# Biochemical Characterisation of DMSP Lyases in Marine Bacteria and Phytoplankton

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# Abstract

Dimethylsulphoniopropionate (DMSP) is the most abundant sulphur molecule in the oceans. Catabolism of DMSP by marine organisms is important both for the global movement of sulphur and as a carbon and sulphur source for microbial life. The molecular basis of DMSP catabolism had been revealed by the discovery of a DMSP demethylase and six different lyases in marine bacteria. However, at the start of this study in 2009 no eukaryotic DMSP lyase had been isolated, purified or characterized from axenic cultures, and there was little information on the enzymology of any of the bacterial DMSP lyase enzymes. The work presented in this thesis identifies the requirement of the three bacterial cupin DMSP lyases DddL, DddQ and DddW for metal cofactors, and also establishes the enzymology and biochemistry of these cupin-containing DMSP lyases. The presence of a typical DMSP lyase in the coccolithophore *Emiliania huxleyi* RCC1217 is also demonstrated for the first time from bacteria-free cultures by application of previously successful techniques used in the purification of bacterial *ddd* genes. Combined these findings provide new insights into the mechanisms of cleavage of DMSP by bacteria and phytoplankton and expand our understanding of the enzyme diversity involved in this process.

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# Abbreviations

| | |
|---|---|
| AUC | Analytical Ultra Centrifugtation |
| CHES | N-Cyclohexyl-2-aminoethanesulfonic acid |
| DEAE | Diethylaminoethyl |
| DNA | Deoxyribonucleic acid |
| DMSP | Dimethylsulphioniopropionate |
| DMS | Dimethyl sulphide |
| EDTA | Ethylenediaminetetraacetic |
| GC | Gas chromatography |
| GF | Gel Filtration |
| HIC | Hydrophobic interaction chromatography |
| HPLC | High Performance Liquid Chromatography |
| ICP-OES | Inductively Coupled Plasma Optical Emission Spectroscopy |
| IPTG | Isopropyl-β-D-1 thiogalactopyranoside |
| LB | Lysogeny Broth |
| NMR | Nuclear Magentic Resonace |
| PCR | Polymerase Chain Reaction |
| qRT-PCR | quantitative Real Time – Polymerase Chain Reaction |
| RNA | Ribonucleic Acid |

| | |
|---|---|
| RPC | Reverse Phase Chromatography |
| SDM | Site Directed Mutagenesis |
| SDS-PAGE | Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis |
| SEC | Size Exclusion Chromatography |
| TCA | Citrate cycle |
| TRIS | Tris(hydroxymethyl)aminomethane |

# 1 Introduction

## 1.1 Dimethylsulphioniopropionate

Dimethylsulphioniopropionate (DMSP) is a tertiary sulphonium compound (Figure 1.1) important in several aspects of life and the biochemistry of the worlds' oceans. DMSP is prevalent in the marine environments with ~$10^9$ tonnes per annum being produced by marine eukaryotic organisms (Kettle *et al*., 2000; Stefels, 2000; Stefels *et al*., 2007).

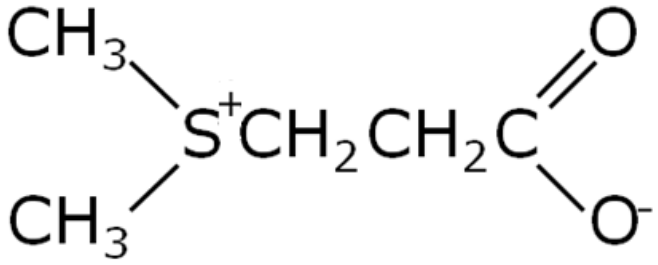


**Figure 1.1 Structural representation of DMSP**

DMSP was originally identified as a metabolite from the red alge *Polysiphonia lanosa* and was subsequently found to be produced by a broad variety of marine eukaryotic organisms including some angiosperms e.g. *Spartina,* (salt marsh Cord Grass) and *Wollastonia biflora* (beach sunflower), phytoplankton of the Haptophyceae such as the coccoliophore *Emiliania Huxleyi (E. huxleyi)*, diatoms including the model diatom *Thalassiosira pseudonana*, macroalgae including *Ulva intestinalis* a green alga, corals including *Acropora millepora* and *Acropora tenuis* (branching coral) and lastly, but importantly, the dinoflagellates. Phytoplankton which contain the highest levels of intracellular DMSP are the small dinoflagellates ([1,082 mM]), and the coccolithophores (*E. huxleyi*) ([166mM] (Stefels, 2000; Otte *et al*., 2004; Broadbent *et al.,* 2002). Indeed, in *E. huxleyi* blooms in the North Sea, DMSP can reach 10% of the total carbon fixed (Archer et al., 2001). It is therefore fair to assume that, following these blooms in the earth's oceans, DMSP is released into the environment through lysis of phytoplankton by viral lysis, natural senescence, or zooplankton grazing. This DMSP is considered a significant source of carbon and sulphur in the microbial food web. Interestingly, in blooms of *E. huxleyi* where the biomass of dinoflagellates exceeded that of the coccolithophores, 75-80% of the total DMSP lyase activity was due to the dinoflagellates. However, this is a complex relationship due to the association and

attachment of bacteria that also possess DMSP lyase activity. Because of this and in order to determine the roles played by both the phytoplankton and bacteria, there is an absolute requirement to collect and produce data from *bona fide* axenic cultures of *E. huxleyi* so that total lyase activity can be truly determined and until this study no such results had been realised.

Despite the importance and abundance of DMSP (it is estimated that 1 billion tonnes is produced annually), the function of DMSP still remains unclear, with a number of suggested roles (see below). One of the most important roles of DMSP in the marine and estuarine ecosystems is osmoregulation in plants and phytoplankton. Early investigations using the coccolithophore *Hymenomonas caterae* demonstrated that intracellular levels of DMSP can reach concentrations of 0.3 mol $L^{-1}$ in order to offset the external osmotic pressure (Vairavamurthy, 1985). Some marine bacteria can import DMSP, use it for growth and incorporate sulphur into amino acids, emphasising its importance as a sulphur and carbon source for marine bacteria (Curson et al., 2011d). Experimental studies have also led to the conclusion that some bacteria have evolved to a state in which DMSP is the only source of sulphur which they are able to exploit (Tripp, 2008).

### 1.2 DMSP – Why is it important?

Aside from the physiological role of DMSP in the organisms that produce it (see Figure 1.2) DMSP is most important and well known because it is the most significant biological precursor of the climatically active gases dimethyl sulphide (DMS) and methanethiol (MeSH) (Kiene and Taylor, 1988). Both of these gases have important biological, chemical and environmental impacts in the global environment, in particular the marine environments, where their production is greatest. Since DMSP is a key molecule in the marine food web it is imported by many marine microorganisms which use it as a preferred carbon and sulphur source (Tripp, 2008). Bacteria in particular are capable of harvesting carbon and sulphur from DMSP for the assimilation of important molecules required for growth. For example, sulphur is a key component of metalloproteins, polypeptides and many cofactors including coenzyme A, biotin, and thymine, which are essential to all organisms. Through the catabolism of DMSP, organisms can

generate either DMS or MeSH, both gases being part of two very important microbial driven pathways.

**Figure 1.2 Organisms that produce DMSP.** From left to right *Emiliania huxleyi, Polysiphonia lanosa, Acropora millepora, Spartina, Braarudosphaera bigelowii*

### 1.3 Why do organisms synthesise DMSP?

There are many suggested roles for DMSP in the eukaryotic organisms that produce it but none have been definitely established due to a lack of molecular genetic studies on the organisms that make it. DMSP was identified in the mid-20$^{th}$ century as a substrate for transmethylation reactions and as a precursor to gaseous sulphurous volatiles (Cantoni, 1956, Challenger, 1948). Putting aside its significance in the global cycling of biological sulphur (see section 1.4), it must play an important role in cellular processes with the organisms producing it since these organisms make it at such high levels. To date, all we know about the function of DMSP in the organisms producing it comes from physiological studies.

#### 1.3.1 DMSP as an osmolyte.

One role to which DMSP has long been associated with is its potential function as a compatible solute. Indeed it has been shown that in *E. coli* DMSP can act as an osmolyte (Yancey, 2001; Murdock *et al*., 2014), and also more recently in *Roseobacter*. Together with the knowledge that marine bacteria can also contain high concentrations of DMSP, through importing it into the cell, it suggests that DMSP does protect the bacteria against salinity stress. DMSP is also considered to be a structural analogue of the nitrogenous glycine betaine (Figure 1.3). Glycine betaine is an established osmoprotectant present in the cytoplasm of some higher plants and bacteria (Simon et al., 2002). It is postulated that in the marine environment, DMSP is favoured due to the abundance of sulphur and scarcity of nitrogen.

Compatible solutes are osmoprotectant compounds, which, as the name suggests, are soluble. They shield the cell from abrupt changes in the concentration of the solute surrounding the cell instigating a rapid change in the movement of water across the cell membrane (see Figure 1.4). Compatible solutes contain no net charge (DMSP is a zwitterion), and do not interact with proteins. As a result, such organic compounds are capable of accumulating within cells at high concentrations whilst not hindering the functions of the cell (Cody et al., 2000). In doing so, they assist cells to acclimate to environmental stresses such as high salinity. Some studies have suggested that whilst some species, namely the green alga *Ulva rigida*, *Ulothrix subflaccida* and *Enteromorpha bulbosa* and the diatom *Fragilariopsis cylindrus*, show DMSP accumulation in response to increased salinity (Lyon *et al*., 2011) other species including the saltmarsh cordgrass *Spartina* do not exhibit any such response (Vairavamurthy, 1985, Curson et al., 2011d, Dell'Ariccia et al., 2014).

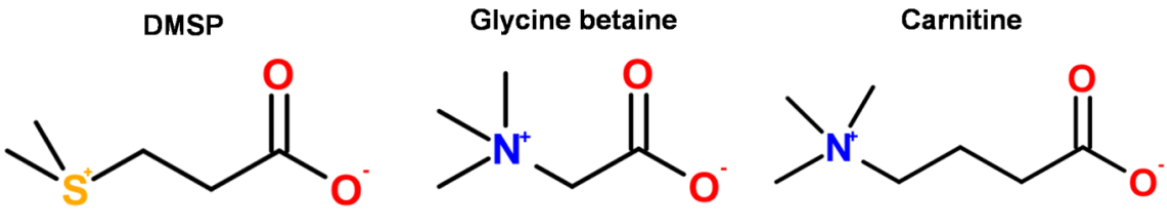



**Figure 1.3 Structures of DMSP and structural analogues, glycine betaine and carnitine known osmoprotectants.**

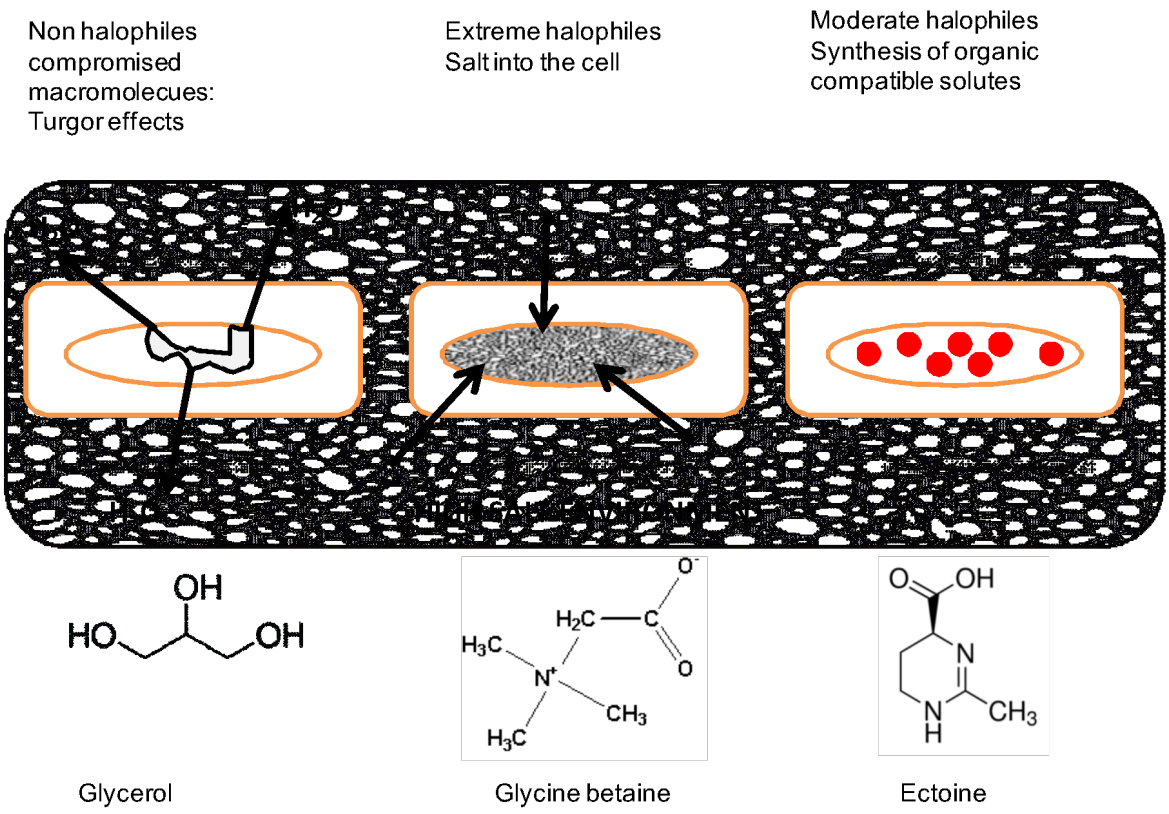



**Figure 1.4 Function of compatible solutes.** Marine organisms are required to adapt to high salinity. In organisms not adapted to high salinity, water will diffuse out of the cell into the high salt concentration external environment and leave behind a cell that will lose its structure and function. To bypass problems associated with osmoregulation, organisms have adapted protective strategies such as the acclimation of osmolytes (Bohnert, 1995).

### 1.3.2 DMSP as a cryoprotectant.

Another role long suggested for DMSP is that of a cryoprotectant. This role was first observed in Antartic macroalgae where an increase in intracellular DMSP concentration was observed as temperatures decreased (Welsh et al, 1999)

### 1.3.3 DMSP as an antioxidant.

It is also suggested that DMSP, or its catabolites, may act as an antioxidant to protect against the effects of oxidative stress by scavenging reactive oxygen species. Oxidative stress ultimately leads to the production of free radicals, atoms

and small molecules carrying unpaired electrons and which have an open configuration. These species can either be positively, negatively or neutrally charged and take part in an array of chemical reactions which damage DNA and effect cellular processes. Antioxidants are molecules capable of reducing or nullifying the oxidative effects of free radicals by being oxidised themselves by the radical. DMSP possesses such properties due to its zwitteronic status and the negative and positive charges it possesses. A study conducted by Sunda *et al*. (2002) measured the ability of DMSP as an antioxidant compared to other known antioxidants, including ascorbate and glutathione, in the coccolithphore *Emiliania huxleyi*. It was demonstrated that DMSP readily scavenges hydroxyl radicals and suggested that algae containing higher intracellular concentrations of DMSP may be more resistant to oxidative stress.

### 1.3.4 DMSP as a grazing deterrent.

It is also considered that DMSP discourages predation either directly or by acting as a precursor to other toxic or unpalatable compounds, such as DMS or acrylate (see section 1.7). These molecules may deter organisms such as zooplankton from ingesting the DMSP-producing organism. The studies conducted by Wolfe et al (1997) suggested that acrylate, a by-product of DMSP cleavage, acts as a grazing deterrent for *E. huxleyi* against protist grazers (Wolfe et al., 1997), but further research indicated that DMSP may actually be the deterrent and not acrylate (Strom, 2003).

### 1.3.5 DMSP as a sulphur storage molecule

It has been suggested that DMSP may act as a biological sulphur resource in the form of reduced sulphur (Stowe-Evans et al., 2004), as an overflow system (Stefels, 2000) and as a methyl donor during nitrogen limitation. In higher plants, the sulphur-containing amino acids methionine and cysteine are maintained at low cellular concentrations, thus preventing any unwanted feedback mechanisms. By producing DMSP under conditions where sulphur incorporation exceeds nitrogen incorporation, algae can protect themselves from such increases in methionine and cysteine levels and allow the continuation of other metabolic pathways (Laub, 2007).

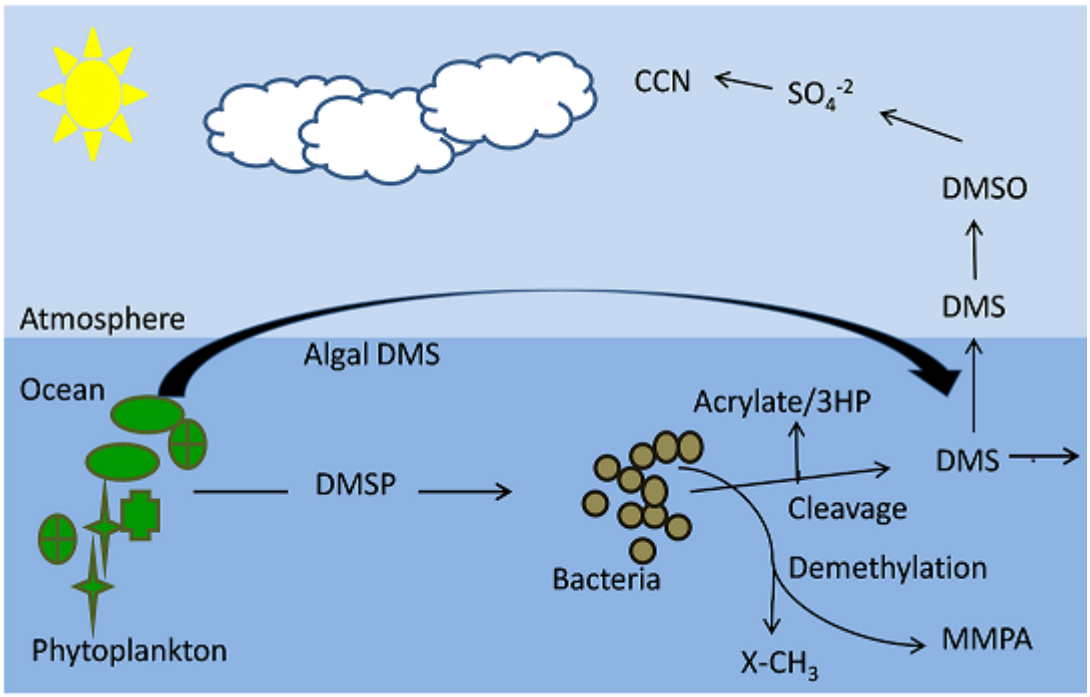



**Figure 1.5 Simplified diagram of the biological transformations of DMSP.** The demethylation pathway, which accounts for the catabolism of ~70% of all DMSP, produces MMPA prior to the generation of MeSH. Some of this is released whilst some is integrated into protein-sulphur by marine bacteria. The DMSP lyase pathway, found in both marine bacteria and phytoplankton that make DMSP, generates the climatically active gas DMS with ~30% of all DMSP being catabolised in this way. Much of this DMS is further degraded by other bacteria, to produce DMSO, for example. However, 10% of the total, (~ 30 million tonnes) escapes into the atmosphere, where its oxidation products (e.g. sulphite and sulphate) are formed by photo-oxidation. These act as cloud condensation nuclei (CCN) increasing cloud albedo, thus influencing local climate conditions. (Sullivan, 2011)

### 1.4. The role of Dimethylsulphide in the environment

Lovelock (1972) suggested that DMS was a significant sulphur source which allowed for the mobilization of sulphur from the Earth's oceans into the atmosphere and back to the land. DMS is now considered as one of the most abundant, biologically derived sulphur-containing molecules transmitted from the oceans to the air, with 35 million tons estimated to be transferred each year Li et

al. (2003), (Andreae, 1997, Kettle, 2000). Estimates of the biological global production of DMS from DMSP lyase-containing organisms varies between 16 and 39 million tonnes, although it has been suggested this is just 5-10% of the total DMS derived from DMSP (Andreae, 1983, Bates, 1992).

### 1.4.1. DMS: Climate cooling, Cloud Condensation Nuclei and the CLAW hypothesis.

DMS was first implicated as a climate-regulating gas in 1987 when Charlson, Lovelock, Andreae and Warren co-wrote a paper (Charlson et al., 1987) that proposed the CLAW hypothesis, in which it was suggested that phytoplankton are capable of influencing the Earth's climate. The suggestion was that the greater the temperature the higher the concentration of DMS produced via phytoplankton. This DMS is liberated from the oceans into the atmosphere and its oxidation products, such as sulphate aerosols, act to increase the amount of sunlight being reflected away from the Earth's atmosphere, either directly or by acting as cloud condensation nuclei (CCN) to increase cloud formation (Figure 1.5, Figure 1.6). This increase in reflected sunlight would result in the reduction of global temperatures and also a reduction in phytoplankton growth and DMS emission, thus creating a negative feedback mechanism. However, the CLAW hypothesis has more recently come under intense scrutiny, and over the last two decades new data, a better understanding of the biogeochemistry of the marine and the global environments and of climate physics has led to the suggestion that the effect of phytoplankton on a global scale is not the primary driver as once thought. Whilst DMS particles may play an important role in the development of clouds, the particles themselves only actually reduce the solar radiation by 0.04 watts $M^{-2}$, an insignificant figure when compared to the effect of industrial emissions. Some now suggest the CLAW hypothesis itself should be retired. (Vogt, 1998, Dawkins, 1999, Simo, 2001, Quinn and Bates, 2011)

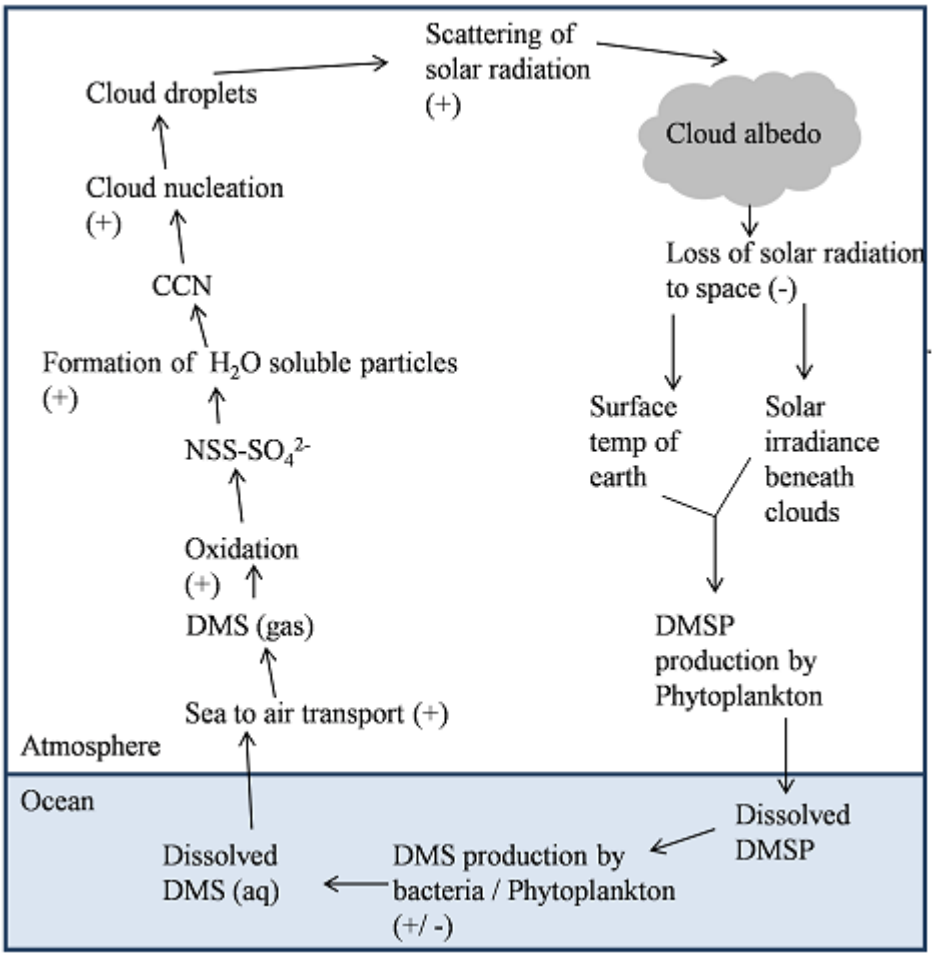



**Figure 1.6 DMS and the CLAW hypothesis.** The atmospheric transport of DMS and its oxidation products provides an important link in the global sulphur cycle. DMS emission into the atmosphere is a source of cloud condensation nuclei and connects DMS production to climate regulation.

### 1.4.2 DMS and its role as a chemoattractant

A rather different role for DMS has been associated with is its capacity to induce the behavioural changes in a variety of marine organisms. As a result, DMS is considered to be a potent chemoattractant for a range of organisms from various marine fauna including microscopic crustaceans, such as copepods, to oceanic birds and mammals, such as seals, with all having been shown to use DMS as a foraging cue (Nevitt, 2008; Savoca and Nevitt, 2014) (Bender and Conrad, 1994, Steinke, 2006, King, 2007). A recent study (Dell'Ariccia *et al*., 2014) has demonstrated that petrel and shearwater seabirds smell DMS and respond to it as

a foraging cue, not just in the sub-antartic waters as proposed by previous hypotheses, but also in temperate waters. However what is still not clear is if any visual cues are also used to establish foraging spots.

### 1.4.3 DMSP and DMS as key nutrients

DMSP and DMS are thought to act as significant sources of carbon and sulphur in the microbial food web. The bulk of DMSP produced has long been linked to the phytoplankton which produce it and to the food web to which the phytoplankton are an integral part (Yoch, 2002). The DMSP stored in marine phytoplankton which is a component of dissolved organic carbon (DOC) is thought to range from 5 to 50 nM with concentrations reaching in excess of 100 nM in blooms of DMSP-producing algae. The DMSP released from phytoplankton represents a significant part of the microbial food web as DMSP constitutes up to 10% of the carbon in some algal species. Some studies indicate that the dissolved DMSP released from coccolithophore blooms alone was so plentiful that it was able to support over 5% of the microbial carbon demand in the North Sea (Simo, 2001). Secondary to this, the DMSP associated with the phytoplankton blooms contributed nearly all the sulphur requirements to the microbes living in close proximity to the blooms. To date, no other compound is known to give such a large amount of carbon and sulphur to the marine microbial food web, but when we consider the intracellular concentrations of DMSP in some species of phytoplankton (see sections 1.1. and 1.3) this is not wholly surprising.

## 1.5 Biosynthesis of DMSP

The ability of plants, green algae, red algae, diatoms, coccolithophores and dinoflagellates to produce DMSP has been recognised for some time (Vairavamurthy, 1985, Hanson, 1994, Barnard, 1984) and more recently it has been revealed that some corals are also able to produce DMSP (Raina, 2013). Although there are no details of any genes involved in DMSP synthesis in any organism, there has been much work to establish the biosynthetic pathways in some plants and phytoplankton. In this section the known pathways through which DMSP is synthesised in plants and algae shall be reviewed. Primarily, we will describe which organisms are known to produce DMSP and the concentrations they make.

Green algae begin with the Virdiplantae clade of the Kingdom along with the higher plant group Embryophyta. Whilst the red algae (*rhodophyta*) belong to the Archaeplastida, this taxon also contains some Virdiplantae. The synthesis of DMSP in higher plants differs to the pathways associated with the divergent green and red algae that use the same route of synthesis. Single-celled eukaryotes, also called microalgae, include dinoflagellates, diatoms and coccolithophores, which are taxonomically distinct to the Archaeplastida and belong to the Chromalvetolata. Rather curiously, there is some suggestion that diatoms and coccolithophores use the same pathway for DMSP synthesis as the green and red algae, and that the dinoflagellates have evolved a third pathway exclusive to them (Dickschat, 2015).

The three biosynthetic DMSP pathways in plants, green algae and the dinoflagellates have evolved autonomously but what is not known is whether taxonomically distinct organisms, for example green algae and coccolithophores, use the same pathway due to horizontal gene transfer or because of an independent evolutionary event.

Given the diversity of organisms producing DMSP (Figure 1.7, table 1.1), and the varied amounts which they produce, it is perhaps not surprising that there are different DMSP biosynthetic pathways which exist in these organisms. Despite a general lack of molecular analysis performed on DMSP-producing organisms, there are, thanks largely to the work of Dr Andrew Hanson, published pathways for DMSP synthesis in the beach sunflower *Wollastonia biflora* (Rhodes *et al*., 1997), the salt marsh cordgrass *Spartina alterniflora* (Kocsis and Hanson, 2000) and the green algae *Ulva lactuca* . Building upon this work, a separate pathway was determined in the dinoflagellate *Crypthecodinium cohnii* (Uchida. A., 1993). The knowledge we have regarding DMSP biosynthesis follows studies using isotopic labelling and recognition of pathway intermediates in DMSP producers. In spite of this biochemical analysis there is no information currently available on the genes and enzymes used for DMSP biosynthesis. To date, there is not a single gene, expressed sequence tag (EST) or protein sequence that has been shown to be involved in the synthesis of DMSP in any organism.

**Table 1.1 Diversity of DMSP Producers and their reported cytoplasmic concentrations**

| | Kingdom | Phylum | [DMSP] mM |
|---|---|---|---|
| ***Spartina alterniflora*** | Plantae | Angiosperm | 70 (Dacey and Wakeham, 1986) |
| ***Ulva lactuca*** | Plantae | Chlorophyta | 121 (Kerrison et al., 2012) |
| ***Crypthecodinium cohni*** | Chromalteolata | Myzozoa | 377 (Dacey and Wakeham, 1986) |
| ***Acropora millepora*** | Animalia | Cnidaria | 529 |
| ***Emiliana huxleyi (CCMP1516)*** | Chromalteolata | Haptophyta | 50 |
| ***Fragilariopsis cylindrus*** | Chromista | Bacillatlophyta | 15 (Lyon et al., 2011) |
| ***Hymenomonas carterae*** | Chromista | Haptophyta | 300 (Vairaramurthy 1985) |
| ***Gymnodium nelsoni*** | Protista | Dinoflagellata | 280 (Dacey and Wakeham, 1986) |
| ***Ochromonas*** | Chromista | Ochrophyta | 529 |

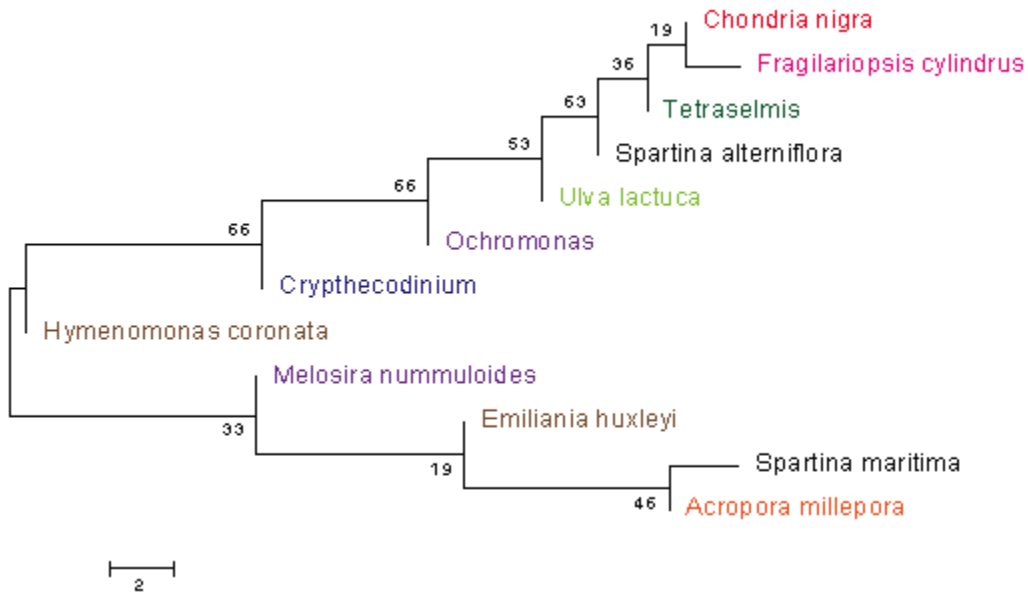



**Figure 1.7 Phylogenetic tree of DMSP-producing organisms.** The protein sequences of 18S rRNA genes of known DMSP producers were aligned using MEGA5 and used to estimate an unrooted phylogenetic tree using LG model gamma distributed with invariant sites. The scale bar indicates the number of substitutes per site. Bootstrap values of 1000 replicates are given at the base of each base pair. Species names are coloured according to the taxa to which they belong. Angiosperm (black), Rhodophyta (red), Chlorophyta (green), Myzozoa (blue), Cnidaria (orange), Ochrophyta (purple), Haptophyta (brown), Bacillariophyta (pink), Prasinophyceae (dark green).

The next section will describe the known DMSP synthesis pathways in DMSP-producing eukaryotes. All of these pathways have a common start point, as all are initiated by the transformation of the substrate L-methionine but differ in the subsequent steps that lead to the production of DMSP.

### 1.5.1 Biosynthesis of DMSP in angiosperms (s-methylation)

Studies of the biosynthetic DMSP pathway in the angiosperms *Wollastonia biflora* (*W. biflora)* and the saltmarsh cordgrass *Spartina alterniflora* (*S. alterniflora)* have revealed that biosynthesis of DMSP initiates with the s-methylation of methionine to produce s-methyl-methionine (SMM). From SMM, these two plants slightly differ in their synthesis pathway and have some differing pathway products. In *W. biflora*, SMM is involved in a transamination-decarboxylation reaction via pyridoxal 5' phosphate (PLP – an active form of vitamin B6) to produce 3-dimethylsulphonio propionaldehyde (DMSP-CHO). A likely intermediate following PLP action is 4-(dimethylsulphonio)-2-oxobutyrate. However, due to its instability, it is yet to be observed (Rhodes *et al*., 1997, Dickschat *et al*., 2015), and a subsequent oxidation step forms the end product DMSP. In *S. alterniflora* SMM is transformed into 3-dimethylsulphoniopropylamine (DMSP-amine) via decarboxylation and further reduced to DMSP-CHO by oxidative deamination before the final oxidation to yield DMSP (Hanson, 1994). Therefore, although the two pathways are very similar, there is an extra intermediate, DMSP-amine, between SMM and DMSP-CHO in *S. alterniflora* (Figure 1.8) (Kocsis *et al*., 1998)

Hanson *et al*. (1994) identified the first pathway intermediate of the plant pathway using pulse-chase experiments with *W. biflora*. Pulse-chase is a method used to examine a cellular process occurring over time by successively exposing cells to a labelled compound which is then detected by its incorporation into other intermediate molecules of the pathway under study. Following pulse-chase experiments using Met $C^{14}$, it was observed that the labelling in SMM increased significantly and then slowly reduced over time which is indicative of central pathway intermediates. Moreover, SMM was also shown to be incorporated into DMSP, suggesting it is a direct precursor of DMSP (Greene, 1962). Hanson *et al* successfully purified and characterised an S-adenosyl-L-methionine:methionine S-methyltransferase (MMT), an enzyme which produces SMM from Methionine in *W.*

*biflora.* This MMT methyltransferase was shown to be a 115-kDa homotetramer, but its identity at the amino acid level and other characteristics are as yet unknown.

The DMSP synthesis intermediates of *S. alterniflora* were established by similar investigations using radioactively labelled precursors. Labelling from [$^{35}$S] methionine was observed in SMM, and similarly labelling of [$^{35}$S] L-methionine and [$^{35}$S] SMM identified that the assimilation of DMSP-CHO occurred before being transformed to produce DMSP. This confirmed the first and last steps of the DMSP pathway are the same in *S. alterniflora* and *W. biflora* (Kocsis *et al*., 1998)

As previously mentioned, the central steps of the DMSP synthesis pathway in these organisms differ and were also established via labelled feeding experiments. Feeding of [methyl-$^{2}H_3$,$^{15}$N] SMM to *W. biflora* revealed that the amino group of SMM is lost via transamination, and not deamination. With this evidence, Rhodes *et al*. (1997) showed that while the labelled methyl group was seen in DMSP, the nitrogen label was seen primarily in glutamic acid but not in the amide nitrogen of glutamate. Secondly, during the proposed transamination step the amino group is conveyed via PLP to 2-oxoglutarate to produce glutamic acid, whereas a deamination reaction would release ammonia and via glutamine enter the amino acid pool.

Feeding experiments verified that SMM is decarboxylated (the removal of a carboxyl group and release of carbon dioxide) to DMSP-amine. The appearance of labelling from either SMM or DMSP-amine in DMSP-CHO indicated this as the last intermediate before DMSP (Kocsis *et al*., 1998). Following a further investigation by Kocsis and Hanson (2000), any PLP-dependent transamination activity was ruled out in *S. alterniflora* when a radioassay using [$^{35}$S] DMSP-amine demonstrated $O_2$–dependent amine oxidase activity to allow for conversion into DMSP-CHO.

### 1.5.2 Biosynthesis of DMSP via the transamination pathway.

Greene (1962) conducted preliminary experiments on DMSP biosynthesis in which labelled methionine was fed to the green alga *Ulva lactuca.* These studies demonstrated that the sulphur atom and the entire carbon backbone of DMSP are

derived from L-methionine (Greene, 1962). Later studies of the red alga *Chondria coerulescens* also showed DMSP production in the same manner. In the 1990's Hanson et al carried out extensive research of the pathway known as the transamination pathway in order to determine its intermediates. They did so via feeding experiments using the green alga *Ulva intestinalis* (Gage *et al*., 1997). Analysis of culture extracts of *U. intestinalis* involved the use of mass spectrometry in order to determine the presence of $^{35}$S-enriched compounds. On the basis of speculative metabolites, a pathway was suggested in which the starting L-methionine undergoes transamination to generate 4-methylthio-2-oxobutyrate (MTOB). This is then reduced to 4-(methylthio)-2-hydroxybutyrate (MTHB). S-methylation of MTHB yields 4-dimethylsulphonio-2-hydroxybutyrate (DMSHB) via a MTHB methyltransferase. The final reaction is catalysed by a putative DMSHB decarboxylase enzyme that generates DMSP from DMSHB. During the same study, Gage *et al*. (1997) confirmed the transamination mechanism by following the feeding of [$^{15}$N] Methionine(Curson et al., 2008). In this work, the consumption of glutamic acid but not the amide nitrogen of glutamate was observed. This demonstrated that the $^{15}$N was acquired via glutamate whilst the amide group of glutamine was not taken up. This is in line with the transamination of methionine but not with oxidative deamination. A deamination reaction would have allowed for labelling of glutamine amide via glutamine synthase. Experiments using cell free extracts showed the transamination of L-methionine to MTOB is dependent on 2-oxoglutarate and the reduction of MTOB to MTHB requires NAD(P)H .

Incubation experiments were conducted on the enantiomers of the intermediates MTHB and DMSHB using cell free extracts of *U. intestinalis.* These demonstrated a favoured consumption of the (R)-enantiomers which helped establish the complete arrangement of the pathway intermediates (Gage *et al*., 1997). As a result S-adenosyl-L-methionine (SAM) was identified as the methyl group donor to enable the methylation of MTHB to DMSHB.

Following the identification of the transamination pathway in *Ulva intestinalis*, similar feeding and labelling assays were performed using the prymnesiophyte *E. huxleyi*, the diatom *Melosira nummuloides* and the prasinophyte*Tetraselmis* (Gage *et al*., 1997). All of these organisms contain the key intermediate of the transamination pathway DMSHB, and in all of them, [$^{35}$S]-labelled DMSHB was

detected following feeding with [$^{35}$S]-Methionine. Also, all [$^{35}$S]-DMSHB was metabolized to DMSP, giving rise to the conclusion that these species follow the same transamination pathway as *Ulva intestinalis*.

### 1.5.3 Molecular identification of DMSP synthesis genes of the transamination pathway.

As previously mentioned, to date there are no known characterised genes for DMSP synthesis. Lyon *et al*. (2011) suggested that two orthologues of the DMSP biosynthesis genes found in the sea ice diatom *Fragilariopsis cylindrus* were also found in coral genomes. These suggested enzymes encoding an NAD(P)H-dependent reductase and a SAM-dependent methyl transferase. However, these candidate genes are yet to be functionally ratified.

In an attempt to identify such DMSP synthesis genes in the transamination pathway Lyon *et al*. (2011) conducted a salinity shift experiment using *Fragilariopsis cylindrus.* Salt concentrations were shifted from low to high and results were analysed using 2D gel electrophoresis. These conditions were predicted to upregulate the expression of genes involved in DMSP biosynthesis and led to the prediction of candidate genes for the enzymes for each of the four steps of the transamination pathway in *Fragilariopsis cylindrus.* However, the role of these genes and putative enzymes in DMSP biosynthesis have yet to be confirmed. Indeed, under the conditions which the diatom was grown, the proteins are only moderately enhanced by elevated levels of salinity and the proposed MTHB methyltransferase (MMT), when cloned and expressed from the diatoms *Thalassiosira pseudonana*, *Phaeodactylum tricornutum* and *Fragilariopsis cylindrus* does not exhibit any MMT activity (Todd, personal comms). More recently, Todd, in unpublished work, has identified a different gene, *mmtD*, in *T. pseudonana* and *P. tricornutum* that shows upregulated expression in elevated salinity conditions and has MMT activity when cloned and expressed in *E. coli***.**

### 1.5.4 The decarboxylation pathway in the dinoflagellate *Crypthecodinium cohnii*

A third DMSP synthesis pathway has also been identified in a dinoflagellate and the route through which the dinoflagellate synthesised DMSP differs significantly to

that demonstrated by the angiosperms and algae. As with Hanson's work in plants and Ulva, Uchida *et al*. (1993) found that after feeding $^{14}C$ and $^{35}S$ labelled isotopic isomers of methionine to the dinoflagellate *Crypthecodinium cohnii* all carbon and sulphur atoms of methionine were integrated into DMSP. The initial transformation of methionine was a decarboxylation reaction generating methanethiopropanamine (MTPA). MTPA is subsequently reduced via oxidative decarboxylation to methylmercaptopropionate (MMPA) and the final step is a S-methylation of MMPA to produce DMSP (Kitaguchi et al., 1999).

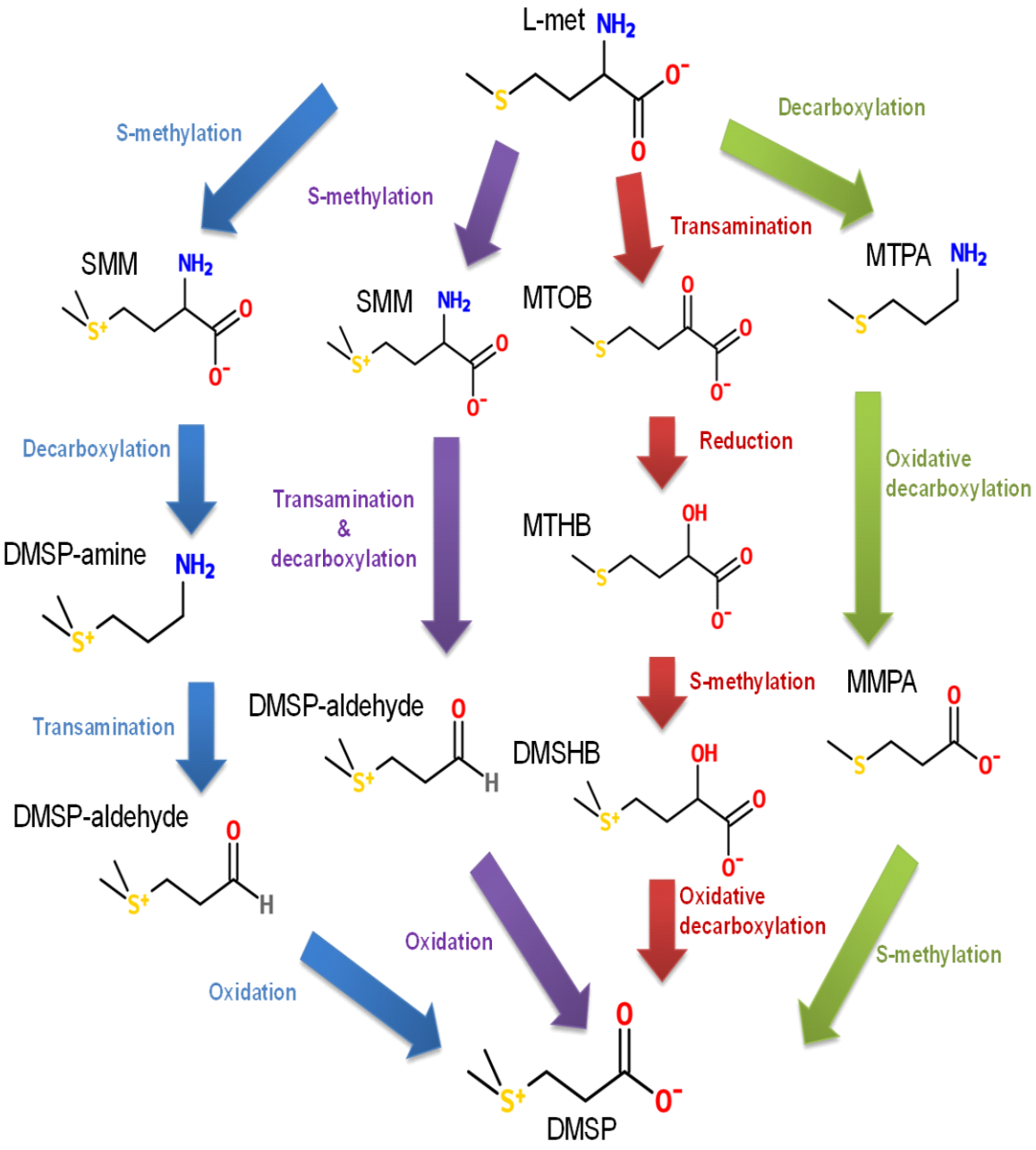



**Figure 1.8: Proposed pathways for the DMSP biosynthesis in angiosperms and some marine algae.** Blue arrows: *Spartina*; Purple arrows; *Wollastonia*; Red arrows: Coccolithophores and *Ulva*; Green arrows: Dinoflagellates. Abbreviations: L-methionine (L-Met); *S*-methylmethionine (SMM); 4-methylthiol-2-oxobutyrate (MTOB); methylthiopropanamine (MTPA); *D*-4-methylthio-2-hydroxybutyrate (MTHB); methylmercaptopropionate (MMPA); *D*-4-dimethylsulphonio-2-hydroxybutyrate (DMSHB). Adapted from Hanson *et al.*, 1994; Trossat *et al.*, 1996a; Uchida *et al.*, 1996; Gage *et al.*, 1997; Kocsis *et al.*, 1998.

## 1.6 DMSP biosynthesis in corals

In recent years DMSP biosynthesis has also been reported in two corals *Acropora millepora* and *Acropora tenuis* (Raina, 2013). It had previously been suggested that the abundance of DMSP found in coral habitats was due to its production by the endosymbiotic dinoflagellete *Symbiodinium* (Raina et al., 2009). However, studies of juvenile corals showed that they were capable of producing DMSP in the absence of their dinoflagellate symbionts, and also that DMSP levels increased in response to thermal stress (Raina *et al*., 2013). It has therefore been suggested that DMSP synthesis in corals may, as predicted for other DMSP-producing organisms, function to provide an anti-stress response. The presence in *Acropora* of homologues of the candidate diatom DMSP synthesis genes (Lyon *et al*., 2011) suggested that corals may synthesise DMSP using the same pathway as that seen in the diatoms, green algae and coccolithophores (Raina *et al*., 2013).

## 1.7 DMS production from marine eukaryotes

DMS production from DMSP has long been associated with marine algae, with the first such reports relating to studies in the early and late 1950's using *Polysiphonia* and *Ulva*. In *Ulva* a DMSP lyase was identified and partially characterized (Challenger, 1948, Cantoni, 1956), and in the years since, this capability has been observed in a number of algal species. However, at the start of this study there had been no studies performed in which a DMSP lyase had been isolated that could be reliably confirmed as coming from an axenic culture by use of molecular techniques such as 16S PCR. Although we feel it is likely that some marine eukaryotes that produce DMSP contain DMSP lyases, the biochemical characterization of DMSP lyase enzymes performed using non-axenic cultures does not provide sufficient evidence, so there is a requirement to confirm this through the use of molecular techniques.

### 1.7.1 Algal DMSP lyases

To date there have been few attempts to isolate and purify the DMSP lyase enzymes in Phytoplankton as highlighted in Table 1.2 the details of the more significant studies are discussed in chapter

**Table 1.2 Characteristics of some DMSP algae enzymes.**

| Class/Phylum | Species | Source | pH optima | Location |
|---|---|---|---|---|
| **Rhodophyceae** | *Polysiphonia lanosa* | Cantoni & Anderson (1956) | 5.1 | Plasma membrane |
| **Dinoflagellata** | *Gyrodinium cohnii* | Kadota & Ishida (1968 | 6.2 | Membrane bound |
| **Chlorophyceae** | *Enteromorpha clathrata (Ulva)* | Steinke & Kirst (1996) | 6.2-6.4 | Membrane bound |
| **Rhodophyceae** | *Polysiphonia paniculata* | Nishiguchi & Goff (1995) | - | Membrane bound |
| **Chlorophyceae** | *Ulva curvata* | De Souza et al (1996) | 8 | Membrane bound (x3)<br>Soluble (x1) |
| **Haptophyceae** | *Phaeocystis* | Stefels (1996) | 10.5 | Extracellular<br>Membrane |
| **Haptophyceae** | *Emiliania huxleyi* (CCMP strains 370,373, 379, 374, 1516) | Steinke et al (1998) | 8 (CCMP 370)<br>6 (CCMP 373)<br>6 (CCMP 379)<br>5 (CCMP 374)<br>5 (CCMP 1516) | Intracellular<br>Membrane<br>Intracellular<br>Membrane<br>Membrane bound |

## 1.8 DMSP catabolism

DMSP catabolism is known to occur in both Eukaryotic organisms that produce DMS (see section 1.7) and in a myriad of other microbes when it is released into the environment via viral lysis, senescence or during damage inflicted by zooplankton (Malmstrom et al., 2004). There are two known pathways for DMSP catabolism. The first of these is the process through which MeSH is released and is known as the demethylation pathway. This process is limited to marine microorganisms and the DMSP demethylase gene only found in marine bacteria. More importantly for this study, the second pathway is known as the DMSP lyase or cleavage pathway, and leads to production of the climate-active DMS (see section 1.4) (Yoch, 2002). The cleavage pathway is catalysed by enzymes known as DMSP lyases. The ddd genes which encode these DMSP lyase enzymes are found in taxonomically diverse marine bacteria, and homologs have been identified in some terrestrial bacteria and also in fungi. It is because of the release of these important sulphurous gases, especially DMS, that there is such a significant interest as to how these two pathways are spread though the marine environment and how indeed they are regulated in bacteria. For example the alphaprotobacterium *Ruegeria pomeroyi* has genes for enzymes of both pathways.

### 1.8.1 DMSP Demethylation.

Vast numbers of marine bacteria demethylate DMSP and it is considered to be the predominant pathway for DMSP catabolism, with approximately 70% of all consumed DMSP passing through the demethylase pathway. The DMSP demethylation pathway has only recently been investigated at a molecular level (Howard et al., 2006; Reisch et al., 2013), although the production of MeSH from DMSP has long been established (Howard et al., 2006, (Gonzalez et al; 1999). Through this pathway, DMSP is at first demethylated by DmdA a methyl transferase isolated from the α-proteobacterium *Ruegeria pomeroyi* DSS-3 (Howard et al 2006) (Figure 1.9). DmdA is a member of the glycine cleavage T-family and transfers a methyl group from DMSP to produce tetrahydrofolate and 3-methylmercaptopropanic acid (MMPA) (Howard et al., 2006). MMPA is further catabolised in a series of step wise reactions using coenzyme-A similar to that displayed in fatty acid β-oxidation (Reisch et al., 2013). MMPA is converted by the

coenzyme A ligase DmdB into the CoA enzyme ester and the DmdC dehydrogenase oxidises it to methylthioacryloyl-CoA. The addition of water by DmdD, an enoyl-CoA hydratase, results in the production of a hemithioacetal intermediate which liberates MeSH to produce a CoA thioester of malonyl semialdehyde, which is subsequently hydrolysed to acetaldehyde and $CO_2$.

The final step within the demethylation pathway releases $CO_2$, MeSH, a free CoA and acetaldehyde (MeCHO). The MeCHO is then oxidised to acetate and from there enters central carbon metabolism (Todd et al 2010a; Reisch et al 2013).

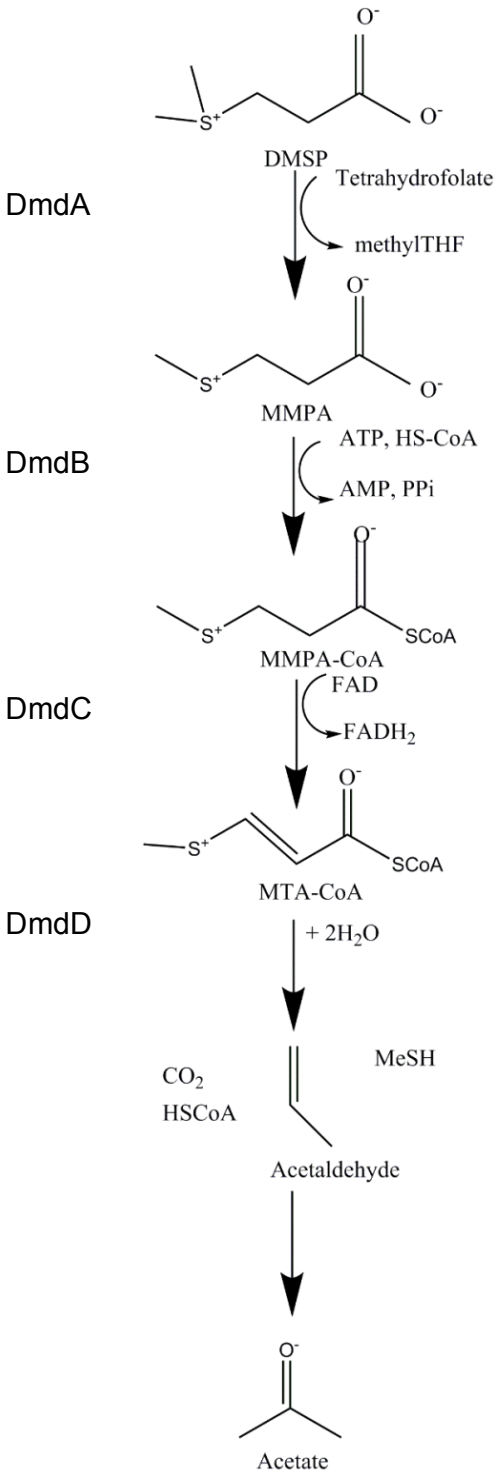



**Figure 1.9 DMSP demethylation pathway in *Ruegeria pomeryoi* DSS-3.** DMSP is demethylated to MMPA by THF. MMPA is then transformed to MMPA-CoA which is dehydrogenated by producing MTA-CoA. MTA is hydrated to acetaldehyde to release MeSH CoA and $CO_2$. Figure adapted from Reisch et al 2011.

### 1.8.2 DMSP cleavage pathway

DMSP can be catabolised to produce DMS and either acrylate or 3-hydroxypropionate (3-HP), depending on the DMSP lyase (see Figure 1.10). This alternative method of DMSP degradation through which marine bacteria can reduce DMSP is known as the cleavage pathway and there is a great amount of interest from an array of scientific fields for this particular component of the global sulphur and carbon cycles. Through this route of catabolism, the climatically active volatile DMS is produced in significant amounts.

The production of DMS is achieved by the DMSP lyase enzyme which was first detected in the eukaryote *Polysiphonia lanosa* (Auling and and Stackebrandt, 1988). However it is clear, following a number of studies, that the biotransformation of DMSP to produce DMS and acrylate is accomplished by a number of very different enzymes so the term DMSP lyase only denotes the total activity which is achieved by the distinct proteins.

To date, six different enzymes have been shown to facilitate the cleavage of DMSP to produce DMS (DddD, DddL, DddP, DddQ, DddY and DddW) (see Figure 1.9). The phenotype which denotes the breakdown of DMSP to DMS has been called $Ddd^+$ (DMSP–dependent DMS). The isolation and description of the six different bacterial *ddd* genes and corresponding lyase enzymes is discussed further here.

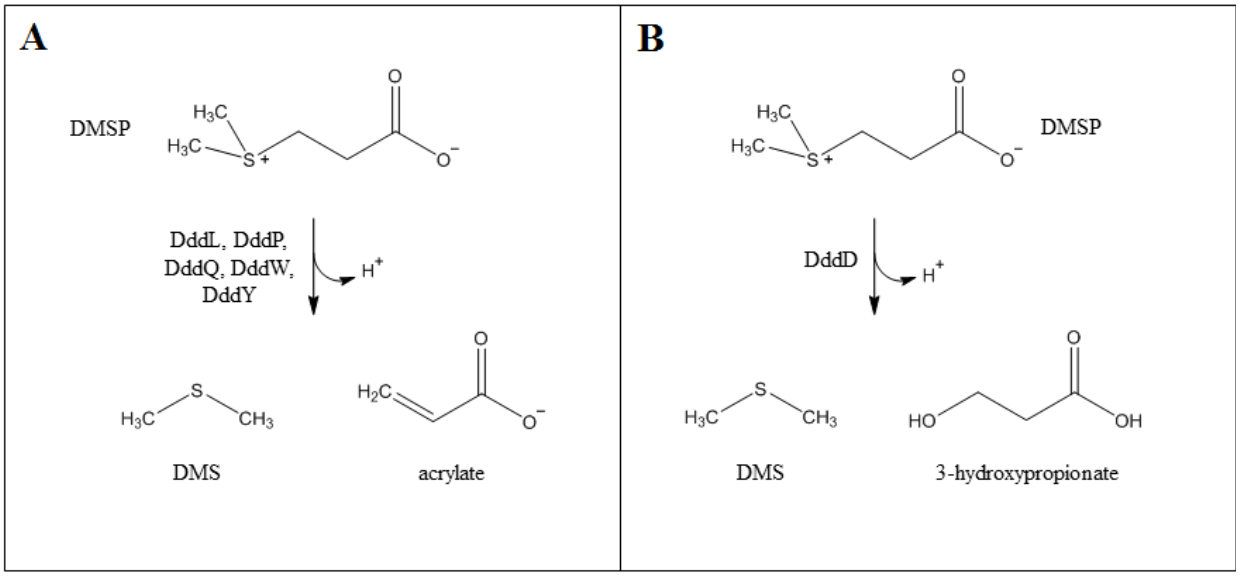



**Figure 1.10 DMSP cleavage pathways.** (A) DMSP is cleaved with the release of DMS and acrylate by the classic DMSP lyases. (B) DMSP is cleaved by DddD to produce DMS and 3-hydroxypropionate.

### 1.8.3 DddD

The first DMSP lyase to be discovered was the DddD gene. However, in a rather sardonic turn of events, instead of a being a DMSP lyase in the 'true' sense by releasing DMS and acrylate, DddD actually transforms DMSP to DMS and 3-HP.

The first genetic study on the bacterial production of DMS was on a γ-proteobacterium *Marinomonas* sp. MWYL1 (Todd et al., 2007), which was isolated from the salt marsh grass *Spartina anglica* (Otte et al 2004). A fosmid library of *Marionomonas* sp. MWYL1 genomic DNA was created in *E. coli* and assayed for the $Ddd^+$ phenotype using Gas Chromatography (GC). One fosmid containing two transcriptional units was shown to confer this phenotype and it was determined that a gene termed *dddD* was essential in order for *Marionomonas* sp. MWYL1 to demonstrate the $Ddd^+$ phenotype of DMS production. In *E. coli*, when DddD was expressed using the T7 promoter in the pET21A vector, the cloned *dddD* gene conferred a $Ddd^+$ phenotype, confirming it as the first gene to encode a DMSP cleavage enzyme. The DddD protein belongs to the Class III CoA transferases and was determined to be an acyl CoA transferase. It shows homology with CaiB of *E. coli* (Todd et al., 2007). CaiB is implicated in an anaerobic respiratory

pathway and the transfer of CoA to the amino acid carnitine which is used as a terminal electron acceptor (Eichler, 1994, Engemann, 2001)

#### 1.8.3.1 DddD distribution

Following the discovery of DddD in *Marinomonas*, Curson et al (2011b) isolated the γ-proteobacteria *Pseudomonas* J465 and *Psychrobacter* J466 from the gut of an Atlantic herring (*Clupea harengus).* Both bacteria were shown to contain DddD homologues.

Using the Basic Local Alignment Search Tool (BLAST), a bioinformatics software tool, further DddD homologues were found in alphaprotobacteria, including members of the abundant marine Roseobacter clade (Figure 1.11), including *Sagittula stellata* E-37. (Johnston et al., 2007, Todd et al., 2010a, Curson et al., 2011a). DddD also appeared in terrestrial bacterial strains, including the β-proteobacteria *Burkholderia cepacia* found in the rhizosphere of a number of angiosperms (Ramette, 2005). The presence of *dddD* in these taxonomically diverse microbes is suggestive of horizontal gene transfer (HGT), and evidence even points to a HGT event taking place between bacteria and algae with a 30% identity to DddD found in the coccolithophore *Emiliania huxleyi* (see chapter 5) (Curson et al., 2011c)

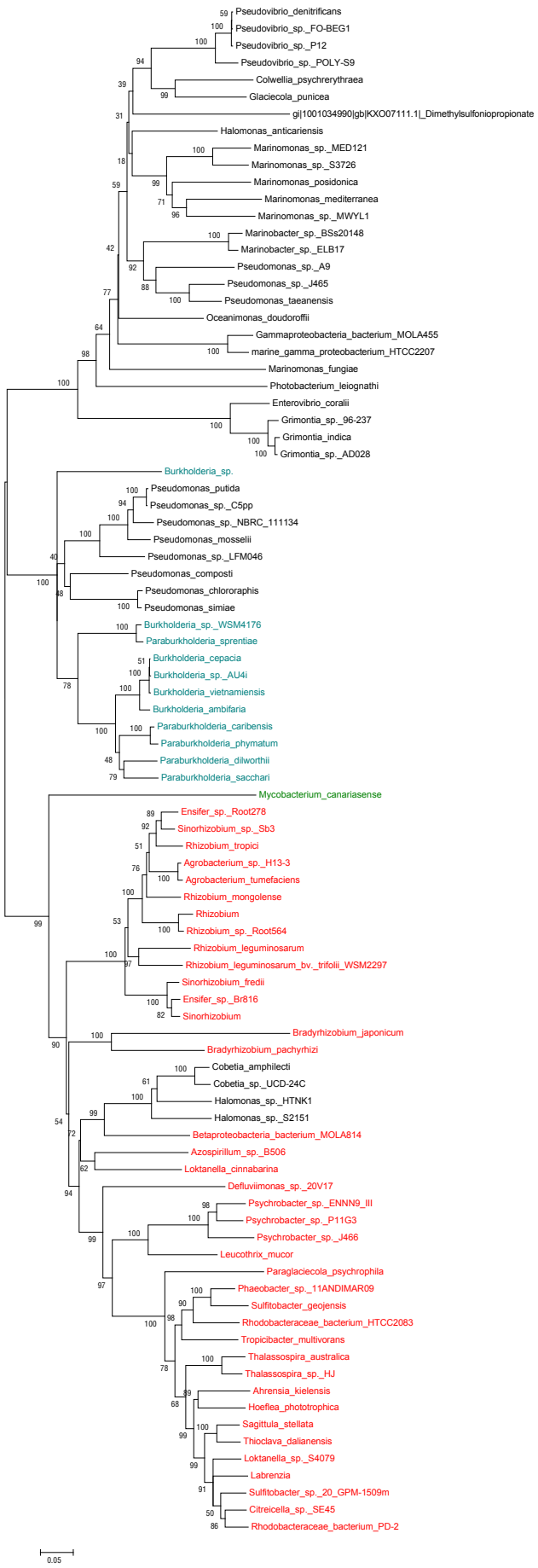



**Figure 1.11 Phylogenetic tree of DddD polypeptides.** The protein sequences of DddD homologues were aligned using MEGA5 and used to build an unrooted phylogenetic tree using LG model gamma distributed with invariant sites. The scale bar indicates the number of substitutions per site. Bootstrap values of 1000 replicates are given at each branch point. Species names are coloured according to their taxonomic status. α-proteobacteria (red), β-proteobacteria (teal), γ-proteobacteria (black), actinomycete (green).

### 1.8.4 DddL

DddL was the first 'true' DMSP lyase to be genetically characterised (Curson et al., 2008), from bacteria isolated from a salt marsh grass on the coast of Georgia, USA (Gonzalez et al; 1999). The bacterium studied was the marine α-proteobacterium *Sulfitobacter* sp. EE-36, a member of the Roseobacters. In order to identify any DMSP lyase genes in *Sulfitobacter* sp. EE-36, a genomic library was constructed in the wide host range vector pLAFR3, transformed into *E. coli 803* and conjugated into the α-proteobacterium *Rhizobium leguminosarium* J391 (Curson et al., 2008). *R. leguminosarium* J391 possesses a large number of sigma factors required for transcription initiation, thus making it more likely that any genes of interest from the *Sulfitobacter* library would be expressed. A cosmid was found which conferred the $Ddd^+$ phenotype to *R. leguminosarium*. Genetic screening using bioinformatics had already determined that any production of DMS was not due to the DddD enzyme as no homologue to this was found. Therefore, in order to identify the DMSP lyase gene from the cosmid, and to establish if *Sulfitobacter* sp EE-36 contained more than one DMSP lyase, the cosmid was mutated using the Tn*5lacZ* transposon. This mutagenesis technique led to the isolation of mutated cosmid derivatives that no longer conferred DMS production. Following sequencing of such mutant cosmids, a single gene, termed *dddL*, was determined to be responsible for the $Ddd^+$ phenotype (Curson et al., 2008). The genetic loci of *dddL* is illustrated in Figure 1.12. HPLC analysis confirmed DddL to be a DMSP lyase as DMS and acrylate were produced in equal amounts.

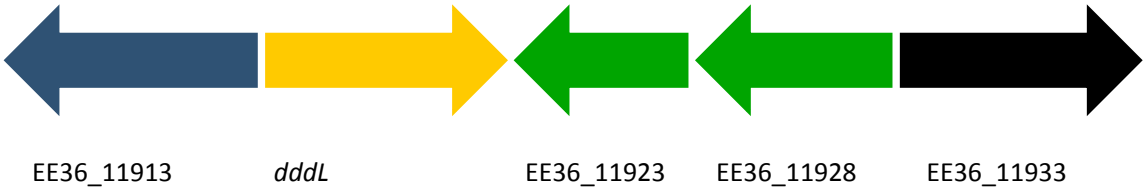



**Figure 1.12 Map denoting arrangement of the *dddL* operon in *Sulfitobacter EE-36*.** The blue arrow denotes the location and orientation of a DNA gyrase, the orange demonstrates the location of *dddL*. Green arrows refer to putative hydrolases whilst the black arrow represents a proposed multi-drug efflux pump. On a structural level studies of DddL have shown that it contains two motifs (Todd et al 2011). These are discussed in more detail in the section which follows.

### 1.8.4.1 Cupins

#### Cupin domains

Cupins are a super-family of domains with a β-barrel structure (Dunwell, 1998, Dunwell et al., 2004). The functions of cupin-containing proteins are highly diverse with a number of examples seen within all three domains of life, and with some polypeptides containing a single domain and others containing multiple copies. A cupin domain typically possesses two conserved motifs. These motifs are generally separated by a region spanning 11 to 100 amino acids. Cupins are usually transition metal-binding proteins with the predicted metal-binding residues being two histidines and one glutamate residue within motif 1 and a single histidine within motif 2, behaving as ligands to bind an array of metals including zinc, nickel, iron and copper (Woo et al., 2000) at these highly conserved amino acid residues (Dunwell et al., 2004, Todd, 2011).

Motif 1: $(X)_5$**H**X**H**$(X)_{34}$E$(X)_6$**G**

Motif 2: G(X)5PXG$(X)_2$**H**$(X)_3$N

The DddL, DddQ and DddW (see chapters 3 and 4 respectively) homologues share a number of the conserved residues of cupin domain proteins and through the experiments conducted as part of this study it has become clear that these cupin domains are significant for the cleavage of DMSP.

Homologues of DddL are found for the most part in other marine α-proteobacteria within the Rhodobacterales, including a few Roseobacters (Figure 1.13). A number of these strains have been established to be $Ddd^+$ due to the probable presence of DddL. One isolate of the γ-proteobacteria *Pseudomonas* found within the coral *Montipora aequituberculata* contains a gene with homology to *dddL* (Raina et al., 2009).

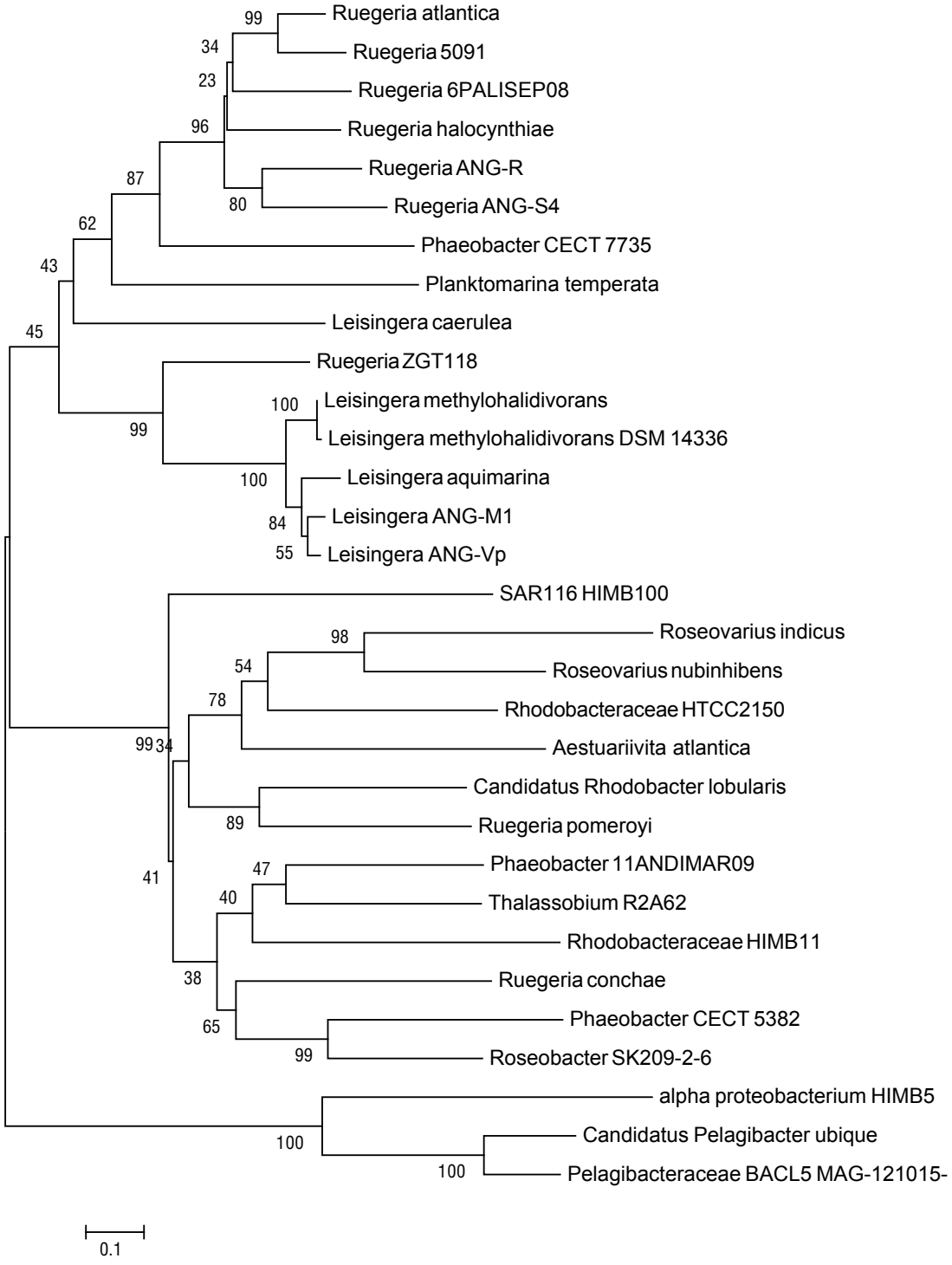



**Figure 1.13 Phylogenetic tree of DddL polypeptides.** The protein sequences of DddL homologues were aligned using MEGA5 and used to estimate an unrooted phylogenetic tree using LG model gamma distributed with invariant sites. The scale bar indicates the number of substitutions per site. Bootstrap values of 1000 replicates are given at each branchpoint. All species are members of the α-proteobacteria.

### 1.8.5 DddP

Following on from the discovery of DddL, Curson et al (2008) hypothesised that the genome sequenced α-proteobacterium *Roseovarious nubinhibins* should possess another entirely different DMSP lyase as it also produces DMS from DMSP (Gonzalez, 2003) but its genome contained neither *dddD* or *dddL*. Methods similar to those used for the isolation and confirmation of *dddL* were employed to identify the gene which conferred the $Ddd^+$ phenotype. A genomic library was produced and transformed into *E. coli* and conjugated into R. *leguminosarium*. Gas Chromatography (GC) was used to screen primary transconjugants for the $Ddd^+$ production of DMS and one cosmid was found to confer this phenotype. This cosmid was mutated using the Tn5lacZ transposon and three mutagenic insertions were identified and located within a single gene. This gene was named *dddP* (Todd, 2009).

Interestingly, the *dddP* gene encodes a member of the M24B family of metallopeptidases, which differs significantly to the enzyme types of DddD and DddL. Metallopeptidases are classified as the most varied group of proteases and generally cleave peptide bonds. Rather obviously, DMSP does not contain any peptide bonds so the enzymatic function of DddP is apparently different to that of other known family members. M24B family enzymes have a pitta bread fold which contains their active sites (Bazan, 1994), with six residues within this fold being thought to form the active site and be involved in direct metal binding. In *R. nubinhibins*, these residues sit towards the C-terminal end and are D295, D297, D307, H371, E406 and E421.

#### 1.8.5.1 Characterising DddP

DddP was the first of the enzymes capable of producing DMS to be biochemically characterized (Kirkwood et al., 2010). To do so, *dddP* was cloned into the expression vector pET21a forming pBIO1658, which was then used to overexpress and purify DddP from recombinant *E. coli*. Using GC, High Performance liquid Chromatography (HPLC) and Nuclear Magnetic Resonance

(NMR), DddP was confirmed to cleave DMSP to DMS and acrylate (Kirkwood et al., 2010).

Given the classification of DddP as a M2B metallopeptidase, the metal content of the enzyme was also examined using metal chelators to try and reduce or knockout DMSP lyase activity. Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) was also employed in an attempt to identify any metal content (Kirkwood et al, 2010). ICP-OES results did not however identify any metal associated with DddP and chelation assays did not diminish lyase activity. In order to determine if the conserved active site and metal-binding residues were significant to the functionality of the lyase, site-directed mutagenesis was performed. Using pBIO1658, the gene was mutated such that each of the six conserved residues D295, D297, D307, H371, E406 and E421 was individually substituted for alanine. Mutation of any one of these residues was shown to abolish the production of DMS by DddP (Kirkwood et al., 2010). These results are therefore somewhat conflicting, with metal analysis and chelation assays indicating that there is no metal present in DddP, but mutation of the predicted metal-binding residues affecting DMSP lyase activity. Recently published data established the crystal structure of DddP in *Roseobacter denitrificans* and that it is in fact a homodimeric metalloprotein which contains a binuclear center of two metal ions in its active site (Hehemann J-H, 2014). Further analysis via ICP-MS and Total reflective X-ray fluorescence suggested that various transition metal ions were tightly bound to DddP but that iron was most abundant in the protein.

### 1.8.5.2 DddP distribution

Homologues of *dddP* are found in more sequenced bacterial genomes of than either *dddD* or *dddL* with most being found in strains of Roseobacter. Homologues have also been identified in α-proteobacteria of the SAR116 clade (Figure 1.14). Curson et al. (2011c) also identified two DddP homologues able to confer the $Ddd^+$ phenotype from the γ-proteobacterium *Oceanimonas doudoroffii*. Interestingly, Todd et al (2009) also found functional copies of DddP in some fungi including *Fusarium* and *Aspergillus* strains.

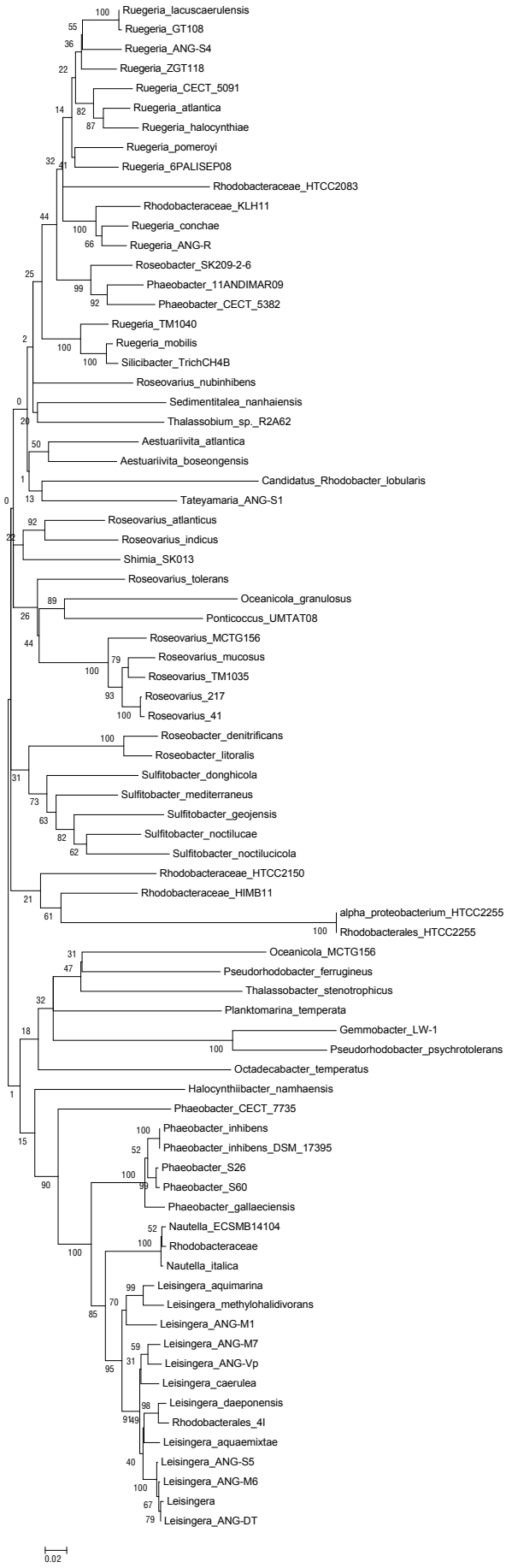



**Figure 1.14. Phylogenetic tree of DddP polypeptides.** The protein sequences of DddL homologues were aligned using MEGA5 and used to estimate an unrooted phylogenetic tree using LG model gamma distributed with invariant sites. The scale bar indicates the number of substitutions per site. Bootstrap values of 1000 replicates are given at each branchpoint. All species are from the α-proteobacteria.

### 1.8.6 DddQ

Work conducted by Todd et al. (2011), which involved knocking out DMSP lyase expression within the Roseobacter *Roseovarius nubinhibins* ISM, revealed that there was a second functional DMSP lyase in this bacterium. Whilst DddP was responsible for ~90% of the DMSP degradation observed in this strain, a small proportion was conferred by another lyase. This was identified using similar techniques used for the identification of DddP (section 1.8.5). Sequencing of a cloned cosmid which conferred a $Ddd^+$ phenotype revealed the presence of two adjacent genes (*dddQ1* and *dddQ2*) located within a predicted ten-gene transcriptional unit. A single *dddQ* gene was also located in *Ruegeria pomeroyi DSS-3*, which when cloned also conferred a $Ddd^+$ phenotype. These three genes, like *dddL*, encode proteins containing a proposed cupin motif. Subsequent cloning of these dddQ genes and expression in *E. coli* confirmed their ability to catabolise DMSP, with the formation of DMS and acrylate as products.

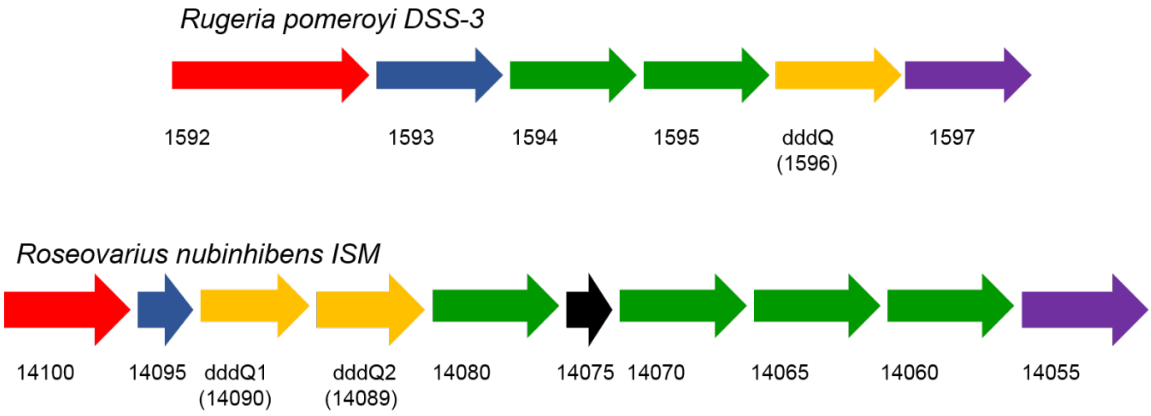



**Figure 1.15 Map denoting arrangement of *dddQ* genes in *Rugeria pomeroyi DSS-3* and *Roseovarius nubinhibens ISM*.** Gene tags are shown without prefixes (SPO and ISM respectively). Gene annotation: Red: members of an amino transferase family, Blue: members of a Zn alcohol dehydrogenase family. Green: members of an Enolase super family Orange: *dddQ* Purple: members of an acetyl ornithin aminotransferase family Black: Cupin 2 like protein (not seen in *R. pomeroyi DSS-3*) Adapted from Todd et al (2011).

The DddQ DMSP lyases were found only within the proteomes of the Roseobacters with a number of other strains containing a DddQ orthologue. From this evidence to date however no other bacterial taxa are thought to contain DddQ lyase.

### 1.8.7 DddW

A third cupin-containing DMSP lyase DddW was identified in the Roseobacter species *Ruegeria pomeroyi* (Todd *et al*., 2012). This will be described in detail in chapter four.

### 1.8.8 DddY

*Alcaligenes faecalis* M3A, a β-proteobacterium isolated from the salt marsh cordgrass *Spartina*, was the first bacterial strain to be biochemically examined for DMSP lyase activity (de Souza and Yoch, 1995a; de Souza and Yoch, 1995b). However, no DMSP lyase gene was identified in this strain until a more recent study (Curson et al., 2011a). A genomic library was created in the wide host range cosmid pLAFR3 and this was subsequently mobilised into *Pseudomonas putida J450* and screened for cosmids that conferred growth on DMSP as a sole carbon source. Two cosmids were sequenced which demonstrated this ability and four contiguous operons containing eight contiguous genes were exposed (Curson et al, 2011a). One gene present in both these cosmids, termed *dddY*, was cloned into pET21a, expressed in *E. coli* and shown to confer a $Ddd^+$ phenotype. Cell-free extracts also confirmed *dddY* to be a *bona fide* DMSP lyase as NMR analysis revealed equivalent amounts of DMS and acrylate were produced from the DMSP substrate.

#### 1.8.8.1 Distribution of DddY in other bacteria.

On bioinformatic investigation, close homologues (>50%) of DddY were not present in the proteomes of any other bacterial strains (Curson et al., 2011c). However there were a number of more distantly related polypeptides (~35% identical; see Figure 1.16) including in the γ-proteobacterium *Shewanella putrefaciens*, and the ε-proteobacterium *Arcobacter nitrofiglis*. On further investigation these *dddY*-containing strains were also shown to have a $Ddd^+$ phenotype. To confirm that these more distant homologues of *Alcaligenes* DddY

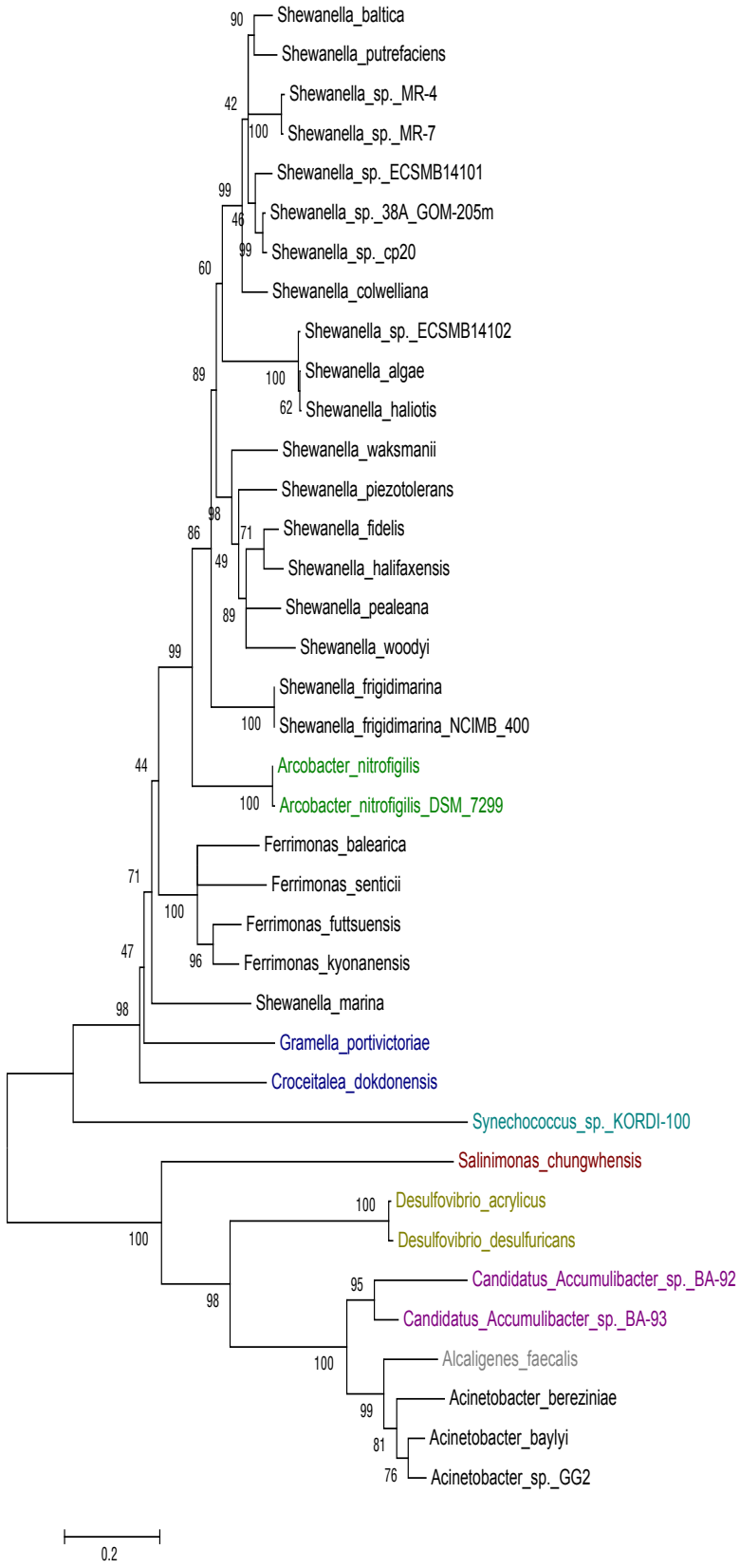



**Figure 1.16 Phylogenetic tree of DddY polypeptides.** The protein sequences of DddY homologues were aligned using MEGA5 and used to estimate an unrooted phylogenetic tree using LG model gamma distributed with invariant sites. The scale bar indicates the number of substitutions per site. Bootstrap values of 1000 replicates are given at each branchpoint. Species names are coloured according to taxonomic group: α-proteobacteria (purple), β-proteobacteria (grey), γ-proteobacteria (black), ε-proteobacteria (green), bacteroidetes (blue), cyanobacteria (teal), δ-proteobacteria (olive), firmicutes (maroon).

were functional DMSP lyases, the *dddY* gene of *Shewanella putrefaciens* CN-32 was cloned into pET21a and expressed in *E. coli*, where it conferred a $Ddd^+$ phenotype (Curson et al., 2011c).

### 1.8.8.2 DddY is a periplasmic lyase

Evidence from earlier biochemical analysis of the DMSP lyase in *Alcaligenes*, before the *dddY* gene was identified, predicted that the lyase protein would be situated at or near the bacterial cell surface (Yoch et al., 1997). The DMSP lyase protein was purified (de Souza and Yoch, 1996) and possessed an N-terminal sequence of:

AQFQ**H**QDDVKPAAISAEEGKGKLVDEQFQEAQKNNEAL

Following its more recent identification, the DddY protein was shown to contain the same sequence as that above, with the exception of a cysteine residue instead of a histidine (highlighted and underlined in bold above). However, significantly, the DddY protein also contains a 21 amino acid N-terminal leader sequence predicted to be a signal peptide to direct its translocation to the periplasm, and which would be removed during translocation. Consistent with this, *in silico* removal of this leader created a polypeptide whose sequence matched that of the N-terminal sequence revealed by de Souza and Yoch (1996). Curson *et al*. (2011a) have since confirmed by cellular fractionation that the DddY polypeptide is located in the periplasm and that higher DMSP lyase activities were seen in the periplasmic fraction compared to the cytoplasmic fraction (Curson et al., 2011a).

## 1.9 Transport of DMSP

In most cases, DMSP must first be transported across the cytoplasmic membrane before the DMSP lyase enzymes within the cytoplasm can act on it as a substrate, the exception being the periplasmic DddY. It is significant, therefore, that genes predicted to encode DMSP transporters are closely associated to some ddd DMSP lyase genes. An example of this is in genes, termed *dddT*, encoding for members of the betaine-carnitine-choline transporter (BCCT) family of bacterial transporters (Todd, 2007, Curson et al., 2010) . These *dddT* genes were observed divergently transcribed from *dddD* in *Marinomonas* MWYL1 and *Pseudomonas* J465, and co-transcribed with *dddD* in *Halomonas* HTNK1 and *Psychrobacter*

J465. The *Halomonas* DddT protein was confirmed as a functional DMSP transporter by monitoring the uptake of [$^{14}$C] DMSP by *E. coli* containing the cloned *dddT* gene (Todd et al., 2010a).

Originally *dddT* genes for DMSP transport were only observed in bacteria containing the *dddD* mechanism for DMSP catabolism but further investigations revealed orthologues of *dddT* flanking two copies of *dddP* in the γ-proteobacterium *Oceanimonas doudoroffi* (Curson et al., 2011b). In other bacteria exhibiting the Ddd$^+$ phenotype but not possessing a functional DddD, some BCCT-type transporters function to import DMSP but are not adjacent to genes known to be involved in DMSP catabolism. For example, Sun et al. (2011) identified genes encoding functional DMSP transporters in *Sulfitobacter* EE-36 (containing *dddL*) and *Roseovarius nubinhibens* ISM (containing *dddP* and *dddQ*).

Sun et al. (2011) also demonstrated that *Burkholderia ambifaria* AMMD and *Sinorhizobium fredii* NGR234, which lack *dddT* but contain *dddD*, have very different ways to import DMSP. In these circumstances genes encoding functional subunits of ABC type transporters were linked to the *dddD* gene. ABC transporters are one of the most prevalent families of paralogous transmembrane proteins seen in prokaryotes and eukaryotes. In Gram-negative bacteria, ABC transporters are typically composed of a periplasmic binding protein, an inner membrane transporter and an ATPase. In *Burkholderia ambifaria* AMMD the genes linked to *dddD* encoded a putative spermidine/putrescine ABC transporter and in *Sinorhizobium fredii* NGR234 they encoded a predicted proline/glycine betaine ABC transporter. The four ABC transporter genes from *B. ambifaria* AMMD were cloned and expressed in *E. coli* and shown to encode a functional DMSP transporter, albeit less efficient than the DddT BCCT type DMSP transporters of *Marinomonas* and *Halomonas* (Sun et al., 2011) (Figure 1.17).

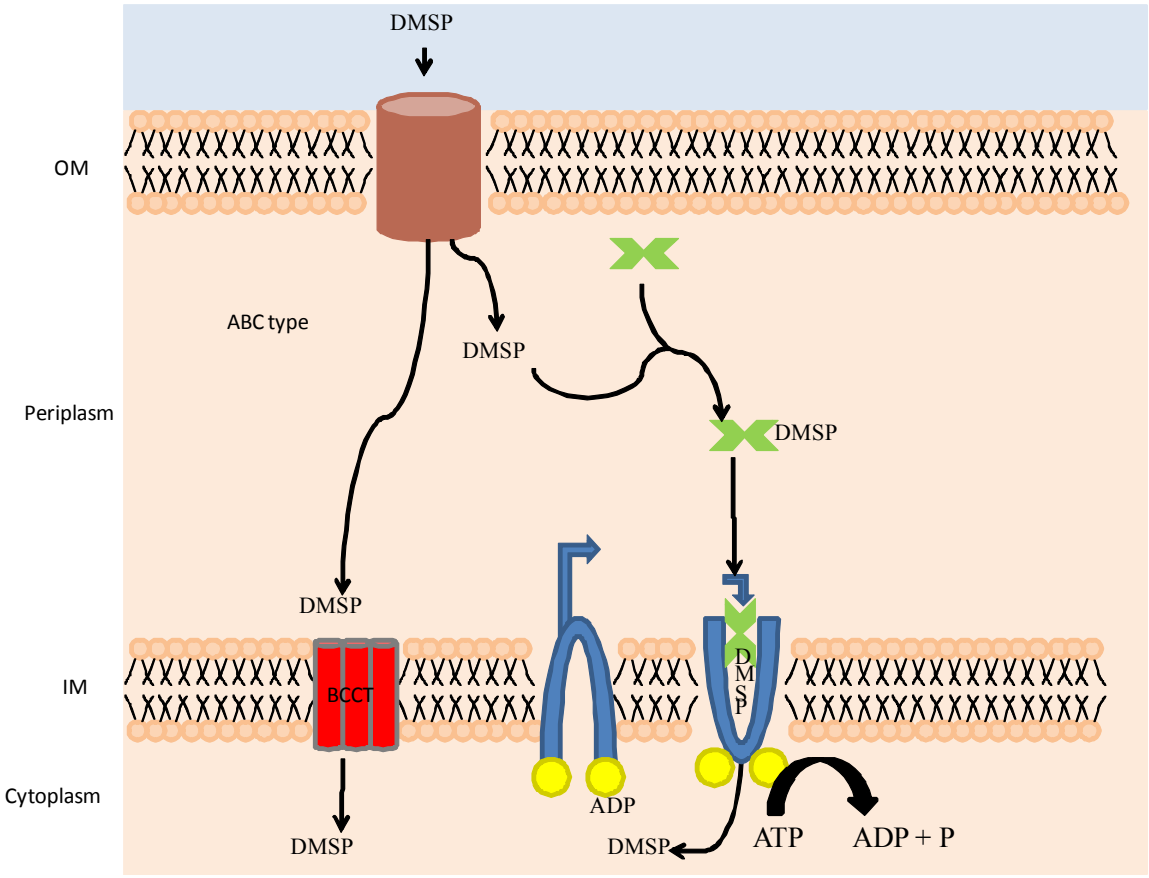



**Figure 1.17 Mechanism of DMSP transport in bacteria.** DMSP crosses the outer membrane by diffusion through non-specific outer membrane porins e.g. OmpF/OmpC. DMSP may then be transported into the cytoplasm by BCCT type transporters (red) or by ABC type transporters which are composed of a periplasmic substrate binding domain (green) and transmembrane domain (blue) which are coupled with ATP binding domains (yellow). Hydrolysis of ATP results in the translocation of DMSP across the inner membrane following presentation of the molecule to the transmembrane domain by the periplasmic binding protein.

Previous sections of the introduction have covered the general background to DMSP, its roles and its catabolism by bacteria and eukaryotes. The following sections will briefly introduce the specific organisms under study here and why they are important in relation to DMSP and more generally in the environment.

## 1.10 Introduction to the Roseobacters

The Roseobacters are defined as a subgroup of the α-proteobacterial order Rhodobacterales, which mostly have large genomes with high GC content and are particularly recognised for their highly versatile metabolic capabilities (Luo and Moran, 2014). They are one of the major clades of heterotrophic bacteria found in ocean surface waters, along with the α-proteobacterial SAR11 and SAR116 clades, and the γ-proteobacteria lSAR86 clade (Giovannoni et al., 1990; Rappe et al., 2000; Suzuki et al., 2001). In the open oceans, between 3-5% of all bacterial cells belong to the Roseobacter group, and this increase to as much as 20% in coastal waters (Moran et al., 2007). Due to the abundance of the Roseobacters in marine environments and the fact that the majority of them have genes for DMSP cleavage and/or demethylation, they play a highly significant role in the total turnover of DMSP.

### 1.10.1 *Ruegeria pomeroyi* DSS-3

*Ruegeria pomeroyi* DSS-3 is considered to be the model Roseobacter for the study of DMSP catabolism, with numerous studies on the molecular aspects of this process having been published (Todd et al., 2011; Todd et al., 2012; Reisch et al., 2011; Reisch et al., 2013, Bullock et al., 2014). *Ruegeria pomeroyi* DSS-3 has genes for both DMSP demethylation and cleavage, including three different DMSP lyase genes *dddP*, *dddQ* and *dddW*, more than any other sequenced Roseobacter, or indeed any sequenced bacterium, and the genes and pathways involved in the downstream catabolism of the products of DMSP demethylation and cleavage are also known (see Figure 1.18).

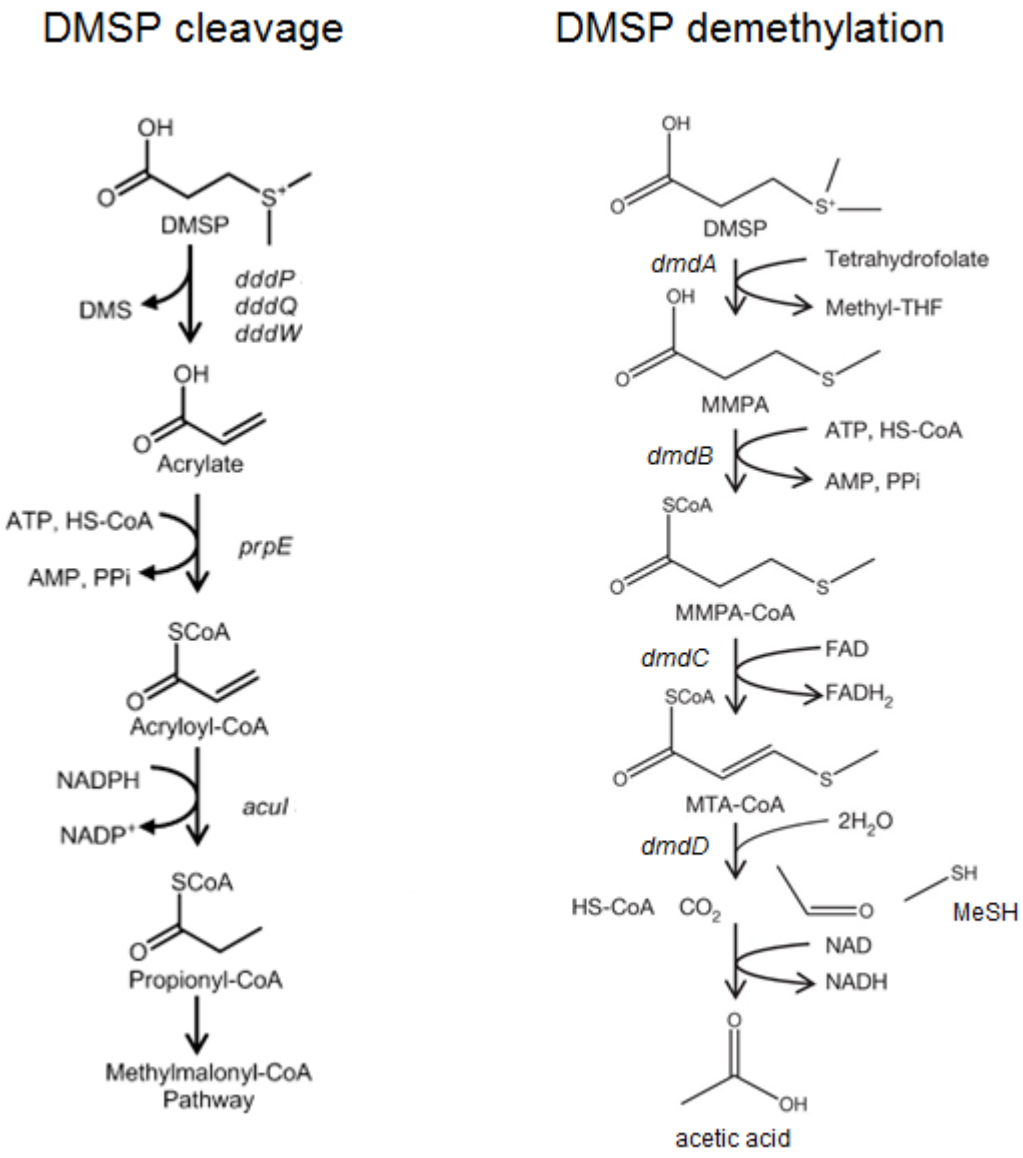



**Figure 1.18 DMSP catabolic pathways in *Ruegeria pomeroyi* DSS-3.** DMSP can be either cleaved or demethylated and the products of these reactions can then be further utilised in downstream catabolic steps to enter the TCA cycle. Figure adapted from figures in Reisch et al. (2011) and Reisch et al. (2013). Abbreviations: MMPA, methylmercaptopropionate; MMPA-CoA, methylmercaptopropionyl-CoA; MTA-CoA, methylthioacryloyl-CoA

## 1.11 Marine Phytoplankton and their importance.

The earth's climate has been subject to major changes over geological time scales. The conditions of the global climate and the changes within it have direct consequences for the Earth, and manipulate the structure and efficiency of ecosystems and the propagation or loss of organisms. Equally, biological activity exerts an influence on local and global climates. By driving the elemental cycles around the Earth, organisms are capable of influencing changes in the climate (Lovelock et al., 1972, Rost, 2004).

The largest ecosystem on our planet is the marine pelagic system (Figure 1.19), and whilst the foundation of this food web is created by in excess of 5000 species there are only a small number of taxonomic groups of phytoplankton which are accountable for the majority of the energy transfer to higher trophic levels from primary producers. These crucial microorganisms can be further distinguished in to 'functional groups', for example in the creation of silicon and calcium shells and coccoliths, as seen by diatoms and coccolithophores, or the formation of organic plates by flagellates, and the fixing of atmospheric nitrogen by cyanobacteria. Each of these groups makes a distinct impression on elemental changes between all regions of the ocean as well as the overlying atmosphere (Inomata et al., 2006).

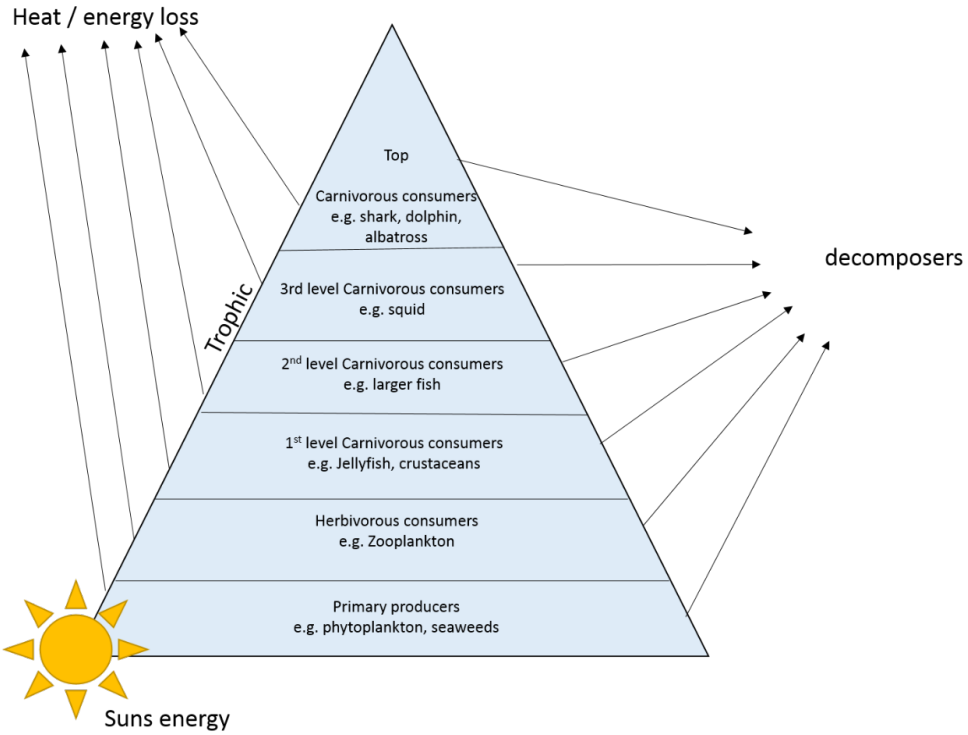



**Figure 1.19 Marine ecosystem pyramid**

## 1.12 Phytoplankton and their effects on the Environment.

Marine phytoplankton affect the global climate by means of three biological processes; The Organic Carbon Pump, the Carbonate Pump, and the production of Cloud Condensation Nuclei (CCN) following the release of DMS from its precursor DMSP (Holligan, 1992, Hanson, 1994, Balch et al., 1992, Charlson, 1987).

Amongst the coccolithophores, *Emiliania huxleyi* is one of the most abundant and widespread species. It is thought to be the most productive calcifying organism on Earth, often forming very large blooms in temperate and sub-polar oceans. The combination of organic matter production and calcification in *E. huxleyi* and other coccolithophore blooms emphasizes their biogeochemical significance in the marine carbon cycle involved in both the Organic Carbon Pump and the Carbonate Counter Pump.

### 1.12.1 The Organic Carbon Pump,

The organic carbon pump is responsible for the transport of carbon dioxide from the atmosphere and the upper mixed water layer to the deeper ocean via synthesis of particulate organic carbon. Primary production via photosynthesis in the photic zone and the vertical export of organic matter to deep water draws down $CO_2$. This is called the organic carbon pump (see Figure 1.20).

### 1.12.2 The Carbonate Counter Pump

In contrast to the organic carbon pump, calcification and the subsequent formation of biogenic calcium carbonate releases $CO_2$. This is named the carbonate counter pump because it counteracts the effect of the $CO_2$ fluxes (see Figure 1.20).

The close connection of the organic carbon pump and the carbonate counter pump is believed to be primarily responsible for generating and maintaining the vertical distribution of total alkalinity (TA) in seawater and for regulating the atmospheric $CO_2$ (Rost, 2004)

$Organic\ carbon\ pump$: $CO_2 + H_2O \rightarrow CH_2O + O_2$

$Carbonate\ counter\ pump$: $Ca^{2+} + 2HCO_3^- \rightarrow CaCO_3 + H_2O + CO_2$

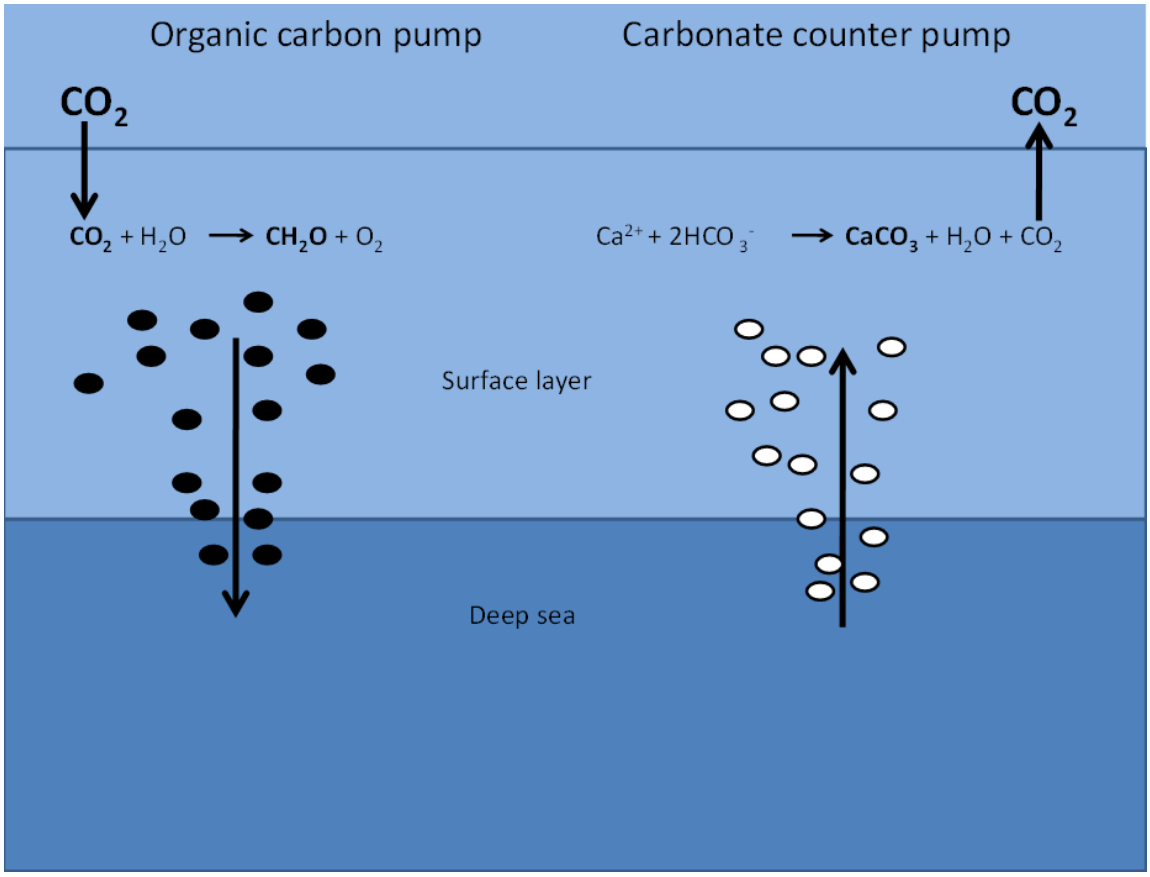



**Figure 1.20 The biological carbon pumps.** Photosynthetic production of organic matter in the surface layer and ensuing transport to the deeper ocean is called the organic carbon pump. It generates a $CO_2$ sink in the ocean. In contrast, calcium carbonate production and its transport to the deeper ocean, is called the calcium carbonate pump. The calcium carbonate pump releases $CO_2$ in the surface layer. Figure was reproduced from Rost and Riebesell (Rost, 2004).

## 1.13 Phytoplankton and the sulphur cycle

Despite the importance of DMSP its function still remains unclear and a number of roles have been suggested (see section 1.3). When DMSP is released into the environment via viral lysis, senescence or grazing by zooplankton, we observe the production of MeSH or the climatically active DMS, depending on the pathway through which DMSP is transformed. There are some DMSP-producing phytoplankton which are known to create DMS (Steinke and Kirst, 1996) but at the start of this study no DMSP lyase had been successfully isolated nor characterized from any eukaryotic organism.

### 1.13.1 The role of the Coccolithophores in the Marine carbon and sulphur cycles

The marine carbon cycle is a crucial constituent of the global climate as the ocean is a chief sink for $CO_2$ emitted to the atmosphere. Conversely, the uptake of $CO_2$ during the past two centuries has in part resulted in acidification of the surface oceans and had major effects on the biogeochemical carbon cycling of the oceans and the ecosystem dynamics of the habitats linked to it. Whilst there is some understanding of the positive and negative feedback of the marine carbon cycle on climate change, they are largely poorly understood and characterized.

Coccolithophores, and particularly *Emiliania huxleyi*, are widespread and abundant around the globe, with *E. huxleyi* considered the most calcifying organism on earth due to it forming huge blooms (Rost, 2004). The innate coupling of the production of organic matter and calcification within coccolithophore blooms emphasizes their significance in biogeochemical measures of the marine carbon cycle.

## 1.14 The Major Objectives of this study.

DMSP is an important source of carbon and sulphur in marine food webs and a precursor of the environmentally important gas DMS. It is essential to fully understand the genetics and molecular mechanisms underlying DMSP catabolism so that environmental understanding of the amounts and functions of this molecule can be recognised.

With the identification of the DMSP demethylase DmdA and the DMSP lyases DddD, DddP, DddL, DddY, DddQ and DddW, it became clear that the molecular mechanisms and enzymes used to break down DMSP were diverse and that the characteristics and mechanisms through which they cleave DMSP required clarifying. With the known DMSP lyases displaying such divergence in their sequence similarities, attention was then refocused on the long-considered eukaryotic DMSP lyases. At the start of this project, no enzymes had been isolated, purified and characterized from any axenic culture of any marine eukaryote.

The overarching aim of this project was therefore to investigate the characteristics of the cupin-containing DMSP lyases DddL, DddQ and DddW and to isolate and purify a novel DMSP lyase from a marine eukaryote.

The research work conducted for this thesis can be divided into two parts focusing on two major objectives:

(1): Expression, purification and characterization of the enzymology and biochemistry of the DMSP cupin-containing lyases DddL, DddQ and DddW.

(2) The application of previously successful techniques used in the purification of bacterial *ddd* genes to the isolation and purification of a DMSP lyase in the eukaryote *Emiliania huxleyi.*

# 2 Materials and Methods

**Table 2.1 Bacterial strains used in this study**

| Strain | Characteristics | Source |
|---|---|---|
| *E. coli* 803 | Used for transformation of large plasmids. | Wood, 1966 |
| *E. coli* BL21 | Used for over expression of Proteins. | Yanisch-Perron *et al*., 1985 |
| *E. coli* JM101 | LacZ,; used for transformation of small plas mids. | Messing 1979 |
| *Rhizobium leguminosarium* J391 | Wild type strain; StrepR mutant | Young 2006 |
| *Rugeria pomeroyi* DSS-3 | Wild type strain | González *et al*., 2003 |
| J470 | *Ruegeria pomeroyi* DSS-3; $Rif^R$ mutant | Todd *et al*., 2010b |

**Table 2.2 Plasmids used in this study**

| Plasmid | Characteristics | Source |
|---|---|---|
| pRK2013 | Used for mobilising plasmids in bacterial conjugation: $Kan^R$ | Figurski and Helinski, 1979 |
| pET21a | Overexpression plasmid, T7 promoter, Optional C-terminal His-tag; AmpR | *Novagen* |
| pBIO1652 | Rhodobacter 2.4.1 dddL PCR product cloned in pET21a | Curson et al 2008 |
| pBIO1753 | Dervivative of pBIO1652 with H152A mutation | This study |
| pBIO1754 | Dervivative of pBIO1652 with H154A mutation | This study |
| pBIO1755 | Dervivative of pBIO1652 with E159A mutation | This study |
| pBIO1756 | Dervivative of pBIO1652 with H190A mutation | This study |
| pBIO1878 | Wide host-range *lacZ* reporter plasmid, based on pMP220; SpcR, TetR | Todd *et al* 2012a |
| pBIO1879 | $Spc^R$ derivative of suicide plasmid pK19*mob*, used for insertional mutagenesis: $Spc^R$; KanR | Todd et al 2010a |
| pBIO1886 | SPO1596 (dddQ) cloned into pET16 | Todd et al |
| pBIO1948 | 1kb PCR product containing SPO0454 cloned in PS4 to make a lacZ fusion | Kirkwood 2012 |

| | | |
|---|---|---|
| pBIO1959 | Dervivative of pBIO1886 with H128A mutation | This study |
| pBIO1960 | Dervivative of pBIO1886 with H130A mutation | This study |
| pBIO1961 | Dervivative of pBIO1886 with E134A mutation | This study |
| pBIO1962 | Dervivative of pBIO1886 with H169A mutation | This study |
| pBIO2243 | Dervivative of pBIO1963 with H83A mutation | This study |
| pBIO2244 | Dervivative of pBIO1963 with H85A mutation | This study |
| pBIO2245 | Dervivative of pBIO1963 with E87A mutation | This study |
| pBIO2246 | Dervivative of pBIO1963 with H121A mutation | This study |

## 2.1 Bacterial methods:

### 2.1.1 Media, growth conditions, Strains and Plasmids

All strains and plasmids used in this study are listed in tables 2.1 and 2.2. Table 2.3-2.5 details any concentrations of supplements added where required**.**

#### 2.1.1.1 Preparation of culture media:

Media was prepared using distilled water ($DH_2O$) and was sterilized via autoclaving. Solid media contained 1.5% agar. All liquid cultures were grown at appropriate temperatures and shaken at 220 rpm.

**Luria-Bertani broth (LB)**

Working stocks of *Escherichia coli* were grown at 37 $^{o}$C in or on Luria-Bertani broth (LB). LB : 10g Tryptone, 5g yeast extract, 5g NaCl,1.5g D-glucose per litre of $DH_2O$, pH 7.2.

**1/2 YTSS**

Working stocks of *Ruegeria pomeroyi* were grown at 28 $^{o}$C in or on a complete media, 1/2 YTSS:

1.25g Tryptone, 2g yeast extract, 20g sea salts (Sigma) per litre of $DH_2O$, pH 7.0

**Marine Basal Medium (MBM**

Working stocks of *Ruegeria pomeroyi* were grown at 28 $^{o}$C in or on minimal media Marine Basal Medium (MBM):

MBM:

Before sterilization; 20g sea salts (Sigma), 250ml Basal media [150ml 1M Tris HCl, pH 7.5, 87mg $K_2PO_4$, 1.5g $NH_4Cl$, 375ml $DH_2O$], 10mM succinate, after sterilization; 50ml FeEDTA [50mg FeEDTA, 100ml FeEDTA], 0.1% vitamin solution [ 2mg biotin, 2mg folic acid, 10mg pyroxidine HCl, 5mg riboflavin, 5mg thiamine, 5mg nicotinic acid, 5mg pathotenic acid, 0.1mg cyanobalamin, 5mg p-aminobenzoic acid per 100ml $DH_2O$]

## 2.2 Antibiotic Concentrations:

**Table 2.3 Antibiotic supplements to media**

| Antibiotic | Solvent | Stock [mg ml$^{-1}$] | [Final µg ml$^{-1}$] |
|---|---|---|---|
| **Ampicillin (Amp)** | $dH_2O$ | 100 | 100 |
| **Gentamycin (Gent)** | $dH_2O$ | 10 | 5 |
| **Kanamycin (Kan)** | $dH_2O$ | 20 | 20 |

| | | | |
|---|---|---|---|
| **Rifampicillin (Rif)** | MeOH | 100 | 100 |
| **Spectinomycin (Spec)** | $dH_2O$ | 50 | 200 |
| **Streptomycin (Strep)** | $dH_2O$ | 200 | 400 |
| **Tetracycline (Tet)** | EtOH (70%) | 5 | 5 |

## 2.3 Inducer Concentrations

**Table 2.4 Inducer supplements to media**

| Inducer | Solvent [mM] | Stock [mg ml-1] | [Final] [mM] |
|---|---|---|---|
| **Acrylate** | $dH_2O$ | 100 | 2.5 |
| **Dimethylsulfoniopropionate (DMSP)** | $dH_2O$ | 100 | 5 |
| **Dimethylsulfide (DMS)** | N/A | N/A | 5 |
| **Methylmercaptopropionate (MMPA)** | $dH_2O$ | | 2.5 |

## 2.4 Carbon Source

**Table 2.5 Concentrations of carbon sources used**

| Carbon Source | Solvent | [Final] [mM] |
|---|---|---|
| **Succinate** | **$dH_2O$** | **10** |
| **Dimethylsulphoniopropionate** | **$dH_2O$** | **5** |
| **DMS** | **-** | **1** |

## 2.5 Long term storage of bacterial strains.

In order to preserve bacterial strains and plasmids cultures were grown overnight to stationary phase in respective rich media (LB or YTSS) and glycerol added (25%). *R. Pomeroyi* required the addition of 15% DMS to serve as a cryoprotectant. Stocks were flash frozen using liquid nitrogen ($LN_2$) and stored at – 20 $^{o}$C or -80 $^{o}$C.

## 2.6 Nucleic acid preparation techniques:

### 2.6.1 Mini preparation of Plasmid DNA using QIAGEN Mini kit spin columns

For the isolation of small scale (< 5 µg), DNA to be used in PCR, transformations, restriction digest analyses, and sequencing plasmid mini-prep spin columns were employed (Qiagen). This protocol uses the alkaline lysis method (Birnboum and Doly,1979). It comprised of separate steps firstly alkaline lysis followed by column purification of covalently closed circular DNA molecules through binding to the silicagel membrane. All centrifugation steps were performed at 16000 x g All buffers and spin columns were supplied with the kit and the procedure performed as detailed by the manufactures specifications as follows:

1. A single colony of *E. coli* containing the required plasmid was inoculated in 5 ml LB liquid, containing the appropriate antibiotics, and incubated at 37 °C overnight.

2. Cells were pelleted (1.5 ml culture for high copy number plasmids; 3 ml for low copy number plasmids) by centrifugation in 1.5 ml eppendorf tubes, and the supernatant was totally removed.

3. Pelleted cells were re-suspended in 250 µl P1 re-suspension buffer, containing 100 µg ml-1 RNase A to remove RNA, and incubated at room temperature for 5 minutes.

6. 250 µl P2 lysis buffer was added and mixed well by inversion. Samples were then incubated at room temperature for no more than 5 minutes.

5. 350 µl P3 (stored at 4 °C) neutralisation buffer was added and mixed well by inversion. Samples were then incubated on ice for 5 minutes to allow for precipitation of protein, genomic DNA and cell debris.

6. Samples was centrifuged for 15 minutes.

7. The resultant supernatant was applied to a Qiaprep spin column and centrifuged for 1 minute.

8. Eluate was discarded and 500 µl PB binding buffer was applied to the column and centrifuged for 1 minute to remove excess nuclease activity and high carbohydrate content from *endA*+ strains such as JM101.

9. Eluate was discarded and 750 µl PE wash buffer was applied to the column and centrifuged for 1 minute.

10. Eluate was discarded and the column and collection tube was centrifuged for 1 minute to remove remaining wash buffer from the column matrix.

11. Spin columns were transferred to clean, labelled 1.5 ml eppendorf tubes and DNA was eluted by pipetting 50 µl dH2O onto the column matrix and allowed to stand for 1 minute. This was then centrifuged for 1 minute, the spin column discarded and eluted DNA stored at -20 °C.

### 2.6.2 Midi preparation of Plasmid DNA using QIAGEN Midi Kit spin columns

Plasmid midi kits (QIAGEN) were employed to isolate large quantities (>5µg) of high purity plasmid DNA for various procedures and for sequencing according to the manufacturers instructions using supplied reagents. This protocol is based on an optimised alkaline lysis method comprising of three distinct steps. Firstly alkaline lysis followed by column purification of DNA molecules and lastly the precipitation of plasmid DNA using isopropanol. All the required constituents were supplied with the QIAGEN kit with the exception of 100% ethanol and isopropanol.

1. A single colony of *E. coli* containing the required plasmid was inoculated to 100ml LB liquid containing the appropriate antibiotic(s) and incubated overnight at 37°C.

2. Cells were pelleted using sterile 50ml falcon tubes and centrifuged for 10 minutes

3. Pelleted cells were resuspended in 4 ml of P1 resuspension buffer containing 100µg $ml^{-1}$ RNaseA to remove RNA and incubated for five minutes at room temperature.

4. 4ml of P2 lysis buffer was added and mixed via inversion until a blue colour was observed (indicating an alkaline pH). The samples were incubated at room temperature for no longer than five minutes.

5. 4ml of N3 a neutralization buffer was added and mixed via inversion. The samples were then incubated on ice for 15 minutes in order to precipitate protein, genomic DNA and membrane debris.

6. Precipitates were pelleted by centrifugation for 15 minutes.

7. A midi preparation column was equilibrated by introducing 4ml of QBT equilibration buffer to the column matrix and allowing it to follow through. Eluate was discarded.

8. The supernatant from step 6 was applied to the equilibrated column.

9. The eluate was discarded and the column washed using 20ml of QC buffer. Any eluates were discarded.

10. Plasmid DNA was released from the matrix and eluted from the column by the addition of 5ml QF elution buffer. Eluates were collected into sterile 50ml falcon tubes.

11. Eluted plasmid DNA was precipitated by adding 3.5ml of 100% Isopropanol and mixed via inversion before being centrifuged for 30 minutes in order to pellet the precipitated plasmid DNA.

12. The resulting DNA pellet was washed with 1ml of 70% ethanol and centrifuged for two minutes. The resulting supernatant was removed completely using a micropipette and the DNA pellet allowed to air dry for 10 minutes.

13. The dry DNA pellet was resuspended in 60µl $DH_2O$ and stored at -20°C until required.

### 2.6.3 Extraction of plasmid DNA by alkaline lysis and phenol chloroform.

The following method was used to isolate plasmid DNA for restriction digestion to analyse prospective clones. All centrifugation steps were performed at 16000 x g. As with mini prep DNA isolation this protocol employs the alkaline lysis method.

1. A single colony of *E. coli* containing the required plasmid was inoculated to 5 ml LB liquid, containing the appropriate antibiotic(s), and incubated at 37 °C overnight.

2. Cells were pelleted by centrifugation in 1.5 ml eppendorfs, and the supernatant was completely removed.

3. Pelleted cells were re-suspended in 200 µl resuspension buffer (50 mM glucose, 10 mM Tris-HCl and 20 mM EDTA, pH 8.0) and incubated for 5 minutes at room temperature.

4.400 µl lysis solution was added, mixed by inversion and incubated on ice for 5 minutes.

5. 300 µl neutralisation buffer (3M Na.acetate, pH 6.8) was added and mixed by inversion. Samples were incubated on ice for 5 minutes to precipitate protein, genomic DNA and cell debris.

6. Samples were centrifuged for 15 minutes.

7. The resultant supernatant was transferred to a clean 1.5 ml eppendorf and 400 µl of phenol-chloroform (phenol:chloroform:isoamyl alcohol 25:24:1, Sigma) was added and mixed by vortexing. Samples were then centrifuged for 4 minutes.

8. The upper aqueous layer was transferred to a clean 1.5 ml eppendorf tube containing 700 µl room temperature 100% ethanol to precipitate plasmid DNA. Samples were then centrifuged for 15 minutes.

9. The supernatant was then discarded and the resultant DNA pellet was washed in 500 µl 70% (v/v) ethanol and centrifuged for 5 minutes.

10. The supernatant was then completely removed using a micropipette, and the DNA pellet was air-dried for 15 minutes.

11. The pelleted DNA was resuspended in 40µl dH2O and stored at -20 °C.

### 2.6.4 Preparation of genomic DNA using the Wizard Genomic DNA purification kit

The Wizard Genomic DNA purification kit (Promega) was used to isolate genomic DNA from *Ruegeria Pomeroyi* required for PCR amplification, cloning and restriction digests. All buffers were supplied with the kit and centrifugation performed in a bench-top centrifuge at 15500 x g.

A single colony was inoculated to 5ml of the rich media 1/2YTSS and grown overnight shaking at 28$^{O}$C.

1. Single colonies were inoculated to 5ml of the rich media 1/2YTSS and grown overnight shaking at 28$^{O}$C.

2. 2 ml of stationary phase cells were pelleted in an eppendorf by centrifugation

3. Pelleted cells were resuspended in 600 µl Nuclei lysis solution, and cells were lysed by incubating at 80 °C for 10 minutes, and cooled to room temperature.

6. 3 µl of RNase solution was added to the lysate, mixed via inversion and incubated at 37 °C for 60 minutes.

5. Samples were cooled to room temperature and 200 µl Protein precipitation solution was added and mixed via vortexing, samples were then incubated on ice for 5 minutes.

6. Samples were centrifuged for 3 minutes and supernatants were transferred to clean 1.5 ml eppendorfs containing 600 µl room temperature isopropanol, to precipitate DNA.

7. Mixtures were gently inverted until the DNA became visible as thread-like strands, and were then centrifuged for 2 minutes.

8. Supernatants were removed and pelleted DNA was washed by adding 600 µl room temperature 70% ethanol (v/v) and mixed by inversion. DNA was pelleted by centrifugation for 2 minutes.

9. Supernatants were removed using a micropipette and air dried for 10 minutes.

10. Dried DNA pellets were then resuspended in 70 µl ddH2O and incubated for 1 hour at 65 °C.

Genomic DNA solutions were then stored at -20 °C.

### 2.6.5 Ethanol precipitation of DNA/RNA

To precipitate DNA/RNA samples of approximately 150µl, 45µl of 3M RNase free sodium acetate (pH5.7; Sigma) was added. To this 405µl RNase free 100% ethanol (Fisher) was added and the samples mixed via inversion. The DNA/RNA was precipitated on ice for 30 minutes. Precipitated nucleic acids were pelleted via centrifugation at 12,000rpm and 4°C for 30 minutes. The supernatant was removed and discarded and pelleted material washed with RNase free ice cold 70% ethanol and gentle pipetting up and down. Samples were then centrifuged at 12,000rpm at 4°C for 15 minutes and the supernatant totally removed using a sterile micropipette tip. DNA/RNA pellets were air dried for five minutes and then resuspended in an appropriate volume of RNase free $H_2O$.

### 2.6.6 DNA quantification

DNA preparations were quantified using absorbance readings at 260nm using a NanoDrop2000 (ThermoScientific).

The purity of the DNA preparations was determined using ratio of absorbances at 260/280nm (protein contamination), and 260/230 (solvent contamination). A 260/268 ratio of 1.8 and a 260/230 ratio in the range of 2.0-2.2 were satisfactory.

## 2.7 Transfer of genetic material

### 2.7.1 Transformations

#### 2.7.1.1 Preparation of competent cells

Competent *E. coli* cells were prepared using cold $CaCl_2$, as described by Sambrook et al (1989).

A single colony of appropriate *E. coli* was inoculated into 5ml LB media and incubated overnight at 37 $^{O}$C. 1ml of the overnight (stationary phase) culture was inoculated the following morning to 100 ml LB and incubated to $OD_{600}$ 0.3-0.4 (~2 hours). The culture was centrifuged at 4 $^{o}$C at 6000rpm for 10 minutes and pelleted cells returned immediately to ice. The supernatant was discarded and pellets resuspended in 15ml ice cold 0.1M $CaCl_2$. The cells were then incubated on ice for 30 minutes. Following incubation cells were pelleted at 4 $^{o}$C at 10,000rpm for 10 minutes, transferred to ice and supernatant discarded. Cells were then re-suspended in 2 ml of ice cold 0.1M $CaCl_2$, and stored at 4 $^{o}$C overnight for use the next day.

#### 2.7.1.2 Transformations using heat shock

Competent *E. coli* cells were transformed using plasmid DNA or ligation mix. In a sterile eppendorf 100 µl of competent cells DNA or ligation mix was added and incubated on ice for one hour. The cells containing the DNA/ligation mix were then heat shocked for 3 minutes at 42 $^{o}$C before being incubated back on ice for 2 minutes. 0.5 ml of warm LB was added and the mix incubated for 1 hour at 37 $^{O}$C. The transformation was plated to LB containing the appropriate antibiotics. For the transformation of large (>10 kb) plasmids *E. coli* strain 803 was used. For all

protein purification experiments *E. coli* strain BL-21 was the host strain for the over-expression plasmid, pET21a. Super competent cells used in site directed mutagenesis were always transformed according to the manufactures instructions.

### 2.7.2 Bacterial Conjugations

Plasmids were transferred by conjugation from *E. coli* strains JM101 or 803 to *Ruegeria pomeroyii* or *Rhizobium leguminosarum* using the patch cross technique (Johnston et al., 1978) the filter cross technique (Beinger and Hopwood 1976) for triparental mating with *E. coli* 803 containing pRK2013 as a helper plasmid (Figurski and Helinski, 1979) to mobilise plasmids which were not self transmissible.

#### 2.7.2.1 Patch cross

Plasmids were transferred by conjugation to *Ruegeria pomeroyi* or *Rhizobium leguminosarum* from *E. coli* using a patch cross (Johnston et al., 1978). *Ruegeria pomeroyi* DSS-3 or *Rhizobium leguminosarum* recipients *E. coli* strain 803 *lacZ*::promoter fusion donor plus the *E. coli* helper strain pRK2013 were cultured on ½ YTSS (*R. pomeroyi*), TY (*R. leguminosarum*) or LB (*E. coli*) agar plates. Each strain was mixed on ½ YTSS agar, or TY agar. Following overnight incubation at 28°C, the cells were streaked on selective media with the appropriate antibiotics for the plasmid and/or strain. These plates were incubated at 28°C for 2 days before selection of transconjugants.

#### 2.7.2.2 Filter cross

For rare conjugation events (e.g. insertional mutagenesis), crosses were performed on nitrocellulose filters (Whatman). Overnight liquid cultures of the *E. coli* donor strain and *E. coli* helper strain and the recipient strain. The mixture was centrifuged at 13,000rpm for two minutes to pellet cells. The supernatant was almost entirely removed except ~100µl, which was used to resuspend the pellet which was spread onto a sterile nitrocellulose filter on a plate of the appropriate medium to support the growth of all three strains. Following two days incubation at 28 $^{O}$C filters were removed to a sterile universal and 1 ml of minimal media was added to the tube and the bacteria washed from the filter via vortexing for ~5

minutes. Serial dilutions were prepared on the washed cells and spread onto selective media. Plates were incubated at 28 $^{o}$C until single colonies appeared.

## 2.8 Polymerase Chain Reaction

A Techne TC-512 machine was used to perform PCR. Standard PCR reactions were composed of:

1-5µl DNA/gDNA

1µl forward primer

1µl reverse primer

10µl master mix ()

$DH_2O$ to a final volume of 20µl

The PCR cycle parameters are detailed in table 2.6

### 2.8.1 Colony PCR

For colony PCR cells of single colonies were re-suspended in 100 µl $DH_2O$ and 1 µl of this was used in each reaction as template. Enzymes buffers and reagents used were the same as those used for standard PCR. PCR reactions were composed of:

1 µl resuspended cells

10 µl Master mix

1 µl Forward primer

1 µl Reverse primer

$DH_2O$ to final volume of 20 µl

## 2.9 Purification of PCR products

PCR products were purified using a High Pure PCR product purification kit (Roche) according to the manufacturers specifications. All buffers were supplied with the kits, and the protocol is detailed below:

1. The PCR product was added to 500µl binding buffer and mixed via inversion.
2. The sample mix was loaded onto a High Pure Spin filter tube and then centrifuged at 13,000rpm for 60 seconds.
3. Flow through from the filter was removed and 500µl of was buffer introduced to the filter and centrifuged for 60 seconds.
4. Flow through was discarded and the filter centrifuged for 60 seconds in order to remove any residual buffer.
5. The column was transferred to a sterile 1.5ml eppendorf tube and 30µl $dH_2O$ was applied to the filter and allowed to soak for 2 minutes before centrifugation at 13,000 rpm for 60 seconds to elute the purified PCR products.

Purified PCR products were stored at -20 $^oC$

## 2.10 Restriction Enzyme digestion of DNA

Digests were performed using Roche or Promega restriction enzymes and conducted using supplied buffers and according to the manufacturers' specifications. Total reaction volumes of 20µl were made up using approximately 1µg DNA, 10U/µl restriction enzyme, and to volume with $dH_2O$. Digestion took place at 37 $^oC$ for 2-3 hours and was stopped by heating to 75 $^oC$ for 10 minutes.

## 2.11 Alkaline Dephosphorylation of Plasmid DNA

Alkaline phosphatise RAPID (Roche) was used to dephosphorylate the cleaved vector DNA prior to use in ligation reactions. Dephosphorylation was performed as per the manufacturer's instructions and using the supplied reagents. Reactions were incubated for 2 hours at 37 $^oC$ and were finished by heating at 75 $^oC$ for 10 minutes.

## 2.12 Ligation reactions

Recombinant plasmids were produced by ligating PCR products which had been cut using the appropriate restriction enzymes to digested vector DNA using T4 DNA Ligase and supplied reagents (Roche) as per manufactures instructions. Standard ligation reactions contained >200ng of vector and insert DNA, 1µl 10x ligase buffer, 1µl T4 DNA ligase made to a total volume of 10µl using $dH_2O$. Ligations were incubated for 16 hours overnight on ice or at 4 $^oC$. The ligase was inactivated by heating to 75 $^oC$ for 10 minutes.

## 2.13 DNA Gel electrophoresis

DNA fragments were separated via electrophoresis in 1% agarose gels containing 500ng/ $ml^{-1}$ Ethidium Bromide (EtBr), 1x TAE buffer (Tris borate, 1mM EDTA, 5mM Tris borate, pH8.0). DNA samples were mixed with 1 volume of 2x loading dye [0.25% bromophenol blue (v/v), 30% glycerol (v/v)] were loaded onto gels alongside 1kb ladder (Invitrogen) and run in SCIE-PLAS mini or midi horizontal gel tanks containing 1xTAE running buffer at 70-100 mV for 1.5 – 2 hours

## 2.14 Gel Extractions

DNA fragments were extracted from agarose gels using a QIAquick gel extraction kit (Qiagen) according to the following protocol:

1. The required DNA fragment was cut from the gel using a clean scalpel and placed into a sterile eppendorf tube containing 300µl QG buffer and incubated at 50 $^oC$ for 10 minutes in order to dissolve the agarose gel.
2. The sample was applied to a QIAquick column and centrifuged at 13,000 for 1 minute.
3. The flow through was discarded and 500µl QG buffer was added to the column and centrifuged for 1 minute.
4. The flow through was discarded and 750µl PE buffer was applied to the column in order to wash it.
5. The column was centrifuged for 1 minute the flow through discarded and the column centrifuged for a further 1 minute to remove remaining buffer.

6. DNA was eluted from the column into a sterile eppendorf by applying 30µl $DH_2O$. This was allowed to soak for 2 minutes before centrifuging for 1 minute.

DNA samples were stored at -20 $^{o}$C until required

## 2.15 Mutagenesis

### 2.15.1 Insertional Mutations in *Ruegeria Pomeroyi*.

In order to produce insertional mutations into *Ruegeria pomeroyi* containing the suicide plasmid pK19mob ($Kan^R$) (Schafer et al., 1994) a modified version was used in which a spec cassette was cloned into the Hind III site of pK19mob. Resulting plasmids were transformed into *E. coli* strain 803 and then mobilized via triparental conjugation using pRK2013 into *R. pomeroyi* DSS-3 ($Rif^R$). Mutants were selected for on the rich medium ½ YTSS containing spectinomycin, kanamycin and rifampicin. The conjugations were performed via filter crossing as follows. *Ruegeria pomeroyi* cells were grown overnight in ½ YTSS at 28$^{O}$C shaken at 220rpm. *E. coli* containing the suicide plasmid and the helper strain (pRK2013) were both grown in LB media at 37 $^{o}$C overnight and shaken at 220rpm. The following day 0.5 ml of *R. Pomeroyi* and 1 ml of both *E. coli* strains were centrifuged and re-suspended in 100 µl of liquid ½ YTSS. The culture mixes were spread upon a nitrocellulose filter on a ½ YTSS plate containing no antibiotics. The plates were incubated at 28 $^{o}$C for ~ 2 days. Cells were then washed from the filter using ½ YTSS and spread onto selective ½ YTSS plates containing spectinomycin, kanamycin and rifampicin. Resulting transconjugants were ratified via PCR and southern blot hybridizations.

### 2.15.2 Site directed mutagenesis

Four separate in-frame point mutations of DddL, DddQ and DddW was produced using the QuikChange Lightning mutagenic PCR kit according to the manufacturer's instructions (*Agilent*). The plasmid DNA template was added at 100 ng.µl-1. The primer pairs used to construct all mutants are shown in Table 2.6 The mutagenised products were transformed into *E. coli* XL10-Gold Ultra competent cells (*Agilent*), selecting ampicillin resistance. Mutant plasmids were

confirmed via sequencing, and the mutagenised inserts were sub-cloned into pET21a or pET16.

**Table 2.6** PCR parameters for quickchange lightening Site Directed Mutagenesis Method

| Segment | Cycles | Function | Temperature | Time (secs) |
|---|---|---|---|---|
| **1** | 1 | Initial denaturation | 95$^{o}$C | 120 |
| **2** | 18 | Denaturation | 95 $^{o}$C | 20 |
| | | Annealing | 60 $^{o}$C | 10 |
| | | Extension | 68 $^{o}$C | 162 |
| **3** | 1 | Final extension | 68 $^{o}$C | 300 |

## 2.16 Protein purification

### 2.16.1 Over-expression and purification of DddQ protein.

Single cell colonies of *Escherichia coli* strain BL21 containing plasmid pBIO1886 were inoculated to 5 ml in LB broth containing 100 µg ampicillin ml−1 and grown at 37 °C shaking overnight. The starter cultures were inoculated to 500mls LB broth containing 100 µg ampicillin ml−1 to an OD600 of 0.4–0.6 before inducing *dddQ* expression, using 0.2mM IPTG. The cultures were incubated at 25 °C until cells reached the stationary phase. Cells were pelleted via centrifugation at 7,000 rpm for 20 minutes and re-suspended in 20 mM Tris buffer (pH 8) at 4 °C. Cells were lysed via French press and cell debris removed by centrifugation at 10,000 rpm for 30 minutes. In order to remove membranes the cell lysate was centrifuged at 40,000 rpm for 60 minutes. 50 % (w/v) $(NH4)_2SO_4$ was added to the lysate and the resulting precipitate was removed by centrifugation at 20,000 rpm for 30 minutes. The supernatant was loaded onto a phenyl sepharose high-performance column (xk16/20; GE Healthcare) equilibrated with 20 mM Tris containing 50 % (w/v) $(NH_4)_2SO_6$. Proteins were eluted using a 0-100 % $(NH_4)_2SO_4$ gradient (flow rate 1 ml min−1) over 205ml (0-50% over 30mls, 50-100% over 75mls, 100% over 100mls) The fractions that contained the DddQ polypeptide were then applied to a

DEAE (HiTrap, 5 ml; GE Healthcare) column equilibrated with 20 mM Tris buffer, pH 8 (flow rate 5 ml min−1). Proteins were eluted in the same buffer with a linear gradient of 0–1 M NaCl and 2ml fractions collected. The fractions containing DddQ were concentrated to 0.5 ml using an Amicon Ultra 4 ml Centrifugal filter and loaded onto a Superdex 200 gel filtration column (10/300GL; GE Healthcare) equilibrated with 20mM TRIS buffer, pH 8 with a flow rate 0.4 ml min−1 and 0.5ml fractions were collected. The DddQ-containing fractions were pooled and stored at 4 °C.

### 2.16.2 Over-expression and purification of DddL protein.

Single cell colonies of *Escherichia coli* strain BL21 containing plasmid pBIO1652 were inoculated to 5 ml in LB broth containing 100 µg ampicillin ml−1 and grown at 37 °C shaking overnight. The starter cultures were inoculated to 1L LB broth containing 100 µg ampicillin ml−1 to an OD600 of 0.4–0.6 before inducing *dddL* expression, using 0.2mM IPTG. The cultures were incubated at 25 °C until cells reached the stationary phase. Cells were pelleted via centrifugation at 7,000 rpm for 20 minutes and re-suspended in 20 mM Tris buffer (pH 8) at 4 °C. Cells were lysed via French press and cell debris removed by centrifugation at 10,000 rpm for 30 minutes. In order to remove membranes the cell lysate was centrifuged at 42,000 rpm for 60 minutes. 50 % (w/v) $(NH4)_2SO_4$ was added to the lysate and the resulting precipitate was removed by centrifugation at 20,000 rpm for 30 minutes. The supernatant was loaded onto a phenyl sepharose high-performance column (xk16/20; GE Healthcare) equilibrated with 20 mM Tris containing 50 % (w/v) $(NH_4)_2SO_6$. Proteins were eluted using a 50–0 % (NH4)2SO4 gradient (flow rate 1 ml $min^{-1}$). The fractions containing DddL were concentrated to 0.5 ml using an Amicon Ultra 4 ml Centrifugal filter and loaded onto a Superdex 200 gel filtration column (10/300GL; GE Healthcare) equilibrated with 20mM TRIS buffer, pH 8 with a flow rate 0.2 ml $min^{-1}$ and 0.5ml fractions were collected. The DddL-containing fractions were pooled and stored at -80°C.

### 2.16.3 Over-expression and purification of DddW protein.

Single cell colonies of *Escherichia coli* strain BL21 containing plasmid pBIO1887 were inoculated to 5 ml in LB broth containing 100 µg ampicillin ml−1 and grown at 37 °C shaking overnight. The starter cultures were inoculated to 500mls LB broth containing 100 µg ampicillin ml−1 to an OD600 of 0.4–0.6 before inducing *dddQ* expression, using 0.2mM IPTG. The cultures were incubated at 25 °C until cells reached the stationary phase. Cells were pelleted via centrifugation at 7,000 rpm for 20 minutes and re-suspended in 20 mM Tris buffer (pH 8) at 4 °C. Cells were lysed via French press and cell debris removed by centrifugation at 10,000 rpm for 30 minutes. In order to remove membranes the cell lysate was centrifuged at 40,000 rpm for 60 minutes. 40 % (w/v) $(NH4)_2SO_4$ was added to the lysate and the resulting precipitate was removed by centrifugation at 20,000 rpm for 30 minutes. The pellet was washed in 20mM TRIS pH 8.0 40% (w/v) $(NH4)_2SO_4$ and the centrifugation repeated. The pellet was re-suspended in 20 mM TRIS pH8 and dialysed against the same buffer overnight. The dialysed sample was concentrated to 2ml using an Amicon Ultra 4 ml Centrifugal filter and loaded onto a Superdex 200 gel filtration column (10/300GL; GE Healthcare) equilibrated with 20mM TRIS buffer, pH 8 with a flow rate 0.4 ml min−1 and 0.5ml fractions were collected. The DddW containing fractions were pooled and stored at -80$^{o}$C

### 2.17 Separation of protein by SDS-PAGE

Proteins were separated for analysis via polyacrylamide gel electrophoresis using sodium dodecyl sulphate (SDS) to supply denaturing conditions. Gels were prepared according to Sambrook (Sambrook, 1989) A 15% resolving gel was prepared (see below) and poured between two glass plates, sealed with a rubber gasket to a level approximately 2cm from the top of the glass plates. The gel was levelled using water saturated butanol and given time to polymerise before an 8% stacking gel was prepared (see below) and poured on top of the resolving gel. A 15 well comb was placed into the stacking gel before it polymerised.

Prior to loading to the gel samples were mixed with SDS-PAGE loading buffer (see below) and were run alongside Precision Plus Protein $^{TM}$ Dual Colour Standard (Bioline)

Gels were run in vertical tanks (ATTO AE-6450) at 170V for 1.5 hours in PAGE running buffer [25mm TRIS, 20 mM Glycine, 0.1% SDS (w/v)]. Gels were then removed from glass plates and stained with Instant Blue or Sybro Ruby.

Resolving Gel

5ml 4x resolving buffer [1.5M TRIIS pH 8.8, 0.4% SDS (w/v)]

5ml $dH_2O$

10ml Protogel 30% Acrylamide solution

250µl 10% Ammonium Persulphate (APS) (w/v)

25µl Tetramethylethylenediamine (Temed)

Stacking Gel

2.5ml 4x stacking buffer [0.5M TRIS pH6.8, 0.4% SDS (w/v)]

5.5ml $dH_2O$

2ml Protogel 30% Acrylamide solution

100µl 10% APS (w/v)

20µl Temed

SDS-PAGE loading buffer (4x stock)

2ml TRIS-HCl (1M, pH6.8)

0.8g SDS

4ml glycerol

0.4ml β-mercaptoethanol

1ml EDTA [0.5M]

8mg Bromophenol blue

3ml $dH_2O$

## 2.18 Bradford's Assay

Protein concentrations were estimated using the Bradford's assay (Bradford 1976). An appropriate volume of sample was added to $dH_2O$ to prepare a total volume of 800µl. To this 200µl Bradfords reagent (Bio-Rad) was added and mixed via inversion. Using bovine serum albumin at concentrations of 0, 10, 20 or 40mg/ml a standard curve was produced plotting their absorbances at 595nm. This curve was then used to determine the concentration of unknown samples based on their absorbance at 595nm.

## 2.19 Assays for DMS production via gas chromatography

Gas chromatography used a flame photometric detector (GC 2010; Shimadzu, Milton Keynes, UK) and a 30 m x 0.53 mm ID-BP1 5.0 m capillary column (SGE Europe, Milton Keynes, UK). The column flow was 2 ml min-1, with a split ratio of 2:1 and a pressure of 13.095 psi. The hydrogen gas flow was 50 ml min-1, and the air and make up (nitrogen) flow were 60 ml min-1. The carrier gas was helium. The injector and detector temperatures were 250°C, with an oven temperature of 40°C. All assays were carried out in 2 ml glass crimp-top vials, sealed with an 11 mm PTFE/rubber/aluminium crimp cap (*Thermo Scientific*), with a total reaction volume of 300 µl, and an injection volume of 100 µl. The retention time of the peak representing DMS was 2.29 minutes. To quantify DMS concentrations in the headspace of vials, the assay was calibrated using the peak areas produced by seven DMSP standards (1 µM, 2 µM, 10 µM, 20 µM, 100 µM, 200 µM and 300 µM) mixed with sodium hydroxide for complete alkaline lysis of DMSP into equimolar concentrations of DMS and acrylate. To complete this 500 mM NaOH was added to each vial, and the appropriate amount of DMSP, dissolved in dH2O was pipetted onto the septum of each vial lid. In doing so the NaOH and DMSP was mixed only when lids were inverted and sealed onto the vials, guaranteeing minimal escape of released DMS. Vials were incubated overnight in the dark at 28°C, and then assayed at room temperature. Assuming complete lysis of all the DMSP to DMS, 1 µM of DMS would be produced from 1 µM of DMSP. This is equivalent to 0.3 nmol total DMS in the 300 µl liquid reaction volume, and of this, 0.15 nmol DMS is transferred to the 1.7 ml vial headspace.

### 2.19.1 Assays *in vivo*

Cell cultures to be assayed for DMS production were grown overnight in appropriate media.. Cells were then pelleted and washed in minimal or rich media. Washed cells were added to vials containing a final concentration of 5mM DMSP, and vials were sealed immediately. Samples were incubated at 28°C, and then assayed at room temperature.

### 2.19.2 Assays *in vitro*

DMSP lyase proteins were over-expressed in *E. coli* BL21 as described above. Assays were performed on lysed cells, or partially pure protein or pure protein. For lysed cells, 1 ml of induced culture was centrifuged to pellet cells, which were then re-suspended in NPI-10 buffer. The re-suspended cells were lysed by sonication for 4 x 10 second bursts at full power. Then, 297 µl lysed cell material was added to 3 µl 100 mM DMSP in a vial and sealed immediately. Samples were incubated at 28°C and then assayed at room temperature.

### 2.20 β-galactosidase assays

β-galactosidase activity was used to measure the transcription of *lacZ* reporter fusions. Similar to the methods described by Sambrook *et al.* (1989). *Ruegeria pomeroyi* or *Rhizobium leguminosarum* were inoculated from a single colony to 5 ml ½ YTSS, or TY medium and grown for ~2 days at 28°C. 1 ml of culture was inoculated to 100 ml MBM or TY containing potential co-inducer molecules and grown at 28$^{o}$C until OD600 nm was between 0.4 and 0.6. 1 ml of culture was used to measure the OD600 nm and to determine the cell density. Aliquots of the culture (0.1 - 0.5 ml depending on activity) were removed and added to a 2 ml eppendorf tube and made up to 1 ml with Z buffer (see below). To lyse the cells 2 drops of chloroform plus 1 drop 0.1% SDS (w/v) was added to each eppendorf tube and vortexed for 10 seconds. The tubes were incubated at 28°C for 5 minutes before the addition of 0.2 ml *O*-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg ml-1) was added to each tube, a timer started and the samples were incubated at 28°C. When sufficient yellow colour had developed, 0.5 ml Na2CO3 was added to stop the reaction and the time was recorded. The tubes were centrifuged for 3 minutes at 13,000 rpm to pellet cell debris and 1 ml of the

supernatant was added to a 2 ml cuvette, with the OD420 nm measured (blanked against Z buffer).

The β-galactosidase activity, in Miller units, was calculated using the following equation:

Miller units = $(1000 \times OD_{420}) /( t \times V \times OD_{600})$

t = time
V = volume of culture used
Z-buffer contains 1 ml 3 M Na2HPO6.7H2O, 0.5 ml 4 M NaHPO6.7H2O, 0.5 ml 1 M KCl, 0.5 ml 0.1 M MgSO6.7H2O, 175 µl mercaptoethanol, made up to a final volume of 50 ml with dH2O. ONPG was freshly prepared each time. OD600 nm and OD420 nm values were measured using a Unicam 8625 UV/VIS spectrophotometer.

### 2.21 Oligonucleotide design

DNA oligonucleotides were ordered from MWG BioTech and designed using DNAStar software (Lasergene) and ARTEMIS (Sanger). Where appropriate oligonucleotides were chosen with a GC content of <60%, a melting temperature (Tm) of approximately 60°C and an optimum length of 20-28 base pairs. For those which incorporated a single nucleotide basechange for site directed mutagenesis this was integrated into the middle of the oligonucleotide. Sequences and uses of primers are listed in Table 6.6

### 2.22 DNA sequencing

Sequencing was carried out by Genome Enterprise Ltd (John Innes Centre, UK) using an Abi Prism 370 Capillary Sequencer. The template Plasmid DNA used for sequencing reactions was isolated using either midiprep or miniprep kits (QIAGEN). Universal M13F and M13R primers were provided by Genome Enterprise Ltd but custom primers for other sequencing reactions were provided at a concentration of 1.5 pmol $\mu l^{-1}$.

## 2.23 *in silico* Analyses

### 2.23.1 Sequence Alignment

Multiple sequence alignments were performed using Multiple Sequence Comparison by Log- Expectation (MUSCLE; Edgar, 2004) through the MEGA 6.0 software (Tamura et al 2013).

### 2.23.2 Phylogenetic Trees

Unrooted phylogenetic trees were estimated from multiple sequence alignments using the optimum model as established by MEGA 6.0 software. The length of each branch represents the distance between sequence pairs and numbers at the base of each branch show bootstrap values.

### 2.23.3 Database Searches

Searches for various sequences were performed using the NCBI online database and the Basic Local Alignment Search Tool (BLAST) BLASTp (protein) BLASTn (genes), BLASTx (nucleotide into protein).

**Table 2.7 Primers used in this study**

| Primer name | Primer sequence | Use |
|---|---|---|
| **Wpet1** | AACTGCAGCATATGACCGCCATGCTCGACAGTTTTC | Amplification of DddW to create pBIO1948 |
| **Wpet2** | ATGGATCCTCAGGCGCTGGCGGTGAACCG | Amplification of DddW to create pBIO1948 |
| **RPDddWH81AFOR** | CCAGTTGCGCCCC**GCC**CGCCATACCCCGCCCG | Site directed mutagenesis of *Rugeria pomeroyi DSS-3* DddW |
| **RPDddWH81AREV** | CGGGCGGGGTATGGCG**GGC**GGGGCGCAACTGG | Site directed mutagenesis of *Rugeria pomeroyi DSS-3* DddW |
| **RPDddWH83AFOR** | GCGCCCCCACCGC**GC****C**ACCCCGCCCGAGTTCTATCTGGC | Site directed mutagenesis of *Rugeria pomeroyi DSS-3* DddW |
| **RPDddWH83AREV** | GCCCAGATAGAACTCGGGCGGGGT**GGC**GCGGTGGGGGCGC | Site directed mutagenesis of *Rugeria pomeroyi DSS-3* DddW |
| **RPDddWE87AFOR** | CGCCATACCCCGCCC**G****CG**TTCTATCTGGGC | Site directed mutagenesis of *Rugeria pomeroyi DSS-3* DddW |
| **RPDddWE87AREV** | GCCCAAGAGATAGAA**C****GC**GGGCGGGGTATGGCG | Site directed mutagenesis of *Rugeria pomeroyi DSS-3* DddW |
| **RPDddWH121AFOR** | CCGGCGATGCGGAA**G****CC**GGCACGGTTGCCGG | Site directed mutagenesis of *Rugeria pomeroyi DSS-3* DddW |

| | | |
|---|---|---|
| **RPDddWH121AREV** | CCGGCAACCGTGCC**GGC**TTCCGCATCGCCGG | Site directed mutagenesis of *Rugeria pomeroyi DSS-3* DddW |
| **RPDddQH128AFOR** | GCTTTACTATCCGTTT**GCC**CAGCACCCGG | Site directed mutagenesis of *Rugeria pomeroyi DSS-3* DddQ |
| **RPDddQH128AREV** | CCGGGTGCTG**GGC**AAACGGATAGTAAAGC | Site directed mutagenesis of *Rugeria pomeroyi DSS-3* DddQ |
| **RPDddQH130AFOR** | CTATCCGTTTCACCA**GCC**CCCGGCCGAAGAGAT | Site directed mutagenesis of *Rugeria pomeroyi DSS-3* DddQ |
| **RPDddQH130AREV** | GATCTCTTCGGCCGG**GGC**CTGGTGAAACGGATAG | Site directed mutagenesis of *Rugeria pomeroyi DSS-3* DddQ |
| **RPDddQE134AFOR** | GCACCCGGCCGAA**GCG**ATCTATTTCATCC | Site directed mutagenesis of *Rugeria pomeroyi DSS-3* DddQ |
| **RPDddQE134AREV** | GGATGAAATAGAT**CGC**TTCGGCCGGGTGC | Site directed mutagenesis of *Rugeria pomeroyi DSS-3* DddQ |
| **RPDddQH169AFOR** | CCCGTCGGGGCACCCG**GCT**GCGACGCGC | Site directed mutagenesis of *Rugeria pomeroyi DSS-3* DddQ |
| **RPDddQH169AREV** | GCGCGTCGC**AGC**CGGGTGCCCCGACGGG | Site directed mutagenesis of *Rugeria pomeroyi DSS-3* DddQ |
| **H152AFOR** | GCACCTATCCGCAGGCCAGCCACAAGGACATCG | Site directed mutagenesis of *Rhodobacter sphaeroides* strain 2.4.DddL |

| | | |
|---|---|---|
| **H152AREV** | CGATGTCCTTGTGGCTGGCCTGCGGATAGGTCG | Site directed mutagenesis of *Rhodobacter sphaeroides* strain 2.4.DddL |
| **H154AFOR** | CGACCTATCCGCAGCACAGCGCCAAGGACATCG | Site directed mutagenesis of *Rhodobacter sphaeroides* strain 2.4.DddL |
| **H154AREV** | CGATGTCCTTGGCGCTGTGCTGCGGATAGGTCG | Site directed mutagenesis of *Rhodobacter sphaeroides* strain 2.4.DddL |
| **E159AFOR** | CCACAAGGACATCGAGGCGAGTTACATCTCGG | Site directed mutagenesis of *Rhodobacter sphaeroides* strain 2.4.DddL |
| **E159AREV** | CCGAGATGTAACTCGCCTCGATGTCCTTGTGG | Site directed mutagenesis of *Rhodobacter sphaeroides* strain 2.4.DddL |
| **H190AFOR** | CCGGCCGGGCCTCGAAGCCCGGATCACGACGGG | Site directed mutagenesis of *Rhodobacter sphaeroides* strain 2.4.DddL |
| **H190AREV** | CCCGTCGTGATCCGGGCTTCGAGGCCCGGCCGG | Site directed mutagenesis of *Rhodobacter sphaeroides* strain 2.4.DddL |

## 2.24 Enzymatic Properties of DddL, DddQ and DddW *in vitro*

### 2.24.1 Establishing pH optima

In order establish the pH optimum of DddL, DddQ and DddW activity. Buffer solutions were used to produce pH ranges 2.0-9.0 HCl or NaOH was added to reach the required pH before assaying for DMS production.

### 2.24.2 Chelation of metal co-factors

To examine the effects of metal availability on DddL, DddQ and DddW function, the metal chelator EDTA was added to the lyases in 20mM TRIS pH8.0 and incubated for 30 minutes at 30°C before the addition of DMSP and assaying for DMS production.

### 2.24.3 Establishing Km and Vmax of DddL, DddQ and DddW

$K_m$ and $V_{max}$ studies were performed with 0.3μM DddL, DddQ and DddW in 20mM TRIS buffer pH 8.0 with DMSP concentrations ranging from 50mM to 1000 mM and incubated at 30 $^{o}$C and the DMS headspace measurements were taken at regular time intervals.

## 2.25 Establishing identity of metal cofactors in as purified protein.

### 2.25.1 Inductively Couple Plasma, Optical Emission Spectroscopy (ICP-OES)

ICP-OES was carried out on samples of DddL and DddQ (concentrations) (DddW in its own chapter) in 2.5% (v/v) nitric acid. A Varian Vista Pro CCD simultaneous ICP-OES with axial torch, concentric seaspray nebuliser (glass expansion) and 50ml cyclonic spray chamber was used to analyse triplicate samples. The power was 1.2KW and the analysed wavelengths were B 249.772, Ca 315.887, Ca 317.933, Co 228.615, Cu 326.754, Fe 238.204, Mn 257.610, Mo 202.032, Ni 231.604, W 207.912, W 220.449, Zn 213.857

## 2.26 Analytical Ultracentrifugation

Analytical ultracentrifugation was performed at 12,000rpm 20$^{o}$C in a Beckman Optima XL-1 analytical ultracentrifuge with absorbance optics and an An50Ti rotor. Scans were recorded every 4 hours to determine when protein samples had reached equilibrium DddL and DddQ [0.3μM] were in 20mM TRIS, 100mM MaCl pH 8. Data was analysed using Ultrascan (Demeler 2005) and fitted to a one component model.

## 2.27 Methods employed for *Emiliania huxleyi* DMSP lyase studies:

## 2.28 Media and growth conditions

### 2.28.1 Growth in liquid media

A preliminary growth experiment in liquid media (Aquil) was carried out to investigate the growth under established culture conditions to enable an estimate of various growth phases (exponential growth, stationary growth and senescence). Consequently the cell count from a starter culture was determined and the volume of inoculum was calculated such that an initial cell count of 5000 cells $mL^{-1}$ was inoculated into media. Five cultures were prepared accordingly and cell counts were measured on a daily base. According to the growth behaviour, sampling strategies were developed in order to harvest cells during the exponential growth phase.

#### 2.28.1.1 Preparation of Aquil media:

##### 2.28.1.1.1 Preparation of synthetic ocean water (SOW)

All salts were dissolved in water from a Milli-Q RO and UF water system (Millipore Corporation) or equivalent water with low metal impurities.

Preparation of Concentrated Stock Solutions (CSS) of minor constituents (NaF, $SrCl_2 \cdot 6H_2O$)
100 mL of each stock was prepared and stocks stored at 4-8 °C.

CSS1 NaF dissolve 0.6 g in 100 mL (6 $g \cdot L^{-1}$)
CSS2 $SrCl_2 \cdot 6H_2O$ dissolve 3.4 g in 100 mL (34 $g \cdot L^{-1}$)

To prepare the synthetic ocean water, 600 mL of high-quality $dH_2O$ was used and each of the anhydrous salts were individually dissolved into it:

| | |
|---|---|
| NaCl | 26.54 g |
| $Na_2SO_4$ | 6.09 g |
| KCl | 0.70 g |
| $NaHCO_3$ | 0.20 g |
| KBr | 0.10 g |
| $H_3BO_3$ | 0.030 g |
| CSS1 (NaF (6 $g \cdot L^{-1}$)) | 0.5 mL |

Next 300 mL of high-quality dH2O was used and each of the hydrous salts listed below were individually dissolved:

| | |
|---|---|
| $MgCl_2 \bullet 6H_2O$ | 11.09g |
| $CaCl_2 \bullet 2H_2O$ | 1.54g |
| $SrCl_2 \bullet 6H_2O$ | 0.5 mL |

The two solutions were dissolved and and made up to 1L.

Before adding nutrients, vitamins and trace metals the seawater solution was passed through an ion exchange column to remove trace metal impurities.

### 2.28.1.1.2 Preparation of Concentrated Stock Solutions (CSS) of major nutrients

The major nutrients were prepared as individual stock solutions at 1000X the final concentration.

Concentrated Stock Solutions (CSS):

100 mL of each stock solution was prepared and stored at 4°C,

| | | |
|---|---|---|
| CSS3 | NaH2PO4·H2O | 0.138 g in 100 mL (1.38 g L-1) |
| CSS4 | NaNO3 | 8.5 g in 100 mL (85 g L-1) |
| CSS5 | Na2SiO3·9H2O | 2.840 g in 100 mL (28.40 g L-1) |

Chelex treatment of stock solutions:

Each solution is passed individually through an ion exchange column.

Preparation of initial Concentrated Stock Solutions (CSS) for Cu and Se:
100 mL of each stock solution was prepared and stored at 4°C:

| | | |
|---|---|---|
| CSS6 | $CuSO_4 \cdot 5H_2O$ | 0.49 g in 100 mL (6.9 g L-1) |
| CSS7 | $Na_2SeO_3$ | 0.19 g in 100 mL (1.9 g L-1) |

Prepare Trace Metal Stock Solution TMSS: [Cu, Mn, Zn, Co, Se, and Mo]
Into 900 mL of highest-quality deionized water the following was dissolved:

| | |
|---|---|
| EDTA (0.1 M) | 29.225 g |
| $FeCl_3 \cdot 6H_2O$ | 0.2642 g |
| $ZnSO_4 \cdot 7H_2O$ | 0.230 g |
| $MnCl_2 \cdot 4H_2O$ | 0.0240 g |
| $CoCl_2 \cdot 6H_2O$ | 0.0120 g |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.0242 g |
| $CuSO_4 \cdot 5H_2O$ | 1 mL of CSS6 |
| $Na_2SeO_3$ | 1 mL of CSS7 |

The volume was made up to 1L.

#### 2.28.1.1.3 Preparation of Aquil Vitamin Stock solutions

| | | |
|---|---|---|
| VSS1 | Vitamin B12 (Cyanocobalamin) | 0.011 g in 10 mL |
| VSS2 | Biotin (vitamin H) | 0.010 g in 100 mL |

Preparation of Final Vitamin Stock Solution (FVSS):
In 90 mL of highest quality deionized water 20 mg Thiamine·HCl. Was dissolved and the following added before being made up to 100mL:

1mL VSS1 - vitamin B12 stock
1mL VSS2 - Biotin stock.

The FVSS was then filter-sterilized through a 0.2 µm acetate filter and stored in aliquots at -20°C.

#### 2.28.1.1.4 Final preparation of Aquil media

Into 1000mL SOW (chelexed) the following was added:

1mL CSS3 - $NaH_2PO_4 \cdot H_2O$ (chelexed) (P-stock)

1mL CSS4 - $NaNO_3$ (chelexed) (N-Stock)

1mL CSS5 - $Na_2SiO_3 \cdot 9H_2O$ (chelexed) (Si-stock)

1mL TMSS - Trace Metal Stock Solution

1mL FVSS - Final Vitamin Stock Sol

### 2.29 Culturing of Emiliania huxleyi strain RCC1217

The axenic 1N Emiliania huxleyi strain RCC1217 was obtained from the Roscoff Culture Collection. Cultures were inoculated into 15L volumes in 20L polycarbonate bottles of filtered synthetic ocean water (SOW) and enriched with Aquil nutrient supplements. The cultures where then incubated at 20oC under a 24 hour light cycle of 135 µmol photons m-2s-1. Cultures were harvested after reaching cell densities of at least $1x10^6$ cells/mL. Stock cultures were also maintained in this media in 20mL volumes under the same light/temperature regime. Stocks were transferred every 7-10 days

### 2.30 Growth rate determination

A preliminary growth experiment was conducted to elucidate the growth rate and phases of RCC1217 using culture conditions described earlier. Initial cells counts of 25,000 cells/mL were inoculated. The growth experiment was conducted using three technical replicates. Exponential growth lasted 5 days and the calculated growth rate was 0.9951.

## 2.31 Identification of $^{14}C$ Acrylate via High Performance Liquid Chromatography (HPLC)

To establish and confirm the production of acrylate by cultures with known enzyme activity were exposed to [1-$^{14}C$] DMSP (1.9 kBq) (American radiolabelled chemicals) to achieve a final concentration of 1mM. Following overnight incubation at 28$^{o}$C cells were lysed by the addition of 5% v/v perchloric acid and incubated on ice for 15 minutes in order to precipitate cell debris. Precipitated material was centrifuged for 10 minutes at 12,000rpm and 100ul of the supernatant was added to HPLC vials. Acrylate was resolved on an ICE-AS6 column (250mmx9mm id; Dionex) eluted isocratically with 0.4mM HCl and a flow rate of 1ml min$^{-1}$ and detected using tandem suppressed ion conductivity. UV detection at 210nm and an online scintillation counter using an ED50 conductivity detector (Dionex), UV975 detector (Jasco) and radiomatic A515 flow detector (Canerra Packard) as described in Todd et al 2010b

## 2.32 Testing of *E. huxleyi* culture axenicity by DAPI staining

In order to determine the axenicity of *Emiliania huxleyi* culture a nucleic acid DAPI staining method was used in which 4',6-diamidino-2-phenylindole (DAPI) was introduced to culture aliquots and followed by epifluorescence microscopy .

The stain DAPI binds to DNA to form a fluorescent complex this allows for detection of possible bacterial contamination of microalgal cultures. The presence of bacteria was checked by examining the filters under UV light using a fluorescence microscope. Bacteria appeared as small bright dots in the background of large algal cells and cultures of *E. huxleyi* RCC1217 were shown not to have any visible bacterial DAPI staining, indicating that they were indeed axenic cultures

## 2.33 Isolation of RNA from *Emiliania huxleyi*

Cells of *Emiliania huxleyi* were grown to exponential phase and harvested via vacuum filtration onto filters and either snap frozen using liquid nitrogen and stored at -80$^{o}$C or used immediately. Filters were placed into 15ml falcon tubes and 1.5ml of preheated (60$^{o}$C) trizol added to the filter and incubated with shaking at

60$^{o}$C for 30 minutes. Glass beads were added to the trizol-filter mix and then beaten for 30 seconds using nitrocellulose filters (Whatman). The mix was transferred into a fresh flacon tube and extraction performed by addition of 1.5ml chloroform to the sample mix and vortexing for 15 seconds before incubating at room temperature for 15 minutes. The sample was centrifuged at 10,000rpm 4$^{o}$C for 30 minutes. Following centrifugation the upper phase layer was transferred to a sterile eppendorf tube and 1 volume (~0.5ml) of ice cold isopropanol added. The mix was incubated at -20oC for 30 minutes and then centrifuged at 10,000 for 30 minutes. The supernatant discarded and the pellet washed in 1ml of ice cold 75% ethanol and centrifuged at 4$^{o}$C 10,000 for 10 minutes. The supernatant was removed and the pellet air-dried for 15 minutes before being re-suspended in 100ul of RNase free water.

# 3 Expression, Purification and Biochemical analysis of the DMSP lyases DddL and DddQ

## 3.1 Aims of this chapter:

The aims of this chapter were to express, purify and characterize the enzymology and biochemistry of the DMSP cupin-containing lyases DddL and DddQ.

The DMSP lyases DddL and DddQ are both cupin-containing proteins. Cupins are usually transition metal-binding proteins and contain two conserved motifs (section 1.8.4.1). The functions of cupin-containing proteins are known to be very diverse and seen in all domains of life.

DddL and DddQ are both considered to be 'true' DMSP lyases in so much that they release DMS, acrylate and a proton, unlike DddD, which while still considered a DMSP lyase, releases DMS and 3–HP (section 1.8.3). The two lyases are also predicted to be metalloenzymes because cupin-containing proteins very frequently require a metal cofactor in order to function.

In order to characterise the DddL and DddQ polypeptides and confirm that they act as DMSP lyases, they needed to be cloned and expressed in *E. coli* and purified to a suitable level of homogeneity. A purification protocol follows for each lyase.

## 3.2 DddL: Preliminary identification and cloning of the *dddL* lyase by Curson et al. (2008)

As previously discussed in section 1.8.4, the first true DMSP lyase to be genetically characterised was isolated from a salt marsh in Georgia, USA, and identified in a member of the Rhodobacteracae *Sulfitobacter* sp. EE-36 (Gonzalez et al., 1999). To identify the *Sulfitobacter* DMSP lyase gene, a library of genomic DNA from *Sulfitobacter* sp. EE-36 was prepared in the wide host range cosmid pLAFR3. The Sulfitobacter library was then mobilized *en masse* into *Rhizobium leguminosarium*. One transconjugant was found which emitted DMS when grown in the presence of DMSP. This cosmid was isolated and transformed into *E.coli* 803 and then retransformed into *R. leguminosarium* and the $Ddd^+$ phenotype confirmed. The DddL gene of *Rhodobacter sphaeroides* 2.4.1 was subsequently cloned into pET21a, a T7 expression plasmid forming pBIO1652. This plasmid should enable the expression of native DddL in *E. coli*. When this vector containing DddL was transformed into *E. coli* BL21, high levels of DMSP-

dependent DMS production were observed when grown in the presence of the IPTG inducer.

### 3.2.1 Overexpression of DddL in *E. coli*

To obtain high concentrations of the DddL polypeptide, a starter culture of the *E. coli* BL21 strain, freshly transformed with pBIO1652, was inoculated into LB liquid medium supplemented with ampicillin (Amp) and grown overnight at 37 ˚C. This was subsequently inoculated 1:100 to 1 litre of LB Amp and incubated for two hours at 37 ˚C until the cells reached an optical density at 600 nm ($OD_{600}$) of 0.4-0.6. The expression of DddL was induced by the addition of 0.2 mM IPTG and the culture was incubated overnight (~16 hours) at 25 ˚C. The cells were harvested via centrifugation, resuspended in Tris buffer (20 mM, pH 8) and lysed using the French Press method. The cell extract was run on a 12.5% SDS PAGE 1D gel and a 26 kDa band, corresponding to the predicted molecular weight of DddL, was seen to be notably more concentrated than any others (see Figure 3.1).

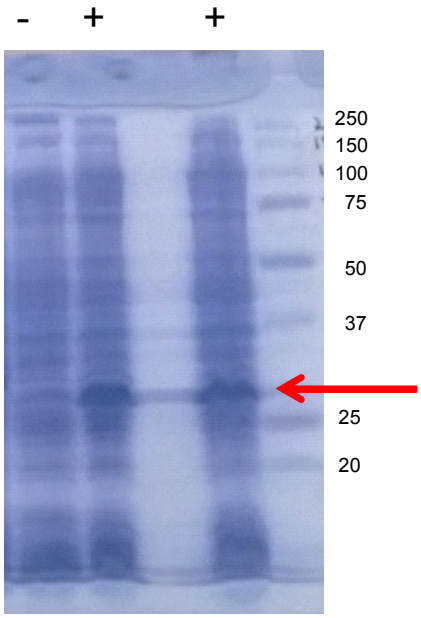



**Figure 3.1 DddL induction of cultures**. Cell extract of DddL non induced (-) and induced (+). The red arrow highlights concentrated DddL following induction via IPTG addition.

### 3.2.2 Purification of DddL from *E. coli*

Proteins are purified from cell extracts using chromatography techniques and can be separated according to differences in the properties individual proteins possess.

The simplest way to purify a protein is by using an affinity column such as the widely used poly His-tagging system. However, we decided not to employ such a technique because the cupin-containing enzymes are predicted to contain divalent metal cofactors. His-epitopes bind to metal matrices and so it is possible that that they could interfere with the function of the likely metal-binding DMSP lyases.

Generally, most protein purification protocols will employ two or more techniques to isolate the target protein and it is generally considered that there are three main steps to successful protein purification. Firstly, cells are lysed using cell disruption techniques such as sonication or French press. The first extraction phase of protein purification requires the removal of unbroken cells from the cell lysate using low speed centrifugation. The second intermediate step requires techniques capable of swift and good resolution of target proteins from contaminating proteins (Ion Exchange chromatography is commonly used for this step). The third and final polishing step requires a system which is capable of high resolution protein separation and a size exclusion column is frequently used for this. Special steps are required if trying to isolate membrane associated proteins. However, as neither DddL nor DddQ are membrane-associated, such techniques do not require mentioning at this point (see chapter 4).

### 3.2.3 DddL is a cytosolic protein

Upon analysis of BL21::1652 cell extracts expressing DddL, although some DddL was found within inclusion bodies, it was judged that the majority of the DddL protein and Ddd activity was found in the soluble extract, and it was determined that sufficient DddL was present in soluble form for purification. From bioinformatic analysis using the subcellular localisation programs PSORTb, TMpred and SignalP, DddL was not predicted to be a membrane-associated protein and was thus treated as a cytosolic protein.

### 3.2.4 Purification of DddL protein

During initial experiments, it was found that the DddL protein precipitated between 40% and 50% ammonium sulphate concentrations. Therefore, for the large scale purification of DddL, the cell lysate was firstly partially precipitated using the ammonium sulphate precipitation method, with the cell lysate saturated to 50%. Ammonium sulphate precipitation was used as an initial purification step in many of the previous studies that purified bacterial DMSP lyases (de Souza and Yoch, 1995a; Ansede *et al*., 1999; Kirkwood *et al*., 2010). By increasing the ammonium sulphate concentration gradually, different proteins will precipitate at different concentrations. By then centrifuging the precipitate, this enriches the purity of the protein sample and precipitated proteins are normally resolubilised if required upon removal of the salt using filtration or dialysis techniques. Following ammonium sulphate precipitation and dialysis to remove salts, the resulting DddL lysate was centrifuged and the supernatant further purified by to two column chromatography steps.

At each stage of purification an absorbance trace was used to determine which of the collected fractions contained protein. These were verified using SDS-PAGE for the presence of the expected 26 kDa band. DMS assays were also conducted to confirm lyase activity was still present.

The supernatant was first run through a phenyl sepharose high performance column. The separation media (stationary phase) used in this type of purification uses Hydrophobic Interaction Chromatography (HIC) and employs the same principles as ammonium sulphate precipitation. HIC takes advantage of the hydrophobic properties of proteins in polar environments, and is favoured over the older technique of reverse phase chromatography (RPC) because it operates using milder binding and elution conditions, which helps to retain the biological activity of target proteins. In HIC, the polarity of the chromatographic system is increased by reducing the ligand density on the stationary phase and via the addition of salt to the mobile phase. Most proteins possess hydrophobic areas on their surface and to dissolve these areas is not energetically favourable to the system, resulting in the formation of hydrophobic cavities in the aqueous mobile phase. The hydrophobic effect is enhanced by addition of salts according to the

Hoffmeister series (a classification of ions in order of their ability to precipitate proteins). The addition of salt drives the adsorption of hydrophobic areas on a protein to the hydrophobic areas on the stationary phase. This produces a thermodynamically favourable system in that it decreases the number and volume of hydrophobic cavities. By reducing the salt concentration, hydrophobic interactions are reduced, resulting in release of target proteins from the stationary phase and elution from the column. Therefore the column was equilibrated with Tris buffer (20 mM, pH 8) containing 50% $(NH_4)_2SO_4$. DddL was then eluted from the stationary phase by applying a 50% - 0% $(NH_4)_2SO_4$ gradient (flow rate 1 ml $min^{-1}$).

The final stage of column chromatography and purification of DddL was performed using a gel filtration column. Gel filtration is the simplest and mildest form of the chromatography techniques, separating proteins according to differences in size as they pass through the gel filtration media. It is a technique appropriate for proteins as they have a tendency to be sensitive to changes in pH, cofactors and other environmental conditions. In contrast to other chromatographic techniques, proteins do not bind to the stationary phase so the makeup of the buffer does not have a direct impact on the resolution of peak separation.

The fractions which contained DddL from the phenyl sepharose column were pooled and concentrated using centrifugal concentrators to a volume of ~500 µl and loaded onto a Superdex 200 column equilibrated with Tris buffer (20 mM, pH 8) and run at a flow rate of 0.2 ml $min^{-1}$. 2 ml fractions were collected and analysed on SDS PAGE gels and assayed via gas chromatography for Ddd activity. As shown in Figure 3.2, the gel filtration step yielded a single visible band on with Coomassie Blue staining.

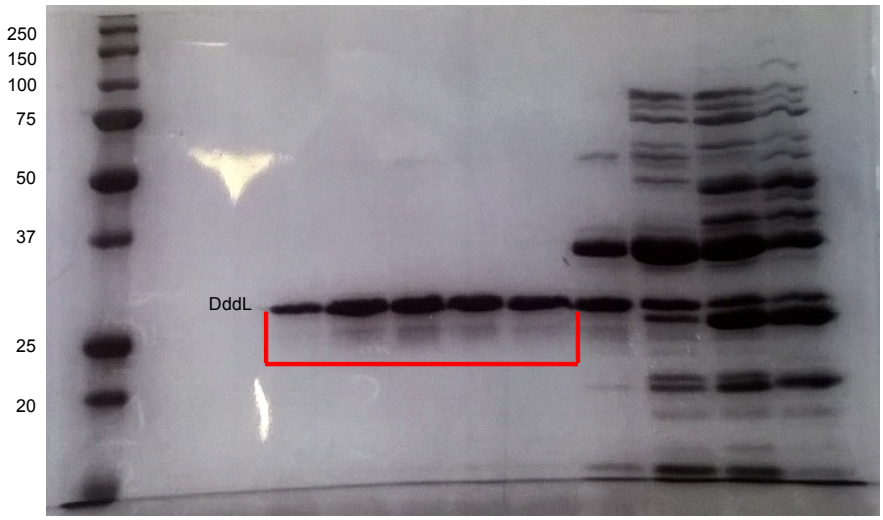



**Figure 3.2 DddL following elution from Superdex 200.** Polypeptides were separated via SDS-PAGE on a 12% acrylamide gel and stained with Coomassie blue. Lane 1 (left to right) contains Precision plus protein standard. Lanes 1-12 contain fractions of eluted protein from the gel filtration column and lanes 3-7 show where the vast majority of DddL eluted from the Superdex column. These fractions were pooled, concentrated and stored at -80$^{o}$C until required.

### 3.3 Identification and cloning of DddQ by Todd et al. (2011)

As discussed in section 1.8.6, following mutation of the DddP lyase in *Roseovarius nubinhibens* ISM, a second DMSP lyase was also found to be active in the bacterium and identified using similar techniques used for the identification of DddP (Todd et al., 2011). This enzyme DddQ is a cupin-containing enzyme like DddL. To obtain high concentrations of the DddQ polypeptide, a starter culture of the *E. coli* BL21 strain containing the pET21a-based *Ruegeria pomeroyi* DSS-3 *dddQ* clone pBIO1886 was inoculated to LB liquid medium supplemented with Amp and incubated overnight at 37 ˚C. This was then inoculated 1:100 to 1 litre of LB Amp and incubated at 37 ˚C for two hours until the cells reached an $OD_{600}$ of 0.4-0.6. DddQ expression was then induced using 0.2 mM IPTG and the culture was incubated overnight (~16 hours) at 25 ˚C. As with DddL, cells were harvested via centrifugation, resuspended in Tris buffer (20 mM, pH 8) and lysed using the French Press method. DddQ was determined, using the methods described

above for DddL, to be a cytosolic protein, and in order to remove membranes, cell lysate was centrifuged once again.

### 3.3.1 Purification of DddQ

The cell lysate was precipitated using $(NH4)_2SO_4$, the precipitate was removed by centrifugation and the supernatant subjected to three column chromatography steps, in a similar format to that described for DddL above. As with all lyase purifications, at each stage of purification, an absorbance trace was used to determine which of the collected fractions contained protein and subsequently verified using SDS-PAGE for the presence of the expected 22 kDa band. DMS assays were also performed confirming lyase activity was still present. It was found that an ammonium sulphate saturation of 50% caused precipitation of the DddQ protein. Therefore, for the large scale precipitation of DddQ, the soluble fraction containing the DddQ protein, was loaded onto a phenyl sepharose high-performance column equilibrated with Tris buffer (20 mM, pH 8) containing 50% (w/v) $(NH_4)_2SO_4$. Proteins were eluted using a 0-100 %B $(NH4)_2SO_4$ gradient over 205 mls (see chapter six).

The fractions containing DddQ were then applied to an ion exchange column containing a DEAE stationary phase. The reversible absorption of charged molecules such as proteins means it is possible to selectively capture and release protein samples from a column matrix. Ion exchange separates proteins based on the charge of the protein which is being isolated. For example, if the target protein has a high negative charge it can be run through a column which possesses a positive charge. The positive charge on the column will bind to the negatively charged protein and other proteins will pass through the column. This type of column is an anion exchange column. The protein is eluted from the column by increasing the salt concentration of the mobile phase. The mobile phase will start to outcompete the protein in binding to the column. This means the column has a higher affinity for the charge of the salts than the protein and so it will release the protein in favour of binding the salts instead. Proteins with weaker ionic interactions will elute at a lower salt concentration. Therefore, eluting with a salt gradient is required for satisfactory purification of target proteins.

The DEAE column was equilibrated with 20 mM Tris buffer, pH 8 (flow rate 5 ml $min^{-1}$). Proteins were eluted in the same buffer with a linear gradient of 0–1 M NaCl and fractions collected. The fractions containing DddQ were pooled and concentrated to ~0.5 ml using an Amicon Ultra 4 ml Centrifugal filter before being loaded onto a Superdex 200 gel filtration column (10/300GL; GE Healthcare), equilibrated with 20 mM Tris buffer, pH 8 and run at a flow rate of 0.4 ml $min^{-1}$, and 0.5 ml fractions were collected.

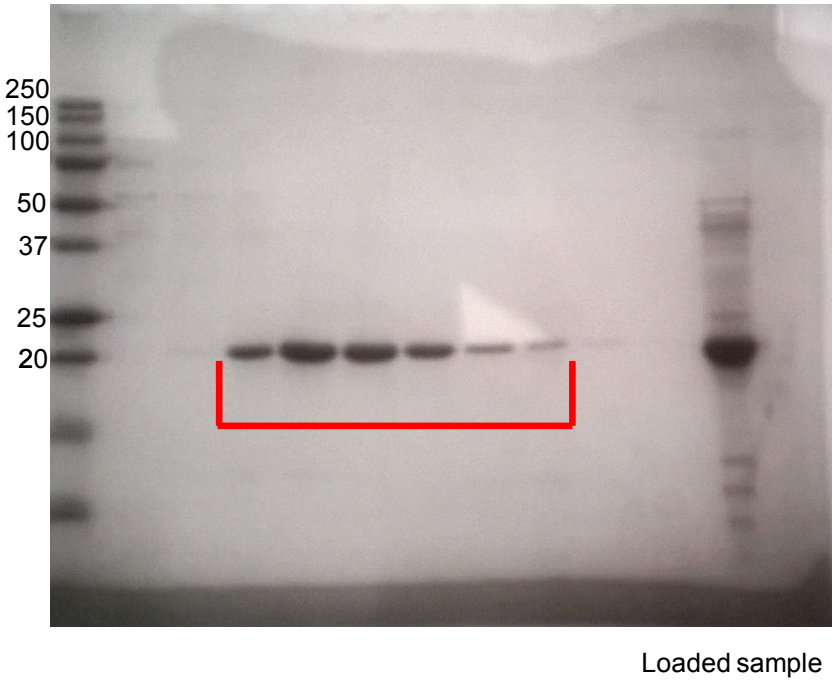



**Figure 3.3 DddQ following elution from Superdex 200. Polypeptides were separated via SDS-PAGE on a 12% acrylamide gel and stained with Coomassie blue.** Lane 1 (left to right) contains Precision plus protein standard. Lane 2-11 contain fractions of eluted protein from the gel filtration column and Lane 12 is a 2 µl sample of the DddQ fractions pooled from the DEAE column which were concentrated and loaded to the gel filtration column.

## 3.4 Association state of DddL

To determine the association states of the native enzymes, analytical ultracentrifugation (AUC) was used. However, at the time of analysis, the DddQ sample was deemed too dilute for accurate determination of its association state so no AUC was performed on this protein.

### 3.4.1 Analytical Ultracentrifugation

AUC has established itself as a superior method for characterising suspensions of molecules and is an essential tool for the quantitative analysis of macromolecule interactions. As AUC is dependent on the principle property of mass and the fundamental law of gravitation there are a wide range of applications to which it can be applied. It can be used to examine the organisation and behaviour of a vast array of molecules in a number of solvents over a broad range of solute concentrations and has a number of advantages. Firstly, in contrast to other frequently used methods, samples used in AUC are characterised whilst in biologically appropriate solutions and in their native state, and because the analysis is performed in free solution, no complications occur caused by interactions with matrices or surfaces. Secondly, also in contrast to other commonly used methods, AUC is a non-destructive process and samples can be recovered for further experimentation following analysis.

As was assumed from its predicted molecular weight, the purified DddL peptide was observed as a 26 kDa band in SDS PAGE. In order to determine the association state of native DddL, sedimentation equilibrium analytical ultracentrifugation was employed. The technique of sedimentation equilibrium analytical ultracentrifugation works by the centrifugation of a protein sample until the contrasting forces of diffusion and sedimentation are equal, with differing species of different masses reaching equilibrium at different times. The data is recorded by a succession of scans which are then applied to recognized models to establish the structural composition of the protein. A sample of DddL (0.3 μM) was centrifuged at 12,000 rpm and 20 ˚C in a Beckman Optima XL-1 analytical ultracentrifuge. Scans were taken every 4 hours so as to determine when equilibrium had been reached, at which point five more scans were recorded. The data was analysed (Ultrascan Demeler 2005) and predicted a molecular mass of

23 kDa (close to the actual size of 26 kDa) suggesting that DddL is a monomer in solution (Figure 3.4)

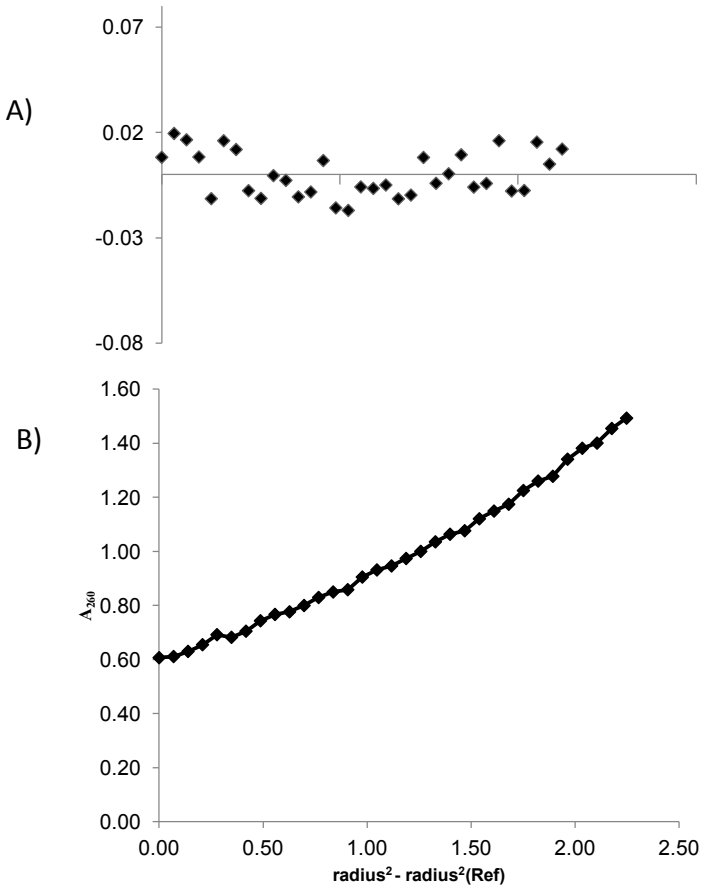



**Figure 3.4 Analytical Ultracentrifugation of DddL:** Absorbance at 260 nm against radius$^2$-radius$^2$following equilibration of purified DddL (0.3 μM). A) Residual difference between experimental data and fitted curve. B) A mean molecular weight of 23 kDa was recorded.

### 3.5 Enzyme kinetics of DddL and DddQ

To establish the $K_m$ and $V_{max}$ values of the DddL and DddQ enzymes, the initial rates of DMS production were measured at intervals using ~0.3 μM purified Ddd protein. Samples were incubated at 30 ˚C in 20 mM Tris (pH 8) with DMSP substrate concentrations between 50 mM and 1000 mM, before being analyzed for DMS production via GC. The data fitted to the Michaelis-Menten equation and a $K_m$ of DddL for DMSP of 69.9 ± 19 mM and a $V_{max}$ of 56.7 ± 4.6 nmol DMS min$^{-1}$

μg DddL protein$^{-1}$ were extrapolated (Figure 3.5). A $K_m$ of DddQ for DMSP of 342.2 ± 48 mM and a $V_{max}$ of 39.2 ± 2.2 nmol DMS min$^{-1}$ μg DddQ protein$^{-1}$ were extrapolated (Figure 3.6).

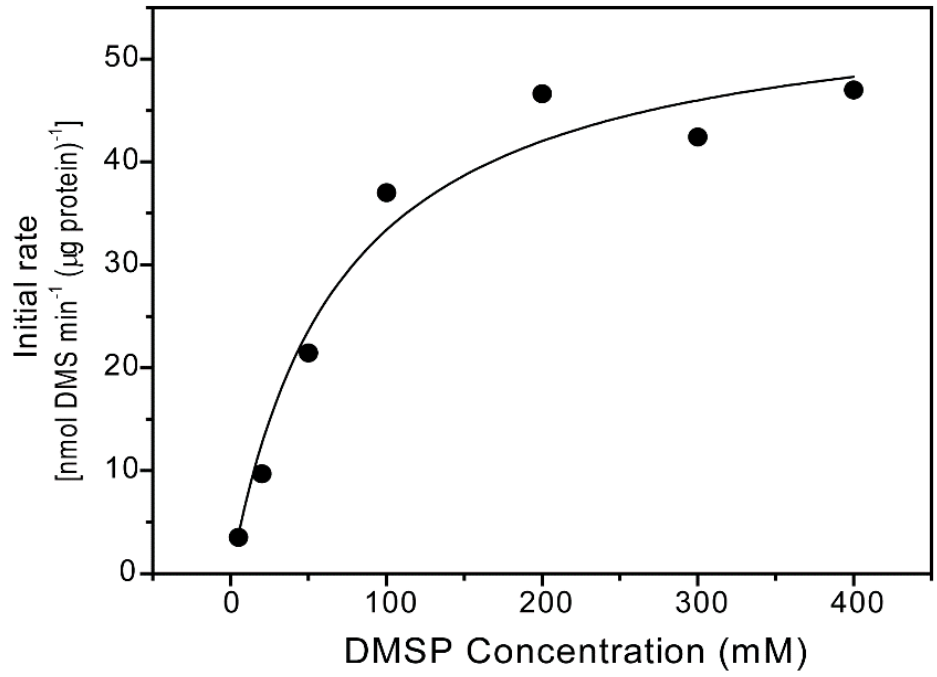



**Figure 3.5 Michaelis-Menten plot for the DMSP lyase activity of DddL.** Data was fitted to the Michaelis-Menten equation using Origin Software (version 8, Origin lab). $V_{max}$ was calculated as 56.7 ± 4.6 nmol DMS min$^{-1}$ μg DddL protein and $K_m$ of 69.9 ± 19 mM. Intial rate (nmol DMS min$^{-1}$ μg DddL protein$^{-1}$) plotted against DMSP concentration. DddL (0.3 μM) was in 20 mM Tris buffer (pH 8).

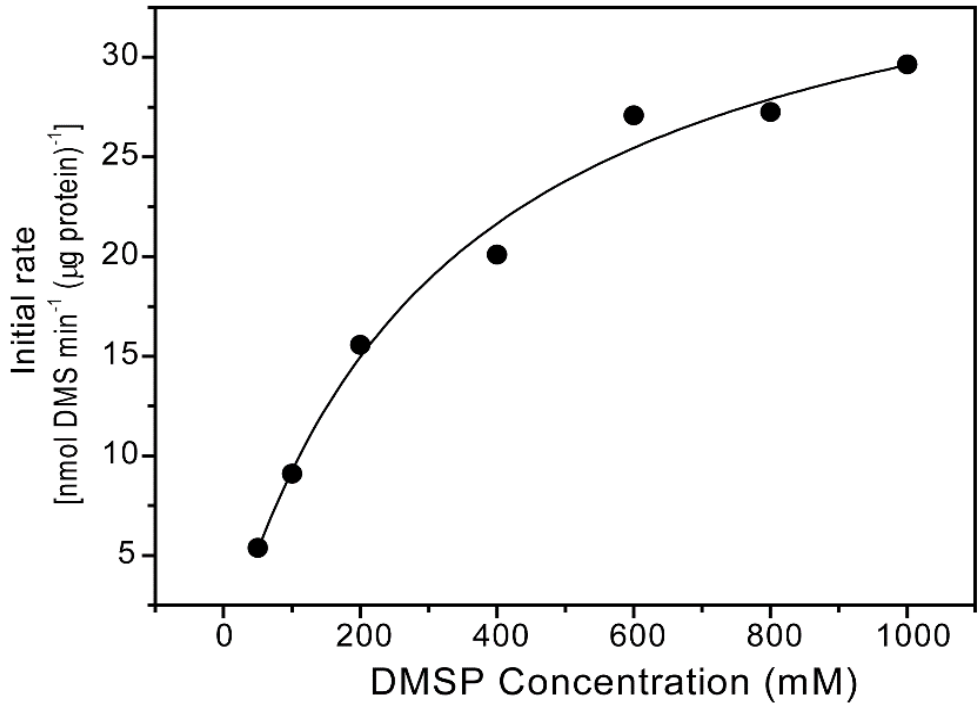



**Figure 3.6 Michaelis-Menten plot for the DMSP lyase activity of DddQ.** Data was fitted to the Michaelis-Menten equation using Origin Software (version 8, Origin lab). $V_{max}$ was calculated as 39.2 ± 2.2 nmol DMS $min^{-1}$ µg DddQ $protein^{-1}$ and $K_m$ of 342.2 ± 48 mM. Intial rate (nmol DMS $min^{-1}$ µg DddQ $protein^{-1}$) plotted against DMSP concentration. DddQ (0.3 µM) was in 20 mM Tris buffer (pH 8)

### 3.6 Metal binding studies

#### 3.6.1 EDTA experiments

As discussed previously, cupin-containing enzymes are known for their ability to bind divalent metals as cofactors that are essential for function (Dunwell et al., 2000, Khuri et al., 2001). As seen in Figure 3.7 DddL, DddQ and DddW (chapter four) all possess conserved residues required for metal binding. Experiments were performed on the DddL and DddQ cupin-containing lyases in order to establish their requirement for metals (see chapter 4 for DddW data). The metal-chelating agent ethylenediaminetetra-acetic acid (EDTA) is widely used for the scavenging of metal ions and is frequently used to deactivate metal-dependent enzymes as it possesses the ability to sequester metals ions. In order to determine if DddL and DddQ require metal cofactors, Ddd assays were performed in the presence of varying EDTA concentrations ranging from 100 µM – 10 mM

EDTA. On addition of EDTA, DddL and DddQ samples were incubated for 30 minutes at 30 ˚C before the addition of 5 mM DMSP and performing GC assays for DMS production (Chapter six). DddL and DddQ activity was completely abolished by the addition of EDTA, strongly suggesting that both DddL and DddQ require metal cofactors for their activity (see Figures 3.8 and 3.9).


**Figure 3.7 Sequence alignments of the predicted cupin motifs sequences of DddQ, DddL and DddW DMSP lyases**. The highly conserved amino acids in all cupins are indicated above the sequences (Dunwell *et al.,* 2004). DddW DMSP lyases (see chapter 4) from *Ruegeria pomeroyi* DSS-3 (R.pomW; *SPO0453*), *Pseudophaeobacter articus*, *Roseobacter* sp. MED193 (R.MEDW; *MED193_09710*;) and *Loktanella hongkongensis*, shown in green are aligned with the DddQ lyases from *R. pomeroyi* (R.pomQ; *SPO1596*) and *Roseovarius nubinhibens* ISM (R.nubQ1, *ISM_14090* and R.nubQ2 *ISM_14085*), shown in blue, and the DddL lyases from *Sulfitobacter* in purple. Residues in red highlight conserved cupin residues.

Having established that both DddL and DddQ require metal cofactors metal add-back experiments were used to determine which metals were required for function. This involved the addition of individual metal solutions to DddL and DddQ proteins [0.32 µg] that had been stripped of the metal cofactor by EDTA addition and dialysis. The metals, Cobalt, Copper, Iron, Manganese, Nickel, and Zinc were used at final concentrations of 20 mM.

These results suggest that both DddL and DddQ are somewhat promiscuous for their specific metal cofactor. For DddL, addition of Zn, Ni, Mn, Fe and Co all gave

significant Ddd activity compared to the apoprotein (Figure 3.10). However, Zn and Ni are clearly favoured since the addition of these metals gave increased DddL activity compared to the other metals when added at the same concentration. In contrast, for DddQ, activity was seen with the addition of Zn, Ni, Mn and Fe, but the highest activity was seen with the addition of Zn and Ni, suggesting these are the favoured metals for this protein (Figure 3.11). Of course this does not confirm the protein preference for the specific metal in the cellular environment, but does give an indication of which metals are capable of providing function to the proteins.

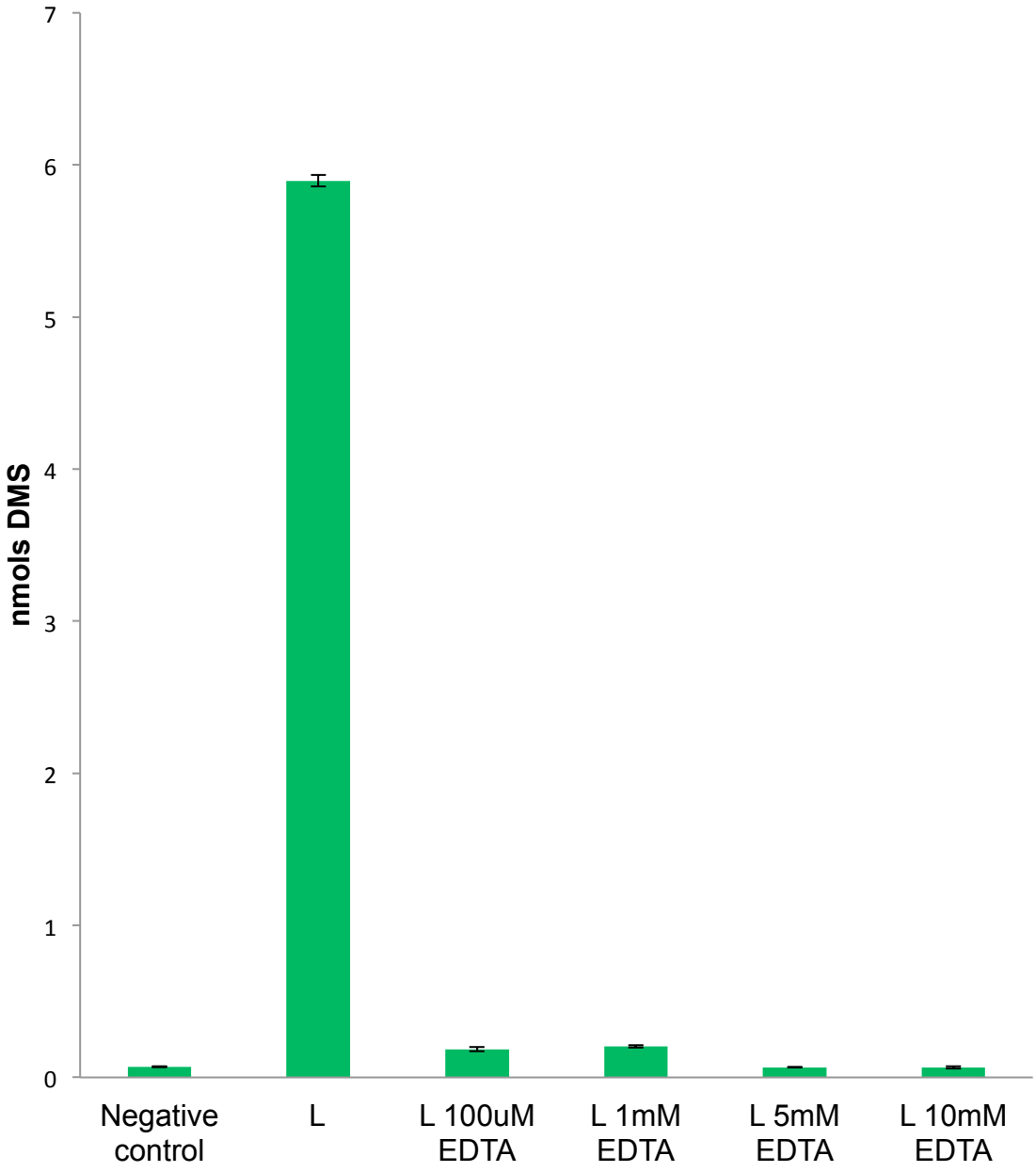



**Figure 3.8 Effect of EDTA on DddL activity.** Ddd assays were performed using varying EDTA concentrations ranging (100μM – 10 mM). On addition of EDTA, DddL and DddQ samples were incubated for 30 minutes at 30 ˚C before the addition of 5mM DMSP and performing assays using GC for DMS concentration determination. A negative control consisting of just buffer and EDTA was used in order to obtain background levels of DMS production.

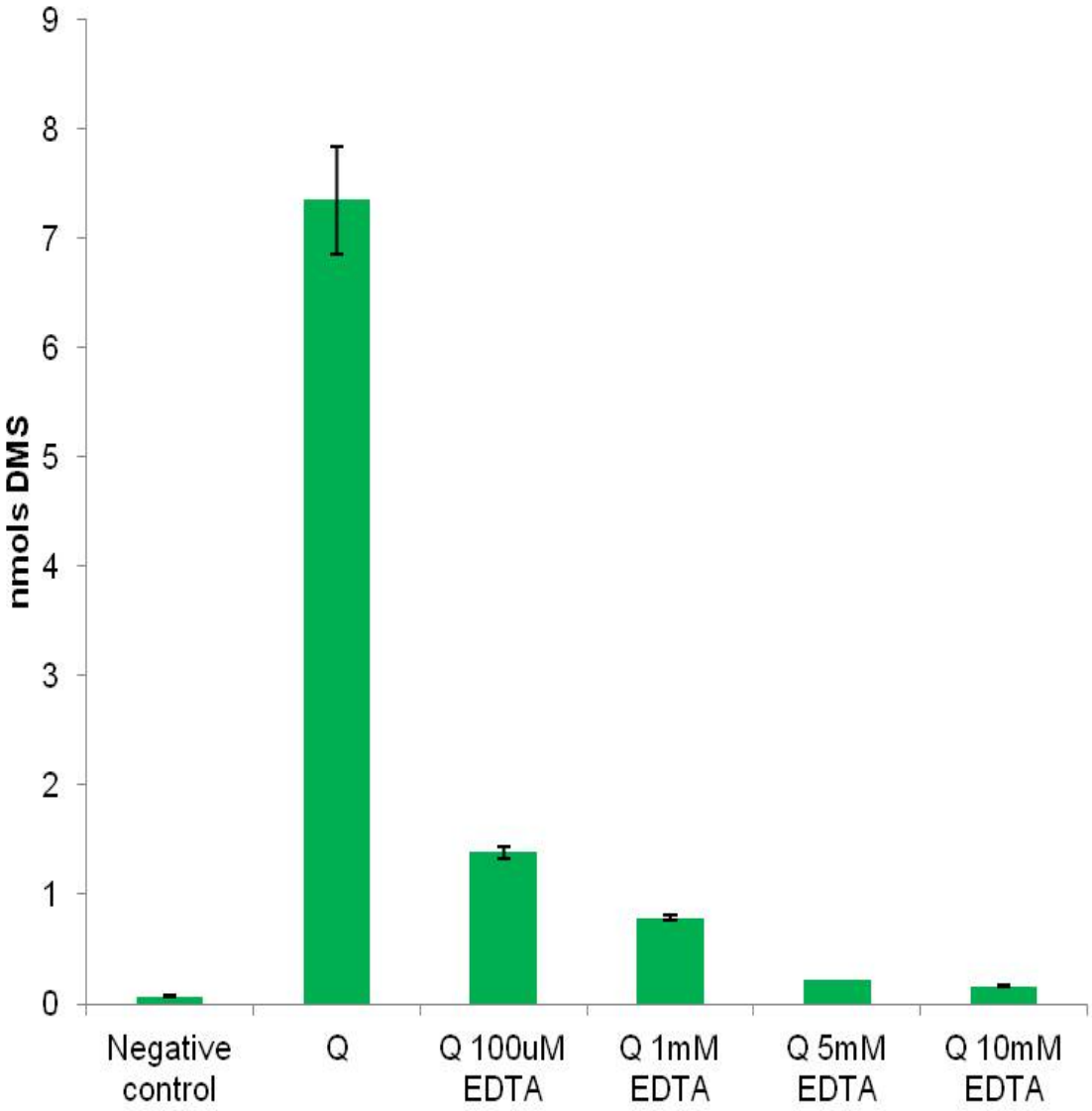



**Figure 3.9 Effect of EDTA on DddQ activity.** Ddd assays were performed using varying EDTA concentrations ranging (100μM – 10 mM). On addition of EDTA, DddL and DddQ samples were incubated for 30 minutes at 30 ˚C before the addition of 5mM DMSP and performing assays using GC for DMS concentration determination. A negative control consisting of just buffer and EDTA was used in order to obtain background levels of DMS production.

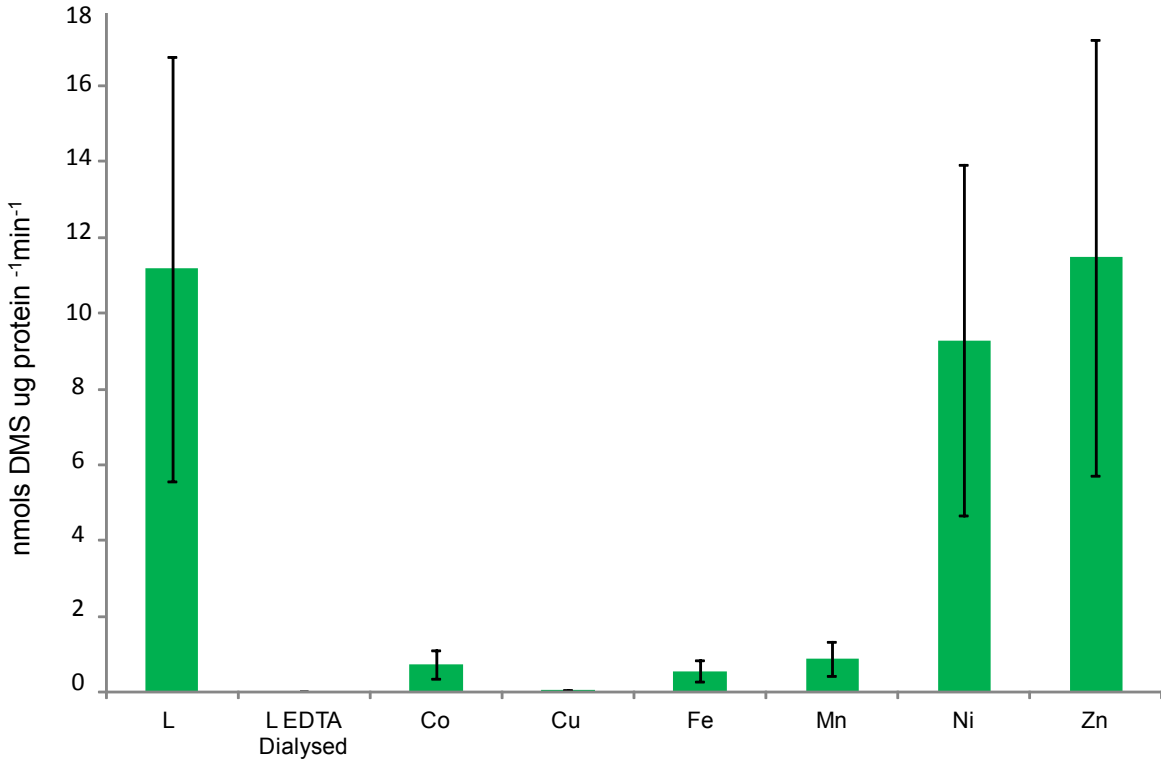



**Figure 3.10 Effect of metal additions to apo DddL activity.** Metal add-back experiments were used to determine which metals were required for DddL to function. This involved the addition of individual metal solutions (20mM) to DddL [0.32 µg] that had been stripped of the metal cofactor by EDTA addition and dialysis.

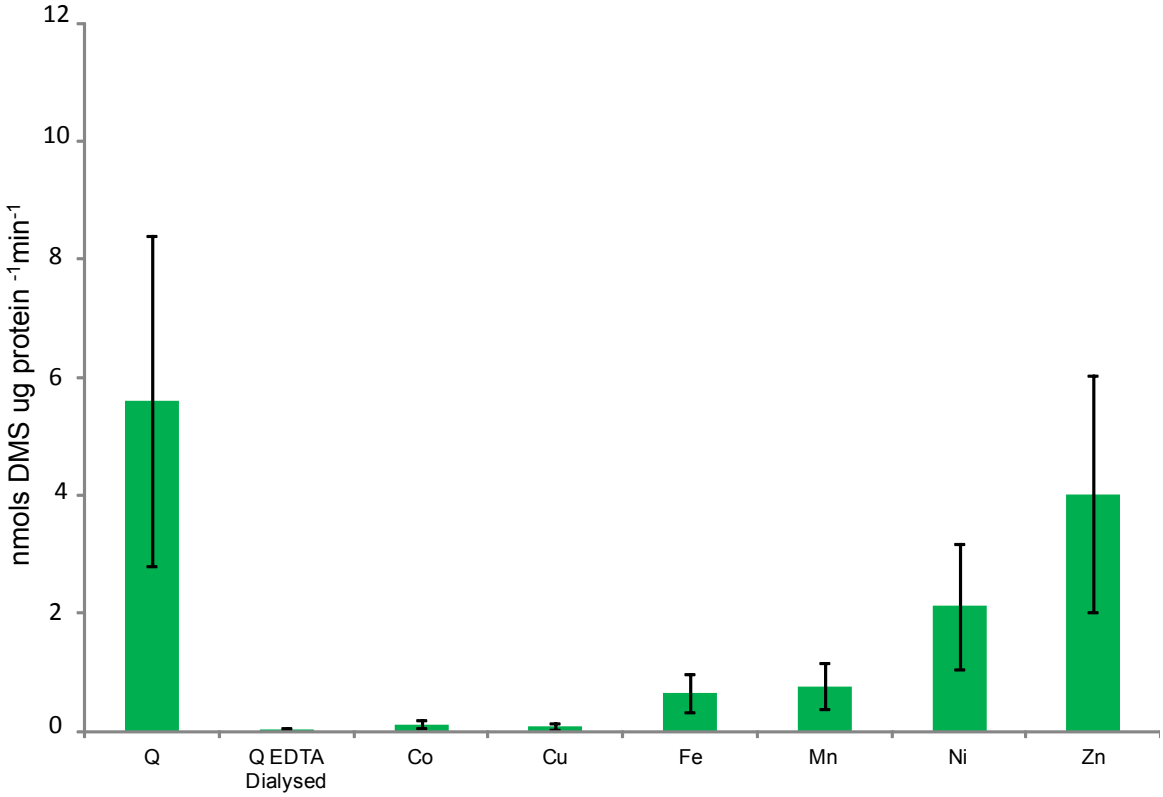



**Figure 3.11 Effect of metal additions to apo DddQ activity.** Metal add-back experiments were used to determine which metals were required for DddQ to function. This involved the addition of individual metal solutions (20mM) to DddL [0.32 µg] that had been stripped of the metal cofactor by EDTA addition and dialysis.

### 3.6.2 Metal cofactor determination by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES).

In an attempt to identify metal cofactors required for DMSP lyase activity more directly, ICP-OES was used to try to detect metal ions in samples of as purified DddL and DddQ. Active samples of DddL and DddQ, containing 2.5% v/v nitric acid, were analysed on a Varian Vista Pro CCP simultaneous ICP-OES. This revealed the presence of the metals Cu and Zn in DddL (Figure 3.12), and Fe and Zn in DddQ (Figure 3.13). Therefore, although Cu and Fe were present as cofactors in purified DddL and DddQ, respectively, Zn was found in both proteins, consistent with the fact that the highest DMSP lyase activities were seen with Zn in the addback experiments (see section 3.6.1). This suggests that Zn is likely the

metal cofactor *in vivo* and is supported by the fact that a recent publication on purified DddQ (Li et al., 2014) also showed DddQ to contain Zn.

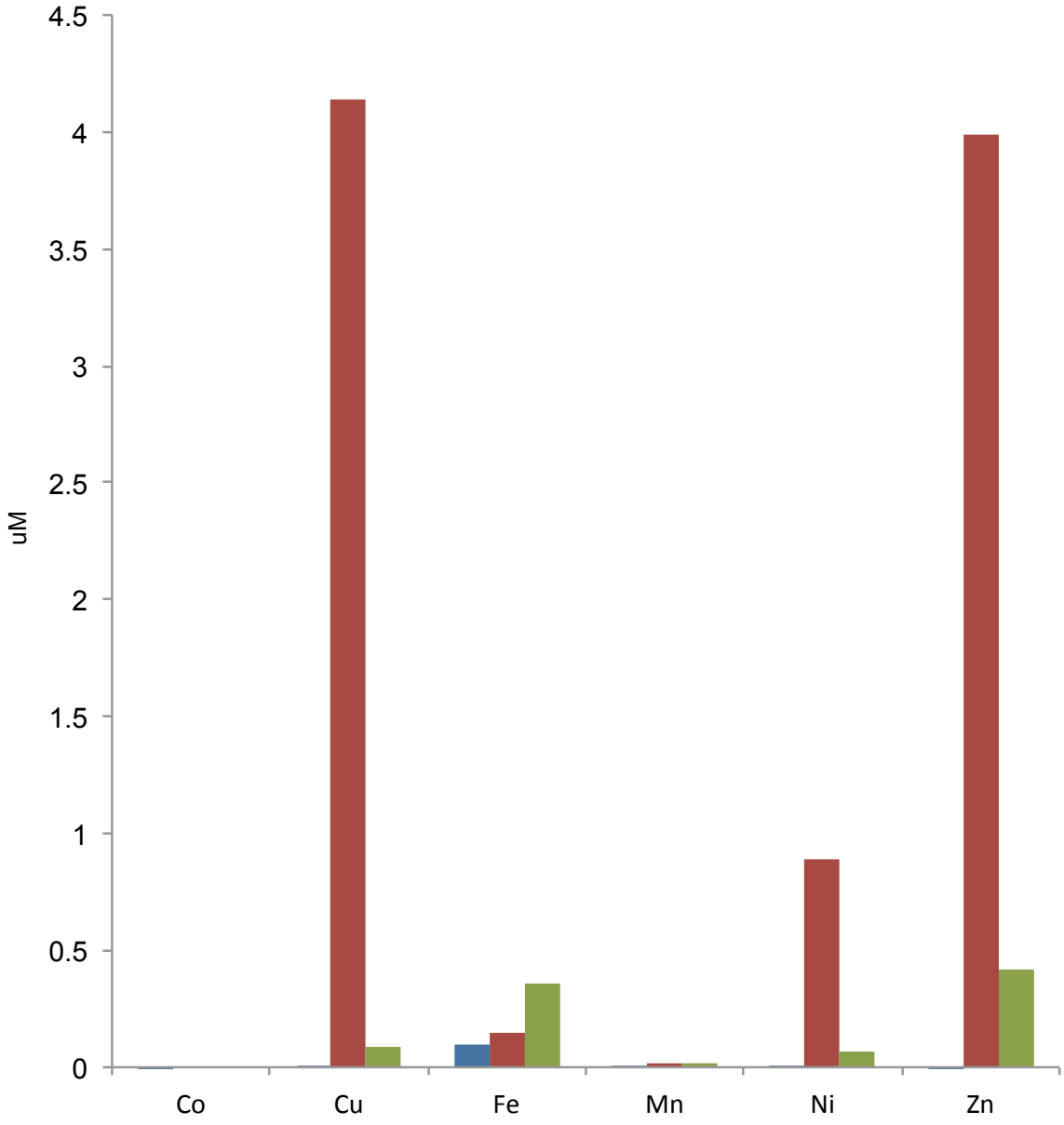



**Figure 3.12 ICP-OES results for DddL.** Blue bar represents metal levels detected in the blank samples used as a negative control. The red bar represents metal levels detected in DddL as isolated and the green represents the metal levels detected in 'apo' DddL (following dialysis).

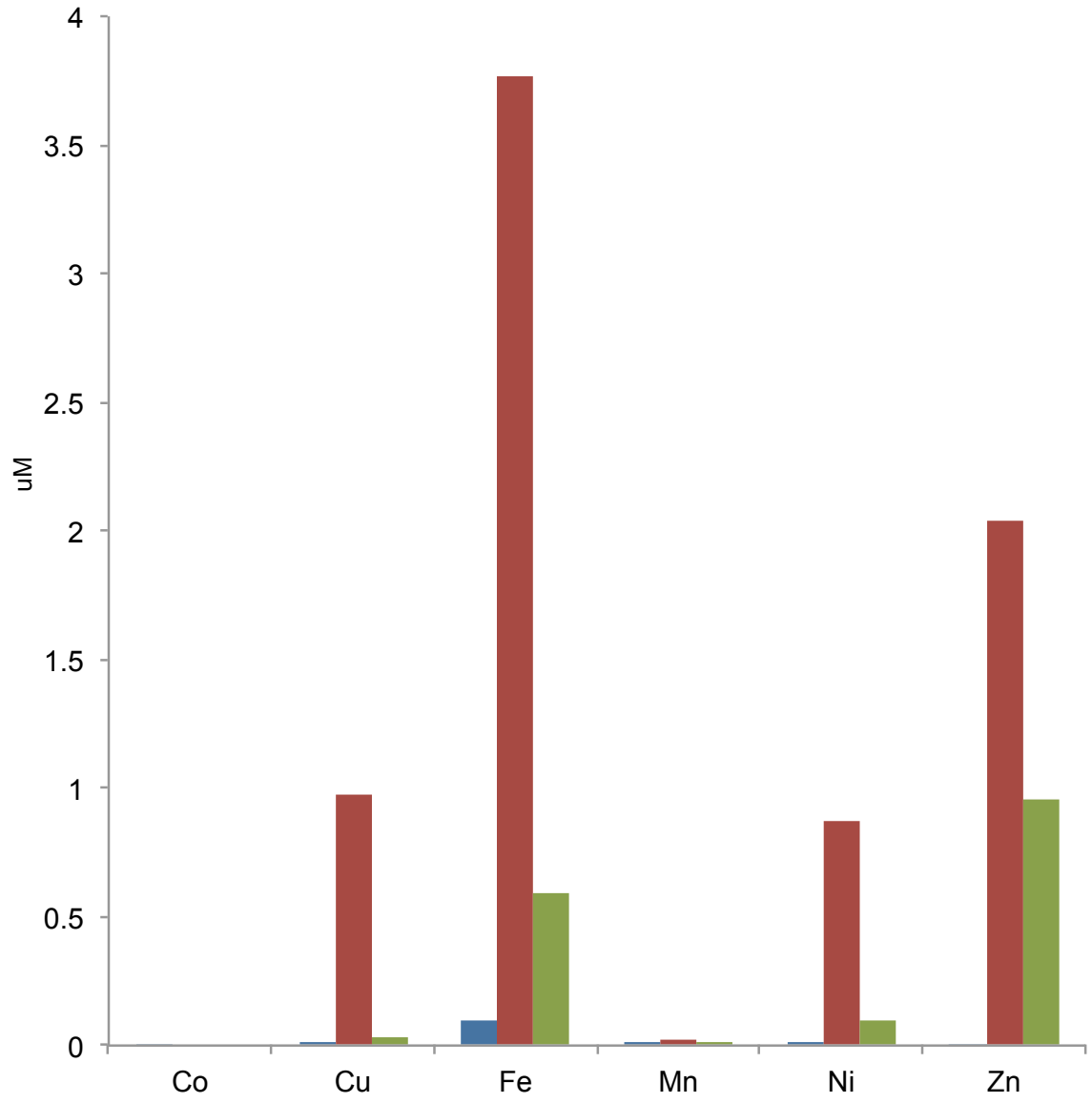



**Figure 3.13 ICP-OES results for DddQ.** Blue bar represents metal levels detected in the blank samples used as a negative control. The red bar represents metal levels detected in DddQ as isolated and the green represents the metal levels detected in 'apo' DddQ (following dialysis).

## 3.7 Site-directed mutagenesis of DddL/DddQ active site residues

Both DddL and DddQ contain a cupin domain consisting of two motifs with conserved residues typical of these motifs that are predicted to be involved in metal binding (see Figure 3.7) In *R. sphaeroides* DddL, these residues are 3 conserved histidines (H152, H154, H190) and a glutamic acid (E159). Each of these residues were mutated by site-directed mutagenesis in the plasmid pBIO1652 resulting in amino acid substitutions of each for alanine in pBIO1753, pBIO1754, pBIO1755 and pBIO1756 (see materials and methods). Mutation of any one of these predicted metal-binding residues resulted in the complete or near complete loss of DMSP lyase function (Figure 3.14). DMSP lyase activity, although greatly reduced, was seen with *E. coli* containing plasmid pBIO1754 (substitution H154A). This suggests that although it is still clearly important for metal binding, the corresponding histidine residue might be less crucial in this respect than the other conserved residues, and that its substitution might not lead to a total loss of metal binding.

DddQ of *Ruegeria pomeroyi* also has the two conserved cupin motifs, and in this protein the predicted metal-binding residues are three conserved histidines (H128, H130, H169) and a glutamic acid (E134). As described for DddL, these residues were substituted to alanine in the pET21a clone pBIO1886 to form pBIO1959, pBIO1960, pBIO1961 and pBIO1962 (see materials and methods). When mobilised into *E. coli* BL21 it was determined that all four mutants no longer made DMS from DMSP (Figure 3.15). These findings from single amino acid substitutions support the involvement of these conserved residues in metal binding which in turn is essential for the DMSP lyase activity of both DddL and DddQ.

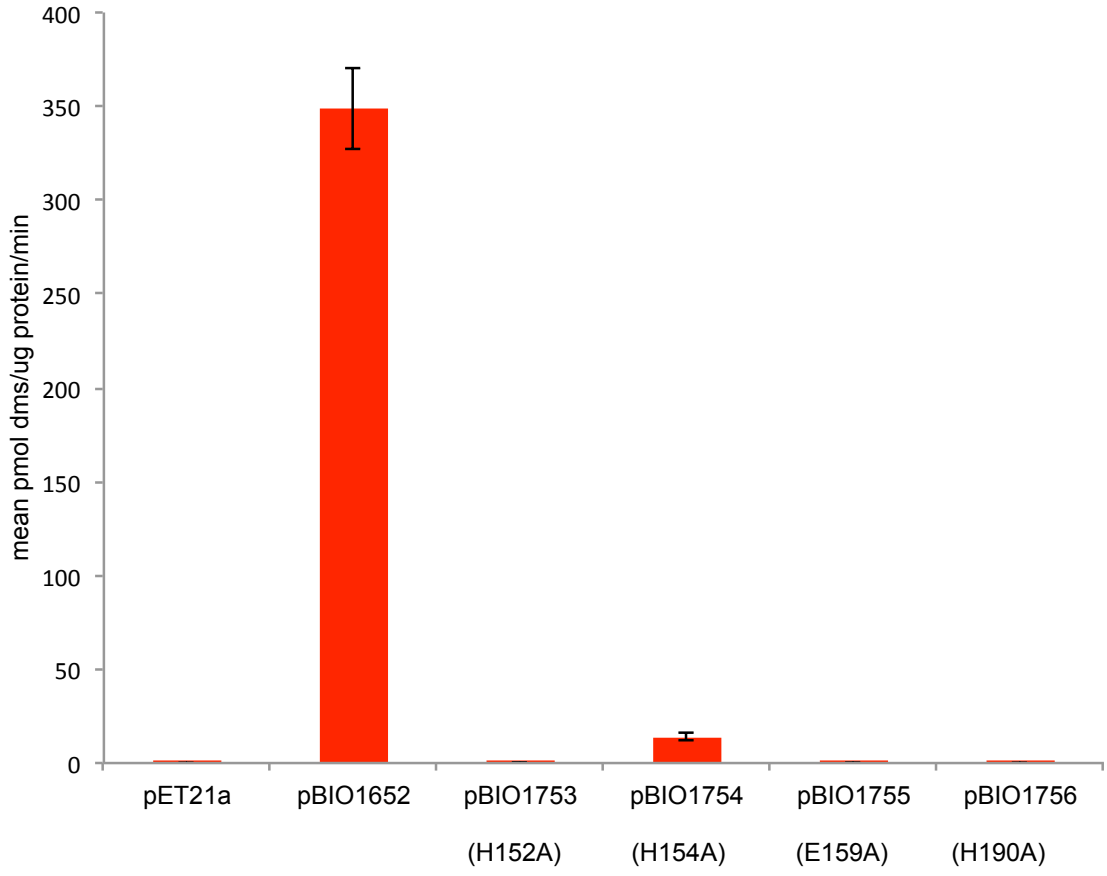



**Figure 3.14 DMS production from DddL with substitutions in metal-binding residues of cupin domain.** The different residues were substituted for an alanine to create four separate in-frame substitutions which were then subjected to DMS production determination via GC.

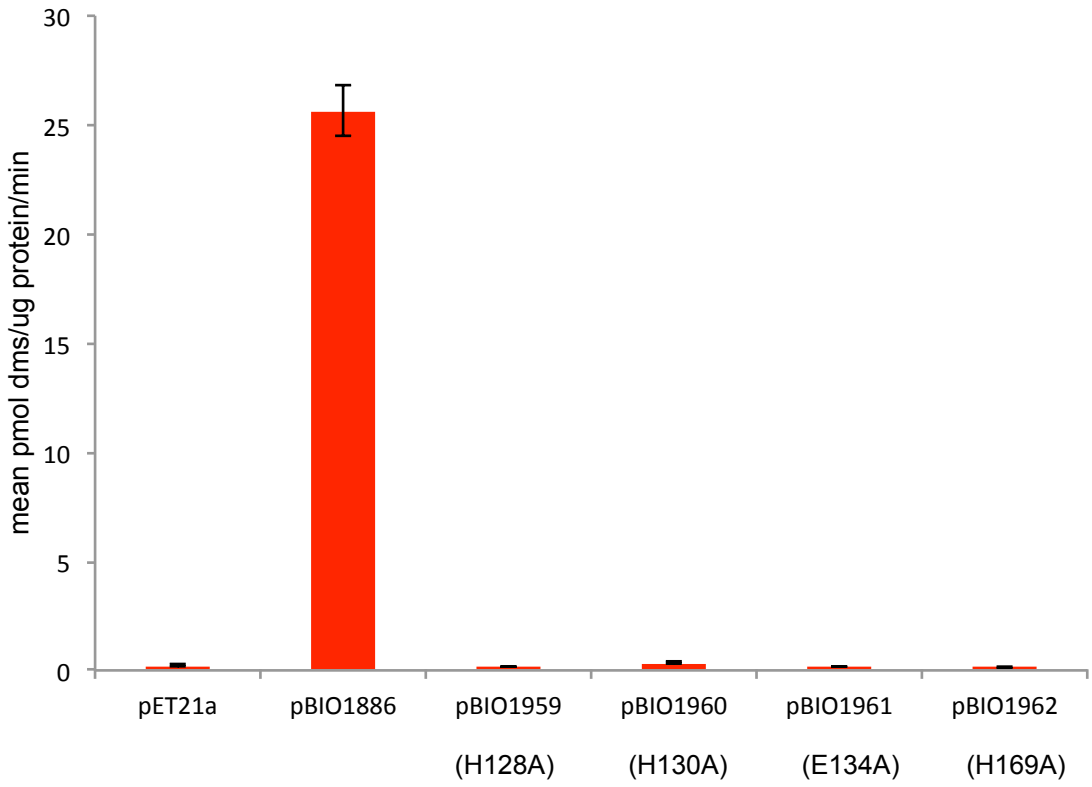



**Figure 3.15 DMS production from DddQ with substitutions in metal-binding residues of cupin domain.** The different residues were substituted for an alanine to create four separate in-frame substitutions which were then subjected to DMS production determination via GC.

## 3.8 Summary

Cupins are very diverse and many are known to require metal binding abilities in order to function.

This study has demonstrated, via analysis of purified protein and through site directed mutagenesis of conserved residues, that DddL and DddQ are indeed cupin-containing metalloenzymes that require metals as cofactors in order to function.

Metal addback assays determined which metals were required for enzyme function and ICP-OES told us which metals the purified enzymes use as essential cofactors for DMSP lyase activity. The substitution of single amino acids in the site-directed mutagenesis of conserved residues supported the hypothesis that the conserved residues were required for metal binding, which is essential for DMSP lyase activity.

Following enzymatic analysis, DddL and DddQ were found to have high $K_m$ values suggesting that either they are rather inefficient enzymes that only function in environments where DMSP concentrations are high or possibly even that DMSP is not the natural substrate for this enzyme.

Further detailed discussion of all the above points can be found in chapter six.

# 4 Identification and characterization of the cupin-containing DMSP lyase DddW

## 4.1 Overview

Some of the work described in this chapter is in the publication Todd et al., (2012), which is included as Appendix I, and there is also another more recent publication Brummett et al. (2015), which is of direct relevance to this work which is discussed in detail in chapter six. This work in this chapter follows on from the work of Dr. Mark Kirkwood, who performed microarray analysis, and the initial identification of *dddW*. In this study we have gone on to confirm the regulation of *dddW* by DMSP, expressed and purified the protein and characterized the enzymology and biochemistry of this cupin-containing DMSP lyase.

The previously discovered DMSP lyase genes *dddD*, *dddP*, *dddL*, *dddY* and *dddQ* were all identified via the screening of genomic libraries for cosmids conferring the $Ddd^+$ phenotype to other bacteria. This chapter describes how the DMSP lyase gene *dddW* was identified through the different approach of microarray analysis rather than via cosmid libraries.

A study of the model marine bacteria *Ruegeria pomeroyi* DSS-3 identified SPO0453 as the gene whose expression was most enhanced by DMSP, being upregulated 37-fold compared to in the absence of DMSP.

SPO0453 was shown to encode a DMSP lyase DddW that, like DddL and DddQ, contained a cupin domain, but which had no significant sequence homology aside from this shared domain. Using LacZ reporter fusions and RT-PCR, we confirmed the DMSP induction of the *dddW* gene and showed that this regulation is mediated by the divergently transcribed LysR transcriptional regulator.

The final part of this chapter includes the cloning and, purification of DddW and the subsequent characterisation of the enzymology, biochemistry and metal-binding ability of this lyase.

## 4.2 Distribution and characteristics of DddW homologues

A BLASTP survey of the NCBI database (as of July 2015), using DddW from *Ruegeria pomeroyi* DSS-3 as the *in silico* probe, found close homologues to polypeptides only in other members of the Rhodobacterales (Figure 4.1), these being *Pseudophaeobacter articus* (65% identity at amino acid level, E = $3e^{-62}$), *Roseobacter* MED193 (68% identity at amino acid level, E = $1e^{-65}$) and *Loktanella hongkongensis* (40% identity at amino acid level, E = $8e^{-42}$). To date, DddW is the least abundant of the DMSP lyases, in terms of the presence of the *dddW* gene in sequenced bacterial genomes.

## 4.3 Microarray analysis of *R. pomeroyi* DSS-3 in response to DMSP addition

To study the catabolism of DMSP in the model roseobacter *R. pomeroyi*, a microarray study of this strain was carried out. *R. pomeroyi* is one of the few known roseobacters that grows well on DMSP as a sole carbon source. It contains *dddP* and *dddQ* DMSP lyase genes and the DMSP demethylase gene *dmdA* (see Chapter one). A microarray analysis was carried out using RNA isolated from *R. pomeroyi* grown in the presence and absence of 5 mM DMSP (with 10 mM succinate as carbon source in both cases). From this study, carried out by Mark Kirkwood, it was found that the *dmdA* demethylase gene was strongly induced (20–fold) in the presence of DMSP. However, the DMSP lyase genes *dddP* and *dddQ* were only modestly enhanced by the presence of DMSP in the growth media. Analysis of the other genes upregulated by DMSP in the microarray revealed that expression of the gene SPO0453 was by far the most enhanced by DMSP, and this gene was termed *dddW*.

## 4.4 What is DddW?

The *dddW* gene encodes a small protein with no significant sequence similarity to proteins of known function, but which has a cupin domain towards its C-terminus. This protein has no sequence identity to DddQ or DddL but shares the conserved metal-binding residues of the cupin domain (see Figure 4.2). Unlike DddQ and DddL, this protein is relatively rare among sequenced bacteria, with close homologues only present in *Ruegeria pomeroyi* and a few other members of the roseobacters.

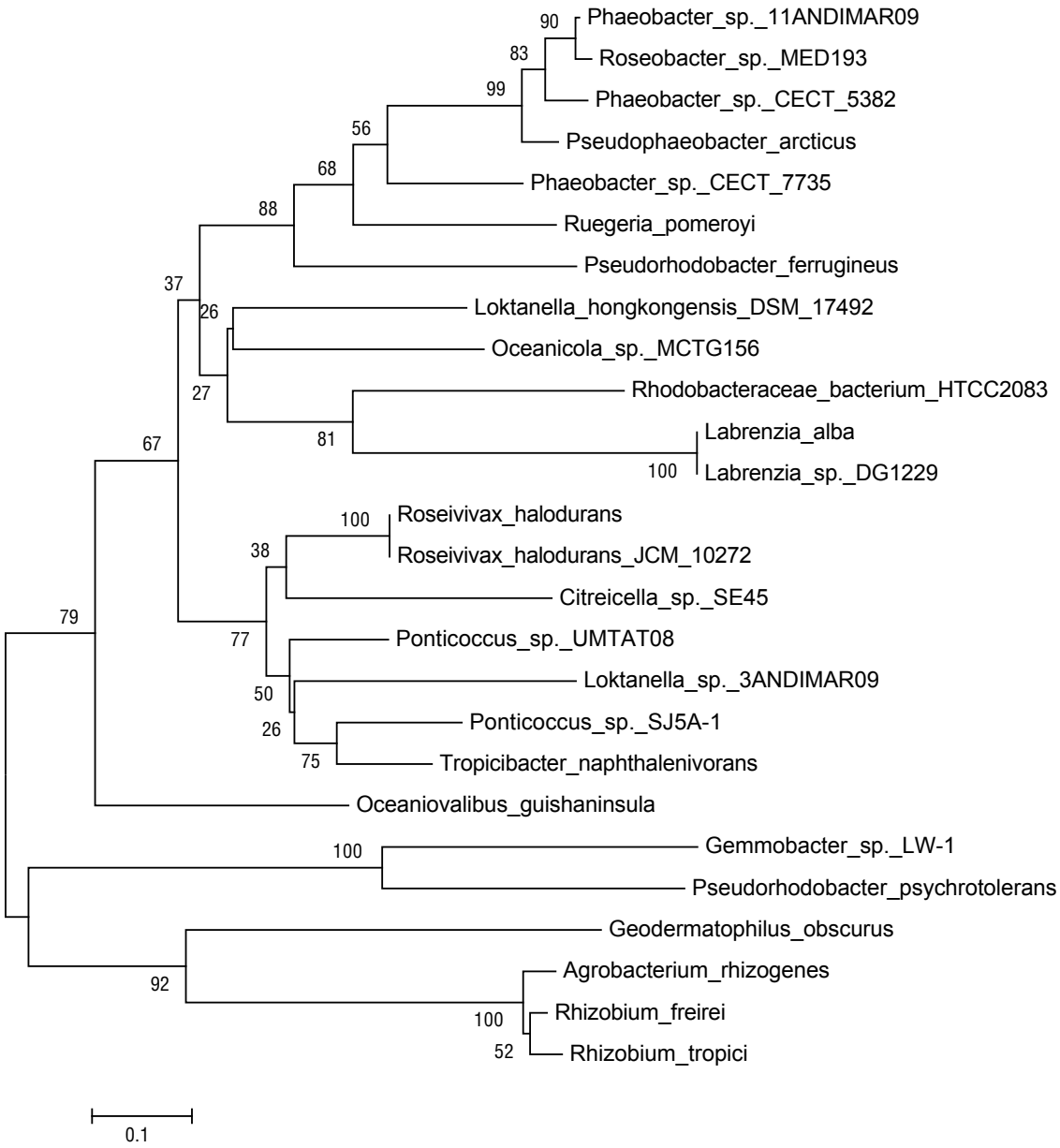



**Figure 4.1 Phylogenetic relationships amongst DddW polypeptides.** Although DddW is the least abundant of the DMSP lyases, with only two ratified lyases (*R. pomeroyi* DSS-3 and *Roseobacter* MED193), some proteins from recently sequenced alpha-proteobacterial genomes show some homology to the known DddW lyases.


**Figure 4.2 Sequence alignments of the predicted cupin motifs sequences of four DddW polypeptides compared with cupin motifs of the DddQ and DddL DMSP lyases**. The highly conserved amino acids in all cupins are indicated above the sequences and highlighted in red (Dunwell *et al.,* 2004). *R. pomeroyi* DSS-3 (R.pomW; *SPO0453*), *Pseudophaeobacter arcticus*, *Roseobacter* sp. MED193 (R.MEDW; *MED193_09710*;) and *Loktanella hongkongensis*, shown in green, are aligned with the DddQ lyases from *R. pomeroyi* (R.pomQ; *SPO1596*) and *Roseovarius nubinhibens* ISM (R.nubQ1, *ISM_14090* and R.nubQ2 *ISM_14085*), shown in green, and the DddL lyases from *Sulfitobacter*, purple.

The *dddW* (SPO0453) gene appears to be in a single gene transcriptional unit (see Figure 4.3) and sits downstream of SPO0452, a putative tellurite resistance gene, which is also induced when grown in the presence of DMSP (3-fold). These are anecdotally considered to be stress response genes and therefore unlikely to be directly associated with the DMSP pathway. Divergently transcribed upstream of SPO0453 is SPO0454 predicted to encode a LysR-type transcriptional regulator (LTTR). LTTRs are a large family of proteins responsible for regulating a vast array of phenotypes, including quorum sensing, motility and metabolism (Maddocks, 2008). Many LTTRs are also autoregulatory and capable of repressing their own expression even when the cognate co-inducer molecule is absent.

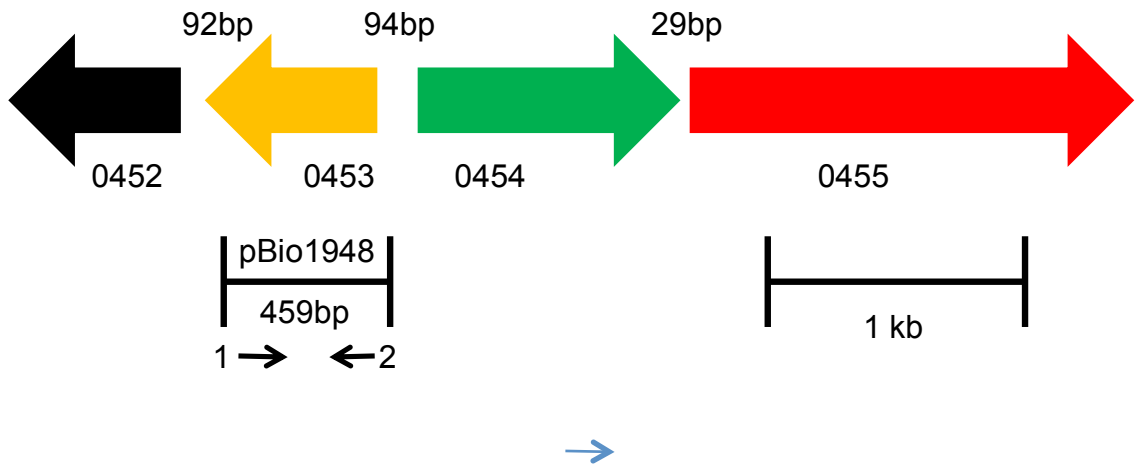



**Figure 4.3 Map denoting genetic loci of *dddW* (SPO0453) in *Ruegeria pomeroyi* DSS-3.** Gene tags are shown without the SPO prefix and intergenic regions are marked. Gene annotation: Black, predicted tellurite resistance protein; Orange, DMSP lyase DddW; Green: predicted LysR-type transcriptional regulator; Red, predicted lysyl tRNA synthetase. The 459 bp fragment was amplified using primers Wpet1 and Wpet2 – arrows 1 and 2. The intact *dddW* was cloned into the pET21a expression vector forming pBIO1948.

### 4.5 Confirmation that *dddW* is induced by DMSP

In order to verify the results obtained from the microarray analysis, two techniques were employed to directly examine the effect of DMSP on transcription of *dddW* (SPO0453).

#### 4.5.1 Assaying *dddW* transcription using *lacZ* reporter fusions

The first approach was to construct a *dddW-lacZ* transcriptional fusion. This was done by using the primers wprom1 and wprom2 to amplify the intergenic region between SPO0453 and SPO0454 from *R. pomeroyi* genomic DNA. This fragment was subsequently cloned upstream of the promoterless *lacZ* reporter gene in the wide host-range promoter probe vector pBIO1878 using the restriction enzymes *EcoR*I and *Pst*I such that the reporter *lacZ* would be under the control of the *dddW* promoter. The resulting plasmid pBIO1945 was introduced via triparental mating into *R. pomeroyi* J470 ($Rif^R$) and transconjugants were selected using rifampicin, spectinomycin and tetracycline. Transconjugants were than assayed for β-galactosidase activity after growing in minimal medium in the presence or absence

of 5 mM DMSP (as described in material and methods). Results confirmed that the *dddW-lacZ* fusion was induced (~10 fold) in the DMSP-containing media consistent with the findings from the microarray (Figure 4.4a) and demonstrated using heterologous expression, that unless a copy of the putative regulator, SPO0454 is present in *trans*, induction via DMSP does not take place (Figure 4.4b). Finally it was established that SPO0454 acts as an auto regulator and its over expression results in it being switched off irrespective of the presence of DMSP (Figure 4.4c).

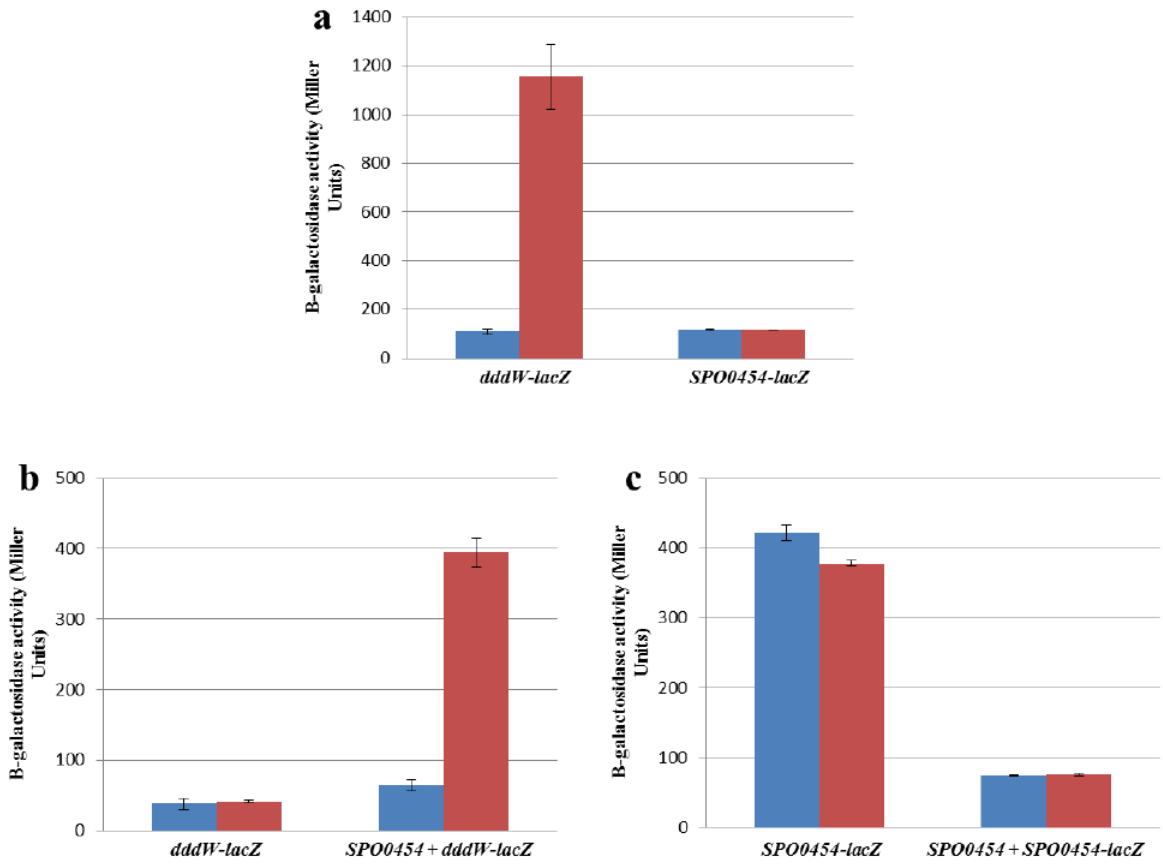



**Figure 4.4 Regulation of the *Ruegeria pomeroyi dddW-lacZ* fusion in response to DMSP and the putative regulator, SPO0454.** β-galactosidase activity of the *dddW-lacZ* (a,b) or SPO0454-*lacZ* (a,c) fusion plasmid when expressed by cultures of *R. pomeroyi* DSS-3 (a) or *Rhizobium leguminosarium*

strain 3841 (b,c) in minimal media in the absence or presence of 5 mM DMSP (blue and red bars respectively). Error bars were calculated from triplicate experiments.

### 4.5.2 qRT-PCR analysis of *dddW* transcription

Induction of *dddW* by DMSP was also confirmed by quantitative real time reverse transcriptase PCR (qRT-PCR). *R. pomeroyi* was grown under the same conditions as those for the microarray dataset, namely overnight incubation followed by induction with or without 5 mM DMSP. Growth was stopped by the addition of phenol:ethanol and cells were frozen in liquid nitrogen. RNA was subsequently extracted, purified, quantified and checked for contaminating DNA or phenol (as described in materials and methods), with any contaminating DNA removed using DNAse. The RNA was then reverse transcribed to cDNA and amplified via PCR. A SYBR green fluorescent system with a C1000 thermal cycler and a CFX real time PCR detection system were used. A housekeeping control gene (i.e. a gene which is constitutively expressed under the conditions used) was employed in order to normalise the relative fold change in expression exhibited by *dddW*. The housekeeping gene used was SPO2904, which encodes a serine/theronine protein phosphatase/nucleotidase. The results demonstrated that *dddW* was induced 6-fold by the presence of DMSP. This level was significantly lower than the levels of increased expression observed from the microarray dataset. However, these experiments provide three independent pieces of data demonstrating that expression of *dddW* is increased in response to DMSP.

## 4.6 DddW encodes a functional DMSP lyase enzyme

As the transcription of *dddW* is enhanced by DMSP, and the DddW polypeptide contains a C-terminal cupin domain similar to DddL and DddQ (see Figure 4.2), we hypothesised that DddW would be a DMSP lyase. In order to test this, we cloned the gene into the expression plasmid pET21a. Using primers Wpet1 and Wpet2, (Figure 4.3) the complete *dddW* gene was amplified using *R. pomeroyi*

DSS-3 genomic DNA as template and cloned into pET21a using *BamH*I and *Nde*I to form plasmid pBIO1948. Clones were ratified by sequencing using T7 primers and by the presence of the correct sized insert on agarose gels after *Nde*I and *BamH*I digestion.

The ratified pBIO1948 plasmid was transformed into *E. coli* BL21 cells for protein expression, selecting for transformants with ampicillin. BL21 cells containing pBIO1948 were grown in LB in the presence of 0.2 mM IPTG, to induce the expression of DddW, and 5 mM DMSP (Figure 4.5), in 2 ml air-tight gas chromatography vials. The vials were grown at 28 ˚C overnight (16 hours) and then assayed for the Ddd phenotype by gas chromatography. It was clear that only *E. coli* expressing the ectopic *dddW* gene made DMS from DMSP. The levels of DMS production from DddW were similar to those described for DddL and significantly higher than for DddQ. DddW activity was also assayed in *E. coli* BL21 cell lysates. Cells were grown as above in 5 ml LB liquid medium and cells were disrupted by sonication. Following centrifugation, the resulting lysate was assayed for Ddd activity in the presence of 5 mM DMSP (see materials and methods). The DMS produced was measured by GC and the protein levels determined using a Bradford assay. The activity observed (35 pmol μgprotein$^{-1}$ min$^{-1)}$ is comparable to that seen in *E. coli* expressing *dddQ* or *dddL* genes from of *Ruegeria pomeroyi* DSS-3 and *Rhodobacter sphaeroides* 2.4.1 respectively. This confirmed that *dddW* (SPO0453) encoded a functional DMSP lyase. Cell lysates were observed to give significantly more DMSP activity than the whole cell samples. This is presumably due to the fact that this negates the need for the transport of DMSP into the cell in order for the enzyme to access its substrate.

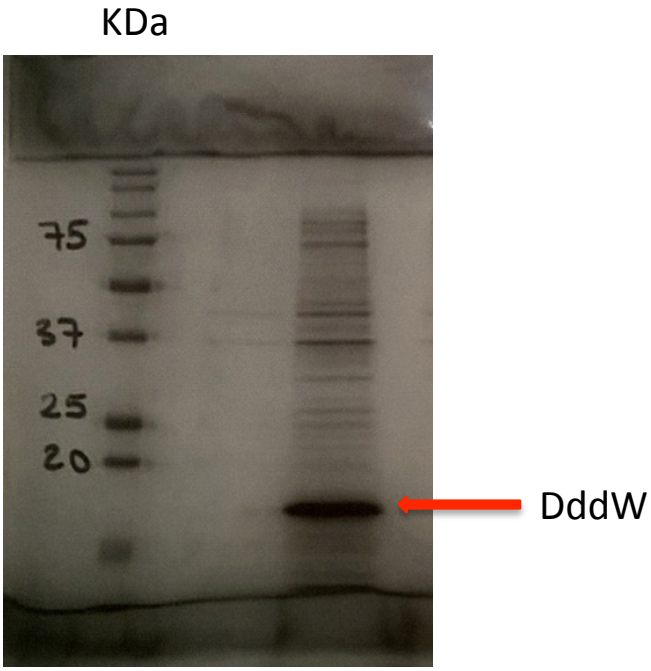



**Figure 4.5 Induction of DddW using IPTG.** DddW (16.9 kDa) expression was induced using 0.2 mM IPTG. Polypeptides were separated via SDS-PAGE on a 15% acrylamide gel and stained with Coomassie blue.

### 4.7 DddW encodes a typical DMSP lyase

To show that DddW is a typical DMSP lyase, generating acrylate and DMS from DMSP, BL21 cells containing pBIO1948 were grown in M9 media made up in $D_2O$ in the presence of [$^{13}$C] DMSP and IPTG. After overnight growth at 28°C, cells were sonicated and 5% v/v perchloric acid was added in order to precipitate the proteins and leave the metabolites in solution. Following centrifugation to remove the proteins, this solution was assayed by $^{13}$C NMR against control lysates of BL21 lacking pBIO1948. These experiments revealed the co–product of DMS, in the DddW-catalysed reaction, to be [$^{13}$C] acrylic acid, confirming DddW as a typical DMSP lyase. This can be clearly seen in Figure 4.6 with a chemical shift for acrylate (at 169.9 ppm) being present, along with unlysed DMSP (at 173.22 ppm), in the *dddW*-containing sample, but not in the control BL21 cells, which only have unlysed DMSP.

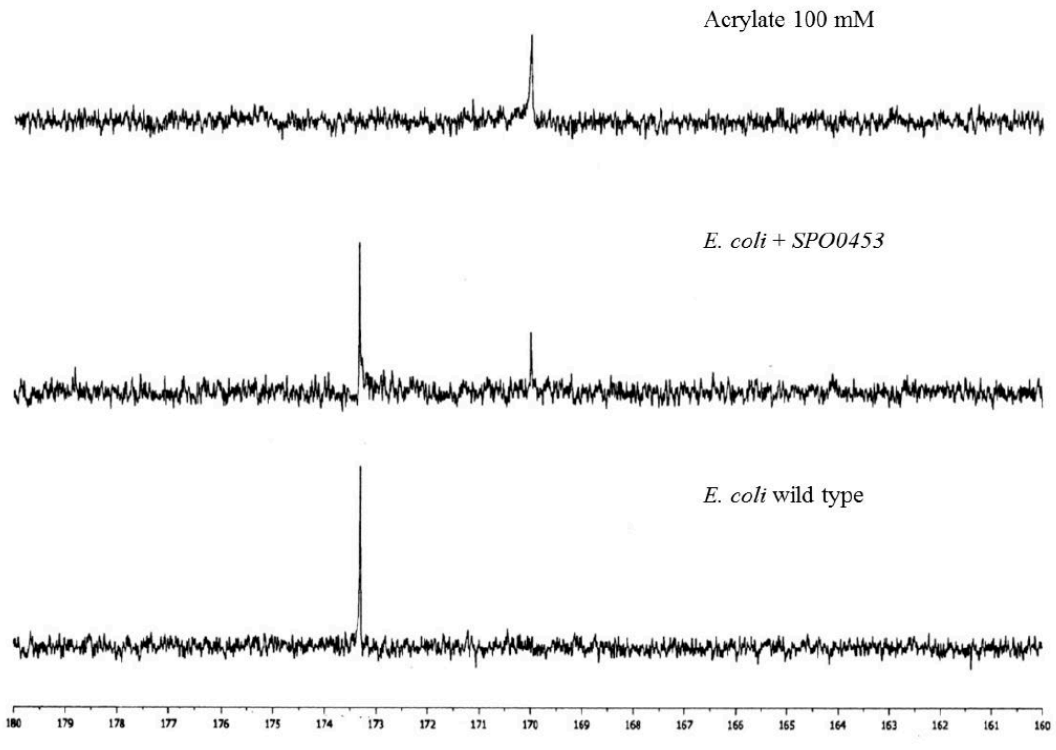



**Figure 4.6 Nuclear Magnetic Resonance (NMR) spectra of *E. coli* BL21 expressing cloned *dddW*, fed with [1-$^{13}$C] DMSP.** NMR spectra for pure sample of 100 mM acrylate is indicated, with a chemical peak shift at 169.9 ppm. The spectra below the acrylate reference from top to bottom shows *E. coli* expressing SPO0453 from the pET21a plasmid and *E.coli* BL21 strain. The peak at 173.22 ppm represents DMSP added at 10 mM. Average scans recorded was 300.

### 4.8 SPO0454 is responsible for the DMSP-regulated transcription of *dddW*

Having shown that transcription of *dddW* is induced by DMSP, the potential regulatory role of the divergently transcribed gene SPO0454, encoding a predicted LTTR, was investigated. It is well known that catabolic genes are often positioned close to their transcriptional regulator. As can be seen in Figure 4.3, SPO0454 is located upstream of and likely co-transcribed with a gene SPO0455, encoding a predicted lysyl tRNA synthetase that is likely an essential enzyme for *R. pomeroyi.* Thus, any mutational analysis of SPO0454 might have affected the expression of the essential downstream genes. Therefore, an alternative approach was used involving the use of *Rhizobium leguminosarum* as a heterologous host. The

primers 454P1 and 454P2 were used to clone SPO0454 and its upstream promoter region into pOT2, a wide host-range vector compatible with pBIO1878 plasmids, generating pBIO1946. This pBIO1946 plasmid was mobilized by conjugation into *Rhizobium* that already contained the pBIO1878-based plasmid, pBIO1945, the *dddW-lacZ* reporter fusion. The β-galactosidase activity of the *dddW-lacZ* fusion was assayed following growth in the presence or absence of 5 mM DMSP, and with or without cloned SPO0454 on pBIO1946. It was found that in the absence of the cloned SPO0454 gene the *dddW-lacZ* fusion was not expressed in minimal media either in the presence or absence of DMSP (Figure 4.4). However, the *Rhizobium* strain which contained both the *dddW-lacZ* fusion and the heterologously expressed SPO0454 showed high levels of β-galactosidase activity, but only in the presence of DMSP. This indicates that the LysR-like SPO0454 does indeed encode a regulator that activates transcription of *dddW* in the presence of DMSP.

### 4.9 Insertional mutation of *dddW* and its effect on DMS production

Having demonstrated that *dddW* is strongly induced by DMSP at the level of transcription and that in encodes a DMSP lyase, its importance in DMSP catabolism in *R. pomeroyi* was then also investigated. To do this, a mutation was made in the *dddW* gene of *R. pomeroyi* and the effect of this mutation on the ability of *R. pomeroyi* to lyse DMSP or use it as a carbon source was examined.

In order to mutate *dddW* in *R. pomeroyi*, an internal fragment of the *dddW* gene was amplified and cloned into the suicide plasmid pBIO1879 using *BamH*I to produce pBIO1949. pBIO1879 is derived from the insertion suicide plasmid pK19*mob* ($kan^R$) (Schäfer et al 1994), which is unable to replicate in hosts other than enteric bacteria such as *E. coli.* In pBIO1879, a spectinomycin resistance cassette has been cloned into pK19*mob* to give an extra selectable antibiotic resistance gene and allow for counter selection in the Roseobacters, several of which are resistant to tetracycline (Todd, 2011). To mutate target genes, an internal fragment of the gene is cloned into the polylinker. When mobilised into the host, it allows for single homologous recombination, with single crossover mutants screened for by selecting for kanamycin and spectinomycin antibiotic resistance (Figure 4.7).

Through triparental mating the plasmid pBIO1949, containing the internal fragment of *dddW*, was introduced into the rifampicillin (rif) resistant strain of *Ruegeria pomeroyi,* J470, and resultant transconjugants screened for $Rif^R$, $Kan^R$ and $Spec^R$. Six possible *dddW* mutants were studied further. To confirm that the six transconjugants were *R. pomeroyi dddW* mutants, genomic DNA was extracted from the possible mutants and checked by PCR amplification. Also, Southern blotting of genomic DNA from the mutant strains compared to the wild type DNA was carried out using an intact *dddW* PCR fragment (PCR product amplified in construction of pBIO1948) as the probe. Of the six possible *dddW* mutants, none of them gave a PCR product in the PCR-based method or contained the wild type restriction fragments in the Southern blotting, confirming that they were all *bona fide dddW* mutants. One of these mutants was selected for further study and designated as strain J497.

The ratified DddW mutant strain J497 was then assayed for DMSP-dependent DMS production to see what effect the mutation had on DMSP production in *R. pomeroyi*. The DddW mutant was grown in minimal media containing 5 mM DMSP for 16 hours, and then assayed for DMS levels by GC. The J497 mutant was found to release 50% less DMS than the wild type strain J470. The results of DMS production assays on previously constructed *R. pomeroyi dddP* and *dddQ* mutants (Todd et al., 2009; Todd et al., 2011) were also compared to those of the *dddW* mutant. It was found that despite *dddW* being the most upregulated gene in response to DMSP; it did not have the largest effect on DMS production in the corresponding mutant. The greatest reduction in DMS production in *R. pomeroyi* was seen in the *dddQ* mutant, with the *dddP* mutant reducing DMS production to levels similar to those seen in the *dddW* mutant.

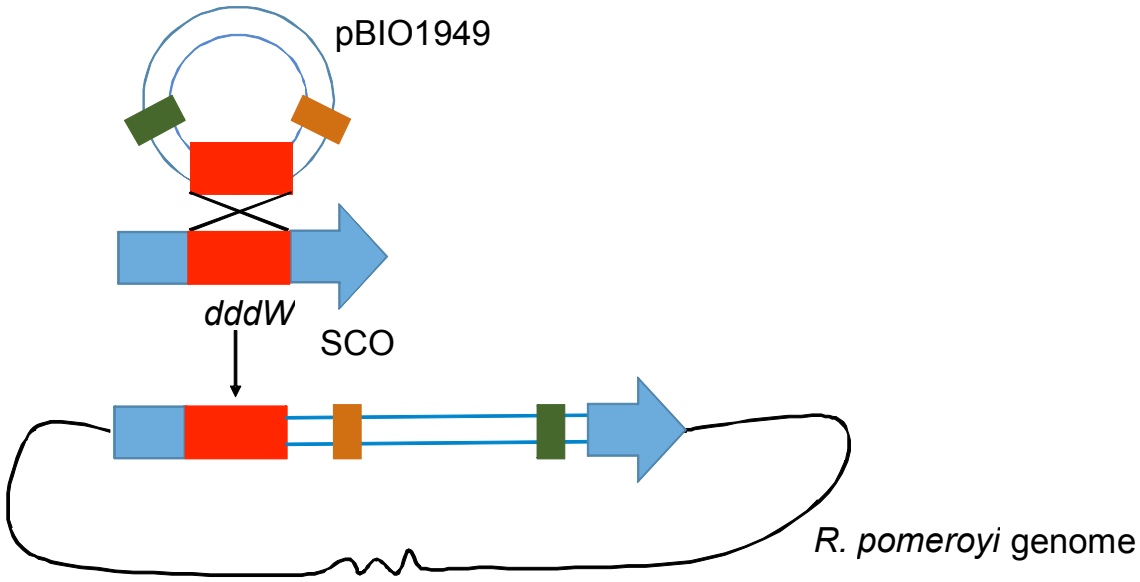



**Figure 4.7 Mutagenesis of *dddW* in *Ruegeria pomeroyi***. The red bar illustrates the internal fragment of *dddW* which was cloned into the pBIO1879 (pK19mob spec$^R$) suicide vector to form pBIO1949, and in the equivalent region of the genomic *dddW* of *Ruegeria pomeroyi* DSS-3 following recombination. The brown and green bars are shown to indicate the orientation of inserted plasmid DNA. SCO, Single Cross Over event via homologous recombination.

### 4.10 Purification of DddW

Knowing that DddW functions as a DMSP lyase and significantly contributes to the lysis of DMSP by the model Roseobacter *Ruegeria pomeroyi*, the biochemistry and enzymology of the protein was then investigated. In order to do this, it was first necessary to purify the DddW protein. As discussed before (chapter three), nickel affinity purification was not used due to DddW likely being a metalloenzyme. Having already established that DddW was active in soluble cell extracts, its expression was scaled up to allow protein purification.

### 4.10.1 Overexpression and protein purification of DddW

A starter culture of *E. coli* BL21 containing the *dddW* pET21a clone pBIO1948 was grown overnight at 37 ˚C in LB Amp liquid medium and then used to inoculate 1:100 to 1 litre of LB Amp. This culture was incubated for two hours at 37 ˚C to reach an $OD_{600}$ of 0.4-0.6 and then expression of DddW was induced by addition of 0.2 mM IPTG and the culture was grown overnight (~16 hours) at 25 ˚C. The cells were harvested via centrifugation and resuspended in 20 mM Tris (pH8) before lysing the cells via French Press (as described in materials and methods). It was established that carrying out an ammonium sulphate precipitation between 20-40% enriched for DddW protein in the pellet, as seen as a 16.9 KDa band on a 1D SDS-PAGE gel, corresponding to the predicted molecular weight of DddW. When resuspended in 20 mM Tris buffer (pH 8), it was shown that DddW was still functional. Therefore, after removal of cell debris by centrifugation, ammonium sulphate was added to the resulting supernatant to a concentration of 40% in order to precipitate DddW protein from the cell lysate. The DddW-containing pellet was resuspended in 20 mM TRIS (pH 8) before being dialysed overnight and loaded onto a Superdex 200 gel filtration column equilibrated with the same buffer. Fractions were taken and run on a 15% SDS-PAGE gel and demonstrated the presence of a 16.9 kDa band of high purity corresponding to the predicted molecular weight of DddW. DMS activity assays via gas chromatography were also performed in order to verify activity was still present in the pure protein sample. This simple two stage purification method yielded DddW judged to be ~ 80% pure (Figure 4.8), and deemed to be of sufficient purity for the purposes of obtaining $K_m$, $V_{max}$ and metal cofactor information on the protein. However, if samples were required for crystallization at a later stage, further purification steps would need to be introduced to achieve greater purity.

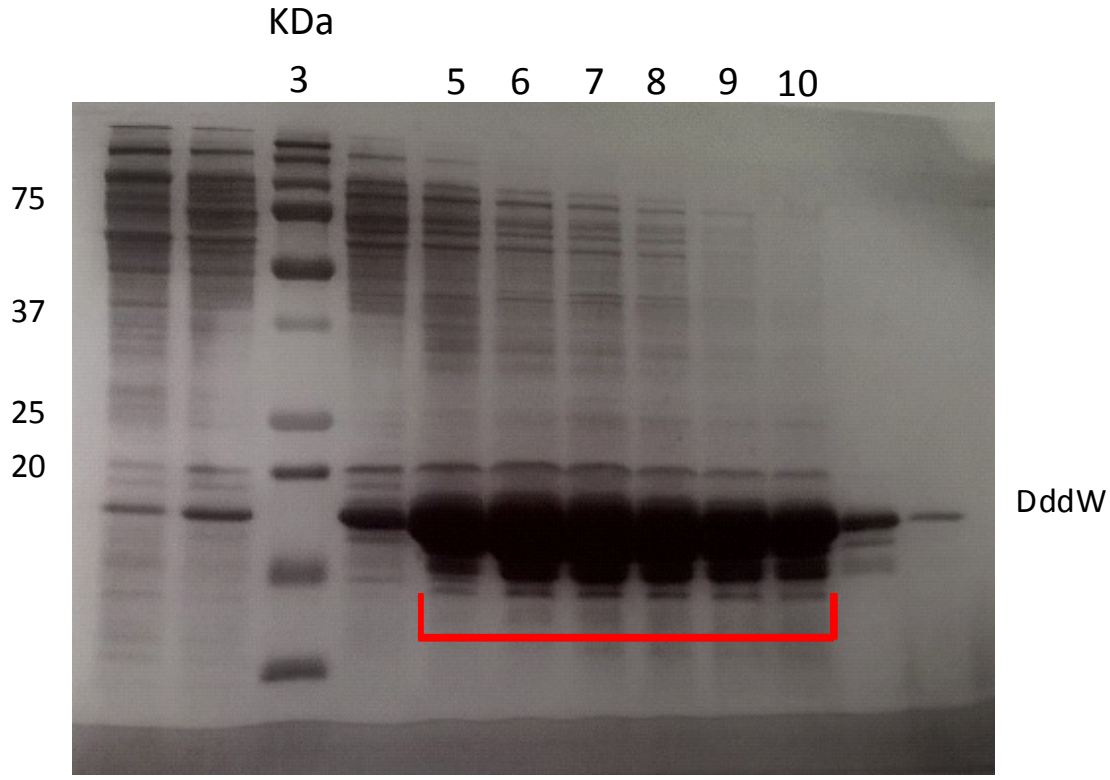



**Figure 4.8 DddW following elution from Superdex 200.** Polypeptides were separated via SDS-PAGE on a 15% acrylamide gel and stained with Coomassie blue. Lane 3 contains Precision plus protein standard. Lanes 1, 2, 4-12 contain fractions of eluted protein from the gel filtration column and Lanes 5-10 show where the vast majority of DddW eluted from the Superdex column. These fractions were pooled, concentrated and stored at -80 ˚C until required.

### 4.11 Enzyme kinetics of DddW

To establish the $K_m$ and $V_{max}$ values of the DddW enzyme, the initial rates of DMS production were measured at intervals using ~0.3 µM purified DddW. Samples were incubated at 30 ˚C in 20 mM Tris (pH 8) with DMSP substrate concentrations between 50 mM and 1M, before being analyzed for DMS production via GC. The data fitted to the Michaelis-Menten equation and a $K_m$ of DddW for DMSP of 32.0 ± 8.8 mM and a $V_{max}$ of 18.3 ± 1.4 nmol DMS $min^{-1}$ µg DddW $protein^{-1}$ were extrapolated (Figure 4.9).

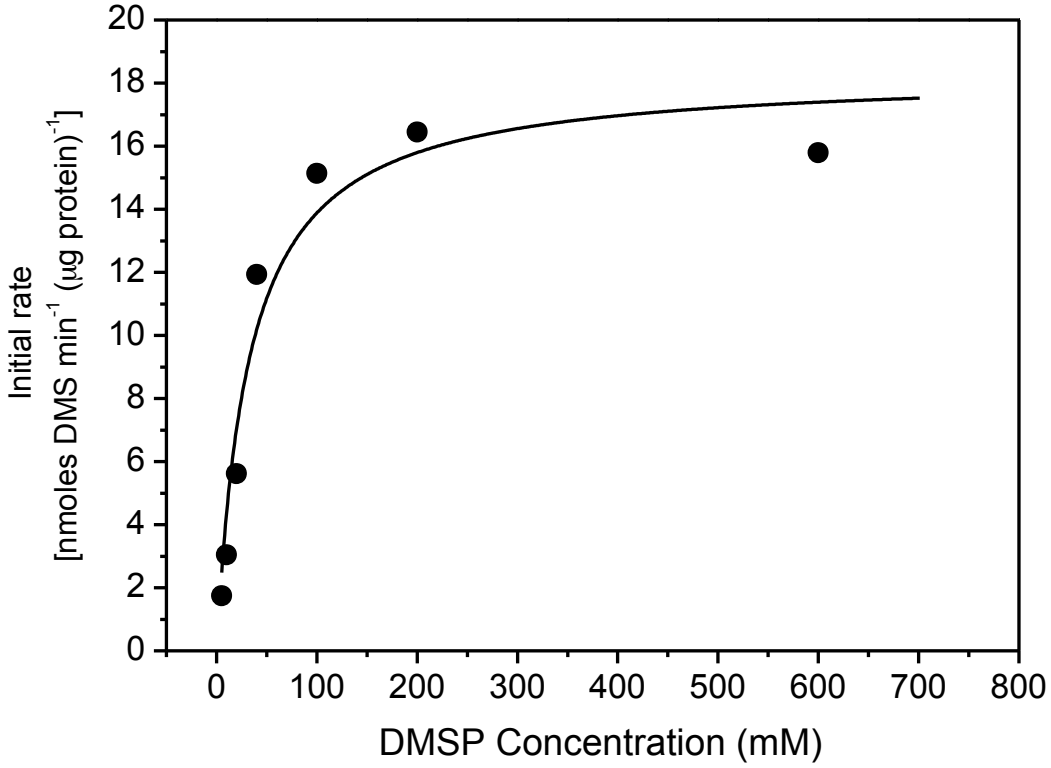



**Figure 4.9 Michaelis-Menten plot for the DMSP lyase activity of DddW.** Data was fitted to the Michaelis-Menten equation using Origin Software (version 8, Origin lab). $V_{max}$ was calculated as 18.3 ± 1.4 nmol DMS $min^{-1}$ µg DddW protein and $K_m$ of 32.0 ± 8.8 mM. Intial rate (nmol DMS $min^{-1}$ µg DddW $protein^{-1}$) plotted against DMSP concentration. DddW (0.3 µM) was in 20 mM Tris buffer (pH 8).

## 4.12 Effect of metals on DMS production / Metal binding studies

As discussed in chapter two, cupin-containing enzymes are known for their ability to bind divalent metals as cofactors required for function (Dunwell et al., 2000, Khuri et al., 2001) and the two cupin-containing lyases DddL and DddQ both possess conserved histidine and glutamic acid residues that were shown to be required for metal binding. As DddW also contains these predicted metal-binding residues (Figure 4.2), the same experiments were performed on DddW in order to establish its requirement for metals. Once again, EDTA assays were performed ranging from 150 μM – 10 mM EDTA. On addition of EDTA, DddW samples were incubated for 30 minutes at 30 ˚C before the addition of 5 mM DMSP and performing GC DMS production assays (chapter 2). DddW activity was reduced by ~50% at 100 uM and by ~90% at 10 mM EDTA. From this, we can conclude that DddW does indeed require metal cofactors for its activity (Figures 4.10 and 4.11).

Having established that DddW had a requirement for metal cofactors, candidate metals were explored using the same metal add-back experiments as those described in chapter two. These involved the addition of metal chloride solutions (Co, Cu, Fe, Mn, Ni, and Zn) to purified DddW, which had previously been treated with EDTA to strip the protein of metals and then dialysed to remove the EDTA.

The results suggest that, similar to both DddL and DddQ, DddW is also rather indiscriminate for which metal cofactor it will employ, with Co and Mn both giving significant Ddd activity compared to the apo protein (Figure 4.12). However, Mn appeared to serve as the most efficient cofactor resulting in highest increase in Ddd activity when added at the same concentration as the other metals. While these results do not confirm the involvement of any particular metal in the function of DddW *in vivo*, they do provide information on which metals can potentially act as cofactors that will allow the protein to function.

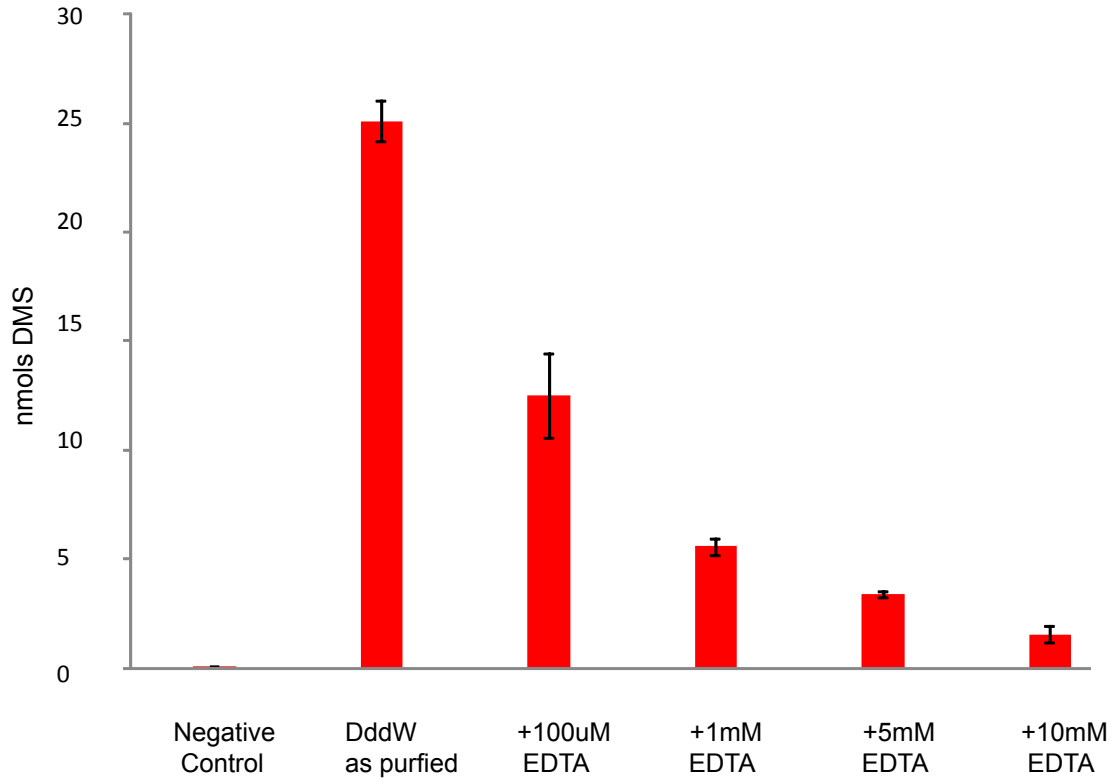



**Figure 4.10 Effect of EDTA on DddW activity.** DddW activity in the presence or absence of EDTA. DddW was with or without EDTA and DMSP added [5 mM]. Using Gas Chromatography, samples were assayed for DMS production. Error bars represent the standard error from triplicate samples. A negative control consisting of just buffer and EDTA was used in order to obtain background levels of DMS production.

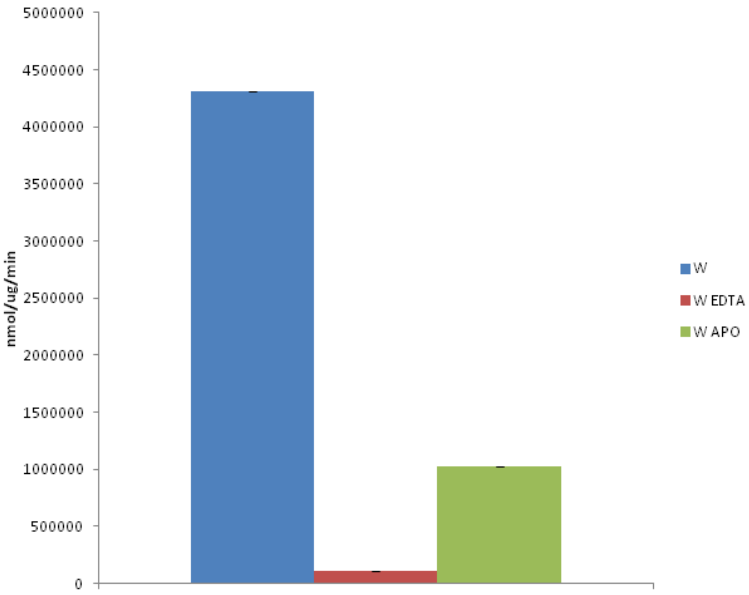



**Figure 4.11 Effect of EDTA and dialysis on the DMSP lyase DddW.** W denotes the protein as purified, W EDTA denotes the protein in the presence of the EDTA chelating agent. W apo denotes protein which has been treated with EDTA and dialysed to remove EDTA. DMSP [5 mM] was added to aliquots of treated DddW and samples were assayed for DMS production via Gas Chromatography. Error bars represent the standard error from triplicate samples.

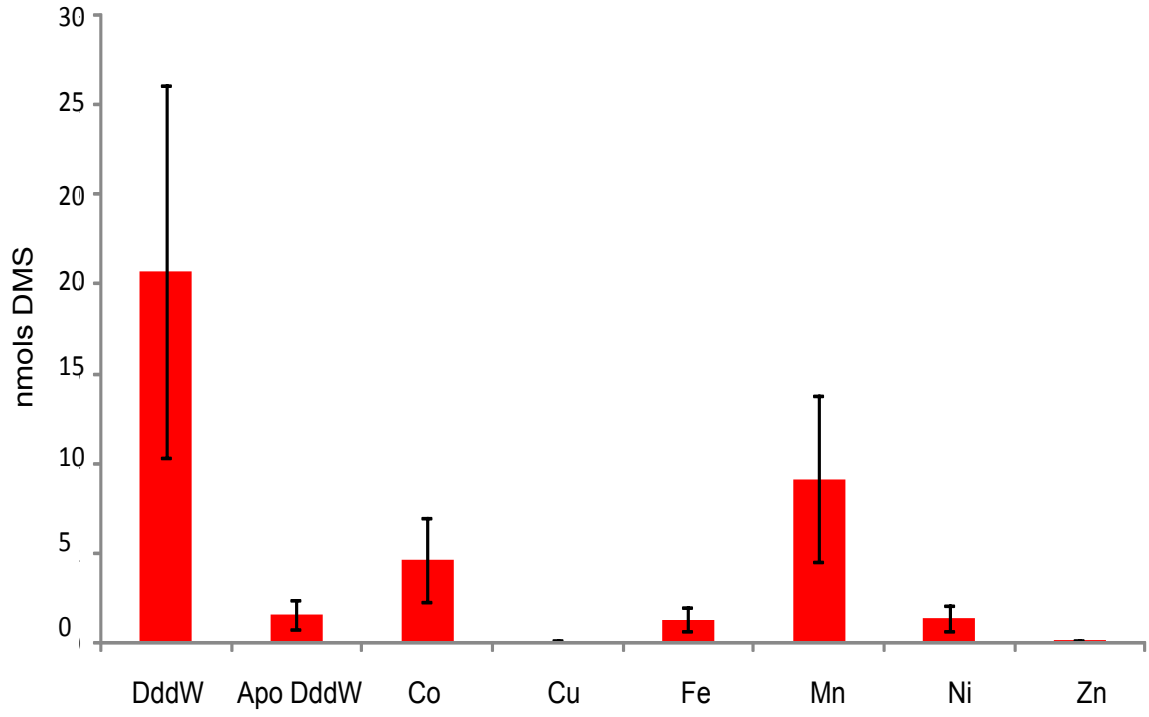



**Figure 4.12 DddW metal addback.** Metal addback experiments were used to determine which metals were required for DddW to function. This involved the addition of individual metal solutions (20mM) to DddW [0.32 µg] that had been stripped of the metal cofactor by EDTA addition and dialysis. Error bars represent the standard error from triplicate samples

## 4.13. Metal cofactor determination by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES).

To further investigate which metal cofactor(s) are present in the DddW protein, purified DddW was analysed by ICP-OES, as for DddL and DddQ in chapter two. As with DddL and DddQ, active samples of DddW containing 2.5% v/v nitric acid were analysed on a Varian Vista Pro CCP simultaneous ICP-OES. The metals

investigated were cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni) and zinc (Zn). ICP-OES results suggested that Fe and Zn are the metal cofactors present in purified DddW, and following EDTA treatment and dialysis the concentrations of these metal ions were seen to drastically decrease, along with the DMSP lyase activity of the treated samples (Figure 4.13). The activity of the 'apo' protein was also significantly reduced compared to the as purified DddW.

## 4.14 Determining DddW association state by analytical ultracentrifugation

In order to determine the association state of DddW, the purified protein was analysed by ICP-OES, as for DddL in chapter three.

As expected from its predicted molecular weight, the purified DddW peptide was observed as a 16.9 kDa band in SDS PAGE. In order to determine the association state of native DddW, sedimentation equilibrium analytical ultracentrifugation was employed. A sample of DddW (0.3 μM) was centrifuged at 12,000 rpm and 20 ˚C in a Beckman Optima XL-1 analytical ultracentrifuge. Scans were taken every 4 hours so as to determine when equilibrium had been reached, at which point five more scans were recorded. The data was analysed (Ultrascan Demeler 2005) and predicted a molecular mass of 30 kDa was predicted (approximately double the size of DddW), suggesting that DddW is a dimer in solution (Figure 4.14)

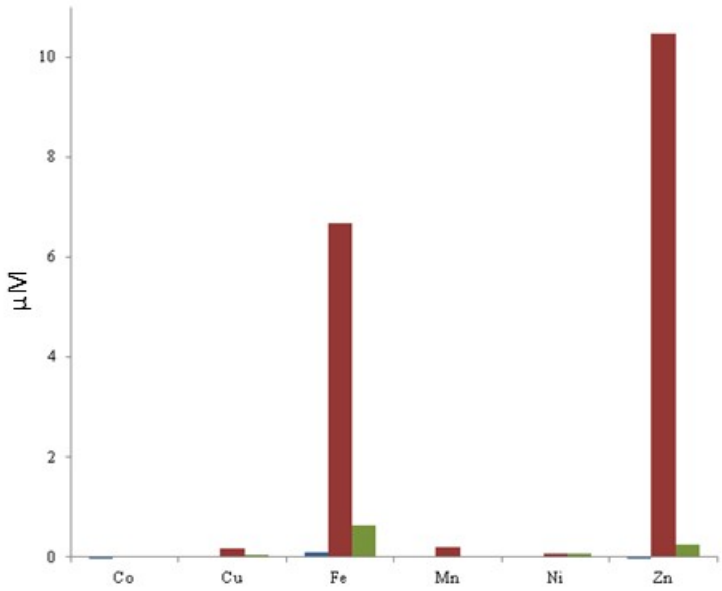



**Figure 4.13 ICP-OES analysis for metal cofactor detection.** The blue bar represents metal levels detected in the blank samples used as a negative control.The red bar represents metal levels detected in DddW as purified and the green bar represents the metal levels detected in 'apo' DddW (i.e. following dialysis).

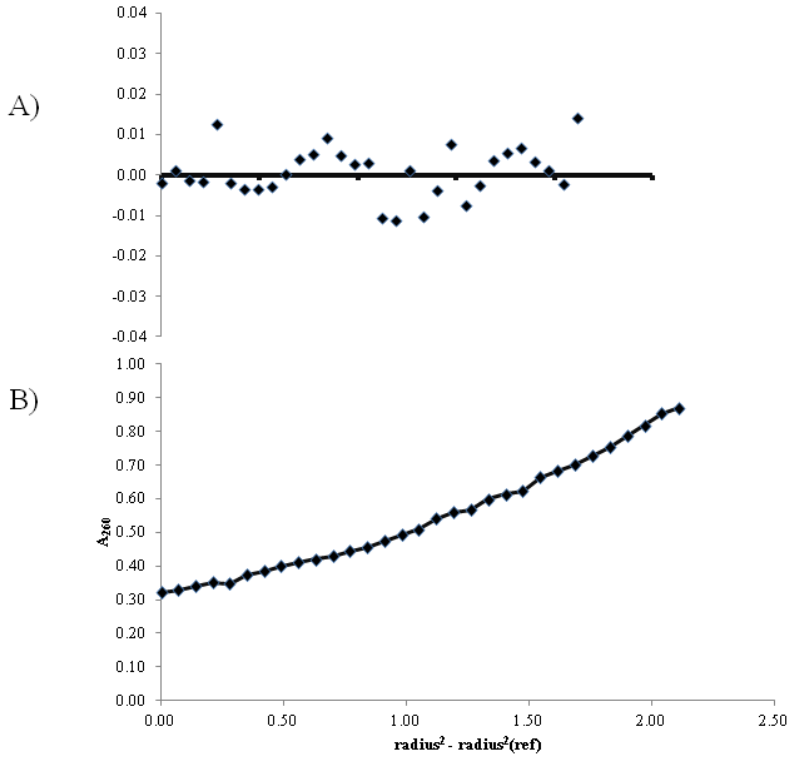



**Figure 4.14 AUC analysis of DddW following chromatographic purification.** Absorbance at 260 nm against radius$^2$–radius$^2$ following equilibration of purified W (76 uM). A) Residual difference between experimental data and fitted curve. B) A mean molecular weight of 30 kDa was recorded suggesting that, in its as purified state, DddW is a dimer.

### 4.15 Site-directed mutagenesis of DddW active site residues

Having shown that DddW is a metalloenzyme that uses Fe and Zn as its cofactors, the role of the predicted metal-binding residues (H83, H85, E87 and H121) in the cupin domain were determined (Figure 4.2). To do this, site-directed mutagenesis PCR was performed on pBIO1889, to create four separate in-frame substitutions encoding for the amino acid alanine (in plasmids pBIO2243, pBIO2244, pBIO2245, pBIO2246 respectively) in DddW (see Materials and Methods). Mutant plasmids were confirmed via sequencing, and the mutagenised inserts were sub-cloned into

pET21a. These plasmids were expressed in *E. coli* BL21 as above and assayed for Ddd activity. In all four mutants, the substitution of each predicted metal-binding residue for alanine completely abolished DddW activity, with no DMS production above buffer levels (Figure 4.15). This suggests that these conserved residues in the cupin domain of DddW are indeed required for metal-binding and DMSP lyase enzyme activity.

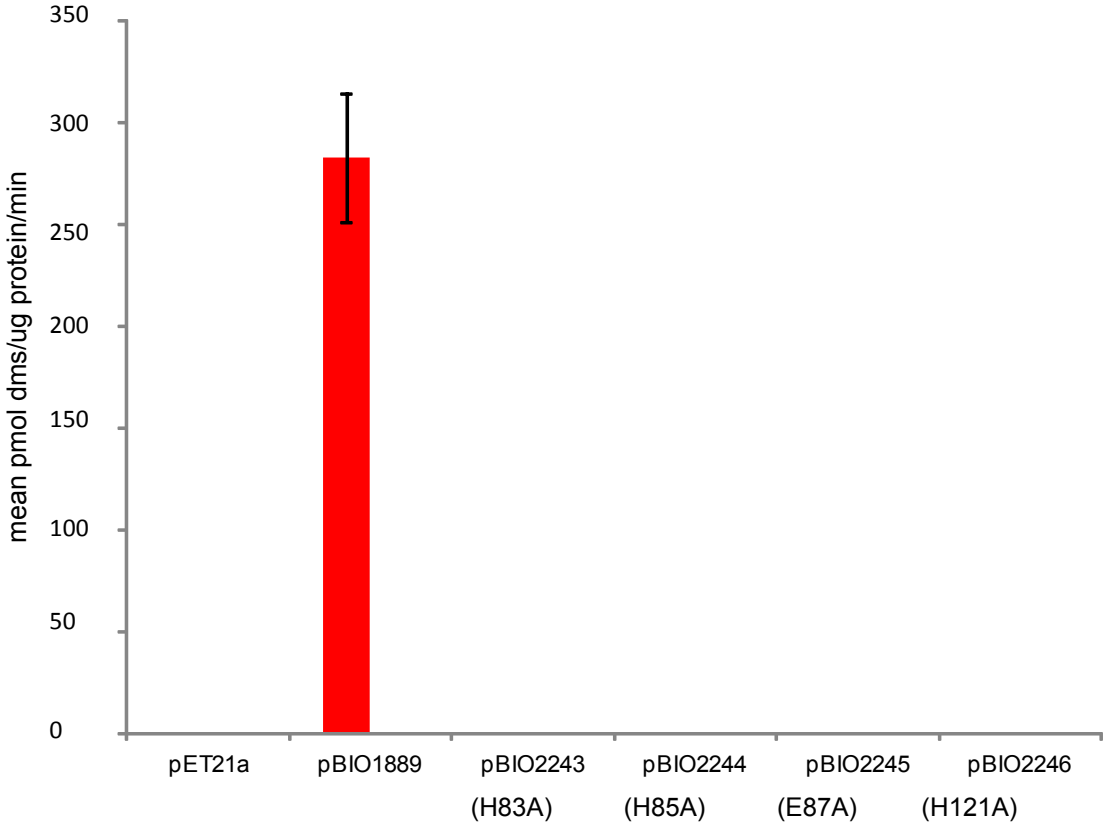



**Figure 4.15 DMS production from DddW with substitutions in metal-binding residues of cupin domain.** The different residues were substituted for an alanine to create four separate in-frame substitutions which were then subjected to DMS production determination via GC.

## 4.16 Summary

By purifying DddW and analysing its enzyme kinetics and protein characteristics, this work has identified DddW as a cupin-containing DMSP lyase that requires a metal cofactor for its function, and that the predicted metal-binding residues of the cupin domain are required for this cofactor binding. Following on from this work, *dddW* clones and site-directed mutants were sent to Adam Brummett and Mishtu Dey at the University of Iowa, USA, where a more detailed study of the enzymology of DddW and its metal dependency was explored (Brummett AE et al, 2015). The findings of this paper are discussed in chapter six.

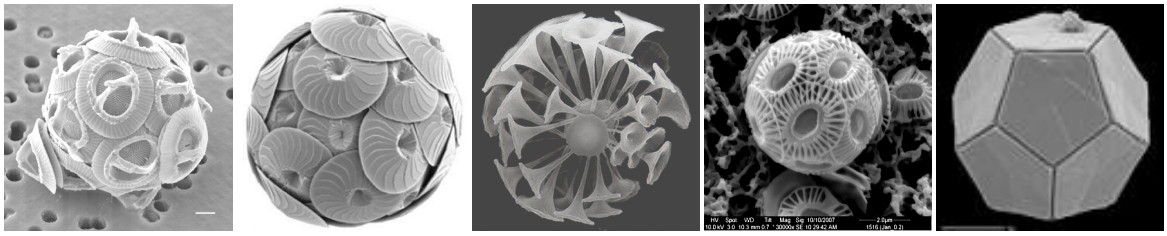
# 5 Biochemical Characterisation of DMSP Lyase Enzymes from the coccolithophore *Emiliania huxleyi*

## 5.1 Biochemical characterization of DMSP Lyase enzymes from *Emiliania huxleyi*

The primary aim of this chapter was to use the techniques applied successfully in the purification of the Ddd proteins from bacteria, as discussed in previous chapters, and apply them to the globally important DMSP and DMS-producing coccolithophore alga *Emiliania huxleyi*. Sequencing of the *E. huxleyi* 1516 genome (Read et al 2013) greatly enabled this study since it allowed any isolated candidate DMSP lyase proteins to be identified via mass spectrometry analysis and then compared to the genome to identify the corresponding gene. However, whilst this sequenced strain of *E. huxleyi* (CCMP1516) was available to us, we decided to use a different strain, *E. huxleyi* RCC1217, because it is known to produce higher quantities of DMS and DMSP than CCMP1516 and because, unlike 1516, it is a haploid strain. Sequencing of the haploid genome of *E. huxleyi* RCC1217 is currently underway at The Genome Analysis Centre (TGAC), Norwich, UK. As this chapter focuses on eukaryotic phytoplankton that were not covered in detail in chapter one, there now follows an introduction to the various phytoplankton groups, including coccolithophores like *E. huxleyi*, and details of what was known about their DMSP catabolism prior to the work described here.

## 5.2 Coccolithophores

The coccolithophores are a group of unicellular marine algae in the division Haptophyta and the class Prymnesiophyceae (Dubois et al., 2004). They range from 2 to 20 μm in size and are the most calcifying of the phytoplanktonic groups; indeed, they get their name from the calcium carbonate liths that they produce to form a shell around themselves (Figure 5.1). Coccolithophores are also known for being able to produce huge blooms in the temperate and sub polar oceans (oceans within close proximity to the polar oceans).



**a)** **b)** **c)** **d)** **e)**

**Figure 5.1 Examples of calcifying coccolithophores**.

*a) Gephyrocapsa oceanic*, b) *Calcidiscus leptoporus,* c) *Discosphaera tubifer,* d) *Emiliania huxleyi* (strain CCMP1516)*, Braarudosphaera bigelowi*

Many coccolithophores can calcify, producing calcium carbonate which results in the formation of microscopic calcite plates. These plates are called coccoliths and when present cover the outer surface of the cell. The plates are constructed in specialised vesicles before being released to the surface of the cell. The production of these calcite plates is highly regulated and is dependent on a continuous flux of $Ca^{2+}$ and $HCO_3$. Calcifying coccoliths play a significant role in the oceanic carbon cycle, as discussed in chapter one.

### 5.2.1 *Emiliania huxleyi*

*Emiliania huxleyi* evolved in the region of 268,000 years ago, and within the last 70,000 years has become the most important coccolithophore in the global ocean, occurring in most of our oceans with the exception of the Arctic and Southern oceans (Crutzen, 1983). *E. huxleyi* is currently the most abundant and globally widespread coccolithophore in the ocean, making up approximately 50% of the coccolithophore population (Brown and Yoder, 1994). *E. huxleyi* is also one of the smallest coccolithophores, possessing spherical cells of just 3-10 µm in diameter. As *E. huxleyi* is capable of living in the euphotic (sunlight) zone of the ocean, due to its tolerance to both temperature and salinity, and its environmental flexibility, it can produce massive blooms (Figure 5.2) covering areas as large as $2 \times 10^5$ $km^2$, with cell concentrations of $3 \times 10^6$ cells $L^{-1}$ (Sukhanova, 1998). Indeed, the ability of *E. huxleyi* to form such large blooms, and its role within the global biogeochemical cycles of carbon and sulfur, make it of particular interest in many

biological disciplines. As a consequence of such large bloom formations, *E. huxleyi* has a significant effect on local and global environments. As mentioned previously, *E. huxleyi* is a producer of calcium carbonate ($CaCO_3$) coccoliths, and, as a result, is responsible for a significant amount of carbon cycling via $CO_2$ consumption during photosynthesis and $CO_2$ generation during calcification (Westbroek P, 1993). *E. huxleyi* also contributes significantly to the transfer of calcite to the seabed via the settling of coccoliths following bloom termination. The calcifying strains of coccolithophores are thought to be responsible for around 50% of the carbonate precipitate in the oceans (Lu, 1993), and of all the coccolithophores, *E. huxleyi* is considered to be the main species involved in the formation of calcium carbonate (Bergamaschi, 2000, Zafiriou, 2003). In recent years, studies have established that various other coccolithophore species do not calcify (Wagener et al., 2009).

**Figure 5.2 *Emiliania huxleyi* bloom.** True colour satellite image of an *E. huxleyi* bloom south of Plymouth. http://www.sanger.ac.uk/about/press/2005/050811.html

In addition to coccolith formation and organic carbon production associated with calcifying strains of *E. huxleyi*, they are also regarded as high producers of the biogenic trace gas DMS and its precursor DMSP, which is generally thought to be

present in high intracellular concentrations (Rosenwald et al., 2002, Call et al., 2003, Simon et al., 2002)**.** They are capable of producing up to one hundred times more DMS than some other DMSP/DMS-producing phytoplankton species, such as the diatoms (Galperin, 2006).

Due to the relative ease of culturing and maintaining *E. huxleyi*, and the speed at which the species grow compared to other taxa, it has become the model coccolithophore employed for physiological, molecular, genomic and environmental studies.

*E. huxleyi* has three cell types (Call et al., 2003). The archetypal cells are diploid (2N) and are coccolith-forming (C type cells). These cells are encased by 10-15 coccoliths. There is, in addition, a mutant 2N cell termed N type (naked), which, whilst sharing the same morphological features as C type cells and the coccolith-forming apparatus, are unable to produce any coccoliths. N type cells are not thought to be part of a regular life cycle. The third cell type is the haploid (1N), termed S type (scale-bearing). These cells form a non-mineralized organic body scale which spreads over the surface of the cell membrane. S type cells are normally smaller (approximately 3-5 μm in diameter) than C type or N type cells (approximately 5-10 μm in diameter). 1N cells lack the coccolith-forming apparatus (Wang et al., 2003), but instead have two flagella which enable motility.

*E. huxleyi* has a complicated life cycle which is haplo-diplontic. This means it is capable of changing between the calcified, non-motile coccolith-bearing diploid and non-calcified, flagellated haploid. Until recently, most studies on *E. huxleyi* have focused on the diploid cells. However, as the structure of the haploid cells is particularly divergent from the diploid variants, it has become apparent that there is a significant lack of understanding surrounding the biology and evolution of this important species. Interestingly, haploid cells were recently shown to be resistant to some viruses which are fatal to diploid cells (Jaluria et al, 2007). This information indicates that haploid cells could therefore be involved with some function in the long term preservation of *E. huxleyi* by promoting survival inbetween bloom periods.

## 5.3 Eukaryotic DMSP-dependent DMS production

Eukaryotic algae were the first reported organisms to make DMS from DMSP, and all the early work was indeed carried out on eukaryotes. However, subsequently all the recent breakthroughs prior to this study came from work on prokaryotic DMS producers. Indeed, questions have been asked about whether marine eukaryotes, often studied using non-axenic cultures, actually have a significant role to play in DMS production, or whether it is the bacteria that are associated with them or in the open ocean that produce the majority of the DMS. The following section will provide a précis of the history of what is known about DMS production and DMSP lyases in marine eukaryotic organisms.

## 5.4 Algal DMSP lyases in history

To date, there have been few attempts to isolate and purify the genes and/or enzymes involved in DMSP catabolism in phytoplankton. Cantoni and Anderson (1956) were the first to isolate DMSP lyase activity and partially characterized the enzyme responsible. The study was carried out using crude extract from an epiphyte *Polysiphonia lanosa*, which belongs to the Rhodophyta division. Cantoni and Anderson investigated the cleavage of DMSP and established DMS, acrylic acid and a proton as the products of the reaction. It was determined that the DMSP lyase was contiguous with particles surrounding the plasma membrane, and although it was not obtained in a soluble state or purified, the optimal pH for the reaction was determined (pH 5.1).

In 1967, a study by Ishida and Kadota (Kadota, 1968) considered the effects of salts on the production of DMS from the mesokaryote dinoflagellate *Gyrodinium cohnii*. This study discovered that the reaction was stimulated by inorganic salt at high concentrations and that the pH optimum was 6.2.

In 1995, two papers were published reporting the isolation, purification and characterization of algal DMSP lyases. The first paper by Nishiguchi and Goff (1995) studied the red alga *Polysiphonia paniculata.* They isolated activity in two different proteins with determined molecular masses of 92 kDa and 36 kDa (Chapter 1, Table 1.4). Magnesium and calcium were found to enhance DMSP lyase activity, whilst the divalent chelator ethylenediaminetetraacetic (EDTA)

decreased the activity, this being the first indication that some DMSP lyases might require metal cofactors. Lyase activity was restored upon the removal of EDTA and addition of magnesium or calcium, similar to what we have reported for the bacterial DMSP lyases DddL, DddQ and DddW in chapters 2 and 3. The second report discussed the isolation of a lyase from *Enteromorpha clathrata* (now synonymous with the genus Ulva) (Steinke, 1996). Following ammonium sulphate precipitation at 35% (indicating high hydrophobicity) and the increase of lyase activity upon addition of detergent, this study, like others, suggested that the DMSP lyase was membrane-bound, and with a pH optima between 6.2-6.4.

A third study by de Souza et al. (1996) identified three hydrophobic and one soluble DMSP lyase isoforms from the membrane of the green macroalga *Ulva curvata* (Slonim, 2002, deSouza and Yoch, 1996)). Currently no ratified bacterial Ddd enzymes have been shown to be associated with membranes. All proteins were determined to be of the same molecular mass (78 kDa), with an optimum of pH 8 and demonstrated cross-reactivity with the DMSP lyase isolated from *Alcaligenes* M3A. However, it is impossible to rule out bacterial contamination in these studies as cultures were likely not axenic and there is no way to determine the origin of the DMSP lyase.

Probably the most significant study with regards to this project was the research performed by Steinke *et al* (1998) in which six strains of *E. huxleyi* were studied and a number of lyases characterized (Steinke et al., 1998). The study was directed towards the investigation and determination of the features of lyase activity with respect to pH and salt (NaCl) requirements. Like previous studies, some of the strains demonstrated lyase activity that was associated with the membrane. It was found that whilst the presence of a DMSP lyase appeared unaffected by the changing environmental conditions, the enzyme activity and characteristics varied a great deal between strains, and the activity levels of the enzyme did not correlate with intracellular DMSP concentrations. However, these findings are not that surprising as *E. huxleyi* is known to be a very divergent species with a complicated life style, as detailed in the section above. The results obtained by Steinke *et al* (1998) suggest that, similarly to the bacterial systems, there are different DMSP lyase isozymes. This study proposes that these

isozymes fall into two groups; those with higher activity, a sharply defined pH range (optimal at pH 6) and no salt requirements, and those with lower lyase activity, some requirements for high NaCl concentrations and a broader pH maxima with an optimal activity at pH 5. One strain was found to fall outside of these groups, with lyase activity increasing with pH up to the maximum tested (pH 8).

Similar to the previous study on *Enteromorpha clathrata, E. huxleyi* strain CCMP379 protein(s) associated with DMSP lyase activity precipitated between 10-30% $NH_4SO_4$, giving further evidence to suggest that some DMSP lyases are membrane-bound. Work over the last few years on marine eukaryotes clearly shows that there are likely DMSP lyases from distinct protein families operating in these diverse organisms. This is exactly what we see in prokaryotic organisms, which may have been the progenitor for these enzymes. However, as mentioned above, it is worth noting that no membrane-associated DMSP lyases have been identified in any bacteria. This perhaps suggests that the lyases may have a different function in different organisms, depending on their subcellular location.

Another interesting observation in *Ulva lactuca* is that the concentration of DMSP is adjusted according to the flow of $Na^+$ and $K^+$ ions in and out of the cell. This indicates that these ion pumps are related to the production and/or breakdown of DMSP in order to maintain good cellular health (King, 1999). If the DMSP lyase was associated with a membrane bound ion exchange system then DMSP conversion could be regulated with the flow of $Na^+$ ions. The alliance of an intracellular ion exchange system to a DMSP lyase could allow the rate at which DMSP is broken down to be controlled on the basis of levels of osmotic stress. Furthermore as reported Nishiguchi and Goff, if the DMSP lyase is a membrane-bound enzyme then its location would be well-suited to it responding to osmotic changes.

## 5.5 Attempts to identify eukaryotic DMSP lyases

In the work described for this project, two different approaches were used in order to identify the presumed DMSP lyase of *E. huxleyi*, which are detailed in the following pages.

### 5.5.1 Bioinformatics analysis of Eukaryotic genomes for potential DMSP lyases

Following the discovery and characterization of the bacterial DMSP lyase genes, a bioinformatic approach was used in an attempt to identify potential DMSP lyases in eukaryotes. This study used the NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990), Joint Genome Institute (JGI) database and National Center for Biotechnology Information (NCBI) databases. With the advancement in sequencing technology, many marine eukaryotic genomes are being sequenced, but there is also data available in databases of expressed sequence tags (EST) and assembled transcriptomes. These datasets potentially offer a means to identify DMSP lyase genes in marine eukaryotes.

The protein sequences of all known bacterial and fungal DMSP lyases were used as probes against sequenced eukaryotic genomes in EST datasets and transcriptome assemblies

The following section describes the bioinformatic methods employed and approaches to the identify DMSP lyase enzymes in marine eukaryotes.

#### 5.5.1.1 DddY

DddY proteins were only found in a relatively small number of beta, gamma, delta and epsilon proteobacteria at significant homology (40%+), as reported by Curson et al (Curson, 2011c). However, the more recent availability of additional genome sequence data shows that there is a DddY homologue (31% identity, E = $2e^{-50}$) in the Cyanobacterium *Synechococcus* sp. KORDI-100. If shown to produce DMS from DMSP, this would be the first report of a photosynthetic Cyanobacterium with the ability to lyse DMSP, but unfortunately the strain was lost since post isolation

so was not available to us for testing. There are no significant homologues of DddY in any available EST or transcriptome datasets, consistent with the fact that *dddY*, unlike many of the other bacterial lyase genes, is absent from marine metagenomic datasets (Curson et al., 2011a, Curson et al., 2011b).

#### 5.5.1.2 DddP

Homologues of ratified DddP enzymes can be found in α-proteobacteria (primarily roseobacters, but also members of the SAR116 clade), γ-proteobacteria, and some Ascomycete fungi, likely as a result of horizontal gene transfer from bacteria. Interestingly, a DddP homologue in *Lyngbya confervoides*, a marine Cyanobacterium that can cause human skin irritation known as 'seaweed dermatitis', and it would be interesting to see if this Cyanobacterium is $Ddd^+$. DddP is also found in ascomycete transcriptome and cDNA datasets, and we know that these proteins are likely functional from previous work on fungal DddPs (Todd et al, 2009) (Kirkwood et al 2010). Interestingly, there is also a gene encoding DddP-like protein in the transcriptome files for the aggregating anemone *Anthopleura elegantissima*. This putative DddP is 37% identical at the amino acid level to DddP2 of *Oceanimonas*. Again, it would be of interest to see if it is a functional DMSP lyase, as some anemones have been shown to contain intracellular DMSP (Borell et al., 2014; van Alstyne et al., 2015), but little known, at least at the molecular level, about DMS production in these marine organisms. However, no homologues of DddP were identified from any available algal sequences.

#### 5.5.1.3 DddQ

Close homologues of DddQ are so far restricted to α-proteobacteria (mainly Roseobacters). No significant sequence hits to DddQ were found in eukaryotes in the EST or transcriptome datasets.

#### 5.5.1.4 DddL

The DddL lyase is found in α-proteobacteria (mainly roseobacters), a few species of γ-proteobacteria and δ-proteobacteria, and one Actinomycete. There are no significant matches to DddL in any eukaryotic EST sequences, but there was one sequence in the transcriptome data, in the marine zooplankton *Calanus*

*finmarchicus*, with 27% identity to DddL of *Sulfitobacter* EE-36. This level of homology is below that seen for ratified bacterial lyases, but as a eukaryote, it might be expected to be distantly related at the sequence level, if it was indeed a DMSP lyase sequence. However, this protein has an AdoMet methyltransferase domain towards its N-terminus, which has not previously been seen in any DMSP lyase enzyme and should not be required for lyase activity, and marine zooplankton are not reported to produce DMS, so it seems unlikely that this sequence would be a DMSP lyase.

#### 5.5.1.5 DddW

DddW is the least abundant of the DMSP lyases with just two ratified lyases (in the Roseobacters *R. pomeroyi* DSS-3 and *Roseobacter* MED193), although, as discussed in chapter three, the recent availability of more bacterial genome sequences indicates additional DddW lyases in alphaproteobacteria. However, searches of transcriptomes or EST datasets did not reveal any DddW homologues in marine eukaryotes.

#### 5.5.1.6 DddD

The atypical DMSP lyase DddD is found in some alpha-, beta- and gammaproteobacteria. DddD is characterized by having tandem CaiB-like type III acyl-CoA transferase domains, with all functional DddD proteins identified so far having two CaiB-like domains (Todd et al, 2007, Curson et al 2011c).

When DddD proteins are subjected to BLAST analysis, there is a clear cut-off below which DddD-like proteins have been shown not to encode functional DMSP lyases. This cut-off is at ~46% identity at the protein level, meaning that DddD homologues in *R. pomeroyi, Streptosporangium viridialblum* and *Dinoroseobacter shibae*, which have all been tested (J. Todd, personal communication), are not functional DMSP lyases. EST sequence data consists of relatively short reads that are not sufficient to distinguish DddD from non-DddD CoA transferases with a single CaiB domain, which are known to share significant sequence similarity with DddD (Todd et al., 2007). There are hits to DddD in transcriptomes of the haptophyte *Prymnesium parvum* with 36% identity to DddD of Halomonas, *Lingulodinium polyedrum* with 26% identity, and the dinoflagellate *Alexandrium*

*tamarense* also showing 26% identity. However, when aligned to known DddD proteins, it is clear that they are more closely related to the non-funtional DddD-like proteins of, for example, *Dinoroseobacter shibae*.

In all these bioinformatic searches using bacterial lyase sequences, by far the most promising proteins identified in marine eukaryotes were two DddD-like enzymes indentified in the genome of *E. huxleyi* strains CCMP1516, EMIHUDRAFT_462647 and EMIHUDRAFT_469489, which showed 35% and 37% identity to DddD of *Halomonas.* These are tandem CaiB domain proteins but, like the eukaryotic DddD homologues described above, are most similar to the non-functional *D. shibae* DddD, and therefore, based on sequence homology alone, were perhaps unlikely to function as DMSP lyases.

### 5.6 The *E. huxleyi* DddD-like proteins

Although it was unlikely that the DddD-like enzymes in *E. huxleyi* were functional enzymes, these proteins were nevertheless investigated as potential DMSP lyases in work conducted by Stephane Lefebvre at the University of Essex in collaboration with Dr Michael Steinke. Both EMIHUDRAFT_462647 and EMIHUDRAFT_469489 were amplified from *E. huxleyi* CCMP 1516 cDNA and cloned into pET21a, but when expressed in *E. coli* BL21 neither protein showed any DMSP lyase activity. Of course, it could be that these eukaryotic proteins require codon optimization or more specific post-translational modifications in order to function, which would not occur when expressed in *E. coli*. For this reason, the two DddD-like proteins were produced using a rabbit reticulase system. This cell free system is employed due to its low background and efficient utilization of exogenous RNAs. However this still did not yield proteins with DMSP lyase activity (personal communication Dr J Todd). It is still possible that these proteins are involved in DMS generation in *E. huxleyi*, but that they require post-translational modifications that only *E. huxleyi* or related marine eukaryotes can produce. This could be determined by gene knockouts or overexpression in *E. huxleyi*, but unfortunately these methods are not currently easily available in *E. huxleyi*.

## 5.7 Cupin-containing proteins in *E. huxleyi*

Since many of the bacterial Ddd enzymes contain cupins and are metal-dependent (see chapters two and three), it was hypothesised that at least one of the eukaryotic DMSP lyases might also contain a cupin domain, especially as it is also known that the known bacterial DMSP cupin enzymes can possess variable sequence identity (chapters three and four). Therefore the *E. huxleyi* CCMP 1516 genome was screened for proteins that contained a cupin that was not combined with other complex domains, suggestive of an alternate function. Using PFAM classification, we scanned the cupin-containing proteins encoded by the *E. huxleyi* CCMP1516 genome and identified one single cupin domain-protein. The corresponding gene, which was only found in *E. huxleyi*, was targeted as a possible DMSP lyase and encodes a small 184 amino acid protein with an estimated molecular weight of 22 kDa and a cupin domain at its C-terminus. The gene was synthesized so as to be codon-optimised for *E. coli*, with a ribosome binding site to allow for expression in the vector pUC57. This clone was assayed in *E. coli* but no $Ddd^+$ activity was observed above that of the buffer control. The simplest explanation for this is that this cupin-containing candidate may simply not function as a DMSP lyase. Alternatively, the protein could require post-translational modifications to function, as described above, that would not occur when expressing in *E. coli*.

## 5.8 Purification of DMSP lyases from *Emiliania huxleyi* RCC1217

As attempts to isolate a eukaryotic *ddd* gene led by bioinformatics were not successful, we decided to try and build on the earlier DMSP lyase purification work (described above) by working with a model organism. Due to its abundance and bloom-forming nature, *E. huxleyi* is considered to be a major producer of DMS. For this reason, this coccolithophore was targeted as the model eukaryotic system to work on for isolating DMSP lyases. *E. huxleyi* RCC1217, a haploid non-coccolith-forming strain, was used because of its smaller gene number, ease of culturing in the lab, and because its genome is currently being sequenced at The Genome Analysis Centre (TGAC), Norwich. Also, most importantly, RCC1217 makes considerably more DMSP and DMS than CCMP1516, thus indicating that it might contain more active or actively expressed DMSP lyases. The following sections

describe the attempts to purify DMSP lyase enzymes from *E. huxleyi* RCC1217, for the first time from an axenic *E. huxleyi* strain which had been confirmed to be free of bacteria.

### 5.9 Growth rate and optimum DMSP lyase activity determinations

A preliminary growth experiment was conducted to elucidate the growth rate and phases of *E. huxleyi* RCC1217 using culture conditions described in Materials and Methods. The cultures were monitored for density and health via Coulter counting, DAPI staining and 16S rRNA PCR. Exponential growth was determined to last five days and the calculated growth rate was 0.9951 (Figure 5.3). DMSP lyase activity was monitored at various points during the life cycle and it was determined that lyase activity was most significant during the stationary phase of growth.

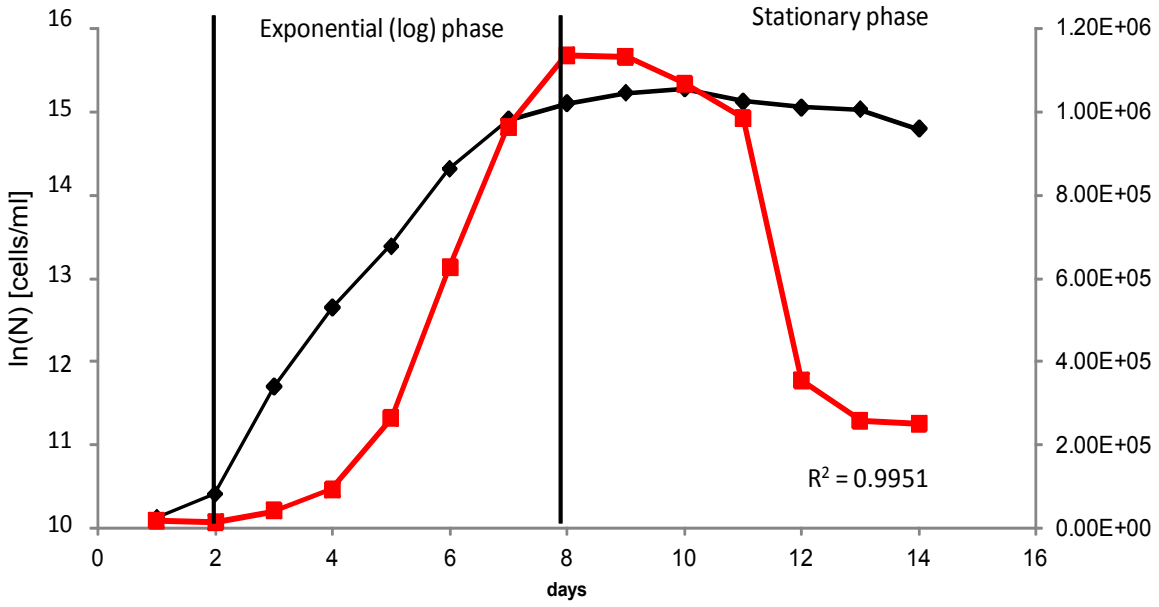
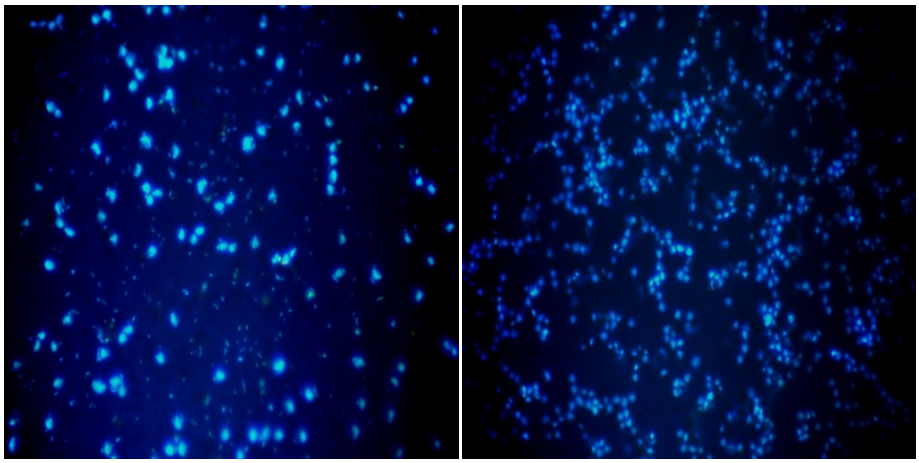



**Figure 5.3 Demonstrating growth of *E. huxleyi* 1217** inoculated with an initial cell count of 25,000 cells/mL (n=3) and mean growth rate (μ) = 0.9951 and DMS production (red).

## 5.10 Testing of *E. huxleyi* culture axenicity by DAPI staining

In order to determine whether cultures of *E. huxleyi* were axenic a nucleic acid DAPI staining method was used in which 4',6-diamidino-2-phenylindole (DAPI) was introduced to culture aliquots and followed by epifluorescence microscopy. The presence of bacteria was checked by examining the filters under UV light using a fluorescence microscope. Bacteria appeared as small bright dots in the background of large algal cells and cultures of *E. huxleyi* RCC1217 were shown not to have any visible bacterial DAPI staining, indicating that they were indeed axenic cultures (Figure 5.4).



A) B)

**Figure 5.4 DAPI staining of *Emiliania huxleyi* cells highlights bacterial contaminants in cultures. A) healthy axenic culture, B) contaminated culture.**

## 5.11 Phylogenetic analysis and confirmation of axenic cultures by 16S rRNA sequencing

The genes for ribosomal RNA (rRNA) are conserved at the molecular level between enzymes. Variations between 16S rRNA sequences of bacteria and of eukaryotic plastids and mitochondria provide us with evidence as to how closely or distantly related various organisms are to one another. As the 16S rRNA gene is short (1.5 kb), it can be easily amplified and sequenced. This enables

comparisons of rRNA from environmental samples or laboratory grown cultures so we can determine whether previously known or new microbes are present in a particular environment. It also helps to confirm the axenic nature of algal cultures. Therefore, to check the axenic status of *E. huxleyi* 1217 cultures, 16S rRNA PCR was carried out on genomic DNA isolated from *E. huxleyi* RCC1217 cultures. The PCR products were purified and sent for sequencing. In axenic cultures, the 16S rRNA sequences would be expected to only have significant identity to plastid rRNA from other haptophytes, including other strains of *E. huxleyi*, rather than the bacterial sequences you would expect if the cultures were contaminated with bacteria. This method confirmed, as with the DAPI staining described above, that the *E. huxleyi* RCC1217 cultures used for the DMSP lyase purification experiments described in the following sections were axenic cultures, not contaminated with bacteria or other algae (Figure 5.5).

### 5.12 Demonstration of DMSP lyase activity from cell extracts

Cell pellets of *E. huxleyi* were lysed using either sonication for small volumes (< 5ml) or the French press method, as described in previous chapters for DddL, DddQ and DddW, for larger volumes. Initially, pellets were resuspended in buffer containing 50 mM sodium acetate pH 4. The lysates were assayed for Ddd activity and were found to be active.

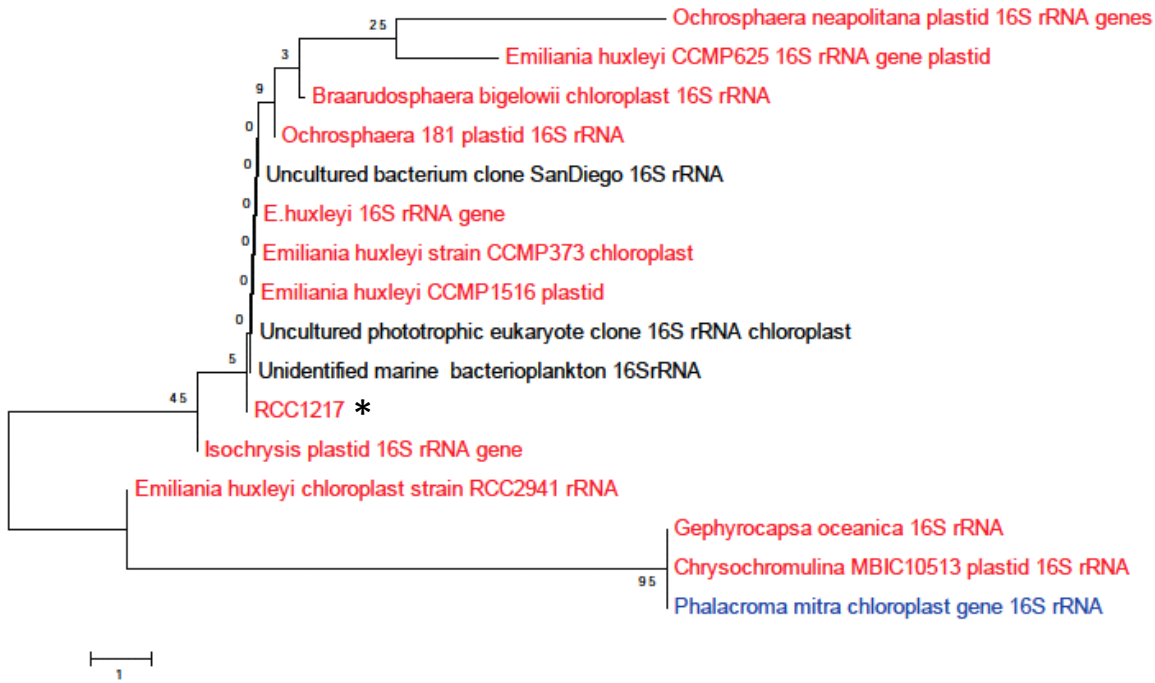



**Figure 5.5 Phylogenetic tree of 16S rRNA sequencing results.** *E .huxleyi* RCC1217 shares homology with other strains of haptophytes (red) and dinophyta (blue); RCC1217 is highlighted with an asterisk. The scale bar indicates the number of substitutions per site. Bootstrap values of 1000 replicates are given at each branchpoint.

## 5.13 Determination of appropriate assay buffer

Cell extracts were lysed in three different buffers including: 1) 50 mM succinic acid, 2) 50 mM sodium acetate and 3) 50 mM N-Cyclohexyl-2-aminoethanesulfonic acid (CHES), tris(hydroxymethyl)methylamine (Tris), Tris sodium citrate and dipotassium hydrogen phosphate (mixed buffer).

To these solutions, 50 µl of active protein solution and 5 mM DMSP was added. Following incubation the samples were assayed for Ddd activity. There was little difference between the lyase activity seen with the three tested buffers as can be seen in Figure 5.6, but highest level of Ddd activity was observed sodium acetate

pH 7 an this buffer was subsequently used for determination of the pH optima (section 5.14).

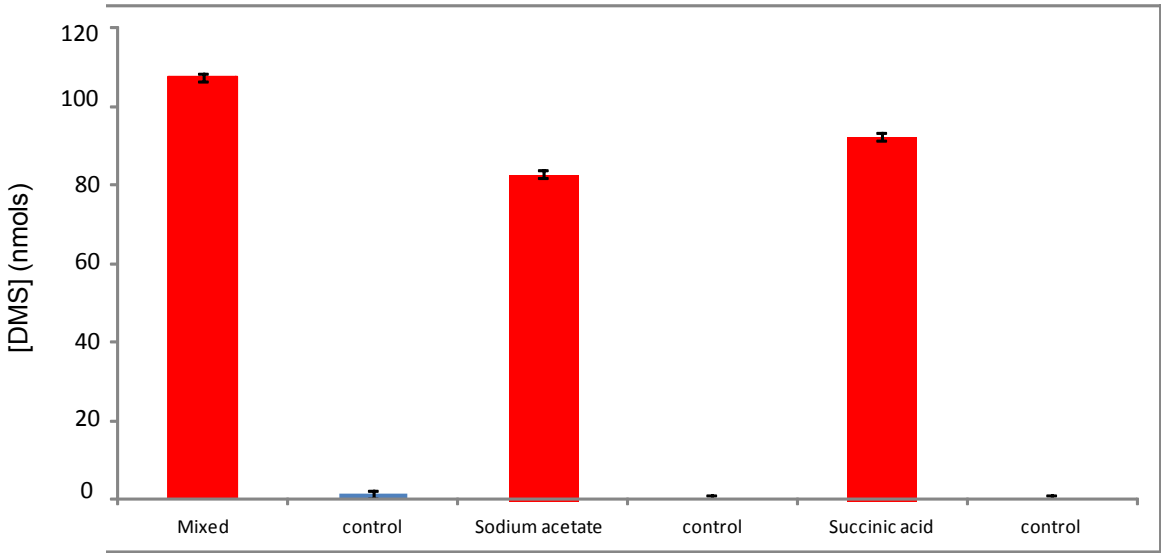



**Figure 5.6 Determination of appropriate buffer for optimum lyase activity** using sodium acetate, CHES, and tris(hydroxymethyl)methylamine (Tris), Tris sodium citrate and dipotassium hydrogen phosphate buffers at 50mM concentrations.

### 5.14 pH optimum of DMSP lyase

Sodium acetate buffer at a range of different pH (2-11) was used to assay the Ddd activity of the *E. huxleyi* RCC1217 lysate as above. As seen in Figure 5.7, the lyase activity peaked at pH 4, but a second peak, seen at pH 7, suggested that different isozymes might be present. Significant activity was seen over a broad range of pH, whilst a considerable fall in lyase activity was seen below pH 3 and above pH 9. Thus, for lyase purification, sodium acetate at pH 7 was used in chromatographic separations.

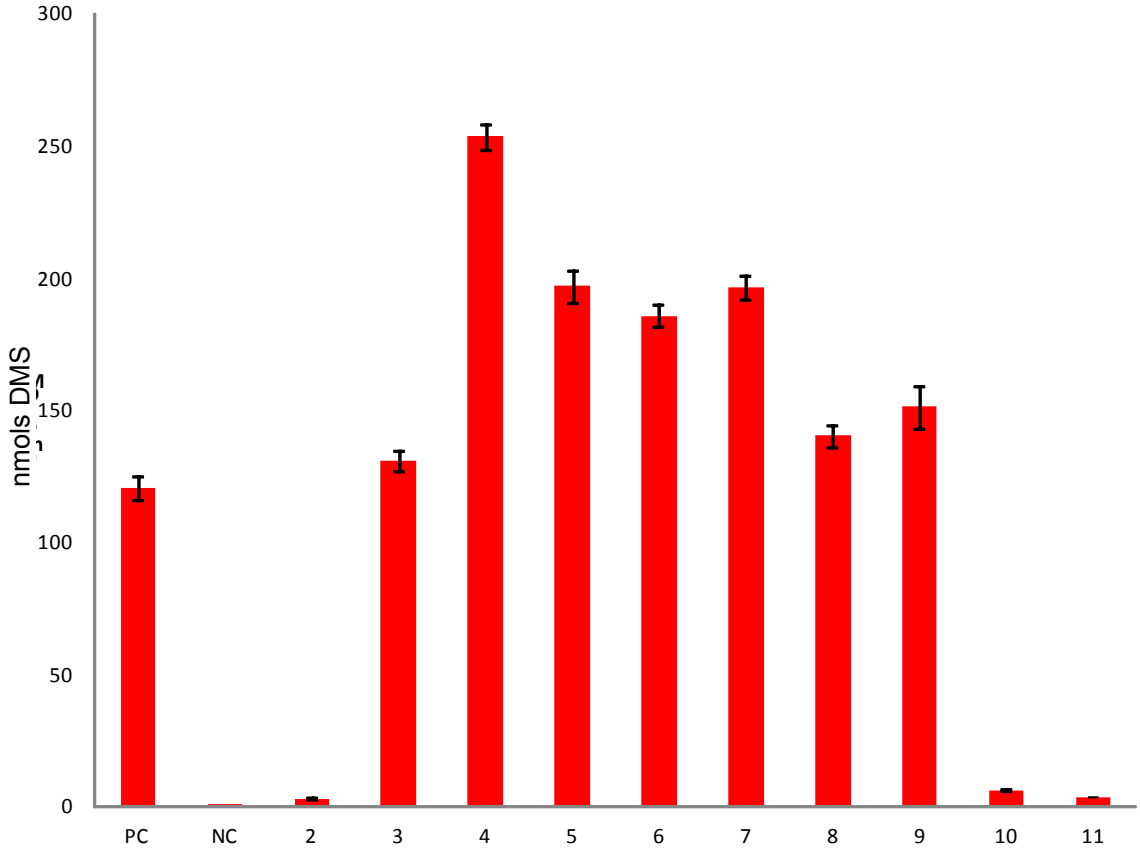



**Figure 5.7** Determination of appropriate pH for optimum lyase activity was achieved using a sodium acetate buffer at various pH values.

## 5.15 Detergent optimization

Some studies have suggested DMSP lyases are associated with the membrane so a number of different detergents used for membrane solubilisation were tested. The extent of the solubilisation and the stability of the solubilised protein is dependent on the detergent type and concentration. It is not possible to give general rules for either of these variables and they require experimental optimisation. However, the presence of detergents can have significant effects on subsequent chromatographic purification steps so should be taken into account. The buffer concentration can have an effect on the solubility and stability of proteins. Phosphate buffers are known for their solubilising properties, with concentrations of 50 mM to 500 mM increasing the solubility of membranes proteins. Results showed that water soluble fractions contained the same high

Ddd activity as the membrane fraction (Figure 5.8). For this reason, further purification techniques treated fractions as non-membrane-associated proteins and detergents were therefore not used.

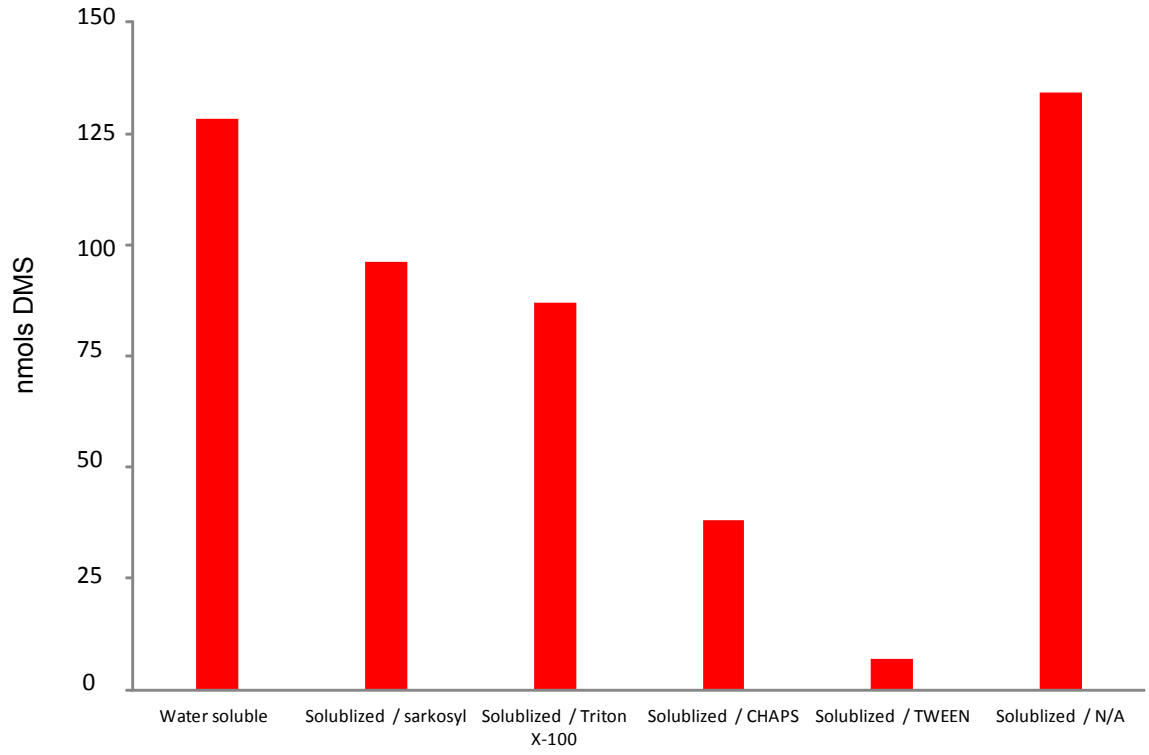



**Figure 5.8 Membrane / detergent solubilisation test** performed using phosphate buffer at 50mM and various detergents (sarkosyl, triton X-100 and TWEEN).

## 5.16 Hydrophobic interaction purification

The first separation technique employed was hydrophobic interaction chromatography. A volume of 20 litres of stationary phase *E. huxleyi* culture was concentrated to ~50 ml. The cells were lysed via sonication and cell debris removed by centrifugation before removal of membranes via ultra centrifugation. Subsequently 50% (w/v) $(NH_4)_2SO_4$ was added to the lysate and precipitated protein removed via ultra centrifugation. The lysate was then loaded onto a phenyl sepharose column equilibrated with 20 mM Tris containing 50% (w/v) $(NH_4)_2SO_4$. Proteins were eluted using a 50–0 % $(NH_4)_2SO_4$ gradient (Figure 5.9).

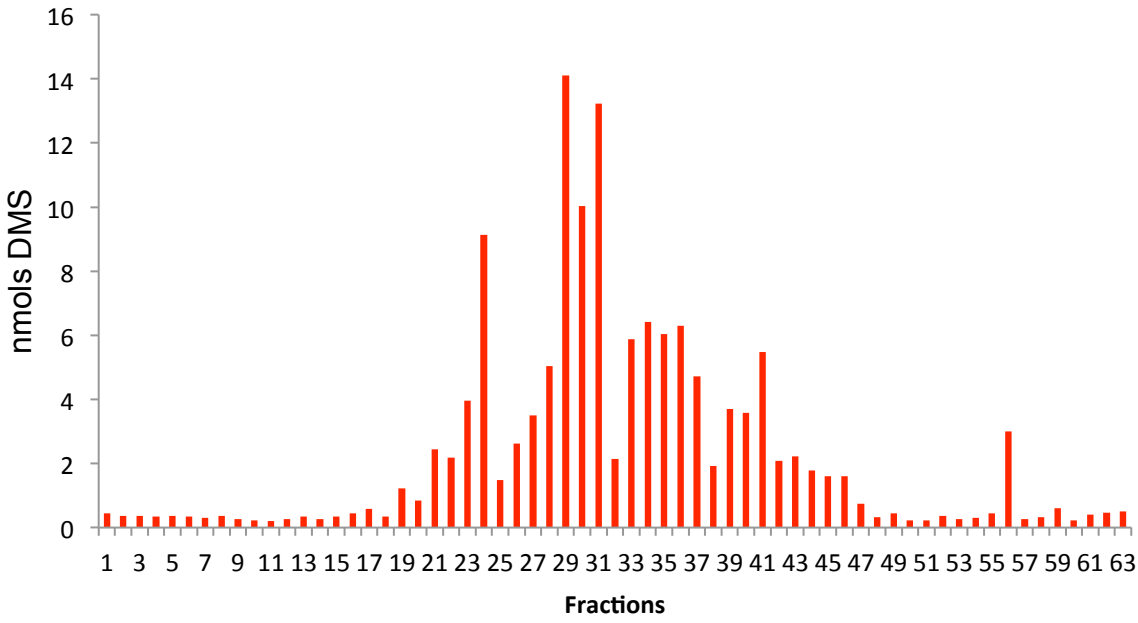



**Figure 5.9 Elution profile of RCC1217 from Phenyl Sepharose Column.** Cell lysate was saturated to 50% (w/v) with $(NH_4)_2SO_4$. Fractions were assayed for DMS production to determine levels of DMSP lyase activity in each fraction.

### 5.17 Anion exchange purification

The next separation method used was an anion exchange column (DEAE). A 5 ml concentrated sample of *E. huxleyi* RCC1217 was applied to the column. Eluted fractions were collected and analysed for Ddd activity via gas chromatography and SDS –PAGE as described in materials and methods. DMSP lyase activity eluted from the DEAE column appeared to pass straight through the column and was present in the flow through. This suggests that there was little interaction between the lyase proteins and the stationary phase. However, there was the suggestion of a smaller second peak of activity was suggested between fractions 8-12 (Figure 5.10).

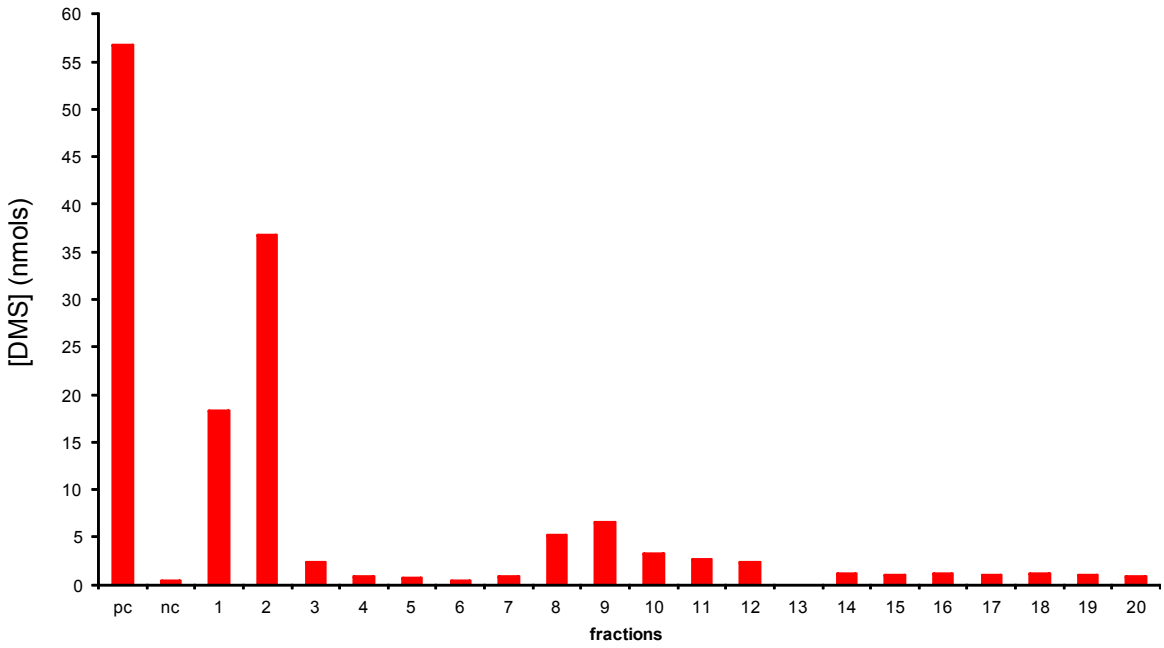



**Figure 5.10 Elution profile of RCC1217 from a DEAE** column relative to active fractions ([DMS] nmoles).

## 5.18 Gel filtration purification

Fractions containing significant activity from the anion exchange step (including the flowthrough) were retained and concentrated to a smaller volume (~5 ml) before being loaded onto a Superdex 200 gel filtration column. Whilst some fractions collected from the gel filtration column gave significant Ddd activity, those with the highest lyase activity eluted close to the expected void volume ($V_O$), so this may be as a result of dimer and trimer structures. The void volume is the elution volume of the molecules that are expelled from the gel filtration medium since they are bigger than the largest pores in the column matrix and hence pass straight through the packed bed. Eluted fractions, as with previous purification steps, were collected and analysed for Ddd activity via gas chromatography (Figure 5.11) and protein identification via SDS-PAGE.

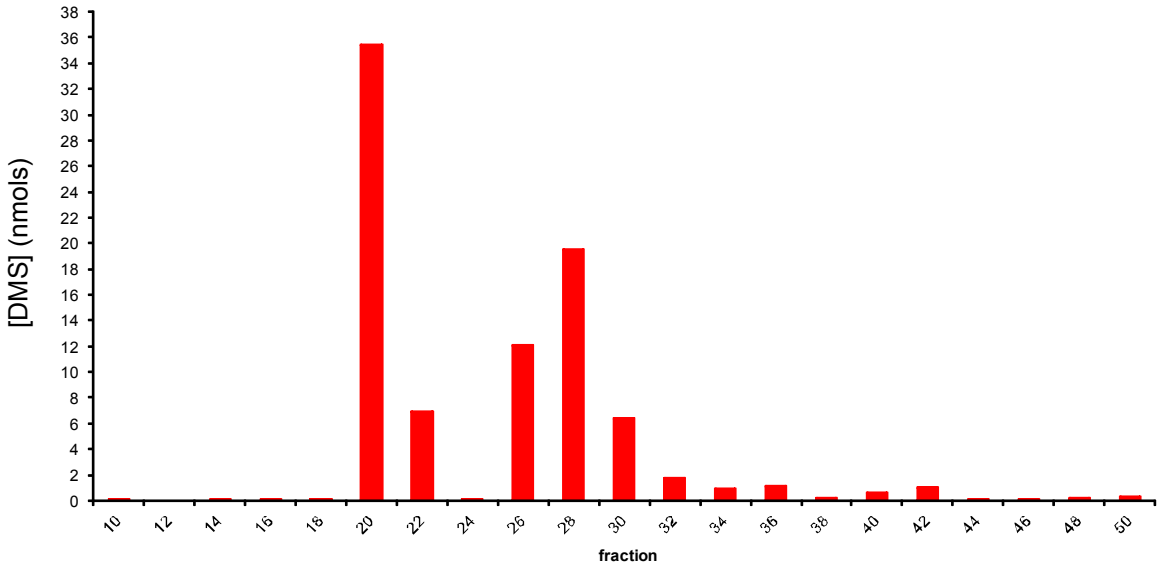



**Figure 5.11 Elution profile of RCC1217 from gel filtration column** relative to active fractions ([DMS] nmoles).

## 5.19 SDS gels

A fraction eluted from the gel filtration column which had significant DMSP lyase activity was loaded onto an SDS-PAGE gel. As Figure 5.12 clearly shows this fraction contained four distinct bands corresponding to proteins of ~120 kDa, ~50 kDa, ~30 kDa, and ~25 kDa.

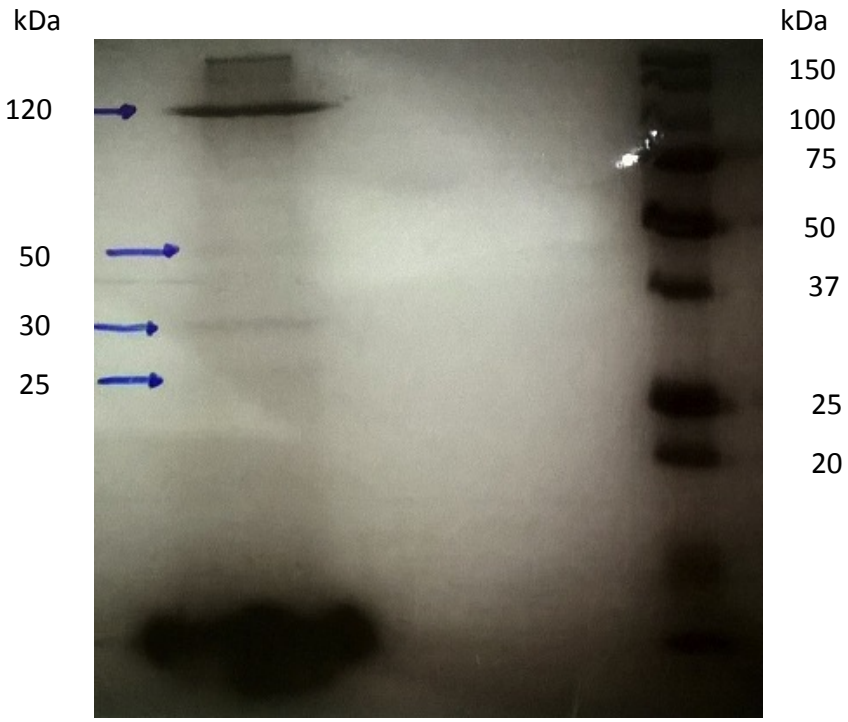



**Figure 5.12 SDS-PAGE analysis of active protein sample isolated from RCC1217** 12% acrylamide gels stained with Coomassie blue. Four bands were detected as highlighted.

After initial identification of bands, further gel analysis was conducted using a sample containing protein with known DMSP lyase activity, and compared against protein-containing fractions with no lyase activity (Figure 5.13). There was a band with a molecular weight between 100 and 150 kDa that was present in the high Ddd activity fractions but not in the fractions without Ddd activity. When this band was excised from gels and assayed for activity, no activity was detected. This suggests that, if this protein is a functional DMSP lyase, it is not capable of refolding to form the functional enzyme under these conditions.

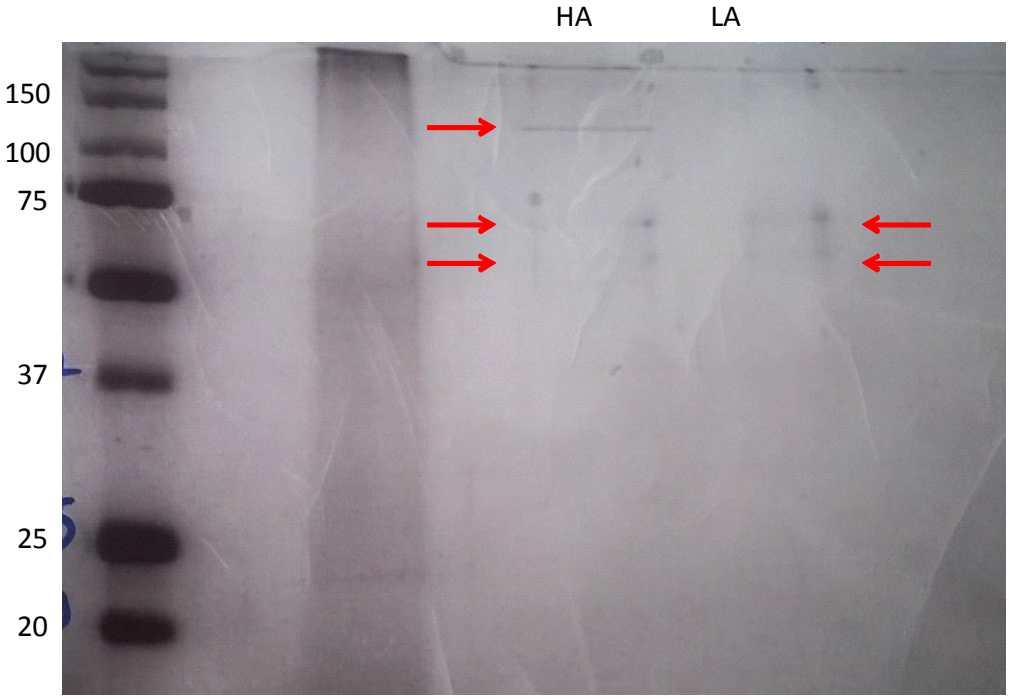



**Figure 5.13 RCC1217 protein separation by SDS-PAGE on 12% acrylamide gel and stained with coomassie blue.** Lane 1 Precision Plus Protein Standard. Lane 4 high Ddd activity fraction (HA), Lane 5 low Ddd activity fraction (LA) (as seen in buffer). A band at ~ 120 kDa in the protein sample which exhibited high Ddd activity is not seen in low activity fractions. Bands are indicated by red arrows.

## 5.20 MALDI-TOF

The four bands present on the SDS-PAGE gel seen in Figure 5.12 were extracted and analysed by MALDI-ToF. MALDI-ToF is a soft ionization technique used for molecules which are fragile and fragment when ionized by other ionization methods. A specific mass spectrograph is obtained and compared to a spectrograph database to see which molecule is most closely matched in order to identify it. Although the *E. huxleyi* RCC1217 genome was being sequenced, data for this genome is still not in a state where we could search against it using our MALDI-ToF data so the existing *E. huxleyi* CCMP1516 genome was used. However, no significant matches were obtained when the MALDI-ToF data was compared against the CCMP1516 genome. It is possible that CCMP1516 lacks this RCC1217 protein and that this approach might prove more successful once

the RCC1217 genome becomes available, but at present this method has not been successful in identifying whether this excised protein band represents a functional DMSP lyase.

## 5.21 Identification of $^{14}C$ acrylate via High Performance Liquid Chromatography (HPLC)

DMSP lyases can be typical or atypical, generating acrylate (DddY, DddP, DddQ, DddL, and DddW) or 3-hydroxypropionate (DddD) respectively as co-products. In order to establish if acrylate was produced from the *E. huxleyi* DMSP lyase(s), [$^{14}C$] DMSP (1 mM) was added to a sample of RCC1217 culture and or fractions from the DEAE gel filtration purification step, all of which had previously been shown to have Ddd activity. After overnight incubation, samples were analysed by HPLC (see Materials and Methods). The results showed that all the *E. huxleyi* cells/protein fractions generated acrylate as a product of the DMSP lyase reaction (Figure 5.14), and therefore this indicates that *E. huxleyi* RCC1217 possesses at least one typical DMSP lyase, producing DMS and acrylate from the DMSP substrate. In addition, no [$^{14}C$] 3HP was detected in these experiments indicating that no 'atypical' DddD-type lyase activity is present in *E. huxleyi*, at least under the conditions tested here.

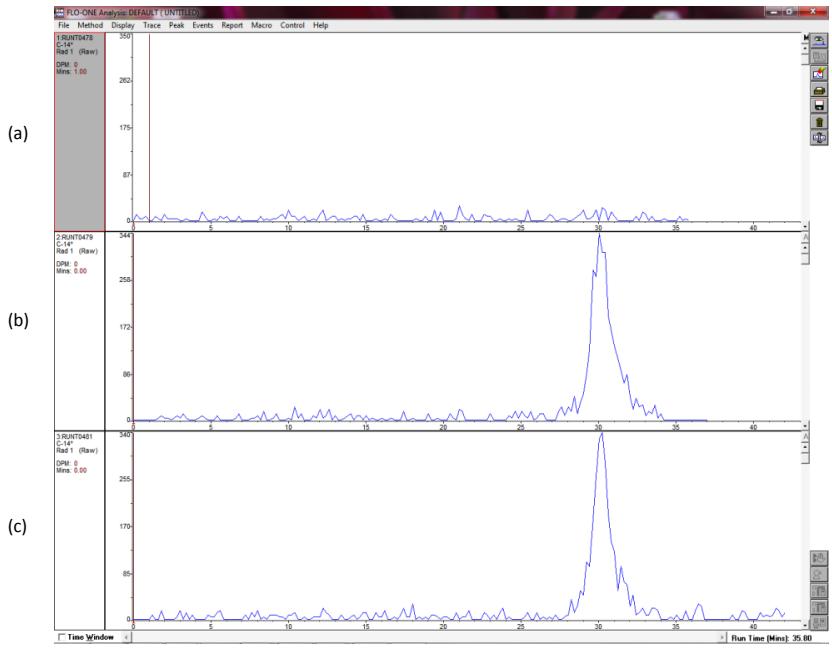



**Figure 5.14 Acrylate production from DMSP by *E. huxleyi* RCC1217** analysis of (a) buffer sample with 1 µL [$^{14}$C] DMSP (1 mM), (b) RCC1217 DEAE purification fraction with 1 µL DMSP [$^{14}$C] DMSP (1 mM), (c) spiked sample (10mM [$^{14}$C] acrylate)

## 5.22 Summary

Studies so far have demonstrated that the RCC1217 DMSP lyase(s) are moderately robust. They are capable of withstanding very low temperatures (when being stored at -80 °C) and a wide range of pH. The identification of acrylate as a product indicates that the lyase active in RCC1217 is a typical DMSP lyase. There are likely multiple DMSP lyases in *E. huxleyi*, as seen in the bacteria e.g. *Roseovarius nubinibens* and *R. pomeroyi* (Segre et al., 2006). It is likely that the DddD homologues identified in *E. huxleyi* do not encode functional DMSP lyases since no activity was detected when attempts were made to express them and *E.*

*huxleyi* cells do not appear to produce 3-hydroxypropionate, a product of the DddD DMSP lyase reaction.

Our methods have shown that DMSP lyase activity could be purified and clarified from *E. huxleyi* RCC1217 cells. However, more sophisticated methods, such as, for example, mass spectroscopy technologies, may be necessary to identify the proteins involved. This is clearly highlighted in the recent nature paper (Alcolombri et al., 2015) identifying that there are indeed multiple DMSP lyases in *E. huxleyi.* The findings of this chapter are placed in context of (Alcolombri et al., 2015) in chapter six.

# 6 Discussion

## 6.1 Foreword

The overall objective of the work described in this thesis was to develop an understanding of the cupin-containing DMSP lyases in marine bacteria and explore the presence of DMSP lyases in the coccolithophore *Emiliania huxleyi.* This has been achieved in several ways. We have confirmed that the cupin motifs within the DMSP lyases of DddL, DddQ and DddW are essential for DMSP lyase activity and likely comprise the active sites for these enzymes. It was also demonstrated that these three cupin-containing Ddd enzymes all require metal cofactors for function and site-directed mutagenesis of predicted metal binding amino acids also abolished enzymatic activity. This study confirmed that a functional DMSP lyase, producing DMS and acrylate as products, exists in *E. huxleyi* strain RCC1217, showing that these important phytoplankton do indeed generate DMS, as widely predicted, but shown here for the first time in cultures free of bacteria.

The following chapter explores the results of my work in more detail, and in relation to recently published data.

## 6.2 Cupin-containing DMSP lyase enzymes

Cupins are found in a vast array of organisms from thermophillic bacteria to plants and animals, and in organisms that inhabit a variety of diverse environments. The prevalence of the conserved cupin domain in prokaryote and eukaryote proteins, which embrace a whole variety of enzymes and binding proteins, led to the conclusion that it is the small beta barrel structure that makes the cupin core (Dunwell, 1998, Woo et al., 2000) and provides a secure scaffold for the protein to survive and function.

The huge diversity exhibited by cupin proteins is certainly reflected in the DMSP lyase cupins. At the amino acid level, other than the conserved residues within the cupin motif, the sequences of these DMSP lyases differ significantly, and it is not until they are observed on a structural level that conformity can be observed.

The cupin superfamily contains a vast number of small polypeptides with, as mentioned previously, a characteristic beta-barrel structure, and they often

possess metal-binding abilities. Members of this family have an a immense range of different functions including enzymatic activation, transcriptional regulation and modification of cell wall carbohydrates (Dunwell et al., 2004). Evidently these functions include cleavage of DMSP. We now know that no fewer than three different cupin type DMSP lyases (DddL, DddQ, and DddW) exist. Given the limited sequence similarity of DddL, DddQ and DddW it is very difficult to predict cupin-containing DMSP lyases from amino acid sequences alone. The presence of a C-terminal cupin domain is not always a prerequisite of DMSP lyase functions, as exemplified in *E. huxleyi* with the non-cupin Alma1 DMSP lyase (Alcolombri et al., 2015) and by the three small cupin-containing proteins that are found co-transcribed with *dddQ* in *R. nubinhibins*, but which do not encode functional lyases (Todd et al., 2011). It may be that a more reliable way to predict cupin-containing DMSP lyases is through 3D structural programs since despite DddL, DddQ and DddW being dissimilar at the amino acid level they are predicted to have very similar overall folds (Li et al., 2014).

### 6.3 Abundance of cupin DMSP lyase genes

With the rise in the use of high throughput sequencing technology, there is a wealth of genomic and metagenomic sequence databases available. The latter of these resources allows us to estimate the abundance of our genes of interest in environments of interest compared to everyday “housekeeping” genes. The largest and most relevant metagenomic data for this study is that of the Global Ocean Sampling (GOS) survey. This resource consists of 7.7 million sequence reads (6.3 billion bp) from bacterial samples taken at various sample sites in the Atlantic and Pacific oceans (Rusch et al., 2007). Homologues of the three DMSP cupin lyase enzymes are all found in the GOS data, being estimated to occur in 1.4% (*dddL*), 5.9% (*dddQ*) and 0.1% (*dddW*) of cells sampled (Carrión et al., 2015). The low number of copies of *dddW* present and the representation of DddW in the GOS is reflected by the fact that there are just two sequenced species containing copies of *dddW, Ruegeria pomeroyi* DSS-3 and *Roseobacter* sp. MED193. Thus, based on these abundances, at least ~6% of the total bacteria in the pelagic regions of our oceans contain these cupin-containing DMSP lyases, indicating their likely importance in marine biogeochemistry.

## 6.4 DMSP lyases of the cupin family are metalloenzymes

DddL, DddQ and DddW all possess conserved metal-binding residues, these metal-binding residues being two histidines and one glutamine residue in motif 1 and a single histidine in motif 2. We have shown through analysis of purified enzyme activity and site-directed mutagenesis of these conserved residues that DddL, DddQ and DddW are indeed metalloenzymes that require metals as cofactors for function. Prior to our studies, it was not known which metals were used as preferred co-factors. Our studies demonstrated that all three cupin lyases are inactivated, or at least activity is diminished dramatically, on the addition of the metal chelator EDTA. Activity is restored to all lyases upon addition of particular metal ions. It was also revealed that the DMSP cupin lyases are somewhat non-specific in terms of which metal they will accept into their binding pocket, such that while they have a preference for one metal, if it is not readily available, another will be accepted in its place. Apo DddL saw activity restored to 100% (relative to as purified protein) on the addition of Zn and ~ 85% on the addition of Ni. DddQ demonstrated that Zn restored the activity of apo protein to ~70% followed by Ni which restored it to ~30%. DddW saw the least amount of activity restored but still significant amounts observed by Mn at ~50% and Co at ~25%. Site-directed mutagenesis and replacement of identified conserved residues with an alanine also abolished enzyme activity telling us that each one is essential for the activity of these enzymes, likely through metal binding. Whilst considering the achievements of this study, it is also interesting to compare our findings in biochemical characterization of DddL, DddQ and DddW to the since published findings of Brummett et al (2015) on DddW and Li et al (2014) on DddQ.

### 6.4.1 DddQ

The *dddQ* lyase genes are among the most abundant in the ocean bacterial metagenomes (Curson et al., 2011c, Carrión et al., 2015), and until this study little was known about its enzymology or biochemistry. The results obtained showed that EDTA almost completely abolished the activity of the DddQ enzyme. Metal addback assays showed zinc to activate the enzyme activity of ‘apo’ DddW. This is in contrast to the work conducted by Li et al (2014) where Co and Mn were seen to dramatically activate the enzyme activity and Zn almost abolished it. A reason for

such differing results could be due to the concentration at which the metal add back experiments were performed. Experiments we conducted routinely used 20 mM concentrations of metals whilst Li et al. performed metal addback experiments at lower concentrations of 2 mM. The addition of zinc at higher concentrations to the purified DddQ protein may cause zinc to bind at a second site, and this might have the effect of enhancing the enzyme activity. However, Brummett et al (2015) working on DddW (see later) also showed that Zn inhibited DddW activity. This begs the question as to why DddQ was crystallized in the presence of zinc, and indeed there is not an available structure for DddQ in the active form. The ICP-OES analysis conducted as part of our studies showed that Zn and Fe were both present, Li et al also demonstrated that Zn was present as the cofactor of DddQ via Atomic Absorption Spectroscopy (AAS) (Li et al., 2014)

The recently published works of Li et al (2014) on DddQ proposed a mechanism (Figure 6.1) in which DMSP initially binds to the Zn atom, causing the movement of a nearby tyrosine (Li et al., 2014). As a result its, oxygen atom is brought closer to the alpha carbon of DMSP, resulting in the release of the proton, acrylate and DMS. The tyrosine involved in this mechanism has also been shown to be a conserved residue in the other cupin-type DMSP lyases (Brummett et al., 2015), which suggests this mechanism could also apply in these DMSP lyase enzymes.

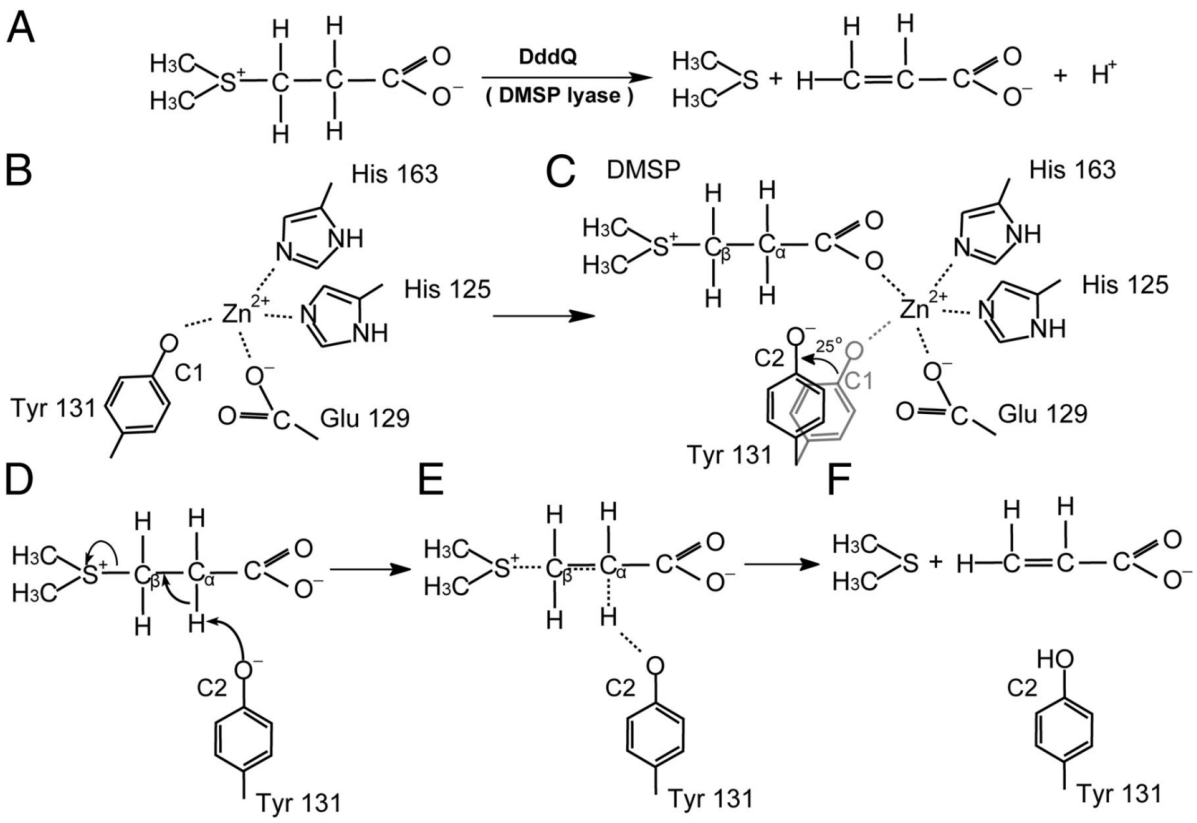



**Figure 6.1 Proposed mechanism for DMSP cleavage by DddQ (Li et al., 2014).** (A) The chemical equation for DMSP cleavage into DMS and acrylate by DMSP lyase. (B) When DMSP is absent, $Zn^{2+}$ in the active site of DddQ is coordinated by the conserved residues (His125, His129, His163, and Tyr131). (C) The oxygen atom from the carboxyl group of DMSP forms a coordination bond with $Zn^{2+}$ and replaces Tyr131, resulting in a deviation of 25° in Tyr131. (D) The cascade reaction is instigated by Tyr131. (E) The intermediate formed by DMSP and DddQ. (F) DMSP is cleaved, and DMS and acrylate are generated. (Li et al., 2014)

### 6.4.2 DddW

DddW was isolated from the model marine bacteria *R. pomeroyi*, a member of the abundant Roseobacter clade. The Roseobacters account for ~30% of bacteria in coastal regions and in ~3% of cells in the surface waters of the open ocean (Gonzalez et al., 1999b). *R. pomeroyi* has the capacity to both demethylate DMSP (DmdA) and cleave DMSP to produce acrylate via three different DMSP lyases (DddP, DddQ and DddW). Previous microarray data showed *dddW* to be the most induced gene in *R. pomeroyi* when grown in the presence of DMSP , with *dddP* and *dddQ* only modestly up regulated (1 to 4-fold) (Todd et al., 2010b,

Brummett AE, 2015). While DddW is not as abundant in marine bacteria compared to other DMSP lyases, it contains a C-terminal cupin domain and is representative of the other cupin-containing DMSP lyases, DddL and DddQ. At the time of this study, DddW was an uncharacterized DMSP lyase at the biochemical level.

Whilst no studies to date have suggested a mechanism for which the lysis of DMSP via DddW takes place, one could assume it would work in a similar way to that of the more prevalent DddQ. A study similar to that conducted by Li et al. (2014) for DddQ would help to confirm these suggestions. Brummett et al (Brummett AE, 2015) conducted a study in which they also confirmed DddW was a metalloenzyme. The addition of chelators and the stripping of the metal cofactors abolished activity, which is wholly consistent with what we found. Furthermore, in agreement with our original findings it was confirmed that the site-directed mutants of the metal-binding residues (H83, H85, E87 and H121), constructed in this study and provided by us, to Brummett et al. have no Ddd activity, indicating a role in metal binding. However, these same mutations did not abolish metal binding (see later). It was also shown that the as purified DddW protein is an Fe-containing enzyme. The study we conducted found that DddW contained Fe, but also Zn, and in fact Zn was present at a higher concentration (see chapter three).

The Brummett et al study carries out a comprehensive study of the potential metal cofactors They show that the DddW enzyme can incorporate Fe, Co, Ni and Cu as active co-factors and it was also determined that Zn had a slight inhibitory effect on the enzyme. This data demonstrates that whilst Fe is strongly favoured, DddW is non-specific in terms of the metals it will bind. This is in contrast to the results we achieved. Our data on metal addback assays suggested Mn and Co were the metals incorporated more readily to the enzyme, with Fe showing little restoration of enzyme activity above the EDTA-treated enzyme, whilst our ICP-OES data demonstrated that Fe and Zn are present as cofactors in the as purified DddW. Brummett et al (2015) also calculated the binding efficiency of the metal cofactor showing that DddW has a high affinity for Fe, Co, Ni and Cu with Fe being the favoured ion and it was also demonstrated that DddW increased activity with Fe. This is reflected in their competition studies where they show DddW preferentially

binds Fe in cocktails of other metals. Further supporting the importance of Fe, they show that the addition of excess Fe to the overexpression media is required for increased Ddd activity. This is highly relevant in an environmental context since Fe is often limiting in marine environments (see later).

Brummet el al (2015), following on from the work of Li et al. (2014) on DddQ, proposed a mechanism for DddW and the lyase activity of the cupin. They proposed that the metal centre of the enzyme is coordinated by the four conserved ligands (H81, H83, E87 and H121), with the remaining coordinating sphere around the Fe occupied by water molecules. A basic histidine can act as a nucleophile, removing a hydrogen atom from the alpha carbon of DMSP to form acrylate. They also discussed that whilst tyrosine is not usually involved in metal-binding in cupin proteins, the DddQ structure did reveal a Zn co-ordinated residue which Li et al. (2014) proposed instigates catalysis, and the Tyr of DddQ (Tyr131) aligns well with Tyr89 of DddW. Therefore the possibility of the involvement of a Tyr89 of DddW in the commencement of an elimination reaction should not be disregarded.

### 6.4.3 DddL

DddL was identified from the marine a α-proteobacterium *Sulfitobacter* sp. EE-36, a member of the Roseobacters isolated from a salt marsh grass on the coast of Georgia, USA (Gonzalez et al; 1996; 1999). Although there are no new papers on the biochemistry of DddL, it is important to note that it likely behaves in a similar way to DddQ and DddW and with a similar enzyme mechanism. Like DddQ and DddW, DddL is a metalloenzyme and metal addback experiments showed that Zn and Ni restored most activity following metal stripping. However, ICP-OES analysis showed Cu and Fe to be the metals present in as purified DddL. Further more detailed study is required to determine which metals are the true cofactors in this DMSP lyase enzyme.

## 6.5 Enzyme kinetics of cupin-containing DMSP lyases

Studies conducted on the enzyme kinetics of DddL, DddQ and DddW demonstrated that the cupin-containing lyases, whilst able to cleave DMSP, do so with relatively high $K_m$ values (L= 69.9 ± 19 mM, Q= 342.2 ± 48 mM, W= 32.0 ± 8.8 mM**).** This seems to be a feature of many of the enzymes that act on DMSP,

as the DddP DMSP lyase enzyme also has a high reported $K_m$ of 14 mM (Kirkwood et al., 2010a) and 13.8 mM (Hehemann et al., 2014), and the DMSP demethylase DmdA has a $K_m$ of 5.4 mM. This, as previously discussed both as part of this study and by other authors, could suggest that they are not efficient enzymes or that DMSP is not the intended substrate of the enzyme but is some other as yet unknown molecule (Alcolombri et al., 2014). It would have been an interesting series of experiments to explore different potential substrates for the enzymes. Future studies should therefore probe the efficiency of these enzymes by studying substrate analogs or inhibitors. Analogues of DMSP such as dimethylsulfoxide or glycine betaine would have been potential candidates to look at. Unfortunately, time constraints meant that these sets of experiments were not completed.

When considering the efficiency of DMSP lyases, it is important to remember that DMSP is above all an osmoprotectant and has many possible roles within cells other than as a carbon or sulphur source. It has been shown that bacteria import and store high amounts of DMSP, for example, up to 70 mM in *R. pomeroyi* (Reisch et al., 2008). The role that DMSP plays in these organisms is unknown but is most likely as a compatible solute. It is considered that DMSP and other osmolytes are expensive molecules to make so therefore why waste energy making them when you do not need to. One would predict that the role of DMSP in the organism containing it should dictate the efficiency of their DMSP lyase enzymes. Therefore, it could be that that these enzymes have evolved to possess high $K_m$ values so that they do not breakdown DMSP efficiently when it is required to act as an osmolyte and indeed only breaks it down when it is in excess and therefore at high intracellular concentrations. For organisms that use it as a sole carbon and energy source, however, this does not appear to be the case, with only DddY having a low $K_m$ (1.4 mM), but even this is relatively high. It could be that the $K_m$ values reported are not representative of the efficiencies of the enzymes in the appropriate cellular environment with the appropriate metal cofactors present.

What is apparent is that in organisms that use DMSP as a carbon and energy source, expression of the *ddd* DMSP lyase genes is tightly regulated, such that expression increases in response to the presence of DMSP or some of its

catabolic products. This is the case in *Marinomonas* (*dddD*), *Halomonas* (*dddD*), *Alcaligenes* (*dddY*) and *R. pomeroyi* (*dddW*) (Todd et al., 2007; Todd et al., 2010; Curson et al., 2011c; Todd et al., 2012). Generally cupin-containing lyase genes, with the exception of *dddW* in *R. pomeroyi*, are not tightly regulated by DMSP. It would be interesting to see if other environmental stimuli are inducers of expression of the cupin-containing lyases.

### 6.6 Other typical DMSP lyases

To date all known cupin-containing DMSP lyases are typical DMSP lyases, producing acrylate as a co-product of the lyase pathway. However, as introduced during chapter one, there are other members of the DMSP lyase family which are also typical DMSP lyases.

#### 6.6.1 DddP

DddP is the most abundant of the known DMSP lyases, predicted to be present in ~20-30% of the bacteria in a variety of marine metagenomes (Carrión et al., 2015). DddP is found in many marine Roseobacters, some terrestrial species of bacteria and fungi (Todd, 2009). For example, the cereal pathogen *Fusarium graminearum* contains a homologue of DddP. Interestingly, DddP is also a metalloenzyme and was predicted to be a member of the M24B metalloproteases (Todd, 2009), and this has recently been confirmed via structural analysis of two Roseobacters DddP proteins (Wang et al., 2015). The structures confirmed that the enzyme was a dimer containing the suspected metalloproteinase (Kirkwood et al., 2010a). A mechanism has since been proposed where the single monomers encompass an active site that includes a binuclear metal centre, with one of these being occupied by Fe (like DddW, Fe is the most abundant metal present in DddP). The metal ions are thought to attract the carboxyl group of DMSP to promote movement of one of the iron ligands. This could then expose the substrate to residues which would remove a proton, leading to a beta elimination reaction as previously described for the cupin mechanism of DMSP cleavage, and, as a result, the release of DMS and acrylate.

### 6.6.2 DddY

To date, DddY has not been part of any comprehensive biochemical studies. It does not bear a resemblance to any of the other DMSP lyases or to any polypeptide of known function, so revealing its enzymatic details is somewhat more challenging. It is not known whether this protein is a metalloenzyme like the cupin-containing lyases and DddP, and it would be interesting to study and establish if this is the case.

## 6.7 DddD, an atypical DMSP lyase

DddD is considered an atypical lyase in that, in addition to DMS, the other product liberated from its DMSP substrate is 3-hydroxypropionate (3-HP) (Todd, 2007). The *dddD* gene is estimated to be present in 0.4% of the bacteria in the ocean metagenome datasets (Carrión et al., 2015). This low abundance is not surprising since *dddD*, unlike many of the other DMSP lyase genes, is not prevalent in marine α-proteobacteria, such as the Roseobacters. Most DddD homologues occur in the γ-proteobacteria, having been isolated in *Marinomonas* MWYL1 in Stiffkey salt marsh. DddD encodes a type III CoA transferase and uses acetyl-CoA (Alcolombri et al., 2014), but, unlike DddP and the cupin-containing lyases, there is no indication that this enzyme requires any metal to function.

## 6.8 The environmental relevance of metals

Metals, notably Fe, are considered vital nutrients for all living organisms, with many essential enzymes requiring metals as cofactors. Highly relevant to marine environments is the fact that many of these metals, most notably Fe and Mn, are severely limiting in these environments. Indeed Fe has been shown to be the major limiting factor in microbial growth in the open ocean. Iron seeding experiments, where Fe was added to the ocean, were found to stimulate microbial growth and productivity. Considering this and our results, it is certainly clear that the availability of metals in the environment will have an effect on the level of Ddd activity, as well the growth of DMS-producing microbes. It could be that in Fe-limited conditions and environments, microbes employ DMSP lyases other than the cupin lyases and DddP, that do not as far as we know have a requirement for

metals, or they may at least use lyases that do not require Fe specifically, but another metal that is more readily available.

### 6.9 Catabolism of DMSP by the coccolithophore *E. Huxleyi*

DMSP catabolism was first detected in the Eukaryote *Polysiphonia lanosa* and many attempts to isolate the DMSP lyase in marine eukaryotes subsequently followed. Many of these studies actually went to great lengths to purify the lyases, with some even isolating purified proteins and getting N-terminal sequences. However, none of these studies led to the identification of a DMSP lyase in any marine eukaryote. The most environmentally relevant and well studied of these marine eukaryotes is *E. huxleyi.*

*E. huxleyi* has long been known to produce DMSP and cleave it to produce DMS, but it was not until this study that *E. huxleyi* was shown to possess at least one 'true' DMSP lyase, releasing acrylate as the other product of DMSP cleavage, and for the first time in cultures that had been established as axenic. Unfortunately our approaches failed to identify any Eukaryotic Ddd enzymes, and there now follows a breakdown of our efforts with a discussion of the limitations.

It is clear from our bioinformatic probing that these are no clear homologues to any of the bacterial Ddd DMSP lyase proteins in any marine eukaryotes. We do not necessarily believe that there are no marine eukaryotes containing known ddd genes, but that this simply reflects a relative lack of eukaryotic transcripts or genomes being published, as compared to those of bacteria. The only possible exception to this is with DddD. DddD-like enzymes are found in *E. huxleyi,* and some other algae, which clearly contain the dual CaiB domain. However, we could not demonstrate any DMSP lyase activity of these enzymes through overexpression in *E. coli* or when using rabbit reticulase to try and account for any possible eukaryotic post-translational modifications. Despite this, it is still possible that these enzymes do have DMSP lyase function, but require expression in *E. huxleyi* or another more similar host. However, given that we and others show that *E. huxleyi* produces acrylate, and not 3-HP, from DMSP, these DddD homologues are unlikely to be functional DMSP lyases.

Our bioinformatic prediction of a cupin-containing DMSP lyase also failed to show Ddd activity when expressed in *E. coli*. There are three possibilities as to why this was not successful (1) The *E. huxleyi* CCMP1516 gene annotation was incorrect and we therefore synthesized a truncated enzyme, (2) It requires eukaryotic post-translational modifications or (3) it is not a DMSP lyase.

The most likely reason as to why the bioinfomatic approach to DMSP lyase identification failed in *E. huxleyi* and other marine eukaryotes is that these organisms contain new *ddd* genes that encode novel DMSP lyases with no homology to the six DMSP lyases previously identified in bacteria and fungi. This would of course make it impossible to identify ddd genes bioinformatically on the basis of similarity to known genes.

The purification techniques employed could potentially have had a greater level of success, since they are not dependent on having any particular candidate protein identified beforehand. Although this approach was also not successful in identifying a DMSP lyase in *E. huxleyi*, we feel that, given more time, it would have been useful to combine the separation methods with in gel extraction and Ddd activity determination on 1D Native PAGE gels, which we demonstrated to have potential in the late stages of this study. It would certainly have been beneficial to combine these techniques with a more robust mass spectrometry identification technique such as MUDPIT. This would enable us to identify many more proteins in our Ddd active samples, which would not necessarily be visible on SDS-PAGE gels. Also, this work would have been greatly helped if there were found to be environmental conditions that enhance the expression of the DMSP lyase activity, allowing for the proteomic identification of induced proteins under these conditions, but no such conditions were known. However, this work has demonstrated that *E. huxleyi* RCC1217 contains at least one typical DMSP lyase, following confirmation that acrylate is produced as the other product, along with DMS, in the DMSP cleavage reaction.

Work performed since this study was completed (Alcolombri et al., 2015) has seen the successful purification and description of a DMSP lyase (Alma1) from *E. huxleyi*. This study successfully used proteomic techniques similar to those we proposed in the above, and on a strain of *E. huxleyi* that has DMSP lyase activity

~2-fold greater than that observed in our *E. huxleyi* RCC1217 strain. Using mass spectrometry identification from chromatographically separated fractions they identified Alma1 as a potential DMSP lyase. The corresponding *alma1* gene was synthesised from the *E. huxleyi* genome and was expressed in *E. coli* and found to have significant Ddd activity, thus confirming Alma1 as the first DMSP lyase enzyme to be identified in a marine alga.

The Alma1 protein sequence shows no homology to any of the known bacterial DMSP lyases to date, explaining why it was not identified from any bioinformatic screening. Alma1 is a tetrameric DMSP lyase enzyme and was shown to be a typical DMSP lyase, generating acrylate and DMS and from DMSP. It was shown to be located within the cells chloroplast membrane. This is consistent with some earlier studies (see chapter one) and suggests that it may have a role in oxidative stress protection, since DMS and acrylate are more effective at scavenging hydroxyl radicals than DMSP. The Alma1 DMSP lyase was confirmed to be a homotetramer with an overall molecular weight of ~160 kDa. It is worth mentioning that during the exploration and attempted purification of a DMSP lyase in strain RCC1217, a potential tetrameric lyase was identified, but with an overall molecular weight of ~225 kDa (chapter five). Interestingly, Alcolombri et al (2015) also found that *E. huxleyi* RCC1217, in comparison to its related diploid strain 1216, displayed 12-fold higher mRNA levels of Alma1, while the DMSP lyase activities differed by ~2.5-fold. Bioinformatic analysis identifies Alma1 as a member of the Asp/Glu hydantoin racemase superfamily, and all enzymes in this racemase superfamily catalyse the removal and/or addition of a proton. Consequently, Alma1 catalyses proton removal resulting in a β-elimination reaction, and the release of DMS and acrylate. This suggests that the mechanism of DMSP degradation in eukaryotes may be similar to that seen in bacterial DMSP lyases such as DddP.

Within the Alcolombri et al. (2015) paper, they find homology suggesting that *E. huxleyi* has seven Alma1-like proteins (Figure 6.2). Alma1, Alma2, Alma3, and Alma6 have all been cloned and checked for Ddd activity. It is interesting that only one, Alma2 has significant activity, ~20-fold higher than Alma1. Alma3 and Alma6 have very low Ddd activity indeed so it would be interesting to see why although

these enzymes are strong homologues of Alma1 and Alma2, they have no activity. Two more Alma proteins, Alma4 and Alma5, which were not tested for activity, are twice the size of Alma1 and contain domains similar to those seen in DddD, so it would be interesting to see if these enzymes are functional.

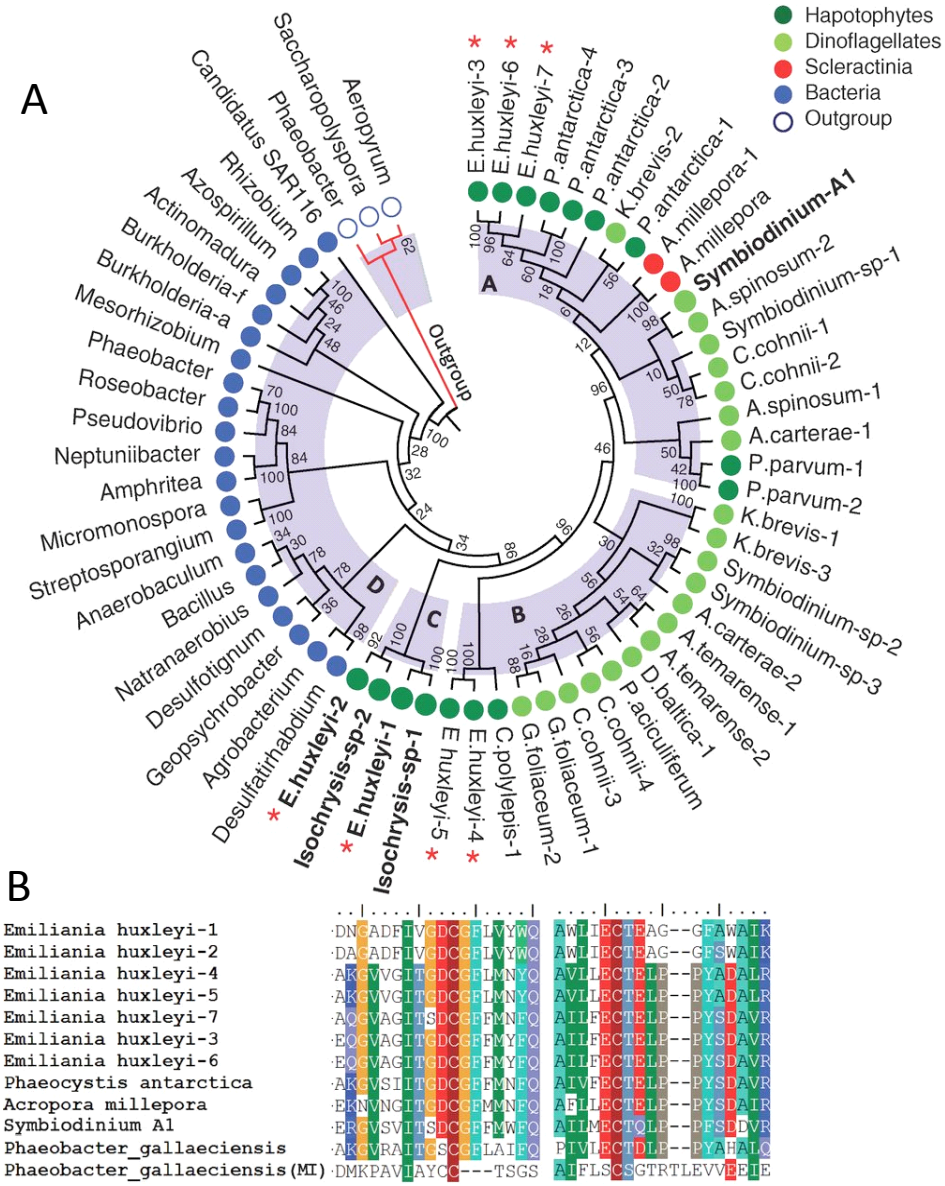



**Figure 6.2 Diagram of the maximum likelihood phylogenetic tree of the Alma1 like proteins (A) and the suggested conserved cupin domains of the proposed functional DMSP lyases (B).** Indentified by Alcolombri et al using NCBI and the Marine Microbial Eukaryote Transcriptome Sequencing Project. The seven *E. huxleyi* paralogs are marked with red stars. Genes with confirmed DMSP lyase activity are marked in bold. Adapted from Alcolombri et al (2015)

Homology searching outside of *E. huxleyi* showed that Alma-like enzymes exist in many marine eukaryotes (Figure 6.2) (Alcolombri et al., 2015). Of these enzymes, only the *Symbiodinium* and *Isochrysis* homologues were shown to be functional DMSP lyase enzymes, and the *Isochrysis* homologue was closely related to *E. huxleyi* Alma1 and Alma2, so does not greatly extend the sequence diversity of functionally confirmed enzymes. However, the *Symbiodinium* Alma-like protein is quite distantly related from all the *E. huxleyi* enzymes and, importantly, was highly active in Ddd assays. Proteins similar to *Symbiodinium* Alma exist in the dinoflagellate *Crypthecodinium cohnii.* Further work is necessary to show if many of the other Alma-like enzymes are active DMSP lyase enzymes but this work is clearly very exciting. Alma-like proteins also exist in some bacteria, but at relatively low levels of sequence identity (up to ~29%) so it remains to be seen whether these are functional DMSP lyase enzymes.

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# 8 Appendix

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SHORT COMMUNICATION

# DddW, a third DMSP lyase in a model Roseobacter marine bacterium, *Ruegeria pomeroyi* DSS-3

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***Ruegeria pomeroyi* DSS-3 is a model Roseobacter marine bacterium, particularly regarding its catabolism of dimethylsulfoniopropionate (DMSP), an abundant anti-stress molecule made by marine phytoplankton. We found a novel gene, *dddW,* which encodes a DMSP lyase that cleaves DMSP into acrylate plus the environmentally important volatile dimethyl sulfide (DMS). Mutations in *dddW* reduced, but did not abolish DMS production. Transcription of *dddW* was greatly enhanced by pre-growth of cells with DMSP, via a LysR-type regulator. Close DddW homologs occur in only one other Roseobacter species, and there are no close homologs and only a few related sequences in metagenomes of marine bacteria. In addition to DddW, *R. pomeroyi* DSS-3 had been shown to have two other, different, DMSP lyases, DddP and DddQ, plus an enzyme that demethylates DMSP, emphasizing the importance of this substrate for this model bacterium.**
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A feature of the abundant marine α-proteobacteria known as the Roseobacters is that they catabolize dimethylsulfoniopropionate (DMSP), an anti-stress molecule made in massive amounts ($\sim 10^9$ tons annually) by marine phytoplankton (Kettle *et al.*, 1999). Strikingly, several Roseobacter strains degrade DMSP by more than one mechanism, either demethylating it or cleaving it, in a process that releases the volatile dimethyl sulfide (DMS; González *et al.*, 1999; Newton *et al.*, 2010). DMS has diverse environmental effects; it is a chemoattractant for different marine animals (Seymour *et al.*, 2010) and its oxidation products form cloud condensation nuclei, affecting levels of reflected sunlight (Charlson *et al.*, 1987).

Recent genetic analyses reveal the molecular basis of this metabolic flexibility, as several different enzymes can act on the DMSP substrate. Indeed, some individual bacterial strains have multiple ways to catabolize DMSP. For example, *Ruegeria pomeroyi* DSS-3 contains DmdA, the DMSP demethylase (Howard *et al.*, 2006), plus two genes, *dddQ* and *dddP*, that encode DMSP lyases that cleave DMSP into DMS plus acrylate, although they are in wholly different polypeptide families (Todd *et al.*, 2009, 2011; Kirkwood *et al.*, 2010).

In a microarray study (MK, unpublished) of *R. pomeroyi* genes whose expression was affected by growth in media with 5 mM DMSP, one of the most markedly induced ($\sim$37-fold) was *SPO0453*, as independently noted by Rinta-Kanto *et al.* (2011). The product of this gene, which we term *dddW,* contained a predicted cupin pocket (Figure 1), a widely distributed motif found in many enzymes, where it forms the active site (Dunwell *et al.*, 2004). Such a feature also occurs in the DMSP lyases DddQ (Todd *et al.*, 2011) and DddL (Curson *et al.*, 2008), but both of these are larger ($\sim$22 kDa and $\sim$26 kDa, respectively) than DddW (16.1 kDa), and share no other significant sequence or predicted structural similarities.

To study *dddW*, it was amplified from *R. pomeroyi* genomic DNA, then cloned into the expression vector pET21a. *Escherichia coli* transformants containing the resulting recombinant plasmid produced DMS at a rate of 35 pmol μg protein$^{-1}$ min$^{-1}$ when the substrate DMSP (5 mM) was added to cell-free extracts, compared with a background value in *E. coli* itself of 0.02 pmol μg protein$^{-1}$ min$^{-1}$ (Supplementary methods). This value obtained for the cloned *dddW* is similar to that obtained when the cloned *dddP* and *dddL* genes of *R. pomeroyi* and *Rhodobacter sphaeroides*, respectively, were examined in the same manner (Curson *et al.*, 2008; Todd *et al.*, 2011). On feeding [1-$^{14}$C]DMSP to cell-free

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**Figure 1** Comparison of cupin-like regions of DddL, DddQ and DddW polypeptides. The sequences of the predicted cupin-like motifs of the DddW polypeptides of *Ruegeria pomeroyi* DSS-3 and *Roseobacter* sp. MED193 were compared with those of the DddL DMSP lyases of *Sulfitobacter* sp. EE-36 and *Rhodobacter sphaeroides* 2.4.1 and the DddQ-type lyases of *Ruegeria pomeroyi* DSS-3 and *Roseovarius nubinhibens* ISM. Corresponding gene numbers are underlined. The highly conserved residues in cupins (Dunwell *et al.*, 2004) are indicated in single-letter code above the comparisons. Residues highlighted in black, dark gray or light gray indicate 100%, >80% or >60% conservation, respectively.

extracts of *E. coli* with cloned *dddW*, 100% of the $^{14}C$ was converted to labeled acrylate after 1-h incubation, confirming that DddW is a *bona fide* 'DMSP lyase'.

To determine its role in *R. pomeroyi*, we made an insertional mutation into *dddW*, using the suicide plasmid pBIO1879 (Supplementary methods). The DddW$^-$ mutant produced DMS from DMSP at a rate ~50% that of wild type, an effect smaller than that seen in a DddQ$^-$ mutant, but similar to that caused by a mutation in *dddP* (Todd *et al.*, 2009, 2011). Thus, DddW, like the other two lyases, contributes to DMSP catabolism in *R. pomeroyi* DSS-3.

To further examine DMSP-dependent regulation of *dddW* expression, we cloned its promoter region into the wide host-range promoter-probe vector pBIO1878, upstream of its *lacZ* reporter. The resulting plasmid, pBIO1945, was mobilized into wild-type *R. pomeroyi* and the transconjugant assayed for β-galactosidase after growth in minimal media that either contained or lacked 5 mM DMSP (Supplementary methods). The *dddW–lacZ* fusion was induced, 10-fold, in the + DMSP media.

Separated by 94 base pairs from the start of *dddW* is a gene, *SPO0454*, which encodes a predicted LysR-type transcriptional regulator. We attempted to mutate *SPO0454* using the same approach as for the insertion into *dddW,* but this was unsuccessful. This may be due to the polar effects of insertions in *SPO0454* on the expression of a downstream gene, *SPO0455*, which is predicted to be co-transcribed with *SPO0454* and which encodes a potentially essential lysyl-tRNA synthetase. We therefore adopted a different approach to show that *SPO0454* regulated *dddW*. First, we conjugated the *dddW–lacZ* fusion plasmid pBIO1945 into the α-proteobacterium *Rhizobium leguminosarum,* which effectively expresses heterologous genes (Young *et al.*, 2006). Into this strain was then transferred pBIO1946, containing intact *SPO0454* including its native promoter, cloned in the wide host-range plasmid pOT2. We then measured the effects of pre-growth in DMSP on *dddW–lacZ* expression (as β-galactosidase activity) in *R. leguminosarum*/pBIO1945 that either contained or lacked the cloned *SPO0454* gene in pBIO1946. In *R. leguminosarum* itself, *dddW–lacZ* was expressed constitutively at a low level in both growth media. However, the presence of *SPO0454* increased β-galactosidase activity ~5-fold, but only when cells were pre-grown with DMSP (Figure 2). Thus, *SPO0454* encodes a transcriptional activator that responds to DMSP. Furthermore, *SPO0454* was auto-regulatory, like other *lysR*-type regulatory genes (Maddocks and Oyston, 2008). This was shown by the behavior in *R. leguminosarum* of an *SPO0454–lacZ* fusion plasmid, termed pBIO1947, that we constructed. This expressed β-galactosidase constitutively in *R. leguminosarum* itself, but was repressed ~5-fold by introducing *SPO0454*, cloned in pBIO1946, irrespective of whether DMSP was in the medium or not (Figure 2).

In addition to its DmdA demethylase (Howard *et al.*, 2006), *R. pomeroyi* DSS-3, remarkably, has three different DMSP lyases, the previously identified DddP and DddQ, and the newly discovered DddW. All these lyases function in *R. pomeroyi*, as mutations in the corresponding genes affect its DMS-emitting phenotype. As judged by the individual mutant phenotypes, in our laboratory conditions, DddW appears to contribute about the same to the flux into DMS production as DddP, but less than DddQ.

Currently, the only other bacterium with a close DddW homolog (65% identical) is *Roseobacter* sp. MED193, whose *dddW* is also transcribed divergently from an *SPO0454* homolog. More poorly conserved homologs (~40% identical to the DddW of *R. pomeroyi* DSS-3) were found in two other Roseobacters, namely *Rhodobacterales bacterium* HTCC2083 and *Citreicella* sp. SE45; nevertheless, DddW is rarer among the deduced proteomes of Roseobacters and other marine bacteria than other Ddd polypeptides (Newton *et al.*, 2010). Consistent with this, there are no very close DddW homologs in marine metagenomic sequences (surveyed in the CAMERA portal at http://camera.calit2.net/), most notably those in the Global Ocean Sampling (GOS) described by Rusch *et al.* (2007). However, four sequences in the GOS had deduced polypeptides with some similarity to DddW (40–50% identical;

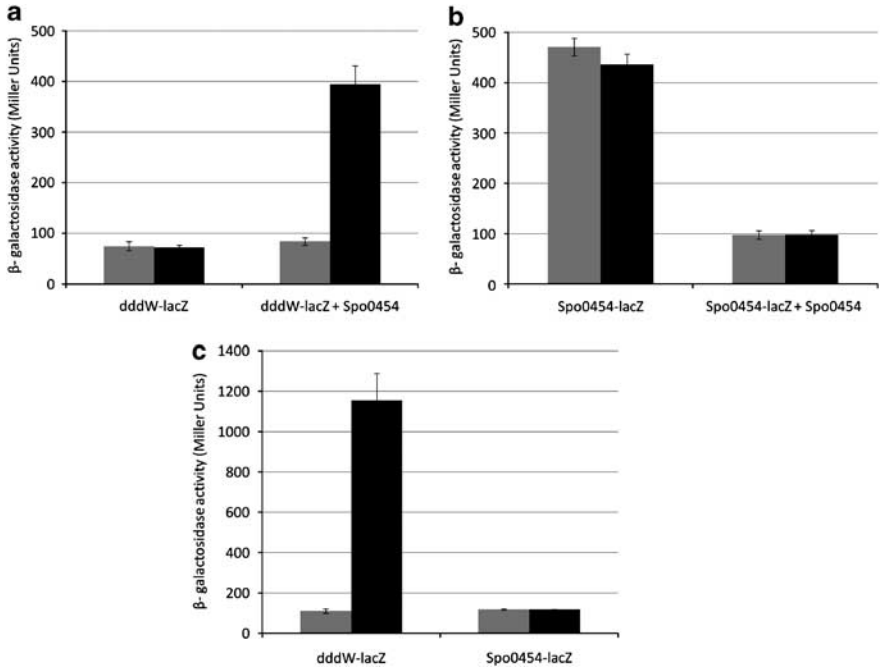


**Figure 2** Effects of DMSP and the regulatory *SPO0454* gene on the expression of the *dddW* and *SPO0454* genes of *R. pomeroyi* DSS-3. Cultures of *Rhizobium leguminosarum* strain 3841 (**a**, **b**) or of *Ruegeria pomeroyi* DSS-3 (**c**) and containing either the *dddW–lacZ* fusion plasmid pBIO1945 (**a**, **c**) or the *SPO0454–lacZ* fusion plasmid pBIO1947 (**b**, **c**) were grown in minimal medium that either lacked (gray columns) or contained (black columns) 5 mM DMSP. These strains were assayed in triplicate for β-galactosidase activities, whose values with standard errors are shown in Miller Units. In the *Rhizobium* background, some of the strains with the fusion plasmids also contained pBIO1946, in which the *SPO0454* gene is cloned in the vector pOT2, as indicated.

probability $<e^{-22}$). These were all from the hypersaline lagoon site at Punta Cormorant in Galapagos, the same site at which homologs of a different lyase, DddL, were seen (Curson *et al.*, 2008). However, the ecological significance, if any, of this is unknown. Indeed, given the relatively low level identity of the metagenomic reads and DddW itself, it remains to be confirmed that these correspond to functional DMSP lyases.

Other Roseobacter strains also have multiple DMSP lyases—*Roseovarius nubinhibens,* for example, has two versions of DddQ plus DddP (Todd *et al.*, 2009, 2011). It will be interesting to know if these different enzymes, plus the DmdA demethylase, are particularly adapted to specific environments that vary in the availability of DMSP substrate (see, for example, González *et al.*, 1999) or other factors, such as temperature, pH or the availability of other nutrients. Furthermore, other bacteria have yet other classes of enzymes that release DMS from DMSP (Todd *et al.*, 2007; Curson *et al.*, 2008, 2011), further emphasizing the genetic diversity of this important environmental biotransformation.

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