5 ml LB media with the relevant antibiotic and a single colony of *E. coli* (803, BL21:DE3, XL10 Gold, DH5 α), followed by incubation at 37 °C overnight with shaking. A 1 ml aliquot was used to inoculate 100 ml of LB with the same antibiotics which was incubated at 37 °C for 2 h or until OD₆₀₀ reached 0.3 – 0.4. The culture was then transferred to two sterile 50 ml falcon tubes and centrifuged at 6,000 RPM for 10 min at 4 °C. The supernatant was removed, and the pellets were resuspended into 10 ml, ice cold 0.1 M CaCl₂ and kept on ice for 30 min. The cultures were centrifuged at 6,000 RPM for 10 min at 4 °C. Afterwards, the supernatant was removed, and the pellets were resuspended in 2 ml ice cold 0.1 M CaCl₂ and stored at 4 °C for a minimum of 2 h or overnight before heat shock transformation.

2.6.2 Heat shock transformation of *E. coli* cells

For transformation of plasmids, 2 μL of vector DNA (100 ng/ μL) was added to 100 μL competent cells. For ligation mixtures, the whole 20 μL reaction was added to the same quantity of cells. If commercial competent cells (DH5 α , NEB) were used, then the manufacturer's instructions were followed. Samples were kept on ice for 1 h and then heat shocked at 42 °C for 45 s. Next, 500 μL of pre-warmed LB was added to each sample, followed by incubation at 37 °C for 1 h with shaking. The samples were centrifuged at 13,000 RPM for 2 min and 300 μL of the supernatant was decanted, and the pellet was resuspended with the remaining supernatant. Samples were plated on pre-warmed LB agar with antibiotics and incubated overnight at 37 °C.

2.6.3 Conjugation by tri-parental mating

Tri-parental mating was used to transfer plasmids into *L. aggregata* and *R. leguminosarum* J391. Typically, mating consisted of a host strain (*L. aggregata*), a donor strain (*E. coli* transformed with plasmid of choice) and a helper strain (*E. coli* 803 with pRK2013) which facilitated plasmid mobilisation into the host from the donor.

Conjugation via patch cross was achieved by first streaking the three strains on fresh YTSS or TY plates with antibiotics at 28 °C for 2 days (*L. aggregata* and *R. leguminosarum* J391) or LB plates with antibiotics at 37 °C overnight (*E. coli*). A single colony was inoculated into 5 ml of relevant media from each of the strains and grown overnight at either 28 °C or 37 °C with shaking. The next day, 1 ml from each culture was harvested by centrifugation at 13,000 RPM for 2 min, the supernatant was removed, and the pellet was resuspended in 1 ml relevant media. This was repeated three times. A sterile loop was used to mix the three strains together on a plate with no antibiotics and the plates were grown overnight at 28 °C. Afterwards, a sterile loop was used to transfer some of the mating to a plate with a cocktail of antibiotics selecting for the host strain (typically rifampicin, gentamicin and nitrofurantoin) with the correct plasmid. These plates were grown at 28 °C for 2 days. Finally, each plate was streaked out to a fresh plate with the same antibiotic cocktail to select for single colonies.

To obtain higher conjugation efficiencies, filter crosses were performed instead of patch crosses. All three strains were incubated overnight in 5 ml of their respective medium with antibiotics at their preferred temperature. Aliquots of 1 ml were taken from the cultures and centrifuged at 13,000 RPM for 2 min, the supernatant was removed, and the cells were washed three times in media. Each of the pellets was resuspended in 100 µL of either YTSS or TY media and sterilised forceps were used to place a sterile filter on a YTSS or TY agar plate. 100 µL of each culture was added to the filter and mixed using a sterile glass spreader. The plates were incubated at 28 °C for 2 days, after which the filters were removed using sterile forceps and placed into sterile universals. Cells were washed off the filter using 50% glycerol and a serial dilution series was made. The dilution series was plated on selective media with the relevant cocktail of antibiotics to only allow for *L. aggregata* or J391 transconjugant growth. Plates were incubated at 28 °C for 2 days and single colonies were picked.

2.6.4 Genomic DNA preparation of filamentous cyanobacteria

Using a sterile pestle and mortar, 1 g of filamentous cyanobacteria was ground with the addition of liquid nitrogen into a fine paste. The sample was then added to a sterile 2 ml Eppendorf. A preparation of 2 % CTAB was produced (2 g CTAB, 8.18 g NaCl, 10 ml 1 M Tris-HCl pH 8, 4 ml 0.5 M EDTA, dH₂O to 100 ml). 900 µL pre-warmed 2% CTAB, 20 µL proteinase K (Qiagen) and 10 µL β-Mercaptoethanol (Qiagen) was added to the cyanobacteria and incubated for 1 h at 65 °C. The sample was centrifuged at 12,000 RPM for 8 min and the supernatant was decanted, before 1 volume of phenol-chloroform-isoamyl (25:24:1) was added and mixed gently. Next, the mixture was centrifuged at 12,000 RPM for 8 min and the supernatant was decanted. A 0.5 volume of chloroform was added to the supernatant and was mixed gently. The mixture was then centrifuged at 12,000 RPM for 5 min. The supernatant was decanted, and a 0.6 volume of isopropanol was added and mixed gently, before centrifugation at 12,000 RPM for 5 min. The pellet was washed in 500 µL 70% EtOH and air dried at room temperature for 10 min. Finally, 50 µL dH₂O was added to the pellet and it was stored at -20 °C for downstream applications.

2.6.5 Library preparation of gDNA

To make the genomic cosmid library of *L. aestaurii* UTEX2515, 2.5 µg of genomic DNA was partially digested with EcoR1 and then ligated to 1 µg of EcoR1 digested and dephosphorylated pLAFR3 cosmid DNA. 0.7 µg of ligated cosmid DNA was then packaged into λ phage using Gigapack III XL packaging extract (Stratagene). This packaged DNA was mobilised into *E. coli* 803 and transformants selected on LB plates with tetracycline. Titres of the packaging mix was used to identify the number of primary transfectants (~50,000). Following amplification, *E. coli* primary transfectants were pooled into a single culture and stored at -80 °C.

2.6.6 Genetic mutation of *Synechococcus*

A volume of 100 ml *Synechococcus* (WH8103 and WH8020) was grown for 1 week under optimal conditions. *E. coli* S17.1Δpir containing the suicide vector pK18MobSacB with the flanking regions of interest was grown in 10 ml LB without antibiotics. *Synechococcus* and *E. coli* were harvested by centrifugation at 4000 RPM, 23 °C for 15 min. Pellets were resuspended into a small volume (~200 µL) of supernatant. *Synechococcus* and *E. coli* cells were mixed, centrifuged at 13,000 RPM for 1 min, before being resuspended in 200 µL of supernatant. The culture was plated onto 0.3% SN agarose plates and incubated at 23 °C for 48 h under a light intensity of 10 µmol photons m⁻² s⁻¹. *Reugeteria pomeroyi* DSS-3 containing pKNG101-BBR-MCS was used to help remove *Synechococcus* sp. cell debris and contained a sucrose suicide cassette so it could be removed from the final culture. *R. pomeroyi* was grown in 10 ml of Marine Broth (MB, Sigma 2216) with antibiotics for 48 h at 23 °C with shaking, before 2 ml was harvested by centrifugation at 13,000 RPM, 23 °C for 1 min. *R. pomeroyi* DSS-3 was then resuspended in 1 ml SN media. 100 ml 0.2% SN agarose flask was melted and cooled to 37 °C before 1 ml of *R. pomeroyi* DSS-3 was added along with antibiotics. Next, 30 ml of the SN agarose was dispensed into a 50 ml falcon tube and 50, 100 or 500 µL of resuspended conjugation mixture was added before being poured into a sterile petri dish and cooled. The plates were parafilmed and incubated in clear plastic bags at 23 °C for 4-6 weeks at a light intensity of 5 µmol photons m⁻² s⁻¹.

Approximately 4 – 5 days before the end of incubation, any colonies were disturbed and spread out with a sterile pipette tip. Afterwards, colonies were pipetted into 5 ml SN media with the selection antibiotic. As the culture grew, 10 mL SN media with antibiotics were added, and the culture was checked by PCR for segregation. An aliquot of 8 ml was removed and 2 ml of SN with 50% w/v sucrose was added. The culture was then incubated under optimal conditions for 24 h. Afterwards, 100 ml of 0.2% SN agarose was cooled to 37 °C and the selection antibiotic was added. The culture was diluted to 10^{-5} , 10^{-6} and 10^{-7} into 100 μ L volumes before being plated. The plates were parafilmed and incubated upright in clear plastic bags at 23 °C for 4 – 6 weeks under a light intensity of 5 μ mol photons $\text{m}^{-2} \text{s}^{-1}$. Colonies were transferred to a new 0.2 % SN agarose plate with antibiotics and disturbed and spread out with a sterile pipette tip to enlarge the colonies. After 5 days the colonies were checked for contamination and any axenic colonies were inoculated into 5 ml SN with antibiotics and incubated under optimal conditions.

2.7 Metabolite analysis

2.7.1 Detection of DMSHB/DMSP and quantification of DMS by gas chromatography (GC)

The DMSP concentration in cultures was determined through gas chromatography (GC). Unless otherwise stated, 100 μ L of 10 M NaOH was added to 200 μ L sample in a 2 ml glass vial, which was then crimp sealed with PTFE/rubber crimp caps and incubated at room temperature in the dark overnight. If DMSHB was being measured, then following crimping, samples were incubated at 80 °C for 10 min to free DMS from the DMSHB. Vials then were incubated overnight at room temperature in the dark. Measurements were carried out using a flame photometric detector (Agilent 7890A GC fitted with a 7693 autosampler) and a HP-INNOWax 30 m x 0.320 mm capillary column (Agilent technologies J&W Scientific).

Readings were plotted against a calibration curve of varying DMSP standards (0.05, 0.1, 0.2, 0.5, 5, 50 100 nmol), which were made up to a final volume of 200 μ L in dH₂O with 100 μ L 10 M NaOH added and treated as described above.

2.7.2 Detection of DMSHB/DMSP and GB by nuclear magnetic resonance (NMR)

NMR was mainly used to detect for the presence of GB, which cannot be detected via GC. Cultures were grown under relevant experimental conditions and 1 ml was centrifuged at 13,000 RPM for 2 min. The pellet was resuspended in 1 ml of D₂O and lysed via sonication for 2 x 20 s (4 x 20 s if the culture was contained cyanobacteria), with incubation on ice for 1 min in between rounds. The cultures were centrifuged at 13,000 RPM for 2 min and the supernatant was decanted into a sterile 1.5 ml microcentrifuge tube. An aliquot of 800 µL of the sample was added to a NMR tube and analysed via ¹H-NMR 500 MHz (Bruker). DMSP and GB standards of varying concentrations were prepared by adding a known concentration of osmolyte into 800 µL D₂O and analysing as described above.

2.7.3 Detection of DMSHB and GB by High Performance Liquid Chromatography (HPLC)

To determine the optimal temperature for purified proteins, reaction mixtures containing 20 mM MTHB, 1.6 mM SAM, purified protein (final concentration of 30 mg/mL), 200 mM Tris-HCl (pH 7.0) to a total volume of 100 µL were prepared. After the mixture was incubated at 10, 20, 30, 40 and 50°C for 5 h, the reaction was terminated by adding 20 µL perchloric acid. The concentration of S-adenosyl homocysteine (SAH) in the reaction mixture was detected by HPLC (SHIMADZU, Japan) on a Sunfire C18 column (Waters, Ireland) with a linear gradient of 2-20% acetonitrile in 50 mM ammonium acetate (pH 5.5) over 24 min at 260 nm. The optimal pH for purified proteins was examined at the protein's optimal temperature (detected earlier) using 200 mM Bis-Tris buffer for pH 5 to 7, 200 mM Tris-HCl buffer for pH 7 to 9, and 200 mM glycine-NaOH buffer for pH 9 to 10. The control group had the same reaction system as the experimental group except that the corresponding buffer was added instead of protein.

To determine the substrate specificity, the reaction mixture included pure protein (final concentration of 30 mg/mL), and either MTHB, glycine, betaine, dimethylglycine, or sarcosine (all at a final concentration of 20 mM). Next, the reaction was mixed with the

reaction buffer containing 200 mM Tris-HCl (pH 7.0) to a total volume of 100 μ L. After the mixture was incubated at the protein's optimal temperature overnight, the reaction was terminated by adding 20 μ L perchloric acid. The amount of SAH in the reaction mixture was detected by HPLC as described above.

2.7.4 Quantification of protein content by Bradford Assay

To normalise results gathered via chemical analysis, the protein concentration of each sample was measured. From each culture, 1 ml was centrifuged at 13,000 RPM for 2 min and the supernatant was removed. The pellet was resuspended in 1 ml of dH₂O and lysed via sonication for 2 x 20 s, with incubation on ice for 1 min between rounds. Afterwards, lysed samples were centrifuged at 13,000 RPM for 2 min and a 20 μ L aliquot of supernatant was added to 980 μ L Bradfords Reagent (BioRad). The samples were transferred to 1 ml cuvettes and absorbance was measured using a spectrophotometer set to OD₅₉₅. A calibration curve was produced using 5 standards of varying concentrations of bovine serum albumin (BSA) (0 – 400 ng/ μ L) and measured as described above.

2.8 Environmental sampling

2.8.1 Sampling of saltmarsh sediment

Saltmarsh environmental samples were collected from surface sediment samples at Stiffkey saltmarsh, UK (latitude 52.9649 °N, longitude 0.9436 °E). Triplicate samples of marine sediment were collected using sterile 50 ml falcon tubes, which were dragged immediately through areas containing a visible microbial mat with no plant material. Samples were transported back to the laboratory and either frozen at -20 °C for DNA and RNA processing or aliquoted out for DMSP and DMS calculations. Each aliquot contained ~ 0.1 g wet weight of sediment analysed as described in Section 2.5.8.

2.9 *In vivo* DMSP analysis

2.9.1 Assaying for DMSP production in *E. coli*

To assay production of DMSP in *E. coli* containing pET16b, pMAL-c2x and pLMB509 plasmids, a starter culture of 10 ml LB was inoculated with a single *E. coli* colony from a freshly streaked plate and then grown at 37 °C overnight. 100 µL of this culture was used to inoculate triplicate 10 ml LB containing either 10 mM taurine or 0.1 mM IPTG as inducers, 0.5 mM methionine and 0.5 mM MTHB as substrates, and relevant antibiotics. The culture was grown overnight at 37 °C and then 200 µL of each culture in technical duplicates was assayed by GC (Section 2.7.1).

2.9.2 Assaying for DMSP and GB production in *L. aggregata*

To assay production of DMSP in *L. aggregata* containing pLMB509 or pRK415 constructs, a starter culture of 10 ml YTSS was prepared by inoculating the media with a *L. aggregata* colony from a freshly streaked plate. The starter culture was grown overnight at 30 °C. If the samples were tested in YTSS, 100 µL of this starter culture was inoculated in triplicate in 10 ml YTSS with either 10 mM taurine or 0.1 mM IPTG for plasmid induction, 0.5 mM methionine, 0.5 mM MTHB or 0.5 mM sarcosine for the substrate, and relevant antibiotics. The culture was grown overnight at 30 °C. 200 µL of each culture in technical duplicates was assayed by GC (Section 2.7.1). If samples were tested in MBM, then 1 ml of starter culture was centrifuged at 13,000 RPM and the pellet resuspended in 1 ml MBM. This was repeated three times. 100 µL of culture was inoculated into 10 ml MBM with the same induction, substrate and antibiotic parameters as mentioned above. The culture was grown for 2 days at 30 °C before 200 µL of each culture in technical duplicates was assayed by GC (Section 2.7.1).

2.10 Statistical analysis

Unless otherwise stated, all reported data are represented by mean values for three biological replicates with two technical replicates per test. Errors bars denote the standard error for that sample. For statistical significance, either a two-tailed paired Students T test or analysis of variance (ANOVA) test was carried out in GraphPad Prism, a P value < 0.05 demonstrating statistically significant differences. In cases of comparison between three or more groups and significant values from Levene's test, ANOVA was carried out, if Levene's test was not significant than Welch's test was used. Otherwise, if comparing between two groups in within-group variation and with

parametric data Students T test was used, otherwise for non-parametric data the Mann Whitney U Test was used.

Chapter 3 – Discovery of a novel MTHB methyltransferase (DsyC) in *Trichodesmium erythraeum* IMS101

3.1 Introduction

3.1.1 Environmental importance of *Trichodesmium* species

Trichodesmium are a genus of diazotrophic, planktonic, colony-forming cyanobacteria consistently found in oligotrophic waters that are warmer than 20 °C, such as the tropical and subtropical regions of the Atlantic, Pacific and Indian oceans (179–183). Here, *Trichodesmium* filaments aggregate into tufts which then combine to form extensive surface blooms which are brown in colour and visible to the naked eye (183).

Blooms occur when large populations of *Trichodesmium* become trapped at the ocean surface forming pigmented layers of dead and decaying cells (184). These blooms fertilise large areas via the release of carbon, nitrogen, and other important nutrients, which heterotrophic bacteria utilise (183). *Trichodesmium* further impacts the marine food web as a food source for copepods, fish, and tunicates (183). Some *Trichodesmium* species also produces and releases secondary metabolites, including toxins, which are harmful to animals and humans (185).

Trichodesmium species have a major role in nitrogen fixation and are one of the dominant diazotrophs in the oceans, fixing around 60 – 80 Tg N annually (183). They are responsible for up to 50 % of new nitrogen in some regions such as the subtropical North Pacific and North Atlantic (186). The model species, *Trichodesmium erythraeum* IMS101 (IMS101), has been shown to have a unique mechanism of nitrogen fixation utilising specialised cells termed diazocytes (187). These diazocytes are cells which have been specialised for nitrogen fixation and contain more gas vesicles and less storage compartments (188). This allows simultaneous nitrogen and carbon fixation via division of labour between different cell types (189). Furthermore, high energy

nitrogen and carbon fixation take place during the day, whereas cell division and diazocyte formation take place at night (190,191).

Contrary to popular opinion, some *Trichodesmium* species are non-diazotrophic and these *Trichodesmium* are abundant and widespread. Large scale metagenomic studies have elucidated some *Trichodesmium* genomes missing key genes related to nitrogen fixation (192). The species *T. miru* and *T. novis* in particular do not contain *nif* genes, demonstrating an evolution away from diazotrophy (192).

Many primary producers, including *Trichodesmium* species, have an associated heterotrophic community formed via attachment or physical colonisation (193). Carbon and nitrogen are passively transferred from host to epibiont and in return the host receives aid in acquiring trace metals, nutrients and reducing hazardous compounds. Many bacteria such as Alphaproteobacteria, Gammaproteobacteria and Bacteroidetes are consistently found with *Trichodesmium* (194). The gammaproteobacteria *Altermonas macleodii* is commonly found with *Trichodesmium* and aids with iron and phosphorous uptake (195). Furthermore, *A. macleodii* also catabolises carbon-based products from *Trichodesmium*, possibly including DMSP, since it contains the *dddP* gene which encodes the DddP DMSP lyase and the *dmdA* gene encoding the demethylase DmdA (195).

3.1.2 DMSP production in *T. erythraeum* IMS101

Multiple studies have been conducted on DMSP production in cyanobacteria with *T. erythraeum* being a particular focus. Concentrations of DMSP in IMS101 were measured in Fe-replete versus Fe-deplete conditions. A 45-fold increase in DMSP production per trichome under iron limitation from 0.05 to 2.27 mmol L⁻¹trichome⁻¹ was observed, suggesting this is a key regulator in DMSP production (130). Iron is a key element used in the active sites of enzymes involved in photosynthesis and nitrogen fixation and limitation likely leads to cellular stress and therefore an increase in DMSP production (130), possibly to counteract an increase in reactive oxygen species (ROS) (196).

Phosphorous limitation also results in an increase in DMSP production in IMS101. DMSP production per trichome in replete conditions versus phosphorus limitation led

to an increase from 0.03 to 0.18 nmol DMSP ($\mu\text{g Chl a}$)⁻¹ (195). In this case it was suggested that IMS101 may be producing DMSP as a nutrient source for *A. macleodii*, which in turn produces siderophores, aiding in the acquisition of phosphorus (195). However, IMS101 does not encode any known genes encoding DMSP biosynthetic enzymes and novel ones were not identified in these studies.

IMS101 under iron and phosphorus limitation produced more DMSP than cultured single-celled and other filamentous cyanobacteria. For example, DMSP production measured in 29 benthic filamentous cyanobacteria, grown in ASN_{III}/2 medium, ranged from 0.007 mmol DMSP ($\text{g}^{-1} \text{ chl a}$) to 0.583 mmol DMSP ($\text{g}^{-1} \text{ chl a}$) (126). Additionally, DMSP production in *Synechococcus* and *Prochlorococcus* surveyed from the Atlantic Ocean, Pacific Ocean and Mediterranean Sea ranged from $2.8 \times 10^{-4} \pm 1.8 \times 10^{-4}$ and $5.1 \times 10^{-3} \pm 7.8 \times 10^{-3} \text{ fg DMSP cell}^{-1}$, respectively (125). However, neither the filamentous nor single celled cyanobacteria were tested under conditions which typically increase DMSP production such as low nitrogen or higher salinities.

3.1.3 Screening for novel gene targets through bioinformatics

A variety of techniques have been used to screen for genes involved in DMSP production such as library screening, RNA-Sequencing (RNA-seq) analysis and homology analysis to previously ratified genes. Library screening has been used in previous DMSP studies to identify both synthesis and catabolism proteins (11,17). A genomic library is constructed by digesting the genomic DNA of the species of interest with a common restriction enzyme such as *EcoR*1 (197). This generates DNA fragments of multiple kbs in length. These are then ligated into a cosmid, which is a plasmid vector designed for handling large inserts and expressed in a heterologous host. This host is then screened for activity and any cosmids that show activity can be sequenced to identify target genes.

RNA-Seq analysis is another method which can detect potential gene targets. In this case, it involves growing up live cultures of a microorganism of interest under different regulatory conditions which may increase or decrease expression of genes related to the process of interest, i.e. high vs low DMSP production conditions. RNA is harvested from the cultures and sent for high-throughput sequencing (198). Genes which are

upregulated under the conditions of interest are then analysed and potential target genes can be identified.

However, library screening and RNA-Seq depend on having access to live cultures and if this is not possible, homology searching may be the only option. Sequence similarity searching with packages such as BLAST is the most widely used and most reliable strategy for characterising sequence targets (199). There are already several reported and published proteins involved in DMSP synthesis which can be used for homology searching. All these proteins are methyltransferases and catalyse the rate-limiting and committing step of DMSP synthesis (7). However, it is challenging to identify methyltransferases specifically involved in DMSP production, since motifs binding of its precursors have not been identified (18). Expression and characterization of enzyme kinetics are then required to determine if a methyltransferase is involved in DMSP synthesis.

As reported earlier, *T. erythraeum* has been shown to produce DMSP in culture; however, the genes and proteins involved in this production have not been elucidated, and targets for DMSP production are currently not known.

3.1.4 Verifying methyltransferases involved in DMSP biosynthesis via complementation of a *Labrenzia aggregata* LZB033 *dsyB* mutant

Labrenzia aggregata LZB033 (LZB033) is an abundant marine alphaproteobacterium first isolated from the East China Sea (11). It was the first species in which a bacterial MTHB methyltransferase enzyme (*DsyB*) was identified and led to a paradigm shift in the recognition of bacteria as significant global contributors to DMSP production (11). LZB033 has now become a model organism used in identifying novel DMSP-producing enzymes due to its quick growth time, ability to synthesis both DMSP and DMS and its genetic versatility. This versatility has led to the generation of several mutants which are essential to complementation assays, including *dsyB*, *GSDMT* and a double $\Delta dsyB/GSDMT$ strain (157), which can be used to identify novel enzymes involved in DMSP or GB synthesis.

Complementation has been used before to ratify function of methyltransferases linked to DMSP production (28). To test for complementary activity, the target gene is cloned

into a plasmid (pLMB509) which can grow in the host strain (LZB033). This is transformed into donor *E. coli* which can donate the plasmid into the host strain. The donor *E. coli* is grown with a helper *E. coli* (pRK2013) which has a plasmid that generates F-pili to facilitate the transfer of the plasmid to the host. All three strains are grown together and then the correctly conjugated host containing the plasmid of interest is selected for with antibiotics.

3.1.5 Aims of the chapter

The primary aim of this chapter is to identify the methyltransferase involved in the key committing step of DMSP synthesis in *T. erythraeum* IMS101. IMS101 was chosen as the model organism due to being the only cyanobacteria with significant reported levels of DMSP production under regulation (130,195) and its abundance in the oceans. However, no genes or proteins have been identified as the cause of DMSP production in IMS101 and the associated DMSP production pathway has not been elucidated. The sub-aims are as follows:

- Use homology-based searching to identify potential candidates for the key methyltransferase in *T. erythraeum* IMS101.
- Identify which pathway *T. erythraeum* IMS101 uses to synthesise DMSP.
- Confirm the activity of any potential methyltransferases in DMSP production via *in vitro* and *in vivo* assays using gas chromatography.

3.2 Results

3.2.1 Identification of a target methyltransferase in *T. erythraeum* IMS101

The IMS101 genome has previously been sequenced (GCA_018457135.1) and therefore it is possible to search via NCBI BLAST with previously ratified DMSP synthesis methyltransferases (**Table 3.1**) to identify targets. The proteins that were tested are summarized in **Table 3.1**, with the majority not resulting in hits with high

similarity. Only a homolog to TpMMT from the filamentous cyanobacterium *Moorea producens* (personal communication), demonstrated close similarity (76 % identity) to a putative SAM-dependent Type 11 methyltransferase, encoded by the gene *Tery_2447*, in *T. erythraeum*.

Table 3.1. Blast outputs of known ratified methyltransferase genes involved in DMSP synthesis against the *T. erythraeum* IMS101 genome. Details of the gene with highest homology is indicated. All hits were to locus ID *Tery_2447*.

Organism	Enzyme	AA % Identity	Coverage	E value	Reference
<i>Thalassiosira pseudonana</i>	TpMMT	24.15%	86%	6.00E-22	Unpublished
<i>Novosphingobium</i> sp. BW1	MmtN	45.00%	6%	3.2	(34)
<i>Chrysochromulina tabinii</i>	DSYB	24.28%	44%	4.00E-10	(28)
<i>Labrenzia aggregata</i>	DsyB	33.33%	37%	1.00E-18	(11)
<i>Moorea producens</i>	Putative SAM-dependent methyltransferase	76.84%	97%	2.00E-160	Unpublished

This protein, tentatively termed DsyC (DMSP synthesis in cyanobacteria), alongside other ratified and putative methyltransferases involved in DMSP synthesis, were subjected to phylogenetic analysis to identify specific clades (**Fig. 3.1**). IMS101 DsyC is a member of its own, distinct clade which includes genes from other cyanobacteria including *Lyngbya aestuarii*, multiple *Synechococcus* and *Prochlorococcus* sp., in addition to heterotrophic bacteria such as *Parvibaculum* sp. and *Alteriqa pengyuania* sp.. This suggests that DsyC is a novel and previously unidentified methyltransferase. However, it is most similar to the prokaryotic MTHB methyltransferase DsyG (200), the GB synthesis methyltransferases TpMMT (unpublished) and the MTHB methyltransferase DsyE, which is also found in some filamentous cyanobacteria (200). Interestingly, DsyC is found in a cluster with methyltransferases that are part of the transamination DMSP synthesis pathway and methylate MTHB through to DMSHB. Furthermore, DsyC is also found in a cluster with methyltransferases of the *de novo* methylation GB synthesis pathway which can catalyse the full conversion of glycine to sarcosine to dimethylglycine and then GB.

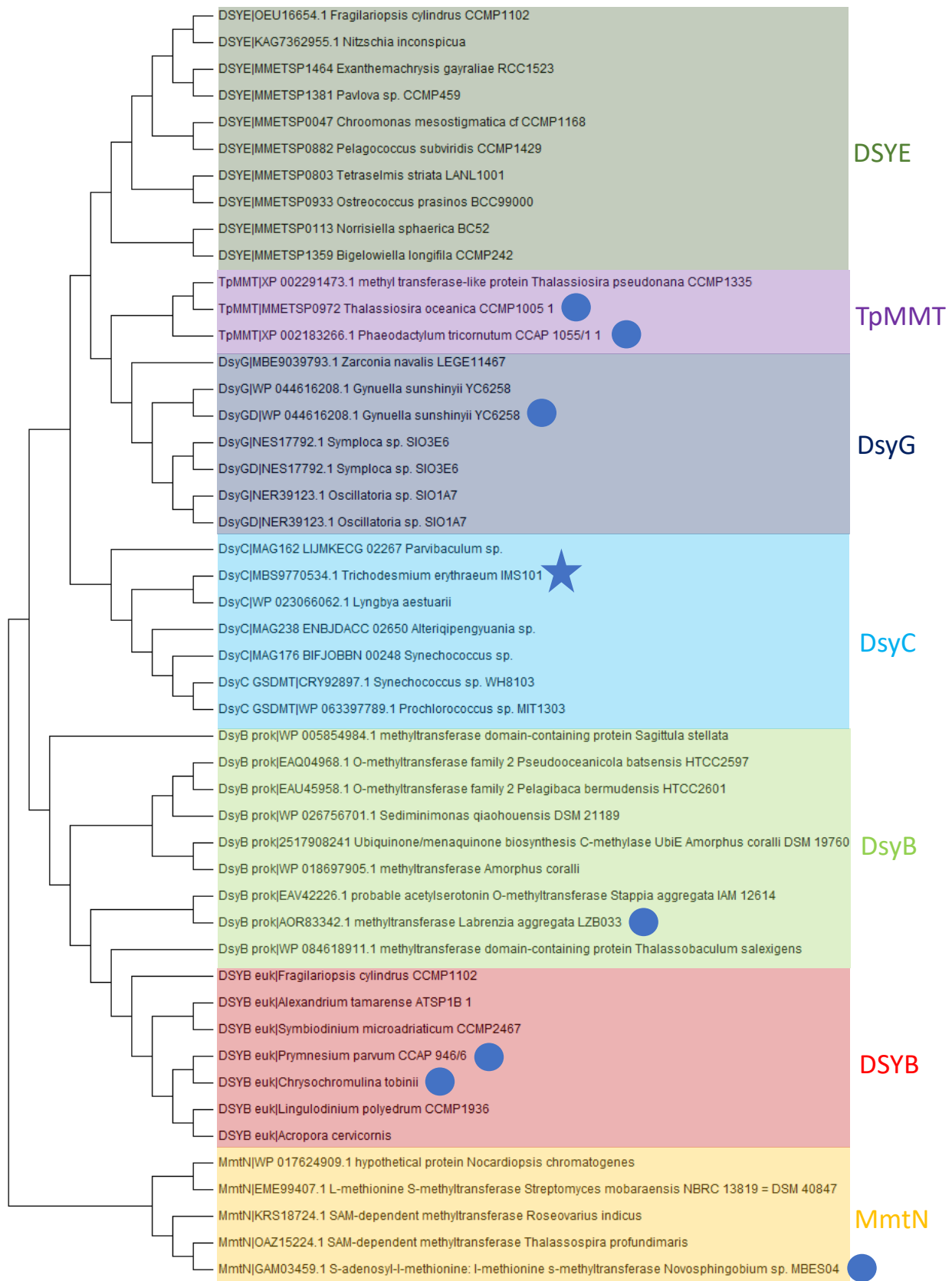


Fig. 3.1 Phylogenetic tree of known DMSP synthesis enzymes. Amino acid tree of homology based on Maximum Likelihood, Bootstraps = 100. Clades are indicated by different colours. *T. erythraeum* IMS101 DsyC is highlighted by the star. Ratified enzymes are highlighted with a circle.

3.2.2 Determining in vivo activity of *T. erythraeum* IMS101 DsyC in *E. coli*

Following identification of the target methyltransferase, codon-optimised *dsyC* gene was subcloned into the IPTG-inducible, expression plasmids pET21a and pET16b for protein expression (**Fig. 3.2 – 3.3**) and confirmation of enzyme activity. The plasmids were transformed into *E. coli* BL21 (DE3), expression was subsequently induced with 0.1 μ M IPTG, and MTHB supplied to determine whether DMSHB was synthesised. *E. coli* containing pET21a only was unable to produce any DMSHB. However, *E. coli* transformed with pET21a-*dsyC* produced approximately 18 pmol DMSP μ g⁻¹ protein and *E. coli* transformed with pET16b-*dsyC* produced around 17 pmol DMSP μ g⁻¹ protein (**Fig. 3.4**). Interestingly, when both the pET21a and pET16b containing *dsyC* was supplied with IPTG they produced far lower quantities of DMSHB, around 3 and 5 pmol DMSP μ g⁻¹ protein, respectively. The reason for this is unclear but may be due to overexpression leading to insoluble inclusion bodies which would render the protein dysfunctional. There was a statistically significant difference between the levels of DMSHB produced between both plasmids containing *dsyC* and the empty pET21a vector ($P < 0.05$, Welch's t-test).

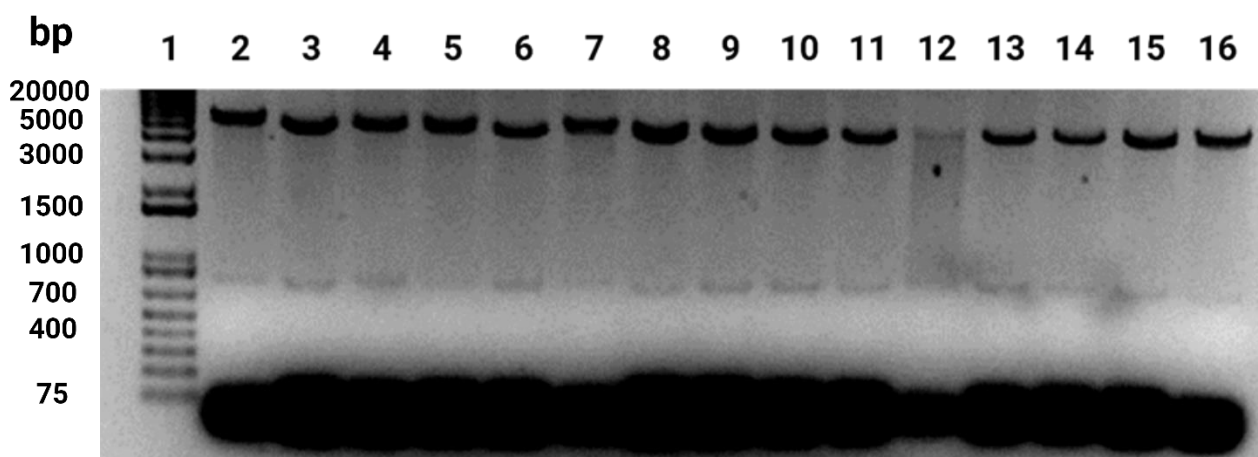


Fig. 3.2 *T. erythraeum* IMS101 *dsyC* cloned into pET21a and pET16b vectors. Lane 1: 1 Kb+ Hyperladder, lanes 2 – 8: pET21a-*dsyC* digested with *EcoR*1 and *Nde*1 (Expected sizes: 5397 bp and 840 bp), lanes 9 – 16: pET16b-*dsyC* digested with *EcoR*1 and *Nde*1 (Expected sizes: 5378 bp and 840 bp).

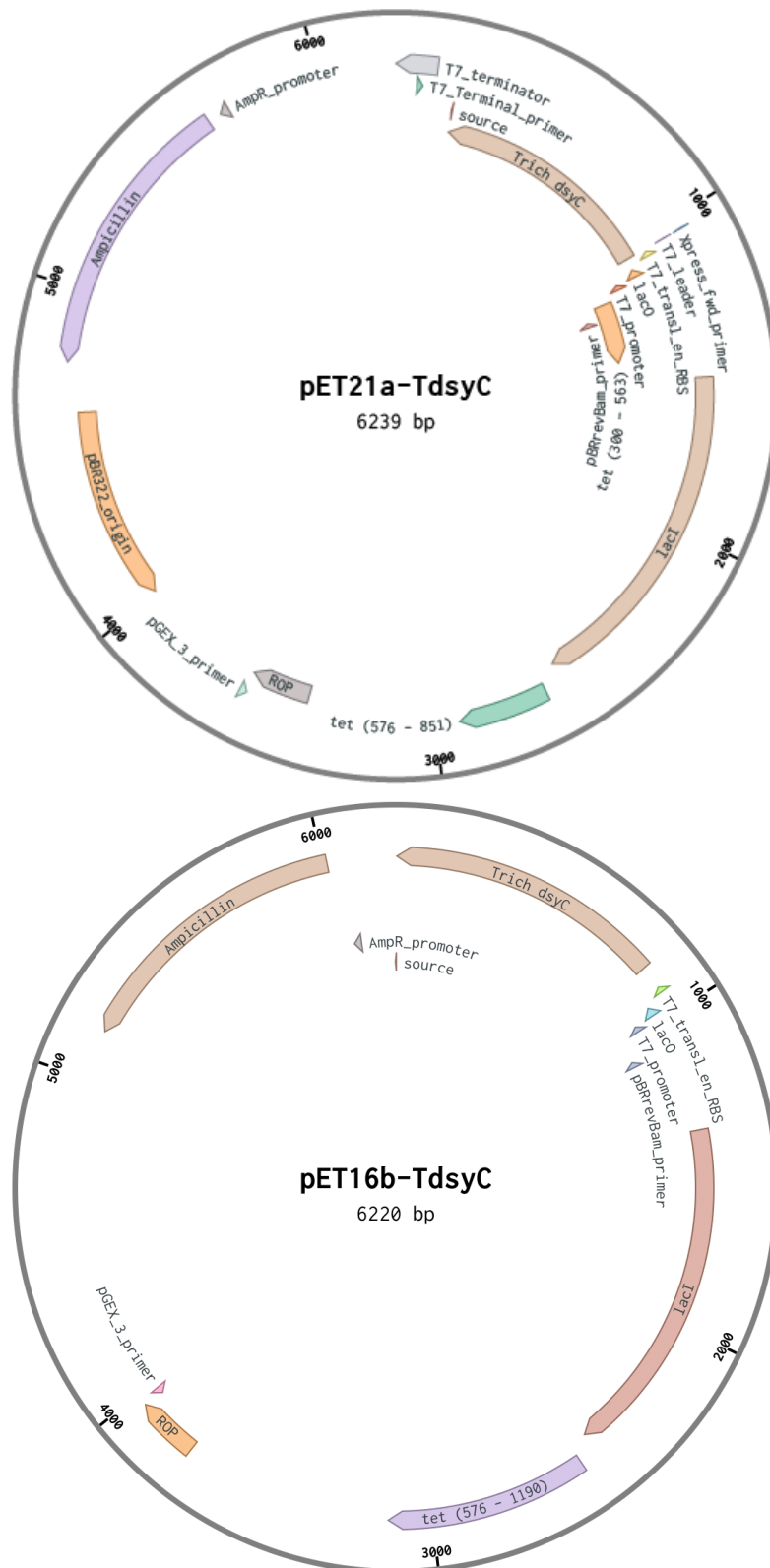


Fig. 3.3 *In silico* constructs of IMS101 *dsyC* in pET21a and pET16b. **A)** pET21a with IMS101 *dsyC* cloned downstream of T7 promoter. **B)** pET16b with IMS101 *dsyC* cloned downstream of T7 promoter.

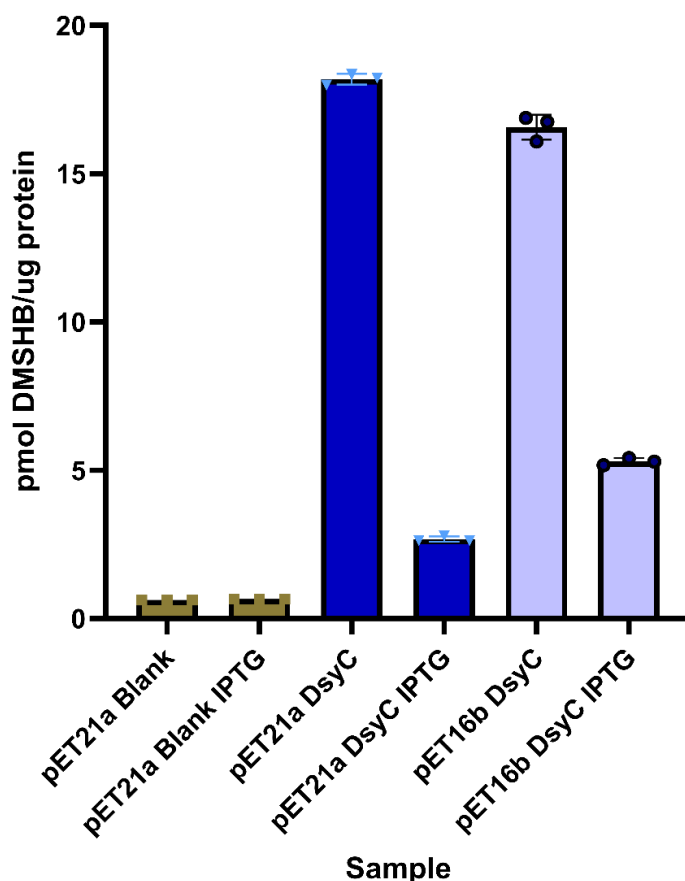


Fig. 3.4 DMSHB production in *E. coli* strains expressing DsyC in pET21a and pET16b. DsyC was assayed for its ability to methylate MTHB into DMSHB in *E. coli* strains containing empty vectors or *dsyC* expressed in either pET21a or pET16b.

3.2.3 Identification of homologous DsyC proteins in other cyanobacteria

Following identification of MTHB methylation activity in IMS101 DsyC, we checked how widespread this protein was in the environment by searching for homologous proteins in other cyanobacteria. We expanded the DsyC clade found in **Fig. 3.1** and the closest 100 hits to IMS101 DsyC were aligned using ClustalW. These were then used to construct a maximum likelihood phylogenetic tree (**Fig. 3.5**). Of greatest similarity to IMS101 DsyC were homologs found in other filamentous cyanobacteria such as *Moorena*, *Symploca*, *Okeania* and *Hormoscilla*. For example, *Okeania* (6 species) share ~86 % identity, *Hormoscilla* (4 species) are ~79 % similar, *Symploca* (6 species) are ~77 % similar and *Moorena* (14 species) are around ~76 % identical. Homologs to DsyC were also present in other strains of *T. erythraeum* including ALOHA_ZT_67, st18_bin1 and MAG_R01. Interestingly, there were no hits for DsyC in any of the other *Trichodesmium* species such as *T. thiebautii* and *T. hildebrandtii*. The wider implications of **Fig. 3.5** are that there may be common ancestry between

DsyC in *T. erythraeum* and homologs found in other filamentous and single celled cyanobacteria.

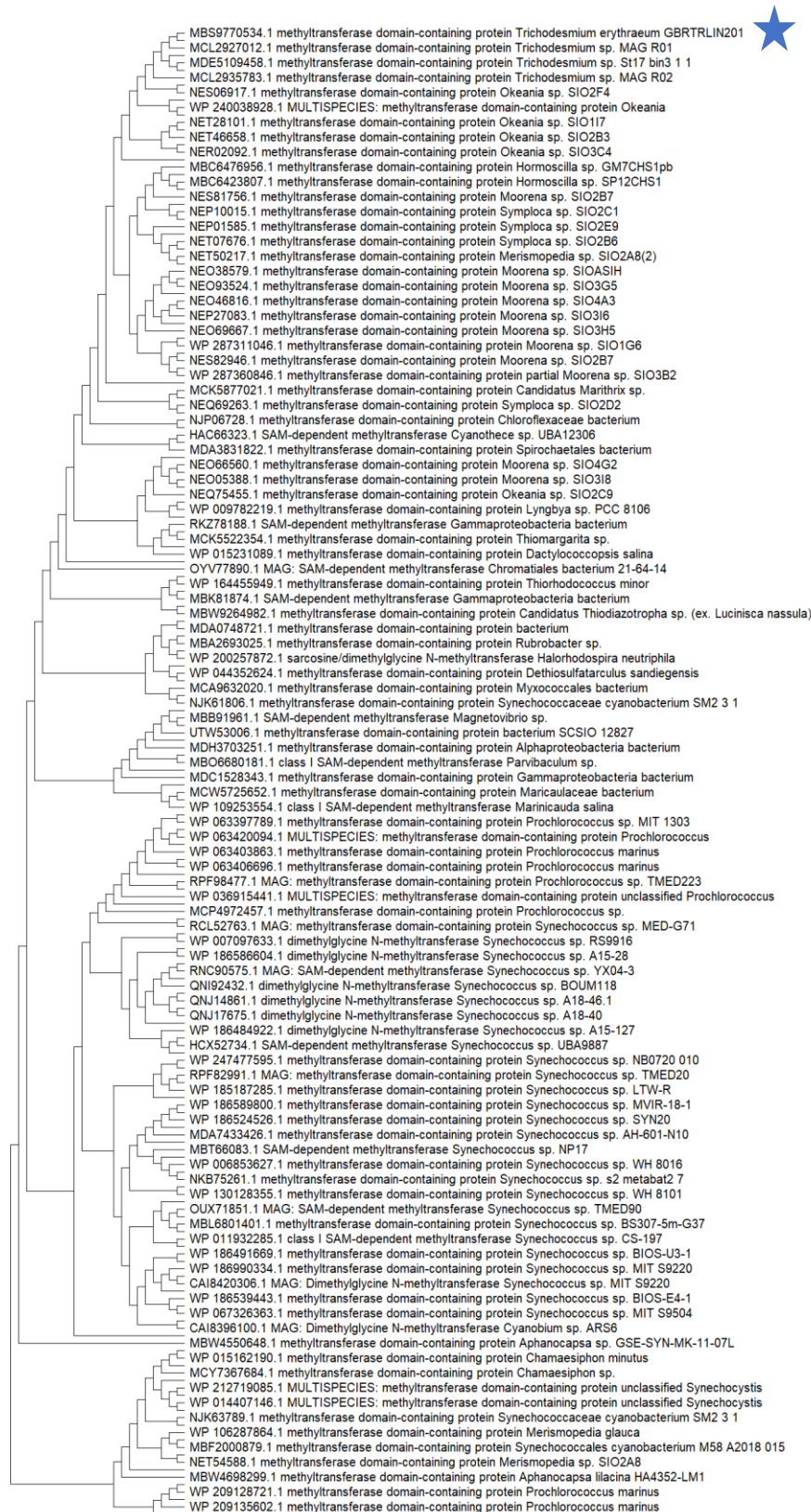


Fig. 3.5 Maximum likelihood phylogenetic tree of *T. erythraeum* IMS101 DsyC against other cyanobacteria. IMS101 DsyC is highlighted with a star.

Homologs to DsyC were also found in *Synechococcus* and *Prochlorococcus* species, although these had lower similarity compared to the prementioned filamentous cyanobacteria DsyC homologs. Homologs were present in multiple *Synechococcus* species from different clades. However, it was only identified in Low Light IV (LLIV) clade *Prochlorococcus* species. The *Synechococcus* and *Prochlorococcus* DsyC homologs were ~39 % similar in identity to IMS101 DsyC. The DsyC homolog in *Synechococcus* WH8102 has already been ratified as a sarcosine-dimethylglycine methyltransferase (SDMT) involved in the GB synthesis pathway (74).

To better understand the relationship between IMS101 DsyC and the DsyC homologs found in *Synechococcus* and *Prochlorococcus* we analysed the synteny between the *dsyC* and *dsyC* homolog gene neighbourhoods (**Fig. 3.6**). IMS101 *dsyC* contains no genes in its neighbourhood which have ratified roles in DMSP or GB metabolism. This is in contrast with the gene neighbourhoods found in *Synechococcus* and *Prochlorococcus*, which are part of the *GSDMT* complex involved in GB synthesis. Immediately upstream of the *GSDMT* genes in *Synechococcus* and *Prochlorococcus* are 3 genes which encode for a putative Betaine-Choline-Carnitine-Transporter (BCCT) likely involved in GB and DMSP transport (44).

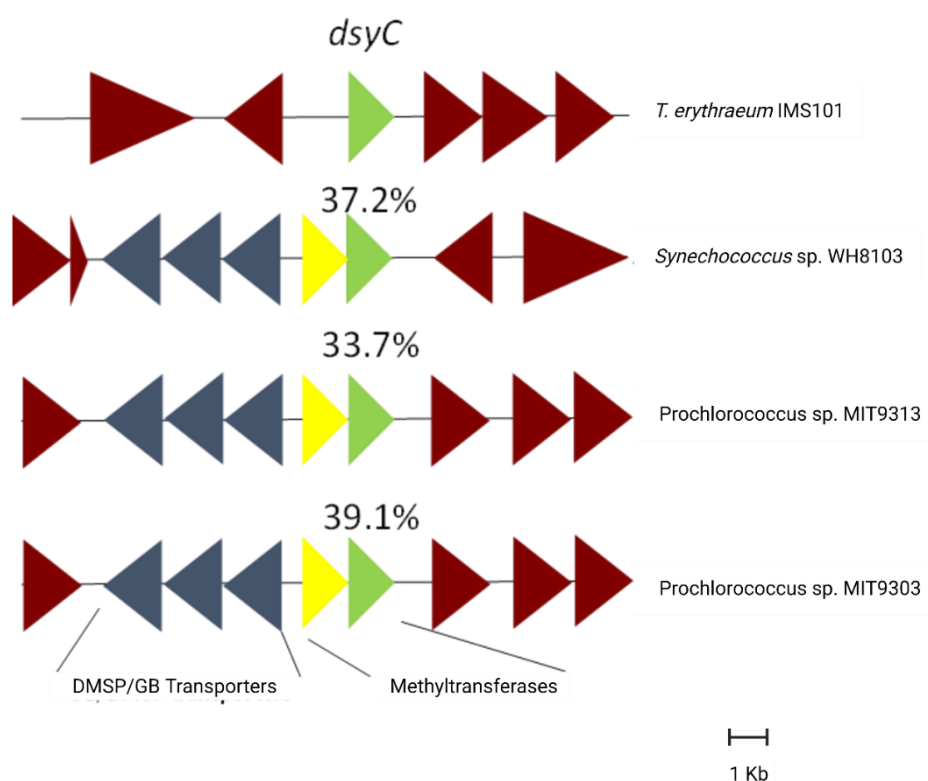


Fig. 3.6 Chromosomal region of IMS101 *dsyC* and putative *dsyC* from *Synechococcus* sp. WH8103, *Prochlorococcus* sp. MIT9313 and *Prochlorococcus* sp. MIT9303. The brown shapes denote genes not known to be involved in DMSP or GB metabolism. Purple triangles denote genes encoding for putative BCCTs. Green triangles indicate *dsyC* or putative *dsyC* homologs with amino acid percentage identity compared to IMS101 *dsyC* shown. The yellow triangle is a *GSMT* gene encoding for a putative GSMT methyltransferase.

3.2.4 Overexpression and purification of *T. erythraeum* IMS101 DsyC

Following identification of MTHB activity in *E. coli* expressing DsyC, we subsequently purified the protein to determine its enzyme kinetics. First, the overexpression system was optimised to obtain maximum amounts of soluble and active DsyC protein (**Fig. 3.7**). Parameters such as temperature (26 °C, 28 °C and 30 °C), IPTG concentration (0.025 mM, 0.05 mM and 0.1 mM) and time (4 h, 6 h and 16 h) under induction were altered to identify which combination led to the highest concentration of active and soluble DsyC, as determined via evaluation of DMSHB production. Overall, none of the adjusted parameters led to higher amounts of active protein (**Fig. 3.7**) than the initial conditions tested (30 °C, 0.1 mM IPTG, 16 h). Production was not observed under 4 and 6 hour growth parameters, suggesting that DsyC has slower reactivity than DsyB (11) and DSYB (28). Overall, this suggests that the pET expression system was very inefficient at producing high amounts of functional protein.

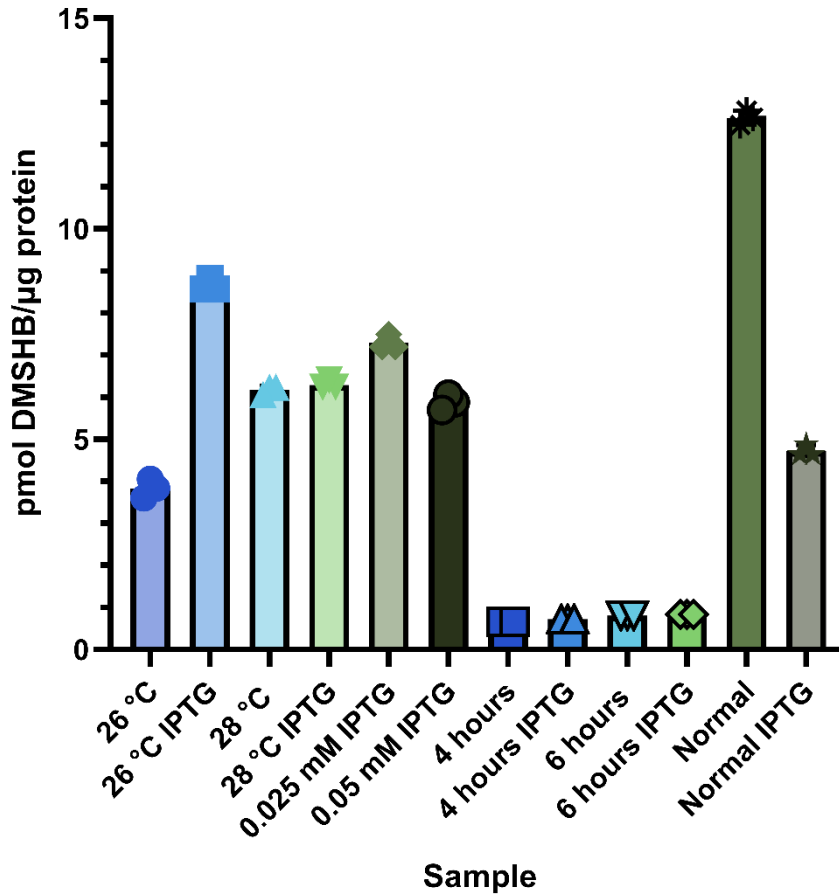


Fig. 3.7 Optimisation of DsyC expression in pET16b vector. The standard conditions to which other samples were compared to were 0.1 mM IPTG, 30 °C and culturing for 16 hours. Differences in these conditions are indicated in the x-axis.

Afterwards, we changed to the pMALC2X system and began purifying DsyC using a maltose binding protein (MBP), which aids in protein solubility. First, both IMS101 DsyC and WH8103 DsyC was cloned into pMALC2X via GoldenGate assembly (**Fig. 3.8**).

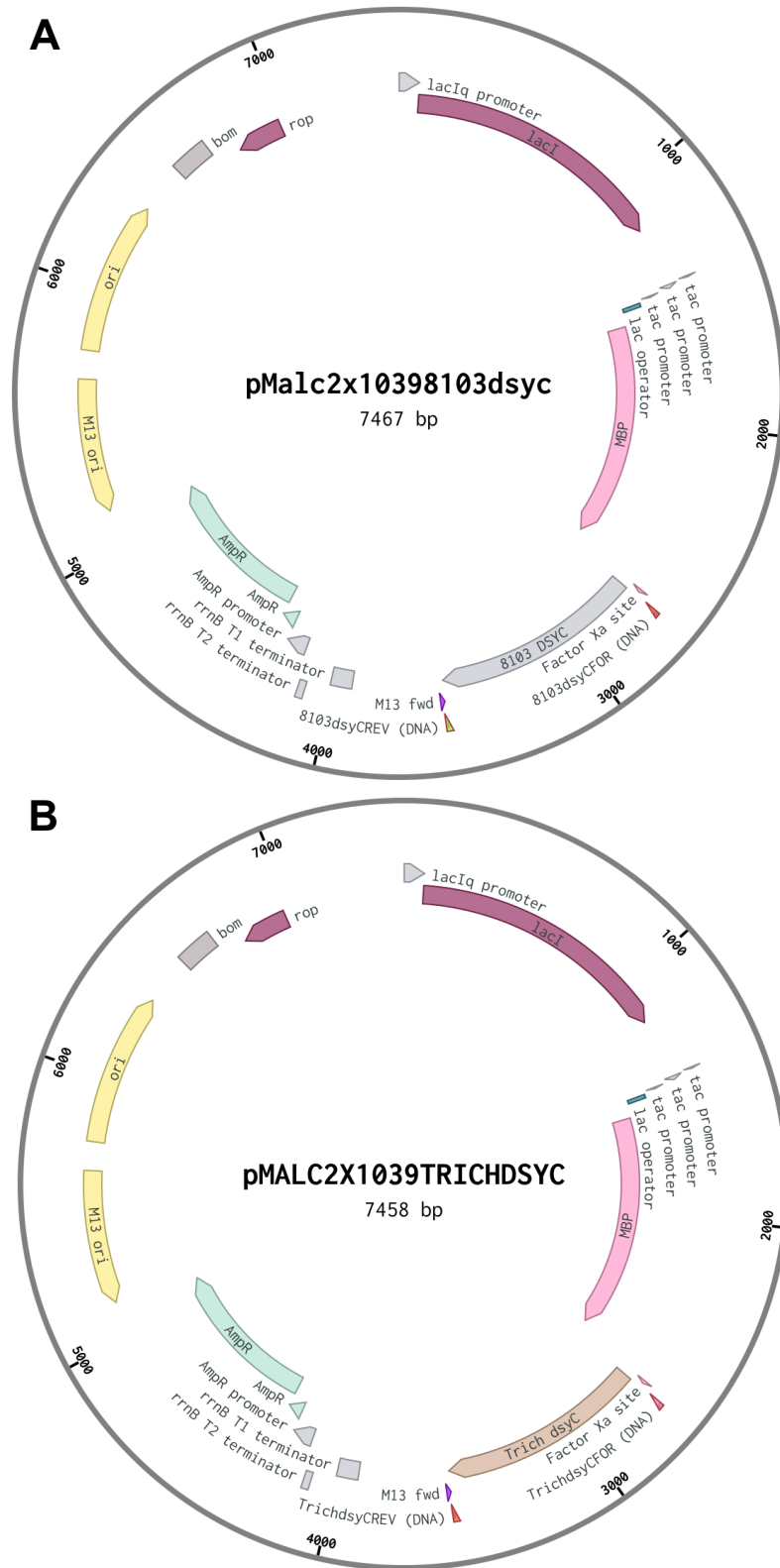


Fig. 3.8 Synthetic constructs of pMALC2X-1039 with DsyC. A) WH8103 DsyC cloned into pMALC2X-1039. **B)** IMS101 DsyC cloned into pMALC2X-1039.

E. coli containing pMALC2X with IMS101 *dsyC* and WH8103 *dsyC* was grown and optimised for production of DMSHB (**Fig. 3.9**). Parameters such as temperature (16 °C, 24 °C and 30 °C) and IPTG concentration (0.1 mM, 0.25 mM and 0.5 mM) were checked. Overall, both DsyC's produced more DMSHB than the negative control (BL21:DE3). IMS101 DsyC was more active towards methylating MTHB than WH8103 DsyC. Additionally, IMS101 DsyC gradually increased in activity with a higher concentration of IPTG and higher temperatures. Conversely, WH8103 DsyC's activity remained constant across all changes in IPTG concentration and temperature of incubation, except for a slight increase in activity when grown at 24 °C.

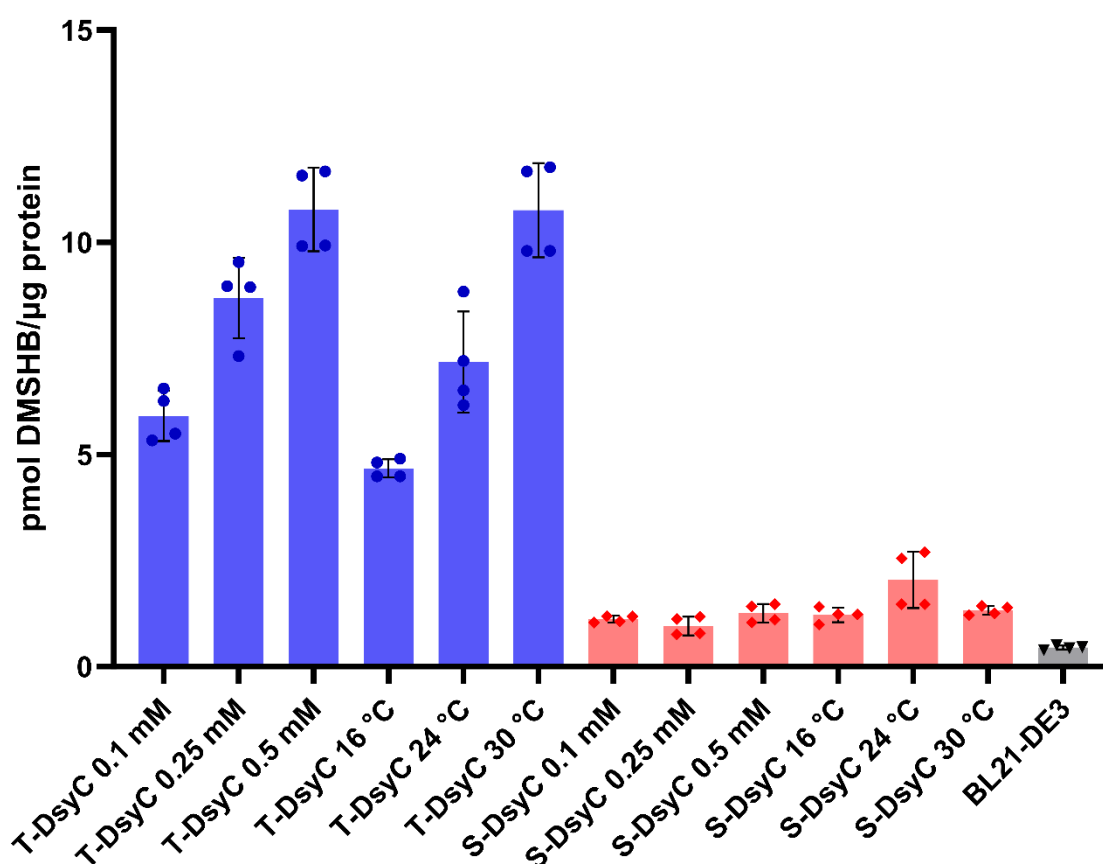


Fig. 3.9 Optimisation of DsyC expression in pMALC2X-1039 vector. The normal conditions to which other samples were compared to were 0.1 mM IPTG and 30 °C. All samples were cultured for 16 hours. Differences in these conditions are indicated in the x-axis. T-DsyC = IMS101 DsyC, S-DsyC = *Synechococcus* sp. WH8103 DsyC.

Afterwards, the cultures were grown under their preferred parameters and lysed to separate the insoluble cell debris from the soluble fractions with functional DsyC, which was then passed through an amylose column. A band corresponding to the expected size of MBP tagged T-DsyC and S-DsyC (75 kDa and 77 kDa respectively) was observed in the soluble, insoluble and purified fractions (**Fig. 3.10**). Normally DsyC proteins are located in the cytosol and in the context of DsyC would have size of 34 kDa. Purification of DsyC removed other proteins, with the only other remaining band being the same size as the MBP (~37 kDa).

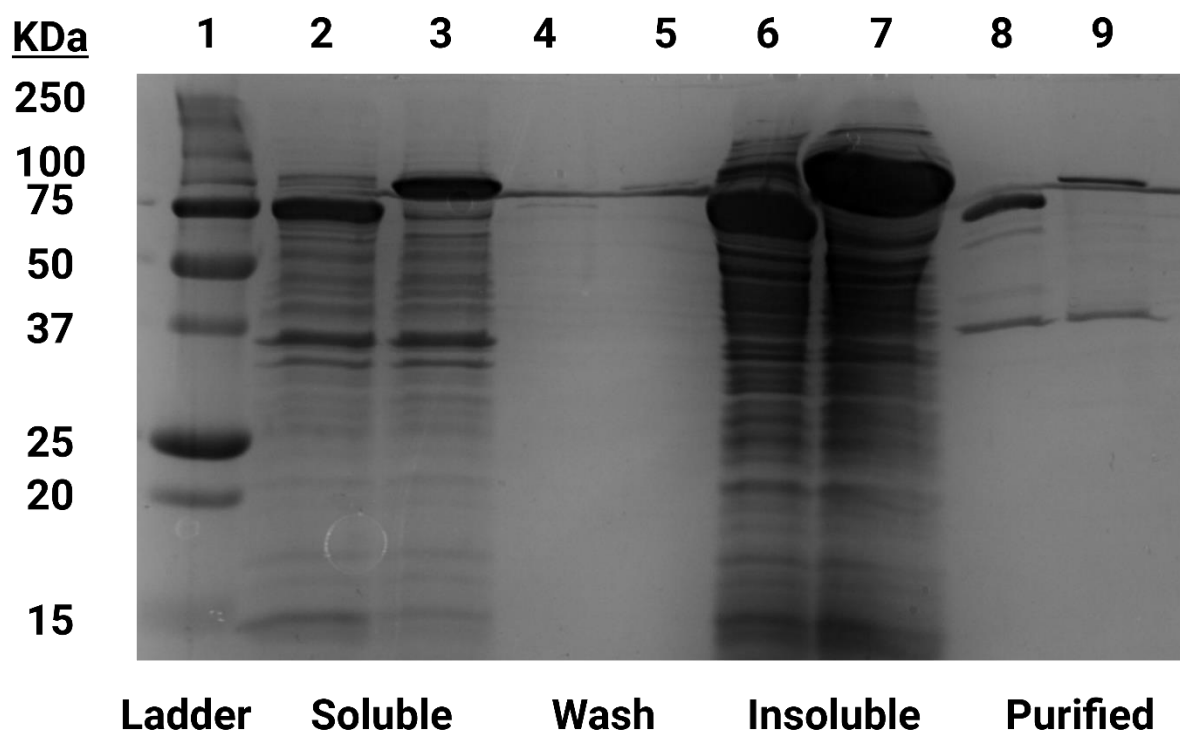


Fig. 3.10 Expression of DsyC in *E. coli* cultures containing pMALC2X-1039. Expected band size for MBP-tagged T-DsyC was 75 kDa and MBP-tagged S-DsyC was 77 kDa. Lane 1: Dual protein plus ladder, Lanes 2 + 3: Soluble fraction of T-DsyC (2) and S-DsyC (3). Lanes 4 + 5: Wash fraction of T-DsyC (4) and S-DsyC (5). Lanes 6 + 7: Insoluble fraction of T-DsyC (6) and S-DsyC (7). Lanes 8 + 9: Purified fraction of T-DsyC (8) and S-DsyC (9).

The purified IMS101 and WH8103 DsyC extract was active when assayed for MTHB methylation to DMSHB (**Fig. 3.11**). Both soluble and purified fractions of the DsyC purification produced significantly ($P < 0.05$, Welch's T test) more DMSHB than the

negative controls (MTHB and SAM). Higher levels of production were seen in IMS101 DsyC than WH8103 DsyC. However, both DsyC's had lower activity in purified fractions. This may have been due to the addition of DTT.

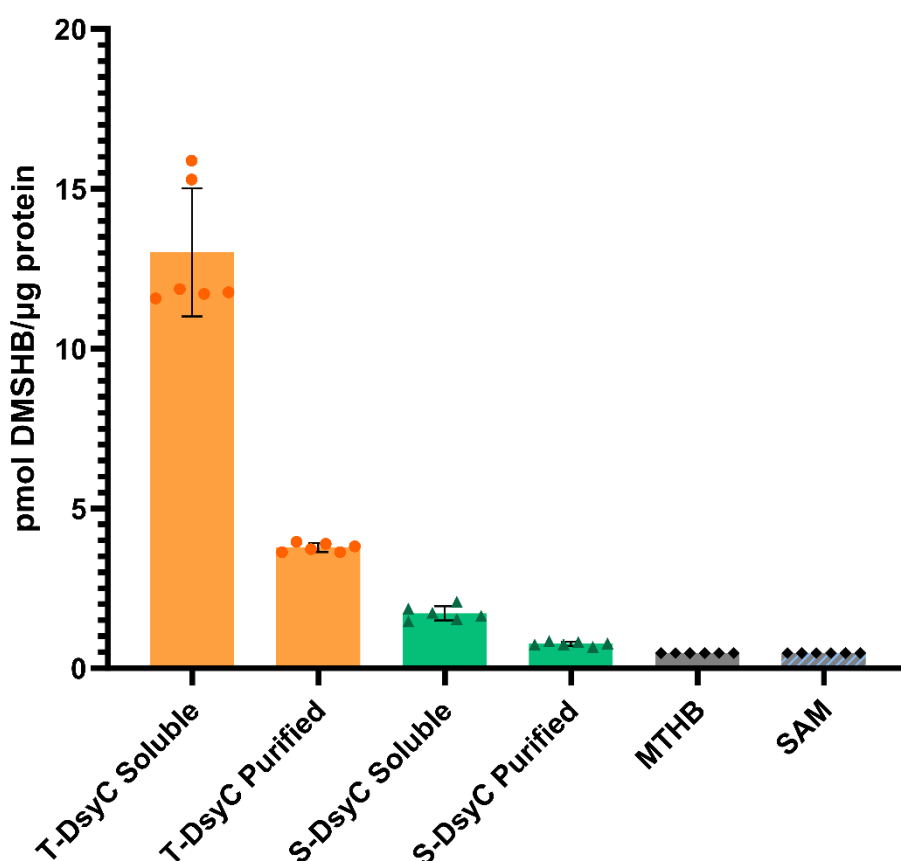


Fig. 3.11 Activity of purified IMS101 and WH8103 DsyC. All protein samples contained MTHB and SAM. The purified protein samples (T-DsyC purified and S-DsyC purified) required the addition of 0.1M dithiothreitol (DTT) for activity.

3.2.5 Assaying for DMSP production in *T. erythraeum* IMS101

We attempted to grow cultures of IMS101 which were generously provided by Tracey Lawson, Essex. However, the culture failed to survive longer than 1 month. Therefore, we were unable to obtain sufficient quantities of culture necessary to run experiments to test production of DMSP under iron/phosphorous replete and deprived conditions. Simultaneously, the Lawson laboratory were also unable to keep their cultures alive.

Successive attempts to order fresh cultures of IMS101 from the Woods Hole Culture Collection failed due to the strains being held at customs for an extended period resulting in unviable cultures upon delivery.

3.2.6 Complementation of *L. aggregata* LZB033 Δ dsyB with *T. erythraeum* IMS101 and *Synechococcus* sp. WH8103 dsyC

Following identification of putative DsyC homologs in multiple *Synechococcus* and *Prochlorococcus* species, we synthesized and tested a DsyC homolog from *Synechococcus* sp. WH8103 (WH8103) via complementation of a LZB033 *dsyB* mutant. WH8103 putative *dsyC* and IMS101 *dsyC* were cloned into the wide host range, taurine-inducible, expression plasmid pLMB509 (Fig. 12).

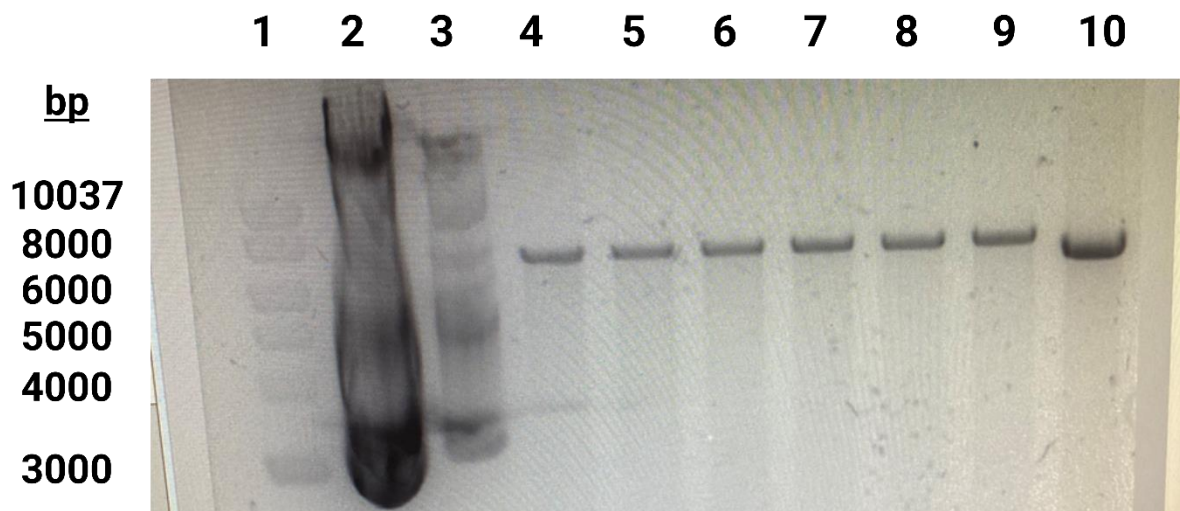


Fig. 3.12 Cloning of IMS101 and WH8103 *dsyC* into pLMB509. Lane 1: 1 kb+ Hyperladder. Lanes 2-3: Undigested pLMB509, Lanes 4-6: pLMB509-T*dsyC*, Lanes 7-9: pLMB509-W*dsyC*, Lane 10: Digested pLMB509. Constructs (pLMB509 – *dsyC*) and pLMB509 digested with *EcoR*I. Expected size of pLMB509-T*dsyC* was 6781 bp. Expected size of pLMB509-W*dsyC* was 6790 bp. Size of pLMB509 was 6162 bp.

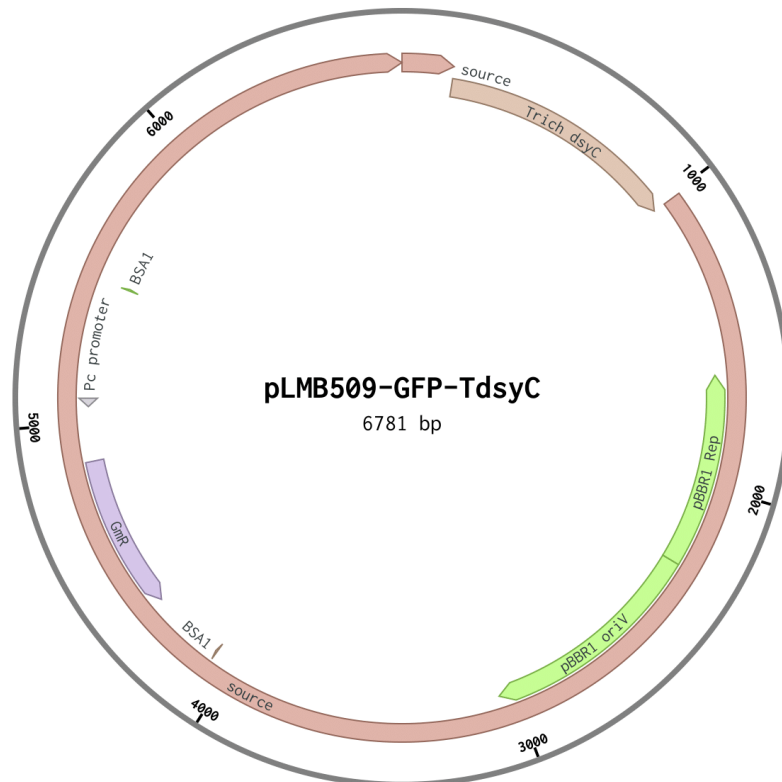
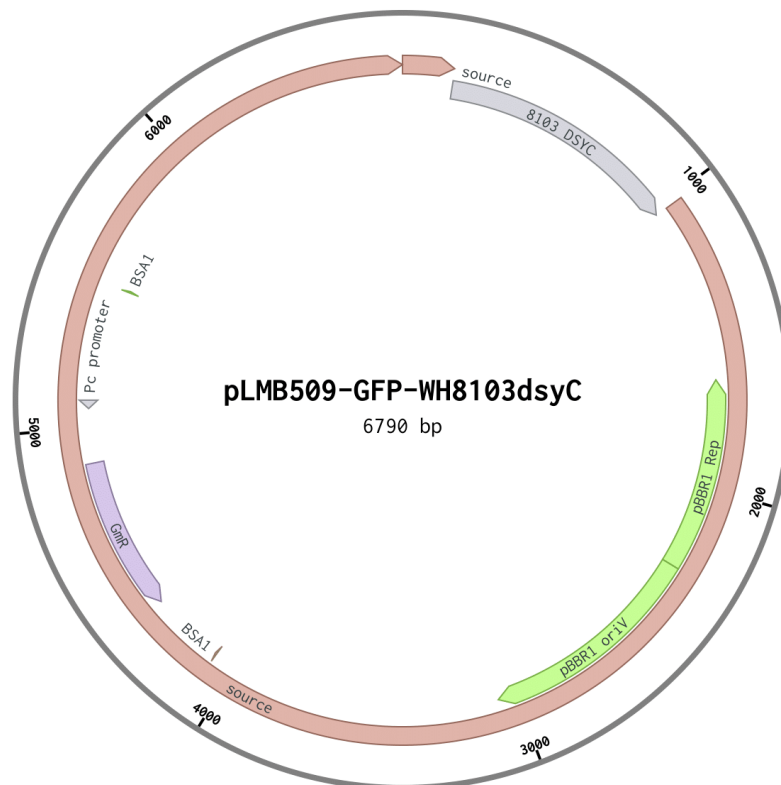
A**B**

Fig. 3.13 *In silico* constructs of IMS101 and WH8103 *dsyC* in plmb509. A) pLMB509 with IMS101 *dsyC* cloned downstream of T7 promoter. **B)** pLMB509 with WH8103 *dsyC* cloned downstream of T7 promoter.

Both pLMB509-TdsyC and pLMB509-WdsyC were crossed into LZB033 via conjugation and assayed for activity towards MTHB. At the same time *dsyB* from LZB033, *DSYB* from *Chrysochromulina tobinii* and *DSYD* from *P. tricornutum* were crossed into $\Delta dsyB$ LZB033 to compare expression to IMS101 and WH8103 DsyC (Fig. 3.14). In comparison to the negative control, *L. aggregata* LZB033 $\Delta dsyB$, all samples tested produced significantly higher levels of DMSP ($P < 0.05$, Welch's T-test). The highest levels of production were seen from *C. tobinii* *DSYB* at ~400 pmol DMSP ($\mu\text{g protein}^{-1}$), followed by *L. aggregata* LZB033 *dsyB* from at ~350 pmol DMSP μg^{-1} , *dsyC* from IMS101 at ~320 pmol DMSP ($\mu\text{g protein}^{-1}$) and then wild-type *L. aggregata* LZB033 at ~300 pmol DMSP ($\mu\text{g protein}^{-1}$). *DSYD* from *P. tricornutum* produced around 10 pmol DMSP ($\mu\text{g protein}^{-1}$) and *dsyC* from *Synechococcus* sp. WH8103 synthesised only a little more than *L. aggregata* LZB033 $\Delta dsyB$, ~3 pmol DMSP ($\mu\text{g protein}^{-1}$).

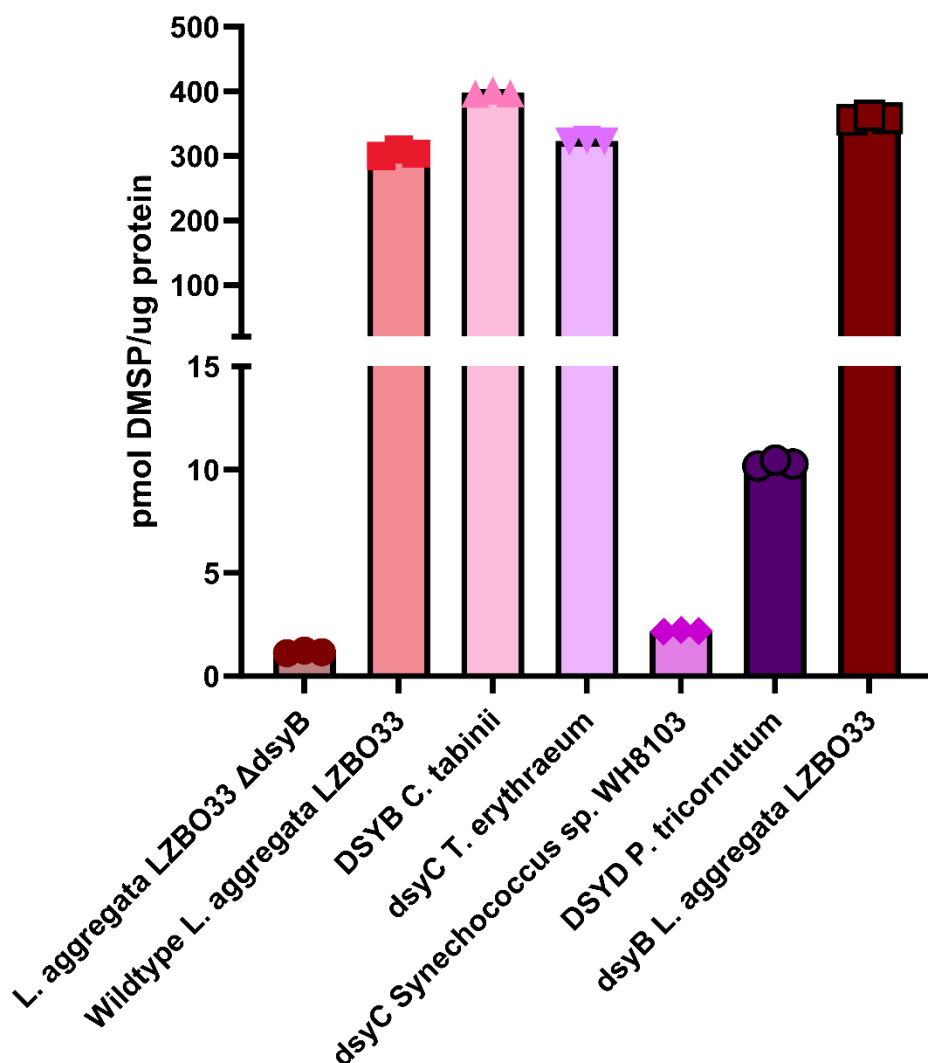


Fig. 3.14 Complementation of *L. aggregata* LZB033 Δ dsyB with DsyC and ratified methyltransferases involved in DMSP synthesis. DSYB, DSYD and DsyB are previously ratified methyltransferases involved in DMSP synthesis. *L. aggregata* LZB033 Δ dsyB was used as a background, negative control as it is expected to produce no DMSP. Wild-type LZB033 was used as a positive control.

3.2.7 Activity of *T. erythraeum* IMS101 DsyC and *Lyngbya* PCC 8106 DsyC with intermediates of GB and DMSP synthesis

Along with IMS101 DsyC, several homologs of DsyC were sent to our collaborators to synthesize and test for their activities with intermediates of GB and DMSP synthesis. Of these homologs, our collaborators tested IMS101 DsyC and *L. aestaurii* PCC 8106 (PCC 8106) DsyC, they did not manage to synthesize other DsyC's such as WH8103 or *Prochlorococcus* sp. DsyC. The activity of IMS101 DsyC and PCC 8106 DsyC was analysed by high performance liquid chromatography (HPLC). Both IMS101 and PCC 8106 DsyC used in this experiment and Section 3.2.8 were purified by nickel affinity column by Chun-Yang Li Ocean University of China. MTHB from the DMSP synthesis transamination pathway and the substrates of the GB *de novo* synthesis pathway, glycine, sarcosine, dimethylglycine and GB were tested. S-adenosyl homocysteine (SAH) was used as a negative control and SAM was used as a positive control.

PCC 8106 DsyC (**Fig. 3.15A**) was active towards all substrates tested but showed much more activity towards MTHB than any of the substrates of the GB synthesis pathway. IMS101 DsyC displayed similar activity as seen in PCC 8106 DsyC, being much more active towards MTHB than the substrates of the GB synthesis pathway. However, the overall activity of IMS101 DsyC (**Fig. 3.15B**) was higher than what was shown for PCC 8106 DsyC, with it being more active towards every substrate tested than PCC 8106 DsyC. We can deduce this from the increased conversion of SAM to SAH under IMS101 DsyC compared to PCC 8106 DsyC and the absorbance given off in Milli Active Units (mAu).

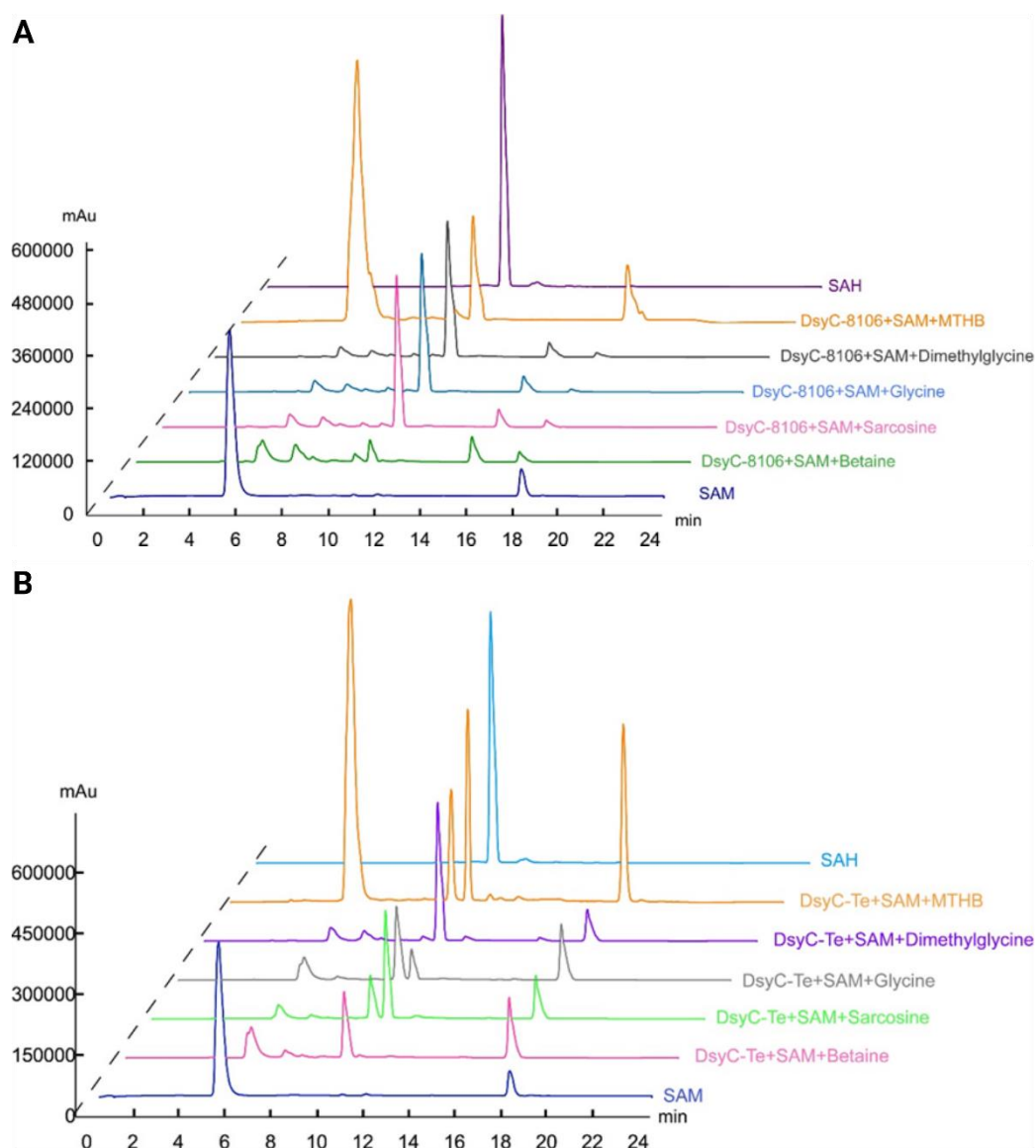


Fig. 3.15 HPLC of *T. erythraeum* IMS101 DsyC and *L. aestuarii* PCC 8106 DsyC with intermediates of GB synthesis. A) PCC 8106 DsyC, B) IMS101 DsyC. SAM was used as a negative control and SAH as a positive control. Analysis by Chun-Yang Li, Ocean University of China.

3.2.8 The optimum temperature and pH of *T. erythraeum* IMS101 DsyC

IMS101 DsyC was tested for its relative activity with varying temperatures and pH. The optimum temperature was 30 °C (**Fig. 3.16A**) with steep reductions in activity for 5 °C temperatures higher or lower than this. Above 50 °C, enzyme activity is non-existent, presumably due to protein denaturation. The optimum pH was 7 (**Fig. 3.16B**). The

activity dropped to 90 % at a pH of 6.5 and 60 % at pH 7.5 but total loss in activity was not encountered until a pH of 9 or at a point untested below pH 5. The optimum temperature and pH was not tested for PCC 8106 DsyC and therefore we could not compare with another DsyC.

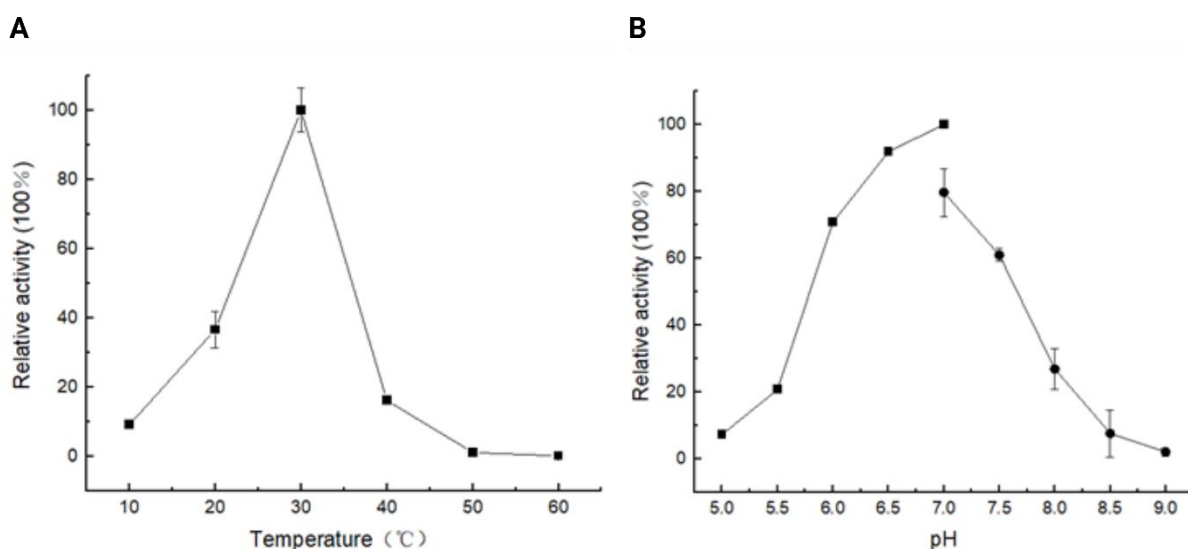


Fig. 3.16 Relative activity of IMS101 DsyC with MTHB at different temperatures and pH.
A) Activity of IMS101 DsyC in relation to temperature. **B)** Activity of IMS101 DsyC in relation to pH. Analysis by Chun-Yang Li, Ocean University of China.

3.3 Discussion

This study identified the protein responsible for the committing step of DMSP synthesis in IMS101, which is encoded by *Tery_2447* and was subsequently termed DsyC. However, *Tery_2447* has been reported to encode for a methyltransferase involved in homoserine betaine (HB) synthesis (32). HB is not as widely studied as DMSP or GB and its function(s) are still unknown although its synthesis requires methylation steps akin to that seen in DMSP or GB synthesis (145). Furthermore, in *Synechococcus* sp. WH8102 the genes *bsmA* and *bsmB* encode for two proteins involved in GB synthesis, a GSMT and SDMT (74). Of these, *bsmB* is a homolog of *dsyC*.

DMSHB production from DsyC is non-specific and its main role may vary from organism to organism. For example, in WH8103 DsyC the production of DMSHB was very low when compared to IMS101 DsyC and PCC 8106 DsyC (**Fig. 3.11**).

Conversely, WH8103 DsyC is a homolog of a ratified SDMT found in WH8102 and may produce more GB compared to IMS101 DsyC and PCC 8106 DsyC. IMS101 DsyC production of DMSHB is far higher than WH8103 and PCC 8106 DsyC and complementation of *L. aggregata* LZB033 $\Delta dsyB$ (**Fig. 3.11**) restored wild-type levels of DMSHB synthesis.

These results suggest that DsyC has a more defined function as both a HB-, GB- and DMSHB-synthesizing methyltransferase. This is a novel finding for this type of enzyme. Structural analysis of DsyC with its respective substrates would be required to determine the specific binding sites. The structures of DMSP, GB and HB are all very similar, with the difference between DMSP and GB being that DMSP contains a sulphur and GB contains a nitrogen with an additional methyl group. HB and GB contain the exact same structure except for an additional branched chain on HB with a hydroxyl group (201).

It is possible that DsyC originated from an enzyme that synthesized a specific osmolyte, such as GB, DMSP or HB and over time specific enzymes evolved to preferentially synthesise the osmolyte that conferred the greatest environmental advantage. For example, some organisms may have favoured DMSP for its availability in times of nitrogen stress (130). Future work could focus on why organisms may produce one osmolyte over another. Furthermore, if there are any similar osmolytes which contain methylation steps in their synthesis pathway then these should be tested on DsyC to determine its promiscuity and preferred osmolyte. Likewise, the enzymes involved in synthesizing these other osmolytes should be tested on substrates from the DMSP, GB and HB synthesis pathways.

Future efforts need to focus on testing DMSP production in *T. erythraeum* cultures under more growth conditions, including higher salinity, low nitrogen and high light (130,195). Other strains should also be tested, including PCC 8106, to determine whether they synthesise HB, in addition to DMSP and GB. Furthermore, kinetics needs to be performed on a range of cyanobacterial DsyCs to determine their relative affinity to these substrates. Finally, structural characterisation of these DsyCs is necessary to identify active sites binding the different substrates as well as targeted mutagenesis of conserved amino acids found in homolog active sites.

DsyC is possibly found in both filamentous and single cell cyanobacteria, including possibly *Synechococcus* and *Prochlorococcus*, the most abundant photosynthetic organisms on Earth (127). Chapter 4 will examine whether DsyC is crucial for DMSP production in these organisms and the rate DMSP and GB production under different conditions.

Chapter 4 – Investigating the relationship between DMSP and GB production in marine and intertidal cyanobacteria

4.1 Introduction

4.1.1 DMSP production in marine cyanobacteria

Previous studies into DMSP research in cyanobacteria are few and far between and do not delve deep into what genes and proteins are responsible for these processes, with very few studies investigating regulation of DMSP production. Furthermore, DMSP production has not been tested in axenic cultures of cyanobacteria. Both open water and benthic cyanobacteria have been studied and estimates have been made for their intracellular concentrations of DMSP.

4.1.2 Aims of the chapter

The main aim of this chapter is to quantify DMSP and GB production in a range of marine cyanobacteria, including axenic cultures, under different environmental conditions via GC and NMR. We will perform this in cyanobacteria containing homologs to DsyC but also in one species which contains another MTHB methyltransferase, DsyG. The focus moved from *T. erythraeum* IMS101 due to the difficulty in acquiring a live culture. In comparison, *Synechococcus* and *Prochlorococcus* were more readily available. Therefore, we could conduct experiments on live cultures.

The specific aims are outlined as followed:

- Assess how much DMSP and GB is produced in axenic *Synechococcus* and *Prochlorococcus* cultures under differing salinity and nitrogen conditions.
- Quantify DMSP production in a cyanobacterium (*Zarconia navalis* LEGE 11467) which contains DsyG.
- Attempt to mutate DsyC in *Synechococcus* sp. WH8103 and WH8020 to clarify its role in DMSP/GB synthesis.

4.2 Results

4.2.1 DMSP production in marine *Synechococcus* species under varied environmental conditions

Following identification of the MTHB methyltransferase DsyC in *Synechococcus* we ordered 4 marine species from the Roscoff Culture Collection (RCC) to test whether they produced DMSP. These samples were not axenic. All species were grown in SN media and then transferred to either low nitrogen (0.5 μ M) or no nitrogen media for 1 week, to maximise the chance of DMSP production. The source of nitrogen removed was NaNO₃. Final absorbance of each species at OD₇₅₀ is recorded in **Table 4.1**. SN media with no inoculum was used as a negative control and the background for quantifying DMSP production.

We first determined DMSP production under low N or N-free conditions. Three of the four strains (WH8103, WH8016 and WH8020) produced DMSP in both nitrogen-free and low nitrogen SN media and gave significantly higher readings than the negative controls ($P < 0.05$, Welch's T test) (**Fig. 4.1**). WH8016 produced more DMSP under N-free conditions than low N conditions, but DMSP levels were similar in both conditions for WH8103 and WH8020. WH8101 did not produce DMSP at detectable levels under these conditions and was not studied further.

Table 4.1 Final absorbance of *Synechococcus* strains in nitrogen limited and nitrogen free SN media.

Strain	Absorbance OD ₇₅₀	
	Nitrogen-Limited	Nitrogen-Free
WH8103	0.035	0.030
WH8101	0.097	0.100
WH8016	0.100	0.088
WH8020	0.088	0.121

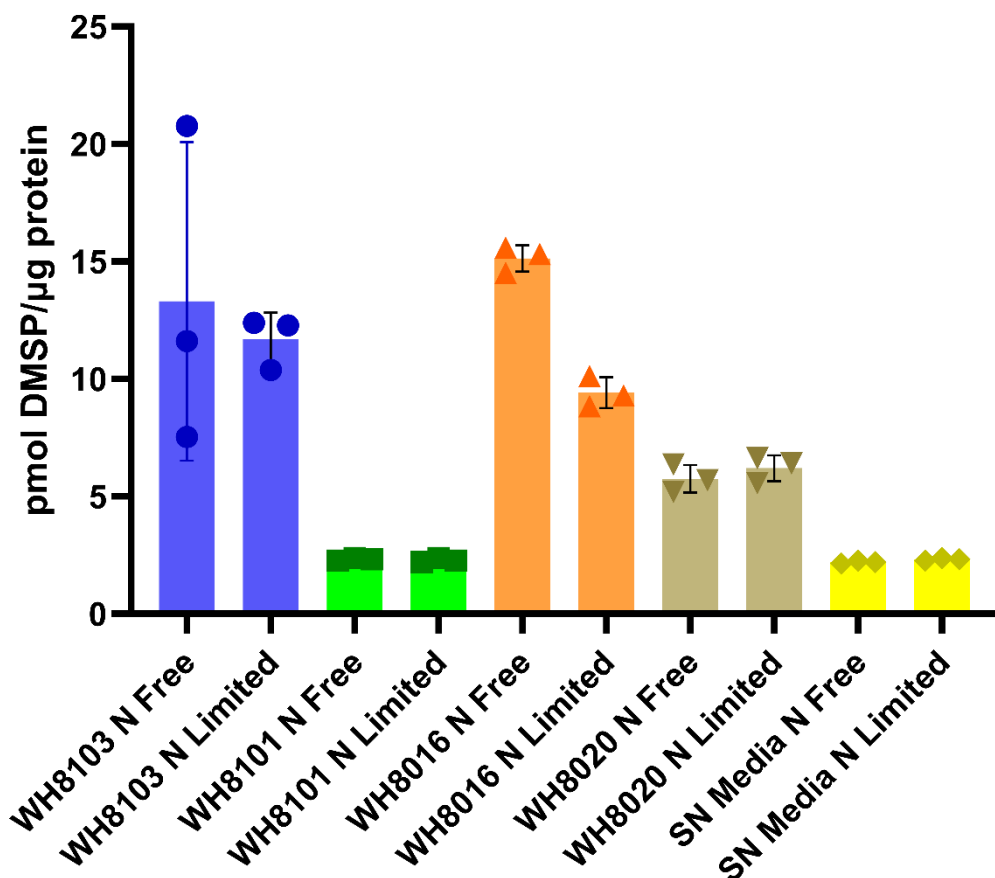


Fig. 4.1 Analysis of DMSP concentrations of *Synechococcus* sp. *Synechococcus* species were grown with low nitrogen (0.5 mM) and no nitrogen and assayed for DMSP production via GC. SN media with no inoculant was used as a negative control, in triplicate.

We next determined production from one of these species, WH8103, under other environmental conditions. We chose one species to take forward due to limitations in photobioreactor capacity. WH8103 was grown under standard conditions (SN media, no modification) as a starter culture to saturation phase (**Fig. 4.2**). Then WH8103 was subcultured into the following conditions: high PSU (SN media, 100 % seawater), low nitrogen (SN media, no NaNO₃), high PSU/low nitrogen (SN media, 100 % seawater and no NaNO₃), low iron (SN media, no ferric ammonium citrate) and no phosphorous (SN media, no K₂HPO₄). SN media containing no *Synechococcus* was used as a negative control and the background measurement.

There was no significant difference ($P > 0.05$, ANOVA) in DMSP production between standard, high PSU, low iron and low phosphorous and the SN media (**Fig. 4.3**). However, there was a significant increase ($P < 0.05$, Welch's T test) in DMSP concentration under both low nitrogen and high PSU/low nitrogen conditions. Production under low nitrogen was ~ 21 pmol DMSP / μg protein, and high PSU/low nitrogen was ~ 19 pmol DMSP / μg protein. There was no significant difference ($P > 0.05$, Student's T test) between amounts seen in low nitrogen versus high PSU/low nitrogen, suggesting that only under low N conditions was DMSP production elevated.

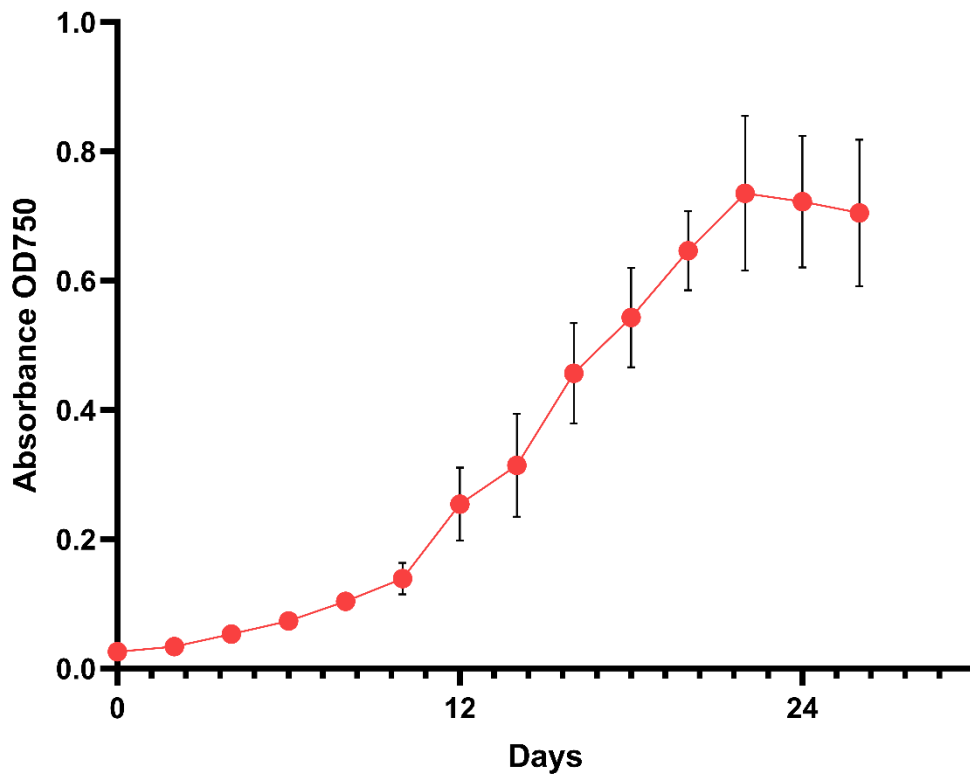


Fig. 4.2 Growth curve of *Synechococcus* sp. WH8103. WH8103 was measured at OD₇₅₀ to saturation phase.

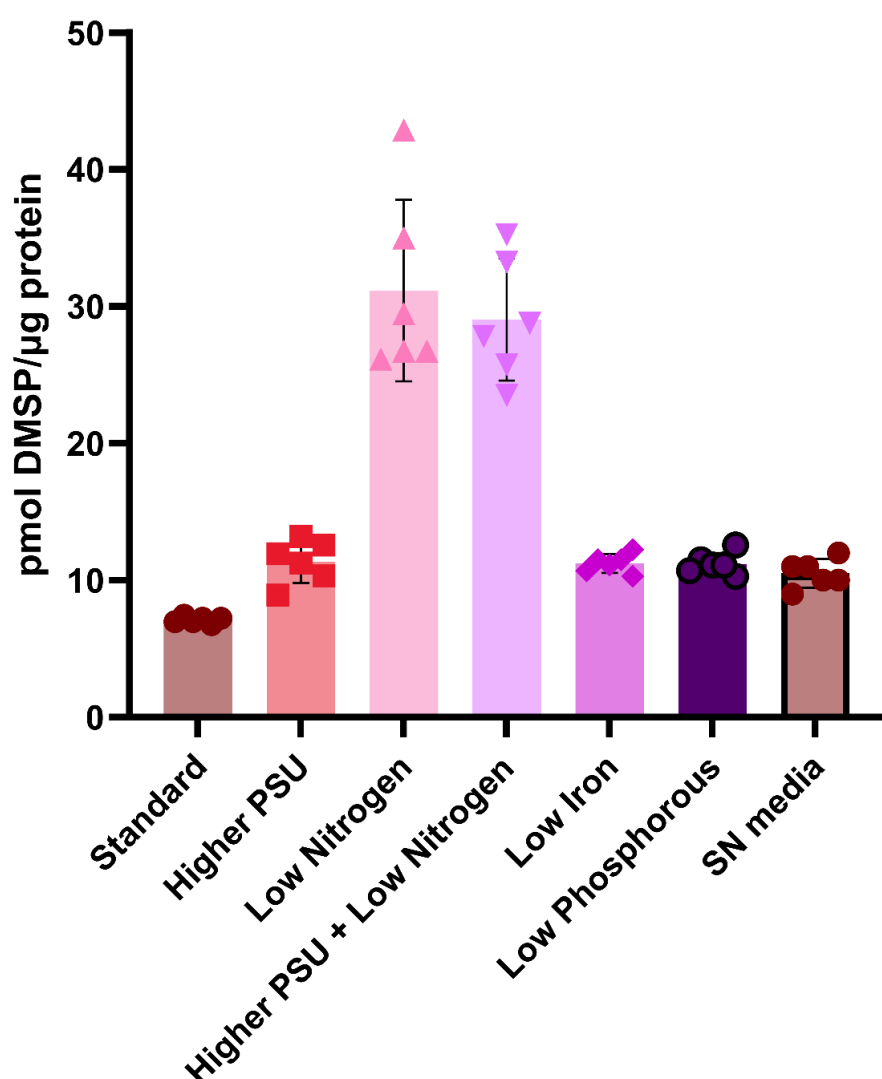


Fig. 4.3 *Synechococcus* sp. WH8103 tested under different environmental conditions. WH8103 was grown under various conditions for 2 weeks and then assayed for intracellular DMSP concentration by GC, in triplicate.

4.2.2 Quantification of DMSP production in axenic strains of *Synechococcus*

DMSP production in the Roscoff *Synechococcus* samples could be derived from contaminants and not the cyanobacterium. Therefore, we tested axenic cultures of *Synechococcus* (CC9311, WH7805, WH8102 and WH7803) provided by Dr Joseph Christie-Oleza, University of the Balearic Islands. Cultures were grown with and

without nitrogen in salinities of 15 PSU, 25 PSU and 60 PSU ASW medium, with 25 PSU representing standard salinity, and then analysed by GC and NMR. Cell numbers were provided via flow cytometry.

In the low nitrogen samples (**Fig. 4.4**), DMSP production per cell varied between species from 0.047 fg DMSP / cell to 0.478 fg DMSP / cell. All 15 PSU samples produced significantly more ($P < 0.05$, Welch's T test) DMSP than the background. Only the 25 PSU samples from WH7803 and WH7805 produced significantly more ($P < 0.05$, Students T-test) than the negative control. None of the 60 PSU samples produced DMSP.

In the normal nitrogen samples (**Fig. 4.4**) DMSP production per cell varied from 0.047 fg DMSP / cell to 0.217 fg DMSP / cell. Only the WH8102 and WH7805 15 PSU and 25 PSU samples produced significantly more ($P < 0.05$, Welch's T test) DMSP than the background. None of the 60 PSU samples were higher than the background.

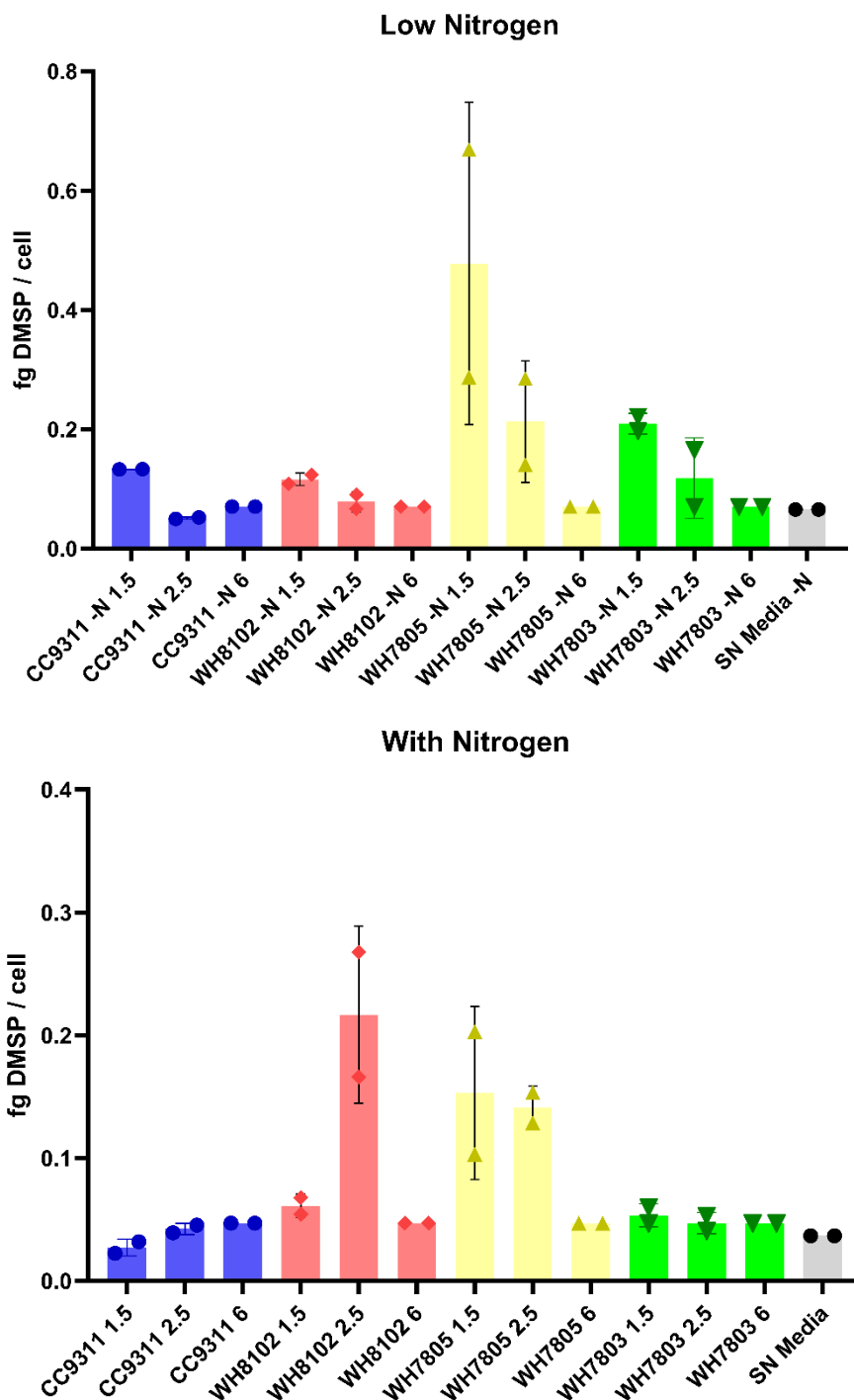


Fig. 4.4 DMSP concentration in axenic *Synechococcus* sp. grown with low (no addition of NaNO_3) and normal nitrogen. *Synechococcus* (CC9311, WH8102, WH7805 and WH7803) were grown in extra salinities of 1.5, 2.5 and 6 % increase in NaCl higher than normal and assayed for intracellular DMSP concentration by GC. SN Media was used as a negative control.

4.2.3 Quantification of DMSP and GB production in axenic strains of *Synechococcus* via NMR

We next tested DMSP and GB amounts in these samples. ^1H -NMR can be used to differentiate between the two compounds since GB standards gave diagnostic chemical shift patterns of 3.81 ppm and 3.19 ppm and DMSP gave diagnostic chemical shift patterns of 2.66 ppm and 2.84 ppm (**Fig. 4.5**). The detection limit for GB and DMSP was 20 μM . All species that contained DsyC (WH7803, WH8102, WH7805), produced significantly ($P < 0.05$, Welch's T test) more GB than CC9311 in both nitrogen-replete and low conditions (**Fig. 4.6**). As expected, all low nitrogen cultures produced significantly less ($P < 0.05$, Welch's T test) GB than the nitrogen replete cultures. There was a significant increase ($P < 0.05$, ANOVA) in GB production as the salinity increased in both WH7803 and WH8102 under nitrogen replete conditions. However, this was not seen in WH7805. CC9311 grown with and without nitrogen did not produce any GB except under 25 PSU with nitrogen, which was not significantly different ($P > 0.05$, Welch's T test) than the background. DMSP was not detected via NMR, possibly due to concentrations being below the detection threshold.

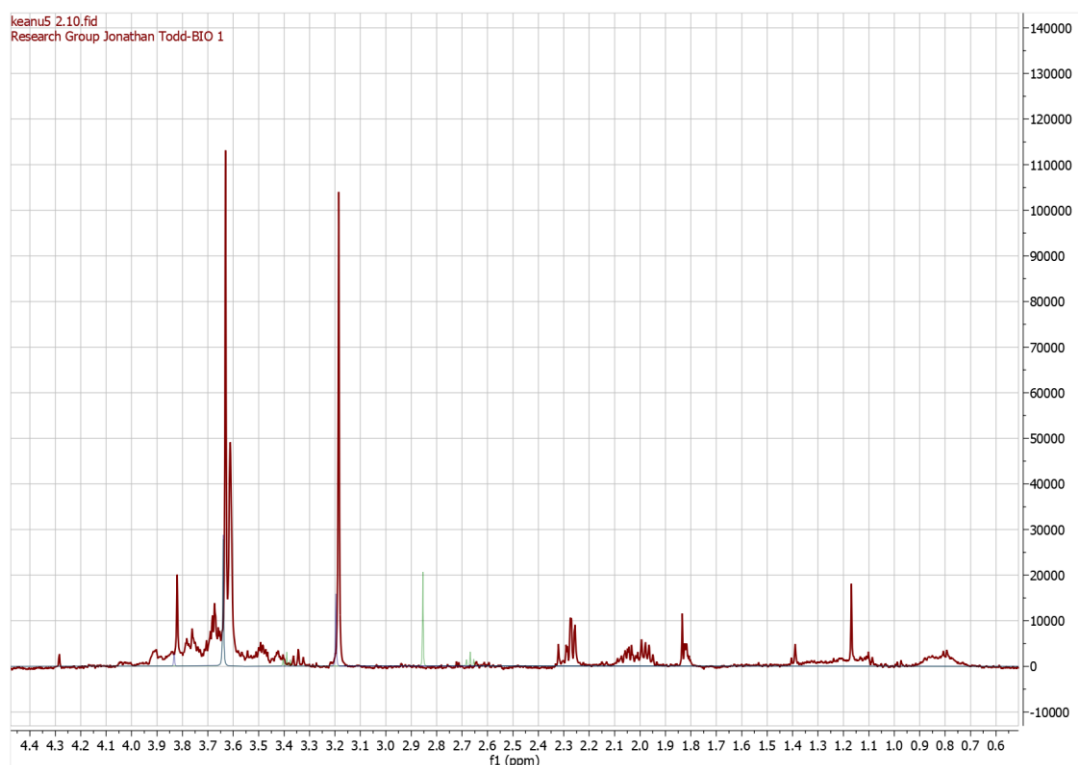


Fig. 4.5 NMR spectra for *Synechococcus* sp. WH7805. GB standard is overlaid in blue. DMSP standard is overlaid in green. *Synechococcus* sample is in red and represents typical spectra seen in samples with DsyC. Analysis by Ash Norcott, UEA.

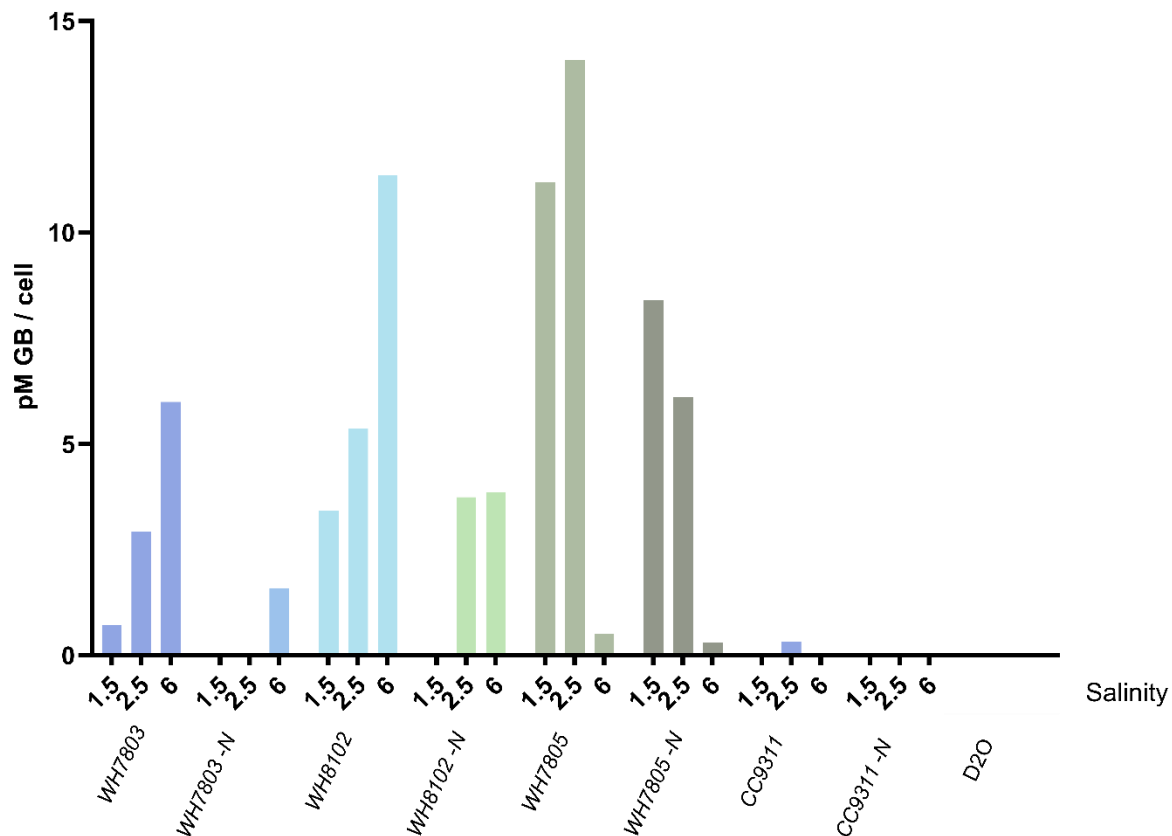


Fig. 4.6 NMR analysis of *Synechococcus* spp. intracellular GB concentrations. *Synechococcus* (WH7803, WH8102, WH7805 and CC9311) were grown with and without nitrogen (-N) and assayed for GB concentrations. The negative control was D2O.

4.2.4 Attempted generation of *Synechococcus* DsyC knockouts

Following clarification that *Synechococcus* species produce DMSP we attempted to generate a *dsyC* deficient mutant to confirm whether this enzyme is required for synthesis and whether loss of this compound results in a growth defect. Homologous regions upstream and downstream of *dsyC* in WH8103 and WH8020 were cloned into the suicide vector *pk18mobsacB* via Gibson Assembly (**Fig. 4.7**). We attempted to conjugate these plasmids into the strains using methods developed by Joseph Christie-Oleza but were unable to obtain any colonies with the correct mutant profile. Our results replicate those in Lu *et al*, 2006 (74) where they were unable to knockout the GSDMT complex in WH8102 by targeting the *bsmA* and *bsmB* genes.

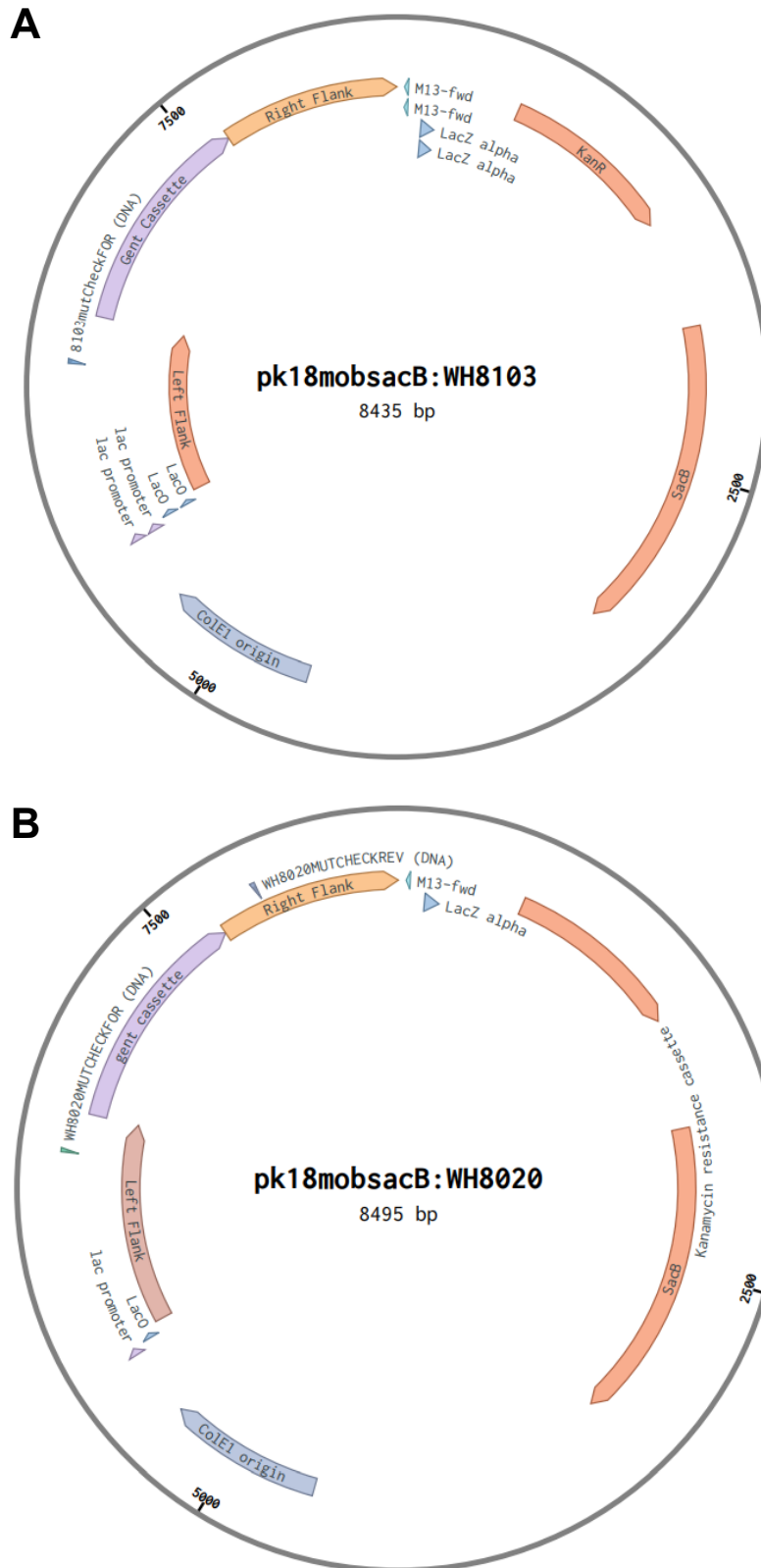


Fig. 4.7 *pk18mobsacB* based constructs for *Synechococcus* sp. mutagenesis. **A)** *pk18mobsacB* with homologous regions flanking *dsyC* in WH8103. **B)** *pk18mobsacB* with homologous regions flanking *dsyC* in WH8020.

4.2.5 DMSP and GB production in axenic species of *Prochlorococcus*

We next tested whether *Prochlorococcus* species containing *dsyC* produced DMSP. Axenic samples of four *Prochlorococcus* species were provided by Assistant Professor Steven Biller (Wellesley College). Cultures were grown under nitrogen replete and starvation conditions. Three of the species (MIT9313, MIT1323, MIT1318) contained putative *dsyC* homologues and were from the LLIV clade. The other species, MIT9312, did not contain any *dsyC* homologue and was from the HL clade. Cell numbers were determined via flow cytometry.

Prochlorococcus (Fig. 4.8) were pelleted and their supernatant (Sup.) was used as negative controls. Measurements were recorded in nmol DMSP per mg wet weight due to the complexity of resuspending the *Prochlorococcus* pellet. In addition, growth medium only (AMP10 and AMP10 -N) samples were also tested. All control samples returned no visible peaks on the GC and their values were similar to the background emission (~3.5 nmol DMSP / mg wet weight.)

Each sample from a *Prochlorococcus* species tested had a significantly higher peak area than the negative controls ($P < 0.05$, ANOVA). MIT9312 contained ~11.5 nmol DMSP / mg wet weight in both nitrogen replete and nitrogen limited conditions. Both MIT1323 and MIT1318 contained more DMSP in nitrogen replete conditions (11.5 nmol DMSP / mg wet weight and 11.3 nmol DMSP / mg wet weight, respectively) than nitrogen limiting conditions (9.3 nmol DMSP / mg wet weight and 9 nmol DMSP / mg wet weight, respectively). However, MIT9313 contained more DMSP under nitrogen limitation (11.6 nmol DMSP / mg wet weight) than in nitrogen replete conditions (4 nmol DMSP / mg wet weight). However, there was no significant difference ($P > 0.05$, Students T test) in intracellular DMSP concentration between any of the *Prochlorococcus* samples grown in nitrogen replete conditions versus nitrogen limiting conditions.

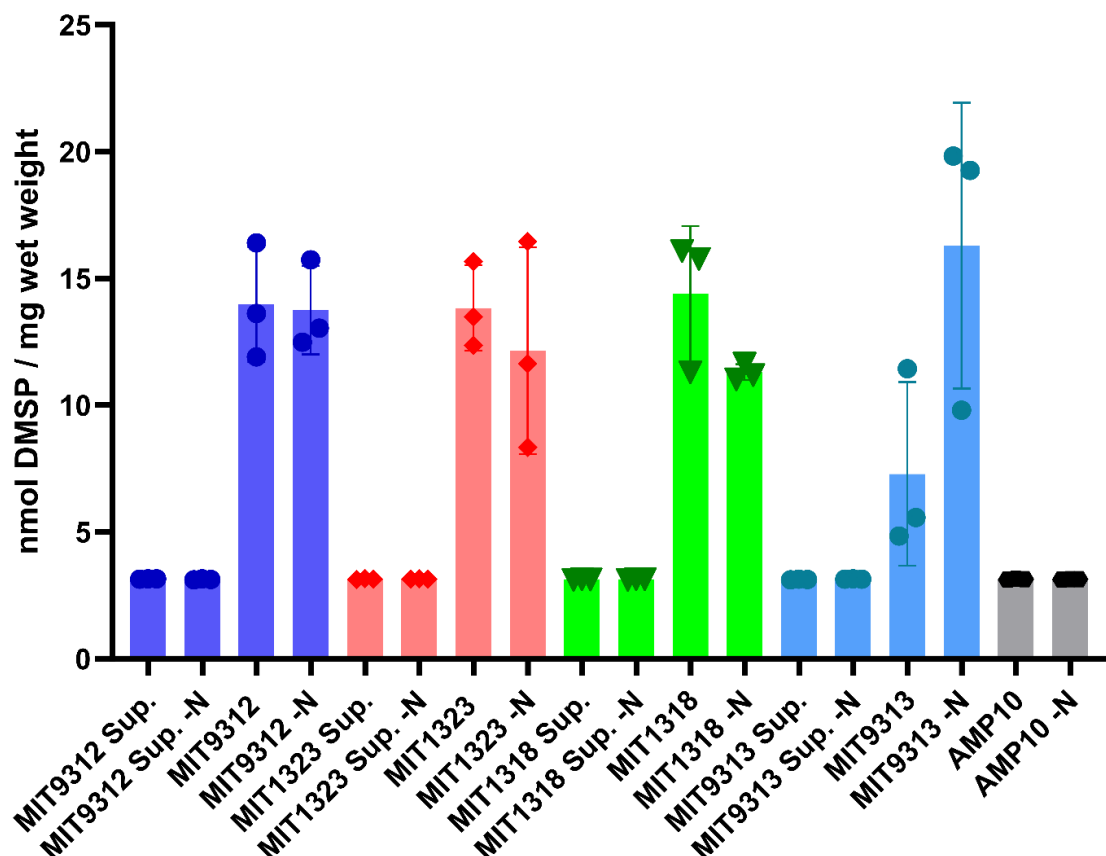


Fig. 4.8 DMSP production in *Prochlorococcus* spp. *Prochlorococcus* (MIT9312, MIT1323, MIT1318 and MIT9313) were grown under nitrogen replete and limited environment and analysed for DMSP content. Negative controls were of culture supernatant (Sup.) and growth medium.

After confirming production of DMSP in multiple *Prochlorococcus* species, we quantified the concentration per cell (**Fig. 4.9**). MIT9312, which does not contain a *dsyC* homolog, produced the least amount of DMSP per cell in both nitrogen replete (0.00029 fg DMSP / cell) and limited media (0.00037 fg DMSP / cell). MIT1323 showed the biggest difference in DMSP production with nitrogen limitation, it produced 0.0006 fg DMSP / cell with nitrogen and had a 10-fold increase up to 0.0058 fg DMSP / cell under nitrogen limitation. Of the *Prochlorococcus* species which contained a *DsyC* homolog (MIT1318, MIT1323 and MIT9313) only MIT1323 gave a significantly higher ($P < 0.05$, Welch's T test) concentration of DMSP per cell than MIT9312, when grown under low nitrogen.

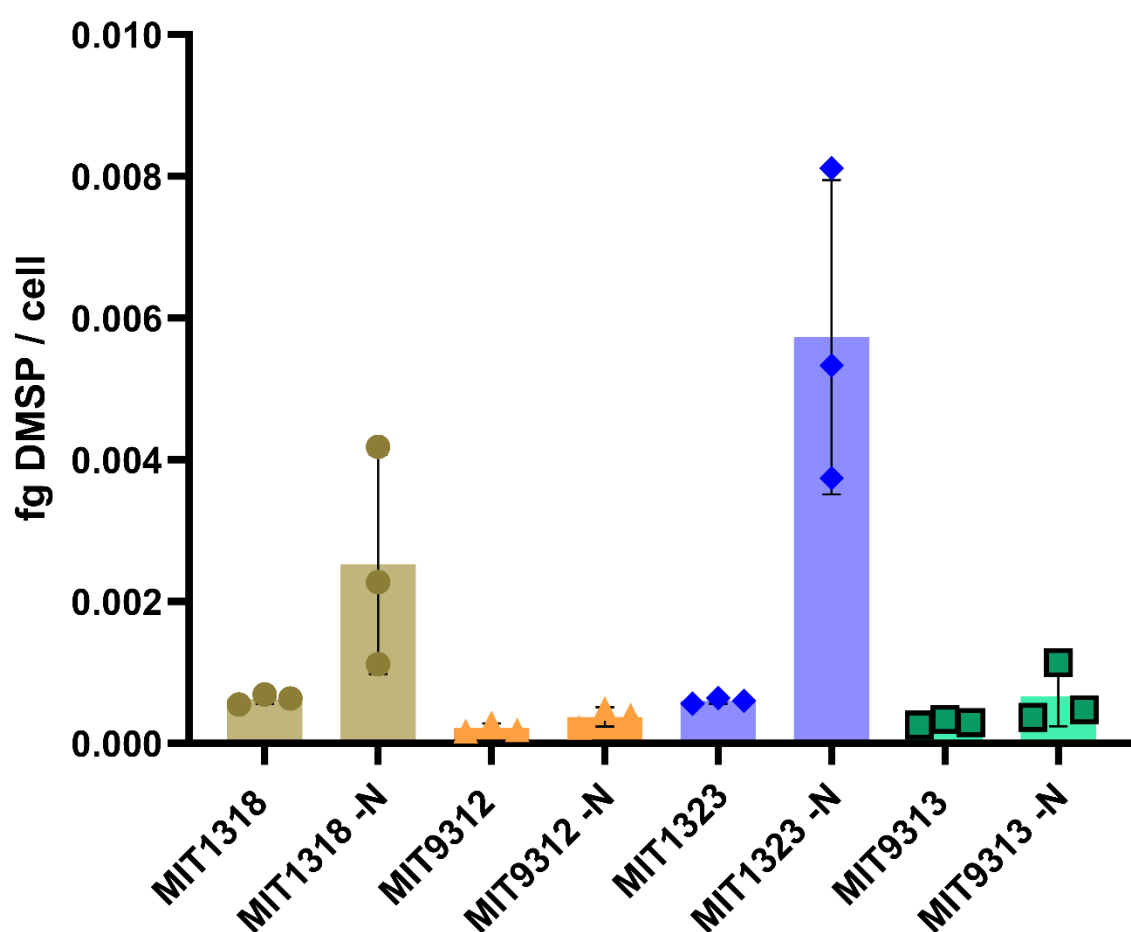


Fig. 4.9 Concentration of DMSP per cell in *Prochlorococcus* spp. *Prochlorococcus* (MIT1318, MIT9312, MIT1323 and MIT9313) were grown under nitrogen replete and limited environments and assayed for DMSP production via GC.

GB and DMSP production were then tested via NMR (**Fig. 4.10**). DMSP was not detected in any of the samples, possibly due to being below the detection threshold (**Fig. 4.11**). The other peaks detected in **Fig. 4.11** are likely not related to DMSP and GB. Intracellular GB was detected in the three *dsyC* containing *Prochlorococcus* species (MIT9313, MIT1323 and MIT1318) at significantly higher ($P < 0.05$, Welch's T test) concentrations than in MIT9312. Furthermore, GB production in these three samples (MIT9313, MIT1323 and MIT1318) was significantly higher ($P < 0.05$, Welch's T test) in the nitrogen replete samples versus nitrogen limited ones. All were also significantly higher ($P < 0.05$, Welch's T test) than in the negative controls. GB concentration of MIT9312 were not significantly different ($P > 0.05$, Welch's T test) than the negative controls, confirming it did not produce GB.

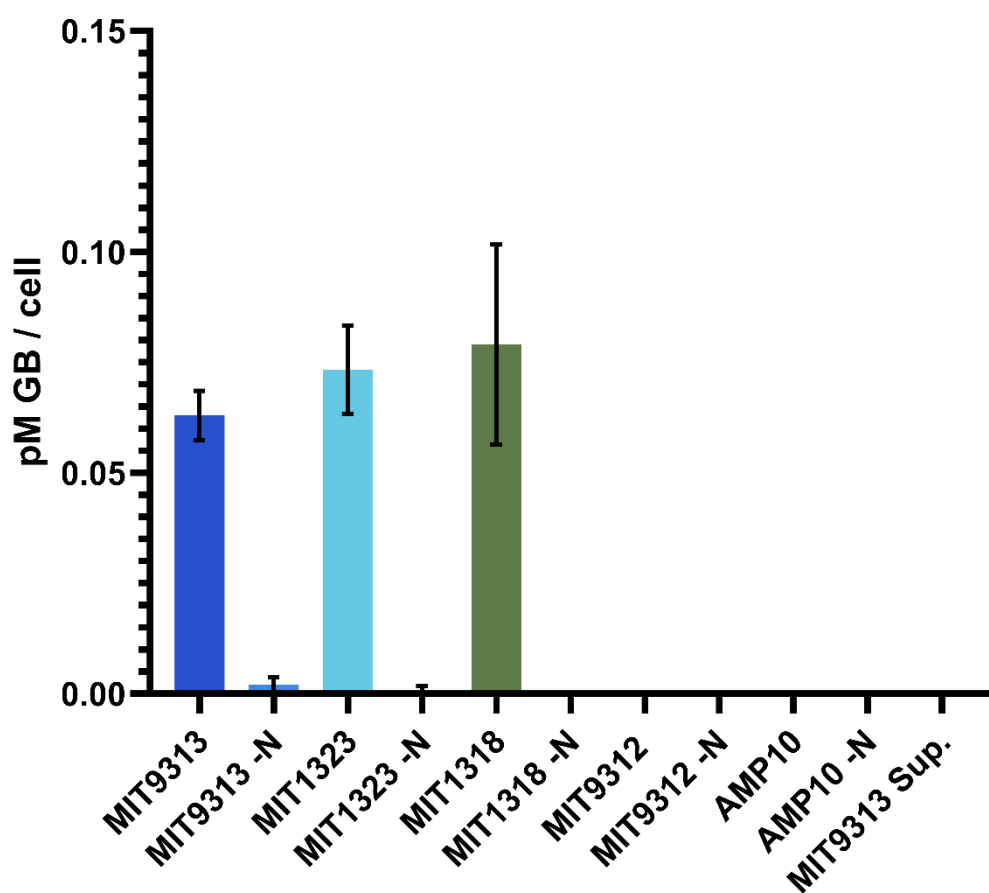


Fig. 4.10 Quantification of intracellular GB concentrations in *Prochlorococcus* sp. via NMR. *Prochlorococcus* (MIT9313, MIT1323, MIT1318, MIT9312) were grown with and without nitrogen (-N) and analysed for GB content. Negative controls were culture supernatant and growth media.

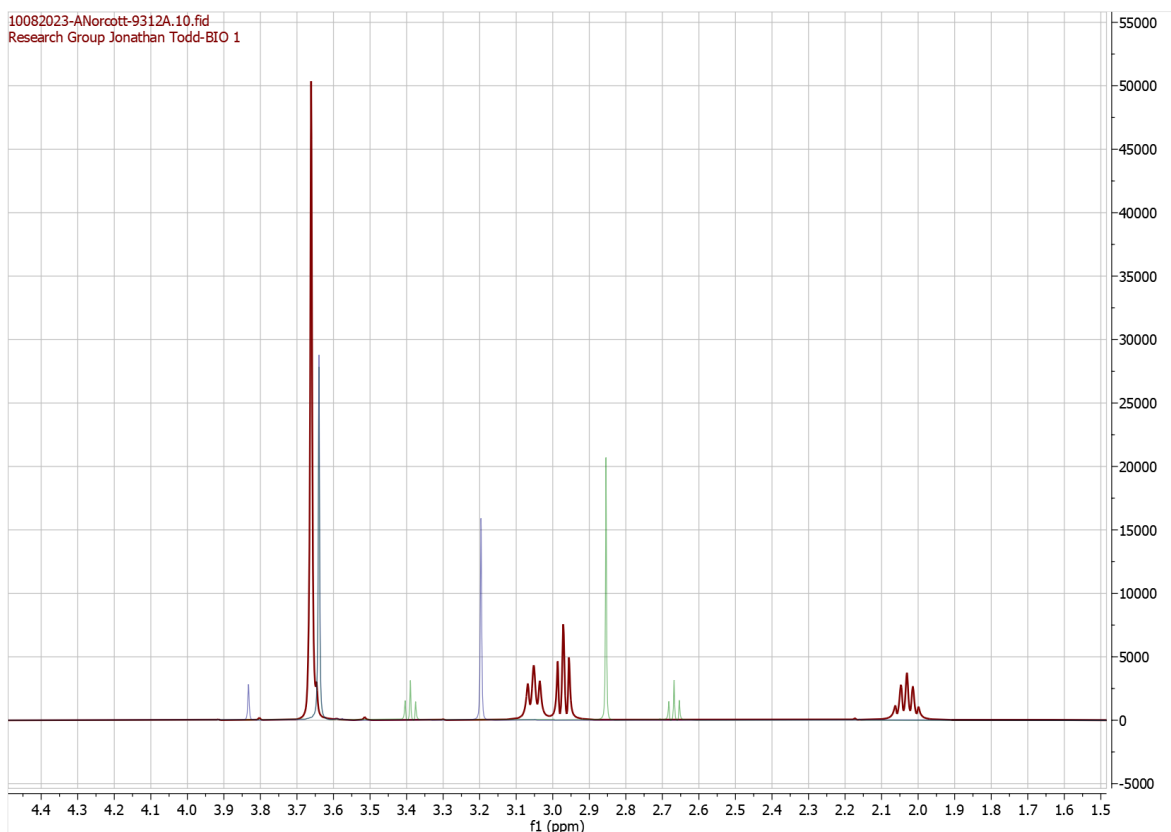


Fig. 4.11 NMR spectra for *Prochlorococcus* sp. The GB standard is overlaid in blue. DMSP standard is overlaid in green. The *Prochlorococcus* sample is in red and represents typical spectra seen in samples with DsyC. Analysis by Ash Norcott, UEA.

4.2.6 DMSP production in *Lyngbya aestaurii* UTEX2515

Lyngbya aestaurii UTEX 2515 (UTEX 2515) was analysed by GC (**Fig. 4.12**) to determine if it produces DMSP and if this was regulated by nitrogen availability. UTEX 2515 contained around 20 and 19 nmol DMSP mg⁻¹ wet weight when grown with and without nitrogen, respectively. This is significantly higher ($P < 0.05$, ANOVA) than the media only negative controls of 3 nmol DMSP mg⁻¹ wet weight. There was no significant difference ($P < 0.05$, Student's T test) between the nitrogen replete and nitrogen limited UTEX 2515 cultures. Interestingly, there was a significant difference ($P < 0.05$, Student's T test) between DMSP amounts in the supernatant. The nitrogen replete supernatant contained around 7 nmol DMSP mg⁻¹ wet weight while the nitrogen limited supernatant was the same as the background (~ 3 nmol DMSP mg⁻¹ wet weight).

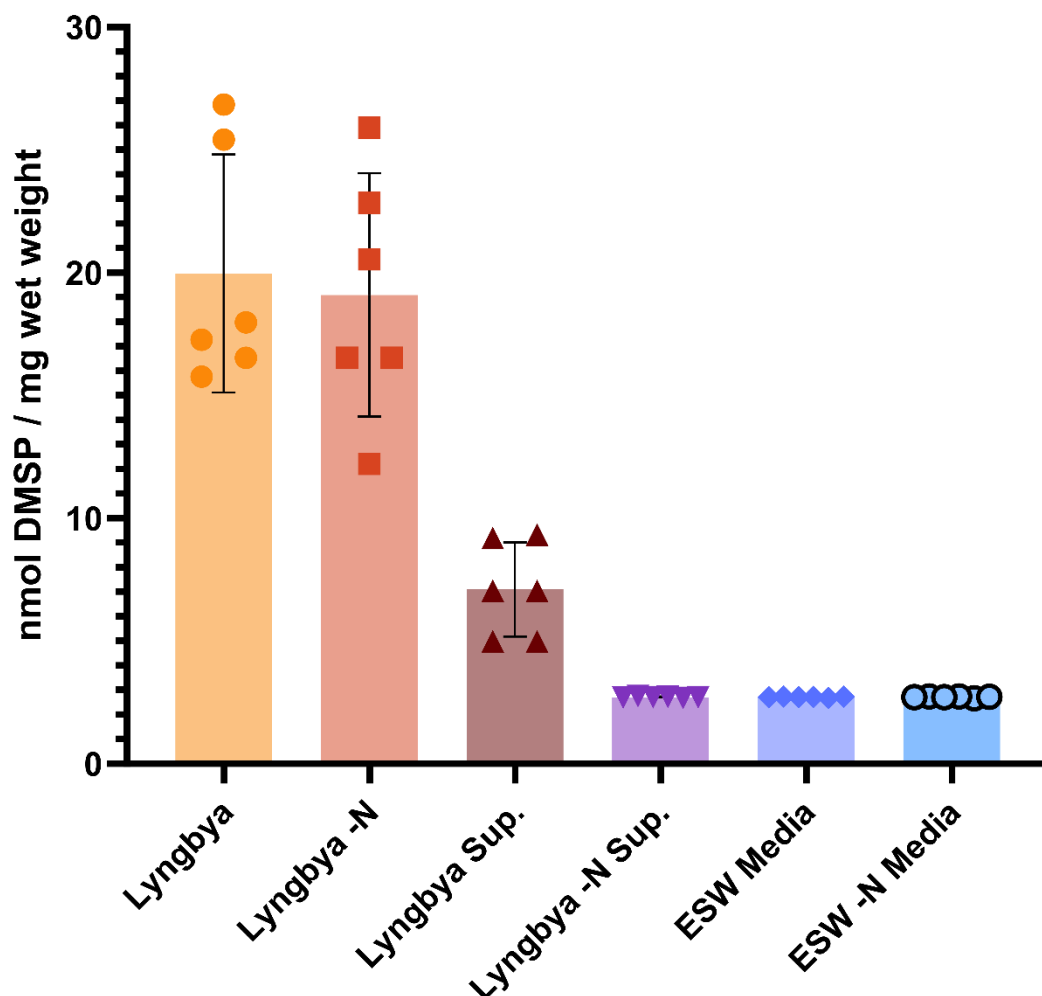


Fig. 4.12 GC analysis of *Lyngbya aestaurii* UTEX 2515. UTEX 2515 was grown with and without nitrogen and assayed for DMSP production. Both nitrogen replete and deplete ESW media were used as negative controls.

4.2.7 DsyG in filamentous cyanobacteria

We next investigated putative MTHB methyltransferases, other than DsyC, which have been identified in some cyanobacteria. The filamentous cyanobacterium *Zarconia navalis* LEGE 11467 (LEGE 11467), which was isolated from an intertidal microbial mat, was grown by Ana Viera, CIIMAR over 18 days in BG11 media with varying concentrations of tropical marine salts (TMS) and nitrate.

In the low salinity (5 g/L TMS)/standard nitrogen (17.65 mM nitrate) and standard salinity (25 g/L TMS)/standard nitrogen samples there was no significant difference ($P > 0.05$, Student's T test) in DMSP concentrations from day 0 to day 7 (**Fig. 4.13**).

However, there was a significant decrease ($P < 0.05$, Student's T test) between day 7 and day 18 in both samples. In the high salinity sample C (50 g/L TMS)/standard nitrogen and standard salinity/low nitrogen sample (0.5 mM nitrate) there was no clear pattern for DMSP production between day 0 and day 18. However, both samples made 2 to 3-fold more DMSP (~ 0.3 nmol DMSP / mg wet weight) on day 0 than the low salinity/standard nitrogen and standard salinity/standard nitrogen samples ($\sim 0.12 - 0.13$ nmol DMSP / mg wet weight).

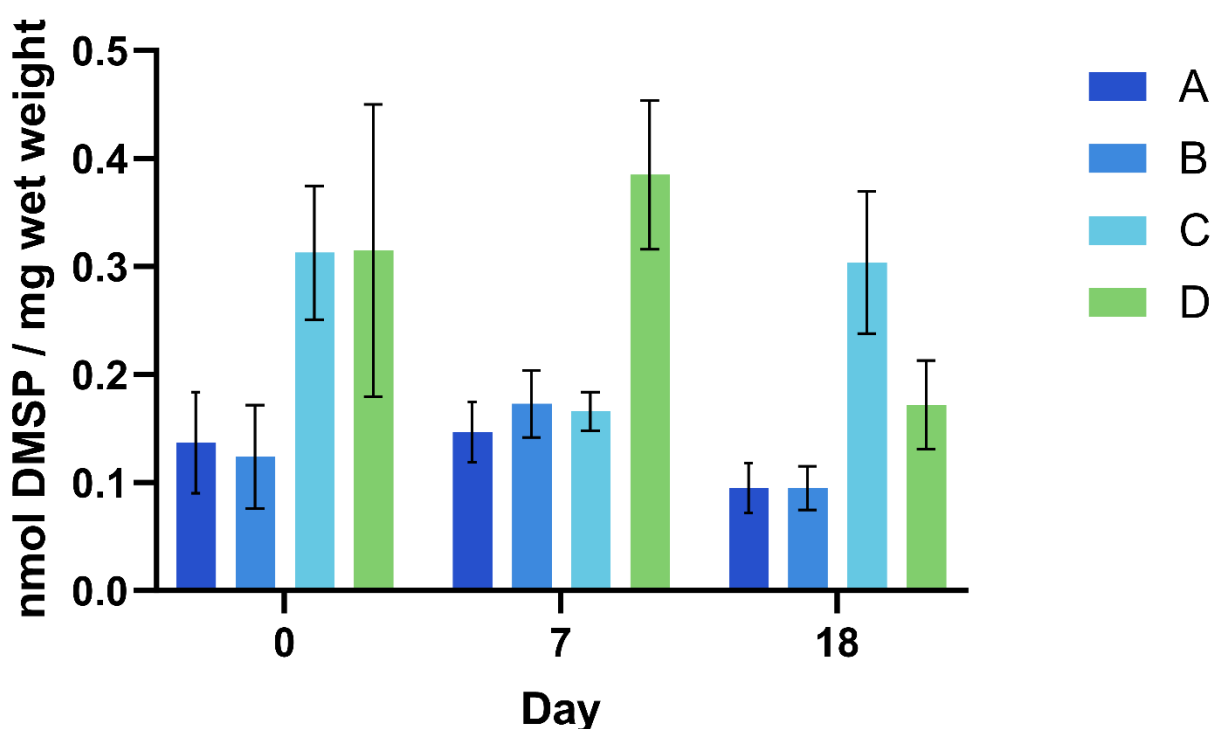


Fig. 4.13 Samples of *Zarconia navalis* LEGE 11467 assayed for DMSP. A to D represents 4 different environmental conditions that LEGE 11467 was grown under. **A)** 5 g/L TMS with 17.65 mM nitrate. **B)** 25 g/L TMS with 17.65 mM nitrate. **C)** 50 g/L TMS with 17.65 mM nitrate. **D)** 25 g/L TMS with 0.5 mM nitrate.

4.3 Discussion

Here we demonstrated that species of the two most abundant cyanobacterial genera *Prochlorococcus* and *Synechococcus* (102) produce DMSP in axenic cultures. One previous study has reported on DMSP production in *Synechococcus* and

Prochlorococcus, with amounts of $2.8 \times 10^{-4} \pm 1.8 \times 10^{-4}$ and $5.1 \times 10^{-3} \pm 7.8 \times 10^{-3}$ fg DMSP/cell, respectively (125). In our analysis, *Prochlorococcus* DMSP concentrations varied from 3.7×10^{-4} to 5.7×10^{-3} fg DMSP/cell, similar to that study. In *Synechococcus*, the DMSP concentrations in our samples was around 10 to 100 times higher than the previous study, between 4.7×10^{-2} to 4.8×10^{-1} fg DMSP/cell. This suggests that per cell *Synechococcus* produced more DMSP than *Prochlorococcus*, although this could be due to their larger volume. This study aligns with previous studies suggesting that cyanobacterial production of DMSP per cell is low but likely increases under environmental stresses such as nitrogen limitation and higher salinity.

All strains of *Prochlorococcus* and *Synechococcus* tested produced DMSP, regardless of if they contained a homolog to DsyC, suggesting DsyC is not critical for DMSP production in these strains. Therefore, it is plausible that there may be another methyltransferase in these cyanobacteria that is indirectly producing a precursor of DMSP, such as DMSHB or S-Methylmethionine (SMM). It is also possible that DsyC and any other unknown DMSP linked methyltransferase are poor enzymes for DMSP synthesis and are primarily involved in synthesizing other metabolites, most likely GB. For example, DsyC in *Synechococcus* sp. WH8102 is a ratified SDMT which synthesizes GB and close homologs found throughout *Synechococcus* and some LLIV clade *Prochlorococcus* (74), similar to the role of IMS101 DsyC in HB synthesis (145).

In both *Synechococcus* and *Prochlorococcus*, this study is the first to highlight that GB production is downregulated under nitrogen limitation, but also showed that there was no relationship between GB and DMSP production in *Synechococcus* and *Prochlorococcus*. Additionally, the species which did not contain the SDMT homolog could not produce GB, suggesting that SDMT is critical for GB production. There was no relationship between DMSP and GB production in *Synechococcus* and *Prochlorococcus*. As production of GB increased with salinity or decreased due to a lack of nitrogen, production of DMSP did not change. It is possible that *Synechococcus* and *Prochlorococcus* shift production to other nitrogen free osmolytes such as glucosylglycerol (GG) and glucosylglycerate (GGA), which are widely found in these genera (74,202). However, this may not reflect cells in marine waters, which would require analysis of samples collected from oceanography cruises.

The filamentous cyanobacterium UTEX 2515 produced similar amounts of DMSP in both nitrogen replete and limited conditions to what was seen in *Synechococcus* and *Prochlorococcus*. There was no difference in DMSP production under nitrogen limitation. However, *Lyngbya* species are nitrogen fixers (203) and therefore may be able to fix enough nitrogen to support the synthesis of its own nitrogenous based osmolytes such as GB (204). Interestingly, extracellular DMSP was detected in the supernatant of the UTEX 2515 nitrogen replete cultures but not in the nitrogen deplete cultures. This indicates the possibility that DMSP is exported to the media by UTEX 2515 when it is nitrogen-replete.

DsyC is not the only DMSP synthesis-linked methyltransferase to be identified in cyanobacteria. DsyG was identified recently (unpublished) and is found in several genera of cyanobacteria such as *Symploca* sp. SIO3E6, *Oscillatoria* sp. SIO1A7 and LEGE 11467. Furthermore, it is also found in the gammaproteobacteria *Gyvuella sunshinyii* (unpublished). LEGE 11467 was assayed for DMSP production and produced marginal quantities which were slightly upregulated by low nitrogen and higher salinity by 2 or 3-fold. However, LEGE 11467 made less DMSP than cyanobacteria which contain DsyC.

Future work should investigate DMSP production in more *Synechococcus* and *Prochlorococcus* species, especially environmental samples, and determine if certain clades produce more or don't produce DMSP at all. Furthermore, any clades which produce DMSP but do not contain any homologs to ratified MTHB methyltransferases should be investigated for novel methyltransferases. DMSP regulation should be examined in UTEX 2515 and LEGE 11467 under low concentrations of iron and phosphorous to determine whether this increases production, as in IMS101 (130,195). Lastly, mutagenesis must be reattempted but this time targeting the BCCT's that are downstream of *dsyC* in *Synechococcus*, to determine whether this affects *Synechococcus* growth and its ability to import DMSP.

Chapter 5 – Characterisation of DMSP production in a saltmarsh intertidal mat

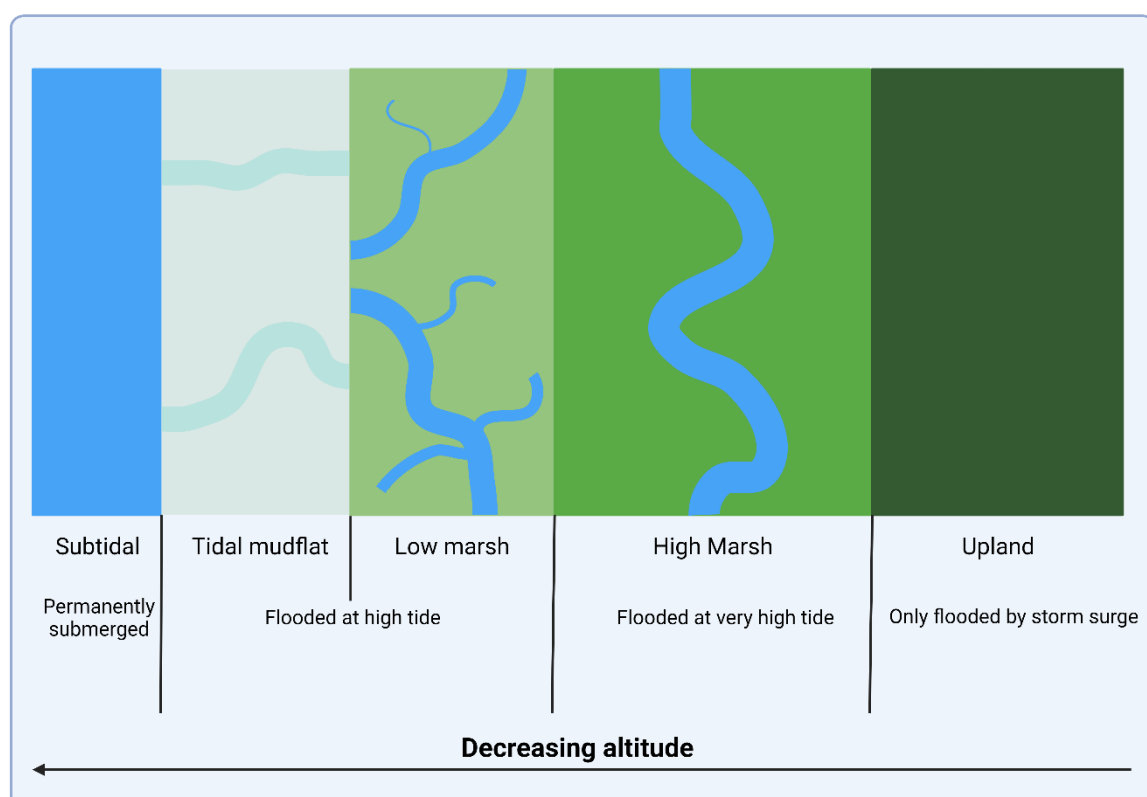
5.1 Introduction

5.1.1 Saltmarsh ecology

Saltmarshes are coastal ecosystems that are transition zones between the land and sea. They are areas of fine sediment which are transported by water but are then stabilised by vegetation (205). They are found globally and are occupied with halophilic vegetation and exposed to low hydrodynamic conditions and tidal flooding (206). Tidal amplitude, in addition to the slope and elevation of the shore are the determinant factors on whether a saltmarsh can form (206). Saltmarshes are classified into three major categories: i) Barrier-connected saltmarshes, ii) Foreland saltmarshes and iii) Estuarine saltmarshes (205). Barrier-connected saltmarshes develop in the lee of spits where the slope of the intertidal and sub-tidal area is shallow on the exposed side. These are typical of North Norfolk and develop adjacent to shingle or sand dunes (205). Foreland saltmarshes develop in front of sheltered alluvial coastal plains with deeper sediment deposits than the barrier connected saltmarshes. Lastly, estuarine saltmarshes are found where rivers merge into the open sea.

The global extent of saltmarshes ranges from 2.2 to 40 Mha (207), but this is likely an underestimate as it only considers local and national mapping and saltmarshes have not been systematically mapped globally (208). Around 40% of currently mapped saltmarshes are found in North America and 25% are found in Australia, with new saltmarshes still being recorded. For example, 290.42km² of saltmarsh was recently mapped in India, representing 0.5% of global saltmarsh coverage (209). However, despite new additions to mapped data, the amount of mapped saltmarsh coverage is gradually decreasing. From 2000 to 2019, 1,452 km² of saltmarsh was lost from hurricanes and coastal erosion, which represents 0.28% of global saltmarshes (210).

Saltmarshes comprise several different zones from the high marsh through to the tidal mudflat (**Fig. 5.1**). Each zone is characterised by specific plant species and vegetation cover (211). Upland areas are typically covered in non-halotolerant plants, such as trees, and represent the boundary area between saltmarsh and terrestrial land. The high marsh is characterized by successional plant species with high cover and density, such as wild rye or reeds (211). In the low marsh, a diverse variety of plant species occur with varying degrees of cover, which is followed by the tidal mudflats which harbour pioneering plant species with a cover greater than 5% (211). Within these tidal mudflats there are microbial communities arranged in thin biofilms containing



colourless sulfur bacteria, purple sulfur bacteria, sulfate-reducing bacteria and cyanobacteria (212).

Fig. 5.1 Schematic of saltmarsh progression. The subtidal zone is permanently underwater, and the tidal mudflat and low marsh are only under water at high tide. The high marsh is only submerged at very high tide and the upland area is only underwater during a storm surge. As the saltmarsh progresses from subtidal to upland the salinity decreases, and the altitude increases.

5.1.2 Stress conditions on saltmarsh microorganisms

Microorganisms inhabiting intertidal zones must tolerate rapid changes in temperature, light, salinity, grazing and physical disturbance (122). Here, microphytobenthic algae form extensive biofilms and are the dominant primary producers (122). High densities of benthic algae are also found in unshaded creeks and salt pans (213), with the risk of desiccation increasing the further from the shore (214).

Cyanobacterial mats are extremely tolerant to desiccation stress. For example, in a salt panne, salinities exceeding 185 PSU were identified in a 'healthy' cyanobacterial mat (215). Within the benthic microbial mats, there are a diverse range of cyanobacteria, which does not alter when flooded at high tide (214). Furthermore, cyanobacteria are found in the subtidal region which is constantly underwater (214).

One strategy which diatoms, cyanobacteria and other photoautotrophic microorganisms employ is to migrate vertically away from the surface to avoid desiccation. This strategy is employed in the areas of a saltmarsh that do not regularly get covered by tidal water or receive too much sunlight (122). Similarly, microbial mats dominated by *L. aestuarii* were shown to have high levels of scytonemin, a natural sunscreen used to block UV-A and UV-B (124). Alternatively, if light levels are too low then the intracellular concentrations of chlorophyll a are increased to facilitate improved light harvesting (122).

Additionally, microbial mats deal with the stresses of living on a saltmarsh by synthesizing and accumulating organic osmolytes. For example, a microbial mat dominated by the cyanobacterium *Coleofasciculus chthonoplastes* was found to synthesize and accumulate glucosylglycerol (GG) and trehalose. GG gradually increased with increasing salinities up to 60 ppt, whereas trehalose was most prominent between 5 – 32 ppt (216). GB, which is almost universally synthesized and accumulated by prokaryotes and eukaryotes, can accumulate within cells up to 1 M concentrations (217). Alternatively, organisms, such as *Spartina*, green seaweeds, algae and bacteria (Carrión *et al*, unpublished) may synthesise DMSP.

5.1.3 Cyanobacteria in saltmarshes

A diverse range of cyanobacteria are found in saltmarshes around the world. Cyanobacterial growth is generally higher in the summer but are often present year-round (218). Within saltmarshes, unicellular and filamentous cyanobacteria occupy both the aerobic and anaerobic environment (119). Typical saltmarsh cyanobacteria include coccoids (*Chroococcus*, *Anacystis*), colonial (*Merismopedia*), heterocystous (*Anabaena*, *Nostoc*, *Calothrix*) and non-heterocystous types (*Oscillatoria*, *Lyngbya*, *Microcoleus*, *Phormidium* and *Schizothrix*) (218). Filamentous cyanobacteria often form thick microbial mats of several mm thick on saltmarsh sediment and can grow vertically up vascular plants to between 5 to 10 cm (215).

These cyanobacteria play crucial roles in the microbial food web via their contribution to net primary productivity, nitrogen fixation, nutrient cycling and ecosystem stabilising (120). Along with algae, cyanobacteria support a variety of organisms such as infaunal invertebrates, large bivalves and fish (121). Furthermore, they are very important members of saltmarsh microbial mats because many other bacteria depend on the excreted, lysed and decomposed products of cyanobacteria for their growth (212).

5.1.4 DMSP production in saltmarsh cyanobacteria

Most of the previous work performed on DMSP production in cyanobacteria has focused on open ocean cyanobacteria, predominantly *Synechococcus*, *Prochlorococcus* and *Trichodesmium* species. Very little work has been conducted on saltmarsh cyanobacteria. In one study, benthic, filamentous cyanobacteria including *Lyngbya*, *Oscillatoria*, *Nostoc*, *Calothrix*, *Anabaena* and *Nodularia* species collected from North Germany were screened for their DMSP production (126). All cyanobacteria were found to be low producers, ranging from 0.007 mmol DMSP (g chl a)⁻¹ to 0.583 mmol DMSP (g chl a)⁻¹ (126).

Other work has been conducted on the widespread benthic cyanobacterium *C. chthonoplastes*. DMSP has been reported in microbial mats in the Netherlands dominated by *C. chthonoplastes* as well as in axenic cultures isolated from Germany (219). In axenic *C. chthonoplastes* cultures, between 36.4 to 38.1 µmol DMSP (g protein)⁻¹ was found which was higher than other reported cyanobacteria such as *Microcoleus lyngbyaceus* (0.36 µmol DMSP (g protein⁻¹)) (219).

Studies into benthic cyanobacterial DMSP production occurred before the widespread adoption of next generation sequencing technologies. Therefore, cyanobacteria were phenotypically identified, and cultures were not verified as axenic. Similarly, studies rarely looked at DMSP producers at a genomic or transcriptomic level, and no genes or their DMSP-producing protein products were identified. Furthermore, these studies took place before widespread knowledge of how DMSP production is regulated, and the cyanobacteria analysed were often grown in nutrient replete environments which do not mimic the environmental stresses the organisms would be exposed to and under which production of DMSP may be elevated.

5.1.5 Aims of chapter

The overall aim of this chapter is to elucidate the scale of DMSP production in an intertidal microbial mat and the organisms and proteins responsible for it. We will do this by conducting the first ever metagenomics and metatranscriptomics study of this environment and link that to DMSP production. Despite previous chapters focussing on open ocean cyanobacteria, we chose to study saltmarshes due to the proximity to the University of East Anglia and characterised DMSP production. There are four sub-aims:

- Identify how much DMSP is produced in an intertidal microbial mat on the North Norfolk coast.
- Identify the key microorganisms in this mat.
- Characterise the most abundant DMSP related transcripts in this environment.
- Determine which proteins and organisms may be responsible for DMSP production.

5.2 Results

5.2.1 Determination of sampling location on Stiffkey saltmarsh

Microbial mats often assemble in areas exposed to high light intensities and high salinities, such as marine intertidal mud flats (220). We therefore sampled the intertidal mud flat of Stiffkey saltmarsh on the North Norfolk coast (U.K.), at coordinates latitude 52.9649 °N, longitude 0.9436 °E (**Fig. 5.2 – 5.3**). We chose to sample an area away from any visible higher plant growth or noticeable, high DMSP producing *Ulva*. Furthermore, we wanted to avoid unnecessary microbial interactions such as that in

the Rhizosphere of plants like *Spartina anglica*, which was found to contain DMSP synthesizing bacteria, as demonstrated in a sample from the nearby Blakeney saltmarsh (34).

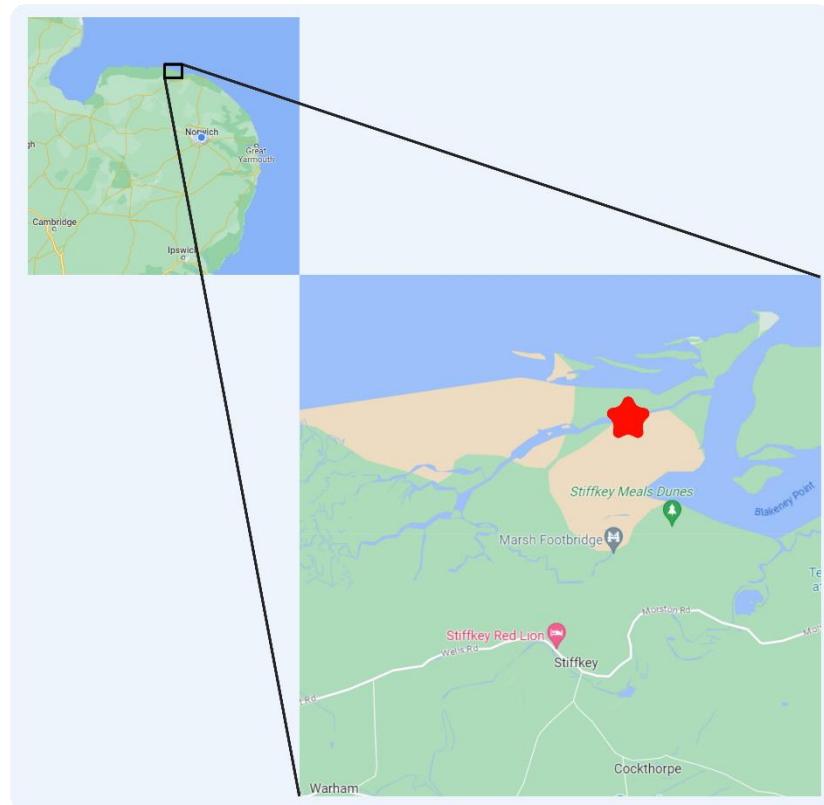


Fig. 5.2 Geographical representation of the sampling location on Stiffkey saltmarsh. The saltmarsh is located north of Norwich on the North Norfolk coastline in the East of England. The red star indicates the local sampling location on the saltmarsh.

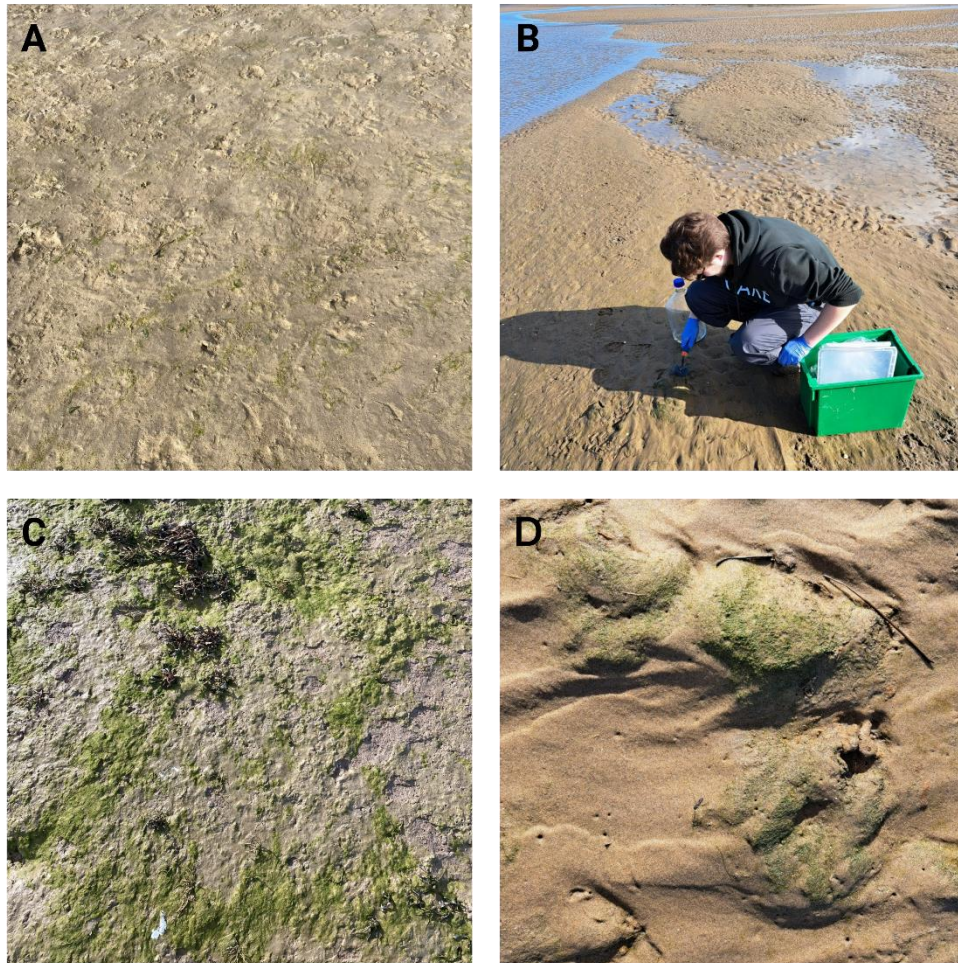


Fig. 5.3 Sampling location on the intertidal mudflat at Stiffkey saltmarsh showing microbial mat growth. A) Patches of microbial mat growth covering a flat area. **B)** Sampling technique digging ground to extract the benthic microbial community. **C)** Microbial mat on a higher altitude plane on intertidal mud flat near to plant life. **D)** Microbial mats growing amongst non-flat vertices on salt marsh sediment.

Sampling away from visible plant and seaweed matter was not difficult as the hostile intertidal mud flat showed very little growth except for the microbial mats. Higher plants only appeared on isolated ridges and higher altitude areas as shown in (**Fig 5.3C**). Three samples were taken from a similar area on the intertidal mud flat around 1 m from each other. Each sample was taken from visible microbial mats which sat on the surface of the sediment and had a distinctive green hue extended up to 2 cm from the sediment. When the microbial mat was extracted from the sediment there was also visible growth up to several cms below the surface (**Fig. 5.4**).

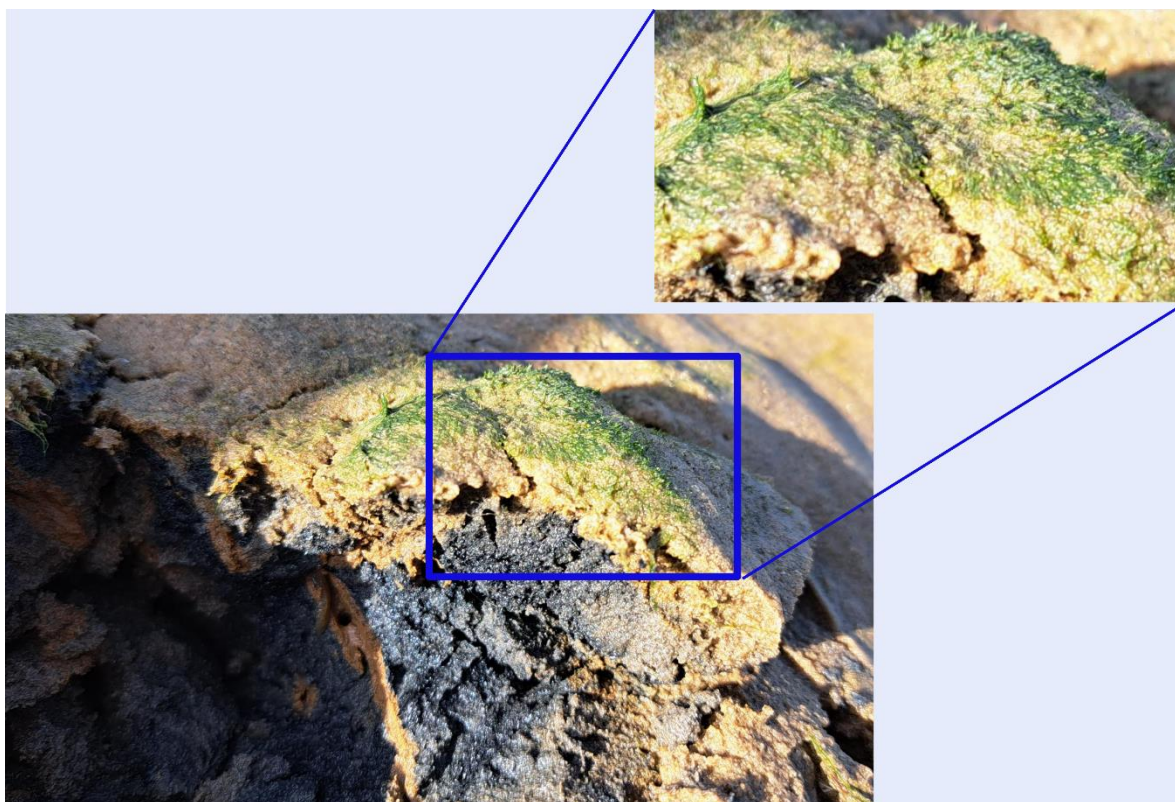


Fig. 5.4 Close up of saltmarsh sediment displaying the microbial mat. The microbial mat grows on top of the sediment, but also develops several cm below the surface.

Samples were taken at low tide in March on a sunny day with little to no cloud cover and therefore it could be expected that cyanobacteria in the microbial mat would migrate to the surface to maximise their light harvesting potential. Possible stresses exhibited on the mat could be from high light intensities but also higher salinity as the sea water had evaporated from the sampling location. DMSP is purported to have a role as an antioxidant (23) and it is widely used as an osmolyte (38) and therefore we would expect higher production under these stressful conditions.

5.2.2 Quantification of DMSP in a microbial mat on Stiffkey saltmarsh

Following sampling, the quantity of DMSP was examined in both the sediment and microbial mat through GC. Triplicate biological replicates were assayed along with controls from the local environment, such as sediment that contained no visible microbial mat and seawater from the nearby river. Statistically significantly different

levels of DMSP were quantified between each of the four environments ($P < 0.05$, ANOVA) (**Fig. 5**). The highest DMSP concentrations per mg of wet weight were observed in the microbial mat, followed by the sediment with visible microbial mat growth, and then in sediment with no visible microbial mat growth. All three samples contained much higher levels of DMSP than in the seawater control. Interestingly, the microbial mat contained more DMSP per mg of wet weight than the microbial mat and sediment. This is likely due to the excess weight from the sediment diluting the DMSP content of the sample. Therefore, the majority of DMSP produced in this ecosystem comes from the microbial mat and not from microorganisms living in the surrounding sediment.

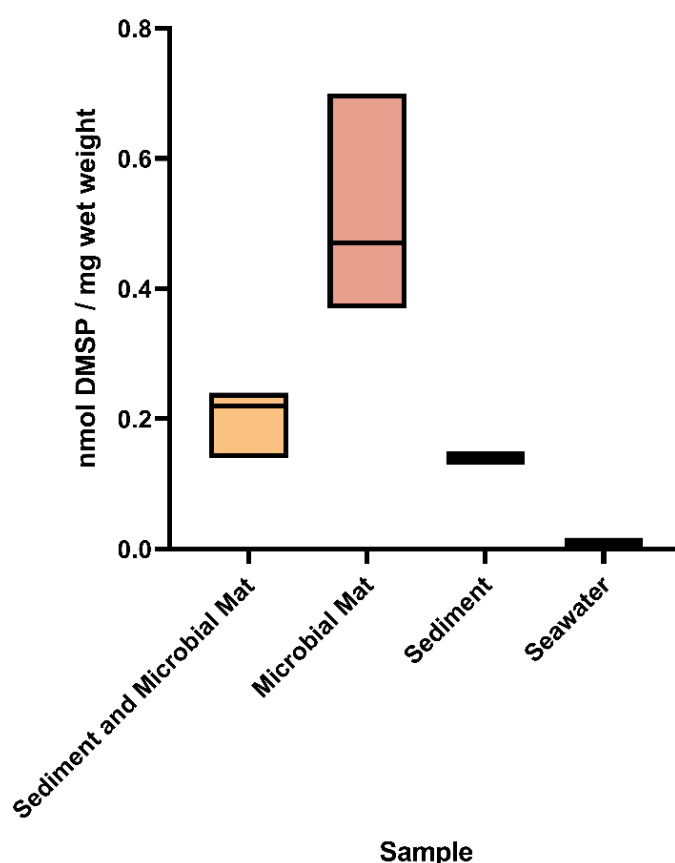


Fig. 5.5 Analysis of DMSP content in Stiffkey Sediment and microbial mat samples. Sediment and microbial mat samples consisted of sediment with visible microbial mat attached. Microbial mat samples were washed of excess sediment. Sediment samples contained no visible microbial mat. Seawater samples consisted of seawater taken from the nearby saline channel.

5.2.3 Determining the microbial community via metagenomics and metatranscriptomics

Following confirmation of DMSP production in the sediment and microbial mat, triplicate DNA and RNA samples were isolated from the microbial mat and adjacent sediment and sent for sequencing with Novogene. The samples were dominated by proteobacteria and bacteroidota (**Fig. 5.6**), with these phyla constituting up to 55 % of representative genomes. Alphaproteobacteria, which contain DMSP-synthesizing members, were between 10-15 % of total genomes and typically less than 10 % of total transcripts. The majority of the alphaproteobacteria were *Rhodobacterales*.

Cyanobacteria, once eukaryotic chloroplasts had been removed, represented around 10 % of relative abundance in genome copies. However, their transcripts were 3 – 4 fold higher than genome copies and had the highest proportion of any prokaryotic order. Interestingly, at the order level, the known cyanobacterial genome abundance dropped to below 10 % with up to 40 % of representative genomes from unclassified cyanobacteria. The dominant cyanobacterium family was Coleofasciculaceae (**Fig. 5.8A**), which has been shown to produce DMSP at higher rates than most other cyanobacteria (219). Interestingly, *Oscillatoria* made up relatively few copies of total cyanobacterial genomes (~3 %) but increased to around 25 – 30 % of total transcripts. Between 20 – 40 % of total cyanobacteria genomes were from unclassified species and these amounted to less than 5 % of total cyanobacterial transcripts.

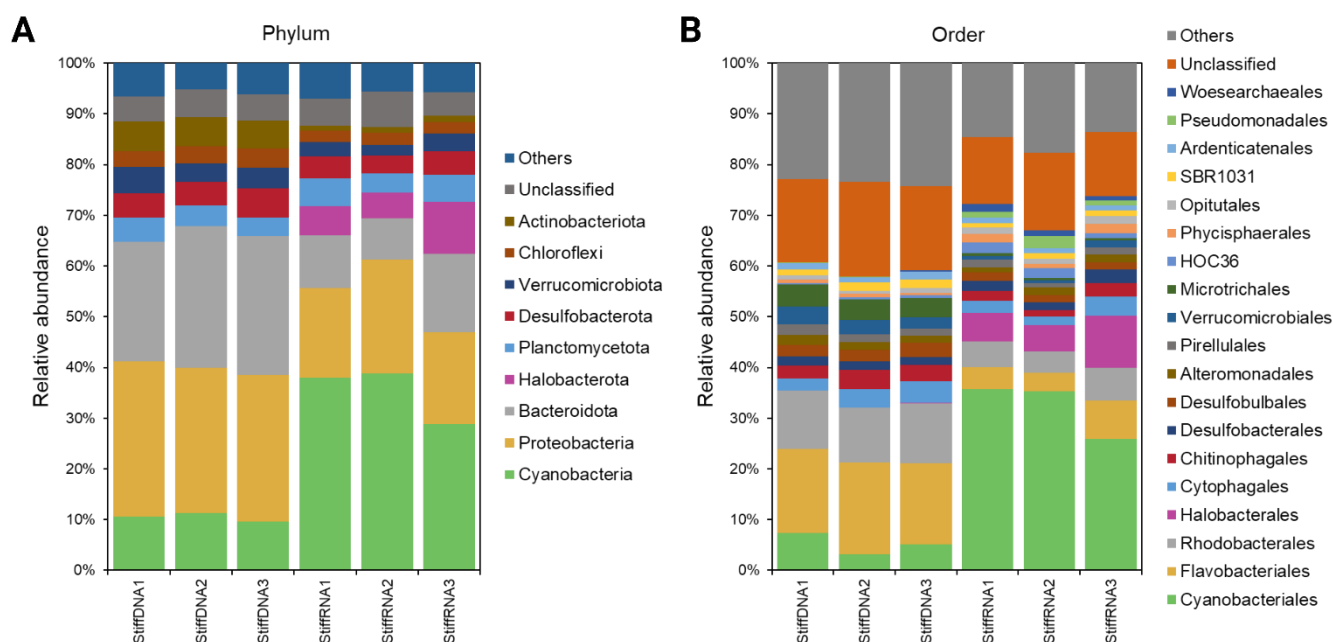


Fig. 5.6 Metagenomic and metatranscriptomic analysis of prokaryotes from Stiffkey saltmarsh sediment samples. A) StiffDNA1-3 shows the relative abundance of phylum based on genomic analysis; StiffRNA1-3 shows the relative abundance of phylum based on transcriptome analysis. **B)** StiffDNA1-3 shows the relative abundance of orders in the sediment based on genomic analysis. Both **A** and **B** are based on amplification of 16s rRNA reads. StiffRNA1-3 is the relative abundance of orders based on transcriptome analysis. Analysis conducted by Xiaoyu-Zhu, UEA.

Next, we examined Eukaryotic DNA and RNA (**Fig. 5.7**). It was important to assess the total proportions and abundances of divisions containing high DMSP producers, such as diatoms, phytoplankton and *Ulva*, as these may contribute to the DMSP pool detected in the samples. Total eukaryotic genomes were around 20 % of total prokaryotic genomes and eukaryotic transcripts were approximately 50 % lower than total prokaryotic transcripts. Chlorophyta represented 50 – 60 % of total genomes. Nearly all were Ulvophyceae of which approximately 30 % were *Ulva* (**Fig. 5.8B**), with the remainder being unclassified Ulvophyceae. However, Ulvophyceae only represented around 2 – 3 % of total eukaryotic transcripts and therefore were not well expressed in this environment.

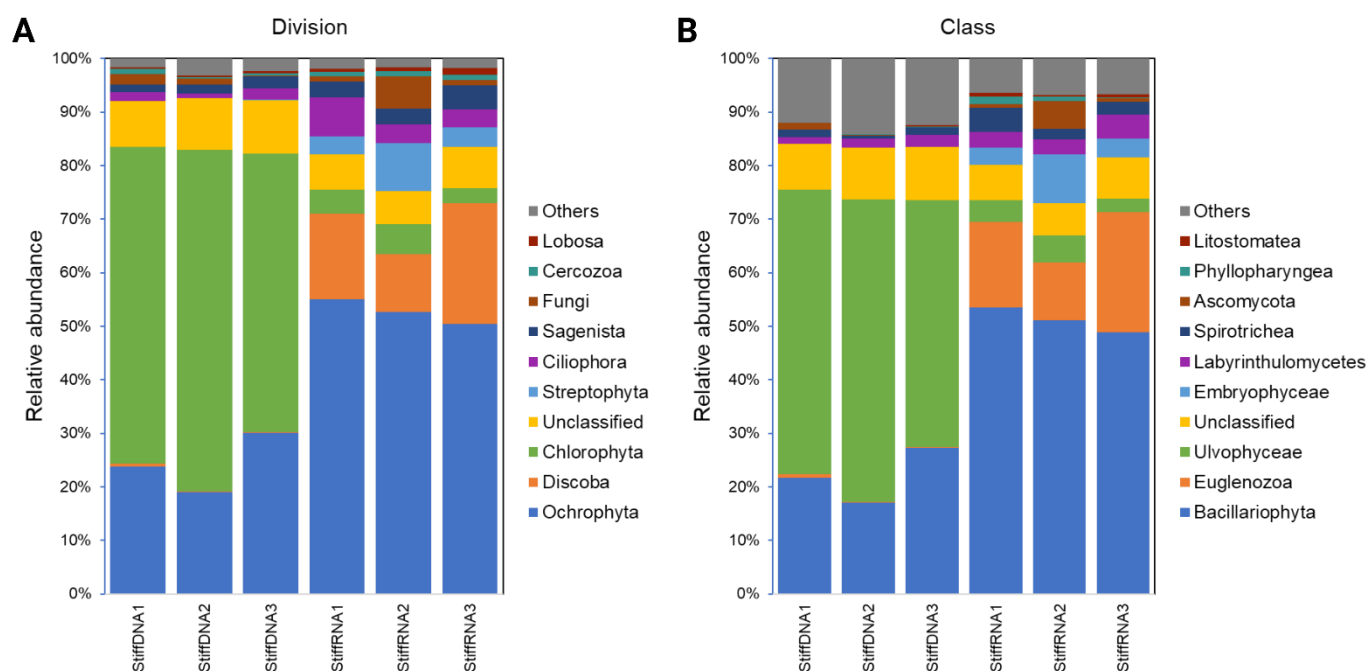


Fig. 5.7 Metagenomic and metatranscriptomic analysis of eukaryotes from Stiffkey saltmarsh sediments. A) StiffDNA1-3 is the relative abundance of phyla based on genomic DNA; StiffRNA1-3 is the relative abundance of phyla based on transcriptomes. **B)** StiffDNA1-3 is the relative abundance of classes based on genome copies; StiffRNA1-3 is the relative abundance of classes based on transcriptomes. Both **A** and **B** are based off of 18S rRNA reads. Analysis conducted by Xiaoyu-Zhu, UEA.

The second most abundant division was Ochrophyta which contains the class Bacillariophyta, comprising up to 50 – 55 % of total genome copies and transcripts. Members of the Bacillariophyta include diatoms which have been shown to produce DMSP, such as *Fragilaria* (221) and *Thalassiosira* (222). However, in our samples these diatoms were negligible. Around 50 % of diatoms were unclassified Raphid-Pennate with a further third being unclassified Bacillariophyta. Combined, these constituted approximately a third of total diatom transcripts in these samples.

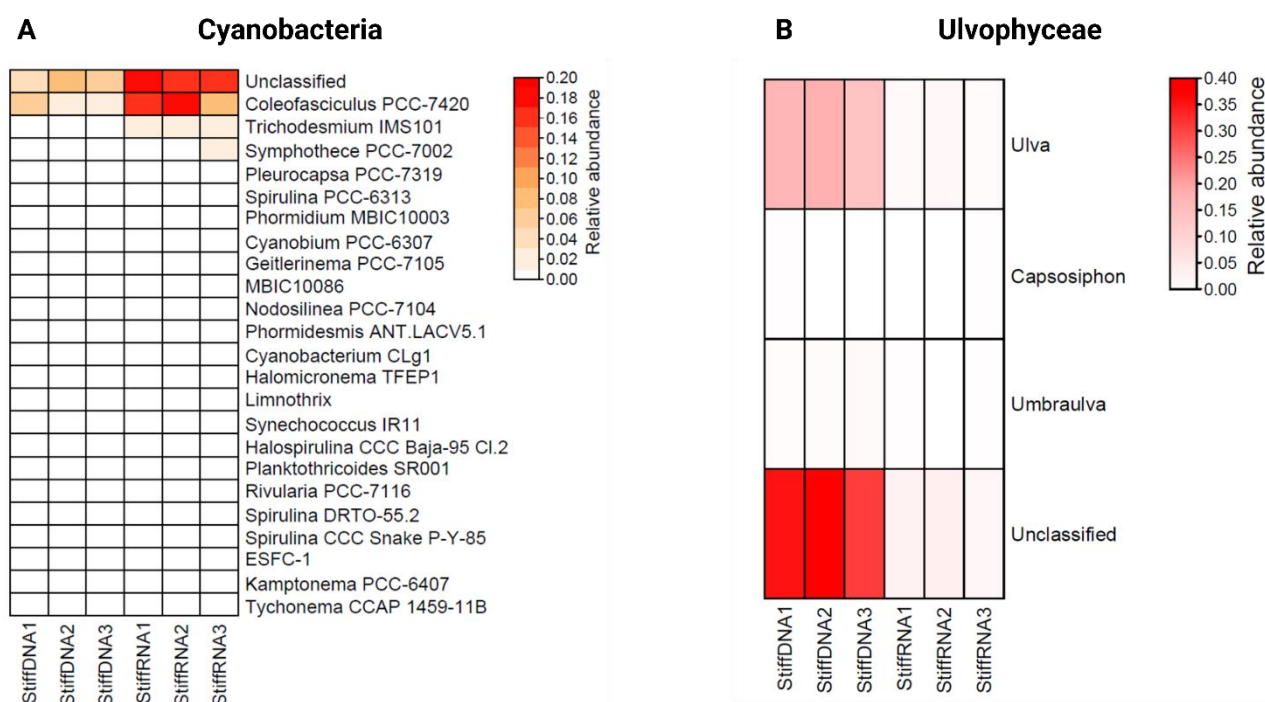


Fig. 5.8 Cyanobacterial and ulvophyceae species identified on Stiffkey saltmarsh ranked by relative abundance. A) Individual species of detected cyanobacteria ranked by abundance of 16S rRNA. **B)** Species of Ulvophyceae ranked by abundance of 18S rRNA.

5.2.4 Determining key DMSP cycling genes present in the microbial mat

We next determined the type and abundance of DMSP cycling genes in this environment. Characterised DMSP synthases, catabolic enzymes and transfer enzymes were searched for in the metagenomic and metatranscriptomic datasets.

The most abundant gene encoding DMSP biosynthesis enzymes was *dsyC* (**Fig. 5.9**), which was found in Desulfobacterales, Cyanobacterales and unclassified microorganisms. However, despite *DSYU* having very few genomic copies it had similar levels of expression to *dsyC*, with both its genomic copies and expression solely found in *Ulva*. Furthermore, there were no hits to any other known DMSP synthesis genes, so despite the prevalence of diatoms in the datasets none of them contained *DSYB* or *TPMMT*. Additionally, none of the abundant alphaproteobacteria contained *dsyB* or *MmtN*. Therefore, DMSP is synthesised via either *DSYU* or *DsyC*, unless there are additional unknown proteins involved.

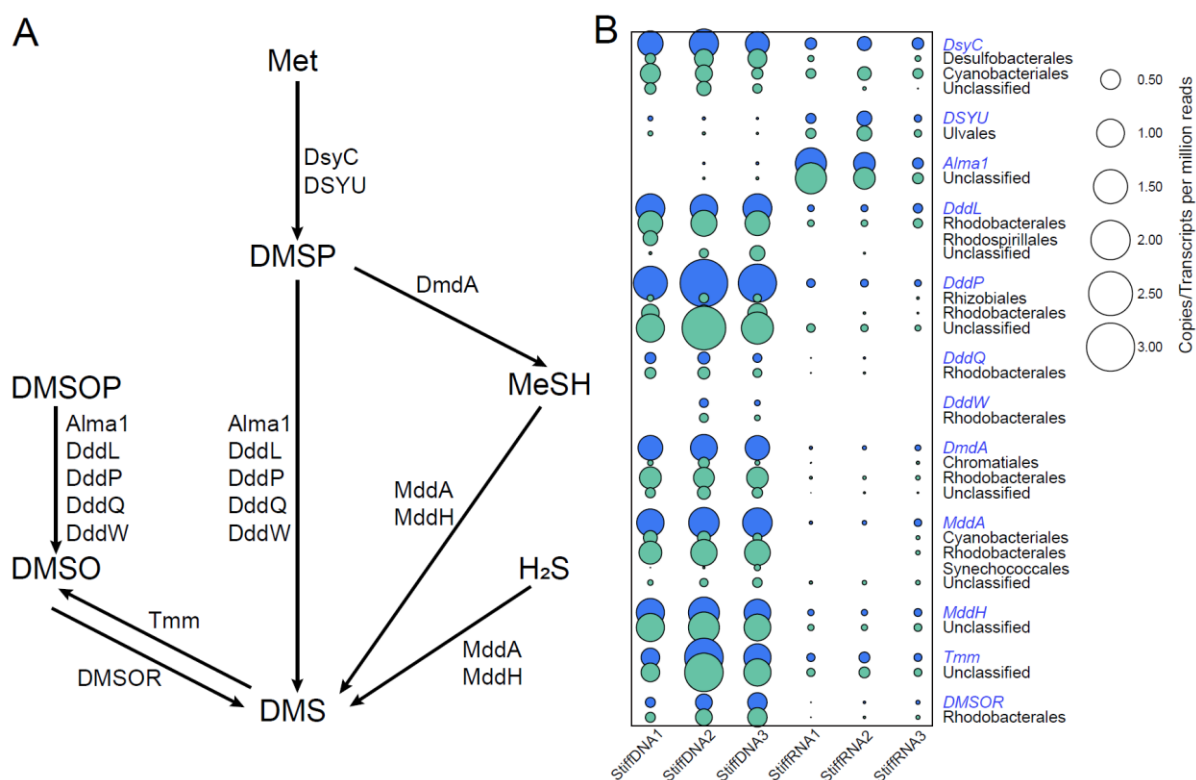


Fig. 5.9 Abundance of known DMSP cycling genes alongside their relative expression from Stiffkey saltmarsh sediment. A) Schematic showing relationship between various DMSP related compounds, including the proteins known to be involved in each pathway. **B)** Relative abundances of genome copies and transcriptome copies of relevant DMSP cycling genes, denoted in either copies or transcripts per million reads. Analysis conducted by Xiaoyu-Zhu, UEA.

Of the DMSP lyases, eukaryotic *Alma1* was the most highly expressed, despite low genomic copy abundance (**Fig. 5.9**). The prokaryotic DMSP lyases, *dddL* and *dddP*, showed higher genomic copy abundance, but were expressed at lower levels. Most of the *dddL* copies and transcripts were from Rhodobacteriales. *dddP* was found partially in Rhodobacteriales but predominantly in unclassified bacteria. Furthermore, the other lyases, *dddQ* and *dddW*, were found in some genomes but very few transcripts of *dddQ* and even fewer of *dddW* were expressed in these samples and only then in Rhodobacteriales.

The demethylase *dmdA* was the most abundant in genomic copy number and predominantly found in Rhodobacteriales and Chromatiales, but its expression was lower than *dddP* and *dddL*. Therefore, organisms in this environment likely favour

catabolism of DMSP to acrylate and DMS rather than MeSH. The MeSH to DMS methyltransferases *mddA* and *mddH* were very abundant in genomes. For example, *mddA* was present in Cyanobacteriales, Rhodobacterales and Synechococcales and both *mddA* and *mddH* were found in unclassified bacteria. *Tmm*, catalysing oxidation of DMS to DMSO, was also abundant in the metagenome, but its expression was low and reserved to unclassified organisms.

Overall, there was poor correlation between the genome copy number and number of expressed transcripts, as shown in **Fig. 5.9**. This was observed across all classes of DMSP metabolism genes that we analysed.

Table 5.1. Percentage of prokaryotic genomes in the sediment which contain the analysed DMSP cycling genes.

Gene	StiffDNA1	StiffDNA2	StiffDNA3	Average
<i>dsyC</i>	2.40%	2.86%	1.97%	2.41%
<i>dddL</i>	3.13%	2.56%	2.92%	2.87%
<i>dddP</i>	4.30%	7.67%	5.08%	5.68%
<i>dddQ</i>	0.46%	0.49%	0.27%	0.41%
<i>dddW</i>	0.00%	0.27%	0.11%	0.13%
<i>dmdA</i>	2.29%	2.44%	2.08%	2.27%
<i>mddA</i>	2.82%	3.15%	3.04%	3.00%
<i>mddH</i>	2.97%	3.24%	2.49%	2.90%
<i>Tmm</i>	1.31%	5.01%	2.59%	2.97%
<i>DMSOR</i>	0.38%	0.95%	1.31%	0.88%

5.2.5 Identifying candidate DMSP cycling genes in cyanobacteria

We next analysed individual MAGs in our dataset to determine sequences for potential DMSP cycling genes. Representative *dsyC* sequences were identified in the cyanobacterium *Coleofasciculus* (Fig. 5.10). *Coleofasciculus* has already been shown to produce DMSP (219) and it is possible that DsyC is responsible for synthesis in this genus.

Other identified sequences include *MddA* in *Trichodesmium*, *Tmm* in the cyanobacterium *Elainellales* as well as a putative DMSP lyase *DddP*. To the best of our knowledge no DMSP lyase has ever been identified in a cyanobacterium before. Therefore, this presents an interesting new development which may expand on our knowledge of the relationship between cyanobacteria and DMSP, if the lyase is functional.

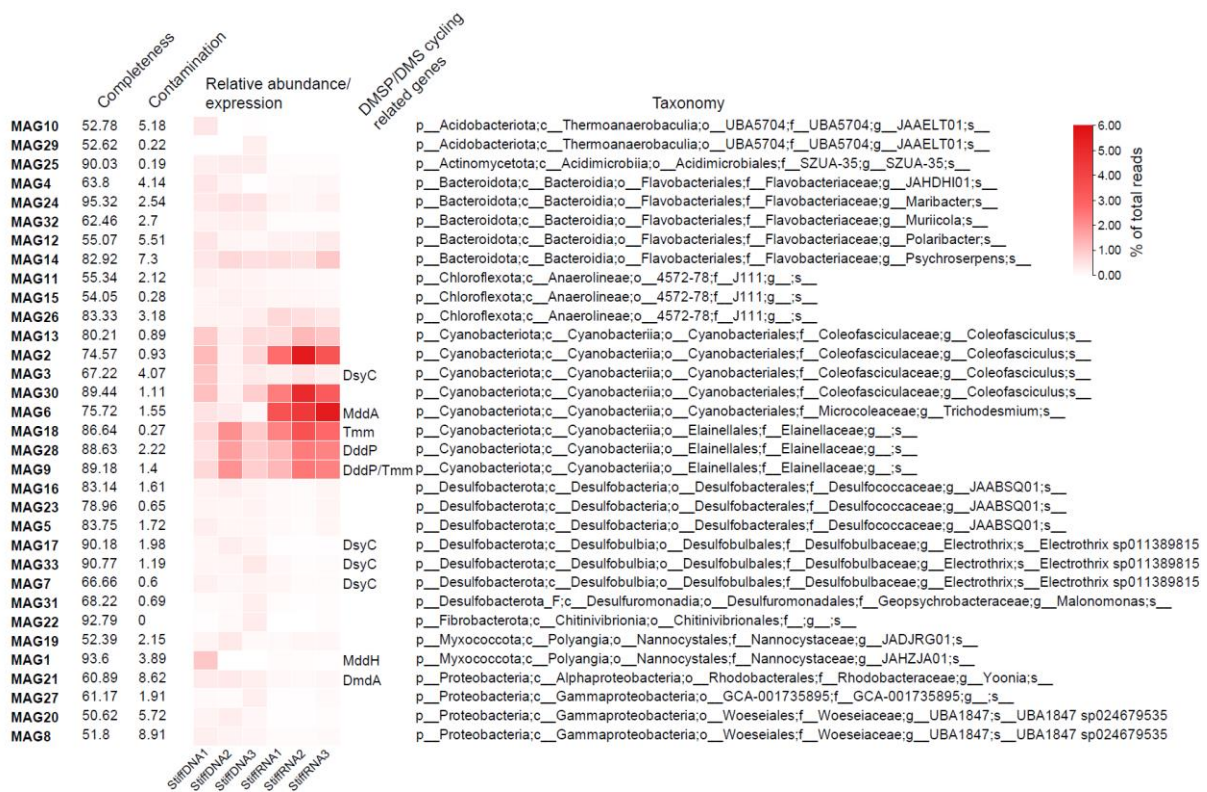


Fig. 5.10 Relative abundance and expression of key DMSP cycling genes in MAGs. StiffDNA1-3 indicates copy number in genomes, StiffRNA1-3 indicates expression levels in the transcriptome. Analysis conducted by Xiaoyu-Zhu, UEA.

Using our past work conducted on ratifying functional DsyC's from other cyanobacteria, we constructed a phylogenetic tree (Fig. 5.11) positioning the DsyC's

identified in this dataset amongst them with *T. erythraeum* IMS101 (IMS101) DsyC at the top. Interestingly, a few species within this dataset contain methyltransferases which are clustered amongst IMS101 DsyC and may represent interesting targets for highly active MTHB methyltransferases. DsyC's from non-cyanobacterial *Electrothrix* and *Planctomycetota* species are clustered near non-functional or less specific MTHB methyltransferases. These may be more active towards substrates of the GB synthesis pathway and may help us elucidate substrate affinity in DsyC enzymes.

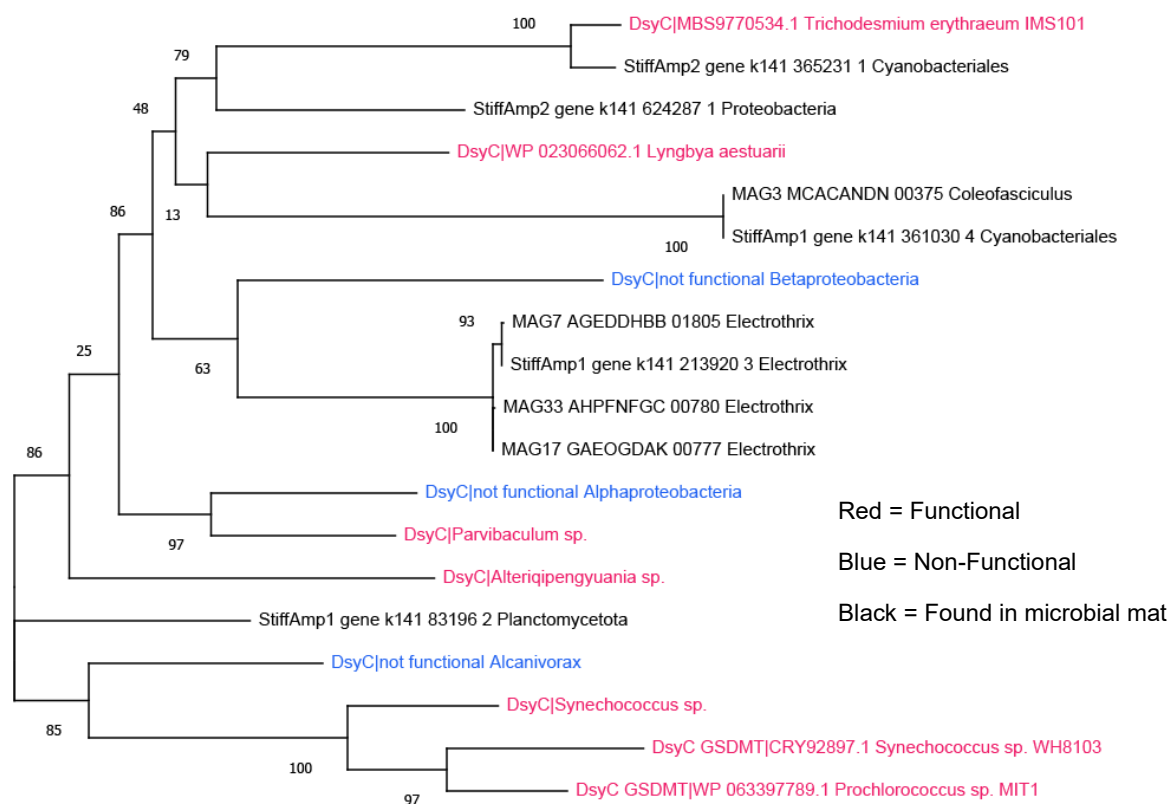


Fig. 5.11 Phylogenetic tree of identified putative *dsyC*'s in the sediment. Branches coloured in red are *DsyC*'s which have been shown to be functional towards MTHB. Branches in blue are *DsyC*'s which have been tested and are non-functional towards MTHB. Branches in black were found in the microbial mat.

5.3 Discussion

Within this chapter, DMSP production has been examined in an intertidal microbial mat. We have confirmed that this microbial mat produces high amounts of DMSP and is probably the main source of DMSP in the intertidal saltmarsh region. Based on previous work, the main producers in this environment are likely *Ulva*. However, cyanobacteria cannot be ruled out as minor contributors. Furthermore, the widespread and abundant gene *dsyC* which encodes for a putative MTHB methyltransferase was one of the most abundant and transcribed genes involved in DMSP synthesis and may contribute significantly to DMSP production.

The levels of DMSP reported in the sediment and microbial mats (400 – 700 nmol g⁻¹) in this study are vastly higher than in other marine sediment environments. For example, 5 nmol DMSP g⁻¹ were found in Mariana trench samples, 128 and 91 nmol DMSP g⁻¹ in surface sediment isolated from saltmarsh pools in Stiffkey and Cley, respectively, and 103 DMSP nmol g⁻¹ from sediment in the Great Yarmouth estuary (34). Ecosystems tested in other studies include Norwegian fjords which were between 20 – 80 nmol DMSP g⁻¹ depending on the depth tested (223) and sediment samples from the South China sea up to 1-2 cm below the surface gave, which varied between 11.25 – 20.90 nmol DMSP g⁻¹ (224). The higher levels of production found in our samples are possibly due to the presence of photoautotrophic algae, the highest known producers (7), which may be missing from the other areas examined.

In this study, *dsyC* was the most abundant known gene involved in DMSP synthesis, found in *Coleofasciculus*, already shown to produce DMSP (219), some *Oscillatoria* species, shown to produce DMSP at lower rates than *Coleofasciculus* (125) and purple sulfur bacteria, also previously shown to produce low amounts of DMSP (225). Our work is the first to possibly identify the methyltransferase responsible for DMSP production in these organisms, although this will need verification by enzyme and complementation studies. The only other gene involved in DMSP synthesis to be identified was *DSYU* in *Ulva*. Its abundance is not well characterised due to it being discovered in the past year (unpublished) and there not studies of how widespread it is in the environment. Interestingly, despite there being Rhodobacterales and diatoms in our samples, *dsyB* (11), *DSYB* (28) or *mmtN* (34) were not detected in our datasets.

These methyltransferases are normally found in saltmarsh sediments, varying between 0.1 – 3.6 % abundance (34).

The most abundant DMSP lyase genes identified in this study were *dddP*, then *dddL*, with *dddQ* and *dddW* found in lower quantities. The dominance of *dddP* is consistent with other sediment DMSP cycling studies (34,226,227) *dddL* has previously been shown to be the second most abundant DMSP lyase gene in Stiffkey saltmarsh sediment (34). However, identification of *dddQ* and *dddW* in saltmarshes in previous studies.

Identification of *mddA* and *Tmm* is consistent with a previous report on saltmarshes on the North Norfolk coast (228). However, our study expands the range of DMSP cycling genes discovered in this environment to include *mddH*, which converts MeSH to DMS (unpublished) and *DMSOR*, which reduces DMSO to DMS (unpublished). Further studies are warranted to explore the various transformations of DMSP related compounds in this environment and how it compares to other intertidal microbial mats.

One consideration not tested in this study is methylation by DsyC of other substrates to produce compounds such as GB and homoserine betaine. Additionally, it is difficult to assign the relative contribution of taxa to DMSP cycling purely based on -omics analysis due to the uncertainty of whether these organisms contain functional proteins involved in DMSP metabolism. Previous work has identified the importance of GSDMTs in many different bacteria and algae (77,89,92,229), and therefore it is important to verify the dual role of DsyC's as SDMT and MTHB methyltransferases. Future work would consider synthesising complete genes found in the metagenomic dataset and testing for activity with substrates from the GB, HB and DMSP synthesis pathways. Furthermore, synthesised genes should be characterized and checked for their kinetic parameters against DsyC from *T. erythraeum* and *Synechococcus* as well as other key methyltransferases from all three DMSP synthesis pathways, such as DsyB, DSYB, TpMMT and MmtN.

Additional future environmental work should include repeating this study over the course of a year and tracking changes in microbial mat community composition and how this affects the total amount of DMSP produced. Changes in transcribed DMSP cycling genes should also be monitored and any interesting DMSP synthesis candidate genes should be synthesized and tested for activity. On top of this, similar

studies should be conducted on other areas of the saltmarsh which may harbour similar microbial mats, such as in the submerged subtidal region and lower marsh regions containing pools.

Chapter 6 – Discussion

6.1 Overview

This project aimed to identify and characterise DMSP production in marine cyanobacteria. Numerous aims were achieved including:

- Identification of a novel MTHB methyltransferase (DsyC) in *T. erythraeum* IMS101.
- Discovery of DsyC in many abundant cyanobacteria including *Synechococcus*, *Prochlorococcus* species, *L. aestuarii* and *C. chthonoplastes*.
- Identifying dual activity in DsyC towards intermediates of GB and DMSP synthesis.
- Confirming production of DMSP *Synechococcus* and *Prochlorococcus* using axenic strains.
- Identifying DsyC and DSYU as the likely two DMSP-linked methyltransferases in an intertidal microbial mat from the Stiffkey salt marsh.

6.2 Identification of a novel DMSP synthesis gene in cyanobacteria

In this study I identified the first MTHB methyltransferase homolog in a cyanobacterium, specifically IMS101 (**Table 3.1**). Through multiple sequence alignment and phylogenetic analysis this methyltransferase was found to be a novel class of MTHB methylating enzyme in a different clade to all previously identified DMSP-linked methyltransferases (**Fig. 3.1**). *In vitro* and *in vivo* studies ratified this enzyme and showed functionality towards methylating MTHB to DMSHB, after which we termed the enzyme DsyC (DMSP synthesis in Cyanobacteria). This study also demonstrated that IMS101, and potentially other cyanobacteria, use the transamination pathway (36) to produce DMSP.

However, DsyC is unusual as it has already been ratified as a methyltransferase involved in HB synthesis (145) and homologs found (**Fig. 3.5**) in *Synechococcus* and *Prochlorococcus* are linked to GB production (74). This study demonstrated a dual and promiscuous function (**Fig. 3.15**) whereby both IMS101 and *L. aestuarii* PCC 8106

(PCC 8106) DsyC methylated MTHB as well as all intermediates of the GB methylation pathway. To the best of our knowledge, a multi-role methylation behaviour of DMSP and GB linked methyltransferases has not been shown before. This also suggests that some GB, HB and DMSP linked methyltransferases may have evolved from a common progenitor with a possible separate function.

L. aestuarii is a common and often dominant component of salt marsh microbial mats (124,204). Previous studies revealed some strains produce DMSP (126) but the protein and genes involved were not identified. This study confirms DsyC is responsible for DMSP and GB production in this abundant and widespread cyanobacterium (**Fig. 3.15**). From an environmental perspective, *dsyC* could be used as a marker to identify other cyanobacteria which are DMSP and GB producers. However, these genes and their protein products will need ratifying before their function can be named. If the majority of cyanobacteria and possibly other species in *L. aestuarii* dominated intertidal microbial mats contain *dsyC* then they would significantly impact the contribution of these environments to DMSP and GB production.

6.3 *Synechococcus* and *Prochlorococcus* species produce DMSP and GB

Production and transport of DMSP by *Synechococcus* and *Prochlorococcus* has been reported but is poorly understood, both in terms of the specific species involved and the enzymes involved in synthesis (49,125,127). In this study I showed that multiple species produce DMSP (**Fig. 4.1 – 4.4, 4.8 - 4.9**) and in axenic cultures, which has not been demonstrated before. Despite the amount of DMSP per cell being low (**Fig. 4.4, 4.9**) compared to other producers such as phytoplankton (130), diatoms (222), algae and plants (21), the abundance of *Synechococcus* and *Prochlorococcus*, in addition to *Trichodesmium*, suggests that cyanobacteria could be major contributors to the global DMSP pool in marine environments.

The DsyC homolog SDMT, discovered in *Synechococcus* sp. WH8102 (74) synthesizes GB from dimethylglycine and sarcosine. Homologs of this gene are found in the low-light adapted clade (LLIV) of *Prochlorococcus* (**Fig. 3.5 and 3.6**). All species of *Synechococcus* and *Prochlorococcus* we tested contained a DsyC/SDMT homolog produced GB whereas species lacking it did not (**Fig. 4.6 and 4.10**). This confirmed

that SDMT was critical for GB production in more species than just WH8102. Therefore, these genes and close homologs detected in environmental samples could be good targets for functional testing.

Surprisingly, reduced GB production under low nitrogen conditions in these species did not result in an increase in DMSP levels (**Fig. 4.4 - 4.5 and 4.9 - 4.10**). This suggests there is no direct relationship between GB production and DMSP production under low nitrogen conditions, although it would be interesting to test osmolyte levels in samples from low N oligotrophic waters. It is possible these species switch from producing GB to another osmolyte such as GG or GGA, both commonly found in these genera, under low N conditions (141). Further analysis will be required to test this hypothesis via HPLC, LCMS or NMR analysis of osmolytes under N limiting conditions.

6.4 High DMSP production in an intertidal microbial mat

DMSP production has not been analysed in intertidal microbial mats, which given their exposure to highly varied salt conditions, could be major producers. Samples of intertidal microbial mat were collected (**Fig. 5.2, 5.3 and 5.4**) and upon confirmation of high levels of DMSP production ($400 - 700 \text{ nmol g}^{-1}$) (**Fig. 5.5**) the samples were analysed via metagenomics and metatranscriptomics (**Fig. 5.6 - 5.10, Table 5.1**). DMSP produced in this microbial mat and sediment was considerably higher than other previously studied sediment samples such as saltmarsh pools, fjord sediment and deep sea sediment ($11.25 - 128 \text{ nmol g}^{-1}$) (34,223,224).

Within the sediment the dominant DMSP-linked methyltransferases were DSYU (unpublished) and DsyC (**Fig. 5.9 and 5.10**). These two proteins have not previously been characterised or found in intertidal salt marsh microbial mats due to their recent discovery. Furthermore, no other methyltransferase involved in DMSP production were found, suggesting that DsyC, DSYU or possibly unknown methyltransferases are solely responsible for DMSP production in this environment. *Coleofasciculus* (formerly *Microcoleus*) is extensively found in microbial mats globally (230) and the species *C. chthonoplastes* has been shown to make DMSP (219), albeit in low concentrations. The identification of DsyC within *Coleofasciculus* is the first evidence of the methyltransferase responsible for DMSP production within these cyanobacteria. This along with identification of DsyC in *L. aestuarii* paves the way for future study of which proteins are responsible for DMSP production in benthic cyanobacteria.

Interestingly, cyanobacteria were not the only microorganisms shown to contain DsyC (Fig. 5.9 and 5.10). DsyC was also found in Desulfobacterales, sulphur bacteria which have previously not been shown to produce DMSP. However, some species have been noted for their use and cleavage of DMSP and reduction of DMS to DMSO (231). DsyC may have crossed over into different families of prokaryotes via horizontal gene transfer (232) since being able to make GB, HB or DMSP in this highly stressful environment would help hosts containing DsyC outcompete other species.

6.5 Future work

Further characterisation of DsyC is required, specifically an understanding of how the structure of each protein differs in relation to its function and preferred substrate. This will allow us to determine proteins involved in DMSP biosynthesis in environmental datasets (TARA Oceans (233), Global Ocean Survey (GOS) (234) and GEOTRACES (235)). Analysis of DsyC proteins from other groups of bacteria will also allow us to determine which organisms produce DMSP, GB, and/or HB. Combined with analysis of DMSP levels and omics analysis in a wide range of marine samples, this will allow us to potentially measure global production levels of DMSP from DsyC containing organisms.

Additional saltmarshes and intertidal zones, which have been previously shown to contain a high proportion of *L. aestuarii* and *C. chthonoplastes*, should also be examined. Since these ecosystems are widespread and large scale this could be another significant source of DMSP production. Investigating DMSP production via rate experiments over different seasons, times of the day and temperatures would provide valuable physical data to combine with the -omics data from this study. Furthermore, analysing the relative contribution of the microorganisms in cycling DMSP in the environment through stable isotope probing or radioisotope labelling would elucidate who the key DMSP cyclers are in this environment.

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8 Supplementary information

Table 1. List of primers used in this study.

Primer	Description	Sequence
M13 Uni (-43)	Forward primer to check inserts in pMALC2X-1039 and pLMB509-GFP.	AGGGTTTTCCCAGTCACGACGTT
M13 Rev (-29)	Reverse primer to check inserts in pMALC2X-1039 pLMB509-GFP.	CAGGAAACAGCTATGACC
T7 Fwd	Forward primer to check inserts in pET vectors.	TAATACGACTCACTATAGGG
T7 Rev	Reverse primer to check inserts in pET vectors.	GCTAGTTATTGCTCAGCGG
WH8103Left Flank_Fwd	Forward primer to clone left flank of <i>dsyC</i> from <i>Synechococcus</i> sp. WH8103.	attcgagctcgggtaccgggACCGTGGAT GCCTTCTCAGGGGAG
WH8103Left Flank_Rev	Reverse primer to clone left flank of <i>dsyC</i> from <i>Synechococcus</i> sp. WH8103.	ttttatgcaCCAGCATCAGTTCCGGTT ATCTCGAAAG
WH8103RightFlank_Fwd	Forward primer to clone right flank of <i>dsyC</i> from <i>Synechococcus</i> sp. WH8103.	cttttcggggAAGATCCACCACACATC CTCCTTGAGGTAG
WH8103RightFlank_Rev	Reverse primer to clone right flank of <i>dsyC</i> from <i>Synechococcus</i> sp. WH8103.	tgcattgcctgcaggtcgactGGTCAGTGC GGATGGCAGCCCCAA
WH8103Gent Cassette_Fwd	Forward primer to clone gent cassette from pBBR1MCS-5 for WH8103 <i>dsyC</i> mutagenesis.	ctgatgctggTGCATAAAACTGTTGT AATTCATTAAGCATTCTGCCGACA TG
WH8103Gent Cassette_Rev	Reverse primer to clone gent cassette from pBBR1MCS-5 for WH8103 <i>dsyC</i> mutagenesis.	ggtggatcttCCCCGAAAAGTGCCACC TGGCGGC
WH8020Left Flank_Fwd	Forward primer to clone left flank of <i>dsyC</i> from <i>Synechococcus</i> sp. WH8020.	aacaatttcacacaggaaacagctatgacatga ttacgcaatcgggtgtgccagggttc
WH8020Left Flank_Rev	Reverse primer to clone left flank of <i>dsyC</i> from <i>Synechococcus</i> sp. WH8020.	ttacaacagttttatgcagggtgcaacacgac gtttg
WH8020RightFlank_Fwd	Forward primer to clone right flank of <i>dsyC</i> from <i>Synechococcus</i> sp. WH8020.	cagggtggcacttttcggggtagagcccgatgatga atgtc
WH8020RightFlank_Rev	Reverse primer to clone right flank of <i>dsyC</i> from <i>Synechococcus</i> sp. WH8020.	cagtgccaaagcttgcattgcctgcaggtcgactct agagagaaggagatttttcgtcaaattattg
WH8020Gent Cassette_Fwd	Forward primer to clone gent cassette from pBBR1MCS-5 for WH8020 <i>dsyC</i> mutagenesis.	aaacgtcgtgttcagccctgcataaaaactgtt gtaattcattaagcattc
WH8020Gent Cassette_Rev	Reverse primer to clone gent cassette from pBBR1MCS-5 for WH8020 <i>dsyC</i> mutagenesis.	acattcatatcgggctctaccccgaaaagtgcc cctgg

IMS101pMAL dsyC_Fwd	Forward primer to clone <i>T. erythraeum</i> IMS101 <i>dsyC</i> from pMALC2X-1039.	gtgcatATGACTGAAGCAGTTAC
IMS101pMAL dsyC_Rev	Reverse primer to clone <i>T. erythraeum</i> IMS101 <i>dsyC</i> from pMALC2X-1039.	gcacgaattcAAGCTCAGTTTTTC
WH8103pMA LdsyC_Fwd	Forward primer to clone <i>Synechococcus</i> sp. WH8103 <i>dsyC</i> from pMALC2X-1039.	gctcatATGGGTACTACCAAC
WH8103pMA LdsyC_Rev	Reverse primer to clone <i>Synechococcus</i> sp. WH8103 <i>dsyC</i> from pMALC2X-1039.	gcttagaattcAAGCTCAACCTGG
WH8020Mut Check_Fwd	Forward primer to check mutation of <i>dsyC</i> in WH8020.	CTCCCAGCTTTGCCTCC
WH8020Mut Check_Rev	Reverse primer to check mutation of <i>dsyC</i> in WH8020.	CCAACAAGGCCATGATGATC
WH8103Mut Check_Fwd	Forward primer to check mutation of <i>dsyC</i> in WH8103.	CAAGCTGAATCCCCTGAAGA
WH8103Mut Check_Rev	Reverse primer to check mutation of <i>dsyC</i> in WH8103.	GGGCACAACGAACGGC
LyngbyadsyC _Fwd	Forward primer to check for <i>dsyC</i> in <i>L. aestuarii</i> UTEX 2515.	GCGCGCATATGCAAACCTGAATAC
LyngbyadsyC _Rev	Reverse primer to check for <i>dsyC</i> in <i>L. aestuarii</i> UTEX 2515.	CGATGAATTCCTATTTTTTGCTAA AATGC

Synthesized genes used in this study

>*Synechococcus* sp. WH8103 *dsyC*

ATGGGTACTACCAACGGTTGTGCTGCTGACTCTGTGGCTGCAACTTACTATGACTCTCAGGATGCGG
ACCAATTCTACGAACAGGTGTGGGGTGGTGAGGACATTCATATTGGCCTGTACGCAACGCCGGACG
AAGCCATCGCAACTGCGTCTGACCGCACCGTTACGCTCTGCTGGATCTGGCAGACCCACTGCCGCA
AGGTGGTTGTGTGGTCGATCTGGGTGCAGGCTATGGTGGTGCATCTCGTCGTCTGGCACGTTGGAG
CGAACGTCCGGTGCACGCTATCAATATTTCTGCTGTTGAGAACGACCGCCATCGTCGTCTGAACGTA
GACGCTGGTCTGGACCAGCAGATTACTGTGCACGACGCATCCTTCGAACAGGTTCCGGTTGCGGAC
GCATCTGCTGACCTGGTGTGGAGCCAAGACGCCATCCTGCACGCTGGTGATCGTGCAACTGTGCTG
GCTGAAGTTTCTCGCCTGCTGAAACCGGGTGGTTGTTTCGTATTCACCGATCCGATGGCAGCCGATG
GTGTTGAAATGGGCCTGCTGCAACCGATTCTGGATCGTATCCACCTGCCGGATCTGGCTTCTCCGGC
ACGTTACAAAGCGTGGGGTGAAGCTGTAGGCCTGACCATGGAAGTTTGGGACGAACGTACCGAAAT
GCTGGTTCGCCACTACGACCGCGTTCGTCAGGATACTGCCTGCGTCGTGCGGAACCTGGAAACCTCC
ATCTCTTCTGGCTATCTGGAACGCATGGACGTGGGCCTGGGTCATTGGGTGGATGGTGGTCAACAG
GGTCGTCTGAGCTGGGGCCTGATGCGTCTGCGTAAACCAGGTTGA

>*Trichodesmium erythraeum* IMS101 *dsyC*

ATGACTGAAGCAGTTACTATCGCCCAGAAATATTACGATAGCACTGAAACTGATAAACTGTATGCTG
CTATCTGGGGCGGTGAACACATTCACTACGGTATTTACACCCGTCCGGACGAACCGATCCATGATGC
GTCACTGAGCACGGTAAAAACCATTGCACAGACCCTGAAAAAATCGACCAAACTCCCGTGTTATC
GATCTGGGTGCAGGTTATGGTGGCGCAGCGCGTTACCTGGCTAAAACTTACGGCTGTAGCGTTTGCT
GTCTGAACCTGTCTCAGCTGCAAAATCAACGTAACGGCCAGCTGAACCAGGAACAGAACCTGGACC
CGCTGGTGGAATTACGCAGGGCAGCTTCGAAGATATCCCATACCCGAACAACCTTTTCGACATCGT
GTGGTCCCAGGATGCTATCCTGCATTCTGGTGACCGTTGTCTGGTGTTCCAGGAGATTAAACGTATC
CTGAAACCGGGTGGTGAGTTCATCTTCACTGATCCGATGGAAAGCGAAACCTTCCCGCCGGGTGCA
CTGCAAACGGCTTTTGATCGTCTGGGTATCAGCGACATGGGCAGCTACCGTTTCTACTCTCAGACCCT
GAAAGACCTGGGTTTCGAGGAACTGAACTTCATCGATCTGTCTCAGAATGTGGCCATCCACTACCGT
CGTTTTCGCGAAGAAGTTCAAAAACGTTACAACGAAGTAGTGAAGCTGACCTCTACGGAATTTGTTG
ACAAAACCCTGGATTCTATCCAGCCGTGGATCGAACGTTATGAGCAGGGCTACATGCAGTGGGCTA
TCCTGCATTTTCGTCTGCTGGAGAAAACTGA

9 Research articles published during the timespan of this doctoral training

1. Carrion, O., Li, C.-Y., Peng, M., Wang, J.-Y., Pohnert, G., Azizah, M., Zhu, X.-Y., Curson, A., **Walsham, K.**, Zhang, X., Monaco, S., Harvey, J., Wang, Q., Chen, X.-L., Gao, C., Wang, N., Wang, X.-J., Wang, P., Giovannoni, S., ... Zhang, Y. Z. (2023). Function and wide distribution of DMSOP cleaving enzymes in marine organisms. *Preprint*.
2. Wang, J., Zhou, S., Curson, A., Vieira, A., **Walsham, K.**, Monaco, S., Li, C.-Y., Rivera, P. P., Wang, X.-D., Hanwell, L., Zhu, X.-Y., Leão, P., Lea-Smith, D. J., Zhang, Y. Z., Zhang, X. hua, & Todd, J. (2023). Novel dimethylsulfoniopropionate biosynthesis enzymes in diverse marine bacteria, cyanobacteria and abundant algae. *Preprint*.